# Susceptibility pays off

Insights into the *mlo*-based powdery mildew resistance

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## SUSCEPTIBILITY PAYS OFF: Insights into the *mlo*-based powdery mildew resistance

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## SUSCEPTIBILITY PAYS OFF: Insights into the *mlo*-based

### powdery mildew resistance

**Michela Appiano** 

Thesis

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# Chapter 1

### **General introduction**

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Plant breeding is the effort to select or modify crop plants to obtain genotypes with (more) favourable traits (Acquaah, 2012). Hereditary traits that breeders purposely want to modify are diverse. Yield and disease resistance, for example, have always been a priority. Recently, the need to select plants that are better adapted to environmental stresses has become more pressing because climate change is modifying the crop production environment. This fact implies that it is important to develop new varieties that can resist biotic (diseases and insect pests) and abiotic (e.g., salt, drought, heat, cold) stresses in their production environment.

By means of this thesis, we want to contribute to understanding and improving breeding activities aimed at obtaining resistance to one particular biotic constraint of Solanaceous and cucurbit crops, which is the powdery mildew (PM) disease.

It's in the interest of breeders to obtain durable resistance that is potentially effective against multiple pathogens. There is a class of genes that combines both features, and it is represented by the so-called susceptibility (S-) genes.

A well-known member of this class of genes is *MLO* (*Mildew Locus Q*). It is mainly studied as key factor to establish a compatible interaction between PM pathogens and plant hosts, but, at least in pepper, it is also involved in susceptibility to bacteria and oomycetes. Moreover, the resistance conferred by impaired *MLO* genes, called *mlo*-based resistance, is known to be durable, as exemplified by the long-lasting effectiveness of the barley *Hvmlo* gene in the field.

#### The PM disease and the pathogens that cause it

PM disease is one of the most important diseases of food and ornamental plants (Glawe, 2008).

#### PM distribution and host range

This disease is worldwide present, and sometimes more widely distributed in semiarid regions than in areas with high rainfall, where other diseases flourish. The fungal species responsible are a conspicuous number (more than 400). They can either infect a broad range of plants or prefer very specific plant species reaching in total almost 10,000 possible hosts. For example, the pathogen, *Blumeria graminis* f.sp. *hordei* (further mentioned as *Bgh*), causing the PM disease in barley is listed among the top ten most detrimental fungal pathogens based on the scientific and economic importance (Dean et al., 2012).

New reports on PM disease between 2010 and 2016 indicate that this disease is still plaguing many countries in the world, therefore very actual. It also appears that the number of host species is enlarging, which means that climate change might be influencing powdery mildew geographic expansion and host range (Figure 1, Harvell et al., 2002; Garrett et al., 2006).

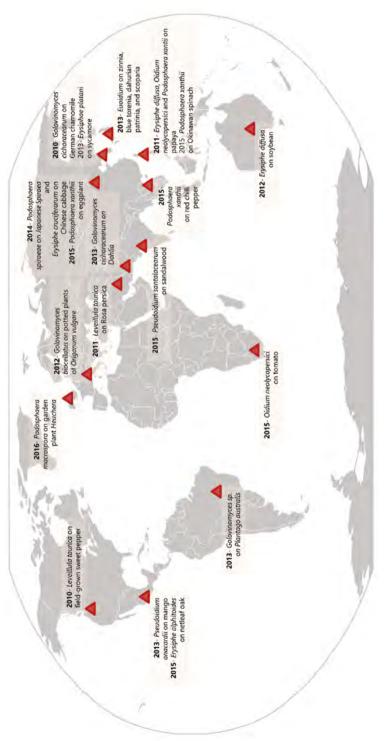


FIGURE 1. Original figure indicating the first new reports of the PM disease on a specific plant species filed from 2010 to 2016 and combined into a world map. Red triangles indicate the country in which the disease appeared.

#### **PM symptoms**

PM disease is easy to recognize by the white powder covering all the aboveground plant organs, predominantly leaves and stems and occasionally flowers and fruits. It can cause various types of damage, such as deformation of stems and leaves, defoliation, reduced yields and lowered quality but also death of the attacked tissue (Figure 2).

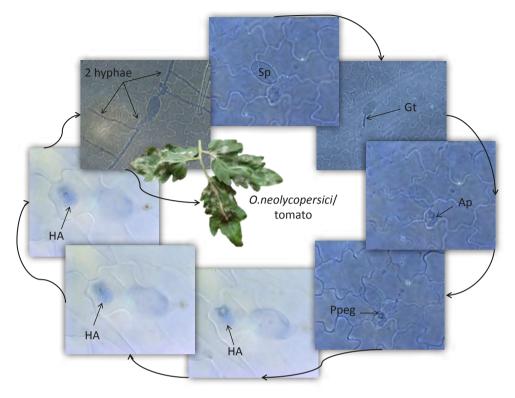


FIGURE 2. Powdery mildew symptoms on A) rose, B) grape, C) apple, D) squash, E) pepper, F) wheat, G) tomato, H) strawberry and I) lettuce. Photos are taken from Google images according to the website link given for each panel.

#### PM lifestyle and life cycle

PMs are obligate biotrophic pathogens as they rely upon the plant host to live, survive, reproduce, and thrive.

Each cycle begins with the landing of an airborne conidia on a host surface (Figure 3). When a conidium germinates, it forms a short hypha, called germination tube. The distal tip differentiates into a swollen, elongated appressorium. From the appressorium, a penetration peg breaks the plant epidermal cell wall and forms a haustorium within the cell surrounded by a plant-derived membrane. The haustorium represents the feeding structure of the fungus and the centre from where effector molecules are secreted. Once nutrients are available, the hyphae grow quickly, in most of the cases only epiphytically, producing secondary appressoria and haustoria, conidiophores and new airborne conidiospores. Altogether, they contribute to the powdery appearance of the disease on plant tissues.



**FIGURE 3.** Life cycle of the PM pathogen *Oidium neolycopersici* on the susceptible tomato cv Moneymaker. Photos composing the figure are original and were taken during our microscopic studies. **Sp**=spore; **Gt**=germination tube; **Ap**=appressorium; **Ppeg**=penetration peg; **HA**=haustorium; **2 hyphae**=secondary hyphae.

There are some PM species that, differently from the majority, penetrate through stomata. One of these PMs is an important pathogen for tomato, pepper, eggplant and other crops, namely *Leveillula taurica* (Braun and Cook, 2012). The haustoria of this fungus are formed in the mesophyll cells. Therefore, the mycelium is partially endophytic. Conidiophores emerge through stomata, mainly on the abaxial leaf surface, while on the adaxial surface, chlorotic spots are usually visible, indicating the development of mildew colonies underneath (Figure 2, panel E) (Zheng et al., 2013).

#### **PM classification**

All the PM fungi belong to the ascomycete order of the Erysiphales.

Mildews known only in their asexual reproductive stage (anamorph state) are called by the genus name *Microidium* (formerly *Oidium*). All PMs with *Oidium* anamorph can be grouped into two genera: *Euodium*, if they exhibit catenate type of conidial development, or *Pseudoidium*, when conidia mature one at the time (Braun and Cook, 2012).

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The latest classification of Braun and Cook (2012), based on holomorph (both the asexual and sexual stages), conidial germination and SEM (Scanning Electron Microscope) data, distinguishes six tribes: *Erysipheae, Golovinomyceteae, Cystotheceae, Phyllactinieae, Blumeriae* and one unnamed tribe to place the anamorph genus *Microidium*.

#### **PM-Omics**

The genome of several powdery mildew species has been recently sequenced, such as *Erysiphe necator, Blumeria graminis* f.sp *hordei* (*Bgh*), *Erysiphe pisi, Golovinomyces orontii* and *Blumeria graminis* f.sp *tritici* (*Bgt*) (Jones et al., 2014; Spanu et al., 2010; Wicker et al., 2013). A number of genomes of other pathogenic fungi are currently underway, like tomato PM *Oidium neolycopersici*, lettuce PM *Golovinomyces cichoracearum*, pepper PM *Leveillula taurica*, cucumber PM *Podosphaera xanthii* and strawberry PM *Podosphaera aphanis* (Bindschedler et al., 2016).

Data obtained from the sequenced genomes indicate that although about 4-times larger than other related Ascomycetes (average size is 36.9 Mbp; Mohanta & Bae, 2015), PM genomes miss many coding genes otherwise present in Ascomycetes species (average number of coding genes is more than 11.000; Mohanta & Bae, 2015).

The genome size increased because of the proliferation of transposons. The close association of transposon with effector-encoding genes most probably led to the rapid evolutionary adaptation of powdery mildews (Hacquard et al., 2013).

A first transcriptome analysis of conidia, germinated conidia, and hyphae of *Bgh* revealed a coordinated change in gene expression patterns between pre- and post-penetration stages (Thomas et al., 2001). Transcripts associated with lipid metabolism, for example, accumulated during fungal germination, while transcripts of genes involved in glycolysis were high in mature appressoria and infected epidermal cells. During the later phase of plant colonization, transcripts related to protein biosynthesis accumulated.

A second transcriptome analysis of epidermal peels of heavily infected *Bgh* leaves, provided insights into the transcripts of candidate effector proteins (Godfrey et al., 2010). This analysis resulted in the identification of a conserved sequence motif (Y/F/W-x-C) in most of the candidate effectors. However, the functional relevance is still under investigation. Another transcriptome study of *G. orontii*-infected *A. thaliana* plants revealed transcripts encoding proteins for the detoxification of reactive oxygen species (Weßling et al., 2012).

Regarding the PM proteome, several attempts have been made to study it (Bindschedler et al., 2009; Bindschedler et al., 2011; Godfrey et al., 2009; Noir et al., 2009). However, the low biomass of the pathogen in the early stages of infection and the dependence on a well-sequenced, well-assembled and well-annotated genome are challenges still to overcome to achieve good proteomic analyses. In a protein-protein interaction of secreted *G. orontii* 

PM effector candidates with their possible Arabidopsis targets, it was found that multiple PM effectors converged onto a limited number of host targets (hubs). These hubs are themselves interconnected with other host proteins and targeted by other pathogens, like oomycetes and bacteria (Weßling et al., 2014).

#### PM epidemiology

An enormous number of conidia are produced during the growing season (Bélanger et al., 2002). Under disease-conducive conditions, a cycle is usually completed within 3–7 days after infecting the host. The rapid rate of asexual reproduction can lead to exponential growth of powdery mildew populations resulting in epidemics.

In regions with mild climates, powdery mildews can survive winter in the form of dormant mycelium within buds of infected plants (Braun and Cook, 2002). Moreover, in these regions sexual fruiting bodies (also called ascocarps) do not seem to occur frequently. When ascocarps are produced, they can survive harsh conditions since they are resistant to low temperatures and drought.

#### PM and climate change

Disease symptoms are the product of the interaction not only between a susceptible host plant and a virulent pathogen but also of the two of them with the environment. The changes associated with global warming (i.e., increased temperatures, changes in the quantity and pattern of precipitation, increased  $CO_2$  and ozone levels, drought, etc.) may affect the incidence and severity of plant diseases. The same changes can influence the further coevolution of plants and their pathogens (Burdon et al., 2006; Chakraborty 2005; Crowl et al., 2011; Garrett et al., 2006).

For example, the effect of changes in temperature has been studied in the pathosystem tomato-PM caused by *O. neolycopersici* (Elad et al., 2009). They observed that disease severity increased dramatically from 18°C to 26°C.

Elevated  $CO_2$  levels are expected to increase plant canopy size and density, which, if combined with increased humidity at the canopy level, will promote PM growth (Manning & Tiedemann, 1995). On the other hand, other studies showed that wheat grown in a high level of  $CO_2$  was more resistant to the pathogen *Blumeria graminis* f.sp. *tritici* (*Bgt*) because plants were able to mobilize the assimilates into defense structures, like papillae (Hibberd et al., 1996a and 1996b). Nevertheless, if able to penetrate, colonies of *Bgt* grew quicker in 2X-CO<sub>2</sub> than in normal conditions (Hibberd et al., 1996a).

Salinity is also a major environmental stress and increased salinization of arable land is expected to have devastating global effects. Given this problem, a recent study addressed at elucidating the interaction between abiotic and biotic stress in tomato indicates that

a mild salt stress (50 mM NaCl) in combination with PM inoculation results in enhanced susceptibility of the cultivated tomato (Kissoudis et al., 2016).

#### PM control

Traditionally, sulphur dust, lime sulphur, and copper sprays have been considered specific remedies for PMs. Fungicides like sterol-biosynthesis-inhibitors (DMI), quinone outside inhibitors (QoI), and quinoline are often used in PM management programs, but some powdery mildew pathogens have developed resistance to a range of these fungicides (Pirondi et al., 2014).

Pruning the plants, spacing them correctly, irrigating properly to reduce the humidity and limiting nitrogen fertilization are among the good agricultural practices to consider. Furthermore, crop rotation, removal of debris, optimal sowing conditions and timing are other good strategies to control this disease.

Recently the biological control of PMs has become possible through the use of mycoparasitic fungi or yeasts. The European project called BIOCOMES, for example, is looking for sustainable biological control agents of the pathogen *Bgt* causing the PM disease on wheat.

For the cucumber PM disease caused by *Podosphaera xanthii*, a recent study reported the efficacy of a biologically active isolate of the epiphytic yeast *Pseudozyma aphidis* (Gafni et al., 2015). This yeast has an antagonistic effect and an antibiosis effect on *P. xanthii*: the crude extract of the yeast affects the fungal spores germination, while application of *P. aphidis* suspension on cucumber leaves can reduce disease severity up to 75%.

Last but not least, powdery mildew can be fought using resistant varieties. In tomato, for example, nine loci have been found to confer resistance to *O. neolycopersici*, five of which are dominant genes (*Ol-1* to *Ol-6*, except *ol-2*), and three are QTLs (Bai et al., 2005; Seifi et al., 2014). The *ol-2* gene is a recessive allele of the *SIMLO1* gene (Bai et al., 2008). The *Ol-4* and *ol-2* genes are, currently, used in breeding programs.

This overview shows the importance of this disease regarding the economic impact and scientific interest. As mentioned above, chemical control of this disease is limited and not always effective. Genetic control, on the other hand, is a valuable and probably durable solution.

#### PM perception and the lines of defence

#### The first layer of defence

#### Plant barriers

The PM infection starts with a conidium landing on the host surface. The first barrier for a PM to overcome is the constitutive layer of the plant defence like cuticle, epicuticular waxes, and cell wall. Unlike other pathogens that use cell wall degrading enzymes, PM pathogens, or at least *Bgh*, presumably use hydrostatic pressure to penetrate this first layer of defence (Pryce-Jones et al., 1999). Such pressure is built up by the appressorium and might activate plant mechanosensors. It might also damage the cell wall, releasing damage-associated molecular patterns (DAMPs) or the fungus itself can produce microbe-associated molecular patterns (MAMPs). However, it is important to notice that because of their biotrophic lifestyle, PM infection strategy is to invade the plant host creating as little damage as possible. Indeed, a recent study pointed out that in the early stages of *O. neolycopersici* infection of a susceptible tomato cultivar, the detection of biotic emissions, such as bigenic volatile organic (Masal et al., 2016).

Part of the early line of defence responses includes cell wall reinforcement below the fungal appressorium. This process, starting with cell polarization, cytoskeleton rearrangement and focal accumulation of defence-related proteins, leads to the formation of a dome-shaped apposition between the epidermal wall and the plasma membrane. This structure is called papilla (Assaad et al., 2004; Collins et al., 2003). Interestingly, papillae are a front line of defence in response to both adapted and non-adapted *formae speciales* of powdery mildew fungi (Aghnoum & Niks, 2010). Their effectiveness depends on timing and structural composition (Chowdhury et al., 2014).

#### MAMP-triggered immunity (MTI)

MAMPs and DAMPs can be detected by pattern recognition receptors (PRR) located in the plasma membrane. The recognition triggers a plethora of defence responses, such as alkalinization of the apoplast and production of reactive oxygen species (ROS), influx of Ca<sup>2+</sup>, and activation of mitogen-activated protein kinase (MAPK) pathways. Finally, biosynthesis and excretion of antimicrobial products like PR (pathogenesis-related) proteins and phytoalexins occur. These responses lead to the MAMP-triggered immunity (MTI).

Chitin is a major component of fungal cell walls. Plant cells have chitin degrading enzymes to digest fungal cell walls during an infection. Chitin fragments, considered as MAMP, are subsequently perceived by the plant triggering an immune response. It was discovered in

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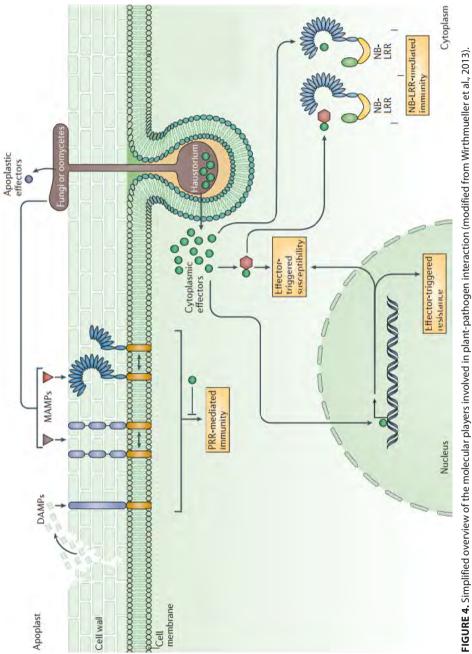
Arabidopsis that chitin is perceived by the membrane-localized PRR CERK1 (Chitin Elicitor Receptor Kinase 1) and LYKs 4/5 (Lysin motif receptor-like kinases 4/5) (Cao et al., 2014; Miya et al., 2007). CERK1 is a plasma membrane protein containing extracellular LysM motif and intracellular kinase domain. Arabidopsis *cerk1* mutants are more susceptible than wild type to the adapted PM *Golonovinomyces orontii* (*Go*) and do not respond anymore to chitin elicitor (Miya et al., 2007). Similarly, *lyk4/lyk5* mutants show complete loss of chitin response. The results of Cao et al. (2014) indicate that AtLYK5, primary receptor for chitin, forms a chitin-inducible complex with AtCERK1 to induce plant immunity.

#### The second layer of defence: ETI

The recognition of MAMPs is counteracted by the action of pathogen-secreted intracellular effectors that prevent the induction of PRR-mediated signaling, initiating the Effector-Triggered Susceptibility (ETS) (Jones & Dangl, 2006). The effectors are microbial molecules known in the past as Avr proteins. Whether recognized or not, the primary role of the Avr protein is to aid the invader in colonizing the host either by suppressing the plant immune signalling or by altering the plant's physiology to the pathogen's advantage (Chen et al., 2010). Effectors are delivered into the host cells by the haustorium. However, the delivery mechanisms of the effectors are largely unknown. Two papers provided a model for the mechanism of PM effectors' delivery into the plant cell (Voegele et al., 2009; Zhang et al., 2013). Effectors are hypothesized to be transferred from the extra-haustorial matrix to the cytosol through retrotranslocon pores located in the endoplasmatic reticulum (ER). A protein called Sec61 is hypothesized to be necessary for pore function. This trafficking is thought to take place in vesicles, dependent or independent of Golgi. The silencing in barley of the HvSec61βa translocon reduces barley's susceptibility to Bqh. This result suggests that Sec61Ba is recruited by the PM pathogen to allow effector's delivery in the cytoplasm.

In turn, plant intracellular receptors also called NB-LRRs- or R-proteins can evolve to recognize these effectors and trigger a new immunity called Effector-Triggered Immunity (ETI). Often, the recognition of the pathogen-Avr and plant-R protein is accompanied by a form of programmed cell death called hypersensitive response (HR). Effectors can be recognized either directly or indirectly by the NB-LRR proteins. In the case of indirect recognition, NB-LRRs 'guard' host proteins called effector target. Modification of this target by the effector results in the activation of the R protein, which triggers the immune response (Dangl & Jones, 2001).

There are several examples of *R*-genes taken from the plant-PM pathosystem. In wheat, the *Pm3b*, a member of the coiled-coil nucleotide binding site leucine-rich repeat (NBS-LRR), is responsible for the race-specific resistance to the PM pathogen *Bgt* (Yahiaoui et al., 2004). The *Pm3* locus is syntenic to the *Mla* locus of barley which confers resistance to





the barley PM *Bgh* (Hartl et al., 1993; Zhou et al., 2001). In both cases, recognition of the specific pathogen avirulence proteins by the cognate NBS-LRRs induces HR (Hückelhoven et al., 2000; Hückelhoven & Kogel, 1998; Bourras et al., 2015). In Arabidopsis, two paralogs *RPW8.1* and *RPW8.2* contribute to the resistance against PM-*Go* (Xiao et al., 2001). The resistance mediated by *RPW8* occurs after the formation of haustoria and it is typically associated with the accumulation of hydrogen peroxide and localized host cell death (Xiao et al., 2001; Göllner et al., 2008). In tomato, the *Ol-4* gene, deriving from the wild tomato species *S. peruvianum* LA2172, was mapped on the short arm of chromosome 6 where a cluster of *R*-genes is found (Seifi et al., 2010). It was also shown to confer PM resistance towards *O. neolycopersici* through fast hypersensitive response (Bai et al., 2005).

This model of continued co-evolution in which pathogens overcome the layers of plant immunity and plants recognize conserved and variable pathogen extracellular and intracellular elicitors is known as the 'zig-zag' model (Figure 4, Dodds & Rathjen, 2010; Jones & Dangl, 2006).

In this simplistic model, it may appear as if MTI and ETI are strictly separated. However, several studies show that there is no clear-cut distinction between the two signalling pathways but a continuum (Thomma et al., 2011). The difference lies mostly in the amplitude and kinetics of the response since both pathways overlap at multiple levels (Qi et al., 2011; Tsuda & Katagiri, 2010).

#### Genes contributing to the susceptibility to powdery mildew

After the discovery of the gene *PMR6*, required for susceptibility to powdery mildew in Arabidopsis (Vogel et al., 2002), Eckardt (2002) pointed out how little research was aimed at studying the so-called plant susceptibility genes (S-genes).

S-genes are plant genes that facilitate the infection and support a compatible interaction with the pathogens (van Schie & Takken, 2014). As described above, adapted pathogens have evolved ways to interfere with the plant immune system. Its consequent failure allows entry of the pathogens. In this process, the host is tricked to collaborate with the biotroph invader. The haustorial complex, for example, is partially fungus-derived, and partially (or fully) plant-derived. Following the haustorial complex, the host plant reroutes nutrients and hormones, like cytokinines, towards the infection sites, which become metabolically active "green islands" (Fotopoulos et al., 2003; Walters, 2015). Hence, the host contributes to the accommodation of the biotroph pathogen in many ways.

Several S-genes have been characterized for their role in PM disease. Based on the reviews of van Schie and Takken (2014) and Lapin & Van den Ackerveken (2013), they can be grouped in three classes. Genes of class I allow a basic compatibility, attracting

the pathogen and allowing its penetration. Genes of class II suppress the plant immune signalling. Genes in class III are involved in the sustained compatibility, providing pathogen accommodation and meeting its metabolic and structural needs.

In the context of powdery mildews, a representative of the class I S-genes is the maize (*Zea mays*) *GLOSSY1* (GL1). Spores of *Bgh* do not germinate, and appressoria do not develop on leaves of this mutant because it lacks long chains of aldehydes normally present in the waxy cuticle (Hansjakob et al., 2011). This cuticle compound is, therefore, particularly important for the successful germination and differentiation of the PM pathogen *Bgh*.

Once the pre-penetration process is completed, the next barrier to overcome is the cell wall. One of the best examples of S-genes required for powdery mildew penetration is the *MLO (Mildew Locus O)* gene. Like the MLO, a number of proteins encoded by other S-genes are involved in the penetration phase. Lifeguard (LFG) proteins, among which the BAX-inhibitor 1 (BI1), but also RAC/ROP G-proteins and GAP, are related to vesicle trafficking and focal orientation of the cytoskeleton towards the infection site (Eichmann et al., 2010; Hückelhoven, et al., 2003; Opalski et al., 2005; Schmidt et al., 2014; Schultheiss et al., 2003; Weis et al., 2013). Probably these proteins help the invagination of the cell membrane, and the accommodation of the fungal haustorium. Interestingly, these genes confer susceptibility, not only towards adapted pathogens, among which powdery mildews, but also resistance towards non-adapted biotrophic and necrotrophic fungi because they cannot bypass the first layer of induced defences.

Once the haustorium is developed, the pathogen needs to maintain the interaction with the host. At this point, other S-genes (class II and III) are involved to ensure a long-lasting intimate relationship with the PM pathogen by suppressing the plant immune responses and providing nutrients for the pathogen's growth. One of these genes is *DMR1*. It encodes a homoserine kinase which is an enzyme involved in the biosynthesis of the amino acids methionine, threonine, and isoleucine. They represent presumably essential metabolites for the sustainment of the pathogen and for the plant itself (Huibers et al., 2013). *PMR4* encodes a callose synthase which negatively regulates the salicylic acid (SA) pathway and may suppress MTI by preventing the accumulation of callose precursors (Ellinger et al., 2013; Nishimura et al., 2003; Huibers et al., 2013). In addition to *PMR4*, the *CESA3* (cellulose synthases) gene may function as a suppressor of the MTI by negatively regulating jasmonic acid (JA) and ethylene (ET) (Ellis & Turner, 2001). Finally, *EDR1* encodes an MAPK kinase-kinase which is a negative regulator of multiple defence responses leading to the MTI (Frye & Innes, 1998; Frye et al., 2001; Gao et al., 2015).

#### Turning susceptibility into a winning factor

S-genes are interesting because, if mutated, they can be turned into powerful resistance factors. For all the S-genes mentioned above, there are mutants or silenced transgenic plants that are resistant to the powdery mildew disease and in some cases also to other diseases. Indeed, one of the powerful effects of the loss-of-function of S-genes is a broad-spectrum resistance against multiple isolates of the same pathogen, but, in some cases, also against different pathogens.

Moreover, contrary to the use of R-genes, the resistance offered by the S-genes is expected to be durable. The introduction of R-genes in cultivated crops represents a longstanding procedure. However, the selective pressure on the pathogen, conferred by the race-specificity of the R-gene, is high. A mutation appearing in the effector is sufficient to avoid the recognition of the R-gene, making the deployed resistance ineffective. Therefore, the resistance conferred by the R-genes is rarely durable. Overcoming the S-gene resistance, on the other hand, implies that the pathogen acquires a new way to infect the host. It is highly improbable for this to happen, also considering how much powdery mildews, as biotroph, are dependent on host factors. The *MLO* and eukaryotic initiator factor 4E (*elF4E*) genes represent well-known examples of durable S-gene resistance. The *mlo*-based resistance was discovered in the 1940's in barley (Lundqvist 1991). It is active against all known isolates of barley PM *Bgh* (Jørgensen, 1992). The *pvr*-based resistance, due to the loss-of-function of *elF4E*, was described in pepper in the 1960's to be effective against potyvirus (Cook, 1960). Since their discovery, *mlo* and *pvr* resistances have been commercially used with great success (Brown, 2015; Moury & Verdin, 2012).

Effectiveness regarding duration and spectrum of action are features that have attracted a lot of attention from public and private sectors. Consequently, the use of impaired S-genes is considered now an alternative and, possibly more successful breeding strategy than the classical use of R-genes (Pavan et al., 2010).

So, how to apply S-genes into a breeding program? The first step consists of searching for homologs in the crop species of interest. Much research has been performed to screen Arabidopsis mutant collections and natural accessions in search for resistant phenotypes towards several pathogens. Therefore, many S-genes are described in Arabidopsis. Nevertheless, the availability of genome sequence information of many important crop species allows the identification of the corresponding homologs.

If the desired S-gene exists in the crop species of interest, the second step is to verify if it is functional towards corresponding crop pathogens. Constitutive gene silencing by RNA interference (RNAi), knock-out expression by cleavage of targeted DNA sequences using sequence-specific nucleases, or overexpression of the candidate S-gene to verify its ability to restore the susceptibility of a resistant mutant, are suitable options in crop species amenable to transformation. Transient gene silencing through viral vectors is an alternative for crops that are recalcitrant to transformation.

If the function is, indeed, conserved, it is important to verify that there are no pleiotropic effects associated to the loss-of-function of the S-gene of interest. Negative effects of the disruption of S-gene function consist of dwarfing, induced lesions, senescence, susceptibility to other pathogens, reduced root colonization by mycorrhizal fungi and lower fertility (Jarosch et al., 1999; Jørgensen, 1992; Kessler et al., 2010; Kumar et al., 2001; Lorrain et al., 2003; Poraty-Gavra et al., 2013; Ruiz-Lonzano et al., 1999; Zheng et al., 2013). At this point, the possible use of the S-gene depends on a careful consideration of benefits and drawbacks.

An elegant example of the application of this strategy is given by Huibers (2013). The authors describe the identification of two Arabidopsis homologs in tomato, the callose synthase *SIPMR4*, and the homoserine kinase *SIDMR1*. Upon RNAi silencing of both genes, tomato plants became resistant to the PM disease. However, silenced *SIDMR1* plants suffered from reduced growth, while silenced *SIPMR4* plants did not present any pleiotropic effect.

If the use of the S-gene is promising, a stable mutant can be obtained through different strategies. The first strategy consists of finding natural mutations among wild relatives of the crop of interest, then crossing with the cultivated species, and performing extensive backcrossing programs. Alternatively, mutations can be induced through chemical or physical mutagens. Afterwards, a screening of large populations to identify mutants with desirable properties is needed. Another option involves targeted knockdown of the gene of interest via RNAi or knock-out by genome editing tools like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR-Cas9 system (Pavletich & Pabo, 1991; Joung & Sander, 2013; Liu & Fan, 2014; Urnov et al., 2010). The latter has been successfully and fast adopted by the scientific community because its application is simpler, less costly and more efficient compared to the former technologies (Liang et al., Zhang, 2014). Moreover, there is confidence that in the future the plants obtained with this editing tool are considered non-transgenic by the European regulatory office (Bortesi & Fischer, 2015).

One final consideration concerns the possibility that the susceptibility factor is encoded by multiple (redundant) genes of a gene family. In this case, it is necessary to evaluate the effect of each homolog on the phenotype and eventually target multiple genes to obtain a complete loss-of-function mutant. The CRISPR/Cas 9 system can be particularly useful to knock out redundant genes (Wang et al., 2014).

#### Molecular components of the mlo-based resistance

The *MLO* gene is a well-known example of S-genes. It encodes a plant-specific protein which typically contains seven transmembrane domains embedded in the plasma membrane.

Disease resistance is characterized by early termination of fungal infection due to failed host cell invasion. The typical histological feature is the occurrence of papillae deposited at the sites of attempted penetration.

The *mlo*-based resistance relies on two pathways, one dependent and the other independent from the actin of the cytoskeleton (Miklis et al., 2007). The molecular components of the actin-dependent pathway are conserved among the plant species investigated, namely barley, Arabidopsis, and grapevine (Consonni et al., 2006; Feechan et al., 2013; Panstruga, 2005). They participate in two distinct secretory systems. In one, a ternary complex is formed between a plasma membrane protein AtPEN1/HvRor2 and two other SNARE proteins to allow vesicle fusion (Collins et al., 2003; Freialdenhoven et al., 1996). In the second secretory system, toxic compounds produced by the glycosyl hydrolase PEN2 are transported across the plasma membrane by a PEN3 transporter (Lipka et al., 2005).

The three PENETRATION (PEN) genes were identified by screening Arabidopsis mutants with increased penetration success of the non-adapted barley powdery mildew *Bgh* (Collins et al., 2003). Later, all three genes have also been found to be required for *mlo*-based powdery mildew resistance in Arabidopsis plants (Consonni et al., 2006). These facts support the hypothesis that non-host immunity and *mlo* resistance rely on functionally overlapping defence pathways (Humphry et al., 2006). Moreover, they are both independent from SA, JA, and ET and share the same histological mechanism of defence response (Ellis, 2006; Trujillo et al., 2004).

#### The charm of the MLO genes dwells in their mystery

With the availability of genomic information, it was found that multiple copies of the *MLO* gene are present in the genome of a plant species. A recent evolutionary study, which reconstructed the likely evolution of the *MLO* family in embryophytes, traced back MLO-like proteins not only to plants but also to unicellular eukaryotes, like green and red algae (Ellis, 2006; Kusch et al., 2016; Trujillo et al., 2004). The number of homologs depends on the plant species investigated. However, in general, the *MLO* gene families of land plants range from small (seven members) to medium size (39 members) (Acevedo-Garcia et al., 2014; Kush et al., 2016). The phylogenetic analyses of the *MLO* gene families resulted in the distinction of seven clades, although not all plant species harbor representatives of all clades (Kusch et al., 2016).

*MLO* genes in Clade IV and V act as powdery mildew susceptibility factors of monocot and dicot species, respectively. In Arabidopsis three of its 15 *MLO* homologs are involved in PM resistance, although *AtMLO2* has a larger effect compared to the other two genes, *AtMLO6* and *AtMLO12* (Consonni et al., 2006).

Studies on the pepper Clade V-*CaMLO2* indicate that this gene is involved in modulating biotic responses not only to powdery mildew but also to bacterial and oomycete pathogens like *Xanthomonas campestris* pv *vesicatoria*, *Pseudomonas syringae* pv *tomato* and *Hyaloperonospora arabidopsis* (Zheng et al., 2013; Kim & Hwang, 2012). Additionally, this gene is a negative regulator of ABA signaling, implicating *CaMLO2* involvement in drought stress responses (Lim & Lee, 2014).

Other Clades harbor *MLO* genes associated with two distinct developmental processes. Arabidopsis null mutants *Atmlo4* and *Atmlo11* show aberrant root thigmomorphogenesis, which is exemplified as unusual root curling upon a tactile stimulus under *in vitro* conditions (Bidzinski et al., 2014; Chen et al., 2009). Both *AtMLO4* and *AtMLO11* are grouped into Clade I.

Another Arabidopsis mutant called *nortia*, which carries a 20-bp deletion in the *AtMLO7* gene, shows reduced fertility and pollen tube overgrowth in the synergid cells (Kessler et al., 2010). These cells, present in the mature embryo sac, guide pollen tubes towards the double fertilization process in angiosperms. The *AtMLO7* belongs to Clade III. The involvement of the remaining *AtMLO* genes in any other processes is still unknown.

In conclusion, MLO proteins seem to be involved in many biological processes, although their core biochemical activity is not known yet. Based on topology and subcellular localization, the MLO proteins resemble G protein-coupled receptors (GPCRs). However, no experimental evidence supports the hypothesis that MLO proteins function as a GPCRs (Kim et al., 2002). A conserved peptide motif is represented by the intracellular calmodulin-binding domain (CaMBD) which interacts *in vitro* with calmodulin (CaM) in a Ca<sup>2+</sup>-dependent manner (Kim et al., 2002). Nevertheless, the precise mechanism of the modulation of CaM by MLO proteins is not clear yet. In an experiment, aimed at investigating genes co-expressed with barley *HvMlo* and Arabidopsis *AtMLO2*, a considerable number of receptor-like kinases (RLK) was found (Humphry et al., 2010). This finding might link MLO proteins to other components of plant immunity, but it requires further experimental evidence to explain how they are interconnected in a functional way.

#### Scope of the thesis

This work is part of a line of studies investigating *mlo*-based resistance in plants, and particularly in crop species.

After the characterization in tomato of *ol-2* as a loss-of-function allele of the *SIMLO1* gene (Bai et al., 2008), one part of the research described in this thesis focused only on *MLO* genes in tomato and on components of the tomato *mlo*-based resistance; the other part on the investigation of *MLO* genes from difference species.

In **Chapter 2**, we used tomato sequence information to complete the characterization of the *SIMLO* homologs. Particular attention was given to the Clade V members of the tomato *MLO* phylogenetic tree. The outcome showed that the simultaneous silencing of *SIMLO1* and two of its closely related homologs confers a higher resistance level than the one obtained by using the *ol-2* allele alone. In **Chapter 3**, we investigated the role of the *SIPEN1*-like genes as factors required for the *mlo*-based resistance in host and non-host interactions strengthening the assumption that "non-host and *mlo*-based resistance may be the two faces of the same coin" (Humphry et al., 2006).

The second line of research was inspired by the fact that *MLO* susceptibility genes are conserved across plant species and therefore can be used in breeding programs if 1) identified and 2) natural or induced mutations are found. In **Chapter 4**, we identified and functionally characterized the orthologs of *SIMLO1* in tobacco and eggplant, two other important Solanaceae crops. We named the newly identified genes *NtMLO1* and *SmMLO1*, in the respective species. We also verified that a Q198R mutation in the protein sequence of NtMLO1 leads to complete loss of function, enlarging the list of point mutations with a detrimental effect on the MLO proteins.

In **Chapter 5**, we proved that despite phylogenetic distance and evolution of different molecular features, monocot and dicot MLO proteins involved in the interaction with powdery mildews are functionally conserved. Also, we confirmed that functional monocot and dicot MLO proteins are both recruited by adapted and non-adapted powdery mildew pathogens during the early phase of the infection, allowing them to penetrate the cell wall. Finally, we set up a suitable complementation assay to validate the function of putative susceptibility *MLO* genes in new species.

In **Chapter 6**, we characterized the resistance observed in an advanced cucumber breeding line due to the insertion of a transposable element (TE) in the CDS of the *CsaMLO8* gene. We verified that this disrupted allele is not functional, contrary to the wild-type *CsaMLO8* allele. Also, we showed that the TE-containing allele represents a natural mutation of the functional *CsaMLO8* because it is present also in a wild cucumber accession.

Finally, in **Chapter 7**, we describe the in-house development of an EMS Micro-Tom population aimed at finding new sources of resistance to different pathogens. Regarding powdery mildew, we discovered a new loss-of-function allele of the *SIMLO1* gene, named *m200*, which confers a higher resistance level than the one provided by the *ol-2* mutant allele. A macroscopic and microscopic comparison between plants carrying these alleles hints at the influence of the genetic background on the resistance level observed. If this is verified, it becomes an important factor to consider in breeding programs.

Based on the results obtained, in **Chapter 8**, I summarize our findings and discuss their implications for a successful use of the *mlo*-based resistance in breeding programs. Furthermore, I discuss the importance of this study as exemplary work to apply also to other S-genes and hopefully achieve a durable broad-spectrum resistance without any drawbacks on other important plant traits.

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# Chapter 2

### Genome-wide study of the tomato SIMLO gene family and its functional characterization in response to the powdery mildew fungus Oidium neolycopersici

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#### Abstract

The *MLO* (*Mildew Locus O*) gene family encodes plant-specific proteins containing seven transmembrane domains and likely acting in signal transduction in a calcium and calmodulin dependent manner. Some members of the *MLO* family are susceptibility factors towards fungi causing the powdery mildew disease. In tomato, for example, the loss-of-function of the *MLO* gene *SIMLO1* leads to a particular form of powdery mildew resistance, called *ol-2*, which arrests almost completely fungal penetration. This type of penetration resistance is characterized by the apposition of papillae at the sites of plant-pathogen interaction. Other *MLO* homologs in Arabidopsis regulate root response to mechanical stimuli (*AtMLO4* and *AtMLO11*) and pollen tube reception by the female gametophyte (*AtMLO7*). However, the role of most *MLO* genes remains unknown.

In this work, we provide a genome-wide study of the tomato *SIMLO* gene family. Besides *SIMLO1*, other fifteen *SIMLO* homologs were identified and characterized with respect to their structure, genomic organization, phylogenetic relationship, and expression profile. In addition, by analysis of transgenic plants, we demonstrated that simultaneous silencing of *SIMLO1* and two of its closely related homologs, *SIMLO5* and *SIMLO8*, confer higher level of resistance than the one associated with the *ol-2* mutation.

The outcome of this study provides evidence for functional redundancy among tomato homolog genes involved in powdery mildew susceptibility. Moreover, we developed a series of transgenic lines silenced for individual *SIMLO* homologs, which lay the foundation for further investigations aimed at assigning new biological functions to the *MLO* gene family.

#### Keywords

MLO gene family, tomato, susceptibility, powdery mildew disease

# Introduction

Many important crop species can be affected by the powdery mildew (PM) disease, resulting in great yield losses in agricultural settings. In barley, recessive loss-of-function mutations occurring in the *HvMLO* (*Hordeum vulgare Mildew Resistance Locus Q*) gene confer resistance to all known isolates of the PM fungus *Blumeria graminis* f.sp *hordei*. Therefore, natural or induced *mlo*-mutant alleles are in use for about seven decades to introduce resistance in spring barley breeding programs (Jørgensen, 1992; Büschges et al., 1997; Reinstädler et al., 2010).

Biochemical analysis showed that the barley HvMLO protein contains seven transmembrane domains integral to the plasma membrane, with an extracellular aminoterminus and an intracellular carboxy-terminus. The latter harbours a calmodulin-binding domain likely involved in sensing calcium influxes into cells (Devoto et al., 1999). Although the domain structure of MLO proteins is related to that of metazoan G-protein coupled receptors (GPCRs), several studies could not confirm the role of MLO proteins as canonical GPCRs (Kim et al., 2002; Lorek et al., 2013). Despite further intensive efforts to explain the biochemical function of the HvMLO protein, its core activity remains elusive (Panstruga, 2005). However, HvMLO might be exploited by the fungus to impair vesicle-associated defence mechanism at plant-pathogen interaction sites, thus facilitating its penetration (Panstruga and Schulze-Lefert, 2003; Opalski et al., 2005; Miklis et al., 2007). This feature makes *HvMLO* a typical representative of susceptibility genes (*S*-genes) (Miklis et al., 2007; van Schie and Takken 2014).

The robustness of barley *mlo*-resistance, due to its non-race-specific spectrum and durability, led in the last years to an extensive quest for identification and functional characterization of the *MLO* genes in other species affected by the PM disease. The search resulted in the identification of multiple *MLO* gene families, ranging from 12 to 39 members in Arabidopsis, rice, grapevine, cucumber, apple, peach, woodland strawberry, tobacco and soybean (Devoto et al., 2003; Feechan et al., 2008; Liu and Zhu, 2008; Shen et al., 2012; Zhou et al., 2013; Pessina et al., 2014; Appiano et al., 2015). Moreover, specific homologs were shown to play a major role in plant-pathogen interactions (Consonni et al., 2006).

A detailed phylogenetic analysis distinguished up to eight clades in which Angiosperm MLO proteins can be found (Feechan et al., 2008; Acevedo-Garcia et al., 2014; Pessina et al., 2014). The MLO homologs involved in the interaction with PM pathogens (Arabidopsis AtMLO2, AtMLO6, AtMLO12, tomato SIMLO1, pea Er1/PsMLO1, grapevine VvMLO3 and VvMLO4, tobacco NtMLO1, pepper CaMLO2, cucumber CsaMLO8, *Lotus japonicus* LjMLO1 and barrel clover MtMLO1) are grouped into clade V. On the other hand, all the known monocot MLO homologs acting as susceptibility factors (barley HvMLO, rice OsMLO3,

and wheat TaMLO\_A1 and TaMLO\_B1) do not cluster in clade V, but in clade IV, which is primarily but not exclusively represented by monocot MLO proteins. For example, grapevine VvMLO14, strawberry FvMLO17 and peach PpMLO12 belong also to clade IV (Elliott et al., 2002; Feechan et al., 2008; Acevedo-Garcia et al., 2014; Pessina et al., 2014).

In Arabidopsis, the PM resistance conferred by the loss-of-function of *AtMLO2* is incomplete and only mutations in all the three *AtMLO* homologs in clade V can completely prevent fungal entry (Consonni et al., 2006). In addition, more recent studies in Arabidopsis indicated that other members of the *MLO* gene family play a role in different biological processes. The homologs *AtMLO4* and *AtMLO11* are together involved in root thigmomorphogenesis, i.e. root responses to mechanical stimuli (Chen et al., 2009), while *AtMLO7* regulates pollen tube reception from the synergid cells during fertilization (Kessler et al., 2010). The biological roles of other *MLO* homologs still remain elusive.

Tomato (*Solanum lycopersicum*) is one of the most economically important vegetables in the world. It can be host of three PM species, namely *Oidium neolycopersici*, *Oidium lycopersici* and *Leveillula taurica* (Seifi et al., 2014). Since 1996, when it was found that all the tomato cultivars were susceptible to *O. neolycopersici*, extensive researches were conducted by our group for sources of resistance (Seifi et al., 2014). An allele containing a 19 bp deletion in the coding region of the PM susceptibility gene *SIMLO1* was found in a wild accession of *S. lycopersicum* var. *cerasiforme*. This mutant allele, named *ol-2*, was shown to confer recessively inherited broad-spectrum resistance to a series of isolates of *O. neolycopersici* (Bai et al., 2008). Through histological analysis, it was shown that its mechanism of resistance is based on the early abortion of fungal pathogenesis at the sites of attempted penetration (Bai et al., 2005). This type of penetration resistance is characterized by papillae apposition, the same as described also for the PM resistance in the *Atmlo2* mutant of Arabidopsis (Consonni et al., 2006). Although papilla formation can significantly reduce fungal development at the host cell entry level, fungal penetration was not fully prevented in the *ol-2* mutant (Bai et al., 2005).

In this study, we exploited tomato sequence information, derived from the tomato genome sequencing Heinz 1706 and the 150 tomato genome resequencing projects (Tomato Genome Consortium et al., 2012; The 100 Tomato Genome Sequencing Consortium et al., 2014), in order to identify tomato *MLO* homologs (*SIMLO*). These were characterized with respect to 1) their genomic organization, 2) relation with *MLO* homologs from other species, 3) occurrence of tissue-specific differentially spliced variants, 4) expression in different tissues in axenic condition, and 5) upon inoculation with the powdery mildew pathogen *O. neolycopersici*. Finally, an RNAi-based reverse genetic approach was followed to investigate the possibility that *SIMLO* homologs other than *SIMLO1* could play additional roles in the interaction with *O. neolycopersici*.

# Results

#### In silico identification and sequencing of the tomato SIMLO gene family

A total of 17 tomato *MLO*-like loci were identified through BLAST interrogation of the tomato genomic sequence database (SGN), using AtMLO protein sequences as query. Two of them (referred to as Solyc09g18830 and Solyc09g18840 in the SGN database) were noticeably shorter than other predicted *MLO* homologs and physically close to each other, suggesting they are different parts of the same gene (Table 1). Search in the tomato EST database and gene prediction analysis in the *S. pimpinellifolium* genome with the FGENESH software allowed identifying a hypothetical full-length *MLO* transcript encompassing Solyc09g18830 and Solyc09g18840. PCR from leaf of the tomato cultivar Moneymaker (MM) confirmed the presence of this transcript, which was named *SIMLO7* (Supplementary Fig. 1). The other 15 predicted *SIMLO* genes were named from *SIMLO1* to *SIMLO6*, and from *SIMLO8* to *SIMLO16*, as reported in Table 1. For all of them, information is available with respect to putative amino acid length and number of introns.

With the exception of *SIMLO4*, information on chromosomal localization could also be inferred (Table 1). Most *SIMLO* homologs are scattered throughout the tomato genome, thus suggesting that segmental duplication events have been a major source for the evolution of the *SIMLO* gene family. Exceptions are represented by two physical gene clusters, one containing *SIMLO6*, *SIMLO10* and *SIMLO15* on chromosome 2, and the other containing *SIMLO3* and *SIMLO16* on chromosome 6.

Sequence and expression of all the predicted *SIMLO* homologs were verified by PCR amplification of cDNAs derived from four different tissues (leaf, root, flower and ripened fruit) of MM. All the *SIMLO* homologs could be amplified at least from one plant tissue. In total, 15 *SIMLO* homologs could be cloned from leaf (with the exception of *SIMLO12*), 10 from flower, 9 from fruit and 8 from root (Supplementary table 1).

Sequence alignment of cloned *SIMLO* transcripts with corresponding SGN predicted coding sequence (CDS), derived from the cultivar Heinz 1706, revealed polymorphisms for *SIMLO7*, *SIMLO8*, *SIMLO10* and *SIMLO15* (Supplementary Fig. 1). The 1339 bp *SIMLO7* cloned transcript corresponds to a short open reading frame (ORF) due to a stop codon at 137-139 bp (Supplementary Fig. 1). The SGN predicted CDS of *SIMLO8* misses part of the third, seventh, eighth and ninth exon present in the corresponding transcript cloned from MM leaf; compared to the SGN predicted CDS of *SIMLO10*, transcript cloned from MM fruit contains a base change at the beginning of the fifth exon, which results in a stop codon (Supplementary Fig. 1). Also the predicted ORF of *SIMLO15* is shorter (375 aa) than the average ORF length of other SIMLOS (Table 1). The sequence cloned from MM leaf has a longer ORF (459 aa) compared to the predicted SGN sequence (Table 3a).

SGN locus name	<i>MLO</i> gene	Chromosome	Position	ORF lenght (aa)	Introns
Solyc04g049090	SIMLO1	4	SL2.40ch04:3870044538705951	507	14
Solyc08g015870	SIMLO2	8	SL2.40ch08:60740406078983	504	13
Solyc06g010030	SIMLO3	6	SL2.40ch06:47867644792828	591	14
Solyc00g007200	SIMLO4	2?	SL2.40ch00:68168926823417	554	14
Solyc03g095650	SIMLO5	3	SL2.40ch03:5027991950288063	517	14
Solyc02g082430	SIMLO6	2	SL2.40ch02:4069460840700995	553	14
Solyc09g018830 Solyc09g018840	SIMLO7	9	SL2.40ch09:1756455517568214	270	10
Solyc11g069220	SIMLO8	11	SL2.40ch11:5093953350946726	506	13
Solyc06g082820	SIMLO9	6	SL2.40ch06:4477967344784035	511	13
Solyc02g083720	SIMLO10	2	SL2.40ch02:4159647441602413	533	14
Solyc01g102520	SIMLO11	1	SL2.40ch01:8307186083075439	475	13
Solyc08g067760	SIMLO12	8	SL2.40ch08:5395706253962884	532	14
Solyc10g044510	SIMLO13	10	SL2.40ch10:2212886822135940	558	14
Solyc07g063260	SIMLO14	7	SL2.40ch07:6299534563002900	563	14
Solyc02g077570	SIMLO15	2	SL2.40ch02:3704509437050486	375	10
Solyc06g010010	SIMLO16	6	SL2.40ch06:46995524706571	477	14

TABLE 1. Features of the SIMLO gene family as inferred by the Sol Genomics Network Database.

In other cases, sequence alignments of cloned *SIMLO* from the different tissues with their corresponding genomic regions showed various types of splice variants, consisting of intron retention, exon skipping and alternative 5' and 3' splice sites, according to the types of alternative splicing described by Keren et al., 2010 (Table 2 and Supplementary Fig. 1).

TABLE 2. Types of differentially spliced events observed in cloned *SIMLO* homologs from different tissues of the tomato cv Moneymaker.

SIMLO	Plant tissue	Type of alternative splicing							
SINILO			Exon skipping	Alternative 5' splice site	Alternative 3' splice site				
SIMLO1	flower								
SIMLO5*	fruit								
SIMLO6	leaf			$\checkmark$	$\checkmark$				
SIMLO9	leaf		$\checkmark$						
SIMLO11*	root								
SIMLO13	leaf		$\checkmark$		$\checkmark$				
SIMLO15	fruit		$\checkmark$	$\checkmark$					
SIMLO15*	root		$\checkmark$	$\checkmark$					
SIMLO15*	flower		$\checkmark$						

The asterisk (\*) indicates SIMLO transcripts that can be either incompletely spliced or alternatively spliced

TABLE 3A. Features and motifs distribution occurring in SIMLO proteins obtained from *in silico* translation of leaf, root, flower and fruit transcripts of the tomato cv Moneymaker. When no deviating transcripts are present for one *SIMLO*, the one from leaf has been used for motif analysis. Cells highlighted in grey indicate the absence of the corresponding motif.

		ORF LENGTH (aa)	MOTIF 1	MOTIF 2	MOTIF 3	MOTIF 4	MOTIF 5	MOTIF 6	MOTIF 7	MOTIF 8	MOTIF 9	MOTIF 10
									Σ	Σ	Σ	ž
	LEAF	507	√	V	V	V	V	V				
SIMLO1	ROOT	507	V	V	V		V	$\checkmark$				
	FLOWER	491	√	√	√	√	√					
SIMLO2	LEAF	504	√			√						
SIMLO3	LEAF	591	√					√			1	
SIMLO4	LEAF	554	√	√	√	√		√	√	√		
	LEAF	517	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$		V				
SIMLO5	FLOWER	517	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$		$\checkmark$				
	FRUIT	540							_			
	LEAF	549	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$						
SIMLO6	ROOT	553	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$		$\checkmark$		$\checkmark$		
5111200	FLOWER	553	$\checkmark$	$\checkmark$		$\checkmark$				$\checkmark$		
	FRUIT	553			$\checkmark$							
SIML07	LEAF	61					_					
SIMLO8	LEAF	561										
	LEAF	448	$\checkmark$			$\checkmark$		$\checkmark$		$\checkmark$		
SIMLO9	FLOWER	511	$\checkmark$	$\checkmark$		$\checkmark$						
	FRUIT	511										
	LEAF	533	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$		$\checkmark$			$\checkmark$	
SIMLO10	ROOT	533	$\checkmark$	$\checkmark$		$\checkmark$					$\checkmark$	
SINILOTO	FLOWER	533	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$			$\checkmark$	
	FRUIT	178		$\checkmark$							$\checkmark$	
	LEAF	475	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$		$\checkmark$		
SIMLO11	ROOT	70										
SINILOTT	FLOWER	475	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$		$\checkmark$		
	FRUIT	475	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$		$\checkmark$		
SIMLO12	FLOWER	532	$\checkmark$	$\checkmark$		$\checkmark$						
	LEAF	63	$\checkmark$									
SIMLO13	ROOT	558	$\checkmark$	$\checkmark$		$\checkmark$		$\checkmark$			$\checkmark$	$\checkmark$
SINILOTS	FLOWER	558	$\checkmark$	$\checkmark$		$\checkmark$		$\checkmark$			$\checkmark$	$\checkmark$
	FRUIT	558	$\checkmark$	$\checkmark$		$\checkmark$		$\checkmark$				$\checkmark$
SIMLO14	LEAF	563		$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$					
	LEAF	459	$\checkmark$				$\checkmark$			$\checkmark$		
	ROOT	56										
SIMLO15	FLOWER	70										
	FRUIT	84								$\checkmark$		
SIMLO16	LEAF	477			$\checkmark$							

	SEQUENCE CONSENSUS	WIDTH	e-VALUE	LOCATION
MOTIF 1	NAFQMAFFFWIWWEYGWKSCFWDNFIPIIIRLVMGVKVQVWCSYMTLPLYARVTQM	56	6.5e-1021	TM6
MOTIF 2	PTWAVAMVCAVIVAISIFIERIIHKLGKWLKKKNKKALYEALEKIKEELMLLGFISLLLTVCQDYISQIC	70	1.5e-1076	TM1
MOTIF 3	LLWIVCFFRQFYRSVNKSDYLTLRHGFIMAHCAPNNYNNFDYYMYRMREDDFDF	54	3.9e-840	2 IC
MOTIF 4	EGKVPFASYEALHQLHIFIFVLAVAHVLYCCTTMWLGMAKMRQWRAWEDETKT	53	6.7e-823	TM3
MOTIF 5	VGISWYLWIFVVLCLLLNINGWHSYFWIPFFPLILILLVGTKLEHIITQMAVEIAE	56	1.0e-402	TM5
<b>MOTIF 6</b>	GSTMKKSIFDENVRDALRKWHMTVKKRKKHKYDRSNTTRSNCPACSMAMDGPNHP	55	8.8e-386	CaMBD
<b>MOTIF7</b>	HRYKTTGHSSRFQGYSDQEASDLENDPTTPMTRAEIATTHIDHDDTEIHVHIPQNGESTRNEDDFSFVKP	70	2.50E-178	C-term
MOTIF 8	PPNVADTMLPCPPNNKDQAKEEEHCRHLGWYERRHLACNE	40	6.30E-149	2 EC
<b>MOTIF9</b>	VNSSAVSSHFYPCSPPDNDMKSAITRDAIHGSSYSNHSTS	40	1.90E-114	2 EC
<b>MOTIF 10</b>	SPCSSRGSFNHLDEKVLSNDHQEDCIVETTNQPGHELSFRNSEVLVTDAEEIVDDEADKIETLFELFQKT	70	2.80E-89	C-term

TABLE 3B. Features details of the consensus motifs reported in Table 3a as predicted by the MEME software package (http://meme-suite.org/tools/meme). For each motif, the MEME e-value for significance and the position of each motif in one of the MLO protein domains (transmembrane –TM-, extracellular –EC-, intracellular

### Characterization of conserved amino acids and motifs of the SIMLO proteins

To examine sequence features of the tomato SIMLO proteins, a multiple sequence alignment was performed using sequences obtained by the conceptual translation of transcripts cloned in different tissues. When no deviating transcripts were observed for a *SIMLO* gene, the sequence obtained from leaf was used for translation, with the exception of *SIMLO12* which is the only homolog that was not cloned from leaf but from flower.

The aligned amino acid sequences of the tomato SIMLO protein family showed a high degree of conservation (92%) of the 30 amino acid residues previously described to be invariable throughout the whole MLO protein family (Supplementary Fig. 2) (Elliott et al., 2005).

Due to aberrant transcripts, the protein sequences of SIMLO7 and SIMLO13 in leaf, SIMLO11 in root, and SIMLO15 in root, flower and fruit, were severely truncated (Table 3a). The predicted ORF of *SIMLO8* in leaf was longer than the one deriving from the SGN prediction, which is missing important domains of the MLO protein. The protein sequence of *SIMLO9* in leaf was shorter (448 aa length) than the ones obtained from the other two tissues (512 aa length) and it is predicted to have five transmembrane (TM) domains, instead of seven as in fruit and flower (Table 3a).

Finally, the SIMLO protein family was also used as input to search for conserved motifs. Ten patterns of consecutive amino acids, having a length ranging from 40 to 70 and shared by at least three MLO sequences (Table 3b), were found. Interestingly, four of these motifs included transmembrane domains, while the others were located in the second intracellular and extracellular domains, in the C-terminus and in the calmodulin-binding domain. The motifs seven and nine were shared only by SIMLO4/SIMLO6 and SIMLO10/SIMLO13 respectively while the motif ten was only present in the amino acid sequences of SIMLO13 of root, flower and fruit. Those motifs might indicate regions of peculiar importance for the specific function of these homologs.

# Phylogenetic analysis of the tomato SIMLO protein family

A phylogenetic analysis was carried out in order to establish the relationships between SIMLO proteins and MLO proteins of other plant species (Arabidopsis AtMLO1-15, pea PsMLO1, *Lotus japonicus* LjMLO1, barrel clover MtMLO1, pepper CaMLO2, tobacco NtMLO1, cucumber CsaMLO8, apple MdMLO18 and MdMLO20, strawberry FvMLO13 and FvMLO15, peach PpMLO9 and PpMLO13, barley HvMLO, rice OsMLO2 and wheat TaMLOA1b and TaMLOB1a). The resulting tree contains eight different clades (Fig. 1). These were named by Roman numerals from I to VIII, in accordance with previous studies performing phylogenetic analysis on the Arabidopsis and apple MLO protein families (Devoto et al., 2003; Pessina et al., 2014).

Five clades, namely clade I, II, III, V and VI, contain both tomato and Arabidopsis homologs; clade IV contains only monocot MLO homologs that were selected for this study; clade VII contains only SIMLO15 together with apple, peach and strawberry MLO proteins (MdMLO18, PpMLO9 and FvMLO15, respectively). No SIMLO homologs could be assigned to clade VIII, which only contains Rosaceae MLO homologs (Fig. 1).

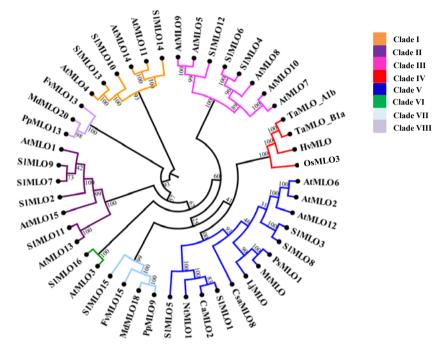


FIGURE 1. Rooted circular cladogram showing the phylogenetic relationships of the tomato SIMLO proteins. A UPGMA-based tree comprises all the sequences of the MLO protein family of Arabidopsis (At) and tomato (SI). Individual sequences of apple (Md), tobacco (Nt), cucumber (Csa), pea (Ps), *Lotus japonicus* (Lj), barrel clover (Mt), pepper (Ca), barley (Hv), rice (Os) and wheat (Ta) are included. Numbers on each node represent bootstrap values based on 100 replicates. Phylogenetic clades are designated with colours and Roman numbers according to the position of AtMLO homologs and apple MdMLO, as indicated by Pessina et al., 2014. The tomato SIMLO protein sequences used for this tree derived all from the translation of the transcripts cloned from leaf of the cv. Moneymaker, except for SIMLO12, which corresponds to the translated sequence of flower. Accession numbers of the sequences used, other than tomato SIMLO, are listed in Supplementary Table 2.

Three tomato MLO homologs, SIMLO3, SIMLO5 and SIMLO8, cluster together with SIMLO1 in clade V, containing all the known eudicot MLO homologs functionally related to powdery mildew susceptibility (AtMLO2, AtMLO6, AtMLO12, PsMLO1, LjMLO1, MtMLO1, CsaMLO8, NtMLO1 and CaMLO2) (Fig. 1) (Elliott et al., 2005; Consonni et al., 2006; Bai et al., 2008; Pavan et al., 2009; Humphry et al., 2011; Várallyay et al., 2012; Zheng et al., 2013; Appiano et al., 2015; Berg et al., 2015).

The tomato homologs SIMLO4, SIMLO6, and SIMLO12 group in clade III together with AtMLO7, which regulates Arabidopsis pollen tube reception by the synergid cells, whereas SIMLO10, SIMLO13 and SIMLO14 are the closest tomato homologs to the root thigmomorphogenesis regulating proteins AtMLO4 and AtMLO11 in clade I (Fig. 1).

Finally, clade II includes four tomato SIMLO homologs (SIMLO2, SIMLO7, SIMLO9 and SIMLO11) together with three Arabidopsis proteins (AtMLO1, AtMLO13 and AtMLO15) and clade VI harbours only AtMLO3 and tomato SIMLO16 (Fig. 1).

# Expression profiles of *SIMLO* homologs in axenic conditions and upon powdery mildew challenge

The expression level of *SIMLO* genes was determined in four different tissues (leaf, root, flower and ripened fruit). These were found to vary considerably among *SIMLO* genes, and it was not possible to assign clade-specific expression patterns (Fig. 2). Concerning clade V, *SIMLO5* and *SIMLO8* were found to be characterized by very low expression levels in all the tissues. Interestingly, *SIMLO1* was found to be less expressed in leaves compared to flowers. Our results are supported by the collection of RNA-seq data, as shown by the FPKM (fragments per kilobase of exon per million fragments mapped) values for the four tissues under investigation of each homolog represented into graphs of Supplementary Fig. 3.

We investigated the expression profile of the *SIMLO* gene family in response to *O*. *neolycopersici*, using L33 as a reference gene (Fig. 3). *SIMLO1* expression significantly increased at six and ten hours after pathogen challenge. No other *SIMLO* homolog in clade V (*SIMLO3*, *SIMLO5*, *SIMLO8*) showed pathogen-dependent up-regulation.

On the other hand, a significant upregulation in response to *O. neolycopersici* was observed for *SIMLO* homologs outside clade V, namely *SIMLO2*, *SIMLO4*, *SIMLO7*, *SIMLO10*, *SIMLO13*, *SIMLO14* and *SIMLO16*. In particular, the expression of *SIMLO4* and *SIMLO14* at ten hours after inoculation was comparable to the one of *SIMLO1*, and ~4-fold and ~3-fold higher than the one of control plants, respectively.

Similar results were obtained repeating the expression analysis using Ef  $1\alpha$  as reference gene (Supplementary Fig. 4).

In order to confirm the strong up-regulation of the above mentioned genes, a second inoculation experiment was carried out, sampling leaf tissues at the same time points (0 hpi, 6 hpi and 10 hpi). The results presented in Supplementary Fig. 5 indicate that indeed *SIMLO1*, *SIMLO4* and *SIMLO14* show a statistically significant up-regulated expression due to the *O. neolycopersici* challenge. The slight down-regulated expression of *SIMLO3* observed after the first pathogen inoculation was not confirmed in the second experiment.

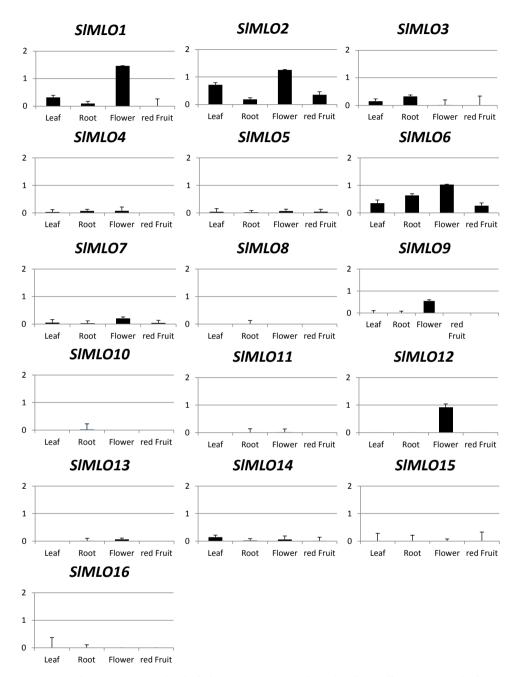


FIGURE 2. Relative expression level of *SIMLO* transcripts evaluated in four different tissues (leaf, root, flower and mature fruit) of the tomato cv. Moneymaker in axenic condition. The expression level of each gene is compared to the abundance of Ef1 $\alpha$  which was used as reference gene. Bars show standard errors based on three technical replicates. Similar trends are reported in Supplementary Fig. 3.

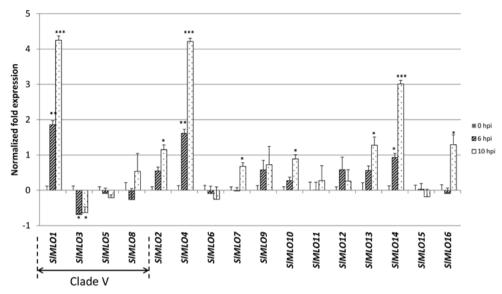


FIGURE 3. Relative expression level of the *SIMLO* gene family in response to *O. neolycopersici* inoculation. Samples were collected at 0, 6 and 10 hrs after inoculation (hpi). Transcript abundance of each *SIMLO* homolog was normalized against the transcription level of the 60S ribosomal protein L33 used as reference gene. Bars show standard errors based on four biological replicates. Asterisks refer to significant differences with respect to non-inoculated plants (0 hpi), inferred by mean comparisons with a Student's t-test (\* p < .05, \*\* p < .01, \*\*\* p < .001). The *SIMLO* genes harbored in clade V, based on the phylogenetic tree of Fig. 1, are indicated by an arrow spanning their corresponding bars. Similar results were obtained by using the elongation factor Ef1a as housekeeping gene (Supplementary Fig. 4)

# Functional characterization of clade V SIMLO homologs

Based on their relatedness with eudicot *MLO* homologs predisposing to PM susceptibility, including *SIMLO1*, the newly identified *SIMLO* homologs in clade V (*SIMLO3*, *SIMLO5* and *SIMLO8*, Fig. 1) were further investigated with respect to their role in the interaction with *O. neolycopersici*. Therefore, specific RNAi silencing constructs for these three homologs were developed, which were used to transform the susceptible cultivar Moneymaker (MM) (Supplementary Fig. 6 and Supplementary Table 3). A silencing construct targeting *SIMLO1* was included as control, which was expected to lead to a resistant phenotype.

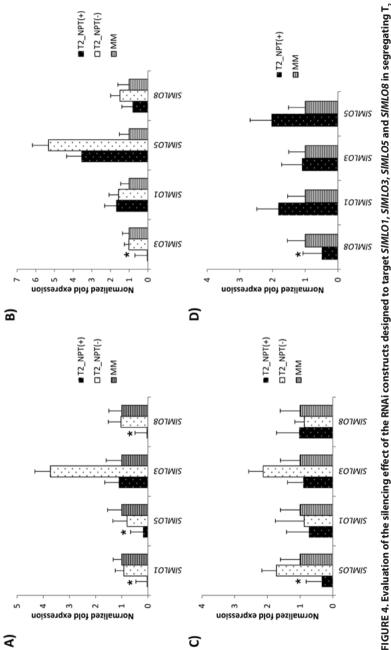
Ten to twenty  $T_1$  plants were obtained for each silencing construct. The expression of the target genes was assessed by means of real-time qPCR (Supplementary Fig. 7) and  $T_1$  plants with a reduced level of expression of the target gene were allowed to self-pollinate to develop  $T_2$  families. In total, two independent  $T_2$  families (each segregating for the presence of the silencing construct) were developed for *SIMLO1* and *SIMLO8*, and three were obtained for *SIMLO3* and *SIMLO5*. Transgenic individuals of each family were further assessed for the silencing levels of target genes and other clade V homologs. This revealed successful silencing of each target genes and no unwanted co-silencing in transgenic

RNAi::*SIMLO3*, *SIMLO5* and *SIMLO8* individuals [Fig. 4, panel B), C) and D)]. Conversely,  $T_2$  transgenic plants of two  $T_2$  families carrying the RNAi::*SIMLO1* silencing construct were characterized by the simultaneous silencing of *SIMLO1*, *SIMLO5* and *SIMLO8* [Fig. 4, panel A) and Supplementary Fig. 8].

As expected,  $T_2$  progenies carrying the RNAi::*SIMLO1* construct segregated for PM resistance:  $T_2$  plants carrying the silencing construct  $[T_2_SIMLO1_NPT(+)]$  were resistant, whereas non-transgenic plants  $[T_2_SIMLO1_NPT(-)]$  were susceptible as MM (Fig. 5, panel A). In contrast, all  $T_2$  progenies segregating for *SIMLO3*, *SIMLO5* and *SIMLO8* silencing constructs visually appeared to be fully susceptible to *O. neolycopersici* (Fig. 5, panel A). The quantification of disease severity on these lines using real-time qPCR supported phenotypic observations, as no significant difference was found between  $T_2_SIMLO3_NPT(+)$ ,  $T_2_SIMLO5_NPT(+)$ ,  $T_2_SIMLO8_NPT(+)$  plants and MM (Fig. 5, panel B and Supplementary Fig. 9). For each  $T_2$  family, transgenic and non-transgenic plants were phenotypically indistinguishable.

The SImIo1 line, harboring a loss-of-function mutation in the *SIMLO1* gene (Bai et al. 2008), is resistant to PM, however lower leaves displayed PM symptoms (Fig. 5, panel A). Compared to the plants of the SImIo1 line, RNAi plants carrying the RNAi::*SIMLO1* construct ( $T_2$ \_*SIMLO1\_NPT*(+) plants) showed no PM symptom and also a significantly lower amount of fungal biomass (Fig. 5 panel B and Supplementary Fig. 9, panel A). Therefore, further microscopic observations were carried out to study the fungal growth on the SImIo1 line and  $T_2_SIMLO1_NPT(+)$  plants.

Since the two T<sub>2</sub> families carrying the RNAi::*SIMLO1* construct showed no difference with respect to the level of reduced expression of the *SIMLO* homologs and fungal biomass quantification (Supplementary Fig. 8 and 9), we used one T<sub>2</sub> family for microscopic study. Compared to MM, fungal growth was significantly reduced in both SImlo1 and T<sub>2</sub>\_RNAi::*SIMLO1\_*NPT(+) individuals due to the formation of a papilla beneath the appressorium (Fig.6). Interestingly, the rate of papilla formation in T<sub>2</sub>\_RNAi::*SIMLO1\_*NPT(+) (93.3% of the infection units) was significantly higher than in SImlo1 (64.4% of the infection units) (Table 4). In some cases, *O. neolycopersici* was still able to penetrate epidermal cells and form haustoria with a rate of 48.9% in SImlo1 and 30% in T<sub>2</sub>\_RNAi::*SIMLO1\_*NPT(+) (Table 4 and Fig. 6). The general development of the spores on the two genotypes was strikingly different: while on the SImlo1 line the fungus could produce mostly up to two secondary hyphae (in 36.7% of the total infection units), on T<sub>2</sub>\_RNAi::*SIMLO1\_*NPT(+) individuals fungal growth was significantly reduced after producing a germination tube (Table 4 and Fig. 6).





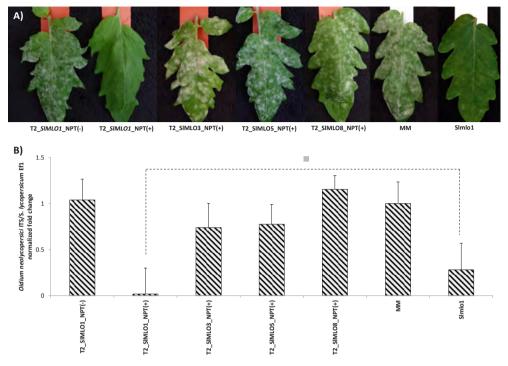


FIGURE 5. Powdery mildew evaluation on plants of segregating  $T_2$  families obtained with silencing constructs targeting *SIMLO* genes to attest their involvement in *O. neolycopersici* susceptibility. Panel A) shows the phenotypic evaluation of the powdery mildew growth on leaves of different  $T_2$  individuals that have been evaluated for the (from left to right) absence of the RNAi::*SIMLO1*, presence of the RNAi::*SIMLO1*, presence of the RNAi::*SIMLO3*, presence of the RNAi::*SIMLO5* and presence of the RNAi::*SIMLO8* silencing constructs, followed by one individual of the cv Moneymaker (MM) and one of the SImlo1 line carrying a loss-of-function mutation in the *SIMLO1* gene. Panel B) shows the relative quantification of the ratio between *Oidium neolycopersici* and plant gDNAs in transgenic individuals [NPT(+)] and not transgenic individuals [NPT(-)] segregating in  $T_2$  families obtained with the silencing constructs above described. Bars and standard errors refer to (from left to right) four individuals of two independent  $T_2$  families not carrying the RNAi::*SIMLO1*, eight individuals of the same two  $T_2$  families carrying the RNAi::*SIMLO1*, 18 individuals of three independent  $T_2$  segregating families carrying the RNAi::*SIMLO3* construct, 18 individuals of three independent  $T_2$  segregating families carrying the RNAi::*SIMLO3* construct, next to 10 MM plants and 10 plants of the SImlo1 line. The asterisk refers to the significant difference in susceptibility between individuals of the  $T_2$ \_SIMLO1\_ NPT(+) and SImlo1, inferred by mean comparisons with a Student's t-test (\* p < .05). TABLE 4. Development of *Oidium neolycopersici* growth on the susceptible genotype Moneymaker and on the two resistant genotypes, SImIo1 carrying a loss-of-function *SIMLO1* gene and plants of a T<sub>2</sub> family selected to carry the RNAi::*SIMLO1* silencing construct which can effectively silence *SIMLO1*, *SIMLO5* and *SIMLO8*.

		Percentag	e of infec	tion units (l	U)		Hypha	e per	IU	
Genotype	Primary AP	Primary papilla	Primary HS	Secondary Papilla	Secondary HS	1	2	3	4	5
ММ	100	0	90.2	0	68.3	76.8	67.1	35.4	6.1	0
Simio1	100	64.4	48.9	23.3	14.4	43.3	36.7	18.9	3.3	0
T <sub>2</sub> _RNAi:: <i>SIMLO1</i> _NPT(+)	100	93.3*	30.0	2.2	0.0	11.1	7.8	3.3	0.0	0

AP= appressorium, HS=haustorium

\* p < .05 compared to SImIo1

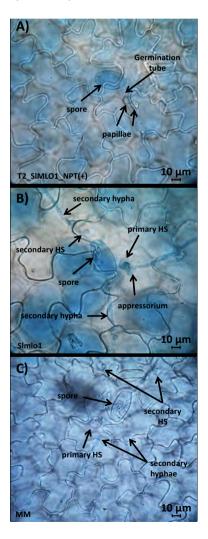


FIGURE 6. Effect of the silencing of *SIMLO1*, *SIMLO5* and *SIMLO8* in tomato cv Moneymaker background compared with the SImIo1 line harboring a loss-of-function of *SIMLO1* gene. Panels A) refers to a transgenic plant carrying the RNAi::*SIMLO1* construct, panels B) a plant of the SImIo1 line and panels C) a plant of the tomato cv. Moneymaker. A), B) and C) show fungal structures (spores, germination tube, appressorium, haustorium –HS- and hyphae) and the plant cellular reaction of papilla apposition at the sites of fungal penetration.

# Discussion

#### Structure and evolution of the SIMLO gene family

In this study, we followed an in silico approach to assign 16 homologs to the tomato *MLO* gene family. This is consistent with the results of previous studies reporting the *MLO* gene families of several diploid species made of a number of homologs variable from 13 to 21 (Devoto et al., 2003; Feechan et al., 2008; Liu and Zhu, 2008; Shen et al., 2012; Pessina et al., 2014; Schouten et al., 2014; Appiano et al., 2015). This suggests that a similar number of *MLO* homologs is likely to be retrieved in future genome-wide investigations involving diploid eudicot species.

Information on chromosomal localization was available for all the *SIMLO* homologs with the exception of *SIMLO4*. However, potato and tomato genomes are highly syntenic (2012) and the closest *SIMLO4* homolog in potato (Sotub02g007200) is positioned on chromosome 2, thus suggesting that *SIMLO4* is also located on tomato chromosome 2.

Cloning of the SIMLO gene family from different tissues of the cultivar MM revealed the occurrence of transcripts deviating from predictions available at the SGN database, indicating that, despite the efforts of the tomato resequencing project, the assembly of genomic regions and the prediction of certain loci are not correct yet. Moreover, several cases of differentially spliced variants among plant tissues were observed, mostly due to intron retention and exon skipping, as it is in the case of SIMLO5, SIMLO9, SIMLO11, SIMLO13 and SIMLO15. Due to the method used in this study to amplify the SIMLO homologs, we cannot exclude that the intron retention is the result of the amplification of non-mature mRNA. However, intron retention was previously reported to be a very common type of alternative splicing in Arabidopsis and rice (Ner-Gaon et al., 2007). There is also a well-documented evidence indicating organspecific regulation of alternative splicing in plants (Palusa et al., 2007). More studies need to be performed to unravel its complexity and functional significance. Certainly, alternative forms of splicing, such as the ones found in this study, can lead to aberrant mRNA isoforms that cause the loss-of-function of a MLO gene. An example is reported by a recent study conducted by Berg et al., 2015 in cucumber. They show that the integration of a transposable element in the genomic region of the CsaMLO8 leads to an aberrant splicing that causes the loss-of-function of this susceptibility gene in a resistant cucumber genotype.

The identification of protein motifs conserved in transmembrane domains of specific SIMLO homologs (Table 3a and 3b) corroborates previous findings in Solanaceae plant species (Appiano et al., 2015). This indicates that transmembrane domains, which are thought to provide a common scaffold invariable for the whole MLO family (Devoto et al., 1999), might also be involved in conferring specific functions to MLO homologs. Future functional studies of targeted mutagenesis of transmembrane MLO protein regions can help to unravel their actual role.

All the SIMLO proteins were found to group in six phylogenetic clades together with other eudicot MLO homologs, including the complete Arabidopsis AtMLO family and certain members of the apple, peach and strawberry MLO family. No SIMLO homolog could be assigned to clade IV, previously shown to contain monocot MLO homologs and a few eudicot homologs (grapevine VvMLO14, strawberry FvMLO17 and peach PpMLO12) (Feechan et al., 2008; Pessina et al., 2014).

Based on their sequence relatedness with Arabidopsis AtMLO proteins of known function, it is logical to argue that one or more of the tomato SIMLO homologs in clade III and clade I could regulate the processes of root response to mechanical stimuli and pollen tube reception, respectively. The RNAi silenced lines of several SIMLO homologs generated in this study could be useful to assign new functions to MLO proteins which have gone unnoticed by the evaluation of the available panel of Arabidopsis Atmlo mutants.

#### Possible pleiotropic effects and co-functioning of SIMLO homologs

RNA-seg data, RT-PCR and real-time gPCR of the SIMLO gene family confirmed the expression of all the 16 SIMLO homologs. Often, it was possible to detect high level of transcript of the same SIMLO homolog in more than one of the four tissues under study (leaf, root, flower and mature fruit). This is in line with the findings of the previous study of Chen et al., 2006, investigating the expression pattern of the Arabidopsis AtMLO gene family in several tissues. Overall, this body of evidence suggest that: a) different MLO homologs may have synergistic or antagonistic roles in regulating the same biological process; b) MLO homologs may have pleiotropic effects on different biological processes. Co-functioning between MLO homologs has been demonstrated to occur in Arabidopsis, where different AtMLO genes co-participate in the same tissue to determine powdery mildew susceptibility and root response to mechanical stimuli (Consonni et al., 2006; Chen et al., 2009). A yet unidentified additional biological function could be hypothesized for the SIMLO1, previously shown to act as a susceptibility gene towards O. neolycopersici (Pavan et al., 2009). This gene was found to exhibit its strongest expression level in tomato flower and moderate expression in root, two tissues which are less or not attacked by the fungus, respectively. Moreover, additional biological roles for SIMLO1 would explain why this gene has not been excluded from evolution, despite promoting susceptibility to PM pathogen. Interestingly, evidence shows that the SIMLO1 orthologs in barley and Arabidopsis are involved in the interaction with pathogens other than powdery mildews, such as necrotrophs and hemibiotroph (Jarosch et al., 1999; Kumar et al., 2001; Consonni et al., 2006). Thus, it is worthwhile to test the RNAi-SIMLO1 plants with more pathogens to broaden its role in plant-pathogen interactions.

## SIMLO homologs involved in powdery mildew susceptibility

In this study, we mainly focused on the *SIMLO* genes grouped in the clade V containing all the *MLO* homologs associated with PM susceptibility in eudicots. The presence of multiple tomato homologs in clade V is in accordance with the existence of three Arabidopsis proteins (AtMLO2, AtMLO6 and AtMLO12) associated with increased fungal penetration (Consonni et al., 2006).

We showed that tomato *SIMLO3*, *SIMLO5* and *SIMLO8*, differently from *SIMLO1*, do not increase their expression upon *O. neolycopersici* challenge. Furthermore, strong silencing of the same homologs in a susceptible tomato background (Moneymaker) did not result in a significant reduction of disease symptoms (Fig. 3, Fig. 4 and Fig. 5).

Plants transformed with a construct meant to silence SIMLO1 showed co-silencing of SIMLO5 and SIMLO8, due to sequence relatedness between these genes (Fig. 4). Interestingly, these plants were also significantly more resistant than plants of the SImIo1 line (Fig. 5). Since the SImIo1 line is only a BC3S2 line carrying the SImIo1 mutation (the ol-2 gene) in MM background, we cannot fully exclude background effects from the ol-2 donor, the resistant line LC-95 of S. lycopersicum var. cerasiforme, which might add to partial susceptibility phenotype of the *Slmlo1* line. On the other hand, our scenario is reminiscent of the one reported in Arabidopsis, where Atmlo2 single mutant displays partial PM resistance, whereas Atmlo2/Atmlo6/Atmlo12 triple mutant is fully resistant (Consonni et al., 2006). Also in grape, more than one VvMLO genes are involved in susceptibility to powdery mildew (Feechan et al. 2008; 2013). Taken together with the knowledge of functional redundancy in Arabidopsis and grape, our data suggest that in tomato SIMLO1, SIMLO5 and SIMLO8 are functionally redundant as PM susceptibility factors with SIMLO1 playing a major role. Our results showed that the contribution of SIMLO5 and SIMLO8 is too small to be observed with an RNAi approach silencing individual genes, but a complementation experiment using the SImIo1 line could be more suitable to observe the minor role of these genes.

It cannot be excluded yet that the other clade V tomato homolog *SIMLO3* is also involved in plant-pathogen interactions. However, it is worthwhile to notice that the SIMLO3 protein is missing three of the six motifs contained in SIMLO1, two of which are also present in SIMLO5 and SIMLO8 (Table 3b). The motif three in Table 3b is located in the second intracellular domain, which is known to be involved together with the third intracellular domain in the protein functionality (Elliott et al., 2005). This would suggest that SIMLO3 might miss important features to be fully functional as susceptibility factor. Overexpressing of *SIMLO3* in the SImlo1 mutant may provide a better evidence on its eventual role as functional susceptibility gene. Interestingly, we noticed that SIMLO4 and SIMLO14, which do not belong to clade V, are up-regulated upon O. neolycopersici infection (Fig. 3 and Supplementary Fig. 4 and 5). SIMLO14 is closely related to AtMLO4 and AtMLO11, which are involved in root thigmomorphogenesis (Chen et al., 2009), while SIMLO4 is related to AtMLO7, involved in pollen tube reception (Kessler et al., 2010). In Arabidopsis, mutation of AtMLO4, AtMLO7 and AtMLO11 does not result in PM resistance. Thus, we expected that silencing of SIMLO4 and SIMLO14 in tomato will not lead to PM resistance too. The up-regulated expression of SIMLO4 and SIMLO14 after challenge with O. neolycopersici might be the result of shared regulatory cis-acting elements. We used a 2kb region located upstream the starting codon of SIMLO1, SIMLO4 and SIMLO14 coding sequences to search for shared regulatory elements through the online database Plant Care (http://bioinformatics.psb.ugent.be/ webtools/plantcare/html/) (Lescot et al., 2002). We found, at least, five common motifs which are associated with upregulation by multiple biotic and/or abiotic stresses: ABRE (CACGTG), involved in abscisic acid responsiveness, CGTCA- and TGACG-motifs, involved in the MeJA responsiveness, HSE (AAAAAATTC), involved in heat stress responsiveness, and TCA (CCATCTTTTT/GAGAAGAATA) element, involved in salicylic acid response. It is intriguing whether SIMLO4 and SIMLO14 can act as a susceptibility gene to PM. Till now, only clade IV and clade V MLO genes have been studied for their role as a susceptibility gene. To further study these PM-induced non-clade V SIMLO genes, a complementation test using the SImIo mutant could be performed.

In conclusion, this study provides a comprehensive characterization of the *MLO* gene family in tomato by analyzing their genomic structure, expression profile and predicted protein motifs. In tomato, there are 17 *MLO* genes which can be grouped into six clades. The expression of these *MLO* genes can be tissue specific and some *MLO* genes showed alternative splicing variants in different tissues. The *SIMLO1* in clade V is confirmed to be the major PM susceptibility factor. In addition, two clade V genes, *SIMLO5*, and *SIMLO8* are suggested to have a partially redundant function, as described in Arabidopsis for *AtMLO2*, 6 and 12 genes (Consonni et al. 2006). To label an *MLO* gene as a PM susceptibility gene, it is recommended to combine phylogenetic analysis and expression profile to select candidates of clade IV (for monocot) and V (for dicot) that are induced by PM infection. However, the upregulation of *MLO* genes outside clade V in response to PM, as shown in this study and in Pessina et al. (2014), raises the possibility that they may act as susceptibility genes. Finally, the RNAi lines generated in this study are useful materials for further assigning new biological functions to the *MLO* gene family members.

# **Materials and methods**

#### Plant material, fungal material and inoculation

In this study, we used the susceptible *S. lycopersicum* cultivar Moneymaker (MM), the SImIo1 line and transgenic T<sub>2</sub> families in which individual *SIMLO* gene was silenced via RNAi in MM background. The SImIo1 mutant (the *ol-2* gene) was a natural mutation discovered in the resistant line LC-95 of *S. lycopersicum* var. *cerasiforme*. The LC-95 line was crossed with the susceptible tomato *S. lycopersicum* cv. Super Marmande and the F2 progeny was used for mapping in 1998 (Ciccarese et al. 1998). Later, we introgressed the *ol-2* allele into *S. lycopersicum* cv Moneymaker (MM) by backcrossing and one BC3S2 line homozygous for the *ol-2* allele (the tomato SImIo1 line) was used in the experiment.

The powdery mildew disease assay was performed by artificial inoculation in the greenhouse. For this, the Wageningen isolate of *O. neolycopersici* (*On*) was used (Bai et al., 2008). A suspension of *O. neolycopersici* conidia was prepared, by rinsing freshly sporulating leaves of infected tomato plants with tap water. This suspension was immediately sprayed on 1 month-old tomato plants. Ten plants for each of the  $T_2$  progenies obtained from the transformation of each silencing construct, 10 Slmlo1 plants and 10 MM plants were used for disease assay. The scoring of powdery mildew symptoms was done 10 days after inoculation, inspecting and collecting the third and fourth true leaves for each plant.

For the evaluation of the expression of the *SIMLO* gene family, two independent inoculations were set up. In both cases, we used the cultivar MM, four and three biological replicates for each of the three time points (0, 6 and 10 hours post inoculation -hpi-) during the first and the second inoculation respectively.

#### Identification and cloning of the SIMLO gene family

Putative tomato MLO protein sequences were identified in the Sol Genomics Network (SGN) (http://solgenomics.net/) database by using the BLASTP and TBLASTN algorithms with Arabidopsis AtMLO protein sequences as a query. Chromosomal localization, sequences of the corresponding genes and introns/exons boundaries were inferred by annotations from the International Tomato Annotation Group (ITAG).

Aiming at cloning and sequencing the *SIMLO* gene family from the cultivar MM, total RNA from leaf, root, flower and ripened fruit was isolated (RNeasy<sup>®</sup> mini kit, Qiagen). The different tissues were collected from five MM plants and pooled together to obtain enough material for the RNA isolation. For each individual *SIMLO* homolog, two primer pairs specifically amplifying overlapping products of around 800bp of the predicted coding sequences (CDS) were designed using the Primer3 plus online software (http:// www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Rozen and Skaletsky, 2000).

The forward primer and the reverse primer of product A and product B, respectively, are located in the respective UTR regions to ensure at least the cloning of the complete CDS. A one-step PCR was performed to obtain the desired product (SuperScript<sup>®</sup> III One-Step RT-PCR System, Invitrogen) (Supplementary Table 1). Indeed, a PCR performed on a cDNA obtained with oligo d<sub>T</sub> primers did not yield any product for many of the homologs under investigation. The use of sequence-specific primers in the one-step PCR, on the other hand, allowed the binding of only the desired mRNA sequences.

Corresponding amplicons were visualized on agarose gel and cloned into the pGEM®-T Easy vector (Promega). Recombinant plasmids were sequenced by using universal T7 and SP6 primers.

In order to reveal gene structures and polymorphisms, *SIMLO* sequences obtained by cloned amplicons were merged using the package Seqman of the software DNASTAR<sup>\*</sup> Lasergene8. The obtained consensus was aligned with the coding region of the *SIMLO* identified *in silico* and the corresponding genomic region using the CLC 7.6.1 sequence viewer software (www.clcbio.com).

Finally, for the motif analysis, the MEME (http://meme.nbcr.net/) package was used to predict consensus patterns of consecutive conserved amino acids in the SIMLO proteins deriving from the *in silico* translation of the cloned transcripts from leaf, root, flower and fruit of the cultivar MM (Bailey et al., 2015).

# **Comparative analysis**

The corresponding SIMLO protein sequences of translated cloned CDS obtained from leaf and flower (in the case of *SIMLO12*) were used as dataset in the CLC 7.6.1 sequence viewer software (www.clcbio.com) for ClustalW alignment and the obtainment of an UPGMAbased comparative tree (bootstrap value was set equal to 100), together with those of the 15 Arabidopsis AtMLO homologs. Moreover MLO proteins experimentally shown to be required for PM susceptibility were added, namely pea PsMLO1, barley HvMLO, wheat TaMLO\_A1b and TaMLO\_B1a, rice OsMLO2, pepper CaMLO2, tobacco NtMLO1, cucumber CsaMLO8, *Lotus japonicus* LjMLO1 and barrel clover MtMLO1. Moreover MLO homologs of the Rosaceae species that cluster in clade VII (FvMLO15, MdMLO18, PpMLO9) and VIII (FvMLO13, MdMLO20 and PpMLO13) were included (Supplementary Table 2). The obtained UPGMA-comparative tree was then displayed as circular rooted cladogram with CLC software.

# Expression analysis of the SIMLO gene family in response to O. neolycopersici

Tissue samples from the third and fourth true leaf of 1-month old tomato plants were collected immediately before fungal inoculation and at two time points after inoculation

(6 and 10 hours). The RNA isolation was performed with MagMAX-96 Total RNA Isolation kit (Applied Biosystem), following the manufacturer's instructions. Included in the protocol is a DNase treatment using the TURBO<sup>™</sup> DNase. An aliquot of the RNA isolated was run on denaturing agarose gel to assess its integrity. Purity and concentration were determined by measuring its absorbance at 260nm and 280nm using the NanoDrop<sup>®</sup> 1000A Spectrophotometer. Following this protocol for RNA isolation, intact and pure RNA was obtained and the concentration was variable between 200ng/µl and 250ng/µl.

cDNAs were synthesized by using the SuperScript III first-strand synthesis kit (Invitrogen) using the oligo(dT)<sub>20</sub> primer, starting from the same amount of RNA (200ng/µl). Specific primer pairs for each of the 16 SIMLO homologs, amplifying fragments ranging from 70 bp to 230 bp, were designed as described above (Supplementary Table 3). The amplification of single fragments of the expected size for each homolog was verified by agarose gel electrophoresis and by the observation of the melting pick. Four tomato reference genes were tested for expression stability in order to determine which ones could be suitable for normalization of the expression of SIMLO homologs. These include the 60S ribosomal protein L33 (GeneBank number Q2MI79), the elongation factor 1a (GeneBank number X14449), actin (GeneBank XP\_004236747) and ubiquitin (GeBank number XP\_004248311) (Schijlen et al., 2007),(Løvdal and Lillo, 2009). Gene expression stability was assayed with the BestKeeper program (Pfaffl et al., 2004), determining as best reference genes the ribosomal protein L33 and the elongation factor 1α. The cDNAs were diluted 10fold and used in real-time qPCR with a Bio-Rad CFX96TM thermal cycler. The thermal cycling conditions used were 95°C for 1 min, followed by 40 cycles at 95°C for 15s, 60°C for 1 min and 72°C for 30s, followed by a melt cycle of 0.5°C increment per min from 65 to 95°C. Comparable amplification efficiencies between target and reference genes were determined using the LinRegPCR software (Karlen et al., 2007). Normalization was performed according to the  $\Delta\Delta C_{\rm c}$  method (Livak and Schmittgen, 2001). Four biological replicates and two technical replicates were used in this experiment. Student's t-tests were applied in order to assess significant differences between the treatments.

#### SIMLO family expression analysis in different tissues

To analyze *MLO* gene expression in leaf, root, flower and ripened fruit approximately equal amount of tissues from five MM plants were pooled and used for RNA isolation and cDNA synthesis as described in the previous paragraph. Before using them as templates, cDNAs were diluted 10-fold. Real-time qPCR was performed using the set of primers reported in Supplementary Table 3 to amplify each homolog in the four tissues above mentioned. Elongation factor 1 $\alpha$  was used as reference gene. Data analysis was performed according to the  $\Delta C_t$  method (Livak and Schmittgen, 2001). Three technical replicates for each sample were performed.

#### **Generation of RNAi silencing lines**

Four primer pairs were designed to amplify and clone fragments from SIMLO1, SIMLO3, SIMLO5 and SIMLO8 into the Gateway-compatible vector pENTR D-TOPO (Invitrogen) (Supplementary Table 3). The cloned sequences of the SIMLO1, SIMLO3, SIMLO5 and SIMLO8 genes are highlighted in Supplementary Fig. 6. After cloning in *E. coli* (strain DH5 $\alpha$ ), the kanamycin-resistant colonies were assessed for the presence of constructs by colony PCR. Positive recombinant plasmids were further analyzed by restriction enzyme digestion and sequencing. Next, amplicons were transferred by LR recombination reaction into the pHELLSGATE12 vector for hairpin-induced RNAi (Wielopolska et al., 2005) following the instructions provided by the manufacturer (Invitrogen), and cloned again in E. coli DH5a. Bacterial colonies growing on a spectinomycin-containing medium were selected for the presence of the silencing construct by colony PCR and sequencing. Recombinant plasmids were transferred into the AGL1+virG strain of Agrobacterium tumefaciens (Lazo et al., 1991) by electroporation, and transformed bacterial cells were selected on a medium containing 100 mg/ml<sup>-1</sup> spectinomycin, 50 mg/ml<sup>-1</sup> carbenicillin and 50 mg/ml<sup>-1</sup> chloramphenicol. Single colonies of A. tumefaciens were picked and the presence of the insert was confirmed by colony PCR. Ten-fold dilutions of overnight culture from single positive colonies were re-suspended in MSO medium (4.3 g/l MS basal salt mixture, 30 g/l sucrose, 0.4 mg/l thiamine, 100 mg/l myoinositol, pH 5.8) to a final OD<sub>600</sub> of 0.5 and used for transformation.

The transformation procedure for tomato cotyledons was carried out similarly to the method described by (Appiano et al., 2015).

Silencing efficiency was assessed, for each of the 4 constructs, on 10 to 20 T<sub>1</sub> plants and on selected T<sub>2</sub> lines by real-time qPCR, as described for the analysis of the *SIMLO* gene family expression in response to *O. neolycopersici*. In addition, the T<sub>2</sub> lines were assessed for the presence of the nptII marker gene and the 35S promoter by PCR, using the primer pair NPTII\_Fw (5'ACTGGGCACAACAGACAATC3') / NPTII\_Rev (5' TCGTCCTGCAGTTCATTCAG 3') and 35S-Fw (5'-GCTCCTACAAATGCCATCA-3') / 35S-Rev (5'- GATAGTGGGATTGTGCGTCA-3'), and visualizing the product on agarose gel.

# **Disease quantification on silenced lines**

 $T_2$  lines originating from selfing of  $T_1$  plants showing high level of silencing were inoculated with *O. neolycopersici* (*On*) by spraying four weeks old plants with a suspension of conidiospores obtained from freshly sporulating leaves of heavily infected plants and adjusted to a final concentration of 4 x 10<sup>4</sup> spores/ml. Inoculated plants were grown in a greenhouse compartment at 20 ± 2°C with 70 ± 15% relative humidity and day length of 16 hours. Two weeks later, infected tissues from the third and fourth true leaf were visually scored and sampled. Plant and fungal DNAs were extracted by using the

DNeasy DNA extraction kit (Qiagen). In total, 15 ng of DNA was used as template for amplification with the primer pair *On*-Fw (5'-CGCCAAAGACCTAACCAAAA-3') and *On*-Rev (5'-AGCCAAGAGATCCGTTGTTG-3'), designed on *On*-specific internal transcribed spacer sequences (GenBank accession number EU047564). The tomato Ef1 $\alpha$  primers (Supplementary Table 3) were used as reference to determine fungal biomass relative to host plant DNA by  $\Delta\Delta$ C, method.

#### Disease tests for microscopic evaluation in histological study

Spores of the Wageningen isolate of *O. neolycopersici* grown in a climate chamber at  $20\pm1^{\circ}$ C, with  $70\pm10\%$  RH and a 16-h photoperiod were water-sprayed on the third leaf of 1-month old tomato plants of the susceptible tomato cv. MM, the resistant line Slmlo1 and transgenic plants of one T<sub>2</sub> family selected by PCR for the presence of the NPTII and 35S marker genes of the RNAi::*SlMLO1* silencing construct. The concentration of the spore suspension was  $3\times10^5$  conidia ml<sup>-1</sup>. After 65 hours, a 4 cm<sup>2</sup> segment was cut from the inoculated leaves. Three samples were taken from four plants of each genotype and from 5 plants of the T<sub>2</sub> family, bleached in a 1:3 (v/v) acetic acid/ethanol solution and 48 hrs later stained in 0.005% trypan blue as described by (Pavan et al., 2008). For each genotype, a total of 90 infection units (IU), defined as a germinated spore that produced, at least, a primary appressorium, were counted. Observations were performed using a Zeiss Axiophot bright field microscope and pictures were taken with an Axiocam ERc5s. For each IU, the number of hyphae, the presence/absence of a primary and secondary haustoria and presence/absence of papillae were recorded.

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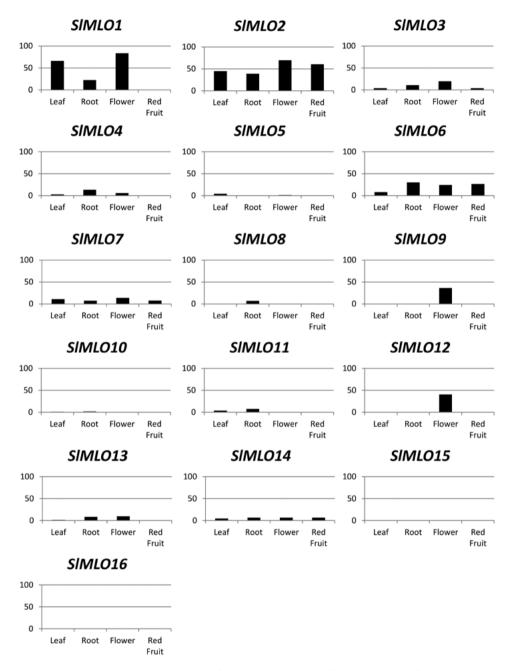
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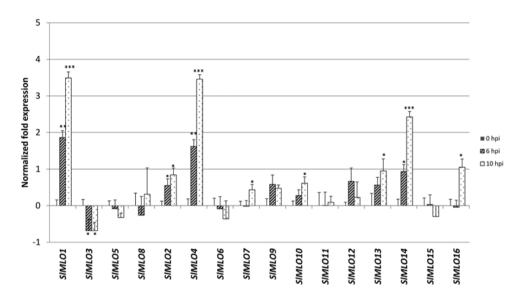
# Supplementary material

**SUPPLEMENTARY FIGURE 1.** Sequence alignments of *SIMLO* homologs cloned from different tissues of the tomato cv. Moneymaker showing deviating transcripts when compared with the predicted CDS sequence, as inferred by the SGN database, against their respective genomic regions. Deviating transcripts are identified for *SIMLO1* in flower, *SIMLO5* in fruit, *SIMLO6*, *SIMLO7*, *SIMLO8* and *SIMLO9* in leaf, *SIMLO10* in fruit, *SIMLO11* in root, *SIMLO13* in leaf and SIMLO15 in all the tissues investigated. Alignments were obtained with default parameters by the CLC sequence viewer software. Due to its size, this figure is only available online at http://journal.frontiersin. org/article/10.3389/fpls.2016.00380

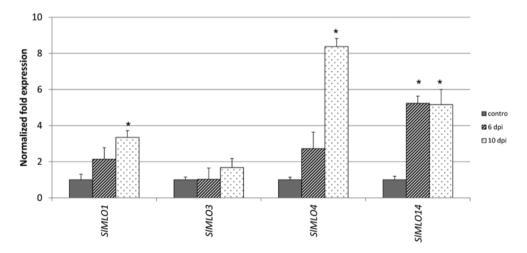
**SUPPLEMENTARY FIGURE 2.** Sequence alignment of a set of 37 MLO proteins cloned from four tissues (leaf, root, flower and fruit) of the cv. Moneymaker. When no deviating transcripts were detected, the translated nucleotide sequence of leaf was used in the alignment, with the exception of *SIMLO12* which was cloned only from flower. Shading was obtained by feeding the alignment, obtained with default parameters by the CLC sequence viewer software, to the online server Boxshade (http://sourceforge.net/projects/boxshade/) and setting as 0.7 the fraction of sequences that must agree for shading. In black shade the amino acids fully conserved, in grey the amino acids substitutions which are predicted to have similar biochemical properties and in red the amino acids identified by Elliot et al., 2005 as invariable for the whole MLO protein family. Transmembrane domains are indicated as inferred by the TMHMM online server (http://www.cbs.dtu.dk/services/TMHMM/) fed with the SIMLO1 protein sequence of leaf. Due to its size, this figure is only available online at http://journal.frontiersin. org/article/10.3389/fpls.2016.00380



**SUPPLEMENTARY FIGURE 3.** FPKM values (fragments per kilobase of exon per million fragments mapped) depicted as columns for each of the four tissues investigated in this study obtained from the RNA-seq data of each of the 16 *SIMLO* homologs as reported by the SGN database.



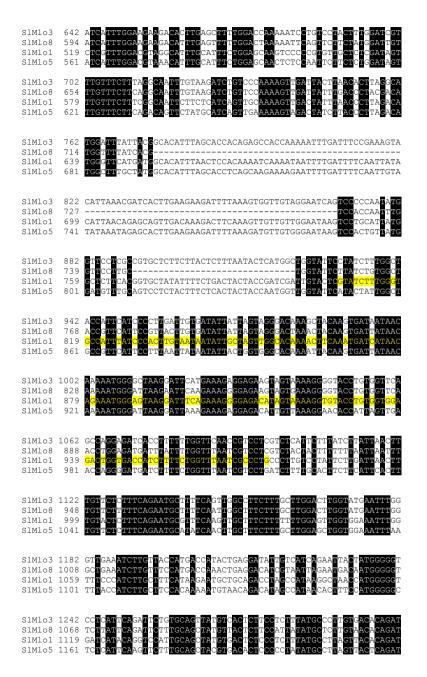
**SUPPLEMENTARY FIGURE 4.** Relative expression level of the *SIMLO* gene family members in response to *O. neolycopersici* infection. Samples were collected at 0, 6 and 10 hrs after inoculation (hpi). Transcript levels of each *SIMLO* homolog were normalized against the transcription level of Ef1 $\alpha$  as reference gene. Bars show standard errors based on four biological replicates. Asterisks refer to significant differences with respect to non-inoculated plants (0 hpi), inferred by mean comparisons with a Student's t-test (\* p < .05, \*\* p < .01, \*\*\* p < .001).



**SUPPLEMENTARY FIGURE 5.** Relative expression level of *SIMLO1*, *SIMLO3*, *SIMLO4* and *SIMLO14* compared to the reference gene Ef 1 $\alpha$  upon inoculation with *O. neolycopersici*. Means are calculated from duplicate technical measurements. Bars show standard errors based on three biological replicates. Asterisks refer to significant differences with respect to non-inoculated plants used as control (0 hpi), inferred by mean comparisons with a Student's t-test (\* p < .05).

SlMlo3 SlMlo8 SlMlo1 SlMlo5	1 ATGGCCGGTGGTGGTGGTGGTGGTGGAAGATCGTTGGAGCAAACGCCAAC 1 ATGGCGGGAGGAGGAGGAGGAAAGATCGTTGGAGCAAACGCCGAC 1 ATGGAGGCAACGCCGAC 1 ATGGCTAGCACAGGCTGTATTAGAACGTGTGATGAACGTCCTCTAGATGACACACCAACA	
SlMlo3 SlMlo8 SlMlo1 SlMlo5	49 TGGGGGGTTGCCGTTGTTTGTTTTGTGTTAGTTGCAATATCTATTGTCATTGAATAA 46 TGGGGGGTTGCCCTAGTTTGTTTTTGCATTGCATTGCAT	1
SlMlo3 SlMlo8 SlMlo1 SlMlo5	109 ATCCACCTTATTGCAAAGTGGTTGAACTCTAAAAATAAAAGTGCCTTGTATGAAGCACTT 106 ATCCACCTTATTGCCAAGTGGTTGAACTCTAAACATAAAAGAGCGATTATATGAAGCACTT 79 ATTCATCACATTGGAGAGTGGTTACTGCAAAAGCGGAAAAAGTGTCTATATGAAGCACTT 121 ATTCATCATCTTGGAGAGTGGTTATGCAAGAAACAAAAGAGACCATTGTATGAAGCACTT	
SlMlo3 SlMlo8 SlMlo1 SlMlo5	169 GAAAAGATCAAAGCAGAGAGCTTATGCTGCTGGGATTCATATCATTGTTGTTAACAGTAAGGA 166 GAGAAGATAAAAGCAGTAGGG 139 GAAAGATCAAAGGTGAACTTATGCTGTTGGGATTCTTATCACTGTTGTTGACAGGTTG 181 GAGAAGATCAAGTGAACATCATGTTATTAGGGTTTATATCCTTATTCTTGACGGTTGTA	1 1 1 1
SlMlo3 SlMlo8 SlMlo1 SlMlo5	229 CAAAGTCCAATTTCGAACATATGTGTATGAGAAAAATTAGGAAATTGATGGCATCGATG 187 CAAGATCCAATTTCAAATATTTGTGTGTATCTGAAAAAATTGCAAGTAGCTGGCATCCATG 199 CAAGATCCAGTTTCTAACTTATCTGTCCCCAAGAGTGTTGGTTATTGATGGCATCCTTG 241 CAGGATCCTATGTCTAAGATATGTATTCCTAGGAGTGTTGGACGCTCTTGGCATCCATG	
SlMlo3 SlMlo8 SlMlo1 SlMlo5	289 AGTAAAAAGAAGAAGAAGATAGTAGTATAATTTCAGAAGATTCGTTGTCGGAGCZ 247 AGTAAACAAAAAGAAGAGCTGAAATGAACAAATATATTTCCGGTGACTTAGAGGC 259 ATGGCAAAGAAGATGCCAAG	
SlMlo3 SlMlo8 SlMlo1 SlMlo5	342       ACACCGCCGGAGACTTCTTATGGATGCTGCCGCGGCGGTGCCGTACGACGAATATTGGCTGC         300       TCATCGCCGGCGACTTTTCACGGCTGACGATGGCGGAGTCCGGCGAGTTTTGGCGGC         280      TCTCAGTAA         322      GACCAATA	2
SlMlo3 SlMlo8 SlMlo1 SlMlo5	402 TGGTGGTGGAGAT <mark>GA</mark> CAAAATGTGCAGGAAAGGGAAAAGTACCATTTGTGTCTGCTGATG 357 TGCCGGAACTGACAAATGTGCAGACAAGGGAAAAGTAGCATTTGTGTCTGCCGATG 289GATGACCCTTGTCTACCAAAGGGAAAGGGCAATTTGCATGTTCATATG 331CTC <mark>GA</mark> TCCA <mark>TGTAGA</mark> ATTAAGGG <mark>GAAA</mark> CTCCAATTTGCTTCAAAATATG	3
SlMlo3 SlMlo8 SlMlo1 SlMlo5	462 AATTCATCAATTACACATTTTCATCITTGTGCTGGCTGGATTTCATGTCCCCCTATTGTG 414 TATTCATCAATATATATTTCATTITTGTGCTGGCTAGTTTTCATGTATTTTATGTG 339 AATACACCAGCTCCATATCTTCATCITTGTATTGGCTGGAGTGCTCATGTATTGTACTGTA 381 AATTCACCAACTCCACATTTTATCITTGTGTTAGCCGTGCACATGTGTGTGTATTGTA	
SlMlo3 SlMlo8 SlMlo1 SlMlo5	522 TAGAACTTIGCCTTIGGGGAGAGCTAAGATCAGAAGTTGGAAGTCAFGGGAAAATGAAA 474 TACCACATTGCCATIGGGAAGAGCTAAGATGAGTCGTTGGAAGATATGGGAAAACGAAA 399 ACGAACTTIGCCTIGGGGAGCGAGGCTAAAGGAAGAGAAAATGGAGGGATGGAGGATGAAA 441 TACCACTTIGGGAATTGCCAAACTAAGCATGAGGACATGGAGGAGCTIGGGAGGATGAAT	
SlMlo3 SlMlo8 SlMlo1 SlMlo5	582 TAAAACAGCTGAATACGAATTCHCTCACGATCCTGAAAGATTTCGATTTAGAAGAGAAAG 534 AAGAACAGCTGAGTACGAATTTHCTCATGATCCAGAGAGACTTCGGATTTGGTAGAGATAC 459 AAAAACAATGGAGTACGAATTCTACAACGACCCTGAGAGATTCAGATTTGGAAGGGAGAG 501 TAAAACAATTGAATACGAATTCTATAACGATCCTGAGAGATTTAGATTTGCAAGAGAAAG	

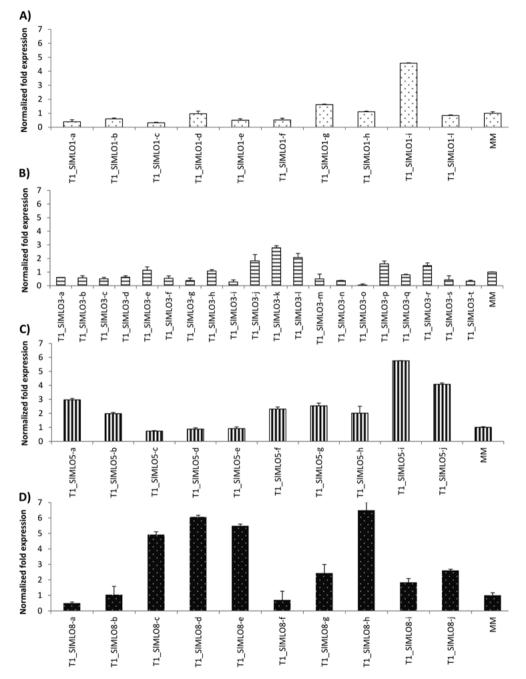
SUPPLEMENTARY FIGURE 6.



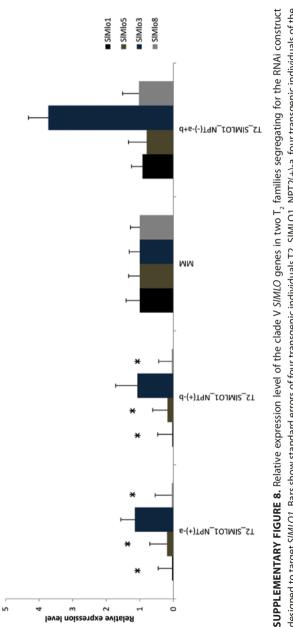
**SUPPLEMENTARY FIGURE 6.** 

SlMlo8 1128	GGGTTCAAACATGARATGAACTATCTTCAATGAAA <mark>GAGTAGCAACAGCATTGAACAAT</mark> TG GGGATCAACAATGAAACGAACAATCTTCAATGAAAGAGTAGCAATGG <mark>GATTGACAAAA</mark> TG GGGTTCATCAATGAAGCCTATCATCTTTGGTGATAATGT <mark>GGCA</mark> ACAGCTCTTAGAAGCTG GGGATCAACAATGAAACCAGTTATCTTTGGTGACAACGTGGCATACGCC <mark>A</mark> TACCCA <mark>CA</mark> TG
SlMlo3 1362 SlMlo8 1188 SlMlo1 1239 SlMlo5 1281	SCA <mark>CCATAGT</mark> GC <mark>CAAAAAACACATCABAGAGATCAACAAGCAACACTCAAATCCAACAAC</mark> GCACCATACAGCGAAAAAACGGGTGAAACATGGGCTATCAGGACATACCAC
S1Mlo8 1248 S1Mlo1 1290	ACCAATGTCAAGTAGACCAGGGACACGTTTCTCATCGCATGTCACGCGTGCATTATT ACCAATGTCAAGTAGCCACCACACGCGTCTGACGCATGTCACGCGTCCATGTCCT CCCTGCAAACAGCAGCAACCAACCACGATTGGGTGCTACCTGCCCGTGTTCACTTATT TCCAGTGAGAAGCAGGCAGGCAGTGTCACCATTGGGTGGAGGTTGCTGTC
SIMlo8 1305 SIMlo1 1347	GCGCGCGACATTATAGGAGTCATATGGCGAGTCTACABAACTCACCTCGTAGATCAAA ACGCGGTATCAGGACGACTGACATGGACGTGGGTCCACGBAGATCGACGTATA ACGCGGTTATCCACAATATAATGAGGACAGTGTTCABGCATCCTCCTCGGACATC CGGTTCABCAAAAAGACGGGCABTTATATCCTCCATCACC
SlMlo8 1358 SlMlo1 1401	CTACGATTTTGATOGGACGAGGAGGAGGGCTCACCTTCACCTCCCGGTTTTACCAGGA ATAATATAGACCATUGGATATTGAGGCCTCACCATCTCCAAATCGTCACGA CAATGTCGAAAATGAAGSCTGGGCTAATGAAAACCAGGA TAATCCTTCGCCTAGGAGGAGCAGTGGAGGTAATCCAGAA
S1Mlo8 1410	GGGAGA
S1Mlo8 1416 S1Mlo1 1446	AGTTATTGACCCTAATTCGTCACAAGGGGTCCCTCTATCACAAGAGGGTCGCCACCAACAA AGTTCACGACCTAATTGTCAGAAATTGAGGCTCGTCGACCAATA GATCCTGCAGCATGCCTCCACTGATCAT
S1Mlo8 1461 S1Mlo1 1483	TGAGATTACTATTGCTGGATCAAGAGATTTTTCGTTTGAGAAAAGAACAACCAGTATATA CGAGATTAATATTGCTCGTTCAAGGGATTTTTCTTTTGATAAAGAACGACTAGTGTATA ATTGAGATTACAATGTCAGATTTTACTTTTGGAAAGAAAT
SlMlo3 1776 SlMlo8 1521 SlMlo1 1524 SlMlo5 1554	A A

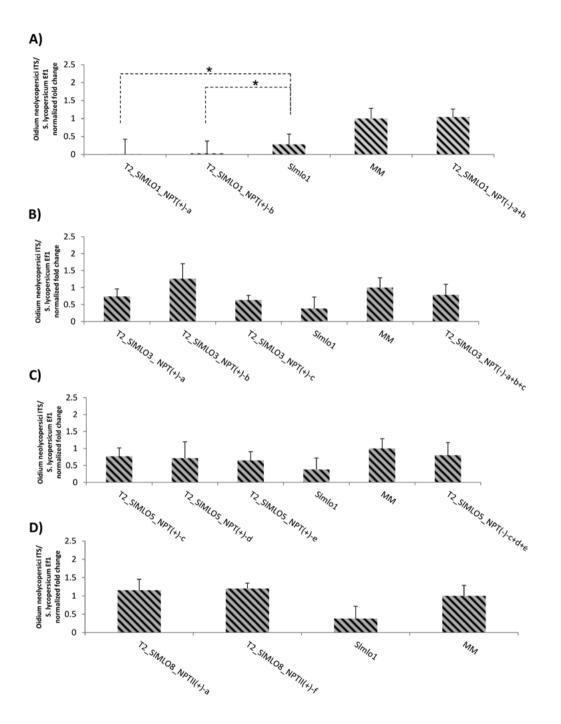
**SUPPLEMENTARY FIGURE 6.** Sequence alignment of the coding sequences of the four *SIMLO* homologs found in clade V, namely *SIMLO1*, *SIMLO3*, *SIMLO5*, and *SIMLO8*. The alignment was generated by CLC sequence view software using default parameter and given to BoxShade online server (http://sourceforge.net/projects/ boxshade/) to allow the shading. The fraction of sequences that must agree for shading was set as 1. Black shade indicates nucleotides that are fully conserved among the four sequences. Regions of each homolog used for the RNAi construct are highlighted in yellow, light blue, green and pink for *SIMLO1*, *SIMLO3*, *SIMLO8* and *SIMLO5* respectively. Primer pairs used to prepare each RNAi construct are listed in Supplementary Table 3.



**SUPPLEMENTARY FIGURE 7.** Relative expression level of the target *SIMLO* gene in T<sub>1</sub> plants obtained with the RNAi silencing construct for *SIMLO1* (panel A), *SIMLO3* (panel B), *SIMLO5* (panel C) and *SIMLO8* (panel D), respectively. Bars shows standard error of three technical replicates.







**SUPPLEMENTARY FIGURE 9.** Fungal biomass measured by relative quantification of the ratio between Oidium neolycopersici and plant gDNAs on independent  $T_2$  families obtained with the silencing constructs targeting SIMLO genes of clade V. Panel A and panel D refer to the two  $T_2$  families tested in this study obtained with the constructs designed to silence SIMLO1 and SIMLO8, respectively. Panel B and panel C refer to the three  $T_2$  families obtained with the constructs designed to silence SIMLO1 and SIMLO8, respectively. Panel B and panel C refer to the three  $T_2$  families obtained with the constructs designed to silence SIMLO3 and SIMLO5, respectively. The SIMLO gene name and extension "a" to "f" of the  $T_2$  families refer to the ones in Supplementary Fig. 7.

For MM and SImIo line in each panel, bars show standard errors based on 10 plants. In panel A, bars show standard errors of four transgenic plants (NPT+) for each of the two  $T_2$  families (T2\_SIMLO1-a and -b) and four plants that do not carry the silencing construct obtained from the two  $T_2$  families. Asterisks refer to significant differences compared to the SImIo1 line (P < 0.01; Student's t-test). In panel B, bars show standard errors of 10 transgenic plants of the T2\_SIMLO3-a and -b families, eight plants of the T2\_SIMLO3-c family and seven plants that do not carry the silencing construct obtained from the three families. In panel C, bars show standard errors of nine transgenic plants for each of the three  $T_2$  families (T2\_SIMLO5-c, -d and -e) and six plants that do not carry the silencing construct obtained from the three families. In panel C, bars show standard errors of 10 transgenic plants for each of the three  $T_2$  families. In panel D, bars show standard errors of 10 transgenic plants for each of the three families. In panel D, bars show standard errors of 10 transgenic plants for each of the two T\_2 families (T2\_SIMLO8-a and -f). For these two T\_2 families, we did not find plants without the NPTII marker.

# Chapter 3

# Functional characterization of a tomato syntaxin involved in powdery mildew resistance

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> <sup>†</sup>these authors contributed equally to the work <sup>\*</sup> corresponding author

> > Intended for publication in Phytopathology

# Abstract

The Arabidopsis syntaxin AtPEN1, as well as its barley and grapevine orthologs, contribute to the resistance conferred by loss-of-function mutations of MLO susceptibility genes (known as mlo resistance) and to non-host resistance against powdery mildew fungi. In this study, a genome-wide survey allowed the identification of 21 tomato syntaxins. Two of them, named SIPEN1a and SIPEN1b, are closely related to AtPEN1. RNAi-based silencing of SIPEN1a in a tomato mlo mutant restored its susceptibility to the tomato powdery mildew fungus Oidium neolycopersici. Moreover, it resulted in a significant increase of the penetration rate of the non-adapted powdery mildew fungus Blumeria graminis f. sp. hordei. Codon-based evolutionary analysis and multiple alignments allowed the detection of amino acids that are specifically conserved in syntaxins involved in pathogen defense. Our findings provide insights on the evolution of plant syntaxins and show that SIPEN1a is involved in tomato mlo resistance to both adapted and non-adapted powdery mildew fungi. Differently from Arabidopsis but similarly to barley, the impairment of a single syntaxin is sufficient to break *mlo* resistance in tomato.

### **Keywords**

mlo resistance, non-host resistance, Blumeria graminis f. sp. hordei, Oidium neolycopersici, tomato syntaxins

# Introduction

In eukaryotic cells, compartmentalization through the endomembrane apparatus and exocytosis requires a highly regulated transport system. Soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins are involved in such a transport, as they mediate the fusion between membranes of cargo-containing small shuttles, referred to as vesicles, with target membranes (14).

SNARE proteins were previously classified according to their localization into vesicleassociated (v-SNARE) and target-membrane-associated (t-SNARE) proteins (26). An alternative classification distinguishes Q-SNARE from R-SNARE proteins by the presence of either arginine or glutamine at the centre of the SNARE domain, respectively (8). Typically, SNARE complexes which determine membrane fusion are tetrameric coiled-coil structures containing one R-SNARE protein anchored to the vesicle and one protein of each Q-SNARE family, conform to the so-called 3Q-plus-1R rule (8). Q-SNAREs are further divided into three families: Qa- (also referred to as syntaxins or SYPs), Qb- and Qc-SNAREs (4, 24). Based on sequence homology, syntaxins can be assigned to five subfamilies which are known as SYP1, -2, -3, -4, -8 (4, 23).

The powdery mildew disease, affecting thousands of plant species, is caused by widespread fungal species of the Ascomycete order of Erysiphales (27). Specific members of the *Mildew Locus O (MLO)* gene family act as susceptibility factors for powdery mildew fungi, as their loss-of-function mutations result in a particular form of resistance, referred to as *mlo* resistance (19). At the histological level, *mlo* resistance is associated with the failure of fungal penetration into host epidermal cells, as a consequence of the formation of thick cell wall appositions termed papillae (25). A number of studies suggests that MLO proteins modulate SNARE-dependent exocytosis at plant-pathogen interaction sites, which allows discharging of antimicrobial compounds (18). In barley, a syntaxin encoding gene, *HvROR2*, was shown to be required for *mlo* resistance (5, 7, 10). Similarly, in Arabidopsis, loss of function of the *HvROR2* ortholog *AtPEN1* comprised the powdery mildew resistance of the *Atmlo2* mutant (6).

Syntaxins have also been shown to play a major role in non-host resistance to powdery mildew fungi. In Arabidopsis, compared to the wild type, the *Atpen1* mutant allowed a significant higher penetration rate of the non-adapted barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*) (5). The Arabidopsis *SYP122* gene encodes a syntaxin closely related to AtPEN1 and its impairment also resulted in a slight increase (10%) of fungal penetration, which was however not reproducible (2).

In barley, the *ROR2* gene is the *AtPEN1* ortholog. The barley *mlo ror2* double mutant showed a higher penetration rate of the wheat powdery mildew fungus *Blumeria graminis f. sp. tritici* (*Bgt*) than the single *mlo* mutant. The further growth of the non-adapted *Bgt* in

the barley *mlo ror2* double mutant was blocked since cells successfully penetrated by *Bgt* showed hypersensitive response (HR) (5, 15, 28).

In this study, we exploited available genomic information to identify and characterize two tomato syntaxins. Using RNA interference technology (RNAi) functional role of the two tomato syntaxins was studied in the interactions of a tomato *mlo* mutant with the powdery mildew fungi *Oidium neolycopersici* (adapted) and *Bgh* (non-adapted). Evolutionary analysis and multiple alignments were performed to identify amino acids differentiate the two tomato syntaxins.

# **Materials and methods**

### Plant and fungal materials

The tomato *Slmlo1* mutant, carrying a loss-of-function mutation of the *SlMLO1* susceptibility gene (3), and the susceptible tomato cultivar (cv) Moneymaker (MM) were used in the disease tests as negative and positive controls, respectively. The tomato *Slmlo1* mutant was also used as the background of RNAi.

The Wageningen isolate of tomato powdery mildew *O. neolycopersici* has been maintained on cv MM plants. The Wag04 isolate of barley powdery mildew *Bgh* was maintained on the susceptible barley cv Manchuria. Infected tomato and barley plants were kept in a greenhouse compartment at  $20 \pm 2^{\circ}$ C with  $70 \pm 15$  % relative humidity at the Unifarm of Wageningen University & Research, The Netherlands.

#### Phylogenetic study of syntaxins in tomato

To retrieve the putative Qa SNARE family in tomato proteome, the Arabidopsis syntaxin AtPEN1 amino acid sequence (Genebank ID:NP\_187788.1) was used as query for a tBLASTn search against the Tomato Genome CDS of Sol Genomics Network (SGN) using default settings. All the 21 putative tomato homologs retrieved, together with the complete Arabidopsis Qa SNARE family and the AtPEN1 grapevine and barley orthologs (VvPEN1 and HvROR2), for a total of 41 sequences, were used for a ClustalW alignment. The gap open cost and the gap extension cost were set equal to 10 and 4, respectively. Afterward, an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree was built setting the bootstrap value equal to 100. All the bioinformatic analyses were performed using the CLC sequence viewer software (http://www.clcbio.com/).

### **Generation of tomato RNAi transformants**

To generated RNAi constructs, the two tomato cDNA sequences showing the highest similarity to AtPEN1, Solyc10g081850.1.1 (named SIPEN1a) and Solyc01g006950.2.1 (named SIPEN1b), were used to design primer pairs for Gateway cloning (Invitrogen), using the Primer3 software (30). These primers, named SIPEN1a\_gate\_Fw/Rev (5'-caccCTGGTTGTGGACCTGGAAGT- 3'/ 5'- TGTCCTCTTCCTTGCTCCTG-3') and SIPEN1b gate\_Fw/Rev (5'- caccACGAGCTGAAAAACCTCGAA-3'/ 5'- ACAACAGACGTCCTCGTCCT-3'), allowed the amplification of regions of 259bp and 250bp, for SIPEN1a and SIPEN1b respectively. Amplification was conducted using PfuUltrall Fusion HS DNA Polymerase (Agilent Technologies). PCR fragments were first cloned into the Gateway-compatible vector pENTR D-TOPO (Invitrogen) and then transferred to Escherichia coli competent cells (XL10-Gold Ultracompetent Cells, Agilent Technologies). Presence of the inserts was assessed by colony PCR, restriction enzyme digestion and sequencing using the universal M13 primer pair. Positive clones were used for a LR reaction, which allowed the inserts to be transferred into the pHELLSGATE12 vector, harbouring the 35S Cauliflower Mosaic Virus (CaMV) promoter for constitutive expression and the marker gene NPTII for kanamycin resistance selection. Plasmids were cloned into E. coli competent cells DH5a and positive colonies were screened by colony PCR and sequencing as described before. Recombinant vectors were finally extracted and transferred to the AGL1+virG strain of Agrobacterium tumefaciens by electroporation.

The tomato *Slmlo1* mutant was used for transformation, as described by Appiano *et al.* (1). For each construct, several T<sub>1</sub> plants were generated and allowed to self-pollinate to obtain T<sub>2</sub> families. To select transgenic plants of each segregating family, T<sub>2</sub> plants were screened with primer pairs NPTII\_Fw/ NPTII\_Rev (5'-TCGGCTATGACTGGGCACAAC-3')/5'-AAGAAGGCGATAGAAGGCGA-3'), and 35S-Fw/Rev (5'-GCTCCTACAAATGCCATCA-3')/ (5'-GATAGTGGGATTGTGCGTCA-3'). By real-time qPCR, expression of each gene in selected T<sub>2</sub> families was assessed using the primer pairs qPEN1a\_Fw/Rev (5'-CGAGATGCTTTGTGCATCAG-3'/5'- CAGTCTCCTTCAGCTCCATTTC-3') and qPEN1b\_Fw/ Rev (5'- TGGTTTAGTTGTTGATGGACCTC- 3'/ 5'- ACCCCCATCCAACTTACTTACTTCC-3'). Selected transgenic T<sub>2</sub> plants of each construct were crossed in order to obtain F<sub>1</sub> individuals in which both genes are silenced. Four-week-old F<sub>1</sub> plants were tested through qPCR for the expression of S*IPEN1a* and S*IPEN1b*, using the above mentioned primer pairs.

# Disease tests with O. neolycopersici and quantification of fungal biomass

 $T_2$  families obtained from individual  $T_1$  plants and  $F_1$  individuals silenced for both *SIPEN1a* and *SIPEN1b* were challenged with *O. neolycopersici*. Inoculation was performed as described by Pavan *et al.*(20), by spraying plants with a suspension of conidiospores obtained from heavily infected leaves of MM plants and adjusted to a final concentration

of 2\*10<sup>4</sup> spores per millilitre. Inoculated plants were grown at 20 ± 2°C with 70 ± 15 % relative humidity and day length of 16 h in a greenhouse of Unifarm of Wageningen University & Research, The Netherlands. Disease evaluation was carried out 15 days after inoculation, powdery mildew symptom was visually scored using the scale of 0 to 3 (3) and fungal biomass was quantified by real-time qPCR as reported by Huibers *et al.* (11). Briefly, plant and fungal genomic DNAs were isolated from infected tomato leaves using the Qiagen DNeasy Plant Mini Kit and amplified with the primer pairs On-Fw (5'-CGCCAAAGACCTAACCAAAA-3')/On-Rev (5'-AGCCAAGAGATCCGTTGTTG-3'), designed on *O. neolycopersici* internal transcribed spacer (ITS) sequences (GenBank accession number EU047564), and Ef-Fw (5'-GGAACTTGAGAAGAGAGCCTAAG-3')/Ef-Rev (5'-CAACACCAACAGCAACAGTCT-3'), designed on the tomato *Elongation Factor* 1α (*Ef1*α) reference gene (17). Relative fold-change of the ratio between fungus and tomato gDNAs was inferred by the 2<sup>-ΔACt</sup> method (16, 21) and results were analyzed by the Student's t-test.

### **Histological analysis**

Three T<sub>2</sub> transgenic plants of each silencing construct (*SIPEN1a* and *SIPEN1b*) were selected by PCR using 35S and *NPTII* primer pairs. Around 18 days after sowing, these plants, together with three plants from the *SImlo1* mutant, were transferred into an infection chamber. Dry inoculum of *B*gh was applied on the third and fourth leaves of four-week old tomato plants using a paintbrush to brush off the conidia from heavily infected barley leaves. At least three samples for each inoculated plant were collected 72 hours post-inoculation (hpi). These samples were stained with trypan blue as described by Freialdenhovenet al. (10) and mounted on glass slides with a 1:1 (v/v) glycerol-water solution. Observation of the slides was done using a Zeiss Axiophot bright field microscope and pictures were taken with an Axiocam ERc5s. For each sample, more than thirty infection units (one infection unit represents a germinated *On* spore) per slide were observed. The pathogen penetration rate was estimated as the percentage of units displaying HR (28). Statistical analysis was carried out using the Student's t-test.

#### Detection of conserved syntaxin motifs and codons

In order to detect conserved motifs in the syntaxin family, the whole 41 syntaxins dataset, which was used for the phylogenetic analysis above, was used as input for the BOXSHADE software (http://www.ch.embnet.org/software/BOX\_form.html), using 1.0 as fraction of sequences value. Furthermore, with the aim of identifying residues specifically conserved in syntaxins acting in defense against powdery mildew fungi, another ClustalW alignment was performed, using proteins previously indicated to be involved in plant-pathogen interactions (AtSYP121, AtSYP122, HvROR12, VvPEN1), and the two tomato syntaxins SIPEN1a and SIPEN1b. This was used as input for the BOXSHADE software. Moreover, the

same dataset was used for a codon-based evolutionary analysis, based on the difference of nonsynonymous-to-synonymous substitutions per nonsynonymous and synonymous sites (dN-dS). This was performed with the Single-likelihood Ancestor Counting (SLAC) method implemented by the Datamonkey web server (www.datamonkey.org). In order to make predictions on the kind of selection pressure (negative, neutral or positive) acting on each codon, the default p-value of 0.1 was used as threshold for significancy.

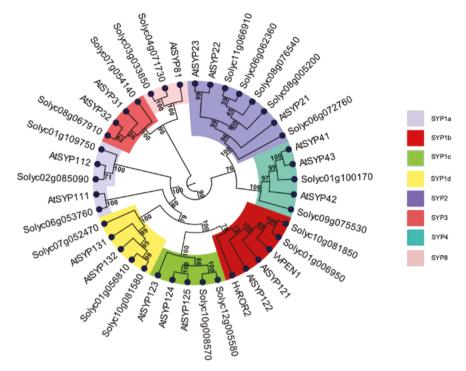
# Results

# In silico identification of tomato syntaxins

In the tomato genome, 21 putative syntaxins were retrieved from a BLAST search using the Arabidopsis syntaxin AtPEN1 amino acid sequence (Genebank ID:NP\_187788.1). With these 21 putative tomato syntaxins, a phylogenetic tree was constructed together with 18 syntaxins of Arabidopsis and two functional AtPEN1ortholog, barley HvROR2 and grapevine VvPEN1 (9). In the phylogenetic tree (Fig. 1), Arabidopsis syntaxins of the five syntaxin subfamilies (SYP1, -2, -3, -4, -8) could be assigned to five separate clades and were named accordingly. Each clade has at least one tomato putative syntaxin. The most represented clade (SYP1), harbouring 21 homologs, was further partitioned in four subclades (indicated as SYP1a-d in Fig.1).

With strong bootstrap support, two tomato putative syntaxins, Solyc10g081850 and Solyc01g006950, were assigned to the subclade SYP1b, including all the homologs functionally shown to be involved in PM resistance, i.e. AtPEN1, HvROR2 and VvPEN1. Therefore, Solyc10g081850 and Solyc01g006950 were renamed *SIPEN1a* and *SIPEN1b*, respectively (Fig.1).

Protein sequences of SIPEN1a and SIPEN1b are highly related with each other (76.8% nucleotide identity and 85.3% amino-acid conservation) and with AtPEN1 (64.2% and 60.4% nucleotide identity, 72.3% and 71.7% amino-acid conservation respectively).

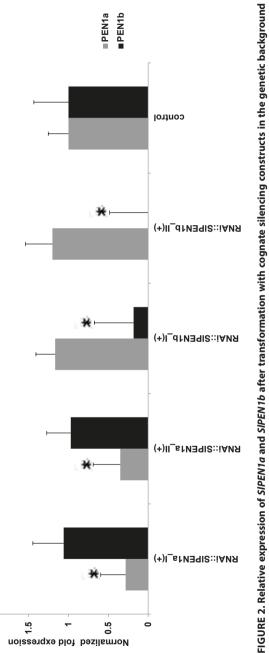


**FIGURE 1. Phylogenetic relationships of 41 syntaxins**. The dataset includes barley HvROR2, grapevine VvPEN1, the 18 Arabidopsis syntaxins (AtSYPs and AtPEN1) and the 21 predicted tomato syntaxins identified in this study (named with the SolGenomics Network database ID code). Numbers at nodes indicate bootstrap support values. Clades are named according to the nomenclature used for syntaxins subfamilies (SYP1, -2, -3, -4, -8). The clade SYP1 is further divided in four subclades (a-d). The subclade SYP1b, containing all the homologs known to be involved in plant-pathogen interactions (AtPEN1, SYSYP122, HvROR2 and VvPEN1), is highlighted in red.

#### SIPEN1a is required for tomato PM resistance

To gain insights on the functional role of *SIPEN1a* and *SIPEN1b*, we prepared RNAi silencing constructs for each of the two genes, which were used to transform the tomato *SImlo1* mutant. Eleven RNAi::*SIPEN1a* and four RNAi::*SIPEN1b* T<sub>1</sub> plants were obtained and self-pollinated to produce T<sub>2</sub> families. Meanwhile, two cuttings per transformant were made and tested with tomato powdery mildew *O. neolycopersici*. Cuttings of all transformants showed more powdery mildew sporulation compared to the background, the tomato *SImlo1* mutant (data not shown).

For each construct, two independent segregating  $T_2$  families (referred to as RNAi::*SIPEN1a*-I, RNAi::*SIPEN1a*-II, RNAi::*SIPEN1a*-II, RNAi::*SIPEN1b*-I and RNAi::*SIPEN1b*-II) were selected and further characterized in a new experiment. Based on the presence of the silencing construct, transgenic  $T_2$  individuals of each  $T_2$  family were selected (15 plants per family), which were further referred to as RNAi::*SIPEN1a*(+) and RNAi::*SIPEN1b*(+). As control for comparison, 5



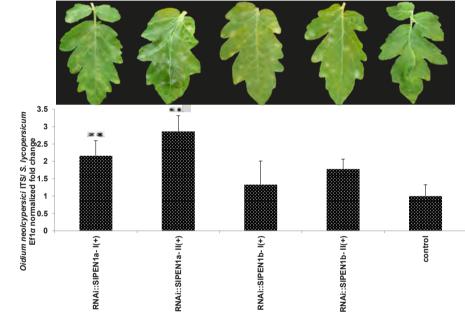


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non transgenic plants per  $T_2$  family were pooled with 5 plants of the *Slmlo1* mutant line since they are genetically identical. The expression level of the *SlPEN1a* and *SlPEN1b* was significantly reduced in the RNAi::*SlPEN1a*(+) and RNAi::*SlPEN1b*(+) individuals compared to the control (Fig.2). Further, no unwanted cross-silencing was found between the two target genes. RNAi::*SlPEN1a*(+) individuals showed clear fungal sporulation (Fig. 3A) and significantly increased fungal biomass (Fig. 3B) compared to the control plants. Although RNAi::*SlPEN1b*(+) individuals showed more fungal colonies than control plants, the fungal biomass was not significantly increased (Fig. 3B). Similar results were obtained by repeating the experiment.

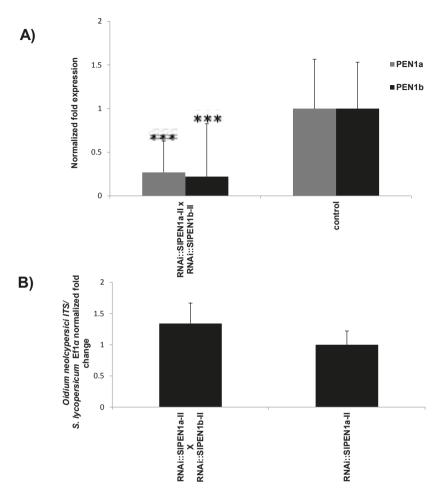


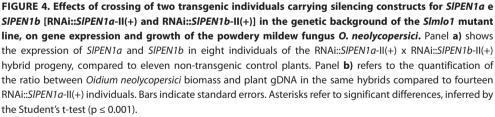
B)



**FIGURE 3.** Effects of RNAi silencing of tomato *SIPEN1a* and *SIPEN1b* in the genetic background of the *SImlo1* **mutant line on the interaction with the powdery mildew fungus** *Oidium neolycopersici*. Panel **a**) shows the phenotype of leaves collected fifteen days after pathogen inoculation. From left to right: selected transgenic individuals of two independent  $T_2$  families carrying the *SIPEN1a* silencing construct [RNAi::*SIPEN1a*-Il(+)], selected individuals of two independent  $T_2$  families carrying the *SIPEN1a* silencing the *SIPEN1b* silencing construct [RNAi::*SIPEN1a*-Il(+)] and RNAi::*SIPEN1a*-Il(+)] and an individual of the *SImlo1* resistant line. Panel **b**) refers to the relative quantification of the ratio between *Oidium neolycopersici* and tomato gDNAs in fifteen transgenic individuals of the same families above mentioned and in control plants, formed by five non-transgenic  $T_2$  individuals and five *SImlo1* individuals. Bars indicate the standard errors. Asterisks refer to significant differences with the control, inferred Student's t-test (\*\* = p<0.01; \*\*\* = p< 0.001).

In order to investigate whether *SIPEN1b* has an additive effect to *SIPEN1a*, we performed an additional experiment using the F<sub>1</sub> progeny obtained by crossing RNAi::*SIPEN1a*-II(+) and RNAi::*SIPEN1b*-II(+) individuals. Eight F<sub>1</sub> individuals with significantly reduced expression of both *SIPEN1a* and *SIPEN1b* were selected (Fig. 4A). Although in these individuals, higher fungal biomass (34%) compared to RNAi::*SIPEN1a*-II(+) individuals was quantified, the difference was not significant (Fig. 4B).

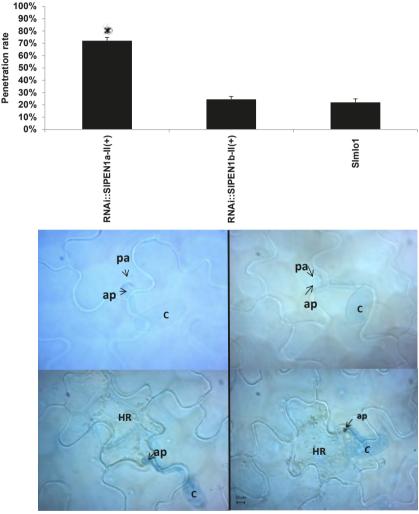




# Role of SIPEN1a and SIPEN1b in non-adapted powdery mildew penetration

Since it has been shown that *PEN* genes are involved in penetration resistance to both adapted and non-adapted powdery mildew, we set-up a functional assay to investigate the role of the two tomato syntaxins, *SIPEN1a* and *SIPEN1b*, in the interaction with barley powdery mildew fungus *Bgh*. An artificial inoculum of this pathogen was used to inoculate RNAi::*SIPEN1a*-II(+) individuals, RNAi::*SIPEN1b*-II(+) individuals, and *SImlo1* plants. The rate

of infection units showing HR dramatically increased from 22% to 72% in RNAi::*SIPEN1a*-II(+) individuals compared to the *SImIo1* mutant line (Fig. 5). A slight increase (2.5%) of HR was also found in RNAi::*SIPEN1b*-II(+) plants, although this value was not statistically different from the one of the *SImIo1* mutant (Fig.5).



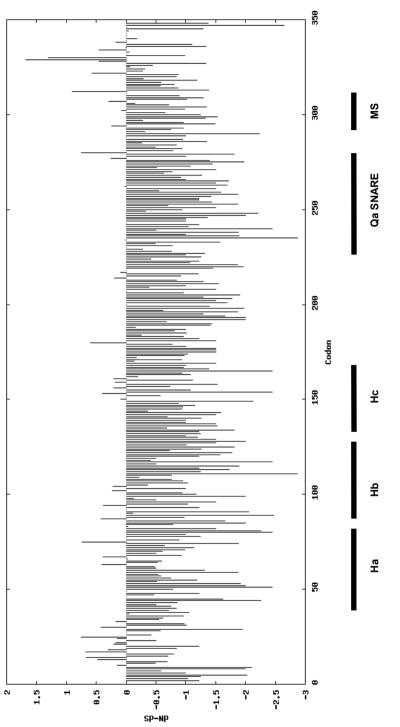
SImIo1 line

RNAi::SIPEN1a-II(+)

FIGURE 5. Effects of RNAi silencing of tomato *SIPEN1a* and *SIPEN1b* on the interaction with the nonadapted powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. The upper panel shows the penetration rate of *Blumeria graminis* f. sp. *hordei* on the *SImlo1* mutant line and transgenic individuals silenced for *SIPEN1a* and *SIPEN1b* [RNAi::*SIPEN1a*-II(+) and RNAi::*SIPEN1b*-II(+)], indirectly inferred as the proportion of infection units showing post-penetrative hypersensitive response. Bars refer to standard errors calculated on three biological replicates. Asterisks indicate significant difference with the *SImlo1 mutant line*, inferred by the Student's t-test (p  $\leq$  0.05). The bottom panel shows the microscopic observation of papillae (pa) and hypersensitive response (HR) in epidermal cells of the *SImlo1* mutant line (left) and RNAi::*SIPEN1a*-II(+)individuals (right) challenged with the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. c=conidium of; a=appressorium.

# Possible molecular features required for the function of syntaxins in powdery mildew penetration

Aiming to investigate the magnitude and direction of natural selection acting on syntaxins involved in defense against powdery mildew fungi, we used SIPEN1a, AtPEN1, HvROR2 and VvPEN1 nucleotide sequences for a codon-based single-likelihood ancestor counting (SLAC) evolutionary analysis. This is based on the dissimilarity level between non-synonymous substitution (dN) and synonymous substitution (dS) values. The SIPEN1b and AtSYP122 were also included since they might play a minor role in tomato and Arabidopsis powdery mildew penetration. Evidence for negative selection was found on 77 codons, associated with amino acid residues scattered in syntaxin protein domains (Qa-SNARE, membrane-spanning, and the three helix domain known as Ha, Hb, Hc) (Fig.6 and Supplementary Tab. S1). Alignment of the same subclade SYP1b proteins revealed that 65 of these residues are invariable throughout the dataset, suggesting they might play a crucial role in protein function. In order to identify syntaxin residues specifically important in pathogen defense, we performed an additional alignment with the same set of syntaxins used for phylogenetic analysis. In total, we found that 24 out of 65 residues that are invariable in the SYP1b sub-clade are not conserved in other syntaxins (Supplementary Fig. S1). In addition to these 65 invariable residues, eight amino acid differences were found between AtPEN1/SIPEN1 and AtSYP122/SIPEN1b (Supplementary Fig. S2). These eight amino acids do not belong to the 77 codons under negative selection (Fig.6 and Supplementary Tab. S1). Intriguingly, three of the eight, A24, Q31 and H72, are also biochemically different (highlighted in red in Supplementary Fig. S2). In particular, H72 belongs to the regulatory motif Ha.





# Discussion

#### The tomato syntaxin family

In this study, a genome-wide survey allowed the identification of 21 syntaxins in the tomato genome. The number of tomato homologs is consistent with the results of previous genome-wide studies analyzing 18 syntaxins in *A. thaliana*, 14 in *O. sativa* and 22 *P. trichocarpa* (14, 22, 29). The identification of ten putative tomato SYP1 members corroborates previous findings indicating that this subfamily, containing homologs localized in the plasma membrane, is more represented in plants than the other subfamilies SYP2, -3, -4, and -8 (14) (Supplementary Tab. S2).

It is known that, specific SYP1 homologs in Arabidopsis are involved in other physiological processes besides defence against pathogens. Specifically, SYP111 (called *KNOLLE* protein) controls cytokinesis (13), whereas SYP123 and SYP132 mediate root hair tip growth (12). Phylogenetic analysis presented in this study clearly suggests that the tomato syntaxin Solyc06g053760 is an ortholog of Arabidopsis SYP111 (Fig.1). Evolutionary relatedness between SYP132 and a couple of tomato syntaxins, Solyc01g056810-Solyc10g081580 (Fig.1), might indicate that these genes are SYP132 co-orthologs due to a recent duplication event after the split of two lineages from an Arabidopsis-tomato common ancestor.

### Involvement of tomato syntaxins in plant-pathogen interaction

Two tomato syntaxins, SIPEN1a and SIPEN1b, are grouped in a phylogenetic clade also containing homologs previously reported to play a major (AtPEN1, HvROR2 and VvPEN1) or minor (AtSYP122) role in penetration resistance to PM fungi (Fig.1). Silencing *SIPEN1a* compromised the penetration resistance to both adapted and non-adapted powdery mildew in the tomato *SImlo1* mutant line (Fig. 3 and 5), showing that *SIPEN1a* is likely the functional ortholog of *HvROR2*, *AtPEN1* and *VvPEN1*. Our data showed that *SIPEN1b* has a minor role in powdery mildew penetration resistance, which is in line with a previous study indicating that the Arabidopsis syntaxin AtSYP122 had a marginal influence on non-host powdery mildew resistance (2). Thus, it is very likely that the SIPEN1b is functionally related to AtSYP122.

Similarly to barley, we found that the impairment of the tomato SIPEN1a syntaxin in the *Slmlo1* mutant background macroscopically restores disease susceptibility to tomato powdery mildew. In contrast, in Arabidopsis, the *Atmlo2 pen1* double mutant, although being characterized by wild-type levels of fungal penetration, still displays a macroscopically resistant phenotype, due to enhanced post-penetration defense responses (6). AtPEN1 was shown to act as a negative regulator of the hormone salicylic acid, known to enhance post-penetration defense mechanisms against biotrophic fungi

(15, 31). It might be that, differently from AtPEN1, SIPEN1a and barley HvROR2 have minor or no role in the regulation of salicylic-acid-mediated defense pathways. In Arabidopsis, the *Atpen1 Atsyp122* double knock-out mutant displays a severely stunted and necrotic phenotype (2). In the present study, tomato individuals, silenced for both *SIPEN1a* and *SIPEN1b* homologs, were devoid of markedly visible pleiotropic effects which might be due to residual gene expression.

#### Amino acids of syntaxins playing a potential role in penetration resistance

In this work, a codon-based evolutionary analysis allowed us to detect 77 codons which are under significant negative selection in the SYP1b subclade containing all the six syntaxins associated with defence against powdery mildew fungi (AtPEN1, AtSYP122, HvROR2, VvPEN1, SIPEN1a and SIPEN1b). Moreover, alignment of all the 41 syntaxin sequences pointed out the occurrence of 24 residues specifically conserved in the SYP1b subclade (Supplementary Fig. S1). Three of these residues are located in the Qa-SNARE domain, whereas ten are found in the Ha, Hb or Hc helices of Qa-SNARE proteins. These auto-inhibitory motifs, when folded in a closed conformation, prevent the exposure of the Qa-SNARE domain and thus the formation of SNARE complexes (5).

Here we hypothesize a regulatory function of these conserved residues since, the HvROR2 $\Delta$ 31 barley mutant, harbouring a 31 amino acids deletion (S118-E148), strongly enhances its binding to HvSNAP34, a main protein of the SNARE complex(5). Interestingly, the deletion causing a constitutively open state of the protein, covers most of the Hc helix and involves four of the 24 conserved residues (namely G123, P124, T133 and G138 in HvROR2 sequence) identified in this study.

In addition to the above discussed 77 codons conserved in the SYP1b subclade, three amino acids with different biochemical functions were found between AtPEN1/SIPEN1 and AtSYP122/SIPEN1b (Supplementary Fig. S2). These three amino acids might play a role in AtPEN1/SIPEN1 for their involvement in *mlo* resistance to powdery mildew fungi. Future functional analyses on syntaxins, such as targeted mutagenesis, might unravel the role of these amino acids identified in this study.

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# **Supplementary tables**

**TABLE S1. Residues under significant negative selection in SYP1b syntaxins**. Residue numbers refer to amino acid position in the AtPEN1 protein. DN-dS values, corresponding p-values and residue position with respect to characteristic syntaxin domains (the three helix domain Ha-Hb- Hc, the Qa-SNARE domain and the membrane-spanning (MS) domain are reported.

Corresponding residue in AtPEN1	dN-dS	p-value	Domain
F5	-3.15883	0.06131	-
S8	-3.1351	0.02213	-
F9	-3.28782	0.09351	-
N43	-3.52804	0.05074	Ha
L44	-2.54257	0.05726	Ha
D50	-3.83057	0.01861	Ha
V51	-3.1351	0.01235	Ha
E52	-3.00033	0.03562	Ha
L58	-2.94697	0.01581	Ha
E73	-2.94552	0.03696	Ha
H79	-3.83057	0.01861	Ha
N80	-3.52804	0.05074	-
A81	-2.35132	0.03704	-
V84	-3.1351	0.01235	-
R88	-3.87355	0.01415	Hb
K90	-3.21478	0.06364	Hb
V95	-2.35132	0.03704	Hb
A98	-3.1351	0.01235	Hb
E110	-4.49517	0.00675	Hb
L112	-2.69996	0.02732	Hb
R114	-2.95551	0.01719	Hb
N116	-3.83057	0.01861	Hb
R120	-2.46381	0.03418	Hb
G124	-2.8307	0.04851	Hb
G126	-2.35132	0.0434	-
P127	-3.1351	0.01235	-
G128	-2.35132	0.03841	-
R133	-2.8257	0.02516	Hc
R135	-2.39318	0.07344	Hc
T136	-2.35132	0.03704	Hc
G141	-2.35132	0.03868	Hc
L142	-2.48174	0.03486	Hc
L146	-1.80166	0.08323	Hc
D148	-3.33705	0.05724	Hc
F153	-3.83057	0.01861	Hc
R157	-2.39325	0.07349	Hc
Y164	-3.83057	0.02885	Hc
V168	-2.35132	0.03704	-
T174	-2.35132	0.03704	-
V175	-2.35132	0.03704	-

Corresponding residue in AtPEN1	dN-dS	p-value	Domain
T176	-2.35132	0.03704	-
P180	-2.35132	0.03704	-
L188	-2.21353	0.04439	-
T191	-3.1351	0.01235	-
G192	-3.1351	0.01249	-
E193	-2.58975	0.09884	-
E195	-2.93078	0.03733	-
F197	-3.08635	0.06377	-
L198	-2.18715	0.05751	-
A201	-2.35132	0.03704	-
1202	-2.77566	0.03544	-
E204	-2.9842	0.03601	-
G206	-2.35132	0.04295	-
1217	-2.27694	0.05266	-
E219	-2.92783	0.03741	-
R220	-1.6729	0.09756	-
L231	-2.45666	0.06387	Qa SNARE
E233	-4.49517	0.00675	Qa SNARE
L234	-2.95785	0.01765	Qa SNARE
Q236	-2.94552	0.04219	Qa SNARE
F238	-3.83057	0.01861	Qa SNARE
L244	-2.13677	0.06406	Qa SNARE
V245	-3.1351	0.01235	Qa SNARE
E246	-3.45719	0.06013	Qa SNARE
G249	-2.35132	0.04494	Qa SNARE
Q251	-2.92688	0.04273	Qa SNARE
L252	-2.24543	0.04253	Qa SNARE
1255	-2.22416	0.05519	Qa SNARE
E256	-2.93077	0.03733	Qa SNARE
V259	-2.35132	0.03704	Qa SNARE
R261	-2.64477	0.03867	Qa SNARE
A262	-2.35132	0.03704	Qa SNARE
G269	-2.35132	0.04325	Qa SNARE
L273	-3.09493	0.0138	Qa SNARE
R277	-2.82596	0.0199	Qa SNARE
R284	-2.12025	0.06354	-
C288	-3.49295	0.06064	Membrane-spanning

**TABLE S2.** Distribution of Arabidopsis thaliana, Oryza sativa, Populus trichocarpa and Solanum lycopersicum syntaxins in subfamilies.

SNARE type	SNARE subfamily	A. thaliana	O. sativa	P. trichocarpa	S.lycopersicum
	SYP1	9	7	11	10
	SYP2	3	3	3	5
Qa	SYP3	2	1	3	2
SNARE	SYP4	3	1	3	2
	SYP8	1	2	2	2
	tot	18	14	22	21

# Supplementary figures

AtPEN1						SFSRFRSGEPSPRRDVAGG0	
VvPEN1						SFSRFRSEEPPPS	
HvROR2 SlPEN1a						WKRAGAGGDGDLESG	
SIPEN1a SIPEN1b						FSRFRNEEQSPNQESAG	
AtSYP122	1	MN=====				SFSRYRENDHDQDSHG SFKTSVADGSSPPHS	-H=NIEWSKYKAA
AtSYP124	1	MN				SFKKYTDLKQQAQMD	
AtSYP123						SFKRYTDLNHQVQLD	
AtSYP111						SFMSYVDLKKAAMKDMEAGI	
AtSYP131						SLEFSRDRSNRS	
AtSYP125						SFKKNQAQLG	
AtSYP132						SFELPRGQSSREG	
AtSYP112						SFLSYVELKKQARTDMES	
Solyc12g005580						SFKKYQDLKKQTEVD	
Solyc10g008570	1	MN			DLFSP	SLKKYQDLKQQVQMD	DLELGTGGTGP
Solyc10g081580						DDNFDAPRHQSNRNG	
Solyc01g056810	1	M					
Solyc07g052470						S-SFIAGKDNASKES	
Solyc06g053760						SFTSYIDLKKAAMKDVEASI	
Solyc02g085090						SFLSYMELKKQAHLDLET	
Solyc01g109750	1	M			TK:	SFLSYVELKKQAMMDVEA	-GPDIEMGQ
Solyc06g062360							
Solyc11g066910							
Solyc08g005200							
Solyc08g076540							
AtSYP22							
AtSYP23							
AtSYP21							
Solyc06g072760							
AtSYP41 AtSYP43						SLTGTRSG-G	
						SSSTLTEHNSLTGAKSGI	
Solyc01g100170 AtSYP42						ASDSFC	
Solyc09g075530						KDS(	
Solyc03g033850							
Solyc04g071730							
AtSYP81							
AtSYP31							
Solyc07g054140							
AtSYP32							
Solyc08g067910							
					н	•	
						a	
						a	
AtPEN1	40	GGVN	DKFFED	VESVKEE			RLNETLSSCH
AtPEN1 VvPEN1					KE	LI	
	28	AGVN	DKFFE <mark>D</mark>	V <mark>e</mark> sikee	KE		SLQQKLHDAH <mark>E</mark> QS
VvPEN1	28 37	AGVN AGAS	ldkffe <mark>d</mark> Ldrffe <mark>d</mark>	V <mark>E</mark> SIKEE VESIKDD	LKE LRE	LL	SLQQKLHDAH <mark>E</mark> QS RIQRSLHDGN <mark>E</mark> SG
VvPEN1 HvROR2	28 37 33	AGVN AGAS GGVN	LDKFFE <mark>D</mark> LDRFFED LDKFFE <mark>D</mark>	V <mark>E</mark> SIKEE VESIKDD V <mark>E</mark> TIKDE	KE RE LRE		SLQQKLHDAHEQS RIQRSLHDGNESG KIHTQLHNSH <mark>E</mark> QS
VvPEN1 HvROR2 SlPEN1a	28 37 33 30	AGVN AGAS GGVN GGVN	LDKFFE <mark>D</mark> LDRFFED LDKFFED LDKFFE <mark>D</mark>	VESIKEE VESIKDD VETIKDE VEAIKDE	LKE LRE LRE LKE	]	SLQQKLHDAHEQS RIQRSLHDGNESG KIHTQLHNSHEQS KIYAQLQSSN <mark>E</mark> KS
VvPEN1 HvROR2 SlPEN1a SlPEN1b	28 37 33 30 35	AGVN AGAS GGVN GGVN GSCHGGNN	LDKFFED LDRFFED LDKFFED LDKFFED LDTFFLD	VESIKEE VESIKDD VETIKDE VEAIKDE VEAIKDE	KE LRE LRE LKE LKN LKE		SLQQKLHDAHZQS RIQRSLHDGNESG KIHTQLHNSHZQS KIYAQLQSSNEKS DRLCHNLRSSNEQS
VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122	28 37 33 30 <u>35</u> 29 32	AGVN AGAS GGVN GSCHGGNN E-TMN D-SGN	LDKFFED LDRFFED LDKFFED LDKFFED LDKFFED LDKFFED	VESIKEE VESIKDD VETIKDE VEAIKDE VENVKDN VENVKDN VESVKED	LKE LRE LKE LKE LKN LKR MKG		SLQQKLHDAHEQS RIQRSLHDGNESG KIHTQLHNSHEQS CKIYAQLQSSNEKS ORLCHNLRSSNEOS TIYKSLQDSNEEC DEIHKRLQDANESS
VvPEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP123 AtSYP123 AtSYP111	28 37 33 30 <u>35</u> 29 32 37	AGVN AGAS GGVN GGVN GSCHGGNN E-TMN D-SGN KADKMDEN	DKFFED DRFFED DKFFED DTFFLD DKFFED DKFFED DEFFGY SSFLEE	VESIKEE VESIKDD VETIKDE VEAIKDE VEAVNED VENVKDN VESVKED AEYVKAE	KE LRE LKE LKE KKE KG KA KA		SLQQKLHDAHQQS SRIQRSLHDGNESG KIIHTQLHNSHEQS SKIYAQLQSSNEKS ORLCHNLRSSNEOS JTLYKSLQDSNEEC DEIHKRLQDANEES SETLARIEQYHES
VvPEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP131	28 37 33 30 <u>35</u> 29 32 37 30	AGVN AGAS GGVN GGVN GSCHGGNN E-TMN D-SGN KADKMDEN SGDLG	LDKFFED LDRFFED LDKFFED LDKFFED LDKFFED LDEFFGY LSSFLEE LSGFFKK	VESIKEE VESIKDD VETIKDE VEAIKDE VENVNED VENVKDN VESVKED AEYVKAE VQEIEKQ	KE KE KE KE KE KG KA KA KA KA KA		SLQQKLHDAH QS RIQRSLHDGNSG SKIHTQLHNSHQS KIIYAQQSNEKS DRLCHNLRSSNEOS DEILKRLQDANES DEIHKRLQDANES DEILARLEQYHES DKHLNKLQGAHET
VvPEN1 HvRCR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP131 AtSYP131 AtSYP125	28 37 33 30 35 29 32 37 30 24	AGVN AGAS GGVN GSCHGGNN E-TMN D-SGN KADKMDEN SGDLG E-TMN	LDKFFED LDRFFED LDKFFED LDKFFED LDTFFLD LDEFFGY LSSFLEE LSGFFKK LDKFFED	VESIKEE VESIKDD VETIKDE VEAIKDE VENVKDD VENVKDN VESVKED AEYVKAE VQEIEKQ VENVKDD	LKE LRE LKE LKE LKE KA MKG ZEK ZEK ZEK	11 MB 14 14 14 14 14 14 14 14 14 14 14 14 14	SLQQKLHDAHBQS RIQRSLHDGNESG XHITQLHNSHBQS XHITQLQSNEKS DRLCHNLRSSNEOS TLYKSLQDSNEC DEIHKRLQDANES SETLARIEQYHES XHLNKLQGAHEET CALYKLQDSNEC
VvPEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP131 AtSYP132 AtSYP132	28 37 33 30 35 29 32 37 30 24 29	AGVN AGAS GGVN GGVN GE-TMN D-SGN KADKMDEN SGDLG GDLG GGDQG	DKFFED DRFFED DKFFED DTFFLD DKFFED DEFFGY SSFLEE SGFFKK DKFFED EDFFKK	VESIKEE VESIKDD VETIKDE VEAIKDE VENVKDD VENVKDD VESVKED AEYVKAE VQEIEKQ VENVKDD VQVIDKQ	KE RE KE KKE KG KGL KGL KGC KGC KGC KGC		SLQQKLHDAH QS RIQRSLHDGN SG KIHTQLINNH QS SKIYAQLQSSN KS SRLCHNIRSSN QS TILYKSLQDSN EC DE HIKRLQDAN EC SETLARIEQYH ES SKHLNKLQGAN ET KLLYKKLQASH ES
VvPEN1 HvROR2 SIPEN1a SIPEN1a AtSYP122 AtSYP123 AtSYP123 AtSYP131 AtSYP132 AtSYP132 AtSYP132	28 37 33 30 35 29 32 37 30 24 29 39	AGVN AGVN GGVN GSCHGGNN D-SGN KADKMDEN SGDLG GGDQG FSPADEEN	LDKFFED LDRFFED LDKFFED LDTFFLD LDEFFGY LSSFLEE LSGFFKK LDKFFED LEDFFKK	VESIKEE VESIKDD VETIKDE VEAIKDE VEVVNED VEVVNED VESVKED AEYVKAE VQEIEKQ VENVKDD VQVIDKQ IETIKTL	KE RE KE KK KGL		SLQQKLHDAH QS SRIQRSLHDGM SG SKITAQLQSSNEKS SKITAQLQSSNEKS SRLCHNLRSSN OS STLYKSLQDSNEC DEIHKRLQDAN ES SETLARIEQYH ES SKHLNKLQGAN ET SKLLKKLQASNE S
VvPEN1           HvROR2           SlPEN1a           SlPEN1b           AtSYP12           AtSYP124           AtSYP123           AtSYP131           AtSYP132           AtSYP132           AtSYP132           SlYP152           Slyp152           Slyp1550	28 37 33 30 35 29 32 37 30 24 29 39 33	AGVN AGVN GGVN GSCHGGNN D-SGN KADKMDEN SGDLG GGDLG GGDLG GGDQG GG-TE-SID G-TE-SID	DKFFED DRFFED DKFFED DTFFLD DEFFGY SSFLEE SGFFKK LDKFFED LDKFFED LCKFFED	VESIKEE VESIKDD VETIKDE VEAIKDE VEVVNED VEVVNED AEYVKAE VQEIEKQ VQEIEKQ VQVIDKQ IETIKTL VENVKED	KE LRE LRE LKE KG /GL /GL /GL /GL /GL /GL /GL /KG		SLQQKLHDAH QS RIQRSLHDGN SG KITAQLOSSN KS RIQRSLHNSH QS KITAQLQSSN KS RICHNESSN OS FILVRSLQDSN EC DEIHKRLQCAHET KHLNKLQCAHET KLLKKLQASH ES HLLLDLQNLN ET HLLDLQNN EC
VvPEN1           HvROR2           SlPEN1a           SlPEN1b           AtSYP122           AtSYP123           AtSYP131           AtSYP132           AtSYP125           AtSYP132           AtSYP130           Slpc10g005580           Solyc10g005870	28 37 33 29 32 37 30 24 29 39 33 33 34	AGVN AGVN GGVN GGVN GCVN CSCHGGNN E-TMN SGDLG GDLG FSPADEEN GDLG FSPADEEN G-TE-SID SHNE-SID	LDKFFED LDRFFED LDKFFED LDKFFED LDEFFGY SSFLEE SGFFKK LEDFFKK LSGFFQE LSGFFQE LSGFFQE LAKFFED	VESIKEE VESIKDD VETIKDE VEAIKDE VEVVNED VEVVNED VENVKDN VESVKED VEVVKAE VQEIEKQ VQUIDKQ UETIKTL VENVKED	KE LRE LRE LKE KG /GL /GL /GL /GL /GL /GL /GL /GL /GL /KG	11 MM MM M1 12 14 17 17 17 17 17 17 17 17 17 17 17 17 17	SLQQKLHDAH QS RIQRSLHDGN SG KKITQLQSSNEKS SKITAQLQSSNEKS SKLTNLKSLQDSNEC DEIHKRLQDSNEC DEIHKRLQDSNEC KHLNKLQGAH ET KKLLKKLQASHES KHLLLDLQNLNET KFHKKLQSSNES
VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP123 AtSYP125 AtSYP125 Solyc12g005580 Solyc10g00870 Solyc10g081580	28 37 33 30 29 32 37 30 24 29 39 33 34 34	AGVN AGVN GGVN GSCHGGNN D-SGN KADKMDEN SGDLG FSPADEEN G-TE-SID SINE-SID SGELG	DKFFED DRFFED DKFFED DFFFLD DKFFED DEFFGY SSFLEE SGFFKK SGFFKK SGFFKK SGFFKE AKFFED AKFFED	VESIKEE VESIKDD VETIKDE VENTKDE VENVKDN VESVKED AEYVKAE VQEIEKQ VENVKDD VQVIDKQ VENVKED VENVKED VENVKED	KE RE KE KR KR KG		SLQQKLHDAH QS RIQRSLHORNSG XKITAQLQSNNKS RICHNLRSNN OS TTLYKSLQDNN EC DEIHKRLQDAN ES XKHLNKLQGAH ET XLJYKKLQDSN EC XKLLKKLQASH ES XKHKKLQESN ES XKHKKLQESN ES XKLHKRLQDSN ES
VvPEN1           HvROR2           S1PEN1a           S1PEN1b           AtSYP12           AtSYP123           AtSYP123           AtSYP131           AtSYP125           AtSYP122           AtSYP125           S0jv212g005580           S0jv212g005580           S0jv210g008570           S0jv210g008580           S0jv210g08580           S0jv210g05880	28 37 33 30 29 32 37 30 24 29 39 33 34 34 34 22	AGVN AGVN GGVN GGVN GCVN GSGN KADKMDEN SGDLG E-TMN GGDQG FSPADEEN G-TE-SID SHNE-SID SGELG SELG	DKFFED DRFFED DKFFED DKFFED DEFFGY SSGFKK SSGFKK SGFFKK SGFFQE AKFFED DKFFED DFFKK	VESIKEE VESIKDD VETIKDE VENIKDE VENVKDN VESVKED VEVVKE VQEIEKQ VEVVKED VQUIEKQ VENVKED VQUEKQ	KE IRE IRE IKE IKA IKG IGL		SLQQKLHDAH QS RIQRSLHDGM SG KKITQLQSSNEKS FRLCHNLESSN OS FRLCHNLESSN OS FRLCHNLESSN OS KHLKRLQDAN ES KHLKRLQASH ES KHLKRLQASH ES KHLKRLQSN ES KLHKRLQDSN ES ELLQKLQDAH ES - LALSLIDAM ES
VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP123 AtSYP123 AtSYP123 AtSYP123 AtSYP123 AtSYP124 Solyc12g005580 Solyc10g008550 Solyc10g008150 Solyc01g052470	28 37 33 30 35 29 32 37 30 24 29 39 33 34 34 34 233	AGVN AGVN GGVN GGVN GCVN CCVN KADKMDEN SGDLG FSPADEEN GGQCG FSPADEEN SHNE-SID SGELG QSDSG	DKFFED DRFFED DKFFED DKFFED DEFFED DEFFGY SSFLEE SSFLEE SGFFKK SGFFEE AKFFED DFFKK	VESIKEE VESIKDD VETIKDE VENTKDE VENVKDN VENVKDN VESVKED AEVVKAE VENVKDD VENVKDD VENVKED VENVKED VENVKED	KE IRE KR KR KRG KGL KGL KGL KGC KCC		SLQOKLHDAH QS RIQRSLHORN SG KITAQLQSSN KS KITAQLQSSN KS RICHNIESSN QS TILYKSLQDSN EC DIHKRLQDAN ES WHINKLQGAN ET KLIKKLQDSN ES KHLNKLQDSN ES KLHKRLQDSN ES HLLLQKLQDAH ES -LALSLIDAH ES GLKKTLANAN ET
VvPEN1 HvROR2 S1PEN1a S1PEN1b AtSYP122 AtSYP124 AtSYP121 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 S0Jyc12g005580 S0Jyc12g005810 S0Jyc10g081580 S0Jyc06g053760 S0Jyc06g053760	28 37 33 30 35 29 32 37 30 24 29 39 33 34 34 34 2 33 35	AGVN           AGVN           GGVN           GCGVN           GCGVN           KADKMDEN           SGDLG           SGDLG           GDLG           GGDLG           SGDLG           SG	DKFFED DRFFED DKFFED DKFFED DKFFED DEFFGY SSFLEE SGFFKK SGFFKK SGFFKK AKFFED AKFFED DFFKK DSFNKQ TAFLEE	VESIKEE VESIKED VETIKDE VETIKDE VEVVNED VENVKDN VESVKED VESVKED VEVVKED VENVKED VENVKED VENVKED VENVKED	KE IRE I	L MM M L_ L_ L_ L_ VV VV VI L_ L_ L_ L_ L_ L_ L_ L_ L_ L_ L_ L_ L_	SLQQKLHDAH QS RIQRSLHDGN SG KKITAQLOSSN KS PRICENLESSN CS PEILCHLESSN CS FILVKSLQDSN EC DE IHKRLQDAN ES ETLARIEQYH ES KHLKLQAH ES KLLKKLQASH ES KLLKKLQASH ES KLHKLQDAH ES -LALSLIDAH ES GLLKKLDAN ET
VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP131 AtSYP132 AtSYP132 AtSYP132 AtSYP132 Solyc10g008570 Solyc10g008570 Solyc10g05840 Solyc01g05840 Solyc01g058470 Solyc02g052470	28 37 33 29 32 37 30 24 29 39 33 34 34 2 33 34 34 35 34	AGVN           AGVN           GGVN           GGVN           GSCHGGNN          D-SGN           KADKMDEN           SGDLG           FSPADEEN           G-TE-SID           SHNE-SID           SGELG           QSMDQG           QSMDQG           LSRTDEDN	DKFFED DRFFED DKFFED DKFFED DEFFGY SSFLEE SGFFKK SGFFQE AKFFED AKFFED DFFKK DDFFKK SGFFQE SGFFQE SFFKE SGFFQE	VESIKEE VESIKD VETIKDE VETIKDE VEXIKDE VEXVNED VESVKED VESVKED VEIVKED VENVKED VENVKED VENVKED VENVKED VENVKED VENVKED VENVKEL EEVKKE	KE RE KE KE KR KG KR		SLQOKLHDAH QS RIQRSLHDGN SG KITAQLQSSN KS KITAQLQSSN KS SKITAQLQSSN KS STLYKSLQDSN EC DIHKRLQDAN ES KHLKKLQASH ES KHLKKLQSSN ES KHLKKLQSSN ES EELLQKLQDAH ES GGLKTLKDAN ET ELLGKLQDAH ES GGLKTLKDAN ET
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VyPEN1           HyROR2           SIPENIa           SIPENIA           SIPENIA           AtSYP12           AtSYP124           AtSYP123           AtSYP111           AtSYP131           AtSYP132           AtSYP132           AtSYP132           AtSYP132           AtSYP132           AtSYP130           Solyc12005580           Solyc12008580           Solyc010081580           Solyc010081580           Solyc01005810           Solyc02085090           Solyc02085090           Solyc0100580           Solyc0406052360           Solyc0406052360           Solyc04005200           Solyc04005200           Solyc04005200           Solyc04005540           AtSYP23           AtSYP41           AtSYP43           Solyc040701700           AtSYP43           Solyc040707503           Solyc04071730           AtSYP41           AtSYP43           Solyc04071730           AtSYP41           AtSYP41           AtSYP43           Solyc040	288 377 333 359 322 377 300 244 299 333 44 299 333 344 299 333 344 299 333 344 299 333 344 299 333 344 299 333 344 299 333 344 299 333 344 299 333 344 299 333 344 299 322 347 347 299 332 344 299 332 344 299 333 344 299 322 347 347 299 322 347 347 347 347 347 347 347 347 347 347	AGVN AGVN GGVN GGVN GGVN GD-SGN KADKMDEN SGDLG D-SGN KADKMDEN SGDLG GD-SGN GD-SGN GD-SGN GD-SGN GD-SGN GD-SGN GSGN G	DRFFED DRFFED DRFFED DFFFLY SSFLEE SGFFKK SGFFKK SGFFKK SGFFKK DFFKK SGFFQE AKFFED DFFKK SGFFQE SKFFED ISTEDP- LSTEDP-	VESIKEE VESIKED VEIKDE VESIKED VENVKDN VEVVKDN VEVVKDN VESVKED VESVKED VEIKKEN VEIKKEN	KE           RE           KR           KK           KG           GL           KG           GKA           GGL           KG           GGL           KG           GR           BR           GR           CR           GR           CR           GR		SLOCKLHDAH OS SLOCKLHDAH OS KITAQLOSSN KS KITAQLOSSN KS KITAQLOSSN KS KITAQLOSSN KS SKITAKLOSSN CS TILYKSLODSN EC SKILKKLOAN ES KILKKLOSSN ES KILKKLOSSN ES KILKKLOSSN ES KILKKLODSN ES KILKKLODSN ES SKILKKLODSN ES SKILKKLODSN ES SKILKKLODN ES ILLDLOLNN ET NILMDLOLN KT DTTQAVA DTTQAVA DTTQAVA DTTQAVA DTTQAVA DTTQAVA DTTQAVA DTTQAVA DTTQAVA SLPPAWDVSEIS SLPPAWDVSESIS SLPPAWDVSESIS SLPPAWDVSESIS SLPPAWDSESIT SLPPAWDSESI

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VyEEN164KTLENNISKELESRMD							
VyEENI         64         KTLLNANS         KELRSRMD         St. SLALKKAKLIKUELEAL           HVROR2         73         KSLHDASA         RALRSRMD         AD RAAIKKAKUKUELEAL           SIPENIA         69         KTLINAKON KOLRSKMD         DO ISL-ALKKAKUFUKUELEAL           SIPENIA         66         KTLINAKON KOLRSKMD         DO ISL-ALKKAKUFUKUELEAL           ALSYP122         75         KTLINAKON KOLRSKMD         DO ISL-ALKKAKFFUKUELEAL           ALSYP124         65         KTVUNAKO KE-LRARMD         DO ISL-ALKKAKFFUKUELEAL           ALSYP123         66         KTVUNAKO KE-LRARMD         SO VEVVWINKOKUEAL           ALSYP123         66         KTVUNAKO KE-LRARMD         GD AM-VLRAVKI KUALEAL           ALSYP123         66         KSVTKAPA KAIKKTME         SO VEVKWINKIKUELEAL           ALSYP124         66         KSVTKAPA KAIKKTME         SO VEVKWINKIKUELEAL           ALSYP125         60         KTVINAKKI KE-LRARMD         GD AM-VLKRAVKIKUELEAL           ALSYP124         65         KSVTKAPA KAIKRME         SO VEVKWINKIKUELEAL           Solyclog065500         71         KUHNAKIK KE-LRARMD         SO VEVKWINKIKUELEAL           Solyclog065800         71         KAVKASA KA-TIKRME         SO VEVKWINKIKUELEAL           Solyclog065810 <td></td> <td></td> <td>KUT HNAKA</td> <td>KDIPSKMD-</td> <td>GD</td> <td>WCV</td> <td>AT KKYKWIKAKI EY</td>			KUT HNAKA	KDIPSKMD-	GD	WCV	AT KKYKWIKAKI EY
HW0R2         73         KSLHD&SAR_RA-LEGERMD         ADR AANIKKAKVVKLELSL           S1PEN1a         69         KTLENKKN KD-LEKKMD         ND KL-ALKKAKFIKVVKLEAL           S1PEN1b         66         KTLENKKN KD-LEKKMD         DD KL-ALKKAKFIKVVKLEAL           AKSYP122         75         KTLENKKN KD-LEKKMD         DD KL-ALKKAKFIKVKLEAL           AKSYP123         68         KTVHDKAK KK-LEAKKMD         DD KD-VLKKVKHKIKKLEAL           AKSYP131         67         KAVTKAPA KK-LEARMD         SD TE-VLKKVKHKIKKLEAL           AKSYP131         67         KAVTKAPA KK-LEARMD         SD TE-VLKKVKHKIKKLEAL           AKSYP131         67         KAVTKAPA KK-LEQRME         SD TE-VLKKVKHIKKLEL           AKSYP132         66         KTVHNAKKK KE-LEARMD         SD VT-VLKWVKHIKKLEL           AKSYP132         66         KSVTKAPA KA-TKREMD         SD VT-VLKWVKHIKKLEL           Solycl0g06550         71         KLVHNAKK KK-LEARMD         SD VT-VLKWVKHIKKLEL           Solycl0g06580         71         KAVTKASA KA-TKRMD         SD VT-VLKWVKHIKKLEAL           Solycl0g06580         71         KAVTKASA KA-TKRMD         SD VLWVKHKIKLEAL           Solycl0g06580         71         KAVTKASA KA-TKRMD         SD VLWVKHKIKKLEAL           Solycl0g065300         71         KAVTKASA K							
SIERIA         69         KTLHNKWKD-LRKKMD         DISL-RLKKAKFIKVRLEAL           SIPENIB         66         KTLHNKWKD-LRKKMD         DISL-RLKKAKFIKVRLEAL           ALSYP122         75         KTLHNKAKKEND-LRSKMD         DISL-RLKKAKFIKVRLEAL           ALSYP124         65         KTUHNAKKENE-LRAKMD         AICTARRLKONLEAL           ALSYP123         68         KTUHDSKAKKE-LRAKMD         SYPE-VLKRVKMIKKKIKKLAL           ALSYP131         67         KUTHAKKWKE-LRAKMD         SYPE-VLKRVKMIKKKIKALEAL           ALSYP131         67         KUTHAKKWKE-LRAKMD         GD AQ-VLKRVKMIKGKIKELAL           ALSYP131         67         KUTHAKKKE-LRAKMD         GD AM-VLKRVKHIKGKLEEL           ALSYP132         66         KUTHAKKKE-LRAKMD         SN VT-ISKANTVKIKIELEL           ALSYP132         66         KUTHAKKKE-LRAKMD         SN VT-ISKANTVKIKIELEL           Solyc19005580         71         KUHNAKYKE-LRAKMD         SN VT-ISKANTVKIKIKGLEL           Solyc19005580         71         KUHNAKYKER-IRSRMD         SN VT-VLKKAKKEGEL           Solyc19005580         71         KUHNAKYKER-IRSRMD         SN VT-VLKKAKIKKEGEL           Solyc19005580         71         KUHNAKKER-IRSRMD         SN VT-VLKKAKIKKEGEL           Solyc0190052470         70         KSUTKASAKA-IKQRME <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>							
Slpenib         66         KTLENKARKDLRSKMD							
ALSYP122         75         KTLHNNAKKE-LIKKKMD							
ALSYP124         65         KTVHINAKK KE-LIRAKMD							
AtSYP123       68       KTVHDSKAKKK-LRARMD							
ALSYP111         77         KGVHKAES         KS-LIRKIS         NESV9-GLEKAKSIKSKIKSKLEEM							
ALSYP131         67         KAVTKAPAKSIKQRME			-				
ALSYP125         60         KTVHNAKK KE-LIRÅKMDGDFAMVLKVKTIKOKLEAL							
ALSYP132         66         KSVTKAPA KA-IKTYME							
AtSYP112         79         KSTHSTKI RG-LRDRME							~
Solycl2q005580         71 KLVHNAKT KE-IRSRMDSDFSO-VLRVKWHKKKLEAL							
Solvcl0q008570         73 KTVHSAKKKKDTRARMDSDTTVLRVKVIKGKLEGL							
Solvel0q081580         71 KAVTKASA*KAIKQRME				-			
Sol_ccliq056810         14 KAVTKATA*KA-TKQRME			-				
SolyC07Q052470         70         KSVTKASA:KA-TRRME         SOLYC07Q052470           SolyC03Q05070         72         KSLHKPEALKS-MRDRIN							
Solyc06q053760         72 KSLHKPEAKSMRORIMSDIVAVLKKARIKSQLEEM				~			
Solyc02008090         74         KTTHGFKVFRGIRDRMDSDFV9VLRKAKHVKAKLEALSolyc0109750           Solyc0109750         70         KSAPSAKI QGHRDQINSDITTVLRKAKMIKTRLELL							
Solycoliq109750         70 KSAPSAKI QG-HRDQINSDIT			-				· 2
Solycubg0c2is0         31         SGIPQINTAVSTFQRLUNTLGTPKDTPELRDKLHKTRLHIGQL           Solyc01g006910         31         SGIPQINTAVSTFQRLUNTLGTPKDTPELRDKLHKTRLHIGQL           Solyc08g005200         31         SGIFQINTAVSTFQRLUNTLGTPKDTPELRDKLHKTRLHIGQL           Solyc08g005610         31         SGIFQINTAVSTFQRLUNTLGTPKDTPELRDKLHKTRLHIGQL           AcSYP22         30         SGIFQINTAVSTFQRLUNTLGTPKDTPELRDKLHKTRLHIGQL           AcSYP21         38         AGIFRISTAVN-SFFRLUNTLGTPKDTPELRDKLHKTRLHIGQL           AcSYP21         38         AGIFRISTAVN-SFFRLUNTLGTPKDTELRDKLHKTRLYIGQL           AcSYP21         38         AGIFRISTAVN-SFFRLUNTLGTPKDTELRDKLHKTRLYIGQL           AcSYP43         96         VIQGRAFTKMAELCKAHAKALMPSFGDGKEDQNUERLTELTEISTQEITFL							
Solvelige         31 SGIFQINTAVSTFQRLUNTLGTPKDTEPLRDKLHKTRLHIGQL           Solvedg005200         31 SGIFQINTAVSTFQRLUNTLGTPKDTEPLRDKLHKTRUHIGQL           Solvedg076540         31 SGIFQINTAVSTFQRLUNTLGTPKDTEPLRBKLHKTRUHIGQL           AcSYP22         30 SGIFQINTGVSTFQRLUNTLGTPKDTEPLRBKLHKTRUHIGQL           AcSYP23         35 SGIFQINTGVS-TFQRLUNTLGTPKDTEPLRBKLHKTRUHIGQL           AcSYP23         35 SGIFQINTGVS-TFQRLUNTLGTPKDTEPLRBKLHKTRUHIGQL           AcSYP24         38 AGIFRISTAVM-SFFRLUNSIGTPKDTEPLRBKLHKTRUHIGQL           AcSYP21         38 AGIFRISTAVM-SFFRLUNSIGTPKDTELRBKLHKTRUHIGQL           AcSYP41         38 VUTQRARTKMA-FELGKAHAKALMPSFGDGKEDQNIELTQEVTFL							
Solyc08q005200       31 SGIFQINTAVSFFQRLVNTLGTPKDTEPLREKLHKTRVHIGQL         Solyc08q076540       31 SGIFQINTAVSFFQRLVNTLGTPKDTEPLREKLHKTRVHIGQL         AcSYP22       30 SGIFQINTGVSFFQRLVNTLGTPKDTEPLREKLHKTRVHIGQL         AcSYP23       35 SGIFQINTGVSFFQRLVNTLGTPKDTEPLREKLHKTRVHIGQL         AcSYP21       38 AGIFRISTAWN-SFFRLVNTLGTPKDTEPLREKLHKTRVHIGQL         Solyc06q072760       43 VGVFQINTALTNFQRLVNTLGTPKDTEPLREKLHKTRVHIGQL         AcSYP41       88 VNIQRARTKMAELGKAHAKALMPSFGDGKE-DQNUESLTOPTI							
Solyc080076540         31 SGIFQINTAV9FFQRLVNTLGTPKDTPELREKLHKTRVHIGQL           AtSYP22         30 SGIFQINTGVSFFQRLVNTLGTPKDTPELREKLHKTRVHIGQL           AtSYP23         35 SGIFQINTSVSFFQRLVNTLGTPKDTPELREKLHKTRLHIGQL           AtSYP21         38 AGIFRISTAVMSFFRLVNTLGTPKDTPELREKLHKTRLHIGQL           AtSYP21         38 AGIFRISTAVMSFFRLVNTLGTPKDTLELREKLHKTRLJIQLAL           AtSYP21         38 AGIFRISTAVM-SFFRLVNTLGTPKDTLELREKLHKTRLJIQLAL           AtSYP41         88 VNIQRARTKMAELGKAHAKALMPSFGDGKEDQRIEALTHEITQLFET           AtSYP43         96 VYIQRARTKMAELGKAHAKALMPSFGDGKEDQRIEALTHEITDL							
AtSYP22         30 SGIFQINTGVSTFQRLVNTLGTPKDTEFLREKLHKTRLHIGQL           AtSYP23         35 SGIFQINTGVSTFHRLVNTLGTPKDTEFLREKLHKTRLHIGQL           AtSYP21         36 AGIFRISTAVM-SFRLVNTLGTPKDTEFLREKLHKTRLJISEL           Solyc06q072760         43 VGVFQINTALTNFQRLVNTLGTPKDTLELREKLHKTRLJISEL           AtSYP41         80 VNIQRARTKMAELGKAHAKALMPSFGDGKEDQHUESLTGEVTFL           AtSYP43         96 VYIQRARTKMAELGKAHAKALMPSFGDGKEDQRUESLTGEUTFL           Solyc01g100170         87 ANVHRVRTKM5ELAKAHAKALMPSFGDGKEDQRUESLTGEUTFL           Solyc03g075530         84 ASIHQAQVKLADLKKCHAKALTPSFGDGKEDQRUELTHEITDL           Solyc03g075530         84 ASIHQAQVKLA-DLKKCHAKLTPSFGDGRE-DQRUELTWEITDL           Solyc03g075530         84 ASIHQAQVKLA-DLKKCHAVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE           Solyc04q071730         53 KTLESIGTLEQFLMKHKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE           Solyc04q071730         53 KTLESIGTLEQFLMKHKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE           ASYP81         53 KTLDSIKELELFMLKHRKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE           Solyc07g054140         80LAKRSSIFDDPSKEIQELTVIIKOLTSLNVGVSI           ASYP32         84 RMVLRSRTDLFSVAKKTSVFDDPTDEIQELTVVIKQEISALNSALVU           Solyc08g067910         83							
AtsYP23         35         SGIFQINTSVS-TFHRLVNTLGTPKDTPELREKLHKTRLYIGQL           AtsYP21         38         AGIFRISTAVM-SFRRLVNIGTPKDTLELREKLHKTRLYIGQL           Solyco6g072760         3         VGYEQINTALTNFQRLVNIGTPKDTLELREKLHKTRLYIGQLEL           AtsYP41         8         VNIQRARTKMA-ELGKAHAKALMPSFGDGKEDQNIESLTQEITFL           AtsYP43         96         VYIQRARTKMA-ELGKAHAKALMPSFGDGKEDQNIESLTQEITFL           Solyco1q100170         87         ANVHRVRTKMS-ELAKAHAKALMPSFGDGKEDQNIESLTQEITFL			· · ~				
Atsyp21       38 AGIFRISTAVN-SFFRLVNSIGTPKDTLELRDKLQKTRLQISEL         solyc06g072760       43 VGVFQINTALTNFQRLUNTIGTPKDTLQLRKKLHSTRQQIAEL         AtsyP41       88 VNIQRARTKMAELGKAHAKALMPFGCDGKEDQRUESLTQEITFL         AtsyP43       96 VYIQRARTKMAELGKAHAKALMPFGCDGKEDQRUESLTQEUTFL         Solyc01g100170       87 ANVHRVRKMSELAKAHAKALMPFGCDGKEDQREIELTHEITDL         Solyc03g075530       84 ASIHQAQVKLADLKKCHAKALMPFGDGKEDQREIELTHEITDL         Solyc03g033850       16 KTLESIGTLEQFLMKKKDVVDLHTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE         Solyc04g071730       53 KTLESIGTLEQFLMKKKDVVDLHTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE         AtsYP81       53 KTLDSIKELELPHLKHRKDYVDLHTTEQERDSIEQEVAAFIKACKEQIDITGLMMALSI         Solyc07g054140       80      SIFDDPSKEIQELTYIKNDITSLNVGVSI         AtsYP32       84 RVLRSRTDLFSVAKRTSVFDDPTDEIQEIZVVIKQEISALNSALVI         Solyc07g054140       83      SVFDDPTDEIQEIZVVIKQEISALNSALVI			· · ~	· · ~			· ~
Solyc06g072760       43 VGVFQINTALT-NFQRLVNTLGTFKDTLQLRHKLHSTRQQIAEL         AtSYP41       88 VNIQRARTKMAELGKAHAKALMPSFGDGKE-DQHQIETLTGUTFL         Solyc01y100170       87 ANVHRVRTKMSELGKAHAKALMPSFGDGKE-DQHQIETLTGUTFL         Solyc01y100170       87 ANVHRVRTKMSELGKAHAKALMPSFGDGKE-DQHQIETLTGUTFL         Solyc03y030350       86 ASIHQAQVKLA-DLKKAHAKALMPSFGDGRE-DQRVIEVLTMEITDL         Solyc03y03350       16 KTLESIGTLQFLMKKKDYUDLHRTTEQERSIEHEVTIFVKSCKQIDVLRNSINE         Solyc04y071730       35 XTLESIGTLGFLMKKKDYUDLHRTTEQERSIEHEVTIFVKSCKQIDVLINSINE         AtSYP81       53 KTLDSIGTLGFLMKKKDYUDLHRTTEQERSIEHEVTIFVKSCKQIDVLINSINE         AtSYP81       53 KTLDSIGTLGFLMKKKDYUDLHRTTEQERSIEHEVTIFVKSCKQIDVLINSINE         AtSYP81       53 KTLDSIGTLGFLMKKKDYUDLHRTTEQERSIEHEVTIFVKSCKQIDVLINSINE         AtSYP81       53 KTLDSIKELLFMLKRRDYUDLHRTTEQERSIEHEVTIFVKSCKQIDITGLINALSUK         Solyc07g054140       80LAKRSSIFDDPSKEIQELTTSIKNDITSINVGVSI         AtSYP32       84 RMVLRSRTDLFSVAKKTSVFDDPTQEIQELTAVIKQDITALNSAVVI         Solyc08g067910       83LAKRTSVFDDPTTEIQELTAVIKQDITALNSAVVI							
ALSÝP41     88 VNIQRARTKMAELGKAHAKALMPSFGDGKE-DGKIELSLTÖEITFL       ALSÝP43     96 VYIQRARTKMAELGKAHAKALMPSFGDGKE-DQRUETLTGUTFL       Solycolg100170     87 ANVHRVRTKMS-ELAKAHAKALMPSFGDGKE-DQRUETLTGUTFL       Solycolg100170     87 ANVHRVRTKMS-ELAKAHAKALMPSFGDGKE-DQRUETLTGUTFL       Solycolg100170     87 ANVHRVRTKMS-ELAKAHAKALMPSFGDGKE-DQRUEALTHEITDL       Solycolg075530     84 ASIHQAQVKLA-DLAKAHSKALMPTFGDNKGIHREVEMLTHEITDL       Solycolg03033850     16 KTLESIGTLEOFLMKKKDYVDLHRTTEQERDSTEHEVTIFVKSCKEQIDVLNSINE       Solycolq071730     53 KTLESIGTLEOFLMKKKDYVDLHRTTEQERDSTEHEVTIFVKSCKEQIDVLNSINE       ALSYP81     51 KTLDSIKELLFMLKHRKDYVDLHRTTEQEKDSIGEVAAFIKACKEQIDILINSIR       ALSYP31     71IAKSSIFDDFSKIQELTTSIKMALSI       Solyc03g054140     80SIFDDFSKIQELTVIKQEISALNSALVU       Solyc08g067910     83LAKRTSVFDDPTEIQELTAVIKQDITALNSAVU							
ALSYP43     96     VYIQRARTKMAELGKAHAKALMPSFGDGKEDQRIELTTQEVTFL       Solyc019100170     87     ANVHRVRKMSELGKAHAKALMPSFGDGKEDQRIELTTETTQEVTFL       Solyc03070     87     ANVHRVRKMSELGKAHAKALMPSFGDGKEDQRIELTTETTQE       Solyc03070     84     ASIHQAQVKLADLKKCHAKALMPSFGDGRE-DQNUELTTETDL       Solyc0303850     16     KTLESIGTLEQFLMKHKKDYVDLHRTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE       Solyc04g071730     53     KTLESIGTLEQFLMKHKKDYVDLHRTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE       ASYP81     53     KTLDSIKELELFMLKHRKDYVDLHRTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE       Solyc07g054140     80    TIFNBRTVEIQELTVIIRKOLTSUNGVSI       ALSYP32     84     RMVLRSRTDLFSVAKKTSVFDDFTQEIQELTVVIKQEISALNSALVI       Solyc08g067910     83    SVFDDFTGIQELTAVIKQDITALNSAVVI			· ~	~		~	· ~ ~
Solyc01q100170         87 ANVHRVRTKMSELAKAHAKALMPSFGDGKEDGKELTALTHEITDL AtSYP42         85 FNIQKVRDKMNELAKAHSKALMPSFGDGKE-DQNVIEVLTHEITDL           Solyc03q075530         84 ASIHQAQVKLADLKKCHAKALTPSFGDGKE-DQNVIEVLTHEITDL           Solyc03q033850         16 KTLESIGTLEQFLMKKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLNSINE           Solyc04q071730         53 KTLESIGTLEQFLMKKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLNSINE           ASYP81         53 KTLESIGTLEQFLMKKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLINSINE           ASYP81         71           Solyc07q054140         80          LAKRSSIFDDFSKEIQELTVIIRNDITSLNVGVSL           ASYP32         84 RMVLRSRTDLFSVAKKTSVFDDF7GEIQELTVIKQEISALNSALVU           Solyc08q067910         83							
AtsYP42     85     FNIQKVRDKMNELAKAHSKALMPTFGDNKGIHREVEMLTHEITDL       Solyc09g075530     84     ASIHQAQVKLA-DLKKCHAKALTP5FGDGRE-DGNVETULTMEITDL       Solyc09g075530     84     ASIHQAQVKLA-DLKKCHAKALTP5FGDGRE-DGNVEULTMEITDL       Solyc09g075530     84     ASIHQAQVKLA-DLKKCHAKALTP5FGDGRE-DGNVEULTMEITDL       Solyc04g071730     53     KTLESIGTLEQFLMKKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLNSINE       Solyc04g071730     53     KTLDSIKELELFMLKHRKDYVDLHRTTEQEKDSIEHEVTIFVKSCKEQIDVLINSINE       AtsYP81     53     KTLDSIKELELFMLKHRKDYVDLHRTTEQEKDSIEUEVTIFVKSCKEQIDVLINSINE       Solyc07g054140     80    ILAKRSSVFDDPSKEIQELTVLIRKDITSIKNOVSI       AtsYP32     84     RMVLRSRTDLFSVAKRTSVFDDPTEIQELTVVIKQEISALNSALVI       Solyc08g067910     83	Solvc01g100170						
Solyc09q075530       84 ASIRQAQVKLA-DLKKCHAKALTPSFGDGRE-DGNUELUTMEITDI         Solyc03q033850       16 KTLESIGTLEQFLMKHKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE         Solyc04q071730       53 KTLESIGTLEQFLMKHKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE         AcSYP81       53 KTLDSIKELELFMLKHRKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE         Solyc07q054140       80							
Solyc03033850         16 KTLESIGTLEOFLMKHKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE           Solyc04071730         53 KTLESIGTLEOFLMKHKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE           ALSYP81         53 KTLESIGTLEOFLMKHKKDYVDLHRTTEQERDSIEEEVTIFVKSCKEQIDULANSINE           ALSYP81         53 KTLESIGTLEOFLMKHKKDYVDLHRTTEQEKDSIEQEVAAFIKACKEQIDULINSINE           ALSYP81         71           Solyc030054140         80           ALSYP32         84 RMVLRSRTDLFSVAKRTSVFDDFTEIDEITVSIKKDIGSLANSALVU           Solyc08g067910         83	Solvc09g075530						
Solyc04g071730     53 KTLESIGTLEQFLMKHKKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLRNSINE       AtSYP81     53 KTLDSIKELELFMLKHRKDYVDLHRTTEQERDSIEHEVTIFVKSCKEQIDVLINDSIN       AtSYP81     71       Solyc07q054140     80       AtSYP32     84 RMVLRSRTDLFSVAKRTSVFDDP5KEIQELTVSIKNDITSLNVGVSI       AtSYP32     84 RMVLRSRTDLFSVAKRTSVFDDP7EIQELTVVIKQEISALNSALVVI       Solyc08g067910     83							
ALSÝP81     53 KTLDSIKELELFMLKHRKDYVDLRTTEQEKOSIEQEVAAFIKACKEQIDILINSIRN       ALSÝP31     71       Solyc07g054140     80       Solyc07g054140     80       KSP32     84       RMVLRSRTDLESVAKKTSVFDDF7GEIQELTVIKQEISALNSALVU       Solyc08g067910     83							-
AtSYP31 71TIFNDRTVEIQELTVLIRNDITGLNMALSI Solyc07g054140 80LAKRSSIFDDPSKEIQELTVLIRNDITGLNWALSI AtSYP32 84 RMVLRSRTDLFSVAKRTSVFDDPTQEIQELTVVIKQEISALNSALVI Solyc08g067910 83LAKRTSVFDDPTEIQELTAVIKQDITALNSAVVI							
Solyc07g054140 80LARRSSIFDDPSKEIQELTTSIKNDITSLNVGVSD AtSYP32 84 RMVLRSRTDLFSVAKKTSVFDDFTQEIQELTVVIKQEISALNSALVD Solyc08g067910 83LARRTSVFDDPTTEIQELTAVIKQDITALNSAVVD					A.	· ~	· ~ ·
AtSYP32 84 RMVLRSRTDLFSVAKRTSVFDDPTQEIQELTVVIKQEISALNSALVI Solyc08g067910 83LAKRTSVFDDPTTEIQELTAVIKQDITALNSAVVI		. –		LAKRS			N
Solyc08g067910 83LAKRTSVFDDPTTEIQELTAVIKQDITALNSAVV			RMVLRSRT	DLFSVAKRT			
		83					
							~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
					Г	Hc	1

AtPEN1		ANAANRSLP-GC <mark>GPC</mark> SSSDRTRTSVLN <mark>G</mark> RKK MDSMDS NRLRELISSEYRETVQRR
VvPEN1		SNAAN <mark>R</mark> SLP-GC <mark>GPG</mark> SSSD <mark>RTRT</mark> SVVN <mark>G</mark> RKK RDSMDA TSI <b>R</b> NQISSE <mark>Y</mark> RET <mark>V</mark> QRR
HvROR2		ANAAN <mark>R</mark> SVA-GC <mark>GPG</mark> SSTD <mark>RTRT</mark> SVVA <mark>G</mark> RKK RDAMES SSL <mark>R</mark> SRITSE <mark>Y</mark> RETVARR
SlPEN1a		SNAAN <mark>R</mark> SVP-GC <mark>GPC</mark> SSSDRTRTSVVNG RKK QESMNQ NEL <mark>R</mark> QRMASEYRETVQRR
SlPEN1b		SNASN <mark>R</mark> SLP-GC <mark>GPC</mark> SSSDRTRTSVVNG RKK QESMNQ NEL <mark>R</mark> QKMASEYRETVQRR
AtSYP122		ANEVN <mark>R</mark> SLP-ES <mark>GPC</mark> SSSDRO <mark>RT</mark> SVVN <mark>G</mark> RKK KDEMEK SRVRETITNE <mark>Y</mark> KETVGRM
AtSYP124		ANANSRNVS-GCGPGSSTDRTRTSVVSG GKK KDLMDS QGLRARMNAEYKETVERR
AtSYP123		SNAAQRKVA-GCGP <mark>G</mark> SSAD <mark>R</mark> TRTSVVSG GKK KDMMDD QRL <mark>R</mark> TKMATEYKET <mark>V</mark> ERR
AtSYP111		ANKEIKRLS-GTPVYRSRTAVTNG RKK KEVMME QGLRQKMMSEYKETVERR
AtSYP131		ENLENRTKP-GCGKGTGVDRTRTATTIA KKK KDKISE QTLRQNIQQEYREVVERR
AtSYP125		ANANSRNVP-GCGPGSSTDRTRSSVVSG GKK KDLMDS QGLRARMNNEYKETVERR
AtSYP132		ENLANRQKP-GCAK <mark>C</mark> SGVD <mark>R</mark> SRTATTLS KKK KDKMAE QVL <mark>R</mark> ENIQQEYRDV <mark>V</mark> DRR
AtSYP112		RNVANRTSFKECSCVDRTRTSITNG RKK RDTMSE HRLRERIFADYREDLKRK
Solyc12g005580		SNAAHRKIS-GCGP <mark>G</mark> SSAD <mark>R</mark> TRTSVVSG GKK KVLMDD QGL <mark>R</mark> TRMNDEYKET <mark>V</mark> ARR
Solyc10g008570		SNVANRKNL-GCGP <mark>C</mark> SSAD <mark>R</mark> TRTSVVSG GKK KVLMDD QAL <mark>R</mark> AKMNSEYKDT <mark>V</mark> ARR
Solyc10g081580		ENLANRNKP-GCGK <mark>G</mark> SAVD <mark>R</mark> SRTATTVS KKK KDKMAE QTL <mark>R</mark> ENIHHEYREV <mark>V</mark> ERR
Solyc01g056810		ENLSNRSKP-GCGK <mark>C</mark> SAVD <mark>R</mark> SRTATTVS KKK KDKMSE QTL <mark>R</mark> ENIHNEYREV <mark>V</mark> ERR
Solyc07g052470	109	ENLANLQKP-GCGK <mark>G</mark> TSVD <mark>R</mark> SRTNMTNS TKK RDVMTE QTL <mark>R</mark> QRIDNEYREV <mark>V</mark> ERR
Solyc06g053760	111	SNAINRRLS-GCKE <mark>G</mark> TLVD <mark>R</mark> TRSAVTNG RKK KELMMD QGL <mark>R</mark> QRMMTEYKET <mark>V</mark> GRR
Solyc02g085090		SNVGNRKLSVAYAQ <mark>G</mark> SVVD <mark>R</mark> TRVSMSNG RVK RDIMND QALREKILSDYKDCLRRR
Solyc01g109750		SNLDNRGVSGSPVDRTRISVTNG RIK RDMMND QCLRENIVAEHKEGLRKQ
Solyc06g062360		DTSTKLKQASETDHRVEVSASKKITDAK AKD QAVLKE QKAQRLAAERETAYTPFI
Solyc11g066910	76	DTSAKLKQASETDHRIEVSASKKITDAK AKD QAVLKE QKAQRLAAERETSYSPFV
Solyc08g005200	76	DTSAKLKQASETDHHADVSASKKITDAK AKD QAVLKE QKAQRLAAERETAYTPFI
Solyc08g076540		DTSAKLKQASETDHRVEVSASKKITDAK AKD QAVLKE QKAQRLAAERETAYTPFV
AtSYP22		DTSAKLKEASETDHQSGVNPSKKIADAK ARD QAVLKE QKAQQTAAERETTYTPFV
AtSYP23		DTSAKLKEASETDHQRGVNQKKKIVDAK AKD QAVLKE QKAQRLAAERETVYAPLV
AtSYP21		NTSAKLKEASEADLHGSASQIKKIADAK AKD QSVLKE QKAQRLAAEREITYTPVV
Solyc06g072760		ETSANLKQAIGSNRHSQSSVTKKIANAK AKD QSVLKK QRAQQLAAQREAAFTPSI
AtSYP41		KSEKQLQRLSASGPSEDSNVRKNV-QRS ATD QLLSME RKKQSTYLKR
AtSYP43		KSEKQLQRLSAAGPSEDSNVRKNV-QRS ATD QNLSME RKKQSTYLKR
Solyc01g100170		RSEKKLQRLSAAGLSEDSNVRKNV-QRS ATD QSLSME RRKQSTYLKR
AtSYP42		KSEKRLQMLSTRGPSEESNLRKNV-QRS ATD QNLSME RRKQSTYLKR
Solyc09g075530		KSQKKLQKLSASGSCEDSNVRKNV-QRS ATD QNLSVE RRMQSLYLKQ
Solyc03g033850		ANSKGWLGLKGDNLNADTIAHKHGVVLI SEK HSVTSQ DQLRAIRFQDAINRVTPRRN
Solyc04g071730		ANSKGWLGLKGDNLNADTIAHKHGVVLI SEK HSVSSQ DQLRAIRFQDAINRVTPRRK
AtSYP81		ANSKGWLGLPADNFNADSIAHKHGVVLI SEK HSVTAQ DQLRATRFQDIINRAMPRRK
AtSYP31		TLQNMELADGNYSQDQVGHYTAVCDD KTR MGATKQ QDVLTTRSENMKAHENRKQL
Solyc07g054140		ALQDMDVADGTHSKDTIVHCTAICDD KTR MAATKS QEALTIRTKNMKAHEDRKQI
AtSYP32		LFRSSQNDEGNNSRDRDKSTHSATVVDD KYR MDTTKE KDVLTMRTENMKVHESRRQL
Solyc08g067910	120	LHSNARNESGNSDTTSHSTTVVDD KNR MTATKE KEVLTMRTENMKVHENRRQM

VPENI         160         VT_VIENDERTV         DD           SIPENIA         165         VT_VIENDERDET         DT           SIPENIA         165         VT_VIENDERDET         DT           SIPENIA         165         VT_VIENDERDET         DT           SIPENIA         165         VT_VIENDERDET         DT           SIPENIA         164         VT_VIENDERDET         DT           ALSYP124         164         VT_VIENDERDET         DE           ALSYP123         164         VT_VIENDERDET         DE           ALSYP123         165         VT_VIENDERDET         DE           ALSYP123         165         VT_VIENDERDET         DE           SOLVED09570         165         VT_VIENDERDET         DE           SOLVED09570         167         VT_VIENDERDET         DE           SOLVED09570	VPENI         160         VFTW GENB DERTY         DD           SI PENIA         165         VFTW GENB DERTY         DD           SI PENIA         165         VFTW GENB DERTY         DD           SI PENIA         165         VFTW GENB DERTY         DD           SI PENIA         164         VFTW GENB DERTY         DD           SI PENIA         164         VFTW GENB DERTY         DD           ALSYP111         163         VFTW GENBADERT         DD           ALSYP123         164         VFTW GENBADERT         DD           ALSYP123         164         VFTW GENBADERT         DD           SI PENIA         DS         VFTW GENBADERT         DD           SI PENIA												
HMORE         164         VTVM GROBERT	HMORE         164         VTVM GROBERT	AtPEN1											
SIPENIA         165 VFTV/GRS/DECTDT           SIPENIA         165 VFTV/GRS/DECTDT           ARSFY122         17 CFTV/GRS/DECTET           ARSFY123         164 FTV/GRS/DECTET           ARSFY123         164 FTV/GRS/DECTET           ARSFY123         164 FTV/GRS/DECTET           ARSFY133         163 FTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           BS1/SC10205580         167 VTV/GRS/DECTET           BS1/SC10205580         167 VTV/GRS/DECTET           BS1/SC10205580         167 VTV/GRS/DECTET           BS1/SC10205580         171 VTV/GRS/DECTET           BS1/SC10205580         171 VTV/GRS/DECTET           BS1/SC10205580         171 VTV/GRS/DECTET           BS1/SC10205780         134 POAVESSTRAETET           BS1/SC10205600         171 VTV/GRS/DECT	SIPENIA         165 VFTV/GRS/DECTDT           SIPENIA         165 VFTV/GRS/DECTDT           ARSFY122         17 CFTV/GRS/DECTET           ARSFY123         164 FTV/GRS/DECTET           ARSFY123         164 FTV/GRS/DECTET           ARSFY123         164 FTV/GRS/DECTET           ARSFY133         163 FTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           ARSFY132         162 VTV/GRS/DECTET           BS1/SC10205580         167 VTV/GRS/DECTET           BS1/SC10205580         167 VTV/GRS/DECTET           BS1/SC10205580         167 VTV/GRS/DECTET           BS1/SC10205580         171 VTV/GRS/DECTET           BS1/SC10205580         171 VTV/GRS/DECTET           BS1/SC10205580         171 VTV/GRS/DECTET           BS1/SC10205780         134 POAVESSTRAETET           BS1/SC10205600         171 VTV/GRS/DECT		160	YFTVT	GEN <mark>P</mark> DEKTV	7							
B1ERNIA         162         YTYT GERNOEANI	B1ERNIA         162         YTYT GERNOEANI												
A.SYP122         171         CFT/SET         DEATL         ER           A.SYP123         164         YFT/SEGADESCI         EX           A.SYP123         164         YFT/SEGADESCI         EX           A.SYP131         163         YFT/SEGADESCI         EX           A.SYP132         164         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           Soly120905580         167         YFT/SEGADESCI         EX           Soly1209052470         166         YFT/SEGADESCI         EX           Soly2019052470         166         YFT/SEGADESCI         EX           Soly201905230         164         YFT/SEGADESCI         EX           Soly201905230         164         YFT/SEGADESCI         EX           Soly2019057560         162         YFT/SEGADESCI         EX           Soly2019057560         162         YFT/SEGADESCI         EX           Soly2019057560         163         YFT/SEGADESCI         EX           Soly2019057560         17         YFT/SEGADESCI         EX <td>A.SYP122         171         CFT/SET         DEATL         ER           A.SYP123         164         YFT/SEGADESCI         EX           A.SYP123         164         YFT/SEGADESCI         EX           A.SYP131         163         YFT/SEGADESCI         EX           A.SYP132         164         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           Soly120905580         167         YFT/SEGADESCI         EX           Soly1209052470         166         YFT/SEGADESCI         EX           Soly2019052470         166         YFT/SEGADESCI         EX           Soly201905230         164         YFT/SEGADESCI         EX           Soly201905230         164         YFT/SEGADESCI         EX           Soly2019057560         162         YFT/SEGADESCI         EX           Soly2019057560         162         YFT/SEGADESCI         EX           Soly2019057560         163         YFT/SEGADESCI         EX           Soly2019057560         17         YFT/SEGADESCI         EX<td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></td>	A.SYP122         171         CFT/SET         DEATL         ER           A.SYP123         164         YFT/SEGADESCI         EX           A.SYP123         164         YFT/SEGADESCI         EX           A.SYP131         163         YFT/SEGADESCI         EX           A.SYP132         164         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           A.SYP132         165         YFT/SEGADESCI         EX           Soly120905580         167         YFT/SEGADESCI         EX           Soly1209052470         166         YFT/SEGADESCI         EX           Soly2019052470         166         YFT/SEGADESCI         EX           Soly201905230         164         YFT/SEGADESCI         EX           Soly201905230         164         YFT/SEGADESCI         EX           Soly2019057560         162         YFT/SEGADESCI         EX           Soly2019057560         162         YFT/SEGADESCI         EX           Soly2019057560         163         YFT/SEGADESCI         EX           Soly2019057560         17         YFT/SEGADESCI         EX <td></td>												
Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENEROLI-         Ex           Scilycl0q05570         169         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05570         168         YITM GENEROLI-         Ex           Scilycl0q0570         162         YITM GENEROLI-         Ex           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q005200         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q073310         138         PCALE	Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENEROLI-         Ex           Scilycl0q05570         169         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05570         168         YITM GENEROLI-         Ex           Scilycl0q0570         162         YITM GENEROLI-         Ex           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q005200         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q073310         138         PCALE	SlPEN1b	162	YY <mark>TVT</mark>	GEN <mark>P</mark> DEAVI								DT
Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENEROLI-         Ex           Scilycl0q05570         169         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05570         168         YITM GENEROLI-         Ex           Scilycl0q0570         162         YITM GENEROLI-         Ex           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q005200         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q073310         138         PCALE	Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENEROLI-         Ex           Scilycl0q05570         169         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05570         168         YITM GENEROLI-         Ex           Scilycl0q0570         162         YITM GENEROLI-         Ex           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q005200         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q073310         138         PCALE	AtSYP122	171	CFTVT	GEY <mark>P</mark> DEATI								ER
Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENEROLI-         Ex           Scilycl0q05570         169         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05570         168         YITM GENEROLI-         Ex           Scilycl0q0570         162         YITM GENEROLI-         Ex           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q005200         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q073310         138         PCALE	Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENEROLI-         Ex           Scilycl0q05570         169         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05580         110         YITM GENEROLI-         Ex           Scilycl0q05570         168         YITM GENEROLI-         Ex           Scilycl0q0570         162         YITM GENEROLI-         Ex           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q0570         162         YSM GENEROLI-         EX           Scilycl0q005200         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q07540         134         PQAVLESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q0710         138         PCALESSTRAEL         DV           Scilycl0q073310         138         PCALE	AtSYP124	161	YFTI	GEQADEQT	E							EN
Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN	Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN		164	YFTVT	GQKADEETV	7							EK
Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN	Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN	AtSYP111	169	YFTV	GEHANDEM:	E							EK
Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN	Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN		163	VFTVT	GORADEEA:	E							DR
Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN	Acisyrili         113         YITM GENERALDUM         Ex           Scilycl0q0550         169         YITM GENERODLI         Ex           Scilycl0q05570         169         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05580         110         YITM GENERODLI         Ex           Scilycl0q05570         168         YITM GENERODLI         Ex           Scilycl0q05240         184         YAM GENERODLI         EX           Scilycl0q05250         134         PQAVIESSTRAES         DV           Scilycl0q005200         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q07540         134         PQAVIESSTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0q0710         138         PCASTRAES         DV           Scilycl0qq071730         173         RKMTRIN		156	YFTIT	GEKADEOT	c							DN
Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         POM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL	Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         POM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL	AtSYP132	162	VYTVT	GERADEDT	c							DE
Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         POM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL	Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL		173	YFLAT	GEEPSNED	1							EK
Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL	Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL		167	YETV	GEKADDGL	- 							EN
Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL	Solyc1g081580         167         VFTW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc07g052470         166         VITW_GNAADEETI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         171         TYRE GREENEEVI         DR           Solyc01g05200         174         PUM/DESTINGET         DV           Solyc01g062000         174         PUM/DESTINGET         DV           Solyc01g07200         146         PUM/DESTINGET         DV           ActY223         138         HESPLPSYTSAEL         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         PUM/DESTINGET         DV           ActY224         134         DESL_COQOTADE         DL           ActY224         134         DESL_COQOTADE         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL           ActY241         184        LACLORE-DEN         DL		169	YETV	GENADDEL.	- 							DN
Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         PRAVLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         TLEQQKE-DGM         EDI           Solyc01q100170         183         ELQQKEGPDSV         EDI           Solyc03q05330         180         ELLINGKGQDEV         EDI           Solyc04q071700         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q0701730         173         RKNTKENAAETSV	Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         PRAVLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         TLEQQKE-DGM         EDI           Solyc01q100170         183         ELQQKEGPDSV         EDI           Solyc03q05330         180         ELLINGKGQDEV         EDI           Solyc04q071700         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q0701730         173         RKNTKENAAETSV		167	VETV	CNRADEET:	- 							DR
Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         PRAVLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         TLEQQKE-DGM         EDI           Solyc01q100170         183         ELQQKEGPDSV         EDI           Solyc03q05330         180         ELLINGKGQDEV         EDI           Solyc04q071700         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q0701730         173         RKNTKENAAETSV	Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         PRAVLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         TLEQQKE-DGM         EDI           Solyc01q100170         183         ELQQKEGPDSV         EDI           Solyc03q05330         180         ELLINGKGQDEV         EDI           Solyc04q071700         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q0701730         173         RKNTKENAAETSV		110	VYTV	CNRADEET:	- 							DR
Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         PRAVLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         TLEQQKE-DGM         EDI           Solyc01q100170         183         ELQQKEGPDSV         EDI           Solyc03q05330         180         ELLINGKGQDEV         EDI           Solyc04q071700         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q0701730         173         RKNTKENAAETSV	Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         PRAVLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         HKPSLPSSTISSE         EDV           AtSYP21         144         TLEQQKE-DGM         EDI           Solyc01q100170         183         ELQQKEGPDSV         EDI           Solyc03q05330         180         ELLINGKGQDEV         EDI           Solyc04q071700         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q071730         173         RKNTKENAAETSV         ESI           Solyc04q0701730         173         RKNTKENAAETSV		166	1777717	CTRREET.	- 							NN
Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         HRFSLPSSTISSE         EDV           AtSYP21         144         HRFSLPSSTISSE         EDV           AtSYP21         144         HRFSLPSSTISSE         EDV           AtSYP21         144         HRFSLPSSTISSE         EDV           Solyc01q100170         183         ELQQKREQDEV         EDI           Solyc01q100170         183         ELQQKREQDEV         EDI           Solyc01q001730         173         HRMTKENAAETSV         ESI           Solyc01q001730         173         HRMTKENAAETSV         ESI           Solyc01q001730         173         HRMTKENAAETSV         ESI           Solyc01q0054140         176         FSISNAKESTHFWQRLA	Solyc02q085090         171         YYNE GREPNEEVI         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EEK           Solyc01q007260         134         PQAVLPSSTIDGE         EDV           Solyc1q007640         134         PQAVLPSSTIDGE         EDV           Solyc08q005500         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           Solyc08q005200         134         PQAVLPSSTIDGE         EDV           AtSYP21         134         HRFSLPSSTISSE         EDV           AtSYP21         144         HRFSLPSSTISSE         EDV           AtSYP21         144         HRFSLPSSTISSE         EDV           AtSYP21         144         HRFSLPSSTISSE         EDV           Solyc01q100170         183         ELQQKREQDEV         EDI           Solyc01q100170         183         ELQQKREQDEV         EDI           Solyc01q001730         173         HRMTKENAAETSV         ESI           Solyc01q001730         173         HRMTKENAAETSV         ESI           Solyc01q001730         173         HRMTKENAAETSV         ESI           Solyc01q0054140         176         FSISNAKESTHFWQRLA		160	VETV	CEUDDEEU.								DK
Solyc01q109750         162         YSMA         CREPSEEAI         ————————————————————————————————————	Solyc01q109750         162         YSMA         CREPSEEAI         ————————————————————————————————————		171	VVNE	GERFDEEV.								DK
BolyCUGQUEZIGU         14         PONUPSITURE/           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           ALSYE22         133         POSALPSITURE/         DV           ALSYE21         141         TKEN-PERSITURE/         DV           ALSYE21         144         FKENFERSIEL         DV           ALSYE41         164         SQL*CUGQUEZ-DCM         DU           ALSYE41         164         SQL*CUGQUEZ-DCM         DL           ALSYE42         181        LUQQKECPERV         DU           ALSYE42         181        LUQQKECPERV         DL           SolyCUG905530         180        LELQSEGNERV         DL           SolyCUG901730         17         RKWITKENARAKAN         SS           SolyCUG905410         175         FSTINAREN-REPREZICA/CUCRELARASASSUPEPRWSTOGC         SS           SolyCUG9067910         176         FSSISTEREASIPERMQREPLARASASSUPEPRWSTOGC         SSS           SolyCUG9067910         176         FSSISTEREASIPERMQREPLA	BolyCUGQUEZIGU         14         PONUPSITURE/           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           SolyCUGQUEZIGU         14         PONUPSITURE/         DV           ALSYE22         133         POSALPSITURE/         DV           ALSYE21         141         TKEN-PERSITURE/         DV           ALSYE21         144         FKENFERSIEL         DV           ALSYE41         164         SQL*CUGQUEZ-DCM         DU           ALSYE41         164         SQL*CUGQUEZ-DCM         DL           ALSYE42         181        LUQQKECPERV         DU           ALSYE42         181        LUQQKECPERV         DL           SolyCUG905530         180        LELQSEGNERV         DL           SolyCUG901730         17         RKWITKENARAKAN         SS           SolyCUG905410         175         FSTINAREN-REPREZICA/CUCRELARASASSUPEPRWSTOGC         SS           SolyCUG9067910         176         FSSISTEREASIPERMQREPLARASASSUPEPRWSTOGC         SSS           SolyCUG9067910         176         FSSISTEREASIPERMQREPLA												
Solycliq066010         134         PQAVLPSSYTASEV         DV           Solyc08q05200         134         PQAVLPSSYTASEV         DV           AcSY221         133         PQSALPSSYTASEV         DV           AcSY221         134         PQSALPSSYTASEV         DV           AcSY211         141         TKE-PTSYTASEV         DV           AcSY211         141         TKE-PTSYTASEV         DV           AcSY211         144         TKE-PTSYTASEV         DV           AcSY211         144         TKE-PCA         DV           AcSY241         144        LQQXEGODV         DL           AcSY241         181        LQQXEGODV         DL           Solyc03q07531         186        LLQXEGODV         DL           Solyc03q07531         186         FKSTKRANAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc07q054140         176         FSSSNAKESTKPENQRFLAAKASVEPEPHSSSSNEPEGLQCLLPHTTAENGOS	Solycliq066010         134         PQAVLPSSYTASEV         DV           Solyc08q05200         134         PQAVLPSSYTASEV         DV           AcSY221         133         PQSALPSSYTASEV         DV           AcSY221         134         PQSALPSSYTASEV         DV           AcSY211         141         TKE-PTSYTASEV         DV           AcSY211         141         TKE-PTSYTASEV         DV           AcSY211         144         TKE-PTSYTASEV         DV           AcSY211         144         TKE-PCA         DV           AcSY241         144        LQQXEGODV         DL           AcSY241         181        LQQXEGODV         DL           Solyc03q07531         186        LLQXEGODV         DL           Solyc03q07531         186         FKSTKRANAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc04q071730         173         RKNTKSNAAETSV         SS           Solyc07q054140         176         FSSSNAKESTKPENQRFLAAKASVEPEPHSSSSNEPEGLQCLLPHTTAENGOS	301yc01g109750											EK
Solyc08905200         134 PQAVLPSSYTASEV         DV           ALSYP22         134 PQAVLPSSYTASEV         DV           ALSYP22         138 PQALPSSYTASEV         DV           ALSYP23         138 HFXPLSSYTASEV         DV           ALSYP23         141 TEX-IPTSYNAPEL         DV           ALSYP21         141 TEX-IPTSYNAPEL         DV           Solyc06907576         146 SQET-MSSRSIEI         DV           ALSYP41         184IRQQKS-DGM         DL           ALSYP43         184IRQQKS-DGM         DL           Solyc049075530         180IRQGKSGDEV         DL           Solyc049075530         113 ENTIKANAARSV         SS           Solyc04907130         113 ENTIKANAARSV         SS           ALSYP11         113 ENTIKANAARSV         SS           ALSYP11         113 ENTIKANAARSV         SS           ALSYP11         113 ENTIKANAARSV         SS           ALSYP11         116 FSTNAAD-SP         SS           ALSYP11         117 FSTNLSRE-NPP-NORPLAAKAAAAANAANAANAANAANAANAANAANAANAANAANA	Solyc08905200         134 PQAVLPSSYTASEV         DV           ALSYP22         134 PQAVLPSSYTASEV         DV           ALSYP22         138 PQALPSSYTASEV         DV           ALSYP23         138 HFXPLSSYTASEV         DV           ALSYP23         141 TEX-IPTSYNAPEL         DV           ALSYP21         141 TEX-IPTSYNAPEL         DV           Solyc06907576         146 SQET-MSSRSIEI         DV           ALSYP41         184IRQQKS-DGM         DL           ALSYP43         184IRQQKS-DGM         DL           Solyc049075530         180IRQGKSGDEV         DL           Solyc049075530         113 ENTIKANAARSV         SS           Solyc04907130         113 ENTIKANAARSV         SS           ALSYP11         113 ENTIKANAARSV         SS           ALSYP11         113 ENTIKANAARSV         SS           ALSYP11         113 ENTIKANAARSV         SS           ALSYP11         116 FSTNAAD-SP         SS           ALSYP11         117 FSTNLSRE-NPP-NORPLAAKAAAAANAANAANAANAANAANAANAANAANAANAANA			~									DV
b.1yc086076540         134 PGAULPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP21         141 TEX-IPSYNAPEL         D-           ALSYP31         134	b.1yc086076540         134 PGAULPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP23         138 PGALPSSYTAGEV         D-           ALSYP21         141 TEX-IPSYNAPEL         D-           ALSYP31         134		124	DOALT	DOCUMBOR	7							= . DV
Solyc06g072760         146         SQEINSSRSIEI-	Solyc06g072760         146         SQEINSSRSIEI-		134	FQAVL	rssiTASE\	/ <b></b>							DV
Solyc06g072760         146         SQEINSSRSIEI-	Solyc06g072760         146         SQEINSSRSIEI-		134	PQAVL	PSSYTASE								DV
Solyc06g072760         146         SQEINSSRSIEI-	Solyc06g072760         146         SQEINSSRSIEI-		133	FQSAL	PSSYTAGEV	/							D-
Solyc06g072760         146         SQEINSSRSIEI-	Solyc06g072760         146         SQEINSSRSIEI-		138	HKPSL	PSSYTSSE	L — — -							DV
AcSYP43         192	ALSYP43         192		141	TKE-I	PTSYNAPE1								DT
AcSYP43         192	ALSYP43         192		146	SQEI-	-NSSRSIE:	L							QI
ALSYP42       181      LQQCKEGQDEV	AcSYP42       181      LQQCKECQCEV		184	LR	QQKEDGI	1							DL
ALSYP42       181      LQQCKEGQDEV	AcSYP42       181      LQQCKECQCEV		192	LR	LQKEDGA	7							DL
ALSYP42       181      LQQCKEGQDEVDL         Solyc03q075530       180      LLQSECHOCL	AcSYP42       181LQQKEGODEV		183	LQ	QQKEGPDG	7							DL
Solyc03q033850       136       RKSTTKSNAAESA	Solyc03q033850         136         RKSTTKSNAAESA		181	LQ	QQKEGQDE	7							DL
Solyc03q033850       136       RKSTTKSNAAESA	Solyc03q033850       136 RKSTTKSNAAESA	Solyc09g075530	180	LR	LQSEGHDGI								DL
AtSYP31       166       FSTKNAVD-SP	AtSYP31       166       FSTKNAVD-SP	Solyc03g033850											SI
AtSYP31       166       FSTNNAVD-SPQVNAKSVPEPPPWSSTCQSLTAIDAQGSN         AtSYP32       137       FSTNISRE-NP	AtSYP31       166       FSTNNAVD-SPQVNAKSVPEPPPWSSTCQSLTAIDAQGSN         AtSYP32       137       FSTNISRE-NP	Solyc04g071730	173	RKNTT	KSNAAETSV	7							
Solyc07g054140 175 F5TNLSRE-NPLKQFTAEPEPWSTCQ5LTAIDAQGSM At5YP22 133 F5SNASKESTNPFVRQRPLAKANASESV-PLPWANGSSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DS	Solyc07g054140 175 F5TNLSRE-NPLKQFTAEPEPWSTCQ5LTAIDAQGSM At5YP22 133 F5SNASKESTNPFVRQRPLAKANASESV-PLPWANGSSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DS		173	PKRVI	KEATP:	E							NT
Solyc07g054140 175 F5TNLSRE-NPLKQFTAEPEPWSTCQ5LTAIDAQGSM At5YP22 133 F5SNASKESTNPFVRQRPLAKANASESV-PLPWANGSSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DS	Solyc07g054140 175 F5TNLSRE-NPLKQFTAEPEPWSTCQ5LTAIDAQGSM At5YP22 133 F5SNASKESTNPFVRQRPLAKANASESV-PLPWANGSSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DSSSS Solyc08g067910 176 FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DS	AtSYP31	166	FSTKN	AVDSP			PQNNA	KSVPEP	PPWSS	SSNPF	GNLQQPLI	LPPLNTGAPPGS
AtSYP32       193       FSSNASKESTNPFVRQRPLAKRAAASESV-PLPMANGS	AtSYP32       193       FSSNASKESTNPFVRQRPLAKRAAASESV-PLPMANGS	Solyc07g054140	175	FSTNL	SRENP			LK	QPTAEP	PPWST	cos	]	LTAIDAQGSN
Solyc08g067910         176         FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DS	Solyc08g067910         176         FSSSTSKEASNPFMRQRPLASRNTASTSASPPPWAN-DS												
AtPEN1         188         ISTGESRF_QKALCEQ-GRGRVLDTINE	AtPEN1         188         IS	Solvc08g067910	176	FSSST	SKEASNPEN	4ROF	RPLA	SRNTA	CHCACD	PPWAN	-DS		PSSS
VVPEN1         176         ISTGESTFP OKAL CDFGRVLDTISE         Resverties           HVG02         185         LAETGESTFP OKAL CDGRVLDTISE         GRAVADLERS           SIPENIA         181         IISTGOSTFP OKAL CDGRQVMDTVME         GRAVKELERN           SIPENIA         178         IISTGOSTFP OKAL CDGRQVMDTVME         GRAVKELERN           SIPENIA         178         IISTGOSTFP OKAL CDGRQVMDTVME         GRAVKELERN           AtSYP124         177         IISTGOSTFP OKAL CDGRQVMDTVME         HAVKELERN           AtSYP123         180         IISSGESTFP OKAL CDGRQVMDTVSE         HAVKELERN           AtSYP131         179         IISSGESMFP OKAL CDGRQUMDTVSE         HAVKELERN           AtSYP131         179         IIETGDSMPI OKAL CDGRQUMDTVSE         HAVKELERN           AtSYP131         179         IIETGDSMPI OKAL CDGRQUMDTVSE         HAVKELERN           AtSYP132         178         IIETGDSGI OKAL CDGRQUMDTVSE         HAVKELERN           AtSYP132         183         IISSGESSFF OKAL CDGRQUMDTVSE         HAVKELERN           Solyc120005580         183         IISSGESSFF OKAL CDGRQUMDTVSE         HAVKELERN           Solyc120005500         183         IISSGESCSFI OKAL CDGRQUMDTVSE         HAV	VvPEN1         176         LISTGESTTFDOKELOGO-GREVLDTISE         Resverties           HVROR2         185         JAETGESTTFDOKELOGO-GREVLDTISE         GRAADOOGRECUGVVAE         GRAVALERS           SIPENIA         181         IISTGOSTTFDOKELOGO-GROUNDTIME         GRAVELERN           SIPENIA         178         IISTGOSTTFDOKELOGO-GROUNDTIME         GRAVELERN           AtSYP124         177         IISSGESNFPOKADO-GROUNDTIME         HAVKELERN           AtSYP123         180         IISSGESNFPOKADO-GROUNDTISE         HAVKELERN           AtSYP131         179         IISSGESNFPOKADO-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP132         178         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP132         178         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           Solyc12005580         183         IIETGOSOII OKAREQO-GROUNDTISE         HAVKELERN           Solyc030510         185         IIETGOSOII OKAREQO-GROUNDTISE         HAVKELERN <td>1 1 1 1 1 1 1 1 1</td> <td></td> <td></td> <td></td> <td>~</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td>	1 1 1 1 1 1 1 1 1				~							
VVPEN1         176         LISTGESTFFORMALOGO-GRAVLOTISE         Resvention           HVROR2         185         LABTGESTFFORMALOGO GRAVLOVAE         0.         HeaVALERS           SIPEN1a         181         LISTGQSTFFORMALOGO GRAVLOVAE         0.         HEAVKELERN           SIPEN1b         178         LISTGQSTFFORMALOGO GRAVUNCE         0.         HEAVKELERN           AtSYP124         177         LISTGESTFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP123         180         LISTGESTFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         179         LISSGESNFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         179         LISTGBSNFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         179         LISTGBSSOITORALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         180         LISGGASS	VvPEN1         176         LISTGESTTFDOKELOGO-GREVLDTISE         Resverties           HVROR2         185         JAETGESTTFDOKELOGO-GREVLDTISE         GRAADOOGRECUGVVAE         GRAVALERS           SIPENIA         181         IISTGOSTTFDOKELOGO-GROUNDTIME         GRAVELERN           SIPENIA         178         IISTGOSTTFDOKELOGO-GROUNDTIME         GRAVELERN           AtSYP124         177         IISSGESNFPOKADO-GROUNDTIME         HAVKELERN           AtSYP123         180         IISSGESNFPOKADO-GROUNDTISE         HAVKELERN           AtSYP131         179         IISSGESNFPOKADO-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP132         178         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP132         178         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           Solyc12005580         183         IIETGOSOII OKAREQO-GROUNDTISE         HAVKELERN           Solyc030510         185         IIETGOSOII OKAREQO-GROUNDTISE         HAVKELERN <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>.010401</td> <td></td> <td></td> <td></td> <td></td>								.010401				
VVPEN1         176         LISTGESTFFORMALOGO-GRAVLOTISE         Resvention           HVROR2         185         LABTGESTFFORMALOGO GRAVLOVAE         0.         HeaVALERS           SIPEN1a         181         LISTGQSTFFORMALOGO GRAVLOVAE         0.         HEAVKELERN           SIPEN1b         178         LISTGQSTFFORMALOGO GRAVUNCE         0.         HEAVKELERN           AtSYP124         177         LISTGESTFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP123         180         LISTGESTFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         179         LISSGESNFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         179         LISTGBSNFFORMALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         179         LISTGBSSOITORALOGO GRAVITANS         0.         HEAVKELERN           AtSYP131         180         LISGGASS	VvPEN1         176         LISTGESTTFDOKELOGO-GREVLDTISE         Resverties           HVROR2         185         JAETGESTTFDOKELOGO-GREVLDTISE         GRAADOOGRECUGVVAE         GRAVALERS           SIPENIA         181         IISTGOSTTFDOKELOGO-GROUNDTIME         GRAVELERN           SIPENIA         178         IISTGOSTTFDOKELOGO-GROUNDTIME         GRAVELERN           AtSYP124         177         IISSGESNFPOKADO-GROUNDTIME         HAVKELERN           AtSYP123         180         IISSGESNFPOKADO-GROUNDTISE         HAVKELERN           AtSYP131         179         IISSGESNFPOKADO-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP132         178         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP131         179         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           AtSYP132         178         IIETGOSOII OKAREQ-GROUNDTISE         HAVKELERN           Solyc12005580         183         IIETGOSOII OKAREQO-GROUNDTISE         HAVKELERN           Solyc030510         185         IIETGOSOII OKAREQO-GROUNDTISE         HAVKELERN <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td>.515451</td> <td></td> <td></td> <td></td> <td></td>								.515451				
HVROR2         185         NEDTEGCRLL ORALA DOC REGULGVUAE         C.         HEAVADLERS           SIPENIA         181         IISTEGCTEPORIO C.G. GREGVUGVVAE         C.         HEAVADLERS           SIPENIA         181         IISTEGCTEPORIO C.G.G.GREUDTWE         C.         HEAVKELERN           AtSYP124         177         IISTEGCTEPORIO C.G.G.GREUDTIRE         C.         HEAVKELERN           AtSYP124         177         IISSEESTEPORIO C.G.G.GREUDTIRE         C.         HEAVKELERN           AtSYP124         177         IISSEESNEPORIO C.G.G.GUNDTIRE         C.         HEAVKELERN           AtSYP131         179         IIETODSOITOKIRRQ-GROQUMDTIRE         C.         HEAVKELERN           AtSYP132         172         IIASEESNEPORIO C.G.G.GUNDTIRE         C.         HEAVKELERN           AtSYP132         178         IIETODSOITOKIRRQ-GROQUIDTIRE         C.         HEAVKELERN           AtSYP132         183         IISSEESNEPORIO C.G.G.GUNDTIRE         C.         HEAVKELERN           AtSYP132         184         IIETODSOITOKIRRQ-GROQUIDTIRE         C.         HEAVKELERN           Solycl0g008570         185         IISSEESNEPORIO C.G.GQUNDTIRE         C.         HEAVKELERN           Solycl0g052470	HVROR2         185         AE=TGCGTFLORKIA_DOCREVIGUVAE         COL         HEAVADLERS           SIPENIA         181         ISTGCGTFLORKIA_CO-GREQUMDIVME         COL         HEAVADLERS           SIPENIA         181         ISTGCGTFLORKIA_CO-GREQUMDIVME         COL         HEAVKELERN           AtSYP124         177         ISTGCGTFLORKIA_CO-GREQUIDIVE         COL         HEAVKELERN           AtSYP124         177         ISSCESNFPCKA_CO-GREQUIDIVE         HEAVKELERN           AtSYP124         177         ISSCESNFPCKA_COC-GREQUIDIVE         HEAVKELERN           AtSYP131         179         IETGDSOITCKARQE-GREQUIDITAR         HOTVKELERN           AtSYP131         179         IEGDSOITCKARQC-GREQUIDITAR         HAAVKELERN           AtSYP132         172         IEGDSOITCKARQC-GREQUIDITAR         HAAVKELERN           AtSYP132         178         IEGDSOITCKARQC-GREQUIDITAR         HAAVKELERN           Solyc10g008570         183         ISSCESSFOCKQCQC-GREQUIDITAR         HAAVKELERN           Solyc10g008570         185         ISSCESSFOCKQCQC-GREQUIDITAR         HAAVKELERN           Solyc01g050810         183         IEGDSOITCKARQCG-GREQUIDITAR         HAAVKELERN           Solyc01g008570         185	AtPEN1	188	LIS	TGES	RF	LOKA	IOE0-					RHDAVKDIEKN
SIPENIE         178         IISFGSFIF OKALCEO-GROUNDTIME         CONSTRUCT           AtSYP124         177         IISFGSFIF OKALCEO-GROUNDTIME         CONSTRUCT         HEAVKEIERN           AtSYP124         177         IISSGESNFLOKALQEO-GROUNDTISE         CONSTRUCT         HEAVKEIERN           AtSYP123         180         IISSGESNFLOKALQEO-GROUNDTISE         CONSTRUCT         HEAVKEIERN           AtSYP131         179         IIETOBSSQIOKALREO-GROUNDTIAE         CONSTRUCT         HEAVKEIERN           AtSYP131         179         IIETOBSSQIOKALREO-GROUNDTIAE         HEAVKEIERN           AtSYP132         178         IIETOBSSQIOKALREO-GROUNDTIAE         HEAVKEIERN           AtSYP122         178         IIETOBSSQIOKALREO-GROUNDTIAE         HEAVKEIERN           AtSYP121         189         IISSGESSFFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl2005580         183         IISSGESSFFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl008570         183         IISSGESSFFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl009056810         126         IIETOBSOII OKALGEQ-GROUNDTISE         DAKKEIERN           Solycl0080570         184         IISSGES-SFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl020056800 <td>SIPENIE         178         IISFGSEFF OKALCE-C-GROWNDIME.         COME           AtSYP12         187         IISFGSSCESFF OKALCE-C-GROWNDIME.         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         DAVKEIERN           AtSYP13         179         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         172         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         178         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         183         IIESGESSFFOKACEQ-GROGIMDIAE         DAVKEIERN           Solyc12g005580         183         IIETOBSOI OKALCEQ-GROGIMDISE         DAVKEIERN           Solyc10g08150         183         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g085610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOSSOI OKALCEQ-GROGIMDIAE         DAVKEIERN      <tr< td=""><td></td><td></td><td></td><td></td><td></td><td></td><td>IQ<mark>E</mark>Q-</td><td>GRGRVL</td><td>DTINE</td><td></td><td>Q</td><td></td></tr<></td>	SIPENIE         178         IISFGSEFF OKALCE-C-GROWNDIME.         COME           AtSYP12         187         IISFGSSCESFF OKALCE-C-GROWNDIME.         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         DAVKEIERN           AtSYP13         179         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         172         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         178         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         183         IIESGESSFFOKACEQ-GROGIMDIAE         DAVKEIERN           Solyc12g005580         183         IIETOBSOI OKALCEQ-GROGIMDISE         DAVKEIERN           Solyc10g08150         183         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g085610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOSSOI OKALCEQ-GROGIMDIAE         DAVKEIERN <tr< td=""><td></td><td></td><td></td><td></td><td></td><td></td><td>IQ<mark>E</mark>Q-</td><td>GRGRVL</td><td>DTINE</td><td></td><td>Q</td><td></td></tr<>							IQ <mark>E</mark> Q-	GRGRVL	DTINE		Q	
SIPENIE         178         IISFGSFIF OKALCEO-GROUNDTIME         CONSTRUCT           AtSYP124         177         IISFGSFIF OKALCEO-GROUNDTIME         CONSTRUCT         HEAVKEIERN           AtSYP124         177         IISSGESNFLOKALQEO-GROUNDTISE         CONSTRUCT         HEAVKEIERN           AtSYP123         180         IISSGESNFLOKALQEO-GROUNDTISE         CONSTRUCT         HEAVKEIERN           AtSYP131         179         IIETOBSSQIOKALREO-GROUNDTIAE         CONSTRUCT         HEAVKEIERN           AtSYP131         179         IIETOBSSQIOKALREO-GROUNDTIAE         HEAVKEIERN           AtSYP132         178         IIETOBSSQIOKALREO-GROUNDTIAE         HEAVKEIERN           AtSYP122         178         IIETOBSSQIOKALREO-GROUNDTIAE         HEAVKEIERN           AtSYP121         189         IISSGESSFFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl2005580         183         IISSGESSFFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl008570         183         IISSGESSFFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl009056810         126         IIETOBSOII OKALGEQ-GROUNDTISE         DAKKEIERN           Solycl0080570         184         IISSGES-SFOKALGEQ-GROUNDTISE         DAKKEIERN           Solycl020056800 <td>SIPENIE         178         IISFGSEFF OKALCE-C-GROWNDIME.         COME           AtSYP12         187         IISFGSSCESFF OKALCE-C-GROWNDIME.         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         DAVKEIERN           AtSYP13         179         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         172         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         178         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         183         IIESGESSFFOKACEQ-GROGIMDIAE         DAVKEIERN           Solyc12g005580         183         IIETOBSOI OKALCEQ-GROGIMDISE         DAVKEIERN           Solyc10g08150         183         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g085610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOSSOI OKALCEQ-GROGIMDIAE         DAVKEIERN      <tr< td=""><td>VvPEN1</td><td>176</td><td>LIS</td><td>TGES</td><td>TF</td><td>LOKA</td><td>IOEO-</td><td>GRGRVL GRGRVL</td><td>DTINE</td><td>·</td><td>Q</td><td>BHESVKELERN</td></tr<></td>	SIPENIE         178         IISFGSEFF OKALCE-C-GROWNDIME.         COME           AtSYP12         187         IISFGSSCESFF OKALCE-C-GROWNDIME.         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         CHEAVKEIERN           AtSYP12         180         IISSGESNF OKALCE-GROWNDISE         DAVKEIERN           AtSYP13         179         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         172         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         178         IIETOBSOI OKALREO-GROGIMDIAE         DAVKEIERN           AtSYP12         183         IIESGESSFFOKACEQ-GROGIMDIAE         DAVKEIERN           Solyc12g005580         183         IIETOBSOI OKALCEQ-GROGIMDISE         DAVKEIERN           Solyc10g08150         183         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g085610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOBSOI OKALCEQ-GROGIMDIAE         DAVKEIERN           Solyc01g055610         184         IIETOSSOI OKALCEQ-GROGIMDIAE         DAVKEIERN <tr< td=""><td>VvPEN1</td><td>176</td><td>LIS</td><td>TGES</td><td>TF</td><td>LOKA</td><td>IOEO-</td><td>GRGRVL GRGRVL</td><td>DTINE</td><td>·</td><td>Q</td><td>BHESVKELERN</td></tr<>	VvPEN1	176	LIS	TGES	TF	LOKA	IOEO-	GRGRVL GRGRVL	DTINE	·	Q	BHESVKELERN
ALSYP124         177         IISSGESNFP OK NCPC_C-GRGQUIDTISE         CHDAVKEIEKN           ALSYP13         180         IISSGESRF OK NCPC_C-GRGQUIDTISE         CHDAVKEIEKN           ALSYP111         185         IITDNAGGEFF TRACCH-GKGVLETVVE         CHDAVKEIEKN           ALSYP131         179         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           ALSYP131         179         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           ALSYP132         178         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           ALSYP132         188         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           Solyc120005580         183         IIEGGSSFF OKACGC-GRGQUIDTISE         CHDAVKEIEKN           Solyc10908510         183         IIEGGSOI OKARCGO-GRGQUIDTISE         CHDAVKEIEKN           Solyc0190580         183         IIETGDSOI OKARCGO-GRGQUITISE         CHDAVKEIEKN           Solyc0190580         183         IIETGNSOI OKARCGO-GRGQUITISE         CHDAVKEIEKN           Solyc019052470         182         IIETGNSOI OKARCGO-GRGQUITISE         CHDAVKEIEKN           Solyc020850700         187         VSEECRALIVESRCEVILLDNEISTE         CHDAVKEIEKN           Solyc019052470         182         IIETGNS	AtsYP124         177         IISSCESNF 0K KCCPC-CRCQUIDTISE         OK CCPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP13         180         IISSCESRF 0K CCPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP111         185         IITDNAGCEFT TRACCH-CKCVLETVVE         OHAVKEIEKN           AtsYP131         179         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP132         178         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP132         178         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP132         183         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           Solyc12000580         183         IIETCDSQI 0KM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc10008570         185         IIETCDSQI 0KM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc0105681         183         IIETCDSQI 0KM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc0105640         184         IIETCDSQI OKM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc0105640         184         IIETCDSQI OKM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc01052470         182         IIETCDSQI OKM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc020850760         184 </td <td>VvPEN1 HvROR2</td> <td>176 185</td> <td>LIS</td> <td><mark>TG</mark>ES <mark>TG</mark>EG</td> <td>TF RL</td> <td>LQKA LQRA LOKA</td> <td>IQ<mark>E</mark>Q- IAEQÇ IOEQ-</td> <td>GRGRVL GRGRVL GRGEVL</td> <td>DTINE DTISE GVVAE</td> <td>I</td> <td>Q R QE</td> <td>RHESVKELERN RHGAVADLERS RHEAVKELERN</td>	VvPEN1 HvROR2	176 185	LIS	<mark>TG</mark> ES <mark>TG</mark> EG	TF RL	LQKA LQRA LOKA	IQ <mark>E</mark> Q- IAEQÇ IOEQ-	GRGRVL GRGRVL GRGEVL	DTINE DTISE GVVAE	I	Q R QE	RHESVKELERN RHGAVADLERS RHEAVKELERN
ALSYP124         177         IISSGESNFP OK NCPC_C-GRGQUIDTISE         CHDAVKEIEKN           ALSYP13         180         IISSGESRF OK NCPC_C-GRGQUIDTISE         CHDAVKEIEKN           ALSYP111         185         IITDNAGGEFF TRACCH-GKGVLETVVE         CHDAVKEIEKN           ALSYP131         179         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           ALSYP131         179         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           ALSYP132         178         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           ALSYP132         188         IIETGDSOI OKARCEQ-GRGQUIDTISE         CHDAVKEIEKN           Solyc120005580         183         IIEGGSSFF OKACGC-GRGQUIDTISE         CHDAVKEIEKN           Solyc10908510         183         IIEGGSOI OKARCGO-GRGQUIDTISE         CHDAVKEIEKN           Solyc0190580         183         IIETGDSOI OKARCGO-GRGQUITISE         CHDAVKEIEKN           Solyc0190580         183         IIETGNSOI OKARCGO-GRGQUITISE         CHDAVKEIEKN           Solyc019052470         182         IIETGNSOI OKARCGO-GRGQUITISE         CHDAVKEIEKN           Solyc020850700         187         VSEECRALIVESRCEVILLDNEISTE         CHDAVKEIEKN           Solyc019052470         182         IIETGNS	AtsYP124         177         IISSCESNF 0K KCCPC-CRCQUIDTISE         OK CCPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP13         180         IISSCESRF 0K CCPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP111         185         IITDNAGCEFT TRACCH-CKCVLETVVE         OHAVKEIEKN           AtsYP131         179         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP132         178         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP132         178         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           AtsYP132         183         IIETCDSQI 0KM CPC-CRCQUIDTISE         OHAVKEIEKN           Solyc12000580         183         IIETCDSQI 0KM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc10008570         185         IIETCDSQI 0KM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc0105681         183         IIETCDSQI 0KM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc0105640         184         IIETCDSQI OKM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc0105640         184         IIETCDSQI OKM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc01052470         182         IIETCDSQI OKM CPC-CRCQINDTISE         OHAVKEIEKN           Solyc020850760         184 </td <td>VvPEN1 HvROR2 SlPEN1a</td> <td>176 185 181</td> <td>LIS</td> <td><mark>TG</mark>ES <mark>TG</mark>EG</td> <td>TF RL</td> <td>LQKA LQRA LOKA</td> <td>IQ<mark>E</mark>Q- IAEQÇ IOEQ-</td> <td>GRGRVL GRGRVL GRGEVL</td> <td>DTINE DTISE GVVAE</td> <td>I</td> <td>Q R QE</td> <td>RHESVKELERN RHGAVADLERS RHEAVKELERN</td>	VvPEN1 HvROR2 SlPEN1a	176 185 181	LIS	<mark>TG</mark> ES <mark>TG</mark> EG	TF RL	LQKA LQRA LOKA	IQ <mark>E</mark> Q- IAEQÇ IOEQ-	GRGRVL GRGRVL GRGEVL	DTINE DTISE GVVAE	I	Q R QE	RHESVKELERN RHGAVADLERS RHEAVKELERN
ALSYP111         185         IITDNAGGEFFIRMQEH-GKGKVLETVVEICO-WDAVRELEKS           ALSYP131         179         IIETCDSSQI QKARGEQ-CRGQIDTIAE         CHAVRELEKS           ALSYP132         178         IIETCDSSQI QKARGEQ-CRGQIDTIAE         CHAVRELEKS           ALSYP132         178         IIETCNSQI QKARGEQ-CRGQIMDTIAE         CHAVRELEKS           Solycl2000550         185         IISSGESCSDLVKTFEVKPE         CHAVRELEKS           Solycl20008570         185         IISSGESCSDLVKTFEVKPE         CHAVRELEKS           Solycl2005580         185         IISSGESCSDLVKTFEVKPE         CHAVRELEKS           Solycl200560510         185         IISSGEGCQC-GRCQIMDTISE         CHAVRELEKS           Solycl2005601         126         IIETCDSQII QKNQCQ-CRCQIMDTIAE         CHAVRELEKS           Solycl2005601         126         IIETCDSQII QKNQCQ-CRCQIMDTIAE         CHAVRELEKS           Solycl2005601         126         IIETCDS	AtsYP111         185         IITDNAGGEFTIRAQEH-GKGVLETVVEICO_CHDAVRELEKS           AtsYP131         179         IIETCDSQI QKARGQ-CRGQIDTIAE         CHDAVRELEKS           AtsYP132         178         IIETCDSQI QKARGQ-CRGQIDTIAE         CHDAVRELEKS           AtsYP132         178         IIETCDSQI QKARGQ-CRGQIDTIAE         CHDAVRELEKS           AtsYP132         189         IISGSGSCSDLVKTFEVKPE         CHDAVRELEKS           Solyc10g000870         185         IISGSGSCSDLVKTFEVKPE         CHDAVRELEKS           Solyc10g000870         185         IISGSGSCSDLVKTFEVKPE         CHDAVRELEKS           Solyc10g00870         185         IISGSGS	VvPEN1 HvROR2 SlPEN1a SlPEN1b	176 185 181 178	LIS	<mark>TG</mark> ES <mark>TG</mark> EG	TF RL	LQKA LQRA LOKA	IQ <mark>E</mark> Q- IAEQÇ IOEQ-	GRGRVL GRGRVL GRGEVL	DTINE DTISE GVVAE	I	Q2 RF QE	RHESVKELERN RHGAVADLERS RHEAVKELERN
ALSYP111         185         IITDNAGGEFFIRMQEH-GKGKVLETVVEICO-WDAVRELEKS           ALSYP131         179         IIETCDSSQI QKARGEQ-CRGQIDTIAE         CHAVRELEKS           ALSYP132         178         IIETCDSSQI QKARGEQ-CRGQIDTIAE         CHAVRELEKS           ALSYP132         178         IIETCNSQI QKARGEQ-CRGQIMDTIAE         CHAVRELEKS           Solycl2000550         185         IISSGESCSDLVKTFEVKPE         CHAVRELEKS           Solycl20008570         185         IISSGESCSDLVKTFEVKPE         CHAVRELEKS           Solycl2005580         185         IISSGESCSDLVKTFEVKPE         CHAVRELEKS           Solycl200560510         185         IISSGEGCQC-GRCQIMDTISE         CHAVRELEKS           Solycl2005601         126         IIETCDSQII QKNQCQ-CRCQIMDTIAE         CHAVRELEKS           Solycl2005601         126         IIETCDSQII QKNQCQ-CRCQIMDTIAE         CHAVRELEKS           Solycl2005601         126         IIETCDS	AtsYP111         185         IITDNAGGEFTIRAQEH-GKGVLETVVEICO_CHDAVRELEKS           AtsYP131         179         IIETCDSQI QKARGQ-CRGQIDTIAE         CHDAVRELEKS           AtsYP132         178         IIETCDSQI QKARGQ-CRGQIDTIAE         CHDAVRELEKS           AtsYP132         178         IIETCDSQI QKARGQ-CRGQIDTIAE         CHDAVRELEKS           AtsYP132         189         IISGSGSCSDLVKTFEVKPE         CHDAVRELEKS           Solyc10g000870         185         IISGSGSCSDLVKTFEVKPE         CHDAVRELEKS           Solyc10g000870         185         IISGSGSCSDLVKTFEVKPE         CHDAVRELEKS           Solyc10g00870         185         IISGSGS	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyClig060910       ISD       SSDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyC08g005200       ISD       ASDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyC08g005201       ISD       SSG	SolyClig065910       SDDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyClig065700       150       ASDKSQEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         AtSYP22       148      QRALLVESKRQEVLIDMEIAFNEATIEE       EQGIQEIHQQ         AtSYP21       156       EELRISQQRALLVESKRQEVVFLDMEITFNEATIEE       EQGIDEIHQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtsyP122 AtsyP124	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyClig060910       ISD       SSDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyC08g005200       ISD       ASDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyC08g005201       ISD       SSG	SolyClig065910       SDDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyClig065700       150       ASDKSQEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         AtSYP22       148      QRALLVESKRQEVLIDMEIAFNEATIEE       EQGIQEIHQQ         AtSYP21       156       EELRISQQRALLVESKRQEVVFLDMEITFNEATIEE       EQGIDEIHQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SIPEN1a SIPEN1b <u>AtsyP122</u> AtsyP124 AtsyP123	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyClig060910       ISD       SSDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyC08g005200       ISD       ASDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyC08g005201       ISD       SSG	SolyClig065910       SDDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyClig065700       150       ASDKSQEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         AtSYP22       148      QRALLVESKRQEVLIDMEIAFNEATIEE       EQGIQEIHQQ         AtSYP21       156       EELRISQQRALLVESKRQEVVFLDMEITFNEATIEE       EQGIDEIHQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SIPEN1a SIPEN1b AtsyP122 AtsyP123 AtsyP111 AtsyP131	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP131 AtSYP125	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 S1PEN1a S1PEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP131 AtSYP132 AtSYP132	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvEEN1 HvROR2 S1PEN1a S1PEN1b AtSYP122 AtSYP123 AtSYP123 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP122 AtSYP132	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 S1PEN1a S1PEN1b AtSYP122 AtSYP124 AtSYP124 AtSYP121 AtSYP111 AtSYP131 AtSYP125 AtSYP132 AtSYP132 Solyc12g005580	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyClig060910       ISD       SSDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyC08g005200       ISD       ASDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyC08g005201       ISD       SSG	SolyClig065910       SDDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyClig065700       150       ASDKSQEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         AtSYP22       148      QRALLVESKRQEVLIDMEIAFNEATIEE       EQGIQEIHQQ         AtSYP21       156       EELRISQQRALLVESKRQEVVFLDMEITFNEATIEE       EQGIDEIHQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP132 Solyc12g005580 Solyc10g008570	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyClig060910       ISD       SSDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyC08g005200       ISD       ASDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyC08g005201       ISD       SSG	SolyClig065910       SDDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyClig065700       150       ASDKSQEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         AtSYP22       148      QRALLVESKRQEVLIDMEIAFNEATIEE       EQGIQEIHQQ         AtSYP21       156       EELRISQQRALLVESKRQEVVFLDMEITFNEATIEE       EQGIDEIHQQ         AtSYP21       156       EELRISQ	VvEEN1 HvROR2 SlFEN1a SlFEN1b AtSYP122 AtSYP123 AtSYP123 AtSYP111 AtSYP131 AtSYP132 AtSYP132 Solyc12g005580 Solyc10g081580	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyClig060910       ISD       SSDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyC08g005200       ISD       ASDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyC08g005201       ISD       SSG	SolyClig065910       SDDKSQEQRALLVESKRQEVVLIDMEIAFNEATIEE         SolyClig065700       150       ASDKSQEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         SolyClig076540       150       SSGKSPEQRALLVESKRQEVLIDMEIAFNEATIEE       DGIQEVQQQ         AtSYP22       148      QRALLVESKRQEVLIDMEIAFNEATIEE       EQGIQEIHQQ         AtSYP21       156       EELRISQQRALLVESKRQEVVFLDMEITFNEATIEE       EQGIDEIHQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP131 AtSYP132 AtSYP132 AtSYP132 Solyc12g005580 Solyc10g081500 Solyc01g056610	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP123 AtSYP123 AtSYP111 AtSYP131 AtSYP132 AtSYP132 AtSYP132 Solyc10g008570 Solyc10g008570 Solyc10g008570 Solyc10g058470 Solyc07g052470	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP124 AtSYP123 AtSYP111 AtSYP132 AtSYP132 AtSYP132 AtSYP132 Solyc12g005580 Solyc10g08570 Solyc10g056810 Solyc0fg053760	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
SolyGlig06501     ISD     SSDKSQEQRALLVESKRQEVVLDNEIAFNEATLEE       SolyGlig05200     ISD     ASDKSQEQRALLVESKRQEVLUDNEIAFNEATLEE     DGGUEVQQQ       SolyGlig076540     ISD     SSG	SolyClig065910       SDSDKSQEQRALLVESKRQEVVLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig065910       SDSDKSQEQRALLVESKRQEVLUDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         SolyClig076540       ISO       SSGKSPEQRALLVESKRQEVLLDMEIAFNEAILEE DUGGQEVQQQ         AtSYP22       148      QRALLVESKRQEULLDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQQRALLVESKRQEUVELDMEIAFNEAVIEE EQGIGEIQQQ         AtSYP21       156       EELRISQ	VvEEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP132 AtSYP132 Solyc102008570 Solyc10008570 Solyc1090856810 Solyc029055910 Solyc029055090	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
Solyco8g005200         150         ASDKSQEQRALLVESRQDVLYLDNEIAFNEATIEE         DLGIQEVQQQ           Solyco8g076540         150         SSGKSPEQRALLVESRQEVLLDNEIAFNEATIEE         DLGIQEVQQQ           AtSYP22         148	Solyc08g005200         150         ASDKSQEQRALLVESRQDVLYLDNEIAFNEATIEE         DLGIQEVQQQ           Solyc08g076540         150         SSGKSPEQRALLVESRQEVLLDNEIAFNEATIEE         DLGIQEVQQQ           AtSYP22         148	VvEEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP123 AtSYP123 AtSYP123 AtSYP111 AtSYP131 AtSYP125 AtSYP122 Solyc12g005580 Solyc10g018580 Solyc10g058100 Solyc01g005810 Solyc02g05500 Solyc02g08500 Solyc02g08500 Solyc02g08500 Solyc02g08500 Solyc02g08500	176 185 181 178 187	LIS LAE LIS LIS LIS	<mark>TG</mark> ES TGEG TGQS TGQS	ETF ERL ETF ETF	lqka lqra lqka lqka lqka lqka	IQ <mark>E</mark> Q- IAEQQ IQEQ- IQEQ- IQEQ-	GRGRVL GRGRVL GRGEVL GRGQVM GRGQVM GRGQVM	DTINE DTISE GVVAE DTVME DTIME DTINE	I I I	QE QE QE QE QE	HESVKELERN HGAVADLERS HEAVKELERN HEAVKEIERN HDAVKDIEKS
Solyc08g0/6540     150     SSGKSPEQRALLVESRRQEVILLDNEIAFNEATIEE     EQGIQEIQQQ       AtSYP22     148	Solyc08g0/6540     150     SSGKSPEQRALLVESRRQEVILLDNEIAFNEATIEE     EQGIQEIQQQ       AtSYP22     148	VvEEN1 HvROR2 SIFEN1a SIFEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP111 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 Solyc12g005580 Solyc10g05580 Solyc10g05580 Solyc00g055870 Solyc00g052370 Solyc00g052370 Solyc00g052370 Solyc00g052350 Solyc00g052350	176 185 181 178 177 180 185 179 172 178 189 185 183 126 182 182 184 182 184 185	LIS LIS LIS LIS LIS LIS LIS LIE LIS LIS LIS LIS LIS LIS LIS LIS LIS LIS SSD		E TF TF TF TF TF TF TF TF TF TF TF TF TF T	LQKA LQKA LQKA LQKA LQKA LQKA LQKA LQKA		GRGRVL GRGRVL GRGQVM GRGQVM GRGQIL GRGQIL GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGVUF GRGVUF GRGVUF	DTINE DTISE GVVAE DTVME DTISE DTISE DTISE DTISE DTLAE EVKPE DTISE GTLAE DTISE GTLAE STVEE ETVVE AAKTE 			HSAVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKELERN HDAVKEIEKN HDAVKEIEKN HDAVKELEKN HDAVRDLEKK HDAVKELERN HDAVKELERN HDAVKELERN HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS
AtsYP22     148	AtsYP22     148	VvEEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP123 AtSYP131 AtSYP132 AtSYP132 Solyc10g008570 Solyc10g008570 Solyc10g058160 Solyc01g056810 Solyc07g052470 Solyc06053760 Solyc06g053760 Solyc0755555 Solyc075555 Solyc07555555555555555555555555555555555555	176 185 181 178 187 177 180 185 179 172 178 189 183 185 183 126 182 184 187 178 150	LIS LIS LIS LIS LIS LIS LIE LIE LIS LIS LIS LIS LIS LIS LIS LIS LIS SSD		E TF E TF E TF E FF C FF C QI E QI E QI E SF C QI E QI	LQKA LQKA LQKA LQKA LQKA LQKA LQKA LQKA	I QEQ- I AEQC I QEQ- I QEM- S I QEH- S I QEU- S I QEQ- I QQEQ- I QQEU- I QEQ- I QQEU- I QUE- I Q	GRGRVL GRGRVL GRGQVM GRGQVM GRGQUM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQVL GRGQVL GRGQVL GRGQVL GRQVE RRQEVV	DTINE GVVAE DTVME DTIME DTINE DTISE DTLSE DTLAE DTLAE DTLAE DTLAE DTLAE STVEE DTLAE STVEE AAKTE 			HSAVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKEIERN HDAVKEIERS HDAVKEIERS HDAVKEIERS HDAVKEIERS HDAVKEIEKN HDAVKELERN HDAVKELERK HDAVKELERK HDAVKELERS HDAVKELERS HDAVKELERS HEAVKIIKS HEAVEICS HEAVEICS HEAVEICS
AtsYP23         154         NGDRHPEQALLVESKRQELVLLDNEIFTNEAVIEE         EQGIDIQOQ           AtsYP21         156         ESLRISQQQALLUSKRCQUVLDNEITFNEAVIEE         EQGIDIQOQ           Solyc06g072760         160         SSSISPESSSILLESKRQDVVQLEHEIVFNKAIIEE         EQGMEIDQQ           AtsYP41         195         ENNLNGSRYRAEDDDFDDWTSEHQMSKIKKSEEVSVE         EKEIQVVES           Solyc01g100170         196         ENNNGSRYRAEDDDFDDWTSEHQMSKIKKSEETE         EKEIQVVES           Solyc01g100170         196         ENNNGS	AtsYP23         154         NGDRHPEQALLVESKRQELVLLDNEIFTNEAVIEE         EQGIDIQOQ           AtsYP21         156         ESLRISQQQALLUSKRCQUVLDNEITFNEAVIEE         EQGIDIQOQ           Solyc06g072760         160         SSSISPESSSILLESKRQDVVQLEHEIVFNKAIIEE         EQGMEIDQQ           AtsYP41         195         ENNLNGSRYRAEDDDFDDWTSEHQMSKIKKSEEVSVE         EKEIQVVES           Solyc01g100170         196         ENNNGSRYRAEDDDFDDWTSEHQMSKIKKSEETE         EKEIQVVES           Solyc01g100170         196         ENNNGS	VvEEN1 HvROR2 SIFEN1a SIFEN1a AtSYF122 AtSYF123 AtSYF123 AtSYF123 AtSYF111 AtSYF131 AtSYF125 AtSYF125 AtSYF125 S0Jyc12g005580 S0Jyc10g08570 S0Jyc10g05800 S0Jyc07g052470 S0Jyc06g053760 S0Jyc06g053760 S0Jyc06g062360 S0Jyc06g062260 S0Jyc11g066910 S0Jyc08g005200	176 185 181 178 177 180 185 179 172 178 189 183 185 183 126 182 184 187 178 150 150	LIS LIS LIS LIS LIS LIS LIE LIE LIE LIE LIE LIE LIE LIE SSD SSD SSD		TF F TF TF TF TF TF TF TF TF TF T	LQKA LQKA LQKA LQKA LQKA LQKA LQKA LQKA	QEQ-         IAEQC           IAEQC         IAEQC           IQEQ-         IQEQC           IQEQC-         IQEQC           IQEQC- </td <td>GRGRVL GRGRVL GRGQVM GRGQVM GRGQIL GRGQIL GRGQIL GRGQIL GRGQIM GRGQIM GRGQIM GRGQIM GRGQVL GRGVU GRGVVE RRQEVL RRQEVL</td> <td>DTINE DTISE GVVAE DTIME DTIME DTINE DTISE CTVVE DTISE DTIAE DTISE CREAT DTISE CREAT DTISE CREAT DTISE CREAT DTISE CREAT DTISE DTISE CREAT DTISE</td> <td></td> <td></td> <td>HSAVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKELERN HDAVKEIEKS HDAVKEIEKS HDAVKELEKS HDAVKDLEKK HDAVKELEKS HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELESS HDAVKELSS HD</td>	GRGRVL GRGRVL GRGQVM GRGQVM GRGQIL GRGQIL GRGQIL GRGQIL GRGQIM GRGQIM GRGQIM GRGQIM GRGQVL GRGVU GRGVVE RRQEVL RRQEVL	DTINE DTISE GVVAE DTIME DTIME DTINE DTISE CTVVE DTISE DTIAE DTISE CREAT DTISE CREAT DTISE CREAT DTISE CREAT DTISE CREAT DTISE DTISE CREAT DTISE			HSAVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKELERN HDAVKEIEKS HDAVKEIEKS HDAVKELEKS HDAVKDLEKK HDAVKELEKS HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELESS HDAVKELSS HD
AtsYP21     156     ESLFRISQQQALLLQSRQEVVFLDNEITFNEAILEE EQGTREIEDQ       Solyc06g072760     160     SSSISPESSSILLESKRQUVVQLEHEIVFNEAILEE EQGMIEIQQQ       AtsYP41     195     ENNLSRNSSSILLESKRQUVVEHEIVFNEAILEE EQGMIEIQQQ       AtsYP43     203     ENNLNGSRYR-PEEDDFGDML-NEHQMSKIKKSEESVSE EKEIQQVVES       Solyc01g100170     196     EMNLNGSHSR-RDDDDDDLDGFNEHQMSKIKKSEETSIE EKEIQQVVES       AtsYP42     194     EFNVNGKMSRLDEEDELGGMGFDEHQTIKLKEGHVSAE EREIQVVES       Solyc03g033850     152     SLDPDMKRDSEVRDNDVSQAPMRVQEQ-LLDDETRALQVELNSLLDSVQETETK       Solyc04g071730     189     NLDPMKRDSEGLGPDFDQAPFRLQQQLLDDETRALQVELSNLLDGKQTETK       AtSYP81     186     TLGNSESIEPDEIQAQFRRLQQQLLDDETRALQVELSNLLDGKRQTETK       AtSYP81     216     016	AtsYP21     156     ESLFRISQQQALLLQSRQEVVFLDNEITFNEAILEE EQGTREIEDQ       Solyc06g072760     160     SSSISPESSSILLESKRQUVVQLEHEIVFNEAILEE EQGMIEIQQQ       AtsYP41     195     ENNLSRNSSSILLESKRQUVVEHEIVFNEAILEE EQGMIEIQQQ       AtsYP43     203     ENNLNGSRYR-PEEDDFGDML-NEHQMSKIKKSEESVSE EKEIQQVVES       Solyc01g100170     196     EMNLNGSHSR-RDDDDDDLDGFNEHQMSKIKKSEETSIE EKEIQQVVES       AtsYP42     194     EFNVNGKMSRLDEEDELGGMGFDEHQTIKLKEGHVSAE EREIQVVES       Solyc03g033850     152     SLDPDMKRDSEVRDNDVSQAPMRVQEQ-LLDDETRALQVELNSLLDSVQETETK       Solyc04g071730     189     NLDPMKRDSEGLGPDFDQAPFRLQQQLLDDETRALQVELSNLLDGKQTETK       AtSYP81     186     TLGNSESIEPDEIQAQFRRLQQQLLDDETRALQVELSNLLDGKRQTETK       AtSYP81     216     016	VvEEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP132 Solyc10g008570 Solyc10g008570 Solyc10g008570 Solyc01g058100 Solyc02g052370 Solyc02g052370 Solyc02g052360 Solyc02g052360 Solyc02g076540 Solyc08g076540	1766 185 181 178 177 180 187 172 178 189 183 126 182 183 126 182 184 187 178 187 150 150	LIS LIS LIS LIS LIS LIS LIE LIE LIS LIS LIS LIS LIS LIS SSD SSD ASD SSG		TF RL TF TF TF TF TF TF TF TF TF TF TF TF TF TF T	LQKA LQKA LQKA LQKA LQKA LQKA LQKA LQKA	QEQ-1           IAEQC           IQEQ-1           QEQ-1           QEQ-1<	GRGRVL GRGRVL GRGQVM GRGQVM GRGQUL GRGQUM GRGQIL GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQUM CRCQVL CRVQIF GVVE RRQEVU RRQEVV	DTINE DTISE GVVAE DTIME DTINE DTISE ETVVE DTISE CVAPE DTISE GTLAE CVAPE DTISE GTLAE CONTINE CO			HSAVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKEIERN HDAVKEIERS YDAAKEIES HDAVKEIES HDAVKEIES HDAVKEIES HDAVKEIES HDAVKEIES HDAVKEIES HDAVKEIERS HDAVKEIERS HDAVKEIERS HDAVKEIERS HDAVKEIERS HDAVKEIES HDAVKEIS
AtSYP41     195     ENNLSRNRYR-PEEDDFCOML-NEHQMSKIKKSEEVSVE EKEIQQVVES       AtSYP43     203     ENNINGSRYR-AEDDFDDWTSEHQMSKIKKSEETSIE EKEIQQVVES       Sclyc01g100170     196     ENNINGSMSR-RDDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVVES       AtSYP42     194     EFNVNGKMSR-RDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSMSRLDEEDLGGMGFDEHQMIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSDDFNDVGFTELQMATGKDEQTAL EKEIQVISS       Sclyc03g07553     152     SLDPDMKRDSEVRDNDVSQAAPMRVQEQ-LLDETRALQVEINSLIDSVQETETN       Sclyc04g071730     189     NLDPMKRDSEGLGDFDQAAPTRVQEQ-LLDETRALQVEINSLIDSVQETETK       AtSYP81     186     TLGNSESIEPDELQAQPRRLQQQCLIDETRALQVEINSLIDGRQTETK       AtSYP81     216     016DBRATERDEOSTARD	AtSYP41     195     ENNLSRNRYR-PEEDDFCOML-NEHQMSKIKKSEEVSVE EKEIQQVVES       AtSYP43     203     ENNINGSRYR-AEDDFDDWTSEHQMSKIKKSEETSIE EKEIQQVVES       Sclyc01g100170     196     ENNINGSMSR-RDDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVVES       AtSYP42     194     EFNVNGKMSR-RDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSMSRLDEEDLGGMGFDEHQMIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSDDFNDVGFTELQMATGKDEQTAL EKEIQVISS       Sclyc03g07553     152     SLDPDMKRDSEVRDNDVSQAAPMRVQEQ-LLDETRALQVEINSLIDSVQETETN       Sclyc04g071730     189     NLDPMKRDSEGLGDFDQAAPTRVQEQ-LLDETRALQVEINSLIDSVQETETK       AtSYP81     186     TLGNSESIEPDELQAQPRRLQQQCLIDETRALQVEINSLIDGRQTETK       AtSYP81     216     016DBRATERDEOSTARD	VvEEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP123 AtSYP123 AtSYP123 AtSYP123 AtSYP125 AtSYP125 AtSYP122 Solyc12g005580 Solyc10g008570 Solyc10g056810 Solyc0052470 Solyc02g00509 Solyc02g00500 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g0692360 Solyc02g0692360 Solyc08g005200 Solyc08g076540 AtSYP22	1766185 18111787 17771777 17771777 17800 17991772 1788 18991772 1788 18991772 1788 1893185 189518555 189518555 1895185555 1895185555555555	LIS LIS LIS LIS LIS LIS LIS LIE LIS LIS LIS LIS LIS LIS SSD SSD SSD SSD SSD		TF	LQKA LQKA LQKA LQKA LQKA LQKA LQKA LQKA	QEQ-1AEQQ           QEQ-1AEQQ           QEQ-1AEQQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ-1QEQ           QEQ-1QEQ	GRGRVL GRGQVM GRGQVM GRGQVM GRGQIL GRGQIL GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQIM GRGQVC RRQEVL RRQEVL RRQEVL RRQEVL	DTINE DTISE GVIAE DTIME DTISE DTISE DTISE DTISE DTISE DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR DTISE CHAR CHAR CHAR CHAR CHAR CHAR CHAR CHAR	I		HSJVKELERN HGAVADLERS HGAVADLERS HGAVKELERN HDAVKELERN HDAVKEIEKS HDAVKEIEKS HDAVKELEKS HDAVKELEKS HDAVKELEKS HDAVKELEKS HDAVKELEKS HDAVKELEKS HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERS HBAVKELES HBAVKELS HBAVKELES HBAVKELS HBAVKELS HBAVKELES HBAVKELS HBAVK
AtSYP41     195     ENNLSRNRYR-PEEDDFCOML-NEHQMSKIKKSEEVSVE EKEIQQVVES       AtSYP43     203     ENNINGSRYR-AEDDFDDWTSEHQMSKIKKSEETSIE EKEIQQVVES       Sclyc01g100170     196     ENNINGSMSR-RDDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVVES       AtSYP42     194     EFNVNGKMSR-RDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSMSRLDEEDLGGMGFDEHQMIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSDDFNDVGFTELQMATGKDEQTAL EKEIQVISS       Sclyc03g07553     152     SLDPDMKRDSEVRDNDVSQAAPMRVQEQ-LLDETRALQVEINSLIDSVQETETN       Sclyc04g071730     189     NLDPMKRDSEGLGDFDQAAPTRVQEQ-LLDETRALQVEINSLIDSVQETETK       AtSYP81     186     TLGNSESIEPDELQAQPRRLQQQCLIDETRALQVEINSLIDGRQTETK       AtSYP81     216     016DBRATERDEOSTARD	AtSYP41     195     ENNLSRNRYR-PEEDDFCOML-NEHQMSKIKKSEEVSVE EKEIQQVVES       AtSYP43     203     ENNINGSRYR-AEDDFDDWTSEHQMSKIKKSEETSIE EKEIQQVVES       Sclyc01g100170     196     ENNINGSMSR-RDDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVVES       AtSYP42     194     EFNVNGKMSR-RDDDLDGFEHQMSKIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSMSRLDEEDLGGMGFDEHQMIKKSEAFTVE EKEIQQVISS       Sclyc03g07553     193     ENNEKKSDDFNDVGFTELQMATGKDEQTAL EKEIQVISS       Sclyc03g07553     152     SLDPDMKRDSEVRDNDVSQAAPMRVQEQ-LLDETRALQVEINSLIDSVQETETN       Sclyc04g071730     189     NLDPMKRDSEGLGDFDQAAPTRVQEQ-LLDETRALQVEINSLIDSVQETETK       AtSYP81     186     TLGNSESIEPDELQAQPRRLQQQCLIDETRALQVEINSLIDGRQTETK       AtSYP81     216     016DBRATERDEOSTARD	VvEEN1 HvROR2 S1PEN1a S1PEN1b AtSYP124 AtSYP124 AtSYP123 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 S01yc10908570 S01yc10908570 S01yc069053760 S01yc069053760 S01yc069053760 S01yc06905230 S01yc1066910 S01yc089076540 AtSYP23 AtSYP23	1766185 1851178 17771800 17771800 179178 1855179 172178 189178 1833126 1843183 185318 1843187 178 1843187 178 1843187 178 1843187 1845187 1843187 1843187 1843187 1843187 1843187 1843187 1843187 1843187 1843187 1845	LIS LIS LIS LIS LIS LIS LIE LIE LIS LIS LIS LIS LIS SSD SSD SSD SSG SSG		TF	LQKA LQRA LQRA LQKA LQKA LQKA LQKA LQKA LQKA FQKA FQKA FQKA FQKA FQKA FQKA FQKA F	QEQ_1AEQUE           QEQ_1AEQUE           QEQ_1QEQ_1           QQQ_1QEQ_1           QQQ_1QEQ_1           QQQ_1QEQ_1           QQQ_1QEQ_1           QQQ_1QEQ_1           QQQ_1QEQ_1           QQQ_1QEQ_1           QQUQES           LLVES           QLQES           LLVES           QLQES	GRGRVL GRGRVL GRGQVM GRGQVM GRGQUM GRGQL GRGQL GRGQL GRGQL GRGQL GRGQL GRGQL GRGQL GRGQL GRGQL GRGQL GRGQL KRQEVV RRQEVV RRQEVV KRQELV KRQELV	DTINE DTISE GVVAE DTIME DTISE DTISE DTISE DTISE DTISE DTISE DTISE STVEE ETVVE LUDRE LLDNE LLDNE	I I		HESVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKELERN HDAVKELERS HDAVRELEKS HDAVRDLEKS HDAVRDLEKS HDAVRDLEKS HDAVRELERS HDAVRELESS HDAVRELSS H
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Solyc01g100170     196     ENNLNGSHSR-RDDDLDDLGFNEHQMAKLKSEAFTVE: CREIQOVUSS       AtsYP42     194     ENNNNGKMSRLDEEDLGGMGFDEHQTIKLKEGHYSAE     EREIQOVUSS       Solyc03g075530     193     ENNEKKSSFLDDFNDVGFTELQMATGQKDEQFTAE     EREIQVLSS       Solyc03g03850     152     SLDPDMKRDSEVKDNDVSQAAPMRVQEQ-LLDETRALQVELNSLIDSVQETETN       Solyc04g071730     189     NLDPMKRDSEGLGDFDTQAAPTRVQEQ-LLDETRALQVELNSLIDSVQETETK       AtSYP81     186     TLGNSESIEPDEIQAQPRRLQQQLLDETRALQVELSNLIDGRQTETK       AtSYP81     216     01DBRALTMAEGRARTERSLOOTUKENSLIGRARTERSE	Solyc01g100170     196     ENNLNGSHSR-RDDDLDDLGFNEHQMAKLKSEAFTVE: CREIQOVUSS       AtsYP42     194     ENNNNGKMSRLDEEDLGGMGFDEHQTIKLKEGHYSAE     EREIQOVUSS       Solyc03g075530     193     ENNEKKSSFLDDFNDVGFTELQMATGQKDEQFTAE     EREIQVLSS       Solyc03g03850     152     SLDPDMKRDSEVKDNDVSQAAPMRVQEQ-LLDETRALQVELNSLIDSVQETETN       Solyc04g071730     189     NLDPMKRDSEGLGDFDTQAAPTRVQEQ-LLDETRALQVELNSLIDSVQETETK       AtSYP81     186     TLGNSESIEPDEIQAQPRRLQQQLLDETRALQVELSNLIDGRQTETK       AtSYP81     216     01DBRALTMAEGRARTERSLOOTUKENSLIGRARTERSE	VvEEN1 HvROR2 SIFEN1a SIFEN1a AtSYF122 AtSYF122 AtSYF123 AtSYF123 AtSYF123 AtSYF125 AtSYF125 AtSYF125 AtSYF122 Solyc12g005580 Solyc10g008570 Solyc10g05800 Solyc10g05800 Solyc07g052470 Solyc06g052360 Solyc02g085090 Solyc02g085090 Solyc02g085000 Solyc02g085000 Solyc02g05200 Solyc02g05200 Solyc11g066910 Solyc08g076540 AtSYF22 AtSYF23 AtSYF22 Solyc06g072760	1766 1855 1811 1778 1777 1777 1800 1855 1799 1722 1875 1833 1855 1833 1266 1822 1844 1877 1500 1500 1500 1500 1500 1500 1500 15	IS		2         TF           2         TF <tr td=""></tr>	LQKA LQRA LQRA LQKA LQKA LQKA LQKA LQKA LQKA FQKA FQKA FQKA FQKA FQKA FQKA FQKA F	QEO_         AEO_           AEO_         QEO_           QEO_         QEO_           QEO_ <td>GRGRVI GRGRVI GRGVU GRGQVM GRGQVM GRGQVM GRGQVM GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI RQEVV RQEVV KRQELV KRQELV KRQELV</td> <td>DTINE DTISE DTVME DTIME DTIME DTISE DTISE DTISE DTISE DTISE DTISE GTLAE STVEE ETVVE LIDNE: LIDNE: LIDNE: LIDNE: LIDNE: STDE</td> <td>I</td> <td></td> <td>HSAVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKELERN HDAVKEIEKS HDAVKEIEKS HDAVKELEKS HDAVKDLEKK HDAVKDLEKK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERS HEAVKELERS HEAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELESS HDAVKELSS HDAVKS HDAVKELSS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HDAVKELSS HDAV</td>	GRGRVI GRGRVI GRGVU GRGQVM GRGQVM GRGQVM GRGQVM GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI GRGQVI RQEVV RQEVV KRQELV KRQELV KRQELV	DTINE DTISE DTVME DTIME DTIME DTISE DTISE DTISE DTISE DTISE DTISE GTLAE STVEE ETVVE LIDNE: LIDNE: LIDNE: LIDNE: LIDNE: STDE	I		HSAVKELERN HGAVADLERS HGAVADLERS HDAVKELERN HDAVKELERN HDAVKEIEKS HDAVKEIEKS HDAVKELEKS HDAVKDLEKK HDAVKDLEKK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERK HDAVKELERS HEAVKELERS HEAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELERS HDAVKELESS HDAVKELSS HDAVKS HDAVKELSS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HDAVKELSS HDAV
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ALSIPSI 210 QL-TKRKSALEMAPSQUMMMSLLQQUTVPKQENYSQS-AVALHSVESR Solyc07g054140 211 QL-TKRLASDMPPSNELEMSKUDQDVPKQESYSQS-AVALHSVESR ALSYP32 235 QLVPMKPGEGESSPLLQSQQQQQQQQVVPLQDTYMQG-AEALHTVEST	ALSIFSI 210 QL-"KRKSALEMAPSQUMEMSLLQQUTVFRQENYSQS'AVALHSVEST Solyc07g054140 211 QL-RRRLASDMPPSNELEMSMLQDQVPRQESYSQS'ATALQNVEST ALSYF32 235 QLVFWRFGEGESSPLLQQSQQQQQQQUVFLQDTYMQS'ATALQNVEST Solyc08g067910 218 QLFPRKQGDGDTQPLLQDQQQQQQQQUVFLQDSYMQS'ATALQNVEST	VvEEN1 HvROR2 SIPEN1a SIPEN1a AtSYP122 AtSYP122 AtSYP123 AtSYP123 AtSYP123 AtSYP123 AtSYP125 AtSYP125 AtSYP122 Solyc12g005580 Solyc10g008570 Solyc10g005810 Solyc006g053760 Solyc02g00500 Solyc02g00500 Solyc02g00500 Solyc02g00500 Solyc08g005200 Solyc08g005200 Solyc08g076540 AtSYP22 AtSYP23 AtSYP21 Solyc06g072760 AtSYP41 AtSYP43 Solyc01g100170 AtSYP42 Solyc03g033850 Solyc04g071730	$\begin{array}{c} 1766\\ 185\\ 181\\ 178\\ 181\\ 177\\ 180\\ 177\\ 180\\ 177\\ 172\\ 178\\ 185\\ 178\\ 185\\ 182\\ 184\\ 187\\ 178\\ 185\\ 182\\ 184\\ 187\\ 178\\ 185\\ 182\\ 184\\ 187\\ 178\\ 185\\ 182\\ 184\\ 187\\ 178\\ 180\\ 195\\ 150\\ 100\\ 195\\ 150\\ 195\\ 196\\ 195\\ 196\\ 193\\ 192\\ 189\\ 193\\ 152\\ 189\\ 182\\ 188\\ 182\\ 180\\ 180\\ 180\\ 180\\ 180\\ 180\\ 180\\ 180$	IS- IS- IS- IS- IS- IS- IS- IS- IS- IS-			LQKA LQKA LQKA LQKA LQKA LQKA LQKA LQKA	QEO.           IAEQC           IAEQC           IAEQC           QEO.           PEEE           -AEDD           -REDD           -RED.           -RED. <t< td=""><td>GRGRVL GRGRVL GRGV</td><td>DTINE DTISE DTIME DTIME DTIME DTISE DTISE DTISE DTISE CONTINE DTISE CONTINE STVEE AAKTE LLDNE LLDNE LLDNE LLDNE FERQ QELHE FFSEQUEHT</td><td>ISF</td><td></td><td>HSJVKELERN HGAVADLERS HGAVADLERS HGAVKELERN HEAVKELERN HDAVKELEKS HDAVKELESS HDAVKELSS HDAVKE</td></t<>	GRGRVL GRGRVL GRGV	DTINE DTISE DTIME DTIME DTIME DTISE DTISE DTISE DTISE CONTINE DTISE CONTINE STVEE AAKTE LLDNE LLDNE LLDNE LLDNE FERQ QELHE FFSEQUEHT	ISF		HSJVKELERN HGAVADLERS HGAVADLERS HGAVKELERN HEAVKELERN HDAVKELEKS HDAVKELESS HDAVKELSS HDAVKE
Solycu/gus4140 211 QLKRKLASDMPPSNELEMSMLQUQVPKQESYSQS ATALQNVEST AtsyP32 235 QLVPWKPGEGESSPLLQ0SQQQQQQQQVPKQDTYMQG AEALHTVEST	Solycu/gu54140 211 QLKRKLASUMPPSNELEMSMLQDQVPKQESYSQS ARALQNVEST AtSYP32 235 QLYPMKPGEGESSELLQSQQQQQQQQUVPLQDTYMQS ARALQNVEST Solyc08g067910 218 QLFPRKQGDGDTQPLLQDQQQQQQQQUVPLQDSYMQS ARALQNVEST	VvEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP123 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 Solyc10g008570 Solyc10g008570 Solyc010g05870 Solyc010g05870 Solyc010g05800 Solyc010g0552470 Solyc06g052360 Solyc010g0553760 Solyc06g052360 Solyc06g052360 Solyc06g052360 Solyc06g072650 Solyc06g072650 AtSYP23 AtSYP21 Solyc06g072760 AtSYP41 AtSYP43 Solyc01g100170 AtSYP42 Solyc03g033850 Solyc03g033850 Solyc03g033850 Solyc03g033850	1765 1815 1811 1788 1877 1800 1855 1831 1799 1722 1889 1833 1855 1833 1266 1893 1833 1266 1833 1855 1833 1855 1833 1855 1833 1855 1855	IS		TF	LQKA LQKA LQKA LQKA LQKA LQKA LQKA FQKA FQKA FQKA FQKA FQKA FQKA FQKA F	QEO           IABQC           IABQC           IQEQ           IQEQ           QEQ           QUES           LLVES           LLVES           LLVES           LLVES           PEELE          DD           -AEDDD          DDAPRWAPR           APMRV	GRGRVL GRGRVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL GRGVL RRQVL RRQVL RRQVL RRQVL RRQVL RRQVL BRQVL BRQVL BRQVL BRQVL BRQVL GRGV	DTINE DTISE CVVAE DTIME DTIME DTIME DTISE ETVVE DTISE CVVEE DTISE GTLAE STVEE ETVVE LLDNE LLDNE LLDNE LLDNE FFELQUE FFELQUE FFELQUE DETRE	I		HESVKELERN HGAVADLERS HCAVKEIERN HEAVKEIERN HDAVKEIERS HDAVKEICS HDAVKEIERS HDAVKEIES HDAVKEIS HDAVKEIES HDAVKEIES HDAVKEI
ALSIF32 235 QLVFWKPGEGESSPLLQQSQQQQQQQQQQQVVPLQDTYMQG AEALHTVEST	ALSIF3Z 235 QLVFWARGEGESSFLLQQSQQQQQQQQQQQMVFLQDTYMQGAEALHTVEST Solyc08g067910 218 QLFPRKQGDGDTQPLLQDQQQQQQQQVFLQDSYMQSAEALQNVEST	VvEEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP123 AtSYP123 AtSYP111 AtSYP131 AtSYP125 AtSYP125 Solyc10g008570 Solyc10g008570 Solyc10g058100 Solyc01g10956110 Solyc06g053760 Solyc06g053760 Solyc06g053760 Solyc06g052360 Solyc08g005200 Solyc08g076540 AtSYP22 AtSYP23 AtSYP21 Solyc06g072760 AtSYP42 Solyc06g072760 AtSYP42 Solyc03g033850 Solyc03g033850 Solyc04g071730 AtSYP81	1766 1855 1811 1788 1877 1800 1779 1722 1788 1899 1722 1788 1899 1722 1788 1899 1722 1788 1899 1722 1788 1899 1830 1799 1830 1500 1500 1500 1500 1500 1500 1500 15	IS- IS- IS- IS- IS- IS- IS- IS- IS- IS-			LQKA LQKA LQKA LQKA LQKA LQKA LQKA CQKA CQKA CQKA CQKA CQKA CQKA CQKA C	QEO_           IABQQ           IABQQ           IABQQ           ICEO_           ICEO_           QEQ           LLVES           LLLQS           LLLQS           LLLQE	GRGRVL GRGRVL GRGV	DTINE DTISE DTISE DTIME DTIME DTISE ETVIE DTISE ETVIE AAKTE LLDNE LLDNE LLDNE TLDNE FLDNE FLDNE FDEQUEHEQ FNEHQUEHEQ FNEHQUEHEQ DETRA	ISF		HESVKELERN HGAVADLERS HEAVKELERN HEAVKELERN HDAVKELERN HDAVKELERS HDAVKELESS HDAVKELSS HDAVKS HDAVKELSS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HD
	201ACn8dn9/310 51% ÖFLAKKÖGDGDLÖLTYÖDÖÖÖÖÖÖÖÖIALTÖDZAWÖSÄVEYTÖNAE21	VvEEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP123 AtSYP131 AtSYP131 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 Solyc01g08570 Solyc01g08570 Solyc02g0553760 Solyc02g052370 Solyc08g076540 AtSYP23 AtSYP23 AtSYP23 AtSYP23 Solyc01g100170 AtSYP41 AtSYP41 AtSYP43 Solyc01g10170 AtSYP42 Solyc03g03850 Solyc03g03850 Solyc03g03850 Solyc03g03850 Solyc03g037530 Solyc03g03850 Solyc03g037530 Solyc03g03850 Solyc03g037530 Solyc03g037530 Solyc03g037530 Solyc03g037530 Solyc03g03850 Solyc03g037530 Solyc03g03850 Solyc03g0753440	1766 1855 1811 1788 1877 1800 1779 1722 1788 1899 1722 1788 1899 1722 1788 1899 1722 1788 1899 1722 1788 1899 1830 1799 1830 1500 1500 1500 1500 1500 1500 1500 15	IS- IS- IS- IS- IS- IS- IS- IS- IS- IS-			LQKA LQKA LQKA LQKA LQKA LQKA LQKA CQKA CQKA CQKA CQKA CQKA CQKA CQKA C	QEO_           IABQQ           IABQQ           IABQQ           ICEO_           ICEO_           QEQ           LLVES           LLLQS           LLLQS           LLLQE	GRGRVL GRGRVL GRGV	DTINE DTISE DTISE DTIME DTIME DTISE ETVIE DTISE ETVIE AAKTE LLDNE LLDNE LLDNE TLDNE FLDNE FLDNE FDEQUEHEQ FNEHQUEHEQ FNEHQUEHEQ DETRA	ISF		HESVKELERN HGAVADLERS HEAVKELERN HEAVKELERN HDAVKELERN HDAVKELERS HDAVKELESS HDAVKELSS HDAVKS HDAVKELSS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HD
SOTACO980.0110 − 518 ÖFLAKKÖRDEDIÅLFFÖDÖÖÖÖÖÖÖÖÖ ALFÖD21WÖSEVESL		VvEEN1 HvROR2 SIPEN1a SIPEN1b AtSYP122 AtSYP124 AtSYP123 AtSYP123 AtSYP111 AtSYP131 AtSYP132 AtSYP132 AtSYP132 AtSYP132 AtSYP132 Solyc10g008570 Solyc10g068500 Solyc01g10956010 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc02g085090 Solyc08g0752300 Solyc08g076540 AtSYP23 AtSYP42 Solyc06g072760 AtSYP42 Solyc03g03380 Solyc03g03380 Solyc03g03380 Solyc03g054140 AtSYP31	1766 1855 1811 1788 1877 1800 1779 1722 1788 1899 1722 1788 1899 1722 1788 1899 1722 1788 1899 1722 1788 1899 1830 1799 1830 1500 1500 1500 1500 1500 1500 1500 15	IS- IS- IS- IS- IS- IS- IS- IS- IS- IS-			LQKA LQKA LQKA LQKA LQKA LQKA LQKA CQKA CQKA CQKA CQKA CQKA CQKA CQKA C	QEO_           IABQQ           IABQQ           IABQQ           ICEO_           ICEO_           QEQ           LLVES           LLLQS           LLLQS           LLLQE	GRGRVL GRGRVL GRGV	DTINE DTISE DTISE DTIME DTIME DTISE ETVIE DTISE ETVIE AAKTE LLDNE LLDNE LLDNE TLDNE FLDNE FLDNE FDEQUEHEQ FNEHQUEHEQ FNEHQUEHEQ DETRA	ISF		HESVKELERN HGAVADLERS HEAVKELERN HEAVKELERN HDAVKELERN HDAVKELERS HDAVKELESS HDAVKELSS HDAVKS HDAVKELSS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HDAVKELSS HDAVKELSS HDAVKS HDAVKS HD

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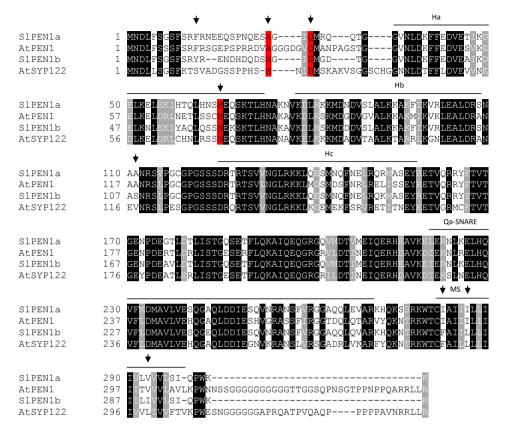
AtPEN1	231	R	HOVE	LDMAV	VEHQ	GAOII	DD	SH	GR	SSFIRG	TDO	QTA <mark>R</mark> VYQKNT	R
VvPEN1	219	K	⊡ H <mark>Q</mark> VF	LDMAV	QAQ		DDI	SQ				QTA <mark>R</mark> KHQISS	R
HvROR2	229	LLE		NDMAV	AAQ		DDI	EGH \	GR	RSFVDR	REQ	QVA <mark>R</mark> KHQKSS	R
SlPEN1a	224	K		MDMAV	ESQ			ESQ				EVA <mark>R</mark> KHQKSS	R
SlPEN1b	221 230	K		LDMAVI	ESQ EHO		DD					QVA <mark>R</mark> KHQKNT	R
AtSYP122 AtSYP124	220	I		LDMAVI LDMAAI	VESQ		DD IDI	EGN ESH	CV.	CCEUDD	TDO	VKARFYOKNT	R
AtSYP123	223	T.0		LDMAA	EAO			SN	SK	SSEVMR	TDO	QDAREYQKSS HGAKVLQRNN	R
AtSYP111	229	LÞ	HOVE	LDMAV	ĒSQ	EQ I	DE	енн	IN	SHYVAD	ANE	KTAKSHQRNS	R
AtSYP131	222	LD	LQ <mark>Q</mark> V <mark>E</mark>	LDMAV	DAQ	GEM I		ENM	SS	VDHVQS	NNQ	TKAVKSQKSS QDAREYQKSS	R
AtSYP125	215	LÞ		LDMAA	EAQ	GQQ 1	VN⊥	ESH	AK	SSFVRR	TDQ	QDAREYQKSS	R
AtSYP132	221	LD	2.2	LDMAV	DAQ			sQ	SS	VDHVQS	NTA	QRAKSLQKNS	R
AtSYP112	227 226	N		LDMAVI LDMAAI	ETQ			EAN ESH				YYANQMKKKT	
Solyc12g005580 Solyc10g008570	228	III I I	HOIF	LDMAA	EAQ EAQ			ESH	AH	SSEVER	TEO	QEAREIQKSS TEARELOKSS	R
Solyc10g081580	226	Lo		LDIAV	DAQ	DM I	DN I	ESQ	ST	VDHVOS	TTA	TEARELQKSS QKAKKLQKNS	R
Solyc01g056810	169	1 Lo	<b>Q</b> QI <b>F</b>	LDMAV	DAQ	DM I	DNI	ESQ	SA	VDHVQS	NTA	QKAKSLQRNS	R
Solyc07g052470	225	LD	I H <mark>Q</mark> IY	ldmav	EAQ			ETQ	RY	VDHVNM	TDA	QTAKSLQKKS	R
Solyc06g053760	230	i Le	IH <mark>Q</mark> IF	LDMAV			DDI	EHH.	VN	AQYVND	AKN	KTAKKYQKSS	R
Solyc02g085090 Solyc01g109750	222 205	LDK		LDMAVI				EHN	AI	GSFISG	TNS	FYAKQQQKKG DRANRM-KRT	R
Solyc06g062360	197	G		LDMAV <mark>N</mark> KDLAVI				EQN GSN				AKAAKTQRSN	
Solyc11g066910	197	G	NDIF	KDLAVI	HEO	GTM I	ם מכ	GSN	ENS	SHAATAO	RTO	AKAAKTORSN	s
Solyc08g005200	197	G	VNEIF	rkdlavi rkdlavi	HEQ	GTM I	DD	GSN	ENS	SHAATAL	RSQ	AKAAKTQRSN AKAAKTQRSN	S
Solyc08g076540	197	ΙGΒ	VNEIF	KDLAVI	HEQ	GAM I	DD	GSN	ENA	AHAATAQ	RSQ	AKAAKTQRSN	S
AtSYP22	192	G	NEIF	FKDLAVI	NDQ	GVM I	DD	GTH	DNS	SRAATSQ	KSQ	VQAAKTQKSN	S
AtSYP23 AtSYP21	201 203	G	HEIF	FKDLAVI	HDQ	GNM I		GTH	DNS	SYAATAQ SHAATTQ	KSH	VRHQRHK RKAAKTQRSN	D
Solyc06g072760	203	G									AKO TVQ	TKASKIQQCN	s
AtSYP41	243	VND		IKDLSAI								QKAERTQRHG	
AtSYP43	252	VSE	AQIM	KDLSAI KDLSVI	IDQ	GTIVI	DR I	DYN	QN	ASTVDD	LKQ	QKAERTQRQG KKAERSQKRG	G
Solyc01g100170	245	VND				GTIVI	DR I	DHN	QN	/ASTVED	LKQ	KKAERSQKRG	G
AtSYP42	244	VND		IKDLSAI								QKAERTQREG	
Solyc09g075530	240	VN 🗆	AQIM	IKDLSVI	IDQ			DHN	QSI	/SASVEE	FKQ	QKAERSQRKG	G
Solyc03g033850 Solyc04g071730	206 243	VID		HLMSTH HLMSTH			ELI.	YEQ YEQ	VEA	ATQNVEL ATQNVEL	NKE	SQAIQRNSSS SQAIQRNSSS	
AtSYP81	236	vv	SALN	HLMATH	L LOO	AOO I	SF	YDO	VEA	TKNVEL	NKE	SQAIQRNSSS	R
AtSYP31	261	пΤр		PQLATM		GEL :	IR I	DDN	DES	SLVNVEG	RSA	LQHLTRISSN	RWLMMKI
Solyc07g054140	256	IS		THLATM		GELS	ER I	DDN	DES	SLTNVEG	QGA	LKYLNRISSN ARYLNSISSN	SRKV
AtSYP32	286	II HID											
			SSIF	TQLATM	I SQQ	GEIA:	ER I	DQN	EDI	LANVEG	QSQ	ARYLNSISSN	RWLMMKI
Solyc08g067910	266		SSIF GSIF	FTQLATM FNQLATI	I SQQ SQQ	GEIA: GEVa:		DQN DEN	ED1 DD1	rlanveg rltnveg	QSQ QGA	ARYLNSISSN LKYLNSISSN	RWLMMKI RW
Solyc08g067910	266	II H D	GSIF	FNQLATI	SQQ	GEV	[R	DEN	DD1	LTNVEG	QGA	LKYLNSISSN	RW
Solyc08g067910 AtPEN1 VvPEN1	266 28 27	1 H 5 - 3 -	GSIF	FNQLATI	SQQ	GEV	ER	DEN	DD1	TLTNVEG	QGA	LKYLNSISSN	RW KWTCIA KWTCYG
Solyc08g067910 AtPEN1 VvPEN1 HvROR2	266 28 27 28	5 - 3 - 3 -	GSIF	FNQLATI	SQQ	GEV	ER	DEN	DD1	PLTNVEG	QGA	LKYLNSISSN	RW KWTCIA KWTCYG KWTFIG
Solyc08g067910 AtPEN1 VvPEN1 HvROR2 SlPEN1a	266 28 27 28 27	5 - 3 - 3 - 8 -	GSIF	FNQLATI	SQQ	GEV	ER	DEN	DD1	PLTNVEG	QGA	LKYLNSISSN	RW KWTCIA KWTCYG KWTFIG KWTCIA
Solyc08g067910 AtPEN1 VvPEM1 HvROR2 SlPEM1a SlPEN1b	266 28 27 28	5 - 3 - 3 - 8 - 5 -	GSIF	FNQLATI	SQQ	GEV	ER	DEN	DD1	LINVEG	QGA	LKYLNSISSN	RW KWTCIA KWTCYG KWTFIG KWTCIA KWTCFA
Solyc08g067910 AtPEN1 VvPEN1 HvROR2 SlPEN1a	266 28 27 28 27 27 27 28 27	5 - 3 - 3 - 8 - 5 - 4 - 4 -	GSIF	FNQLATI	SQQ(	GEV		DEN		rltnveg	QGA	LKYLNSISSN	RW KWTCIA KWTCYG KWTFIG KWTCIA KWTCFA KWTCFA KWTCYA
Solyc08g067910 Atpen1 VvPEN1 HvROR2 SlPEN1a SlPEN1b AtSYP122 AtSYP124 AtSYP123	266 27 28 27 28 27 27 28 27 27 27 27	5 - 3 - 3 - 3 - 5 - 4 - 7 -	GSIF	FNQLATI	SQQ:	GEV		DEN		rltnveg	QGA	LKYLNSISSN	RW KWTCIA KWTCIG KWTCIA KWTCFA KWTCFA KWTCYA KWTCYA
Solyc08g067910 AtPENI VvPENI HvR0R2 SlPENIa SlPENIb AtSYP122 AtSYP124 AtSYP123 AtSYP111	266 28 27 28 27 27 27 28 27 27 27 27 27 28	5 - 3 - 3 - 8 - 5 - 4 - 7 - 3 -	I GSIF	FNQLATI	SQQ	GEV		DEN		rltnveg	QGA	LKYLNSISSN	RW KWTCIA KWTFIG KWTCIA KWTCFA KWTCFA KWTCYA KWTCYA KWACIA
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Solyc07g054140 AtSYP32		GACTACGATGAAGATA
Solyc08g067910	347	-FVLIAFLMIFLFFVA
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AtPENI           VvPEN1           HvROR2           S1PEN1a           S1PEN1b           AtSYP122           AtSYP124           AtSYP123           AtSYP131           AtSYP125           AtSYP122	296	PILFNTLLRP
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AtPEN1		
VVPEN1		
HVROR2		
SIPENIa		
SIPENIb		
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AtSYP124		
AtSYP123		
AtSYP111		
AtSYP131		
AtSYP125		
AtSYP132		
AtSYP112		
Solyc12g005580	301	WWW
Solyc10g008570	408	NFLETRRFFDGMVYKDVVAWSSMITGYVRIGKPKISLELYGEMIDLGFEPNGFTLSAVIK
Solyc10g081580		
Solyc01g056810		
Solvc07q052470		
Solyc06q053760		
Solyc02q085090		
Solyc01q109750		
Solyc06g062360		
Solyc06g062360 Solyc11g066910		
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**FIGURE S1. Multialignment of plant syntaxins.** The dataset includes all the Arabidopsis syntaxins (AtSYPs and AtPEN1), barley HvROR2, grapevine VvPEN1 and 21 putative syntaxins retrieved from the tomato proteome in this study (indicated by the SolGenomics Network database ID code). The position of characteristic domains of syntaxins (the three helix domain Ha, Hb and Hc, the Qa-SNARE domain and the membrane-spanning (MS) domain are indicated. Gray color indicates residues physio-chemically conserved throughout the dataset; green color indicates residues physio-chemically conserved in SYP1 syntaxins. Yellow color highlights 24 residues specifically conserved in syntaxins of the SYP1b subclade described in this study.



**FIGURE S2.** Four syntaxins multi alignment. The dataset includes Arabidopsis AtPEN1 and AtSYP122 and tomato SIPEN1a and SIPEN1b. The position of characteristic syntaxin domains (the three helix domain Ha, Hb and Hc, the Qa-SNARE domain and the membrane-spanning (MS) domain are indicated. The arrows indicates the amino acid differences between functional syntaxins (AtPEN1 and SIPEN1a) and not functional ones (AtSYP122 and SIPEN1b). Red colour indicates substitutions in which the biochemical properties of the residues are not preserved.

# **Chapter 4**

# Identification of candidate *MLO* susceptibility genes in cultivated Solanaceae and functional characterization of tobacco *NtMLO1* and eggplant *SmMLO1*

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> <sup>†</sup> these authors contributed equally to the work \* corresponding author

Chapter composed of published data (*NtMLO1*) and submitted data (*SmMLO1*), both in Transgenic Research

Link to the published paper here: http://link.springer.com/article/10.1007/s11248-015-9878-4

# Abstract

Specific homologs of the plant <u>Mildew Locus Q</u> (MLO) gene family act as susceptibility factors towards the powdery mildew (PM) fungal disease, causing significant economic losses in agricultural settings. Thus, in order to obtain PM resistant phenotypes, a general breeding strategy has been proposed, based on the selective inactivation of MLO susceptibility genes across cultivated species. In this study, PCR-based methodologies were used in order to isolate MLO genes from cultivated solanaceous crops that are hosts for PM fungi, namely eggplant, potato and tobacco, which were named SmMLO1, StMLO1 and NtMLO1, respectively.

Based on phylogenetic analysis and sequence alignment, these genes were predicted to be orthologs of tomato *SIMLO1* and pepper *CaMLO2*, previously shown to be required for PM pathogenesis. Full-length sequences of the tobacco *NtMLO1* and eggplant *SmMLO1* homologs were used for two heterologous transgenic complementation assays. In both cases restoration of PM susceptibility was observed, which indicates that NtMLO1 and SmMLO1 are PM susceptibility factors in their species. The same assay showed that a single nucleotide change in a mutated *NtMLO1* allele leads to complete gene loss-of-function. Results here presented, also including a complete overview of the tobacco and potato *MLO* gene families, are valuable to study *MLO* gene evolution in Solanaceae and for molecular breeding approaches aimed at introducing PM resistance using strategies of reverse genetics.

#### **Keywords**

Eggplant, tobacco, MLO, powdery mildew, resistance, plant breeding

# Introduction

Powdery mildew (PM) is a major fungal disease affecting thousands of plant species, caused by ascomycete fungi belonging to the order of Erysiphales (Glawe 2008). Chemical control of PM accounts for a large proportion of fungicides used in agricultural settings (Hewitt 1998). Therefore, the use of cultivars harbouring genetic sources of PM resistance is generally envisaged as a valuable strategy to reduce farming costs and to cope with public concerns related to environmental pollution and human health.

The Mildew Locus O (MLO) gene family encodes for plant-specific proteins harbouring several transmembrane domains, topologically reminiscent of metazoan G-protein coupled receptors (Devoto et al. 2003). Specific homologs of the MLO family act as susceptibility genes towards PM fungi. Indeed, their inactivation, through loss-of-function mutations or silencing, has been associated with a peculiar form of PM resistance, referred to as mlo resistance (Pavan et al. 2010). This is associated with the enhancement of exocytosis defence pathways at plant-pathogen interaction sites, which are thought to contribute to the prevention of fungal penetration into host cells (Assaad et al. 2004). Initially discovered in barley, mlo resistance has been later shown to occur in other plant species as well, specifically Arabidopsis, tomato, pea, pepper and bread wheat (Bai et al. 2008; Büschges et al. 1997; Consonni et al. 2006; Humphry et al. 2011; Pavan et al. 2011; Wang et al. 2014; Zheng et al. 2013). This eventually led to the formalization of a breeding approach based on the systematic inactivation of MLO susceptibility genes across cultivated species affected by the PM disease (Dangl et al. 2013; Pavan et al. 2010; Pavan et al. 2011). Proof of concept for this strategy has been recently provided by the work of Wang et al. (2014), reporting the introduction of PM resistance in bread wheat following targeted mutagenesis of three MLO homoeoalleles. In contrast with most genetic sources of PM resistance, experimental data clearly indicate that *mlo* immunity is not specific towards particular fungal isolates and is extremely durable. For example, loss-of-function mutations of barley HvMLO confer resistance to all known isolates of the PM fungus Blumeria graminis f. sp. hordei, and is successfully employed in barley breeding since 1979 (Lyngkjaer et al. 2000). Similarly, pea er1 PM resistance, originating from the loss of function of *PsMLO1*, was first reported more than sixty years ago and is the only resistance source worldwide used for breeding purposes (Harland 1948; Humphry et al. 2011; Pavan et al. 2013).

Following the completion of the respective genome sequencing projects, a number of *MLO* homologs variable between 12 and 19 has been identified in the diploid species Arabidopsis, rice, grapevine, peach, woodland strawberry and cucumber (Devoto et al. 2003; Feechan et al. 2008; Liu and Zhu 2008; Pessina et al. 2014; Schouten et al. 2014). Remarkably, when placed in MLO protein family phylogenetic trees, all dicot MLO

isoforms experimentally shown to be required for PM susceptibility group in the same clade, referred to as clade V in scientific literature (e.g. Feechan et al. 2008; Pavan et al. 2011; Acevedo-Garcia et al. 2014). This shows that evolutionary studies on MLO proteins may predict candidates for being PM susceptibility factors.

Concerning solanaceous crops, we have functionally characterized the two *MLO* orthologs *SIMLO1* in tomato and *CaMLO2* in pepper, whose inactivation is causally associated with PM resistance (Bai et al. 2008; Zheng et al. 2013). In this work, we report the isolation, through a PCR-based approach, of three *MLO* genes from other cultivated Solanaceae, namely eggplant, potato and tobacco, which are likely to share a relation of orthology with *SIMLO1* and *CaMLO2*. The tobacco *NtMLO1* and the eggplant *SmMLO1* homologs were chosen for a transgenic complementation assay, resulting in their functional characterization and identification of a loss-of-function mutant allele of *NtMLO1*. Finally, newly available tobacco and potato genome sequences (Sierro et al. 2014; The Potato Genome Consortium 2011) were exploited to provide a comprehensive overview of the *MLO* gene families in these species.

# **Materials and methods**

#### PCR-based isolation and phylogenetic characterization of MLO putative orthologs

Young leaves of eggplant (*Solanum melongena* cv. Half Lange Violette), potato (*Solanum tuberosum* cv. Desiree) and tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) were collected for RNA extraction, which was performed using the Trizol reagent (Invitrogen). After RNA purification with the NucleoSpin RNA II kit (Macherey-Nagel), cDNA was synthesized using the SuperScript III RT first-strand cDNA synthesis kit (Invitrogen) with oligo(dT) primers.

Aiming to identify sequences of *SIMLO1* putative orthologs, the primer pairs Sol-F1 (5'-CATTTGACATTTCCCCTTCTTC-3') / Sol-R1 (5'-GCACCATGCATGAGTACCTCT-3') and Sol-F2 (5'-TTGGCAGTTGCTCATGTATTG-3') / Sol-R2 (5'- ATGGTGCCAGCTTCTAAGAG-3') were designed on the untranslated and coding sequences of the *SIMLO1* gene (GeneBank accession number NM\_001247885), respectively, (Primer3, Rozen and Skaletsky 2000) and used for PCR amplification of cDNAs. Amplicons obtained with the Sol-F2/Sol-R2 primer pair were purified using the NucleoSpin Extract II kit (Macherey-Nagel) and ligated (molar ratio 1:1) into the pGEM-T easy vector (Promega). Recombinant plasmids were cloned in *E. coli* DH10 $\beta$  chemically competent cells and recovered by using the Qiaprep spin miniprep kit (Qiagen). Sequencing reactions were performed using universal T7 and SP6 primers (Eurofins MWG Operon).

Inordertoobtainfull-length coding sequences of potato and tobacco *MLO* genes, sequences overlapping with those of the amplicons above mentioned were retrieved by BLAST search, using the tomato *SIMLO1* coding sequence as query against expressed sequence tags (ESTs) and predicted coding sequence repositories, both available at the Sol Genomic Network (SGN) database (http://solgenomics.net), and then used for local alignment. The expression and sequence of candidate genes was verified by PCR amplification of cDNAs, using the primer pairs StMLO1-F (5'- ATGGCTAAAGAACGGTCG -3') / StMLO1-R (5'-TTATTTGTTTCCAAAAGT-3') and NtMLO1-F (5'-ATGGAGGCAACTCCGACTTG-3') / NtMLO1-R (5'-TCAACTCATTTTGTTGCCAAATG-3'), cloning and sequencing, which were performed as above described.

In order to amplify a full-length *MLO* sequence in eggplant, the following primer pair was used: SmMLO1-F2 (5'-ATGGCTAAAGAACGGTCG-3') / SmMLO1-R1 (5'-TTATTTGTTTCCAAAAGTAAAATCTGA-3'). The corresponding PCR product was cloned and sequenced as indicated above.

Full-length eggplant, potato and tobacco *MLO* genes (named *SmMLO1*, *StMLO1* and *NtMLO1*, respectively) were translated *in silico*. Corresponding protein sequences were used, together with those of dicot MLO proteins experimentally associated with PM susceptibility [*Arabidopsis thaliana* AtMLO2 (GenBank accession code NP172598), AtMLO6 (NP176350) and AtMLO12 (NP565902), *Solanum lycopersicum* SIMLO1 (NP001234814), *Capsicum annuum* CaMLO2 (AFH68055), *Pisum sativum* PsMLO1 (AC007297), *Lotus japonicus* LjMLO1 (AAX77015) and *Medicago truncatula* MtMLO1 (ADV40949) and those of the remaining twelve homologs of the *Arabidopsis thaliana* AtMLO protein family, for ClustalW alignment and the construction of a Unweighted Pair Group Method with Arithmetic Mean (UPGMA) phylogenetic tree. Bootstrap values were calculated from 100 replicates. All of these bioinformatic analyses were performed using the CLC sequence viewer software (http://www.clcbio.com/).

# Generation of transgenic plants overexpressing NtMLO1 and SmMLO1

Two different *NtMLO1* PCR products, differing for a single nucleotide polymorphism, were inserted into the Gateway-compatible vector pENTR D-TOPO (Invitrogen) and cloned in *E. coli* competent cells. For functional analysis of *SmMLO1*, the full-length sequence was amplified using primer pair Fw1aGATE (5`-CACCATGGCTAAAGAACGGTCG-3`)/ RV5(5`-TTATTTGTTTCCAAAAGTAAAATCTGA-3`), and ligated according to the manufacturer's instructions in pENTR D-TOPO (Invitrogen).

Presence of the inserts was assessed by colony PCR, restriction enzyme digestion and sequencing using the universal M13 primer pair.

Inserts were then transferred by LR recombination into the binary plasmid vector pK7WG2, harboring the 35S Cauliflower Mosaic Virus (CaMV) promoter for constitutive expression and the marker gene *nptll* for kanamycin resistance selection. Plasmids were inserted into *E. coli* competent cells and positive colonies were again screened by colony PCR and sequencing, as above. Recombinant vectors were finally extracted and transferred to the AGL1-*vir*G strain of *A. tumefaciens* by electroporation.

A selected PM resistant tomato line, named SImIo1, described by Bai et al. (2008) and carrying a loss-of-function deletion in the SIMLO1 coding sequence, was used for transformation. This was performed according to the method described by McCormick et al. (1986). Briefly, seeds were surface-sterilized and sown on half-strength Murashige and Skoog (MS) agar supplemented with sucrose (10 g/l). Cotyledons were excised from 10-day-old seedlings, cut in two parts and submerged in an A. tumefaciens suspension with an OD<sub>600</sub> value of about 0.125. Infected cotyledonary explants were placed abaxially on the GCF10 medium (4.3 g/l MS basal salt mixture, 8 g/l agar, 30 g/L sucrose, 108.73 mg/l Nitsch vitamins, 1.5 mg/l zeatin riboside, 0.2 mg/l indole-3-acetic acid, pH 5.8) supplemented with 1 ml/l acetosyringone at 25°C for 48 h. Then, they were transferred to the GCF10 medium to which 100 mg/ml timentin and 50 mg/ml kanamycin were added and sub-cultured onto fresh medium every 3 weeks until shoot buds were observed. These were excised from the callus and transferred to the GCF11 medium (4.3 g/l MS basal salt mixture, 8 g/l agar, 30 g/L sucrose, 108.73 mg/l Nitsch vitamins, 1.9 mg/l zeatin riboside, pH 5.8) with 100 mg/ml timentin and 50 mg/ml kanamycin. After meristem development, the explants were transferred to the root-inducing medium MS30B5 (4.3 g/l MS basal salt mixture, 8 g/l agar, 30 g/L sucrose, 112 mg/L vitamin B5, 50 mg/ml kanamycin, pH 5.8). Once roots were developed, plantlets were finally located on woolen rock and grown in a greenhouse compartment.

For each of the two transformations with a different NtMLO1 gene sequence, 20 T, plants and two T, families (each composed by fifteen individuals derived from self-pollination of individual T<sub>1</sub> plants) were assayed for the presence of the construct, using the primer pair ntpIIF (5'- TCGGCTATGACTGGGCACAAC-3') ntpllR (5'-AAGAAGGCGATAGAAGGCGA-3'), designed on / the *ntpll* gene sequence, and the primer pair 35S-F (5'-GCTCCTACAAATGCCATCA-3') / 35S-R (5'-GATAGTGGGATTGTGCGTCA-3'), designed on the 35S promoter sequence. DNA of individual T, plants was isolated from using 2% CTAB in a protocol adapted for a 96-well plate (Doyle and Doyle 1987). Expression of the transgene was assessed by qPCR using the primer pair NtMLO1 gFw (5'-GTGGAAATAAGTCCAGCATTATG-3')/ NtMLO1 gRev (5'-CACCCAAAGGTACGAGTACAATC- 3').

The expression of the *SmMLO1* gene in eight transformed T<sub>1</sub> individuals was assessed by qPCR using the following primer pairs: mut-Fw (5'- TGTGCCTGTGGTTGAAACAG -3')/mut-

Rev (5'- TAGCCAAATCTGCAGCGTTC – 3') and wt-Fw (5'- TCACTTATTGCGCGGTTACC – 3')/ wt-Rev (5'- TTTGCTGGTTGTGAGCATGG -3'). Four T<sub>1</sub> individuals (T1\_K, T1\_M, T1\_P and T1\_Q) were allowed to generate segregating T<sub>2</sub> families. DNA isolation and PCR with nptII and 35S primer pair to test for the presence of the transgene in individual T<sub>2</sub> plants were performed as above.

#### Disease tests and Oidium neolycopersici quantification on transgenic plants

Three cuttings per  $T_1$  individuals and plants of the  $T_2$  families expressing the *NtMLO1* gene were challenged with an isolate of the tomato PM fungus *Oidium neolycopersici* maintained at the Plant Breeding Department of the University of Wageningen, The Netherlands.

Similarly,  $T_2$  families of four  $T_1$  individuals expressing *SmMLO1* containing at least six transgenic plants were tested for their response to the same pathogen. Twelve non-transgenic individuals segregating from these families were included.

The *Slmlo1* mutant line and the susceptible cultivar Moneymaker (MM) were used as controls. Inoculation was performed as described by Pavan et al. (2008), by spraying plants with a suspension of conidiospores obtained from freshly sporulating leaves of heavily infected plants and adjusted to a final concentration of  $4 \times 10^4$  spores/ml.

Inoculated plants were grown in a greenhouse compartment at  $20 \pm 2^{\circ}$ C with  $70 \pm 15\%$  relative humidity and day-length of 16 hours. Disease evaluation was carried out fifteen days after inoculation, based on a visual scoring as described by Bai et al. (2008) and/or analytically, by the relative quantification of the ratio between fungal and plant gDNAs. The latter was performed by the qPCR assay reported by Huibers et al. (2013). Specifically, plant and fungal genomic DNAs were extracted from *O. neolycopersici* infected tomato leaves (Qiagen DNeasy Plant Mini Kit) and used for amplification with the primer pairs On-F (5'-CGCCAAAGACCTAACCAAAA-3') / On-R (5'-AGCCAAGAGATCCGTTGTTG-3'), designed on *O. neolycopersici* internal transcribed spacer (ITS) sequences (GenBank accession number EU047564), and Ef-F (5'-GGAACTTGAGAAGGAGCCTAAG-3') / Ef-R (5'-CAACACCAACAGCAACAGTCT-3'), designed on the tomato *Elongation Factor* 1a (*Ef1a*) gene (Løvdal and Lillo 2009). Relative quantification was performed by the 2<sup>-ΔΔCt</sup> method (Livak and Schmittgen 2001; Pfaffl 2001).

#### In silico characterization of the tobacco and potato MLO gene families

In order to retrieve tobacco and potato MLO homologs, nucleotide sequences of *NtMLO1* and *StMLO1* and corresponding translated sequences were used as query for BLAST (BLASTn and tBLASTn) search against the Sol Genomics Network (SGN) and the Potato Genomics Resource (Spud DB) databases, using default parameters.

The number of transmembrane domains was predicted using the online software TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). The putative number of introns was obtained using the online service FGENESH of Softberry (http://www.softberry.com/). Chromosomal localization and gene position of potato *MLO* genes were inferred by the annotations of the Potato Genome Consortium. Finally, the MEME (http://meme.nbcr. net/) (Bailey et al. 2009) package was used to predict functional motifs in the NtMLO and StMLO protein families.

Predicted tobacco NtMLO and potato StMLO proteins were used to integrate the phylogenetic tree described in the previous section, according to the same methodologies above mentioned.

#### Results

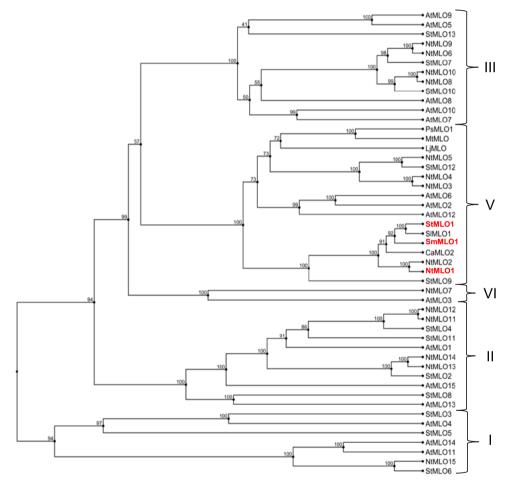
#### Identification of MLO gene sequences from cultivated Solanaceae

Two primer pairs, one designed on the untranslated sequence and the other on the coding sequence of tomato *SIMLO1*, were used to amplify homologous sequences from eggplant, potato and tobacco cDNAs. PCRs performed with the Sol-F1/R1 primer pair failed, thus suggesting the occurrence of polymorphic sequences in untranslated regions. In contrast, PCR performed with the Sol-F2/R2 primer pair, designed within the *SIMLO1* coding sequence, resulted in single amplification products of 876 bp. Full-length sequences of a 1560 bp tobacco gene, named *NtMLO1*, and a 1557 bp potato gene, named *StMLO1*, were obtained by assembling partial gene sequences of PCR products with overlapping sequences retrieved by the interrogation of the SGN database. Amplification and sequencing of *StMLO1* and *NtMLO1* from potato and tobacco cDNAs provided evidence for their actual expression in leaves and validated their sequences. These were deposited in the GenBank database with the accession codes KM244715 (*StMLO1*) and KM244716 (*NtMLO1*).

In order to clone an eggplant *MLO* gene putatively involved in PM susceptibility, several primers were designed, based on the identification of conserved regions from the alignment of *SIMLO1*, *StMLO1* and *NtMLO1*. These primers were then tested on eggplant cDNA. The SmMLO1-F2/SmMLO1-R1 primer pair produced a single PCR amplification product. The corresponding sequence of 1572 bp was named *SmMLO1* and deposited in the GenBank database with the accession code KM244717.

# Bioinformatic analyses support the identification of solanaceous *MLO* functional orthologs required for PM susceptibility

StMLO1, NtMLO1 and SmMLO1 protein sequences were used to perform a phylogenetic analysis. With strong bootstrap support, they were found to group in the phylogenetic clade V, containing all the dicot MLO homologs so far experimentally shown to be required for PM susceptibility (AtMLO2, AtMLO6, AtMLO12, SIMLO1, CaMLO2, PsMLO1, LjMLO1 and MtMLO1) (Fig. 1), thus indicating they could possibly be functionally related.



**FIGURE 1. UPGMA-based tree of full-length MLO proteins.** The dataset includes the tobacco NtMLO, potato StMLO and Arabidopsis AtMLO protein families, tomato SIMLO1, pepper CaMLO2, eggplant SmMLO1, pea PsMLO1, lotus LjMLO1 and barrel clover MtMLO1. Phylogenetic clades are designated with Roman numbers based on the position of AtMLO homologs, according to the nomenclature indicated by Feechan et al. (2008). Homologs identified by means of a PCR-based approach in this study (SmMLO1, StMLO1 and NtMLO1) are indicated in bold red. Numbers at each node represent bootstrap support values (out of 100 replicates).

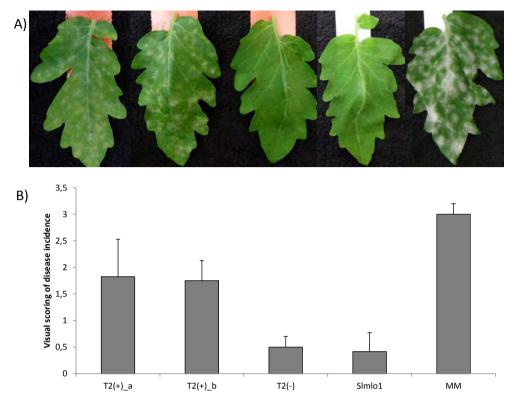
Previous studies highlighted the presence of amino acid residues highly conserved either in the whole MLO protein family or in MLO orthologs involved in the interaction with PM fungi, which are predicted to play a key functional role (Elliott et al. 2005; Panstruga 2005). All of these residues were found to be present in the StMLO1, NtMLO1 and SmMLO1 protein sequences (Supplementary Fig. 1), providing further evidence for the identification of *MLO* genes required for PM susceptibility.

Finally, another strong bioinformatic indication for the identification of solanaceous *MLO* susceptibility genes was provided by aligning the coding sequences of *StMLO1*, *NtMLO1* and *SmMLO1* with those of the PM susceptibility genes *SlMLO1* and *CaMLO2*, functionally characterized in tomato and pepper, respectively (Bai et al. 2008; Zheng et al. 2013) (Supplementary Fig. 2). Indeed, this revealed a very high percentage of nucleotide identity (81,4% between tomato and tobacco, 87,5% between tomato and eggplant and 94,8% between tomato and potato), suggesting that all of these solanaceous *MLO* genes are orthologs.

# Tobacco NtMLO1 complements tomato SIMLO1 in a functional complementation assay

In order to characterize *NtMLO1* at the functional level, we set up an assay based on its transgenic overexpression in the previously described tomato line SImIo1, which carries a loss-of-function mutation in the tomato *SIMIo1* homolog and is thus resistant to the PM fungus *O. neolycopersici* (Bai et al. 2008). We hypothesised that overexpression of *NtMLO1* would have restored PM susceptibility in the tomato SImIo1 mutant line, thereby demonstrating functional conservation between *NtMLO1* and *SIMLO1*.

After transformation, cuttings of 20 T<sub>1</sub> transgenic individuals were challenged with *O. neolycopersici*. Fifteen of the tested T<sub>1</sub> individuals showed restoration of PM symptoms (data not shown). In order to confirm this result, two T<sub>2</sub> families of the fifteen individuals (T<sub>2</sub>\_a and T<sub>2</sub>\_b) derived from self-pollination of two different T<sub>1</sub> plants were also inoculated, together with MM (the susceptible control) and the SImIo1 mutant line (the resistant control). The presence of the overexpression construct in segregating T<sub>2</sub> families was assessed by PCR amplification with primer pairs designed on the *nptll* gene and the 35S promoter (Supplementary Fig. 3). T<sub>2</sub> individuals not carrying the overexpression construct [T<sub>2</sub>(-)], as well as individuals of the SImIo1 mutant line, showed no *NtMLO1* expression and an average of disease score of about 0.5. In contrast, T<sub>2</sub> individuals of the two families positive for the presence of 1.8 and 1.7, respectively (Fig. 2 and Supplementary Fig. 4).



**FIGURE 2. Effects of the transgenic expression of** *NtMLO1* **in a tomato** *mlo* **loss-of-function genetic background.** Panel **A)** refers from left to right as follows: one individual of a  $T_2$  family positive for the presence the *NtMLO1* overexpression construct  $[T_{2-}a(+)]$ ; one individual of another independent  $T_2$  family positive for the presence of the *NtMLO1* overexpression construct  $[T_{2-}b(+)]$ ; one  $T_2$  individual negative for the presence of the overexpression construct  $[T_2(-)]$ ; one individual of the tomato Slmlo1 mutant line, carrying a loss of function deletion in the *SlMLO1* gene; one individual of the susceptible cultivar Moneymaker (MM). Panel **B**) reports the average visual scoring of disease incidence observed on the individuals of the same two  $T_2$  families  $[T_{2-}a(+)]$ ; individuals of both  $T_{2-}a$  and  $T_2-b$  families negative for the presence of the 35S::*NtMLO1* construct  $[T_2(-)]$ ; individuals of the Slmlo1 mutant line; individuals of the cultivar MM. The scale from 0 (completely resistant) to 3 (fully susceptible) reported by Bai et al. (2008), was used for scoring. Bars and standard errors refer to  $11T_2(+)_a$  plants,  $10T_3(+)_b$  plants,  $9T_3(-)$  plants, 10 Slmlo1 plants and 10 MM plants.

# A NtMLO1 point mutation causing the substitution of a conserved glutamine residue results in gene loss of function

During the preparation of the 35S::*NtMLO1* overexpression vector, we accidentally cloned another insert, carrying a single nucleotide polymorphism in the tobacco *NtMLO1* gene. This resulted in the substitution of a glutamine residue, located in the protein second intracellular loop and previously reported to be invariable throughout the whole MLO protein family, with arginine (Q198R, Fig. 3). We could not get the same arginine-coding

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insert by repeating the cloning procedure several times from tobacco cDNA, so we assumed that this resulted from a mutation due to an error by the Tag polymerase used for amplification. Nonetheless, in order to study the effect of this substitution on protein function, we developed transgenic lines carrying an overexpression construct for this insert. Following O. neolycopersici inoculation, none of 20 individual T, plants developed disease symptoms. Individuals of two independent T, families positive for the presence of the construct  $[T_{,}(+)_Q198R$ -a and b] were found to express the transgene, as assessed by qPCR (Supplementary Fig. 4). Nevertheless, following O. neolycopersici challenge, no PM symptoms were visible on [T<sub>2</sub>(+)\_Q198R] individuals, which were phenotypically undistinguishable from those of the SImIo1 line (Fig. 4A). In order to test whether the mutated NtMLO1 sequence maintained some residual functional activity, even so still resulting in a macroscopically resistant phenotype, we quantified, in transgenic individuals of the two T, families, the relative fold-change of the ratio between O. neolycopersici and tomato gDNAs. Compared to the SImIo1 line, no significant difference was found (Fig. 4B), indicating that the point nucleotide mutation causing the substitution of glutamine with arginine in the NtMLO1 protein sequence leads to complete gene loss of function.

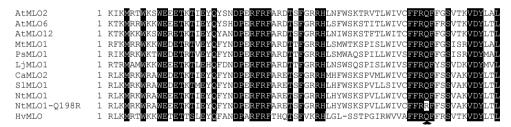


FIGURE 3. Alignment of part of the second MLO intracellular loop from several MLO proteins experimentally shown to be required for powdery mildew susceptibility (Arabidopsis AtMLO2, AtMLO6 and AtMLO12, tomato SIMLO1, pepper CaMLO2, pea PsMLO1, lotus LjMLO1, barrel clover MtMLO1 and barley HvMLO), and NtMLO1 proteins derived from the conceptual translation of the two inserts obtained during the cloning procedure (NtMLO1 and NtMLO1-Q198R). The latter is characterized by the substitution of an invariable glutamine with arginine, whose position is indicated by an arrow.

A) 1,6 0. neolycopersici ITS/S. lycopersicum Ef1 B) 1,4 normalized fold change 1,2 1 0,8 0,6 0,4 0,2 0 T2 (+)\_Q198R T2 (-)\_Q198R Simio1

FIGURE 4. Effects of the transgenic expression of a *NtMLO1* mutant sequence, resulting in the substitution of a glutamine residue with arginine in the protein second intracellular loop (Q198R). Panel A) shows the phenotype of a plant of the tomato loss-of-function Slmlo1 line (right) and transgenic individuals from two different T<sub>2</sub> families (left and centre) assessed for transgene overexpression. Panel B) shows the relative quantification of the ratio between *Oidium neolycopersici* and plant gDNAs in transgenic individuals of the same T<sub>2</sub> families assessed for the presence or absence of the overexpression construct [T<sub>2</sub>(+)\_Q198R and T<sub>2</sub>(-)\_Q198R, respectively] and in the tomato Slmlo1 mutant line. Bars and standard errors refer to 11 and 7 transgenic individuals for NtMLO1\_Q198R-a and b, respectively, and 10 Slmlo1 plants.

# Eggplant SmMLO1 complements tomato SIMLO1 in a functional complementation assay

In order to characterize *SmMLO1* at the functional level, we set up an assay based on its transgenic overexpression in the tomato line *Slmlo1*. This carries a loss-of-function mutation in the tomato homolog *SlMLO1* and is thus resistant to the fungus *O. neolycopersici,* causing PM in tomato (Bai et al. 2008; Seifi et al. 2014). We assumed that restoration of PM symptoms upon *SmMLO1* overexpression would have proven functional conservation with the susceptibility gene *SlMLO1*.

After transformation, eight individual  $T_1$  plants were obtained. All of them expressed the transgene (Fig. 5, panel A). Four randomly selected  $T_1$  plants (T1\_K, T1\_M, T1\_P and T1\_Q) were allowed to self-pollinate and give segregating  $T_2$  families. Following inoculation with *O. neolycopersici*, non-transgenic  $T_2$  individuals and the line *Slmlo1* showed no fungal sporulation, thus displayed a resistant phenotype (DI=0). In contrast, all the transgenic individuals were heavily infected as the susceptible control MM, indicating that *SmMLO1* is a PM susceptibility gene. The lowest and the highest average DI scores were associated with transgenic T2\_K (2,1) and T2\_M (2,5) individuals (Fig. 5, panel B and C).

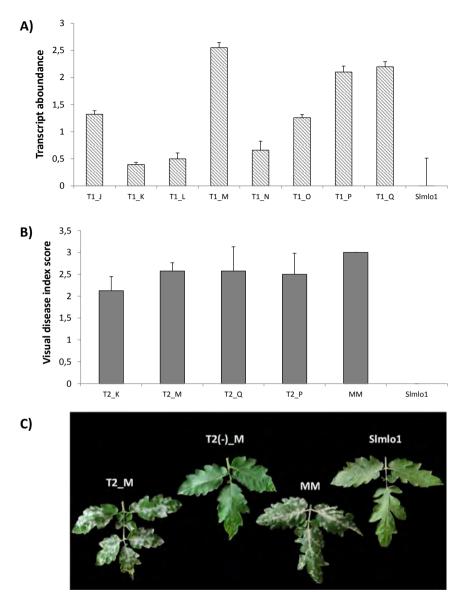
#### In silico characterization of tobacco and potato MLO families

Recently released sequences from potato (group *Phureja DM1*) and tobacco (cv. *Basma Xanthi*) prompted us to perform a genome-wide search aiming to characterize the *MLO* gene families in these species. This search revealed a total of 15 and 13 predicted tobacco *NtMLO* and potato *StMLO* loci, respectively, which were named according to the nomenclature specified in Tables 1 and 2. A predicted tobacco coding sequence, referred to as mRNA\_127718\_cds in the Sol Genomics Database, was found to be identical to *NtMLO1*. No sequence fully matching with *StMLO1* could be identified by the interrogation of the Potato Genomics Resource database, but in its place a partial gene sequence showing 100% of identity with the same gene.

For tobacco and potato MLO proteins, amino acid length and number of transmembrane domains were inferred (Supplementary Table 1 and Table 2). In addition, information on chromosomal localization and intron number was available for predicted *StMLO* genes (Table 2).

The tobacco NtMLO and potato StMLO protein families were used as input to search for conserved motifs, using an approach similar to the one previously reported by Deshmukh et al. (2014). We looked for motifs with length ranging from 40 to 70 residues and shared by at least three homologs. For each of the two families, seven motifs were identified. Of these, five were found to be at least partially matching with those identified in the soybean protein family (Deshmukh et al. (2014) (Supplementary Table 3).

A comparative analysis was carried out in order to establish phylogenetic relationships between the NtMLO and the StMLO protein families and MLO proteins from other dicot plant species. The analysis resulted in the distinction of five clades, designated with Roman numbers based on the position of Arabidopsis AtMLO homologs, according to the nomenclature indicated by Feechan et al. (2008) (Fig. 1). Besides NtMLO1 and StMLO1, additional NtMLO (NtMLO2, NtMLO3, NtMLO4 and NtMLO5) and StMLO (StMLO9 and StMLO12) homologs were found to group in clade V together with all dicot MLO proteins previously associated with PM susceptibility.



**FIGURE 5. Effect of the transgenic expression of** *SmMLO1* **in a tomato** *mlo* **loss-of-function genetic background**. **A)** Relative quantification of *SmMLO1* expression levels in eight T1 individuals transformed with *SmMLO1* (T1\_J,T1\_K,T1\_L,T1\_M,T1\_N,T1\_O,T1\_P andT1\_Q) and four individuals of the tomato line Slmlo1 used as background for transformation. **B**) Average disease index (DI) recorded fifteen days after artificial inoculation with the powdery mildew fungus *Oidium neolycopersici*. The graph refers to: transgenic T<sub>2</sub> individuals of four independent lines (T2\_K,T2\_M,T2\_Q and T2\_P) transformed with *SmMLO1*; the line *Slmlo1* (used as background for transformation). The scale from 0 (completely resistant) to 3 (fully susceptible) reported by Bai et al. (2008), was used for scoring. Bars refer to standard errors of at least six replicates **C**) Phenotype of a selected transgenic individual of the line T2\_M, a non-transgenic individual of the line T2(-)\_M, a MM individual and an individual of the line *Slmlo1*.

#### Discussion

In previous studies, we functionally characterized tomato *SIMLO1* and pepper *CaMLO2* as two solanaceous *MLO* susceptibility genes, as their inactivation was causally associated with PM resistance (Bai et al. 2008; Zheng et al. 2013). Starting from this information, we followed here a combined approach based on database search and PCR amplification, which resulted in the isolation of three *MLO* genes from other widely distributed solanaceous species affected by the PM disease, namely eggplant (*SmMLO1*), potato (*StMLO1*) and tobacco (*NtMLO1*). PM disease represents one of the most important fungal diseases of tobacco and eggplant (Bubici and Cirulli 2008; Darvishzadeh et al. 2010) and in conducive environments may lead to important economic losses in potato cultivation (Glawe et al. 2004).

A chain of evidence, based on phylogenetic relatedness (Fig. 1) and sequence conservation with other known PM susceptibility genes and proteins (Supplementary Fig. 1 and Supplementary Fig.2) was provided, suggesting the identification of solanaceous orthologs of *SIMLO1* and *CaMLO2*. Aiming at the functional characterization of *NtMLO1* and *SmMLO1*, we set up an assay based on their heterologous overexpression in a tomato *mlo*-mutant genetic background, taking advantage from the availability of a tomato resistant line and routine protocols for tomato genetic transformation (Bai et al. 2008). Success of such an assay, as demonstrated by the restoration of symptoms in transgenic plants (Fig. 2), provides a final evidence for the role of *NtMLO1* and *SmMLO1* as a PM susceptibility genes.

While completing this work, newly released sequences of potato and tobacco became available. Thus, a genome-wide search was performed, which allowed to retrieve additional *MLO* homologs and, presumably, to characterize the complete tobacco and potato *MLO* gene families. Phylogenetic analysis using these sequences highlighted the presence of additional NtMLO and StMLO proteins in clade V, previously shown to group dicot MLO homologs acting as PM susceptibility factors (Fig. 1). Functional redundancy of MLO homologs belonging to this clade has been shown to occur in *Arabidopsis thaliana*, as the simultaneous inactivation of the three homolog genes *AtMLO2*, *AtMLO6* and *AtMLO12* is required to result in complete PM resistance. Thus, functional analyses, such as the transgenic complementation test above mentioned, might lead to the identification of additional solanaceous MLO homologs playing a role in the interaction with PM fungi.

Interestingly, due to a polymerase error during the cloning procedure, we also had the opportunity to verify the crucial role of a glutamine residue localized in the second intracellular MLO domain. This amino acid has been shown to be invariable throughout the whole MLO protein family and therefore predicted to be fundamental for the role of MLO proteins as PM susceptibility factors (Elliott et al. 2005). Indeed, its replacement

with arginine in tobacco NtMLO1 (Fig. 3) resulted in complete failure of transgenic complementation, as inferred by visual scoring and relative quantification of fungal gDNA with respect to plant gDNA (Fig. 4). This result represents a complement to earlier investigations addressed to the functional characterization of MLO proteins (Reinstädler et al. 2010; Pavan et al. 2013).

A growing body of experimental evidence supports the view that *mlo*-based resistance can be conveniently pursued as a strategy to cope with the PM disease in practical breeding (Pavan et al. 2010). Therefore, we predict that results here provided might be of great interest for future activities aimed at the introduction of PM resistance in Solanaceae. Targeted identification of mutations of *MLO* susceptibility genes can be achieved through conventional approaches of TILLING (targeted induced local lesions in genomes) or RNA interference (McCallum et al. 2000; Matthew 2006). In addition, cutting-edge technologies of genome editing are also available to the breeder, based on zinc finger nucleases (ZFNs), clustered regularly interspaced short palindromic repeat (CRISPR) and transcription activator-like effector nucleases (TALEN) (Gaj et al. 2013, Terns 2014). Noteworthy, a TALEN-based approach has been recently successfully applied to introduce PM resistance in bread wheat through simultaneous targeting of three *MLO* alleles, as mentioned in Wang et al. (2014).

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## **Supplementary Tables**

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Gene	SGN database sequence ID	Predicted TM	Amino-acid length	Clade
NtMLO1	mRNA_127718_cds	6	525	V
NtMLO2	mRNA_127185_cds	6	532	V
NtMLO3	mRNA_63807_cds	7	597	V
NtMLO4	mRNA_106507_cds	6	605	V
NtMLO5	mRNA_52113_cds	7	520	V
NtMLO6	mRNA_44723_cds	6	554	111
NtMLO7	mRNA_90912_cds	7	489	VI
NtMLO8	mRNA_125509_cds	7	555	111
NtMLO9	mRNA_33476_cds	7	455	Ш
NtMLO10	mRNA_91715_cds	7	410	111
NtMLO11	mRNA_52133_cds	7	492	Ш
NtMLO12	mRNA_46569_cds	7	490	Ш
NtMLO13	mRNA_79933_cds	6	508	Ш
NtMLO14	mRNA_23316_cds	7	505	Ш
NtMLO15	mRNA_44406_cds	7	558	I.

SUPPLEMENTARY TABLE 1. Characteristics of the tobacco *NtMLO* gene family members identified in this study

SUPPLEMENTARY TABLE 2. Characteristics of the potato *StMLO* gene family members identified in this study

Gene	Spud DB database sequence ID	Chr.	Position from – to (bp)	Predicted introns	Predicted TM	Amino-acid length	Clade
StMLO1*	-	-	-	-	6	519	V
StMLO2	PGSC0003DMG400013720	8	8,453,442-8,457,924	11	6	517	II
StMLO3	PGSC0003DMG400018975	10	17,809,877-17,818,901	13	3	456	1
StMLO4	PGSC0003DMG400020286	9	36,181,654-36,187,569	13	7	477	
StMLO5	PGSC0003DMG400003574	2	39,242,676-39,247,920	13	4	455	1
StMLO6	PGSC0003DMG400012451	7	53,305,494-53,314,628	14	7	565	Ι
StMLO7	PGSC0003DMG400013667	2	38,189,087-38,196,233	13	7	552	
StMLO8	PGSC0003DMG400018271	1	79,235,994-79,239,439	12	5	414	Ш
StMLO9	PGSC0003DMG400020605	3	36,041,611-36,048,004	13	6	366	V
StMLO10	PGSC0003DMG400023159	2	18,902,752-18,910,333	11	7	550	
StMLO11	PGSC0003DMG400027665	6	57,855,801-57,859,958	12	7	507	II
StMLO12	PGSC0003DMG400030134	6	9,616,811-9,623,870	13	7	589	V
StMLO13	PGSC0003DMG400033623	8	42,249,518-42,256,102	14	7	532	III

\* Features of StMLO1 refer to the homolog identified by a PCR-based approach for which no corresponding sequence is found in Spud DB

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	ЧзріМ	ənlav-ə	LOJMJS	264WFO2	2£MLO3	₽0JM}S	SOIMIS	901M}S	ZOIMis	801MJS	601M}S	0LOJM <del>J</del> S	LIOIMIS	Stmloid	2101MJS	Sequence motif	Corresponding motif number in soybean (Deshmukh <i>et al.</i> 2014)	l motif rbean I. 2014)
MOTIF 1		70 1.6e-397	$\geq$	$\geq$	$\geq$	>	$\geq$	>	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	PTWAVAVVCTVIVAISLAIERIIHKLGKWLKKKNKKALY EALEKIKEELMILGFISLLLTVLQSYISKIC	.NKKALY 4 IC	
MOTIF 2	70	8.4e-376	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	1	$\geq$	$\geq$	$\geq$	$\geq$	LIHFILFQN AFEI AFFFWIWWEYGFKSCFHDNFGFIIIRLV IGVIVQFLCSYSTLPLYALVTQMGSHMKK	FGFIIIRLV 3 KK	
MOTIF 3		70 1.6e-295	$\geq$	$\geq$	I.	$\geq$		$\geq$	$\geq$	1	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	KFDFQKYIKRSLEDDFKVVVGISPVLWGFVVFLFLLLNVHG WHAYFWIAFIPLIIILAVGTKLQHVITQMA	1 11 11	
MOTIF 4	51	1.0e-245	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	GKVPLLSLEALHQLHIFIFVLAVFHVLYSAITMALGGL KIRQWKxWEDEIK	AALGGL 2	
MOTIF 5	55	1.4e-176 √	$\geq$	$\geq$	i.	$\geq$	1	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	RFTHETSFGRRH×SFWTKSPILFWIVCFFRQFFRSV×KS DYLTLRHGFIMAHLAP	FRSV×KS 5	
<b>MOTIF 6</b>	40	3.9e-038	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	1	1	$\geq$	$\geq$	$\geq$	$\geq$	SIFDEQVQKALHGWHKKAKKRRGHKxxRSxTTxSTSSSx		
<b>MOTIF 7</b>	40	2.00e-11				>	'		$\geq$	'	'	$\geq$	'	'	'	PESVADTLLPCPAKNKAAAEEEHRRRLLWEERRILAGAEP	RILAGAEP -	
	ЧłріМ	ənl <b>av-</b> ə	ιοτωίη	ZOTWIN	EOTMIN 801WIN	TOTWIN	SOIM#N	20 IW+N 907W}N	801M <sup>1</sup> N 201M <sup>1</sup> N	60TWIN	0101₩3Ν	LIOIMIN	ζιοιμίν	ειοτωίη	₽LOJM}N	Sequence motif	Corresponding motif number in soybean (Deshmukh <i>et al.</i> 2014)	motif bean
MOTIF 1	70	1.1e-580 V	>	>	>	~	~	۲ ۲	>	>	>	$\geq$	$\geq$	$\geq$	>	PSDDLFWFNRPQLVLFLIHFVLFQNAFQLAFFFWIWY EYGLKSCFHDNVEDIIIRLVMGVGIQFLCSYIT	FFFWIWY 3 SSYIT 3	
MOTIF 2	70	3.9e-578	$\geq$	>	Ś	>	>	~ ~	~ /	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	V PTWAVAAVCFVIVAISIAIERIIHKLGKWLKKHKKALY EALEKIKAELMLLGFISLLLTVSQYPISKIC	KHKKALY 4 KIC	
MOTIF 3	70	5.4e-559	$\geq$	>	>	>	>	ト	>	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	V KFDFQKYIKRSLEDDFKVVVGISPPLWVFVVFLFLLL NVHGWHAYFWIAFIPLIILAVGTKLQHVITQMA	VLFLLL 1 TQMA	
MOTIF 4	56	3.5e-408	$\geq$	>	>	>	~ ~	∧ ∕	~ /	7	$\geq$	$\geq$	$\geq$	$\geq$	$\geq$	V PERFRFTRETSFGRRHLSFWTRSPILLWIGCFFRQFF RSVSKSDYLTLRHGFIMAH	.FFRQFF 5	
MOTIF 5		56 1.3e-398	$\geq$	>	>	>	~	$\sim$	7	7	>	$\geq$	$\geq$	$\geq$	$\geq$	V KGKVALISLDALHQLHIFIFVLAVLHVLYSALTMALG RAKIRGWKAWEDETQTHEY	LTMALG 2	
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<b>MOTIF 7</b>	40	8 30e-48	ı					1	1	1 1	-	-	-	,	-			

## **Supplementary Figures**

MtMLO1	1	MAEDKVYERTLEETP <mark>T</mark> WAVAVVCFVLLAIS
PsML01	1	MAEEGVKERTLEETP <mark>H</mark> WAVAVVCLVLLAVS
LjMLO1	1	MDKVAQKKLEETPEWAVAVVCFVMLAIS
AtMLO2	1	MADQVKERTLEETS
AtML06	1	MADQVKEKTLEETS
AtML012	1	MAIKERSLEETPUWAVAVVCFVLLFIS
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StML01		MAKERSMEATPTWAIAVVCFILLAIS
SIML01	1	MEATP <mark>M</mark> WAIAVVCFILLAIS
SmML01	1	MAKERSMEGTP <mark>I</mark> WAVAVVCFILLAIS
CaMLO2	1	MAKERSMEATPIWAVAVVCFILLAIS
NtML01	1	MEATPHWAVAAVCFILLAIS
AtMLO7	1	MITRSRCRRSLLWFLVFHGGATATGAPSGGKELSQTP
AtMLO10		MATRCFWCWTTLLFCSQLLTGFARASSAGGAKEKGLSQTP <b>T</b> WAVALVCTFFILVS
AtML08		MGIIDGSLLRRLICLCLWCLLGGGVTVVTAEDEKKVVHKQLNQTPWAVAAVCTFFIVVS
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AtML05	1	WAGGGGGSTSGEGPRELDQTPWAVSTVCGVIILIS
AtML09	T	MAGGGGGGGGGGGPRQLDQTP
AtMLO3	1	MTDKEESNHSSEVGAVRSLQETPTWALATVCFFFIAVS
AtML011	1	MGEGEENGNEADSNERSLALSP <b>T</b> WSVAIVLTVFVVVS
AtMLO14	1	MREETEPSERTLGLTP <b>W</b> WSVATVLTIFVFVS
AtMLO4	1	MEHMMKEGRSLAETP
AtML01	1	MGHGGEGMSLEFTP
AtML015	1	
	1	MAGGGTTLEYTPTWVALVCSVIVSIS
AtML013	T	MAEARSGSLEYTP <mark>E</mark> WVVAFICFIIVLLS
MtML01	31	IVIEHIIHAIGKWFKKKNKNALYEALEKVKGEIMLMGFISLLETVFQDYISKICISEKVG
PsML01	31	ILIEHIIHVIGKWLKKRNKNALYEALEKIKGELMLLGFISLLETVFQDNISKICVSQKIG
LjMLO1	29	TITEHGTEATEKWIEKRHKKATHEAVEKTKGEUMIMGETSELUTVEKDETSNIGTSKOVA
AtMLO2	30	IVIEHIIHAIGKWFKKKNKNALYEALEKVKGELMLMEFIELLIVFQDYIEKICISEKVG ILIEHIIHVIGKWLKKRNKNALYEALEKIKGELMLLEFIELLIVFQDNIEKICVSQKIG IIIEHGIEAIEKWLEKRHKKALHEAVEKIKGELMLMEFIEFILTVFKDPIENICISKQVA IVLEHSIHKIGTWFKKKHKQALFEALEKVKAELMLLEFIELLITIGQTPIENICISQKVA
AtML06	30	
AtML012	28	IVIENDIHNISSEEKKKKKKALISALEAVKALIMULEEISLIIISSEENSEENSE IMIEYFLHFIGHWEKKKKKKALSEALEKVKALIMULEFISLLIVVLQEPVSEICIPRNIA IFIDQIIHHIGEWLLEKKKKPLYEALEKIKALIMULEFISLLIVVLQEPVSNLOVPKSIG
StML01	27	IFIEQIIHHIGEWLLEKRKKPLYEALEKIKAELMLLGFLSLLUTVLQEPVSNLCVPKSIG
SIML01	21	IFIDQIIHHIGEWLLEKRKKSLYEALEKIKAELMLLGFLSLLUTVLQDPVSNLOVPKSVG
SmMLO1	27	IFIEQIIHHLGEWLLEKHKKPLHEALEK <mark>IK</mark> AE <b>LM</b> LL <mark>G</mark> FISLLLTVVQDPVSNICVPKTVG
CaMLO2	27	IXIDQIMHHLGEWLLKKKKKPLYEALEKIKAEIMLLEFISLLITVIQDPVSNLCVPKSVG IFIPQIIHHLGEWLLKKKKKPLYEALEKIKAEIMLLEFISLLITVVQSPVSNLCVPKSVG HLLEKGLQRLANWLWKKKKNSLLEALEKIKAEIMILEFISLLITFGEPYILKICVPRKAA
NtML01	21	TETROTTHHLGEWLLKKHKKPLYEALEKTKAELMILLGETSLLUTVVOSPVSNLOVPKSVG
AtML07	53	HILEKCIODIANWIWKKHKNSIIFALEKTKAFIMILETSIIPTECEDVIKTCUDDKAA
AtML010	50	VLLEKALHRVATWLWEKHKNSLLEALEKIKAELMILGFISLLLTFGEQYILKICIPEKAA
AtML08	61	VLLBKLLHKVGKVLWDRHKTALLDALEKIKAE HVVLGFISLL TFGQTYILDICIPSHVA
AtML05	37	VLDRALHKVATWIWEKRINSLEALDAIRALMAILEFISLITFGQQYILDICIPEKAA VLDRKLHKVGKVLWDRHKTALLDALEKIKAELMVLGFISLLITFGQYILDICIPEKAA IVIELMIHKIGEVFTERRKKALYEALQAIKNELMVLGFISLLITFGQNYIASLCVASRYG IILELIIHKVGEVFERKKKALFEALEKIKNELMVLGFISLLITFGQNYIASICVPSRYG ICLERLINLLSTRLKKNRKTSLLEAVEKLKSVLMVLGFMSLMINVTEGEVSKICIPIKYA LIVERSIYRLSTWLRKTKRKPMFAALEKMKEELMLLGFISLLITATSSTIANICVPSSFY LIVERSIHRLSNWLQKTKKKPLFAALEKMKEELMLLGFISLLITATSSTIANICVSSFY
AtMLO9	36	IILELIIHKVGEVFERKKKKALFEALEKIKNELMVLGFISLLETFGQNYIASICVPSRYG
AtMLO3	39	ICLERLINLLSTRLKKNRKTSLLEAVEKLKSVLMVLCFMSLMUNVTEGEVSKICIPIKYA
AtML011	38	LIVERSIYRLSTWLRKTKRKPMFAALEKMKEELMLLCFISLLETATSSTIANICVPSSFY
AtMLO14	32	LTV RSTHRISNWLOKTKRKPIFAALEKMKEEUMILEFTSLLUTATSSTIANT VSSSFH
AtML04	31	FLVERAIYRFGKWLKKTRRKALFTSLEKMKEELMLLGLISLLLSQSARWISEICVNSSLF
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AtML01		LAVERLLHYFGTVLKKKKQKPLYEALQKVKEELMLLGFISLLLTVFQGLISKFVKENVL FAVERLIHRAGKHFKNNDQKQLFGALQKIKEELMLVGFISLLLSVGQSKIAKICISKELS
AtML015	28	FAVERLIHRAGKHFKNNDQKQLFGALQKIKEEHQLVGFISLLUSVGQSKIAKICISKELS
AtMLO13	29	LLA <b>B</b> RGLHHLGKCLKRRQQDALFEALQ <b>KLK</b> EE <mark>LM</mark> LL <b>G</b> FI <b>S</b> LM <b>H</b> TVSQAAIRHI <b>C</b> VPPALV
MtML01	91	STWHP-CSTPKTKTASNDENSESENHDRKLLEYFDPNPRRILATKGYDQCADK
PsML01	91	STWHP-CSTSNTKAKAKSDESLDYKTNNDRKLLEYFDPIPRRILATKGYDKCFDK
LjMLO1	89	STWHP-CHPEEKKKGPEGYYDKCAKC
-	00	
AtMLO2	90	SIMP - SAAEEARRIGRADAGRADAGDGDGDRYGRRELLELAESIINRRSLAIRGIDRAER
AtML06	90	STMHP-CSAAEEAKKYGKKDAGKKDDGDGDKPGRRLLLELAESYIHRRSLATKGYDKCAEK ASMHP-CSASEEARKYGKKDVPKEDEEENLRRKLLQLVDSLIPRRSLATKGYDKCAEK ATWHP-CSNHQEIAKYGKDYIDDGRKILEDFDSNDFYSPRRNLATKGYDKCAEK
AtML012	88	ATWHP-CSNHQEIAKYGKDYIDDGRKILEDFDSNDFYSPRRNLATKGYDKCAEK
StML01	87	YSWHP-CKPKADAQSEYEVTSCDKK YSWHP-CMAKEDAKSEYD-DPCLPK
SIML01	81	YSWHP-CMAKEDAKSEYD-DPCLPK
SmMLO1	87	YSWHP-CKAQEDDKPKYD-DPCLEK
CaMLO2	87	YSWHP-CKADEDVKSEYD-DPCLQK
NtML01	Q 1	
	01 110	YSWHP-CKSDEAAKNKYD-DPCLPK
AtML07	113	LSMLP-CLSEDTVLFQKLAPSSLSRHLLAAGDTSINC-KQ ASMLP-CPAPSTHDQDKTHRRRLAAATTSSRC-DE RTMLP-CPAPNLKKEDDDNGESHRRLLSFEHRFLSGGEASPTKCTKE
AtML010	116	ASMLP-PAPSTHDQDKTHRRRLAAATTSSRC-DE
AtML08	121	RTMLP- <b>C</b> PAPNLKKEDDDNGESHRRLLSFEHRFLSGGEASPTK <b>C</b> TKE
AtML05	9.7	HAMSF- <b>G</b> GPYDGPSGESKKPKTTEHLERRVLADAAPAQ <b>G</b> -KK
AtML09	96	HAMSF-CGPYDGPSEDDRKKLKKTDHAMRILYSVQRRSLADAPPVNC-KK

AtMLO3 AtMLO11 AtMLO14 AtMLO4 AtMLO1 AtMLO15 AtMLO13	98 92 91 90 88	NRMLP-CRKTIKSHNDVSEDDDDDDDDDDNHDNSFFHQCSSK NDRFLPCTRSEIQELLESG-STVKRNLLTKSLFFNIFRRRLDVIKRTC-SE NDRFVPCTPSEINELLESTISTVKRTQLTRSLFLHTLRRRLSGIGEDTO-SE NSKFYICSEEDYGIHKKVLLEHTSSTNQSSLPHGIHEASHQC-GH MHMLP-SLDSREAGASEHKNVTAKEHFQTFLPIVGTTRRLLAEHAAVQVGYCSEK EKFLP-CTKPAGAEKSLKDSSHFQFSFTGRHLLAGDAPAGDYCSLK NNMFF-CKKPLEEHHAPKSSHSIINNARHLLSTCESPDHCAAK
PsMLO1 LjMLO1 AtMLO2	145 114 150	GKVALVSAYGIHELHIFIFVLAIFHILQCIITLALGRFKMRRMKKMDDETRTVEYQFYN- GQVALVSAYGIHQLHIFIFVLALFHILQCIITLTLGRIKMRKMKTWDDETRTVEYQFYN- GKDKVAFMSQYGIHQLHIFIFVLAIFHILQCITTLALGRTRMAMMKKWBEETKTLEHQFDN- GKVAFVSAYGIHQLHIFIFVLAVFWVYCIVTYAFGKIKMRTMKSMBEETKTIEYQYSN-
AtMLO12 StMLO1 SIMLO1	141 111 104	GKVAFVSAYGMHQLHIFISVLAVCHVIYCIVTYALGKTKMRRWKKMBEETKTIEYQYSH GKVALVSAYGIHQLHIFISVLAVFHVLYCIITYALGKTKMKKMSABRETKTIEYQYSH GKVQFASSYAIHQLHIFISVLAVAHVLYCIATFALGRLKMRKWRAMBDETKTIEYQFYN- GKVQFASSYAIHQLHIFISVLAVAHVLYCIATFALGRLKMRKWRAMBDETKTMEYQFYN-
CaMLO2 NtMLO1 AtMLO7	110 104 151	GKVQFASSYAIHQLHIFISVLAIAHVLYCIATFALGRLKMRKWRANEDETKTIEYQFYN- GKVQFASSYAIHQLHIFISVLAVAHVLYSIATFALGRLKMRKWRANEDETKTIEYQFYN- GSEPLITLKGLHQLHILLFFLAIFIVYSLITMMLSRLKIRGWKKMEQETLSNDYEFSI- GHEPLIPATGLHQLHILLFFMAAFHILYSFITMMLGRLKIRGWKKMEQETCSHDYEFSI-
AtMLO5 AtMLO9 AtMLO3	137 144 138	GYVELISAEALHQLHILIFFLAIFHVLYSFLTMMLGRLKIRGNKHWENETSSHNYEFST- GYVPLISLNALHQVHIFIFFLAVFHVIYSAITMMLGRAKIRGNKVWEEVI-NDHEMMN- DYVALISLNALHQVHIFIFFLAVFHVIYSAITMMLGRAKIRGNKVWEQEVI-HEQEMMN- GKTSLISEEGLTQLSYFFEVLACHHILCNLAILLLGMAKMRKNSNEKETQTVEYLAAN-
AtMLO14 AtMLO4 AtMLO1	143 136 146	GHEPFVSYEGLEQLHRFIEIMAVTHVTYSCLTMLLAIVKIHSMRIMEDVARLDRHDCLTA GHEPFLSYEGMEQLHRFIEIMAVTHVTYSCLTMLLAIVKIHRMRIMEDVHMDRNDCLTV GREPFVSYEGLEQLLRFIEVLGITHVLYSGIAIGLAMSKIYSMRKWEAQAIIMAESDIHA GKVPLLSLEALHHLHIFIEVLAISHVFFCVLTVIFGSTRIHQMKKMEDSIADEKFDPETA GKVPIMSLSALHELHIFIEVLAVAHIIFCLLTIVFGTMKIKQMKKMEDKVLEKDFDTQS
		G-QVPLVSVEALHQLHIFISVLAVFHVIFCASTMVIGGARIQQHHHHBDMFKRPSQKGTT
PsMLO1 2 LjMLO1 2 AtMLO2 2 AtMLO6 2	204 175 209 206	- DPERFRFARDTTFGRRHLSMWTKSPISLWIVCEFROFFGSISRVDYLALRHGFI - DPERFRFARDTTFGRRHLSMWAQSPILLWIVSEFROFFGSISRVDYMALRHGFI - DPERFRFARDTTFGRRHLNSWSQSPISLWIVSEFROFYGSVDKVDYMVLRHGFI - DPERFRFARDTSFGRRHLNFWSKTRVTLWIVCEFROFFGSVTKVDYLALRHGFI - DPERFRFARDTSFGRRHLNFWSKTITLWIVCEFROFFGSVTKVDYLALRHGFI
StMLO1 SlMLO1 SmMLO1 CaMLO2	170 163 169 169	-DPERFRFARDTSFGRRHLNIWSKSTFTLWITCFFROPFGSVTKUDULTLERGEN -DPERFRFARETSFGRRHMFWSKSPVLLWIVCFFROPFSSVAKUDULTLERGEM -DPERFRFARETSFGRRHLHFWSKSPVLLSIVCFFROPFSSVAKUDULTLERGEM -DPERFRFARETSFGRRHMFWSKSPVLLWIVCFFROPFSSVAKUDULTLERGEM -DPERFRFARETSFGRRHMFWSKSPVLWIVCFFROPFSSVAKUDULTLERGEM
AtMLO7 2 AtMLO10 2 AtMLO8 2	210 208 226	-DPERFRFARETSFGRRHLHYWSKSPVLLWIVCFFROFFSSVAKVDVLTLRHGEM -DHSRLRLTHETSFVREHTSFWTTTPFFFVVGCFROFFVSVERTDVLTLRHGEI -DFSRFRLTHETSFVRQHSSFWTKIPFFFYAGCFLOFFRSVGRTDVLTLRHGEI -DTSRFRLTHETSFVRAHTSFWTRIPFFFYVGCFROMLRSVRKSDVLTLRHGEI
AtMLO9 AtMLO3 AtMLO11 AtMLO14	202 197 208 203	-DPSRFRLTHETSFVREHVNSWASNKFFFYVMCFFRÖILRSVRKSDULTMRHGFI -DPNRFRITRDTTFARRHLSSWTETSFOLWIKCFFRÖFYNSVAKUDULTLRHGFI VAREKIFRRQTTFVQYHTSAPLAKNRILIWVTCFFRÖFGRSVDRSDULTLRKGFI VAREKIFRRQTTFVQYHTSAPLVKNRLLIWVICFFRÖFGHSVVRSDULTLRKGFI
AtMLO1 2 AtMLO15 2	206 193	-KKTKVMKRQSTFVFHHASHPWSNNRFLIWMLCELROFRGSIRKSDYFALRLGFL LRKRRVTHVHNHAFIKEHFLGIGKDSVILGWTQSELKOFYDSVTKSDYVTLELGFI IKKFTHVQEHEFIRSRFLGVGKADASLGWVQSEMKOFLASVNESDYITMRLGFV RRGHHAHAHELFSANHEFFEMHAGGFWRRSVVISWVRSEFKOFYGSVTKSEYIALRQAFI

MtML01	256	MAHLAPGNDAEFDFOKYISRSLEKDFKVVVGISPTIWFFAVLFLLTNTHGWYSSYWLPFL
PsML01	258	MAHLAPGNDAEFDFQKYISRSLEKDEKVVVGISPTINFFAVLFLLTNTHGWYSSYWLPFL MAHLPPGHDAQFDFQKYISRSIEEDEKVVVGISPTINLFTVLFLLTNTHGWYSYYWLPFL
LjML01	229	IAHLAPGSESKFDEQKYISKSVDEDEKVVVGISPTVNFFAVLILLTNTHGWHSYLWLPFI
-		
AtMLO2	263	
AtML06	260	
AtML012	254	MAHLPAGSAARFDDQKYIERSLEQDFTVVVGISPLIWCIAVLFILTNTHGWDSYLWLPFL
StML01	224	
SIML01	217	
	211	MALIPONONFDEOLINNAVDRDERVVVGISPALMLFIVLIFLIIIDRLISILWVPFI
SmML01	223	MAHLTPENOKNFDEQIYINRAVDKORKVVVGISPALOLFTVLYFLTTTDGLYSYLWVPFV MAHLTPONQENFDEQIYINRAVDKORKVVVGISPALOLFTVLYFLSTTDGVYSYLWVPFV MAHLTPONQENFDEQIYINRAVEKDSKFVVEISPALOLFTVLYFLTTTNGLYSYLWVPFI
CaMLO2	223	MALLTPQNQENFDEQIYINRAVDKDEKVVYGISPALWLFTVLYFLSTTDGVYSYLWVPFV
NtML01	217	MARLTPONOENFOROININRAVEKORKFVWEISPALWLFTVLYFLTTTNGLYSYLWVPFI
AtMLO7	264	SAHLAPGRKFNEQRYIKRSLEDDEKLVVGISPVLWASFVIFLLFNVNGWRTLFWASIP
AtML010		AAHLAPGRKFDFQKYIKRSLEDDFKVVVGISPLLWASFVIFLLLNVNGWEALFWASIL
AtML08	280	
AtML05	249	SVHLAPGMKFNEQKYIKRSLEDDEKVVVGISPELMAFVMLFLLFDVHGWYVTAVITMI
AtML09	256	SVULAPGMKFDFQKYIKRSLEDDFKVVVGIRPELWAFVMLFLLFDVHGWYVTAVITMI
AtML03	251	
		VNHHLTLKYDFHSYMIRSMEEEFQRIVGVSGPLWGFVVAFMLFNIKGSNLYFWIAII
AtML011		
AtMLO14		MNHHLTLTYD <b>H</b> HS <b>Y</b> MI <b>R</b> SMEEENQKI <b>Y</b> GVSGPLWGFVVGFMLFNIKGSNLYFWLAII
AtMLO4		TKENLPFTYNEHMYMVRTMEDEEHGIVGISWPLWVYAIVCICINVHGLNMYFWISFV
AtML01	262	MTHCKGNPKLNNHKYMMRALEDDHKQVYGISWYLWIFVVIFLLLNVNGWHTYFWIAFI
AtML015	217	TTHCKTNPKFNHHKYLMRALNSDFKKVYGISWYLWVFVVLFLLLNIVAWHVYFWLAFI
	21/	MSHCRTNPSFDBHKYMLRTLEIDEKKVVSISWYLMLFVVVFLLLNVGGWNTYFWLSFL
AtML013	201	MSECRINPSFDEHREMLETLEIDERKVVSISWILWLFVVVFLLLNVGGWNTIFWLSFL
MtML01	316	PLIIILVGA <mark>KL</mark> OMIITKMGLRIQDRGEVIKGAPVVEPG <b>D</b> HL <b>FWE</b> NSPNLL <b>U</b> FIIHLVLE
PsML01	318	PLIVILLVGAKLQMIITKMGLRIQDRGEVIKGAPVVEPGDHLFWFNRPHLLLFTIHLVLF
LjMLO1	289	PLIIILLVGTKLQMIITNMGLKIQERGDVIKGAPLVEPGDDLFWFNRPRLILSLVHLVLF
AtMLO2	323	PLVVILIVGT <mark>KL</mark> EVIITKLGLRIQEKGDVVRGAPVVQPG <b>D</b> DL <b>FWF</b> GKPRFILFLIHLVLF
AtML06	320	PFIVILIVGT <mark>KL</mark> QVIITKLGLRIQEKGDVVKGTPLVQPG <b>D</b> HF <b>FWF</b> GR <b>P</b> RFI <b>L</b> FLIHLVLF
AtML012	314	
StML01	2.8.4	PLVIILLVGTKLQMIITEMGVRISERGDIVKGVPVVETGDHLFWENREGLVLFLINFVLF
S1MLO1	277	PLVIILLVGT <mark>KL</mark> QMIITEMGVRISERGDIVKGVPVVETGDHL <b>FWF</b> NRPALVLFLINFVLF
SmMLO1	283	PLIIILVGT <mark>KL</mark> QMIITEMGVRISERGDIVKGVPVVETG <mark>D</mark> HL <mark>FWF</mark> NRPGLVLFLINFVLF
CaMLO2	283	PLIIILVGT <mark>KL</mark> QMIITEMGVRISERGDIVKGVPVVEIGDHL <mark>FWE</mark> NRPGLVUFFINFVL
NtML01	277	PLVIILLVGTKLEMIIAEMGVRISKRGDIVRGVPVVETGDHLEWENRPGFVLFLINFVLE
AtML07	322	PLLIILAVGT <mark>KL</mark> QAIMATMALEIVETHAVVQGMPLVQGS <b>D</b> RY <b>FWF</b> DCPQLLLHLIHFAL
AtML010	320	PVLIILAVST <mark>KL</mark> QAILTRMALGITERHAVVQGIPLVHGS <mark>D</mark> KY <mark>FWF</mark> NRPQLL <mark>L</mark> HLLHFALF
AtMLO8	338	PVIIILAVGT <mark>KL</mark> QAIMTRMALGITDRHAVVQGMPLVQGN <mark>D</mark> EY <mark>FWF</mark> GRPHLILHLMHFALF
AtML05	307	PPLLTLAIGT <mark>KL</mark> QAIISDMALEIQERHAVIQGMPLVNVSDRH <b>FWF</b> SRPALVLHIIHFILF
AtML09	314	PPLLTLAIGT <mark>KL</mark> QAIISYMALEIQERHAVIQGMPVVNVS <mark>D</mark> QH <mark>FWF</mark> EKPDLVLHMIHFVLF
AtML03	309	PLIIVLVIGT <mark>KL</mark> EMIVAKMAVTIKENNSVIRGTPLVESN <mark>D</mark> TH <b>FWF</b> SNPRFL <mark>U</mark> SILHYTL
AtML011	320	PVTLVLLVGA <mark>KL</mark> QHVIATLALE-NAGLTEYPSGVKLRPRDELFWENK <mark>P</mark> ELL <mark>L</mark> SLIHFILF
AtMLO14	315	PITLVLLVGA <mark>KL</mark> QHVIATLALE-NASITEYASGIKLRPR <mark>D</mark> EL <mark>FWF</mark> KKPELLLSLIHFIQF
AtMLO4	307	PAILVMLVGT <mark>KL</mark> EHVVSKLALEVKEQQTGTSNGAQVKPRDGL <mark>EWB</mark> GKPEILURLIQFII
AtML01	320	PFALLLAVGT <mark>KL</mark> EHVIAQLAHEVAEKHVAIEGDLVVKPSDEH <b>FWF</b> SKPQIVLYLIHFILF
AtML015	305	PLILLLAVGT <mark>KL</mark> EHIITDLAHEVAEKHIAVEGDLVVRPS <mark>D</mark> DL <b>FWF</b> QSPRLVLFLIHFILF
		ELILLAVGINERIIIDLAREVALATIAVEGDLVVRPSDDI WRQSERLVFLIRFII
AtML013	309	PLILLLMVGA <mark>KL</mark> EYIISSLALDVSEKRSRAE-EAVITPS <mark>D</mark> EL <mark>FWE</mark> HRRGIVLQLIHFILF
MtML01	376	ONABOLAFESWSTYEESINSEFHRTTADNUTRUSUGILIOFLCSYVIL DEVALWTOME
PsML01	378	
		QNAFQLAFFAWSTIE-FSTISCFARTADSVIRTIVGVVIGTLCSTVILPLIALVIONG
LjMLO1	349	QNAFQLAFFSWSTYEFSINSCFHRTTADNVIRVSVGILLQFLCSYVTLPLYALVTOMG QNAFQLAFFAWSTYEFSITSCFHKTTADSVIRITVGVVIQTLCSYVTLPLYALVTOMG QNAFQLAFFAWSACDNDFKINSCFHRSTADVVIRITLGVVTOVLCSYVTLPLYALVTOMG
AtMLO2	383	TNAFQLAFFAWSTYEFNLNNCFHESTADVVIRLVVGAVVQILC <mark>SYVTLPL</mark> YAL <mark>V</mark> TQMG
AtML06	380	TNAFQLAFFVWSTYEFGLKNCFHESRVDVIIRISIGLLVQILC <mark>SY</mark> VTLPLYALVTQMG
AtML012	374	TNAFQLAFFVWSTYEFTLKNCFHHKTEDIAIRITMGVLIQVLCSYITLPLYALVTQMG
	344	
StML01		QNAFQVAFFVWSWWKFGFPSCFHQNAADLAIRLTMGVIIQVHCSYVTLPLYALVTQMG
SIML01	337	QNAFQVAFFFWSWWKFGFPSCFHKNAADLAIRLTMGVIIQVHCSYVTLPLYALVTQMG
SmML01	343	QNAEQVAFFVWSWWKFGFPSCFHKNAADLAIRLTMGVIIQVHCSYVTLPLYALVTOMG QNAEQVAFFVWSWWKFGFPSCFHRNAADLAIRLTMGVIIQVHCSYVTLPLYALVTOMG
CaMLO2	343	QNAFQVAFFVWSWWKFGFPSCFHRNAADLAIRLTMGVIIQVHCSYVTLPLYALVTQMG
NtML01	337	QNAFQVAFFVWSWWKFSYPSCFHQNAADIAIRLTMGVIIQVHCSYVTLPLYALVTQMG
AtML07	382	QNAFQITHFFWIWYSFGLKSCFHKDFNLVVSKLFLCLGALILCSYITLPLYALWTQMG
AtML010	380	QNAHQLTYFFWVWYSFGLKSCFHTDFKLVIVKLSLGVGALILC <b>SYIULPI</b> YALVTQMG
AtML08	398	QNAFQLTYFFWVWYSFGLKSCFHTDFKLVIVKLSLGVGALILCSYITLPFYALWTOMG QNAFQITYFFWIWYSFGSDSCYHPNFKIALVKVAIALGVLCLC <mark>SYITLPF</mark> YALWTOMG
AtML05	367	QNABEITYFFWIWYEFGLRSCFHHHFALIIIRVALGVGVQFLC <mark>SY</mark> I <b>T</b> LPLYALVTQMG

AtML03	369	LNTEEMAFIVWITWQFGINSCYHDNQGIIITRLVLAVTVQFLS <mark>SYITLPL</mark> YAIVTQMG
AtML011	379	QNSFELASFFWFWWQFGYSS <mark>C</mark> FLKNHYLVYFRLLLGFAGQFLC <mark>SY</mark> STL <mark>PL</mark> YALVTQMG
AtMLO14	374	QNAFELASFFWFWWQFGYNSCFLRNHLLVYLRLILGFSGQFLC <mark>SY</mark> STL <mark>PL</mark> YALWTQMG
AtMLO4	367	QNAFEMATFIWFLWGIKERSCFMKNHVMISSRLISGVLVQFWC <mark>SY</mark> GTVPLNVIVTQMG
AtML01	380	QNAFEIAFFFWIWVTYGFDSCIMGQVRYIVPRLVIGVFIQVLC <mark>SY</mark> STL <mark>PL</mark> YAIVSQMG
AtML015	365	QNSFEIAYFFFILFQFGWDSCIMDHVKFVIPRLVIGVIIQLLC <mark>SY</mark> STL <mark>PL</mark> YALWT <mark>QMG</mark>
AtML013	368	QNSFEIAFFFWILFTYGIHSCIMEKLGYLIPRLVMGVLVQVLC <mark>SY</mark> STLPLYALWT <mark>QMG</mark>
		C-MPD
		CaMBD
MtML01	434	
PsML01	436	
LjMLO1	409	
AtMLO2	441	
AtML06	438	
AtML012		TSMRPTIFNDRVANALKKWHHTAKKQTKHG-HSGSNTPHSSRPTTPTHGMSPVHLLHN
StML01		SSMKPIIFGDNVATALRSWHHTAKKRVKHG-LSGHTTPANSRPTTPLHGTSPVHLLRG
SIML01		SSMKPIIFGDNVATALRSWHHTAKKRVKHG-LSGHTTPANSRPTTPLRGTSPVHLLRG
SmMLO1		SSMKPIIFGDNVATALRSWHHMAKKRVKHGRLSGNTTPVSSRPTTPLHGTSPVHLLRG
CaMLO2	401	
NtML01	395	
AtMLO7	440	· · · · · · · · · · · · · · · · · · ·
AtMLO10 AtMLO8		SNMKKAVFDEQMAKALKKWHMTVKKKKGKARKPPTETLGVSDTVSTSTSSFHASGATLLR
		SRMKKSVFDEQTSKALKK <mark>R</mark> RMAVKKKKGVKATTKRLGGDGSASPTASTVRSTSSVRSLQR STMKRSVFDDQTSKALKN <mark>R</mark> HKNAKKKSETPGQTQPPLPNLRPKTGGDIESASPANITASV
AtML05	425	
AtMLO9 AtMLO3	432	
AtML03	437	
AtML011 AtML014		TNYKAALLPQRVRETINGNGKATRRKRRHGLIGDDSIVKTETSTIASLEETDNQVLDVTE TNYKAALLPQRVRETINGNGKATRRKRRHGLYGDDSTIRTETSTIASVDEYNDQVLDVSE
AtMLO4		SRHKKAVIAESVRDSLHS <mark>N</mark> CKRVKERSKHTRSVCSLDTATIDERDEMTVGTLSRSSSMTS
AtML01	438	
AtML015	423	
AtML013		SKFKKGIFDNVVOSTLEG <mark>N</mark> LEDTRNRGESTSEAHRIEMOPTTPESYNVOSENP
MtML01	492	QTFGNSDSLQTSPRTSNYENEQWDVEGGGSTSPRNNQTVASEIEIPIVESFSTTELPVSV
PsML01	494	HTAGNSDSLQTSPEKSDYKNEQWDIEGEGPTSLRNDQTGQHEIQIAGVESFSSTELPVRI
LjMLO1	469	HHNYHNSDSPLASPRESPSNYETEQWYLEPNSPSNHTRGHDQTLQMQVLGSSATEFSPAE
AtMLO2	499	FNNRSVENYPSSPSPRYSGHGHHEHQFWDPESQHQEAETSTHHSLAHESSEPVLASVELP
AtML06	496	APHKRSRSVDESFANSFSPRNSDFDSWDPESQHETAETSNSNHRSRFGEEESEKKFVSSS
AtML012	489	YNNRSLDQQTSFTASPSPPRFSDYSGQGHGHQHFFDPESQNHSYQREITDSEFSNSHHPQ
StML01	459	YPQYNEDSVQASPRTSNVENEGWANEISNDNQEGEILQHASTDHNKQIEITMSDFTFGNK
S1MLO1	452	YPQYNEDSVQASPRTSNVENEGWANENQEGEILQHASTDHNKQIEITMSDFTFGNK
SmML01		YPQYNEDSVQASPRTSNVENEGWANEISTDNKDYQEGHASTSVRPPHAHNQQIEITMSDF
CaMLO2		YPKYNEDNVQAYPRTSNVENEGWANETSTENKDHQEEGQILQHASTSMQHPHTDQHQIEI
NtML01		YPQYSNEESRTSNAENEGWANEIPTSPRRQIENIKDDDHQEGEIHASSSVHQVEIAMSEF
AtMLO7		TGHSSNIIYYKQEDEEDEMSDLEAGAEDAIDRIQQQEMQFHNS
AtML010		SKTTGHSTASYMSNFEDQSMSDLEAEPLSPEPIEGHTLVRVGDQNTEIEYTGDISPGNQF
AtML08		YKTTPHSMRYEGLDPETSDLDTDNEALTPPKSPPSFELVVKVEPNKTNTGETSRDTETDS
AtML05	485	DVKESDQSQSRDLLSGP
AtML09		
AtMLO3		FRFSGRQSPILQEIQIQEKTER
AtML011		TSFEQQRKQQEQGTTELELQPIQPRNDCVPNDTSSRVGTPLLRPWLSISSPTTTIELRSE
AtMLO14		TSPVQDNELELQLIRGACGNSSSVETPILRPCASISSTTFSRLQTETTDSLSRSSSLPMR
AtMLO4		LNQITINSIDQAESIFGAAASSSSPQDGYTSRVEEYLSETYNNIGSIPPLNDEIEIEIG
AtMLO1		AGFAGIQLSRVTRNNAGDTNNEITPDHNN
AtML015	483	NQNPKEKSEIAHHD
AtML013		

MtML01	552	RHEIGTTSSSKDFSFEKRHIGSN
PsML01	554	RHESTSGSKDFSFEKRHLGSN
LjMLO1	529	VHHEITPIGLPEFSFDKAPTSRE
AtMLO2	559	PIRTSKSLRDFSFKK
AtML06	556	VELPPGPGQIRTQHEISTISLRDFSFKR-
AtMLO12	549	VDMASPVREEKEIVEHVKVDLSEFTFKK-
StML01	519	Х
SIMLO1	508	Х
SmMLO1	519	TFGNKX
CaMLO2	519	AMSDFTFGNKX
NtMLO1	513	TFGNKMS
AtMLO7		
AtMLO10	558	SFVKNVPANDID
AtML08	576	KEFSFVKPAPSNESSQDR
AtML05		
AtML09		
AtMLO3		
AtML011	557	PMETLSRSSSLPSEKRV
AtMLO14	552	REC
AtMLO4	545	EEDNGGRGSGSDENNGDAGETLLELFRRT
AtML01		
AtMLO15		
AtMLO13		

**SUPPLEMENTARY FIGURE 1.** Protein multiple alignment of a dataset composed of eggplant SmMLO1, potato StMLO1, tobacco NtMLO1, the fifteen Arabidopsis AtMLO homologs and the susceptibility proteins SIMLO1 (tomato), CaMLO2 (pepper), PsMLO1 (pea), LjMLO1 (lotus) and MtMLO1 (barrel clover). Black shading shows amino acid residues reported to be conserved throughout the whole MLO protein family, whereas grey shading highlights residues shared by MLO proteins experimentally shown to be required for powdery mildew susceptibility

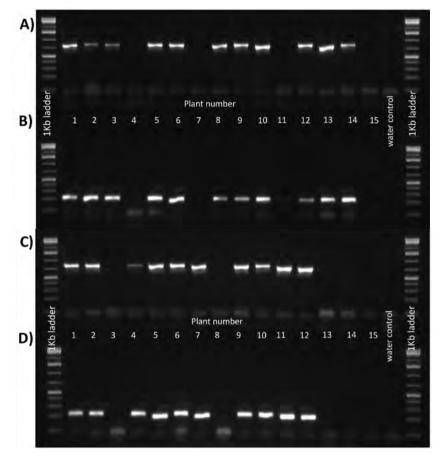
StMLO1 S1MLO1 SmMLO1 NtMLO1 CaMlo2	1 ATGGCTAAAGAACGGTCTATGGAGGCAACCCCTACGTGGGCAATTGCTGTC 1 ATGGCTAAAGAACGGTCGATGGAGGGAACCCCCACTTGGGCCGTTGCCGTC 1 ATGGCTAAAGAACGGTCGATGGAGGCAACTCCGACTTGGGCAGTTGCCGCZ	GTTTGCTTC GTTTGCTTC CGTTTGCTTC AGTTTGCTTC GTTTGCTTC
StMLO1 S1MLO1 SmMLO1 NtMLO1 CaMlo2	61 ATCTTGCTCGCTATTTCCCATTTTTATTGAACAAATTATTCATCATATTGG 61 ATCTTGCTCGCTATTTCTATTTATTGAACAAATTATTCATCACAATGG 61 ATCTTGCTGGCTATTTCCATTTTATTGAACAAATTATTCATCACCCTTGG 61 ATCTTGCTGGCTATTTCCATTTTCATCAACAAATTATTCATCACCTTGG 61 ATCTTGCTGGCTATTTCCATTTKTATTGAACAAATTATTGCATCACCTTGG 61 ATCTTGCTGGCTATTTCC	AGAGTGGTTA AGAGTGGTTA AGAGTGGTTG
StMLO1 S1MLO1 SmMLO1 NtMLO1 CaMlo2	21 CTGCAAAAACCCGAAAAAAGCCTCTATATGCAAGCACTTGAAAAGATCAAAGG 21 CTGCAAAACCCGAAAAAGTCTCTATATGCAAGCACTTGAAAAGATCAAAGG 21 TTGCAAAACCCCAAAAAGCCACTACACGAAGCACTTGAGAAGATCAAAGG 21 TTGAAAAACCTAAAAAGCCTCTTATGAAGCACTTGAGAAAGATCAAAGG 21 TTGAAAAAACCGAAAAAGCCTCTATACGAAGCACTTGAGAAAGATCAAAGG	IGAACTTATG AGAACTTATG AGAACTGATG
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	81 CTGTTGGGATTCTTATCACTGTTGTTGACAGTGTTGCAAGAACCAGTTTC 81 CTGTTGGGATTCTTATCACTGTTGTTGACAGTGTTCCAAGATCCAGTTTC7 81 CTGTTGGGATTCATATCACTGCTGTGACAGTGCTGCAAGATCCAGTTC7 81 TTGTTGGGATTCATATCACTGCTGTTGACAGTGCTGCAAAGCCCAGTGTC7 81 TTGTTGGGATTCATATCATTGTTGTTGACAGTGATACAAGACCCAGTTC7	FAACTTATGT FAACATATGC FAAC <mark>TTATG</mark> C
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	41 GTCCCCAACAGTATTGGTTATTCATGGCATCCTTGTAAGCCAAAGGCAGAG 41 GTCCCCAACAGTCTTGGTTATTCATGGCATCCTTGTATGCCAAAGGAAGA 41 GTGCCCAAAACTCTTGGTTATTCGTGGCATCCTTGTAAGCCACAGGAGAG 41 GTGCCCAAAAGTCTTGGTTATTCTTGGCATCCTTGTAAGTCTGATGAAGC 41 GTCCCCAAAAGTCTTGGTTATTCCTGGCATCCTTGTAAGCCAGATGAAGAC	CGACAAGCCT
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	01 CACTATGACCTTACTTCATCCGACAAAAAGGGAAAAGTCCAATTTGCATC 01 CACTATGATGACCCTTGTCTACCAAAGGGAAAAGTCCAATTTGCATC 01 AACTATGATGACCCTTGTCTAGAAAAGGGAAAAGTCCAATTTGCATC 01 AAATATGATGACCCTTGTCTACCAAAGGGAAAAGTCCAATTTGCATC 01 GACTATGATGACCCTTGTTTACAAAAGGGAAAAGTTCAATTTGCATC	ITCATATGCA ITCATATGCA ITCATATGCA
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	61ATACACCAGCTCCATATC58ATACACCAGCTCCATATC58ATACACCAGCTCCATATC58ATACACCAGCTCCATATC58ATACACCAGCTCCATATC58ATACACCAGCTCCACACACT58ATACACCAGCTCCACACT58ATACACCAGCTCCACACT58ATACACCAGCTCCACACCACT58ATACACCAGCTCCACACT59ATACACCAGCTCCACACT	TACTGTATA TACTGTATA TATTGTATA TACTCTATA TACTCTATA TACTGTATA
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	21       GCAACTTTTGCTTTCGGCAG       CTAAAGATGAGAAAATGGAG       GCATGGAG         18       GCAACTTTTGCTTTCGCCAG       CTAAAGATGAGAAAATGGAG       GCATGGGA         18       GCAACTTTGCTTTCGCCAG       CTAAAGATGAGAAAATGGAGGGCCCGGGC       CTGGGA         18       GCAACTTTGCTTTGGCCAG       CTAAAGATGAGAAAATGGAGGGCCCGGCCGGGA         18       GCAACTTTGCTTTAGGCAG       CTAAAGATGAGAAAATGGAGAGGCCCTGGGA         18       GCAACTTTTGCTTTCGCCAG       CTAAAGATGAGAAAATGGAGAGGCCCTGGGA	GATGAAACA GATGAAACA GATGAAACT GAAGAAACA GATGAAACA
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	<ul> <li>81 AAAACAATTGACTACCAATTCTACAACGACCCTGAGAGATTCAGATTTGC/</li> <li>78 AAAACAATGGACTACCAATTCTACAACGACCCTGAGAGATTCAGATTTGC/</li> <li>78 AAAACAATTGACTACCAATTCTACAACGATCCAGAGAGATTCAGATTTGC/</li> <li>78 AAAACAATTGACTACCAATTCTACAACGATCCAGAGAGGTTCAGATTTGC/</li> <li>78 AAAACAATTGACTACCAATTCTATAACGAYCCTGAGAGGTTTAGCTTTGC/</li> </ul>	AAGGGAGACC AAGGGAGACC AAGGGAGACG
StMLO1 SIMLO1 SmMLO1	41 TCGTTTGGACGTAGGCATATGCATTTCTGGAGCAAGTCGCCCGTGTTGCTC 38 TCGTTTGGACGTAGGCATTTGCATTTCTGGAGCAAGTCCCCCGTGTTGCT 38 TCCTTTGGACGTAGGCATTTGCATTTCTGGAGCAAGTCACGCTCTTGCTC	CTCGATAGTT

NtMLO1 CaMlo2	538 538	TCATTTGGACGTAGGCACTTGCATTATTGGAGCAAGTCTCCAGTGCTGCTCT <mark>G</mark> GATAGTT TCATTTGGACGTAGGCA <mark>TA</mark> TGCATTTTTGGAGCAAGTC <mark>GCCGGTGA</mark> TGCTCT <mark>G</mark> GATAGTT
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	601 598 598 598 598	TGTTTCTTTCGGCAATTCTTC <mark>TCATCAGTTGCAAAAGTTGACTATTTAACCCTTAGACAT</mark> TGTTTCTTCAGGCAATTCTTCTCCTCAGTAGCAAAGGTTGACTATTTAACCCTTAGACAT TGTTTCTTCAGGCAATTCTTC <mark>TCATCAGTAGCAAAAGTTGACTATC</mark> TAACCCTTAGACAT
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	661 658 658 658 658	GGGTTCATGATGGCACATTTAAC <mark>T</mark> CCA <mark>G</mark> AGAATCAAAAGAA <mark>T</mark> TTTGATTT <mark>TCAAA</mark> TATAC GGGTTCATGATGGCACATTTAAC <mark>T</mark> CCA <mark>C</mark> ACAATCAGGAAAATTTTGATTT <mark>CCACA</mark> TATAC
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	721 718 718 718 718 718	AT <mark>TAAC</mark> AGAGCAGTTGA <mark>C</mark> AAAGACTTCAAAGTTGTTGTTGCAATAAGTCCTGCATTATGG ATTAAC <mark>A</mark> GAGAGCAGTTGACAAAGACTTCAAAGTTGTGTGCGCAATAAGTCCAGCATTATGG AT <mark>C</mark> AAT <mark>A</mark> GAGCAGTTGA <mark>A</mark> AAAGACTTCAAATTTGTTGTGGAAATAAGTCCAGCATTATGG
StMLO1 S1MLO1 SmMLO1 NtMLO1 CaMlo2	781 778 778 778 778 778	CTCTTCACGGTACTATATTTTCTAACGACTACCGATGGACTATACTCGTACCTTTGGGTG CTCTTCACAGTACTATATTTTCTAACCACTACCAATGGATTGTACTCGTACCTTTGGGTG
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	841 838 838 838 838	CCATTTATCCC.CTTTAATAATATTTCCTAGTTGGCACAAAACTTCAAATGATCATAACA CCATTTCTCCCACTCATAATAATATTCC CCATTTATCCCCTTASTAATAATATTCC CCATTTATCCCCTTASTAATAATATTCC
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	901 898 898 898 898	GAAATGGGGT <mark>A</mark> AGGA <mark>TTTCA CAAAGGGGAGACATAGTGAAAGGTGTCCCAGTGGTGGAG GAAATGGGAGT<mark>A</mark>AGGA<mark>ATTCCAAC</mark>AGGGGAGACATAGTCACAGGGTGTACCAGTGGTGGAG</mark>
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	961 958 958 958 958	ACTGGTGACCATCTTTTCTGGTTTAATCGCCCTGCCCTG
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	1018 1018 1018	GT <mark>G</mark> CTCTTTCAGAATGCATT <mark>CCAAGT</mark> TGCTTTCTT <mark>CG</mark> TTTGGAGTTGGTGGAAATTTAGT
StMLO1 SIMLO1 SmMLO1 NtMLO1 CaMlo2	1078 1078 1078	TTCCCATCTTGCTTTCATAAGAATGCTGCAGACCTAGCCATAAGGCTAACCATGGGGGTG TTTCCGTCTTGCTTTCACAAGAATGCTGCAGACCTAGCCAT <mark>A</mark> AGGCTAACCATGGGGGTG

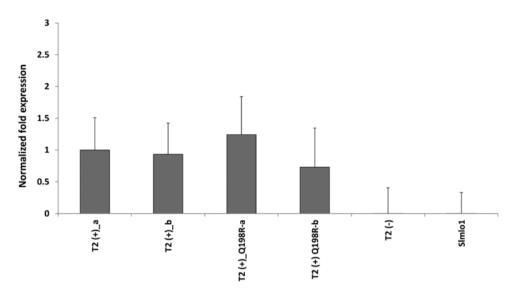
**SUPPLEMENTARY FIGURE 2.** 

StMLO1 1141 S1MLO1 1138 SmMLO1 1138 NtMLO1 1138 CaMlo2 1138	ATCATACAEGTCCATTGCAGCTATGTGACTCTCCCTCTTTATGCCTTAGTTAG
StMLO1 1201 S1MLO1 1198 SmMLO1 1198 NtMLO1 1198 CaMlo2 1198	GGTTCATCAATGAACCCTAT <mark>C</mark> ATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGG GGTTCATCAATGAACCCTAT <mark>C</mark> ATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGG GGTTCATCAATGAACCTATCATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGG GGAACATCAATGAAACCTAT <mark>A</mark> ATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGG GG <mark>TTCATCAATGAACCTATC</mark> ATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGG
StMLO1 1261 S1MLO1 1258 SmMLO1 1258 NtMLO1 1258 CaMlo2 1258	CACCATACA GCGAAAAAACCGGGTGAAACATGGGCTATCA GCACACACACACTCCT CC CACCATACA GCGAAAAAACCGGTGAAACATGGGCTATCA GCACATACCAGCCCT CA CACCATAT GCGAAAAACCGGTGAAACATGGCCGGCTATC GGAAAAACACCAGCCT TC CACAACACGCGAAAAACCGGGTGAAACACGGCCGGCTATCGGAAAACACCACCCT TC CACAACACGCGAAAAACCGGGTGAAACACGGCGGCTATCGGAAAACACCACCCT TC CACAATACAGCGAAAAACCGGGTGAAACATGGGCGGGTATCAGAAAACACCACCCT TC
StMLO1 1318 S1MLO1 1315 SmMLO1 1318 NtMLO1 1318 CaMlo2 1318	AACAGCAGACCAACCACACCACTGCTGGTACCTCCCCTGTTCACTTATTACGCGGTTAT
StMLO1 1378 S1MLO1 1375 SmMLO1 1378 NtMLO1 1378 CaMlo2 1378	CCACAATATAATCAGGATACTCTTCAACCATCTCCTCGGGCATCCAACGTTGAAAATCAA CCACAATATAATCAGGACACTCTTCAACCATCTCCTCGGCACATCCAATGTCGAAAATGAA CCACAATACAAT
NtMLO1 1438	CCTG GTTAATGAAATATCCAATG CAATCAGGAGGGAGAG TCCTGCAGCATGCCTCC GGTG GGTAATGAAAATCAG AGG A AGATCCTGCAGCAT CCTCCACTGATCATAAC GGTG GGTAATGAAATATCT CTG CAATAAAGATTATCAG AGGGACATGCCTCCACA GAATA CCAACCTCTCCTCT GAC AA TTGAGAATATTAAA ATGATGATCATCAGGAG CCTG GGTTAATGAAACATCCACTG GAATAAAGATCATCAG AGGAGGGACAAATCCTG
SIMLO1 1495 SmMLO1 1498 NtMLO1 1498	ACTGATCATAACAACCAA.TTGAGATTACAATGTCAC.TTTCACTTTTGGAAACCAAATAA AAGCAAATTGAGATTACA.TGTCAGATTTACTTTTC.AAACAAATAAATGTAAAAACGA TCTGTGCGACCTCCCCAT.CTCACAACCAGCAAATTG.GATTACAATGTCAGTTTTACT GGAGAAATCCATGCCTCC.GCTCTGTGCACCAGGTTC.GATTGCAATGTCAGATTCACA CAGCATGCCTCCACTTCT.TGCAACATCCGCATACTC.TCAACATCAAATTGAGATTGCA
S1MLO1 1555 SmMLO1 1558 NtMLO1 1558	AAGATCAATGTATATGTAAAAACTAATTTCCTCTGCGTTGTTTAAGTTCATTACTGTAGA ATTTTCTTCTTCATTGTTTAAGTTCATTACTGTAGTTCAAATGGCAATGATTTTGTAAA TTTGGAAACAAATAA TTTGGCAACAAAATGAGTTGA ATGTCAGATTTTACTTTTGGAAACAAATAG
	GAGCATAAATGGATCCTAGATAGTTCAAGTGCCTGCTTGCT
	TCCAAAAAAAAAAAAAA TCGATTTATAGCTACTTTACATCTCA

**SUPPLEMENTARY FIGURE 2.** Nucleotide multiple alignment of full-length coding sequences of eggplant *SmMLO1*, potato *StMLO1*, tobacco *NtMLO1*, tomato *SIMLO1* and pepper *CaMLO2*.



**SUPPLEMENTARY FIGURE 3.** Segregation of the  $T_{2}$  (A and B) and  $T_{2}$  (C and D) families for markers derived from the nptII gene (panels A and C) and 35S promoter (panels B and D), indicating the presence of the 35S::*NtMLO1* construct.



**SUPPLEMENTARY FIGURE 4.** Relative quantification of *NtMLO*1 expression levels in complementation tests, assessed by qPCR. Data refer to 11 and 10 individuals of two T<sub>2</sub> families  $[T_2(+)_a \text{ and } T_2(+)_b]$  positive for the presence of the overexpression construct harboring wild-type *NtMLO*1; 11 and 7 individuals of two T<sub>2</sub> families  $[T_2(+)_Q198R\text{-a} \text{ and } b]$  positive for the presence of the overexpression construct harboring a *NtMLO*1 mutant sequence, resulting in the substitution of a glutamine residue with arginine; 18 non-transgenic individuals from the 4 T<sub>2</sub> families above mentioned  $[T_2(-)]$ ; 10 individuals of the Slmlo1 mutant line, used as background genotype for transformation.

# Chapter 5

# Monocot and dicot MLO powdery mildew susceptibility factors are functionally conserved in spite of the evolution of class-specific molecular features

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### Abstract

#### Background

Specific members of the plant Mildew Locus O (MLO) protein family act as susceptibility factors towards powdery mildew (PM), a worldwide-spread fungal disease threatening many cultivated species. Previous studies indicated that monocot and dicot MLO susceptibility proteins are phylogenetically divergent.

#### Results

We show that monocot and dicot MLO susceptibility proteins evolved class-specific conservation patterns. Many of them appear to be the result of negative selection and thus are likely to provide an adaptive value. We also tested whether different molecular features between monocot and dicot MLO proteins are specifically required by PM fungal species to cause pathogenesis. To this aim, we transformed a tomato mutant impaired for the endogenous *SIMLO1* gene, and therefore resistant to the tomato PM species *Oidium neolycopersici*, with heterologous MLO susceptibility genes from the monocot barley and the dicot pea. In both cases, we observed restoration of PM symptoms. Finally, through histological observations, we demonstrate that both monocot and dicot susceptibility alleles of the *MLO* genes predispose to penetration of a non-adapted PM fungal species in plant epidermal cells.

#### Conclusions

With this study, we provide insights on the evolution and function of *MLO* genes involved in the interaction with PM fungi. With respect to breeding research, we show that transgenic complementation assays involving phylogenetically distant plant species can be used for the characterization of novel *MLO* susceptibility genes. Moreover, we provide an overview of MLO protein molecular features predicted to play a major role in PM susceptibility. These represent ideal targets for future approaches of reverse genetics, addressed to the selection of loss-of-function resistant mutants in cultivated species.

#### Keywords

MLO, powdery mildew, Angiosperms, evolution, plant breeding

## Background

The plant <u>Mildew\_Locus Q</u> (MLO) gene family codes for proteins harboring seven transmembrane domains and a calmodulin-binding site, topologically reminiscent of metazoan and fungal G-protein coupled receptors (GPCRs) [1]. Following the completion of plant genome sequencing projects, a number of homologs varying from 12 to 19 has been identified in the *MLO* gene families of diploid species, namely Arabidopsis, rice, grapevine, cucumber, peach, woodland strawberry and sorghum [1-6].

Specific homologs of the *MLO* gene family act as susceptibility factors towards fungi causing the powdery mildew (PM) disease, worldwide spread and causing severe losses in agricultural settings. Inactivation of these genes, through loss-of function mutations or silencing, indeed results in resistance (referred to as *mlo*-based resistance) in several plant species [7]. The first *MLO* gene described as required for PM pathogenesis was barley *HvMLO* [8, 9]. Since then, *MLO* susceptibility genes have been functionally characterized in rice (*OsMLO3*), wheat (*TaMLO\_A1* and *TaMLO\_B1*), Arabidopsis (*AtMLO2*, *AtMLO6* and *AtMLO12*), tomato (*SIMLO1*), pepper (*CaMLO2*), tobacco (*NtMLO1*), pea (*PsMLO1*), lotus (*LjMLO1*) and barrel clover (*MtMLO1*) [10-17].

Defense mechanisms involved in *mlo*-based resistance prevent fungal penetration in epidermal cells and are associated with the formation of cell wall appositions, referred to as papillae [11]. Similar pre-penetration defense measures also take place in non-host resistance, following the interaction between PM fungal species and plant species beyond their host range. Consistent with the hypothesis of involvement of *MLO* genes in non-host resistance, loss of function of *HvMLO* in the interaction between barley and the wheat PM fungus *Blumeria graminis* f. sp. *tritici* is associated with decreased rate of penetration and lower incidence of epidermal cell death, the latter being a post-penetration defense mechanism [18, 19].

Several studies have been addressed to the characterization of regions of relevance for the functionality of MLO proteins. Multiple alignments have pointed out the occurrence of residues highly conserved within the whole MLO family, which were therefore predicted to provide a common protein structural scaffold [12, 20]. In addition, the occurrence of residues and motifs specifically conserved in putative orthologs of barley HvMLO has been reported [9]. Finally, functionally important residues for MLO susceptibility proteins have been inferred by the association of naturally occurring and induced mutations with partial or complete PM resistance [11, 12, 21-25].

In our previous studies, we showed that phylogenetically related dicot *MLO* genes of the same botanic family are conserved for their function as a susceptibility gene to PM [6, 16]. Notably, monocot and dicot MLO proteins involved in PM susceptibility group in clearly separated phylogenetic clades (e.g. [2, 9]). Here, we show that the evolution of Angiosperm

PM susceptibility genes led to the fixation of class-specific molecular traits. Many of them appear to be the result of negative selection. By means of transgenic complementation assays, we demonstrate that, despite having different conservation patterns, monocot and dicot *MLO* susceptibility genes are essentially conserved with respect to functional features having a role in interactions with PM fungi. Consequences of our findings for plant breeding research are discussed.

#### Results

# Class-specific molecular features of Angiosperm MLO homologs required for PM susceptibility

Previous studies indicated that dicot and monocot MLO proteins with a putative or ascertained role in susceptibility to PM fungi group in two different phylogenetic clades (e.g. [2, 9]). This was confirmed by performing a new UPGMA-based phylogenetic analysis involving all the twelve MLO homologs which have been until recently functionally related to PM susceptibility (Fig. 1). Aiming to detect molecular features responsible for such phylogenetic divergence, the same MLO homologs were used as dataset for multiple

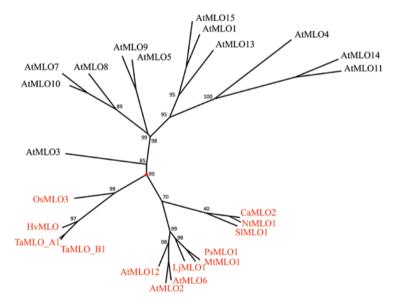


FIGURE 1. Unrooted radial phylogenetic tree of MLO powdery mildew susceptibility proteins. The tree includes, in red, all the monocot and dicot MLO homologs shown to be required for powdery mildew susceptibility (Arabidopsis AtMLO2, AtMLO6 and AtMLO12, tomato SIMLO1, pepper CaMLO2, tobacco NtMLO1, pea PsMLO1, lotus LjMLO1, barrel clover MtMLO1, barley HvMLO, wheat TaMLO\_B1 and TaMLO\_A1b and rice OsMLO3), and the remaining homologs of the Arabidopsis AtMLO family. Numbers at each node represent bootstrap support values (out of 100 replicates).

protein alignment (Fig. 2). Notably, this led to the identification of 41 alignment positions in which residues invariable throughout dicots are absent in monocots, and 84 alignment positions in which residues invariable throughout monocots are absent in dicots. In 44 alignment positions, class-specific residues are replaced in the other class with residues having different properties, according to the chemical features of their side-chain group (hydrophobic, polar basic, polar acidic and polar uncharged).

## Adaptive relevance of class-specific molecular features supported by evolutionary analysis

In order to make inference on the evolutionary events leading to the above mentioned class-specific molecular features, we performed a codon-based Single-Likelihood Ancestor Counting (SLAC) analysis on the difference of nonsynonymous to synonymous substitutions per nonsynonymous and synonymous sites (dN-dS). Tests were conducted to predict the evolution of each codon: neutral/dN=dS or negative (purifying)/dN<dS. We decided to restrict the analysis to a panel of nine dicot *MLO* susceptibility genes, as only four monocot *MLO* homologs have been so far associated with PM pathogenesis and the dN-dS analysis can provide significant results only when using a sequence dataset which is not too small. We found 130 codons under significant negative selection, coding for amino acids scattered throughout MLO protein domains. Among the 130 codons, 27 are translated into class-specific residues, which are therefore predicted to provide an adaptive value (Additional file 3).

		TM1
AtMLO2 AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 SIMLO1 NtMLO1 TaMLO_B1 TaMLO_A1b HvMLO OSMLO3	1 1 1 1 1 1 1 1	20 40 60 A-DQVKETTEEISTWAVAVCFVLLFISIVIEHSIHKIGTWEKKHKQALFEALEK A-DQVKETTEEISTWAVAVCFVLLFISIVIEKIHKIGSWEKKNKKALYEALEK AIKETTEEIPTWAVAVCFVLLFISIVIEKIHKIGSWEKKNKKALYEALEK AEEGVKETTEEIPTWAVAVCVCVLLAISTILEHIHVIGKWEKKNKNALYEALEK AEDKVYETTEEITTWAVAVCFVLLAISTIJEHIHAIGKWEKKNKNALYEALEK A-DKVACKKIEETTWAVAVCFVLLAISTIJEHIHAIGKWEKKNKNALYEALEK ABATPTWAVAVCFVLLAISTIEHIHAIGKWEKKNKNALYEALEK ABATPTWAVAVCFTLLAISTIEQIHHIGEWLLKKHKKLEAVEK ABATPTWAVAVCFTLLAISTFEQIHHIGEWLLKKHKKLYEALEK ADDDEYPPATTEETTWAVAVCFTLLAISTFEQIHHIGEWLLKKHKKLYEALEK AADDEYPPATTEETSWAVANCFTLLAISTFEQIHHIGEWLLKKHKKLYEALEK AADDDEYPPATTEETSWAVANCFTLLAISTFEQIHHIGEWLLKKHKKALEALEK AKDDGYPPATEPETSWAVANCFTLLAISTFEQIHHIGEWLLKKHKKLYEALEK AKDDGYPPATEPETSWAVANCFTULAISTFEQIHHIGEWLLKKHKKALWEALEK AKDDGYPPATEPETSWAVANCFTULAISTFEQIHHIKIGHWEHKHKNALAEALEK AKGGGGRALPETPTWAVAVCAVIVSVLMEHGHKIGHWEHKREKALWEALEK
TM2 80 100 120		
AtMLO2 AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 S1MLO1 NtMLO1 TaMLO_B1 TaMLO_A1b HvMLO OSMLO3	61 61 60	KAELMLIGFISILLT GQTPI-SNICISQKVASTMHPCSAAEEAKKYGKKDAGKKDDGD KAELMLMGFISILLTIGQGYI-SNICIPKNIAASMHPCSASEEARKYGKKDQFKEDE KAELMLIGFISILLTVFQDNI-SKICIPKNIAASMHPCSNHQEIAKYGKNYIDDG- KCELMLMGFISILLTVFQDNI-SKICISCKVGSTMHPCST-SNTKAKAKSDESLD KCELMLMGFISILLTVFQDYI-SKICISKVGSTMHPCST-SNTKAKAKSDESLD KCELMLMGFISILLTVFQDYI-SNICISKVQASTMHPCHP-EEKKKGPEG KAELMLIGFISILLTVTQDPV-SNICVFKSVGYSMHPCKADEDVKS
		140 160 180
AtMLO2 AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1 CaMLO2 SlMLO1 NtMLO1 TaMLO_B1 TaMLO_A1b HvMLO OSMLO3	100 101 95 95 106 106 107	SDKPGRRLLLELA-ESYIHRRSLATKGY K-CAEKG-KWAFVSAMGIHOLHIFIFVLAV -EENLRRKLLQLV-DSLIPRRSLATKGY K-CAEKG-KWAFVSAMGHOLHIFIFVLAV -RKILED-FDSN-DFYSPRNLATKGY K-CAEKG-KWAIVSAMGIHOLHIFIFVLAV -RKILEVF-DP-IPRRILATKGY C-CAEKG-KWAIVSAMGIHOLHIFIFVLAV SE-NHDRKLLEYF-DP-NPRRILATKGY Q-CADKG-KWAIVSAMGIHOLHIFIFVLAI 
		<u>TM3</u> 200 220 240
AtMLO2 AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 S1MLO1 NtMLO1 TaMLO_B1 TaMLO_A1b HvMLO OSMLO3	164 168 139 133 127 127 141 141 140	HVVYCIVTYAFGKIKMRTWKSMEETKTIEYCYSNDPERFRARDTSFGRRHLNFWSKT HVIYCIVTYALGKIKMRRWKKMEETKTIEYCYSNDPERFRARDTSFGRRHLSFWSKS HVIYCIITYALGKIKMRKWKSMERETKTIEYCYSNDPERFRARDTSFGRRHLSFWSKS HILQCIITLAGGRKWRRWKKMEDETRTVEYCFYNDPERFRARDTTFGRRHLSFWSKS HILQCIITLAGGRKWRRWKKMEDETRTVEYCFYNDPERFRARDTTFGRRHLSFWTKS HILQCIITLAGGRKWRRWRAMEDETKTIEYCYNDPERFRARDTTFGRRHLSFWTKS HILQCIITLAGGRKWRRWRAMEDETKTIEYCFYNDPERFRARDTTFGRRHLSFWTKS HILQCIITLAGGRKWRRWRAMEDETKTIEYCFYNDPERFRARDTFGRRHLSFWTSS HILQCIITLAGGRKWRRWRAMEDETKTIEYCFYNDPERFRARDTFGRRHLSFWTSS HILQCIITLAGGRKWRRWRAMEDETKTIEYCFYNDPERFRARETSFGRRHHFWSS SHVLYCIATFALGRLKWRKWRAMEDETKTIEYCFYNDPERFRARETSFGRRHLFWSS SHVLYCIATFALGRLKWRKWRAMEDETKTIEYCFYNDPERFRARETSFGRRHLFWSS SHVLYSVIIMALSRLKWRTWKKRETETASLEYCFANDPARFRTHOTSFVRHLGL-SST HVTYSVIIMALSRLKWRTWKKRETETSLEYCFANDPARFRTHOTSFVRHLGL-SST HVTYSVIITASRLKWRTWKKRETETSLEYCFANDPARFRTHOTSFVRHLGL-SST

FIGURE 2.

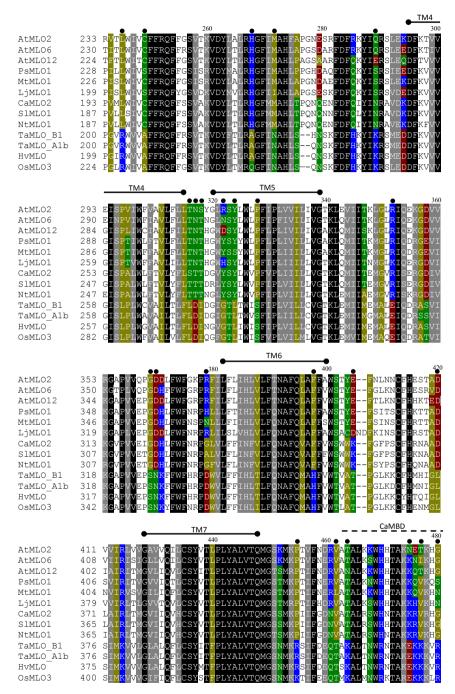
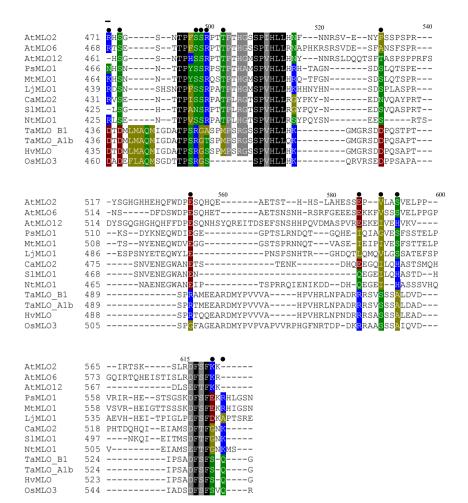


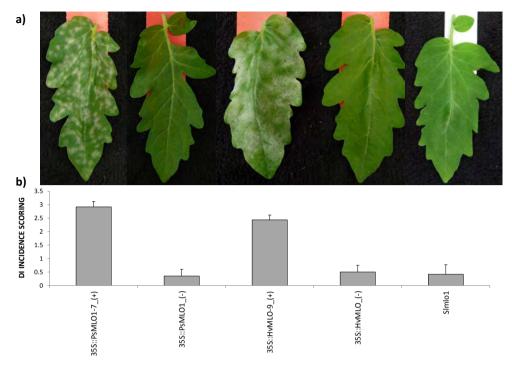
FIGURE 2.



**FIGURE 2. Multiple alignment of MLO powdery mildew susceptibility proteins**. The dataset is composed of all the monocot (barley HvMLO, rice OsMLO3, wheat TaMLO\_B1 and TaMLO\_A1b), and dicot (Arabidopsis AtMLO2, AtMLO6 and AtMLO12, tomato SIMLO1, pepper CaMLO2, tobacco NtMLO1, pea PsMLO1, lotus LjMLO1 and barrel clover MtMLO1) MLO homologs shown to act as powdery mildew susceptibility factors. The positions of the seven MLO transmembrane domains (TM1-TM7) and the calmodulin binding domain (CaMBD) are identical to the ones reported by Feechan *et al.* (2008), *Functional Plant Biology*, **35**: 1255-1266. Black color indicates alignment positions in which invariable residues are present. Grey color indicates alignment positions which there are class-specific residues in monocots, dicots, or both: yellow indicates hydrophobic residues (G, A, V, L, I, F, W, M, P); blue indicates polar basic residues (K,R,H); red indicates polar acidic residues (D, E); green indicates polar uncharged residues (S, T, C, Y, N, Q). Black dots highlight 44 alignment positions in which class-specific residues are substituted in the other class by residue(s) having different biochemical properties.

#### Functional conservation of monocot and dicot MLO susceptibility genes

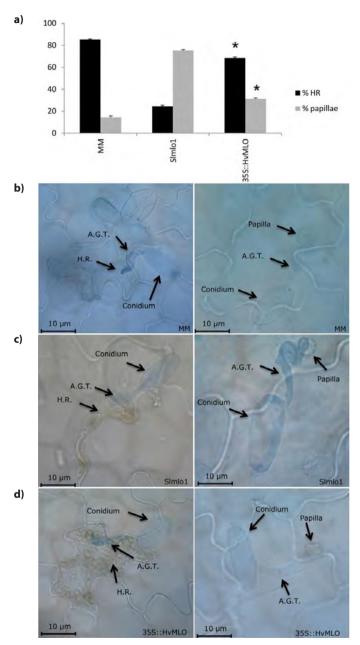
We tested whether different molecular features between monocot and dicot MLO proteins are specifically required by PM fungal species infecting either one or the other class of Angiosperms. To this aim, we developed two constructs for the transgenic expression of a monocot (barley HvMLO) and a dicot (pea PsMLO1) MLO gene in the tomato SImIo1 line, which is homozygous for a loss-of-function mutation in the endogenous gene SIMLO1 and therefore resistant to the tomato PM fungus Oidium neolycopersici. We reasoned that complementation and restoration of PM symptoms would have occurred only in case of functional conservation between SIMLO1 and any of the two tested transgenes. In total, nineteen 35S::PsMLO1 and twenty 35S::HvMLO transformants were obtained. In both cases, eighteen individuals were obtained showing variable transgene expression levels. For each construct, three T, plants displaying high transgene expression (35S::PsMLO1-4,-6 and -7 and 35S::HvMLO-9,-10 and -15) were self-pollinated to generate T, families (Additional Fig. 1). Ten individuals from each T<sub>2</sub> family were tested for the presence or the absence of the transgene and challenged with O. neolycopersici. Transgenic individuals of the three T, families overexpressing PsMLO1 (35S::PsMLO1\_(+)) displayed PM symptoms with an average D.I. (disease index) score ranging from 2.87 to 2.92. Transgenic individuals of the three T<sub>2</sub> families overexpressing HvMLO (35S::HvMLO\_(+)) showed an average D.I. score ranging from 1.8 to 2.4. In contrast, all non-transgenic 35S::PsMLO1 (-) and 35S::HvMLO (-) T, individuals displayed, similar to the SImIo1 plants, hardly any fungal growth (Fig. 3 and Additional file 2). For transgenic plants of the three 35S::HvMLO T<sub>2</sub> families, positive correlation was found between average D.I. and transgene expression level of corresponding T, plants (Fig. 3 and Additional file 1 and 2). Together, these results indicate that monocot and dicot MLO susceptibility genes are functionally conserved with respect to molecular features required for PM pathogenesis.



**FIGURE 3. Transgenic overexpression of pea** *PsMLO1* and barley *HvMLO* in the tomato mutant line SImIo1. Panel a) shows the phenotypes of two selected individuals of the T<sub>2</sub> family 35S::*PsMLO1-7*, segregating for the presence (first from the left) or the absence (second from the left) of the transgene, two selected individuals of the T<sub>2</sub> family 35S::*HvMLO-9*, segregating for the presence (third from the left) or the absence (second from the right) of the transgene, and one individual of the SImIo1 line (first from the right), in response to the tomato powdery mildew fungus *Oidium neolycopersici*. Panel b) from left to right shows average disease index (DI) values relative to transgenic plants (+) of the 35S::*PsMLO1-7* T<sub>2</sub> family, non-transgenic plants (-) of three T<sub>2</sub> families segregating for the 35S::*PsMLO1* construct, transgenic plants of the 35S::*PsMLO1* construct, transgenic plants of the SImIo1 line. Standard deviation bars refer to six 35S::*PsMLO1\_(+)* individuals, nine 35S::*HvMLO\_(+)* individuals, 7 *PsMLO1\_(-)* individuals, 7 *HvMLO\_(-)* individuals and 10 SImIo1 individuals.

## Functional conservation of monocot and dicot *MLO* susceptibility genes in nonhost interactions

We next investigated whether functional conservation between monocot and dicot MLO homologs also holds true in non-host plant-PM interactions. To this aim, we used the PM species *B. graminis* f.sp. *hordei* (*Bgh*) to inoculate plants of the Slmlo1 mutant line, the cultivar Moneymaker (MM), carrying wild-type *SlMLO1*, and two of the 35S::*HvMLO* T<sub>2</sub> families (35S::*HvMLO*-9 and -10, previously described in Fig. 3, Figure S1 and S2). *Bgh* is an adapted PM on barley and a non-adapted PM to tomato. In the Slmlo1 line, 75.4% of infection units were associated with papilla formation and 24.6% with cell death response (Fig. 4). Compared with the Slmlo1 line, transgenic 35S::*HvMLO*-9 T<sub>2</sub> plants displayed a



**FIGURE 4. Functional conservation of** *SIMLO1* and *HvMLO* in the tomato/*Blumeria graminis* **f.sp.** *hordei* (*Bgh*) **interaction.** Panel **a**) shows the ratio of penetrated and non-penetrated epidermal cells, assessed in function of infection units showing hypersensitive response (H.R.) and papillae, respectively, in the following genotypes: the *mlo* mutant line SImlo1; the cultivar MM, with a similar genetic background and carrying wild-type *SIMLO1*; transgenic plants of a T<sub>2</sub> family overexpressing barley *HvMLO* in the SImlo1 genetic background (35S::*HvMLO*-9). Panel **b**), **c**) and d) show, in the same genotypes, fungal structures (conidiospore and appressorium germination tube -A.G.T.-) and cellular events (the formation of papillae and H.R.) arresting fungal growth before and after penetration, respectively.

lower level of papilla formation (31.3%) and a higher level of cell death response (68.7%). In MM, papilla formation and cell death occurred at a rate similar to the one in 35S::*HvMLO*-9 plants (14.6% and 85.4%, respectively). Taken together, this body of evidence indicates that both *HvMLO* and *SIMLO1* predispose to the penetration of a non-host pathogen.

### Discussion

The functional characterization of MLO homologs involved in PM susceptibility is of great interest for basic research on plant-microbe interactions as well as for plant breeding, as loss-of-function genotypes could be conveniently used to introduce durable and broadspectrum resistance in cultivated species [7]. Results of previous investigations indicated that *mlo*-based resistance in a certain plant species can be lost by the heterologous expression of MLO susceptibility genes from related species of the same botanical family. Indeed, restored susceptibility has been observed in barley HvMLO mutants transformed with wheat TaMLO\_B1 and rice OsMLO3, as well as in pea PsMLO1 mutants expressing lotus LiMLO1 or barrel clover MtMLO1[12,13]. Recently, similar evidence was shown on tomato SIMLO1 mutants transformed with pepper CaMLO2 or tobacco NtMLO1 [16, 17]. Here, we investigated whether complementation can also occur by transferring MLO genes from more evolutionary divergent plant species. We found that, in a tomato mlo mutant background, transgenic expression of a MLO susceptibility gene from pea (a distantly related dicot species) and barley (a monocot species) is sufficient to re-establish PM susceptibility (Fig. 3 and Additional file 2). This finding indicates that, despite phylogenetic distance and the evolution of peculiar molecular traits (Fig. 1 and 2), monocot and dicot MLO proteins are essentially conserved with respect to features involved in the interaction with PM pathogens. In support of this conclusion, we show that the monocot gene HvMLO and the dicot gene SIMLO1 both enhance penetration of the non-adapted pathogen B. graminis f.sp. hordei compared to a tomato mlo-mutant (Fig. 4). Moreover, after reviewing scientific literature, we found that only one out of thirty MLO protein substitutions so far associated with PM resistance involves a class-specific residue (a monocot-specific alanine residue in position 350 of the alignment in Fig. 2) (Table 1) [22]. The same residue is replaced in dicots by a glycine (sharing similar nonpolar chemical properties of alanine, Table 1), indicating that, in this case, class-specific conservations are not associated with important changes in protein structure or function.

We cannot exclude that class-specific traits might have minor effects on interactions with PM fungi. Indeed, by comparing three independent  $T_2$  families for each construct, we found that that overexpression of *PsMLO1* results in higher D.I. index scores than the one of *HvMLO* (Fig. 3 and Additional file 2). Clearly, complementation tests with several other monocot and dicot transgenes could help to answer this question.

**TABLE 1.** Amino acid residues in dicot AtMLO2 and monocot HvMLO whose mutation has been associated with PM resistance. For each amino-acid, localization in any of the MLO protein domains, including seven transmembrane (TM) regions, three extracellular loops (E), three intracellular (I) loops, the N-terminus and the C-terminus, is indicated.

Barley HvMLO	Arabidopsis AtMLO2	Substituting residue(s) in the other Angiosperm class	Conservation level (%)	Reference	Type of resistance	Domain
<b>R</b> 10		R/K	69,2	[25]	full	N-terminus
<mark>V</mark> 30		I/V	38,5	[23]	full	TM1
<mark>S</mark> 31		S	100	[23]	full	TM1
	<mark>G</mark> 66	G	100	[11]	full	TM2
<mark>V</mark> 76		V/I	84,6	[24]	partial	TM2
<mark>C</mark> 86		С	100	[12]	full	E2
<b>C</b> 98		С	100	[12]	full	E2
<b>C</b> 114		С	100	[12]	full	E2
W159		W	100	[21]	full	12
W162		W	100	[21]	full	12
<b>E</b> 163		E	100	[21]	full	12
<mark>S</mark> 187	<mark>\$</mark> 220	S/T	76,9	[11, 24]	full	12
D219	D253	D	100	[11, 24]	Partial <sup>a</sup> /full <sup>b</sup>	12
T222		T/V/A	69,2	[23]	partial	12
G226		G	100	[23]	full	12
F240		F	100	[23]	partial	12
D251	D287	D	100	[11, 24]	Partial <sup>a</sup> /full <sup>b</sup>	TM4
L270		F/L/I	69,2	[23]	full	TM4
A306		G	30,7	[22]	partial	13
L307		Q/V/L	69,2	[24]	partial	13
G318		G	100	[23]	full	13
P320		Р	100	[24]	partial	13
P324		T/I/P	76,9	[24]	partial	13
F329		F	100	[24]	full	13
W330		W	100	[24]	partial	13
F331		F	100	[24]	partial	13
R333		R/S/K	84,6	[24]	partial	13
P334		Р	100	[23]	full	13
C367		С	100	[12]	full	E3
P395	P431	Р	100	[11, 24]	full	TM7

1) Numbers adjacent to each amino acid indicate their position in either HvMLO or AtMLO2 proteins.

2) Barley and Arabidopsis residues in the same row correspond to each other in HvMLO/AtMLO2 protein alignment.

3) Percentage of conservation is calculated based on the alignment of 13 MLO proteins functionally associated with powdery mildew susceptibility (AtMLO2, AtMLO6, AtMLO12, SIMLO1, CaMLO2, NtMLO1, PsMLO1, LjMLO1, MtMLO1, TaMLO\_A1b, TaMLO\_B1, OsMLO3 and HvMLO)

4) Amino acid color is according to its chemical properties: non-polar (yellow), polar, uncharged (green), polar, acidic (red), polar, basic (blue).

5) <sup>a</sup> partial resistance observed in barley, <sup>b</sup> full resistance observed in Arabidopsis.

Through the analysis of the dN-dS difference, we provide evidence for negative selection acting on several class-specific residues, which are thus likely to play a major adaptive role (Additional file 3). However, as mentioned before, transgenic complementation tests indicate that these class-specific residues are not crucial for the outcome of the interaction between plants and PM pathogens. Possibly, some of the class-specific residues identified in this study might underlie roles which are not related with the interaction with PM fungi. The implication of MLO susceptibility proteins in other physiological processes would explain why, in spite of being required for pathogenesis, they have been not excluded by evolution. With this respect, it is worth to mention that PM resistance in Arabidopsis and barley *mlo* mutants has been associated with the induction of leaf senescence, a pleiotropic phenotype [11].

We show that *MLO* homologs required for PM pathogenesis can complement a *mlo* mutant background in transgenic assays, irrespective of the phylogenetic distance between the donor and the recipient species (Fig. 3). This would be of great advantage in order to test the function of candidate *MLO* susceptibility genes which are currently being identified by several authors across cultivated species [4, 5]. Moreover, we provide an overview of MLO protein regions which are under negative selection and thus are expected to be of functional relevance. These regions represent ideal targets to select loss-of-function mutants resistant to the PM disease. With this respect, breeders may apply diverse tools, such as conventional targeted mutagenesis approaches of TILLING (targeted induced local lesions in genomes) or advanced technologies of genome editing, based on zinc finger nucleases (ZFNs), clustered regularly interspaced short palindromic repeat (CRISPR) and transcription activator-like effector nucleases (TALEN) [26,27,28].

# Conclusion

This work provides insights on the evolution and function of Angiosperm *MLO* susceptibility genes. We show that complementation assays similar to those carried out in this study are suitable for future activities aimed at the characterization of novel PM susceptibility factors across cultivated species. Moreover, we indicate a series of gene targets for the selection of loss-of-function *ml*o resistant mutants.

### Methods

### **Bioinformatic analyses**

The following MLO proteins, experimentally shown to be required for PM susceptibility, were used as dataset for CLUSTAL alignment using the CLC sequence viewer software (http://clcbio.com): Arabidopsis AtMLO2 [GenBank: NP172598], AtMLO6 [GeneBank: NP176350] and AtMLO12 [GeneBank: NP565902], tomato SIMLO1 [GeneBank: NP001234814], pea PsMLO1 [GeneBank: ACO07297], pepper CaMLO2 [GeneBank: AFH68055], lotus LjMLO1 [GeneBank: AAX77015], barrel clover MtMLO1 [GeneBank: ADV40949], barley HvMLO [GeneBank: P93766], rice OsMLO3 [GeneBank: AAK94907], wheat TaMLO\_B1 [GeneBank: AAK94904] and TaMLO\_A1b [GeneBank: AAK94905]. The alignment was given to Geneious v8 software (http://www.geneious.com, [29] ), to highlight amino acids with different polarity, and the online web service Phylogeny.fr (http://www.phylogeny.fr/) to construct an unrooted radial phylogenetic tree.

In order to make predictions on the type of evolution (negative or neutral) of classspecific molecular features, all the above mentioned dicot *MLO* susceptibility genes were used as dataset for a codon-based evolutionary analysis based on the difference of nonsynonymous-to-synonymous substitutions per nonsynonymous and synonymous sites (dN/dS). This was performed by using the Single-likelihood Ancestor Counting (SLAC) method implemented by the Datamonkey web server (www.datamonkey.org). The default p-value of 0.1 was taken as threshold to call codons under significant negative selection.

### Isolation and cloning of full-length PsMLO1 and HvMLO

Total RNAs from pea (cultivar Sprinter) and barley (cultivar Maythorpe) were isolated by using the RNeasy plant mini kit (Qiagen), and corresponding cDNAs were synthesized by using the SuperScript III first-strand synthesis kit (Invitrogen) and the oligo(dT)<sub>20</sub> primer. Specific primer pairs, named PsMLO1-Fw/PsMLO1-Rev and HvMLO-Fw/HvMLO-Rev (Additional file 4: table S2) were manually designed in order to amplify the *PsMLO1* and *HvMLO* full-length coding sequences, respectively. PCR reactions were performed by using the high-fidelity Phusion DNA polymerase (New England Biolabs) and an annealing temperature of 55°C. Amplicons were ligated into the Gateway-compatible vector pENTR D-TOPO (Invitrogen) and cloned into the *E. coli* One Shot<sup>®</sup> TOP10 cells (Invitrogen), according to the manufacturer's instructions. After selecting positive colonies by colony PCR, using the two gene-specific primer pairs above mentioned, recombinant plasmids were extracted and their inserts were sequenced. A single colony for each construct was selected, in which the inserts resulted to have sequences identical to those of *HvMLO* and *PsMLO1* deposited in the NCBI database.

# Generation and functional characterization of transgenic SIMLO1 mutant tomato plants expressing PsMLO1 and HvMLO

Following the manufacturer instructions (Invitrogen), cloned *HvMLO* and *PsMLO1* gene sequences were inserted by LR recombination into the binary plasmid vector pK7WG2, which harbors the 35S Cauliflower Mosaic Virus (CaMV) promoter and the marker gene *nptll* for kanamycin resistance selection. Plasmids were then transferred to *E. coli* and positive colonies were screened by colony PCR and sequencing, as previously mentioned. Finally, recombinant vectors were extracted and transferred to the AGL1-*vir*G strain of *A. tumefaciens* by electroporation.

The transformation of the tomato *ol-2* mutant line, carrying a loss-of-function mutation of the PM susceptibility gene *SIMLO1*, was performed according to the methods described by [6] and [16]. The evaluation of the expression levels of *PsMLO1* and *HvMLO* in T<sub>1</sub> plants was carried out by real-time qPCR using the primer pairs qPsMLO1-Fw/qPsMLO1-Rev and qHvMLO-Fw/qHvMLO-Rev (Additional file 4). A primer pair designed on the *elongation factor 1a* gene (qEF-Fw/qEF-Rev) was used for relative quantification (Additional file 4).

### Functional characterization of host and non-host interactions

For each of the two transgenes above mentioned, three T<sub>1</sub> individuals showing the highest expression levels were allowed to self-pollinate, resulting in a total of six T<sub>2</sub> families. Individuals of each family were assayed for the presence/absence of the overexpression construct by means of PCR, using the primer pairs NPTII\_Fw/ NPTII\_Rev and 35S-Fw / 35S-Rev designed on the nptII marker gene and the 35S promoter, respectively (Additional file 4). Ten resistant SImlo1 plants carrying the loss-of-function *SIMLO1* allele and ten individuals of each family were challenged with an isolate of the tomato PM fungus *O. neolycopersici* maintained at the Plant Breeding Department of the University of Wageningen, The Netherlands. Inoculation was performed as described by [30], spraying 4 weeks-old plants with a suspension of conidiospores obtained from freshly sporulating leaves of heavily infected plants and adjusted to a final concentration of 4 x 10<sup>4</sup> spores/ml. Inoculated plants were grown in a greenhouse compartment at  $20\pm 2^{\circ}$ C with  $70\pm 15\%$  relative humidity. Disease evaluation was visually carried out 15 days after inoculation, based on the presence of disease signs on the third and fourth leaf, according to the scale from 0 to 3 reported by [10].

For the functional characterization of a non-host interaction, seeds from one of the three  $35S::HvMLOT_2$  families previously tested were surface-sterilized and sown on half-strength Murashige and Skoog (MS) agar supplemented with 50 mg/ml kanamycin for selection of transgenic plants. Seeds were left for 2 days at 4°C and then transferred to a growing chamber for 10 days. Five transgenic seedlings were transplanted in pots and

transferred to a greenhouse compartment. Three barley plants of the PM susceptible cultivar Manchuria, five SImIo1 plants and five MoneyMaker plants were used as controls. An isolate of *B. graminis* f. sp. hordei (*Bgh*) collected in Wageningen (Wag.04) was used for the inoculation. This was performed by rubbing Manchuria leaves heavily infected with *Bgh* on the third tomato leaf. After 72 hours, in which inoculated plants were kept in a climate chamber at 20°C, 16 hours of light/day and 70% RH, a 4 cm<sup>2</sup> segment was cut from the inoculated leaves (third leaf). Three samples were taken from 3 plants of each genotype.

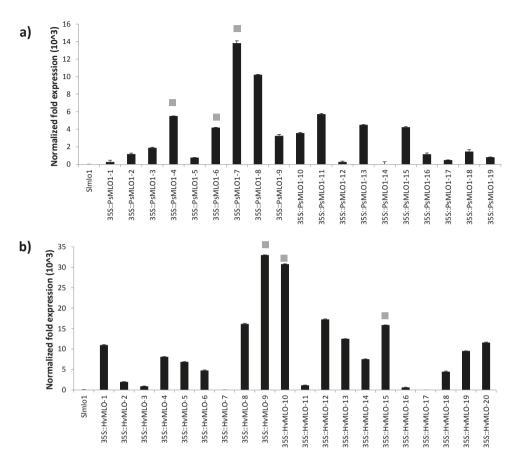
Each leaf segment was bleached is a 1:3 (v/v) acetic-acid/ethanol solution and 48 hrs later stained in 0.005% Trypan Blue as described by [31]. The rate of fungal penetration was estimated by the frequency of infection units showing epidermal cell death. For each genotype, three biological replicates were considered, considering at least 100 infection units.

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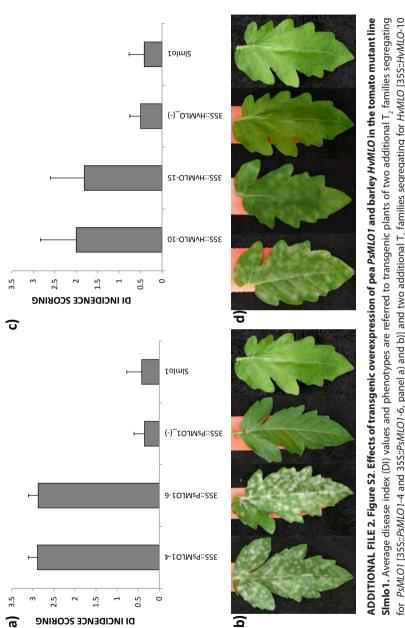
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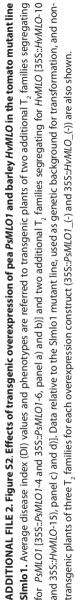
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# **Additional material**



**ADDITIONAL FILE 1. Figure S1. Expression levels of** *PsMLO1* and *HvMLO* after transformation. Panel A) and panel B) show the expression of *PsMLO1* and *HvMLO* in 19 and 20 T<sub>1</sub> individuals, respectively, which were obtained by the transformation of the tomato mutant line Slmlo1, harboring a loss-of-function mutation of the endogenous *SlMLO1* gene. Asterisks indicate T<sub>1</sub> individuals selected for self-pollination and the development of T<sub>2</sub> families.





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**ADDITIONAL FILE 3. Table S1. Codons under significant negative selection in PM susceptibility genes.** Codon numbers refer to positions in the alignment of nine dicot *MLO* genes (*AtMLO2, AtMLO6, AtMLO12, PsMLO1*, *MtMLO1, LjMLO1, CaMLO2, SIMLO1, NtMLO1*) experimentally shown to act as powdery mildew susceptibility genes. Amino acid residues corresponding to each codon in barley HvMLO and pea PsMLO1 are indicated. For each residue, localization in any of the MLO protein domains, including seven transmembrane (TM) regions, three extracellular loops (E), three intracellular (I) loops, the N-terminus and the C-terminus, is indicated. Codons marked in bold are translated into class-specific residues. The threshold p-value was 0.1, representing the default value for Single-likelihood Ancestor Counting (SLAC) analysis implemented by the Datamonkey web server.

Codon	dN-dS	Normalized dN-dS	p-value	Residue in HvMLO	Residue in PsMLO1	Domain
8	-4.82	-2.70	0.021	A9	E8	N-terminus
14	-3.29	-1.85	0.012	T15	T14	TM1
16	-4.12	-2.31	0.004	S17	T16	TM1
18	-3.30	-1.85	0.012	A19	A18	TM1
20	-3.30	-1.85	0.012	A21	A20	TM1
21	-2.88	-1.61	0.045	V22	V21	TM1
24	-2.60	-1.46	0.091	A25	L24	TM1
27	-2.92	-1.64	0.020	V28	L27	TM1
30	-2.47	-1.38	0.044	S31	S30	TM1
31	-2.02	-1.13	0.073	V32	131	TM1
49	-4.87	-2.73	0.007	K50	K49	11
55	-2.47	-1.38	0.037	A56	A55	11
58	-3.16	-1.77	0.037	K59	K58	11
62	-4.83	-2.71	0.006	E63	E62	11
65	-3.49	-1.96	0.012	L66	L65	TM2
72	-3.09	-1.73	0.018	L73	L72	TM2
73	-2.93	-1.64	0.025	L74	L73	TM2
81	-2.47	-1.38	0.040	A83	S81	E1
83	-2.88	-1.62	0.057	185	183	E1
123	-6.11	-3.42	0.072	/	D118	E1
137	-7.01	-3.93	0.023	/	R131	E1
142	-3.30	-1.85	0.085	/	K136	E1
145	-3.26	-1.83	0.034	D112	D139	E1
153	-4.12	-2.31	0.004	V119	V147	E1
162	-2.98	-1.67	0.034	H128	H156	E1
165	-2.98	-1.67	0.034	H131	H159	TM3
166	-2.80	-1.57	0.038	V132	1160	TM3
170	-2.47	-1.38	0.037	V136	V164	TM3
171	-2.71	-1.52	0.029	L137	L165	TM3
172	-2.47	-1.38	0.037	A138	A166	TM3
176	-3.94	-2.21	0.014	V142	1170	TM3
180	-2.56	-1.44	0.046	V146	1174	TM3
186	-2.47	-1.38	0.044	S152	G180	TM3
197	-4.76	-2.67	0.007	E163	E191	12
199	-3.32	-1.86	0.032	E165	E193	12
201	-2.84	-1.59	0.094	T167	R195	12
202	-2.47	-1.38	0.037	S168	T196	12
204	-3.23	-1.81	0.034	E170	E198	12

Codon	dN-dS	Normalized dN-dS	p-value	Residue in HvMLO	Residue in PsMLO1	Domain
207	-2.62	-1.47	0.099	F173	F201	12
209	-2.65	-1.48	0.089	N175	N203	12
211	-2.47	-1.38	0.037	P177	P205	12
214	-4.47	-2.51	0.006	F180	F208	12
215	-3.49	-1.96	0.016	R181	R209	12
218	-2.17	-1.22	0.065	H184	R212	12
219	-2.67	-1.50	0.090	Q185	D213	12
222	-2.98	-1.67	0.034	F188	F216	12
224	-4.09	-2.30	0.007	K190	R218	12
225	-2.28	-1.28	0.063	R191	R219	12
226	-2.98	-1.67	0.034	H192	H220	12
233	-3.63	-2.03	0.030	T198	S227	12
234	-3.55	-1.98	0.052	P199	P228	12
237	-2.62	-1.47	0.036	R202	L231	12
239	-2.24	-1.25	0.060	V204	1233	12
243	-2.98	-1.67	0.034	F208	F237	12
244	-3.47	-1.94	0.017	R209	R238	12
247	-2.64	-1.48	0.090	F212	F241	12
250	-3.79	-2.13	0.016	V215	1244	12
254	-2.98	-1.67	0.034	D219	D248	12
258	-2.94	-1.65	0.022	L223	L252	12
259	-2.26	-1.27	0.069	R224	R253	12
261	-2.47	-1.38	0.040	G226	G255	12
262	-2.98	-1.67	0.034	F227	F256	12
265	-3.29	-1.85	0.012	A230	A259	12
270	-2.77	-1.55	0.097	/	G264	12
280	-2.98	-1.67	0.047	Y243	Y274	12
281	-3.05	-1.71	0.019	1244	1275	12
288	-4.47	-2.51	0.006	D251	D282	12
293	-3.29	-1.85	0.012	V256	V287	TM4
296	-4.13	-2.32	0.021	S259	S290	TM4
297	-2.47	-1.38	0.037	L260	P291	TM4
302	-4.42	-2.48	0.022	V265	F296	TM4
304	-2.47	-1.38	0.037	1267	V298	TM4
308	-2.83	-1.59	0.027	F271	L302	TM4
309	-2.88	-1.61	0.045	L272	T303	E2
321	-2.47	-1.38	0.037	S284	P315	TM5
324	-2.47	-1.38	0.037	P287	P318	TM5
325	-2.78	-1.56	0.099	L288	L319	TM5
328	-4.16	-2.34	0.008	L291	1322	TM5
329	-2.95	-1.65	0.030	L292	L323	TM5
330	-4.67	-2.62	0.008	C293	L324	TM5
331	-2.47	-1.38	0.037	V294	V325	TM5
332	-3.29	-1.85	0.016	G295	G326	TM5
334	-3.35	-1.88	0.033	K297	K328	13
338	-2.36	-1.32	0.054	1301	1332	13

Codon	dN-dS	Normalized dN-dS	p-value	Residue in HvMLO	Residue in PsMLO1	Domain
342	-5.60	-3.136	0.010	M305	M336	13
343	-2.47	-1.38	0.046	A306	G337	13
348	-4.27	-2.40	0.042	D311	D342	13
350	-2.47	-1.38	0.045	A313	G344	13
351	-2.69	-1.51	0.088	S314	E345	13
357	-3.29	-1.85	0.012	P320	P351	13
360	-2.79	-1.57	0.096	E323	E354	13
361	-4.13	-2.32	0.019	P324	P355	13
366	-2.98	-1.67	0.034	F329	F360	13
368	-4.47	-2.51	0.006	F331	F362	13
375	-2.45	-1.38	0.038	L338	L369	TM6
376	-2.54	-1.42	0.095	F339	F370	TM6
381	-3.29	-1.85	0.012	T344	V375	TM6
383	-2.98	-1.67	0.034	F346	F377	TM6
386	-2.47	-1.38	0.037	A349	A380	TM6
392	-2.98	-1.67	0.034	F355	F386	TM6
401	-2.98	-1.67	0.034	P363	F393	E3
408	-2.98	-1.67	0.033	H370	H400	E3
413	-4.47	-2.51	0.006	L375	D405	E3
416	-4.37	-2.45	0.004	M377	1408	E3
417	-2.33	-1.31	0.065	K378	R409	E3
424	-3.20	-1.79	0.067	L385	l416	TM7
425	-4.86	-2.73	0.009	Q386	Q417	TM7
430	-2.98	-1.67	0.047	Y391	Y422	TM7
433	-2.91	-1.63	0.021	F394	L425	TM7
435	-3.19	-1.79	0.014	L396	L427	TM7
438	-2.01	-1.12	0.076	L399	L430	TM7
443	-2.47	-1.38	0.038	G404	G435	C-terminu:
447	-3.92	-2.20	0.054	K408	K439	C-terminu:
448	-2.47	-1.38	0.037	R409	P440	C-terminu
450	-2.74	-1.54	0.059	1411	1442	C-terminu:
451	-2.98	-1.67	0.034	F412	F443	C-terminu
458	-2.47	-1.38	0.037	A419	A450	C-terminu:
459	-3.49	-1.96	0.012	L420	L451	C-terminu
466	-3.29	-1.85	0.012	A427	A458	C-terminu
468	-2.86	-1.61	0.093	E429	K460	C-terminu
472	-4.14	-2.32	0.022	V433	Q464	C-terminu
476	-3.29	-1.85	0.022	D437	S468	C-terminu
483	-2.47	-1.38	0.037	P449	P473	C-terminu
487	-5.72	-3.21	0.001	S453	R477	C-terminu:
496	-3.29	-1.85	0.013	S462	S486	C-terminu:
497	-4.12	-2.31	0.004	P463	P487	C-terminu:
499	-2.98	-1.67	0.034	H465	H489	C-terminu:
500	-3.36	-1.89	0.013	L466	L490	C-terminu
501	-3.59	-2.01	0.023	L467	L491	C-terminu:
502	-2.98	-1.67	0.077	H468	H492	C-terminu:

Name	Fw primer sequence (5'> 3')	Rev primer sequence (5'>3')
PsMLO1	CACCATGGCTGAAGAGGGAGTTAAGGAAC	CTAATTGCTCCCTAAGTGGCGCTT
HvMLO	CACCGACCGATGTCGGACAAAA	TCATCCCTGGCTGAAGGAAAA
qPsMLO1	AGGTTTGCAAGGGACACAAC	TTGTGCATCATGTCCTGGAG
qHvMLO	TTTCATCCCTCTCGTGATCC	CCACTGTCCACACAAAATGC
NPTII	TCGGCTATGACTGGGCACAAC	AAGAAGGCGATAGAAGGCGA
355	GCTCCTACAAATGCCATCA	GATAGTGGGATTGTGCGTCA
Oid	CGCCAAAGACCTAACCAAAA	AGCCAAGAGATCCGTTGTTG
qEf	ACAGGCGTTCAGGTAAGGAA	GAGGGTATTCAGCAAAGGTCTC

ADDITIONAL FILE 4. Table S2. Primer pairs used in this study.

# Chapter 6

A transposable element insertion in the susceptibility gene *CsaMLO8* results in hypocotyl resistance to powdery mildew in cucumber

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## Abstract

### Background

Powdery mildew (PM) is an important disease of cucumber (*Cucumis sativus* L.). *CsaMLO8* was previously identified as a candidate susceptibility gene for PM in cucumber, for two reasons: 1) This gene clusters phylogenetically in clade V, which has previously been shown to harbour all known *MLO*-like susceptibility genes for PM identified in dicot species; 2) This gene co-localizes with a QTL on chromosome 5 for hypocotyl-specific resistance to PM.

### Results

We cloned *CsaMLO8* alleles from susceptible and resistant cucumber genotypes, the latter carrying the QTL for hypocotyl resistance. We found that insertion of a non-autonomous Class LTR retrotransposable element in the resistant genotype leads to aberrant splicing of *CsaMLO8* mRNA. Heterologous expression of the wild-type allele of *CsaMLO8* in a tomato *mlo*-mutant restored PM susceptibility. However, heterologous expression of the *CsaMLO8* allele cloned from the resistant cucumber genotype failed to restore PM susceptibility. Furthermore we showed that inoculation of susceptible cucumber with the PM pathogen *Podosphaera xanthii* induced transcriptional upregulation of *CsaMLO8* in hypocotyl tissue, but not in cotyledon or leaf tissue. This coincides with the observation that the QTL at the *CsaMLO8*-locus causes full resistance in hypocotyl tissue, but only partial resistance in cotyledons and true leafs. We studied the occurrence of the loss-of-function allele of *CsaMLO8* in cucumber germplasm by an *in silico* approach using resequencing data of a collection of 115 cucumber accessions, and found that this allele was present in 31 out of 115 accessions.

### Conclusions

*CsaMLO8* was characterised as a functional susceptibility gene to PM, particularly in the hypocotyl where it was transcriptionally upregulated upon inoculation with the PM pathogen *P. xanthii*. A loss-of-function mutation in *CsaMLO8* due to the insertion of a transposable element was found to be the cause of hypocotyl resistance to PM. This particular allele of *CsaMLO8* was found to occur in 27% of the resequenced cucumber accessions.

### Keywords

Powdery mildew, *MLO*, susceptibility gene, Cucumber (*Cucumis sativus* L.), hypocotyl resistance, non-autonomous transposable element

# Background

Cucumber (Cucumis sativus L.) is an economically important crop, with an annual global production of over 65 megatons [1]. Powdery mildew (PM) is one of the most widespread diseases in cucurbits, and a limiting factor for cucumber production. Two species of fungi have been reported to cause PM in cucumber, i.e. Podosphaera xanthii (synonymous with P. fusca, previously named Sphaerotheca fuliginea) and Golovinomyces cichoracearum (previously named Erysiphe cichoracearum). Of these, P. xanthii is considered to be the main causal agent of PM in cucurbits [2, 3].

Breeding of resistant cucumber varieties has been undertaken for several decennia (e.g. [4–6]), but underlying resistance genes have to date not been functionally characterised. As the genome of cucumber ('Chinese long' inbred line 9930) was published in 2009 [7], and several other cucumber accessions have been resequenced [8, 9], the time is now ripe to identify causal genes for cucumber resistance to mildew diseases.

Traditionally, breeding of disease resistant crops is performed by introgression of resistance (*R*) genes, often from wild relatives of the crop. R proteins, most commonly of the nucleotide-binding, leucine-rich-repeat (NB-LRR) type, are able to recognise either corresponding avirulence (*Avr*) gene products of the pathogen, or degradation products of host factors associated with pathogen attack [10]. This triggers a defence response in the host cell, often associated with a hypersensitive response (HR), leading to cell death [10]. As *R* genes recognise very specific products, introgression and subsequent employment of a new *R* gene puts selective pressure on the pathogen to evolve in such a way that it is no longer recognised by the host plant. Therefore, *R*-gene based resistance is often breached by new, virulent, races of the pathogen quite soon, especially for versatile pathogens, such as powdery mildew fungi [10].

An alternative for *R*-gene mediated resistance is the identification of impaired susceptibility (*S*) genes [11]. Most pathogens require cooperation of their host plant to be able to successfully establish a compatible interaction [12]. This is especially true for biotrophic pathogens such as mildew species, as they greatly rely on a long-lasting interaction with (living) host cells to facilitate their propagation [12]. Therefore, the expression of several host genes is essential for the pathogen. Such genes can be regarded as *S* genes, and can function for instance in facilitating host recognition and penetration, negative regulation of host defences or fulfilling metabolic and structural needs of the pathogen [12]. Loss-of-function mutations in a *S* gene is thought to lead to durable, broad spectrum, recessively inherited resistance [13, 14].

The barley *mlo* gene is one of the best-known examples of an impaired S gene. After it first was found in the 1940s in a mutagenized barley population [15], deployment of loss-of-function *mlo* alleles in barley has resulted in PM resistant barley varieties. These have

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been grown in the field for several decades already without breaching of resistance by virulent new mildew races to date, providing evidence for the durability of *S*-gene based resistance [16]. After the barley *MLO* gene was cloned [17], it was found that *MLO* genes are conserved throughout the plant kingdom and occur in higher plants as a multi-copy gene family [18, 19]. In several plant species, *MLO*-like genes have been found to be involved in PM susceptibility, such as *Arabidopsis*, tomato, pea, pepper, tobacco, bread wheat and potentially also grapevine and peach [20–27]. It has been found that in phylogenetic trees of the *MLO* gene family all *MLO*-like S-genes for PM detected in monocotyledonous species cluster in clade IV, whereas all *MLO*-like *S*-genes identified in dicotyledonous species cluster in clade V. The other clades (I, II, III and VI) harbour *MLO*-like genes that have not been proven to be *S*-genes [19].

The genome of cucumber harbours 13 putative MLO-like genes [28]. Of these, three (i.e. CsaMLO1, CsaMLO8 and CsaMLO11, with respective Cucurbit Genomics Database IDs [Csa1M085890.1], [Csa5M623470.1] and [Csa6M292430.1]) cluster in clade V of the MLO gene family, and can therefore be considered candidate S-genes for powdery mildew resistance [28]. CsaMLO8 is of particular interest, as its position on the genome (Chr5: 24,827,408..24,831,456) co-localizes with pm5.2, a recently identified major QTL explaining 74.5% of the phenotypic variation for 'hypocotyl' resistance in F3 families derived from the resistant cucumber inbred line WI 2757 [29]. 'Hypocotyl' or intermediate resistance of cucumber to PM was previously shown to be a recessively inherited monogenic trait in crossings between several cucumber lines, and was characterised by completely resistant hypocotyl, stem and petiole tissue and partially resistant leaves and cotyledons. Hypocotyl resistance is suggested to play an important role in overall PM resistance of cucumber, as it appears that complete resistance in leaves is not possible without the recessive hypocotyl resistance gene [5]. In breeding practice loss of the hypocotyl resistance allele leads to PM susceptible seedlings. The allele is present in almost all modern pickling cucumber varieties, and most of the resistant long cucumber varieties (Freddy Hermans, personal communications), showing the agricultural significance of hypocotyl resistance in cucumber.

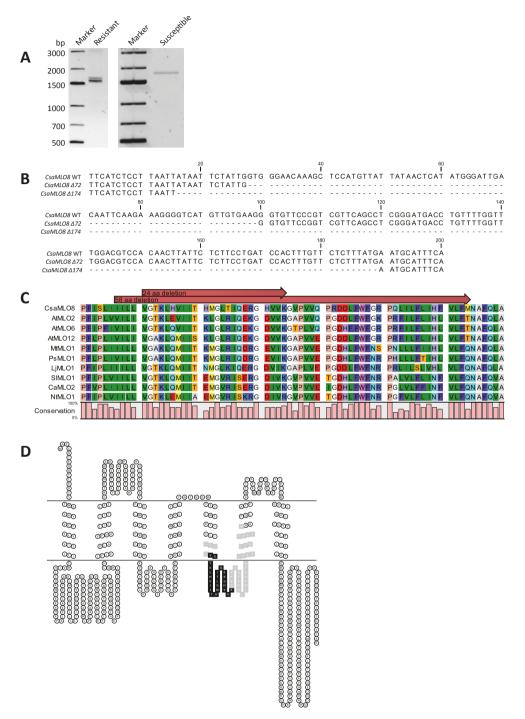
Here, we report the cloning of *CsaMLO8* from both susceptible and (hypocotyl) resistant cucumber genotypes. We show that at the transcript level the allele obtained from the resistant genotype has deletions of 72 or 174 bp due to alternative splicing, caused by the insertion of a LTR retrotransposable element in this gene at the genomic level. Complementation of the tomato *mlo*-mutant with the wild-type and  $\Delta$ 174 alleles of *CsaMLO8* showed that wild-type *CsaMLO8* is a functional susceptibility gene (*S*-gene), whereas the  $\Delta$ 174 allele has lost its function as *S*-gene, thus leading to PM resistance. Furthermore, qRT-PCR showed that *CsaMLO8* is transcriptionally upregulated upon inoculation with *P. xanthii* in hypocotyl tissue, but not in leaves or cotyledon, explaining why loss-of-function of *CsaMLO8* provides particularly resistance in the hypocotyl.

### Results

# Cloning and sequencing of the *CsaMLO8* coding sequence from susceptible and resistant genotypes

We performed RT-PCR using RNA derived from either a susceptible wild-type cucumber cultivar or a resistant breeding line known to be homozygous for the *hypocotyl resistance* QTL as a template. Whereas the product we obtained from the susceptible genotype was of the expected size (i.e. 1726 bp), we obtained two different products from the resistant genotype, both smaller than expected (Fig. 1A). Sequence analysis revealed that the *CsaMLO8* mRNA variant obtained from the susceptible genotype was identical to the predicted coding sequence. The two mRNA products obtained from the resistant genotype however had (non-frameshift) deletions of respectively 72 and 174 bp. The 174 bp deletion variant corresponds to a loss of the complete 11<sup>th</sup> exon of the *CsaMLO8* gene, whereas the 72 bp deletion variant corresponds to the loss of a fragment of the 11<sup>th</sup> exon with canonical splice sites (5'-GT and AG-3') (Fig. 1B). Furthermore, the coding sequence of the resistant genotype has five (synonymous) SNPs compared to the reference genome (Additional file 1).

To determine the impact of the 72 and 174 bp deletions found in the mRNA on the predicted CsaMLO8 protein sequence, the predicted CsaMLO8 protein was aligned to a dataset of MLO proteins encoded by clade V S-genes from several other species i.e. Arabidopsis, barrel clover, pea, lotus, tomato, pepper and tobacco (Additional file 2). It appeared that the region encoded by the deleted area in the 72 and 174 bp deletion variants is highly conserved among different MLO proteins (Fig. 1C). Furthermore, the transmembrane structure of the CsaMLO8 protein (wild-type allele) was predicted using HMMTOP 2.1 software [30]. The predicted transmembrane structure of the wild-type protein was largely consistent with the barley MLO structure determined by Devoto et al. [18, 19]. The 72 and 174 bp deletions correspond to removal of a region of 24 respectively 58 amino acid residues in the (predicted) third cytoplasmic loop of CsaMLO8 (Fig. 1D). The relative transcript abundances of the two CsaMLO8 splice variants characterised by the 72 and 174 bp deletions were determined by gRT-PCR using splice junction spanning primers on different tissues (i.e. hypocotyl, cotyledon and true leaf) of PM resistant cucumber, either inoculated with PM or non-inoculated. It appeared that the 174 bp deletion splice variant was the most abundant isoform, whereas the 72 bp deletion splice variant was less abundant in each tissue regardless whether tissues were inoculated or not (Additional file 3).



#### FIGURE 1. Characterization of CsaMLO8 alleles from resistant and susceptible cucumber genotypes.

(A) cDNA of resistant (left panel) and susceptible (right panel) cucumber genotypes was used as template for PCR with *CsaMLO8* specific primers. Amplified products were analysed on 1.25% agarose gels. Whereas the

product amplified from cDNA of the susceptible genotype gives a single band of the expected size, cDNA of the resistant genotype results in two separate bands, both of a smaller size than expected.

**(B)** Full length *CsaMLO8* amplified from cDNA from susceptible and resistant cucumber genotypes was sequenced. A partial alignment is shown between the (wild-type) sequence as obtained from the susceptible genotype and the sequences from two deletion variants ( $\Delta$ 72 and  $\Delta$ 174) obtained from the resistant genotype. Numbers are relative to the start of the alignment.

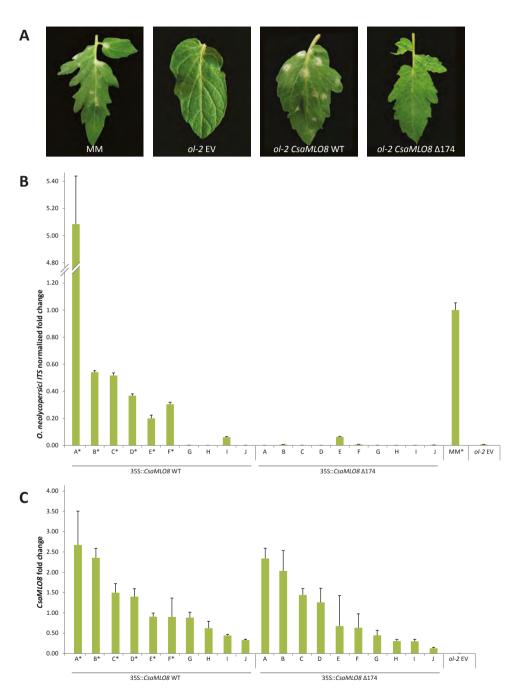
(C) Partial alignment of the CsaMLO8 protein and other proteins encoded by clade V *MLO S*-genes of several species. Amino acid residues are coloured according to the RasMol colour scheme. The 24 and 58 amino acid residues deleted in the proteins encoded by the  $\Delta$ 72 and the  $\Delta$ 174 variants of *CsaMLO8* are indicated by red arrows. A bar graph underneath the alignment indicates the conservedness of each amino acid position.

**(D)** Graphic representation of the transmembrane structure of the predicted CsaMLO8 protein, determined using HMMTOP 2.1 [30]. The plasma membrane is indicated by two horizontal lines. Amino acid residues highlighted in black are predicted to be deleted in the protein encoded by the  $\Delta$ 72 variant of the *CsaMLO8* gene, residues highlighted in black and grey are predicted to be deleted in the protein encoded by the  $\Delta$ 174 variant of the *CsaMLO8* gene.

# Complementation of *SIMLO1* loss-of-function tomato mutant with *CsaMLO8* WT and *CsaMLO8*∆174

The sequence analysis of the transcripts of *CsaMLO8* from susceptible and resistant genotypes led to the hypothesis that *CsaMLO8* is a functional *S*-gene for PM, whereas the 174 bp deletion allele (*CsaMLO8* $\Delta$ 174) has lost its function as *S*-gene. To test these hypotheses, both alleles were overexpressed in a previously described tomato *mlo*-mutant, which carries a mutation in the tomato *SlMLO1* gene and is resistant to tomato powdery mildew, *Oidium neolycopersici* [21].

Cuttings of ten independent transgenic individuals per construct (355::CsaMLO8 WT and 355::CsaMLO8 $\Delta$ 174) were challenged with the tomato PM pathogen O. neolycopersici. Powdery mildew susceptibility was evaluated qualitatively, by looking for PM symptoms on the leaves (Fig. 2A, Additional file 4). Six out of ten individual transformants expressing CsaMLO8 WT were scored as susceptible to PM, whereas none of the transformants expressing CsaMLO8 $\Delta$ 174 were scored as susceptible to PM. PM susceptibility was confirmed quantitatively, by performing qPCR on DNA isolated from inoculated leaves, using O. neolycopersici specific primers. This showed that the biomass of O. neolycopersici in plants scored as susceptible to PM was at least 0.20, relative to the biomass in the susceptible control MM, whereas the biomass in plants scored as resistant was less than 0.20 (Fig. 2B). Furthermore, transcript abundances of the transgenes in each of the transgenic individuals were determined by qRT-PCR using CsaMLO8 $\Delta$ 174 were comparable. The six CsaMLO8 WT transformants scored as susceptible to PM had a higher CsaMLO8 expression than the four CsaMLO8 WT transformants scored as resistant to PM.



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FIGURE 2. Complementation of *ol-2* tomato with *CsaMLO8* WT restores PM susceptibility, whereas complementation with *CsaMLO8*Δ174 does not.

The PM resistant *ol-2* tomato mutant with a deletion in *SIMLO1* [21] was transformed with either a 35S::*CsaMLO8* WT construct, a 35S::*CsaMLO8*Δ174 construct, or an empty vector (EV) control. Cuttings from these transformants were inoculated with a *Oidium neolycopersici* spore suspension. As additional control we used the wild-type, susceptible cv. Moneymaker (MM).

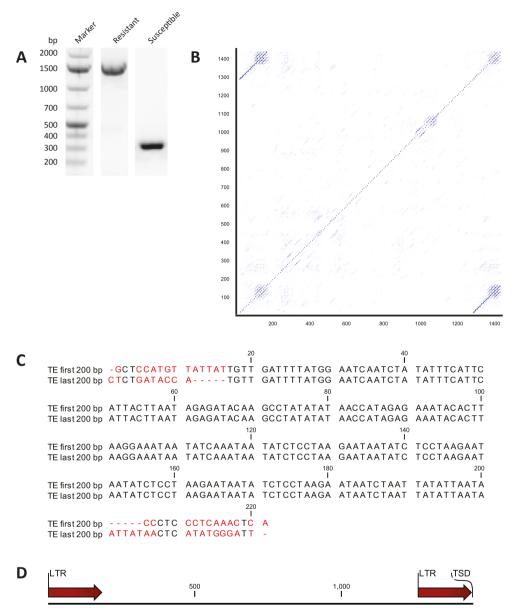
(A) The phenotype of susceptible control MM, resistant EV transformed *ol-2*, and transgenic individuals overexpressing either *CsaMLO8* WT or *CsaMLO8* $\Delta$ 174 in *ol-2* background. Photographs were taken 16 days post inoculation.

(B) Relative quantification by qPCR of the ratio between *Oidium neolycopersici* and plant gDNA in susceptible MM, resistant EV transformed *ol-2*, and transgenic individuals overexpressing either *CsaMLO8* WT or *CsaMLO8* $\Delta$ 174 in *ol-2* background. Fold changes were normalised relative to the susceptible control MM. based on macroscopic evaluation. Bars represent the average fold change over 3 technical replicates. Error bars indicate standard deviation. Asterisks indicate plants scored as susceptible to powdery mildew.

(C) Relative quantification by qRT-PCR of the ratio between *CsaMLO8* expression and expression of tomato housekeeping gene *SIEF-a* in EV transformed *ol-2* and transgenic individuals overexpressing either *CsaMLO8* WT or *CsaMLO8* $\Delta$ 174 in *ol-2* background. Bars represent the average fold change over 3 technical replicates. Error bars indicate standard deviation. Asterisks indicate plants scored as susceptible to powdery mildew.

### Sequencing and characterization of a transposable element in CsaMLO8

To investigate the cause of the deletions in the CsaMLO8 coding sequence, we performed PCR using DNA from both the susceptible and resistant cucumber genotypes as a template, with primers designed to amplify the region that contained the deletions in CsaMLO8. The product amplified from the susceptible genotype had the expected size (i.e. 346 bp), whereas the product amplified from the resistant genotype was larger (ca. 1500 bp, Fig. 3A). Sequence analysis of the amplified product revealed a 1449 bp insertion in the genomic DNA sequence of the resistant genotype compared to the susceptible genotype. This insertion in the DNA of the resistant genotype coincided with the region that contained the deletion in the CsaMLO8 mRNA of this genotype. Characterization of this genomic insertion by a dot-plot (Fig. 3B) revealed the presence of long terminal repeats (LTRs) with a length of ca. 200 bp. An alignment between the first and last 200 bp of the insertion confirmed the presence of 184 bp long LTRs beginning with a 5'-TG-3' and ending with a 5'-TA-3' (Fig. 3C). The LTRs share 100% sequence identity with one another. After the 3' LTR, there is a duplication of the 5 bp of CsaMLO8 before the insertion (Target Site Duplication, TSD, 5'-ATTAT-3'). No open reading frames (ORFs) could be detected in the insertion. Taken together, these findings led us to the conclusion that the insert is most likely a non-autonomous transposable element (TE) of Class I, Order LTR, according to the transposable element classification scheme proposed by Wicker et al. [31].





(A) The genomic region of *CsaMLO8* in which deletions in the coding sequence were observed in the resistant genotype was amplified from DNA isolated from both the susceptible and resistant genotypes. Amplified products were analysed on 1.25% agarose gel. Whereas the product amplified from the susceptible genotype was of the expected size, the product amplified from the resistant genotype was larger than expected.

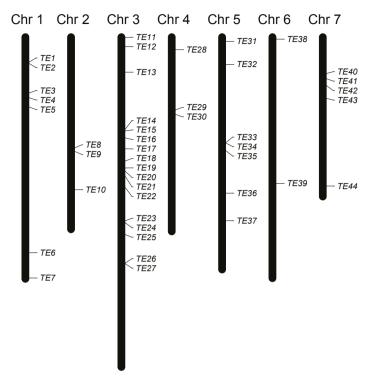
**(B)** The product amplified from the resistant genotype as described in (A) was sequenced, which revealed an insertion with a length of 1449 bp. A dot-plot was made of the insertion to see whether the sequence contains repetitive elements.

(**C**) The first and last 200 bp of the insertion, plus 15 bp of *CsaMLO8* before and after the insertion were aligned to one another, to verify the presence of long terminal repeats (LTRs). Non-aligned parts of the sequence are highlighted in red. It can be seen that the first 184 bp of the insertion are completely identical to the last 184 bp of the insertion. There is a duplication of 5 bp from *CsaMLO8* before and after the insertion (Target site duplication, 5'-ATTAT-3').

(D) Schematic representation of the insertion. The locations of LTRs and the 3' TSD are indicated.

#### Similar TEs in the cucumber genome

In an attempt to identify homologous, potentially autonomous, transposable elements in the cucumber genome, we performed a BLASTn search on the cucumber reference genome (Chinese long inbred line '9930', v2) with the LTR sequence of the TE found in CsaMLO8 as guery. We identified 169 putative homologous LTRs. A previously designed tool [32] was used to screen the genome for regions bordered by two putative homologous LTR sequences. Two putative homologous LTR sequences within a window of 20 kb were considered to be the borders of a putative homologous TE. The 20 kb window was decided upon based on the observation that LTR retrotransposons are generally between 3-15 kb of size [33], the only exception to our knowledge being the very large Ogre retrotransposons found in legumes [34]named Ogre, which is over 22 kb long and makes up at least 5% of the pea (Pisum sativum L., which have ca. 5 kb LTRs and are therefore ca. 22 kb in size. A total of 44 putative TEs was identified, randomly distributed over all seven chromosomes of the cucumber reference genome (Fig. 4, Additional file 5). For 20 putative TEs, the complete sequence in between the LTRs was extracted from the genome, and compared to the sequence of the TE found in CsaMLO8 (Additional file 6). It was found that most of the putative TEs have a length comparable to the CsaMLO8-TE, being between 1 and 2 kb. One putative TE was considerably larger than average, with 7,142 bp, whereas one putative TE was considerably smaller than average, i.e. 367 bp. In only one out of the 20 putative TEs (TE37), an open reading frame (ORF) could be detected. This ORF, with a length of 411 bp, does not lead to a predicted protein with any similarity to known proteins according to a BLASTp search against all non-redundant protein databases, and is therefore considered a false positive ORF. We conclude that we could not detect an autonomous TE that contained the genes that could have been responsible for the insertion of the non-autonomous TE in CsaMLO8.



#### FIGURE 4. There are 44 putative homologous TEs in the cucumber reference genome.

A BLASTn search was performed on the cucumber reference genomes with the LTR sequence of the TE found to be inserted in *CsaMLO8*. Pairs of putative LTRs within 20 kb of one another were considered borders of putative TEs. 44 putative TEs were identified, chromosomal locations of which are indicated.

### Occurrence of the TE-allele of CsaMLO8 in cucumber germplasm

We were interested to see how frequently the TE-allele of *CsaMLO8* we have characterised in our resistant cucumber genotype occurs in the cucumber germplasm. As Qi et al. (2013) resequenced a core collection of 115 very divergent cucumber accessions [8], we decided to perform an *in silico* search for the presence of the mutant *CsaMLO8* allele containing the TE) and/or the wild type (WT) allele among those genotypes. For 21 resequenced accessions (18%) we could only detect reads indicating presence of the TE-allele. For 82 resequenced accessions (71%) we could only find reads indicating presence of the WT-allele. For 10 accessions (9%) we found reads indicating presence of both alleles. For the remaining two accessions (2%), presence of neither of the alleles could be identified (Table 1, Additional file 7). The TE-allele of *CsaMLO8* was present in three out of the four geographic groups of accessions (i.e. East Asian, Eurasian and Indian but not Xishuangbanna) as defined by Qi et al. [8]. One of the 31 accessions in which the TE-allele of *CsaMLO8* was detected (i.e. PI 215589) belongs to the wild form of cucumber, *Cucumis sativus* var. *hardwickii*, whereas the other 30 accessions belong to the cultivated form of cucumber, *C. sativus* var. *sativus*.

# Inoculation with *P. xanthii* induced transcription of *CsaMLO8* in hypocotyl tissue, but not in leaf tissue of susceptible cucumber

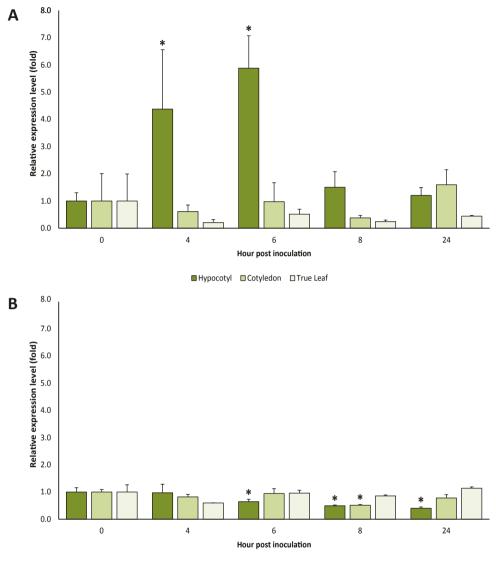
MLO genes involved in PM susceptibility are upregulated in several plant species several hours after inoculation (e.g [26, 35, 36] we sequenced additional mlo resistance alleles, two of which confer only partial resistance. Wild-type MLO dampens the cell wall-restricted hydrogen peroxide burst at points of attempted fungal penetration of the epidermal cell wall, and in subtending mesophyll cells, it suppresses a second oxidative burst and cell death. Although the Bgh-induced cell death in mlo plants is spatially and temporally separated from resistance, we show that the two processes are linked. Uninoculated mutant mlo plants exhibit spontaneous mesophyll cell death that appears to be part of accelerated leaf senescence. Mlo transcript abundance increases in response to Bgh, rice (Oryza sativa). To see whether the same holds true for CsaMLO8, we performed gRT-PCR experiments to quantify CsaMLO8 transcript abundances in hypocotyl, cotyledon and leaf tissues of PM susceptible and resistant cucumber plants, prior to and at 4, 6, 8 and 24 hours after PM inoculation (Fig. 5). For PM susceptible plants, we found that in hypocotyl tissue CsaMLO8 transcript abundance was significantly higher at 4 hpi (P = 0.037) and 6 hpi (P =0.004) compared to the transcript abundance prior to inoculation (0 hpi). The significant difference had disappeared 8 hpi (P = 0.212) and 24 hpi (P = 0.281). Contrastingly, CsaMLO8 transcript abundances in cotyledons and true leaves were not significantly altered at any of the evaluated time points after PM inoculation (P > 0.05) (Fig. 5A). For PM resistant plants, we found that CsaMLO8 transcript abundance was not significantly higher in any tissue at any time point after inoculation compared to the transcript abundance prior to inoculation (P > 0.05). In hypocotyl tissue, transcript abundance was significantly lower at 6 hpi (P = 0.046), 8 hpi (P = 0.006) and 24 hpi (P = 0.009) compared to the transcript abundance prior to inoculation (0 hpi). In cotyledon tissue, transcript abundance was significantly lower at 8 hpi (P = 0.002) compared to the transcript abundance prior to inoculation (Fig. 5B).

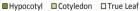
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#### TABLE 1. 31 out of 115 resequenced cucumber accessions have the TE-allele of *CsaMLO8*.

Total reads of 115 recently resequenced cucumber accessions [8] were assayed *in silico* for the presence of reads indicating the presence of either the allele of *CsaMLO8* characterised by the insertion of a TE, or the wild-type allele. The amount of reads indicating presence of either the TE-allele or the WT-allele of *CsaMLO8* is given. Database number, accession names and geographic groups of accessions were obtained from [8].

Accession number NCBI SRA	TE-allele reads	WT-allele reads	Putative genotype	PI or CGN number	Name Accession	Group
SRR543205	9	0	Homozygous	PI 215589	13598	Indian
SRR543216	17	0	Homozygous	V05A0674	Bei Jing Xiao Ci	East Asian
SRR543221	1	9	Heterozygous	V05A1333	Liao Tong Mi Ci	East Asian
SRR543223	19	0	Homozygous	V05A0920	He Cha Huang Gua	East Asian
SRR543224	19	0	Homozygous	V05A1115	Qian Qi Li Huang Gua	East Asian
SRR543225	1	7	Heterozygous	V05A0985	Ye San Bai	East Asian
SRR543226	23	0	Homozygous	V05A0428	Liao Yang Ye San	East Asian
SRR543228	1	0	Homozygous	-	228	East Asian
SRR543230	18	0	Homozygous	V05A0522	Huang Gua	East Asian
SRR543231	5	8	Heterozygous	V05A0552	Qing Dao Qiu Ye Er San	East Asian
SRR543240	1	13	Heterozygous	CGN19828	-	East Asian
SRR543242	22	0	Homozygous	V05A0034	Da Ci Huang Gua	East Asian
SRR543243	12	1	Heterozygous	V05A1427	Qiu Huang Gua	East Asian
SRR543244	1	7	Heterozygous	V05A0291	Leng Lu Huang Gua	East Asian
SRR543246	1	0	Homozygous	-	Bai Ye San	East Asian
SRR543251	4	0	Homozygous	-	2004348	East Asian
SRR543252	11	0	Homozygous	CGN20266	Hok	Eurasian
SRR543253	6	0	Homozygous	-	151G	Eurasian
SRR543257	5	0	Homozygous	CGN20512	752	Eurasian
SRR543258	9	0	Homozygous	CGN20515	Gy 3 (S4)	Eurasian
SRR543264	2	0	Homozygous	-	65G	Eurasian
SRR543265	11	0	Homozygous	-	G8	Eurasian
SRR543267	14	0	Homozygous	V05A0726	Jin Yan Er Hao	East Asian
SRR543269	10	3	Heterozygous	CGN19579	1972 B-2	Eurasian
SRR543271	15	0	Homozygous	CGN19844	2163	Eurasian
SRR543272	2	5	Heterozygous	PI 234517/ CGN20898	SC 50	Eurasian
SRR543274	11	0	Homozygous	CGN21627	Spartan Garden MSU-C7-63	Eurasian
SRR543275	4	0	Homozygous	-	Marketmore76	Eurasian
SRR543276	4	0	Homozygous	-	GY14	Eurasian
SRR543281	9	3	Heterozygous	PI 482412	TGR 580	Indian
SRR543293	6	8	Heterozygous	PI 605943	USM 307	Indian





# FIGURE 5. *CsaMLO8* transcription is induced after inoculation with *Podosphaera xanthii* in hypocotyl tissue, but not in cotyledon or true leaf tissue.

Susceptible (A) and resistant (B) cucumber seedlings were inoculated with a *P. xanthii* spore suspension. Prior to and 4, 6, 8 and 24 hours post inoculation, hypocotyl, cotyledon and true leaf tissue were harvested and immediately frozen in liquid nitrogen. Relative quantification of *CsaMLO8* expression was performed by qRT-PCR. Fold changes were normalised relative to *CsaMLO8* expression prior to inoculation. Bars represent the average fold change over three independent biological replicates. Error bars indicate standard errors of the mean. Asterisks indicate significant differences to the expression prior to inoculation (Student's T test, P < 0.05).

### Discussion

### CsaMLO8 is a functional susceptibility gene for PM in cucumber

Several studies characterised some, but not all, clade V MLO genes as being required for PM susceptibility in different dicotyledonous plant species [20-23, 25-27]. Here we have shown that heterologous expression of the cucumber gene CsaMLO8 in SImIo1 mutant tomato background restored PM susceptibility, providing evidence for the role of CsaMLO8 as a susceptibility gene for PM in cucumber (Fig. 2). As the role of clade V MLO genes in susceptibility to PM seems to be evolutionary conserved between divergent dicotyledonous plant families, e.g. Brassicaceae [20] the ability to cause disease in plants and animals has been gained and lost repeatedly during phylogenesis. In monocotyledonous barley, loss-of-function mlo alleles result in effective immunity against the Ascomycete Blumeria graminis f. sp. hordei, the causal agent of powdery mildew disease. However, mlo-based disease resistance has been considered a barley-specific phenomenon to date. Here, we demonstrate a conserved requirement for MLO proteins in powdery mildew pathogenesis in the dicotyledonous plant species Arabidopsis thaliana. Epistasis analysis showed that mlo resistance in A. thaliana does not involve the signaling molecules ethylene, jasmonic acid or salicylic acid, but requires a syntaxin, glycosyl hydrolase and ABC transporter. These findings imply that a common host cell entry mechanism of powdery mildew fungi evolved once and at least 200 million years ago, suggesting that within the Erysiphales (powdery mildews, Solanaceae [21, 23, 25], Fabaceae [22] tomato, Vitaceae [26], Rosaceae [27, 36] and now also Cucurbitaceae, it is probable that in other economically important species belonging to the family Cucurbitaceae, such as melon (Cucumis melo) and pumpkin (Cucurbita pepo) clade V MLO genes will also play a role in PM susceptibility. Indeed, in a patent application a functional complementation of Arabidopsis Atmlo2, Atmlo2,6 and Atmlo2,6,12 mutants by a melon MLO-like gene was claimed to partially restore PM susceptibility, based on the percentage of diseased leaf area in 4 to 9 primary transformants [37]. Alignment of this melon MLO gene with the three Clade V genes of cucumber revealed that the gene from melon is most similar to CsaMLO8, and less alike to the two other Clade V genes (i.e. CsaMLO1 and CsaMLO11) [28]. This is consistent with our finding that CsaMLO8 is a S-gene for PM. In tomato we observe that complementation of SIMLO1 loss-of-function mutants with CsaMLO8 restores PM susceptibility, with individual transformants with higher CsaMLO8 expression generally being more susceptible to PM than transformants with lower CsaMLO8 expression (Fig. 2). It seems possible that in the case of complementation of Arabidopsis mutants by the melon MLO gene there is also a quantitative effect due to different levels of melon MLO expression in individual transformants, leading to the conclusion that the melon MLO gene only partially restores susceptibility whereas it is actually due to the fact that transgene expression was not high enough to fully complement the loss of AtMLO function.

# Transposon insertion in *CsaMLO8* leads to aberrant splicing and therefore to loss of the *S*-gene function

By cloning *CsaMLO8* from cDNA of a PM resistant cucumber genotype that is homozygous for the hypocotyl resistance QTL, we found evidence for aberrant splicing of CsaMLO8 in this genotype, leading to products with deletions of respectively 72 and 174 bp in exon 11, compared to the WT gene. We showed that these deletions are predicted to lead to loss of 24 respectively 58 amino acid residues in the third cytoplasmic loop of the CsaMLO8 protein, in a highly conserved region between clade V MLO proteins from different species (Fig. 1). As it was previously shown that cytoplasmic loop-loop interplay is required for MLO function [38], we anticipated that such rather big deletions in one of the cytoplasmic loops, if the protein should properly fold at all, would lead to lossof-function of the protein. Indeed, we showed here that expression of the  $\Delta 174$  variant of CsaMLO8 in SImlo mutant tomato background failed to restore PM susceptibility (Fig. 2). This makes cucumber, after barley [17], tomato [21] and pea [22], the fourth plant species in which a natural mutation in an *MLO* gene has been found to lead to resistance. Although we did not try to complement *SImIo* mutant tomato with the 72 bp deletion variant of CsaMLO8, and thus cannot rule out the possibility that it is (partially) functional as an S gene, we expect that the result will be similar to the 174 bp deletion variant, given the conservedness of the deleted region.

To determine the reason for the aberrant splicing of *CsaMLO8* in the resistant cucumber genotype, we set out to amplify and sequence the genomic region of *CsaMLO8* in which the deletions were detected. In this way, we discovered a 1,449 bp insertion in exon 11 of the gene compared to the reference genome. Sequence analysis of the insertion revealed the presence of 100% identical LTRs and TSDs, but no open reading frames or any similarity to known proteins or genes (Fig. 3), leading to the conclusion that the insertion is probably a Class I, Order LTR (retro)transposable element (TE), following the TE classification scheme proposed by Wicker et al. [31]. The fact that the LTRs are completely identical to one another is an indication that the TE is relatively recently inserted. The integration of a transposable element in a *MLO* gene, leading to aberrant splicing of transcripts and in that way to loss of gene function, is reminiscent of the findings in the pea *PsMLO1* gene, where in one of the alleles (found in PM resistant pea cultivar JI 2302) the integration of an *Ogre* LTR retrotransposon lead to aberrant splicing [22].

We analysed putative TEs with similar LTRs (Fig. 4), and found no functional ORFs in these TEs, confirming that we are dealing with a family of non-autonomous TEs. Additionally, a large amount of LTR singlets (i.e. LTR sequences without a partner) were detected, as only 88 out of the 169 detected LTRs could be assigned to a putative TE (Additional file 5). LTR singlets presumably originate from the unequal recombination between two LTRs of a single element [39], or from assembling errors of the reference genome. It is known

that plant genomes are to a great extent shaped by the integration of large amounts of transposable elements, with LTR retrotransposons being the most abundant among them (e.g. [40, 41]. The cucumber genome was shown to be no exception to this, with 24% of the genome consisting of transposable elements and LTR retrotransposons comprising 10.4% of the genome [7]. To our knowledge, the TE we found to be inserted in *CsaMLO8* is the first TE with a reported effect on a cucumber gene. It seems likely that more TEs with an effect on genes in cucumber will be found in the future.

### CsaMLO8 is upregulated upon P. xanthii inoculation in hypocotyl tissue only

Resistance to PM in cucumber has previously been reported to be tissue specific, with an important, recessively inherited gene providing full PM resistance in hypocotyl tissue and partial resistance in leafs [5]. Recently, PM resistance of cucumber was mapped in multiple tissues separately. The strongest QTL for hypocotyl resistance, *pm5.2* was mapped on chromosome 5, in a region containing *CsaMLO8* [29]. In this study, we showed that *CsaMLO8* was, in susceptible cucumber, transcriptionally upregulated in hypocotyl tissue at 4 and 6 hours post inoculation, but not in cotyledon or leaf samples (Fig. 5A). Apparently, the ability of the pathogen to upregulate *CsaMLO8* expression is specific for hypocotyl tissue. Therefore, we postulate that it is very well possible that PM resistance caused by a loss of function allele of *CsaMLO8* would also be specific for hypocotyl tissue.

Interestingly, *CsaMLO8* was not found to be transcriptionally upregulated in hypocotyl tissue (or any other tissue) in the resistant cucumber line (Fig. 5B). This is in sharp contrast with the findings in barley [35] where transcription of the *MLO* gene seemed to be even stronger induced upon PM inoculation in *mlo* loss-of-function mutants compared to wild type plants. In tomato it was found that transcription of the *SIMLO1* gene was slightly upregulated upon PM inoculation in *slmlo1* loss of function mutants, but to a far lesser extent than in wild type plants [21]. Although it remains a question why the pathogen is unable to upregulate *CsaMLO8* expression in our resistant cucumber line several explanations might be offered, e.g. lesser transcript stability of the mutant *CsaMLO8* or differences in the promotor region of the mutant allele of *CsaMLO8* or differences in other genes required for *CsaMLO8* expression compared to the susceptible cultivar.

Previously, RNA-seq experiments on cucumber leaf tissue revealed that of the thirteen *CsaMLO* genes only *CsaMLO1*, another clade V*MLO* gene, was transcriptionally upregulated after inoculation with *P. xanthii* [28]. This is consistent with our finding that *CsaMLO8* is not upregulated in leaf samples after PM inoculation (Fig. 5). It is possible that *CsaMLO1* and *CsaMLO8* are functionally redundant, but are specifically expressed in separate tissues (i.e. *CsaMLO1* specific in leaf tissue and *CsaMLO8* in hypocotyl tissue). To our knowledge there are no other examples of tissue specialization in *MLO*-like *S* genes of other species.

In Arabidopsis, which also has three clade V MLO genes, Atmlo2 mutants were found to be partially resistant, double mutants Atmlo2/Atmlo6 or Atmlo2/Atmlo12 were more resistant than Atmlo2 single mutants, and triple mutants Atmlo2/Atmlo6/Atmlo12 were completely resistant [20]the ability to cause disease in plants and animals has been gained and lost repeatedly during phylogenesis. In monocotyledonous barley, loss-of-function mlo alleles result in effective immunity against the Ascomycete Blumeria graminis f. sp. hordei, the causal agent of powdery mildew disease. However, mlo-based disease resistance has been considered a barley-specific phenomenon to date. Here, we demonstrate a conserved requirement for MLO proteins in powdery mildew pathogenesis in the dicotyledonous plant species Arabidopsis thaliana. Epistasis analysis showed that mlo resistance in A. thaliana does not involve the signaling molecules ethylene, jasmonic acid or salicylic acid, but requires a syntaxin, glycosyl hydrolase and ABC transporter. These findings imply that a common host cell entry mechanism of powdery mildew fungi evolved once and at least 200 million years ago, suggesting that within the Erysiphales (powdery mildews. It is not yet known by what mechanism *MLO* genes are transcriptionally upregulated upon PM infection, although it would seem intuitive to hypothesise that it is an active process caused by an effector of the fungus. Given the tissue specificity of MLO upregulation in cucumber, this might be an interesting model to investigate the mechanism of MLO upregulation by PM fungi.

# The transposon insertion allele of *CsaMLO8* occurs frequently in cucumber germplasm

Interestingly, during the preparation of this manuscript, another group reported the fine-mapping of a QTL for PM resistance on the long arm of chromosome 5, which they called *pm5.1*, to a region of 170 kb containing 25 predicted genes. The main candidate gene in this region was found to be a *MLO* like gene, which appears to be the same as *CsaMLO8* in our study. By cloning and sequencing of this gene from genomic DNA of their resistant parent, line S1003, as well as two additional unrelated resistant lines, S02 and S06, they found that they contained a 1449 bp insert in the 11<sup>th</sup> exon of the gene [42]. Sequence analysis indicates that the location and sequence of the insertion found in their study are completely identical to the LTR retrotransposon described in this study. These researchers did not report on cloning the coding sequence of *CsaMLO8* in their material, nor on complementation experiments.

Additionally, a patent was filed describing an allele of *CsKIP2*, a gene claimed to provide PM resistance, shown to harbour a 72 bp deletion in the coding sequence [43]. Although it is not shown in the patent, the occurrence of this allele is claimed to be caused by the integration of a transposon-like element in the 11<sup>th</sup> exon of the gene. Sequence analysis revealed that *CsKIP2* is in fact the same gene as *CsaMLO8*, and the 72 bp deletion allele

they describe is the same as the 72 bp deletion we found in our material. Interestingly the patent does not describe the 174 bp deletion which we found, but an *in silico* prediction showed that the 174 bp deletion variant would not be amplified by the primers they chose to amplify the partial *CsaMLO8* sequence. In the patent no functional proof is given that this allele of *CsaMLO8* indeed leads to resistance.

As several groups independently found the same allele of *CsaMLO8* in different, to our knowledge unrelated, resistant cucumber genotypes, we were interested to know how often this allele occurs in the global cucumber germplasm. Therefore, we performed an *in silico* screen on a collection of 115 recently resequenced cucumber accessions [8] for the presence and/or absence of the transposable element (TE) allele of *CsaMLO8*. We found evidence for the presence of the TE-allele, either homozygously or heterozygously, in at least 31 out of the 115 accessions (Table 1), indicating that this particular allele of *CsaMLO8* occurs quite often. For some accessions only a small number of reads indicating presence/ absence of the TE allele was found, potentially due to a low read coverage at this locus. It is therefore possible that in some accessions now identified as homozygous for either the TE-allele or the WT allele of *CsaMLO8*, reads indicative of the other allele were missed due to low read coverage, so there might be some heterozygous accessions misidentified as being homozygous for one of the alleles.

As we found that the TE allele of *CsaMLO8* leads to PM resistance, it might have been selected for by cucumber breeders, by selecting for the most resistant plants. Interestingly one of the accessions found to have the TE-allele of *CsaMLO8* was PI 215589, a wild accession of *C. sativus var. hardwickii* collected in India in 1954. This indicates that the TE-allele of *CsaMLO8* does occur in the wild, and might have been introgressed in cultivated cucumber from PI 215589 or a related *hardwickii* accession.

### Conclusions

In this study we provide evidence for a role of *CsaMLO8* as a *S* gene for powdery mildew (PM) susceptibility. We show that complementation by *CsaMLO8* overexpression in *Slmlo1* mutant tomato background restores PM susceptibility. We also show that a mutant allele of *CsaMLO8* cloned from resistant cucumber fails to restore PM susceptibility. As *CsaMLO8* is located in the region where a QTL for hypocotyl specific resistance was detected, we determined *CsaMLO8* expression in different tissues of PM inoculated plants, and found that *CsaMLO8* was only transcriptionally upregulated in hypocotyl tissue. On this basis we conclude that the mutant allele of *CsaMLO8* is causal to the observed hypocotyl resistance towards PM in cucumber.

# Methods

#### Plant materials and fungal strain

Two cucumber genotypes were used in this study: the PM susceptible cv. Sheila and an advanced breeding line, related to the resistant cv. Anaxo, homozygous for a recessively inherited QTL on chromosome 5 conferring hypocotyl resistance (*pm-h*).

Two tomato genotypes were used: PM susceptible cv. Moneymaker (MM), and a PM resistant breeding line *ol-2*, homozygous for a 19 bp deletion mutation in the coding sequence of *SIMLO1* [21].

Unless otherwise indicated, plants were grown under standard conditions in a closed greenhouse.

An isolate of *P. xanthii* (causing PM in cucumber) was obtained from infected cucumber plants in the greenhouse of a seeds company from The Netherlands and maintained on cv. Sheila in a greenhouse compartment at Wageningen University, The Netherlands. The species of the isolate was confirmed by sequencing of the ITS sequence from fungal DNA by primer pair 5'- CGTCAGAGAAGCCCCAACTC-3' (ITS *P. xanthii* Forward) and 5'-AGCCAAGAGATCCGTTGTTG-3' (ITS *P. xanthii* Reverse) (data not shown).

The Wageningen isolate of *Oidium neolycopersici* (tomato PM) was maintained on cv. MM as described [44].

#### Cloning and sequencing of CsaMLO8 CDS

Young leaves of cucumber cv. Sheila and the resistant breeding line were harvested and immediately frozen in liquid nitrogen. Total RNA was isolated by using the RNeasy Kit (Qiagen, Germany). Possible DNA contamination of RNA samples was removed by treatment with DNase I, Amp Grade (Invitrogen life technologies, U.S.A.). cDNA was synthesised using 2 µg of RNA samples with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, U.S.A.).

For amplification of *CsaMLO8* coding sequences, cDNA was amplified with primers 5'- caccCTGCCTCTCCACATGCATAA-3' (Full length *CsaMLO8* Forward) and 5'-GCGCCCTGTACATGAAGAAC-3' (Full length *CsaMLO8* Reverse). As template 50 ng cDNA was used in 50 µl reactions using 1 u *PfuUltra* II Fusion HS DNA polymerase (Agilent Technologies, U.S.A.), 1x reaction buffer, 1mM dNTP and 200 nM of each primer. Cycling conditions were: 1 min. initial denaturation at 95°C, followed by 40 cycles of 20 sec. denaturation at 95°C, 20 sec. annealing at 60°C and 2 min. extension at 72°C. Reactions were finished by 3 min. incubation at 72°C. PCR products were separated by gel electrophoresis in ethidium bromide stained agarose gels. Bands were cut out and purified using QIAquick Gel

Extraction Kit (Qiagen, Germany). Purified products were cloned into Gateway-compatible vector pENTR D-TOPO (Invitrogen life technologies, U.S.A.) and transformed to chemically competent *Escherichia coli* strain One Shot TOP10. Presence of the right fragment was assessed by colony PCR using primers and conditions as above. Plasmids were recovered using the Qiaprep spin miniprep kit (Qiagen, Germany). Sequencing reactions were performed in triplicates using pUC/M13 forward and reverse sequencing primers (GATC Biotech, Germany).

#### Complementation of tomato ol-2 mutant with CsaMLO8 WT and CsaMLO8Δ174

Entry plasmids pENTR:*CsaMLO8* WT and pENTR:*CsaMLO8*Δ174, obtained as described above, were transferred by Gateway LR cloning into binary vector pK7WG2, which harbours the constitutively active 35S Cauliflower Mosaic Virus promotor and the *nptll* marker gene for kanamycin resistance [45]. Recombinant plasmids were transformed to chemically competent *E. coli* strain dh5α. Positive recombinant bacterial colonies were screened by colony PCR using *CsaMLO8* specific primers as described above, and sequenced. Recombinant plasmids were recovered using the Qiaprep spin miniprep kit (Qiagen, Germany). pK7WG2:*CsaMLO8* WT and pK7WG2:*CsaMLO8*Δ174 binary vectors were transformed to electrocompetent cells of *Agrobacterium tumefaciens* strain AGL1-virG by electroporation [46].

Cotyledon explants of *ol-2* mutant tomato seedlings were transformed as previously described [25]. Obtained tomato transformants were assessed for presence of *CsaMLO8*, the *nptll* marker gene and the 35S CaMV promotor sequence by PCR with primers 5'- caccCTGCCTCTCCACATGCATAA-3' (Full length *CsaMLO8* forward) and 5'-GCGCCCTGTACATGAAGAAC-3' (Full length *CsaMLO8* reverse), 5'-GAAGGGACTGGCTGCTATTG-3' (*nptll forward*) and 5'-AATATCACGGGTAGCCAACG-3' (*nptll reverse*), and 5'-TACAAAGGCGGCAACAAACG-3' (35S forward) and 5'-AGCAAGCCTTGAATCGTCCA-3' (35S reverse), with conditions as described above.

For each of the two transformations with a different construct, ten independent transgenic plants were selected, and were assessed for *CsaMLO8* expression by qRT-PCR using primer pair sequences specific for *CsaMLO8* 5'-GCGACGGCATTGAAGAACTG-3' (Forward) and 5'-AGGAGACATGCCGTGAGTTG-3' (Reverse). As housekeeping gene for normalization of *CsaMLO8* expression in tomato, *SIEF-a* was used, with primer pair 5'-ATTGGAAACGGATATGCCCT-3' (*SIEF-a* forward) and 5'-TCCTTACCTGAACGCCTGTCA-3' (*SIEF-a* reverse). qRT-PCR was performed using the CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A.). Each 10 µl reaction contained 300 nM of each primer, 1 µl (50ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were an initial denaturation step of 95°C for 3 min., followed by 40 cycles of 10 sec. denaturation at 95°C and 30 sec. annealing and extension at 60°C, finished by a melt cycle of 0.5°C increment per 10 sec. from 65°C to 95°C.

# Evaluation of PM resistance of *ol-2* tomato, over expressing *CsaMLO8* WT or *CsaMLO8* $\Delta$ 174

Cuttings originating from ten individual transgenic plants per construct (two cuttings per plant) were inoculated with O. neolycopersici. Cuttings of an empty vector (EV) transformed ol-2 plant and the susceptible cultivar Moneymaker (MM) were used as controls. A spore suspension was prepared by washing heavily infected leaves of cv. MM with water, and adjusting the spore concentration to 8 x 10<sup>4</sup> conidiospores/ml. The spore suspension was evenly sprayed on the cuttings. Sixteen days after inoculation the disease severity was assessed by eye, and scored as either susceptible (sporulating powdery mildew colonies visible on leaves) or resistant (no powdery mildew symptoms at all). Additionally, leaf samples were taken for quantification of O. neolycopersici biomass. Infected leaves (the 2nd or 3rd leaf) were sampled for each cutting. Total plant and fungal DNA was extracted using the DNeasy Plant Kit (Qiagen, Germany). Isolated DNA was used for gPCR with primer pair 5'-CGCCAAAGACCTAACCAAAA-3' (Oidium ITS forward) and 5'-AGCCAAGAGATCCGTTGTTG-3' (Oidium ITS reverse), specific for the internal transcribed spacer (ITS) of O. neolycopersici ribosomal DNA, to quantify O. neolycopersici biomass, and with SIEF- $\alpha$  primers as described above for normalization. gPCR was performed using the CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A.). Each 10 µl reaction contained 300 nM of each primer, 2 μl (20ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were identical to those described above for quantification of *CsaMLO8* expression in transformed tomato.

#### Amplification, sequencing and characterization of CsaMLO8-insertion

DNA was isolated from young leaves of cucumber cv. Sheila and the resistant breeding line, which were immediately frozen in liquid nitrogen after harvesting, using the DNeasy Plant Kit (Qiagen, Germany). DNA was amplified with primers 5'-AGCATTTTGCCATCCATACTTCA-3' (*CsaMLO8* insertion region Forward) and 5'-CTGCAAGCACAGGATGAATGTC-3' (*CsaMLO8* insertion region Reverse). As template 30 ng DNA was used in 25 µl reactions using 1.25 u DreamTaq DNA polymerase (Thermo Scientific, U.S.A.), 1x DreamTaq buffer, 0.8 mM dNTP and 200 nM of each primer. Cycling conditions were: 3 min. initial denaturation at 95°C, followed by 35 cycles of 30 sec. denaturation at 95°C, 30 sec. annealing at 57°C and 2 min. extension at 72°C. Reactions were finished by 5 min. incubation at 72°C. PCR products were visualised by staining with GelRed and electrophoresis on agarose gels. PCR products were purified using Qiaquick PCR purification kit (Qiagen, Germany). Sequencing reactions were performed in duplo, using primers 5'-AGCATTTTGCCATCCATACTTCA-3' (*CsaMLO8* insertion region Forward), 5'-ACGAAGAGCGAAACGAAGAA-3' (*CsaMLO8* insertion sequencing Reverse) and 5'-CTGCAAGCACAGGATGAATGTC-3' (*CsaMLO8* insertion sequencing Reverse) and 5'-CTGCAAGCACAGGATGAATGTC-3' (*CsaMLO8* insertion region Reverse)

(GATC Biotech, Germany). Obtained sequences were aligned using CLC Genomics Workbench 7.5 software. The consensus sequence for the amplified region was extracted from the alignment. This consensus sequence was aligned to the genomic reference sequence of *CsaMLO8* to determine the exact location and sequence of the insertion.

A dot plot was constructed for the sequence of the insertion, using CLC Genomics Workbench 7.5 standard settings. The first and last 200 bp of the insertion sequence were extracted and aligned to each other to identify the length and sequence of the LTRs. The sequence of the insertion was scanned for open reading frames using CLC Genomics Workbench 7.5 standard settings, which gave no results.

#### In silico mining of the cucumber reference genome for homologous TEs

The previously determined LTR sequence of the CsaMLO8-TE was used as query to perform a BLASTn search in the genome of the cucumber reference genome (Chinese long inbred line '9930', v2 [7] to identify putative homologous LTRs. The resulting output was stored as a tabular file. A python script described by Wolters et al. (2014) was used to search for LTR matches within 20 kb from each other [32]. Sequences with a length smaller than 20 kb flanked by two LTRs were considered as putative homologous TEs, and were extracted from the genome using the BEDtools suite [47]. The list of putative TEs was manually curated to remove sequences with two LTRs in opposite directions (two instances) and sequences with large (>100 bp) gaps (25 instances). In three instances, putative TEs were found to be nested (i.e. three LTRs were found to be within 20 kb of each other), in which cases the smaller putative TEs were discarded in favour of the bigger, nested model. Putative TEs were aligned to one another and to the CsaMLO8-TE using CLC Genomics Workbench 7.5 software, to determine sequence identity compared to the CsaMLO8-TE. Putative TEs were screened for open reading frames using CLC Genomics Workbench 7.5 standard settings. Putative TEs were used as guery to perform tBLASTx searches to the REPbase database [48].

#### In silico screening of resequenced lines for presence of CsaMLO8-TE allele

Reads of the resequencing project of 115 cucumber accessions by Qi et al. [8] were downloaded from the NCBI short read archive, accession SRA056480. By a simple Bash script, total reads were screened for the presence of 30 bp sequences comprised of:

- 1) The last 15 bp of *CsaMLO8* before the TE insertion and the first 15 bp of the TE insertion, in forward (5'- GCTCCATGTTATTGTTGATTTTATGGA-3') or reverse (5'-TC-CATAAAATCAACAATAATAACATGGAGC-3') orientation;
- 2) The last 15 bp of the TE insertion and the first 15 bp of *CsaMLO8* after the TE insertion, in forward (5'-TATATTAATAATTATAACTCATATGGGATT-3') or reverse (5'-AATCCCATATGAGTTATAATTATTAATATA-3') orientation;

3) The 30 bp of *CsaMLO8* surrounding the TE insertion site, without TE sequence, in forward (5'- GCTCCATGTTATTATAACTCATATGGGATT-3') or reverse (5'-AATCCCATATGAGTTATAATAACATGGAGC-3') orientation.

The number of detected reads per accession with each of the six bait sequences was stored as a tabular file. The total number of reads indicating presence of the TE allele and the total number of reads indicating presence of the WT allele were summated, the genotype of the accessions was determined to be either homozygous TE-allele, homozygous WTallele or heterozygous.

#### CsaMLO8 expression analysis PM-inoculated cucumber

PM susceptible and resistant cucumbers were grown in a climate chamber at 20°C (day) and 16°C(night), with a 16h/8h day/night cycle, and a relative humidity of 90%. 18 days post seeding, plants were inoculated with a *P. xanthii* spore suspension by spray method, using inoculum that was obtained by washing heavily infected cucumber leaves with water. The inoculum was adjusted to a final concentration of  $1.0 \times 10^4$  conidia/ml. The spore suspension was evenly sprayed on leaves, cotyledons and hypocotyl of the seedlings. Prior to inoculation and at 4, 6, 8 and 24 hours post inoculation (hpi), from three individual plants per time point hypocotyl, cotyledon and (first) true leaf samples were harvested separately, and were immediately frozen in liquid nitrogen.

Total RNA was isolated using the MagMAX-96 Total RNA Isolation kit (Ambion, U.S.A.). cDNA was synthesised using 1 µg of RNA samples with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, U.S.A.). Before use in qRT-PCR, cDNA samples were diluted 10-fold.

Quantitative real-time PCR was performed using a CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A.). Primer pair sequences specific for CsaMLO8 5'-GCGACGGCATTGAAGAACTG-3' (Forward) and 5'-AGGAGACATGCCGTGAGTTG-3' (Reverse) were used to quantify CsaMLO8 expression. Primer pairs specific for cucumber housekeeping genes TIP41, CACS and EF- $\alpha$ , as described by Warzybok et al. [49] nitrogen is the most important nutritional factor limiting the yield of cultivated crops. Since nitrogen is essential for synthesis of nucleotides, amino acids and proteins, studies on gene expression in plants cultivated under different nitrogen availability require particularly careful selection of suitable reference genes which are not affected by nitrogen limitation. Therefore, the objective of this study was to select the most reliable reference genes for qPCR analysis of target cucumber genes under varying nitrogen source and availability. Among twelve candidate cucumber genes used in this study, five are highly homologous to the commonly used internal controls, whereas seven novel candidates were previously identified through the query of the cucumber genome. The expression of putative reference genes and the target CsNRT1.1 gene was analyzed in roots, stems and leaves of cucumbers grown under nitrogen deprivation, varying nitrate availability or different sources of nitrogen (glutamate, glutamine or NH3, were used for normalization of *CsaMLO8* expression. Each 10 µl reaction contained 300 nM of each primer, 1 µl (50ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were an initial denaturation step of 95°C for 3 min. followed by 40 cycles of 10 sec. denaturation at 95°C and 30 sec. annealing and extension at 60°C, finishing with a melt cycle of  $0.5^{\circ}$ C increment per 10 sec. from 65°C to 95°C.

Two technical replicates for each sample were tested. *CsaMLO8* expression of each sample was determined by the  $\Delta\Delta C_t$  method [50] provides the necessary accuracy and produces reliable as well as rapid quantification results. But accurate quantification of nucleic acids requires a reproducible methodology and an adequate mathematical model for data analysis. This study enters into the particular topics of the relative quantification in real-time RT-PCR of a target gene transcript in comparison to a reference gene transcript. Therefore, a new mathematical model is presented. The relative expression ratio is calculated only from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus a control. This model needs no calibration curve. Control levels were included in the model to standardise each reaction run with respect to RNA integrity, sample loading and inter-PCR variations. High accuracy and reproducibility (<2.5% variation, normalised by the geometric mean of the three housekeeping genes. Averages and standard errors of *CsaMLO8* transcript abundance were calculated over three biological replicates per tissue/time point combination, and statistical significance of differences in  $\Delta\Delta C_t$  value between time points 4, 6, 8 and 24 hpi and 0 hpi were determined, using Student's T-tests.

#### Relative quantification of CsaMLO8 transcript isoforms in resistant cucumber

cDNA samples of non-inoculated and inoculated (6 hpi) resistant cucumber tissues, obtained as described above, were used to quantify relative transcript abundance of the  $\Delta$ 174 and  $\Delta$ 72 splice isoforms. Quantitative real-time PCR was performed using a CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A.). Four primer pairs were designed to specifically amplify one of the two *CsaMLO8* splice isoforms: 5'-CTCCTTAAT-TAATGCATTTCAGC-3' (Forward) with 5'-CTTGTATGATAACCCCCATTGAG-3' (Reverse) or 5'-TTCATTGTTGCACATCTTGC-3' (Forward) with 5'-AAGCTGAAATGCATTAATTAAGG-3' (Reverse) for specific quantification of *CsaMLO8* $\Delta$ 174 and 5'-ATTCTATTGGGTGTTCCC-GTC-3' (Forward) with 5'-GAACGACGGGAACACCCAAT-3' (Reverse) for specific quantification of *CsaMLO8* $\Delta$ 174 and 5'-ATTCTATTGGGTGTTCCC-GTC-3' (Forward) with 5'-GAACGACGGGAACACCCAAT-3' (Reverse) for specific quantification of *CsaMLO8* $\Delta$ 174 and 5'-ATTCTTGTTGTCATTGTTG-CACATCTTGC-3' (Forward) with 5'-GAACGACGGGAACACCCAAT-3' (Reverse) for specific quantification of *CsaMLO8* $\Delta$ 174 and *S*'-ATTCTTGTTGTTG-CACATCTTGC-3' (Forward) with 5'-GAACGACGGGAACACCCAAT-3' (Reverse) for specific quantification of *CsaMLO8* $\Delta$ 174 and *S*'-ATTCTTGTTGTATTGTTG-CACATCTTGC-3' (Forward) with 5'-GAACGACGGGAACACCCAAT-3' (Reverse) for specific quantification of *CsaMLO8* $\Delta$ 72. Primer pairs specific for cucumber housekeeping genes *TIP41*, *CACS* and *EF-a*, as described by Warzybok et al. [49]nitrogen is the most important nutritional factor limiting the yield of cultivated crops. Since nitrogen is essential for synthesis of nucleotides, amino acids and proteins, studies on gene expression in plants cultivated under different nitrogen availability require particularly careful selection of suitable

reference genes which are not affected by nitrogen limitation. Therefore, the objective of this study was to select the most reliable reference genes for qPCR analysis of target cucumber genes under varying nitrogen source and availability. Among twelve candidate cucumber genes used in this study, five are highly homologous to the commonly used internal controls, whereas seven novel candidates were previously identified through the query of the cucumber genome. The expression of putative reference genes and the target CsNRT1.1 gene was analyzed in roots, stems and leaves of cucumbers grown under nitrogen deprivation, varying nitrate availability or different sources of nitrogen (glutamate, glutamine or NH3, were used for normalization of *CsaMLO8* expression. Each 10  $\mu$ I reaction contained 300 nM of each primer, 1  $\mu$ I (50ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were an initial denaturation step of 95°C for 3 min. followed by 40 cycles of 10 sec. denaturation at 95°C and 30 sec. annealing and extension at 60°C, finishing with a melt cycle of 0.5°C increment per 10 sec. from 65°C to 95°C.

Two technical replicates for each sample were tested. *CsaMLO8* expression of each sample was determined by the  $\Delta\Delta C_t$  method [50] provides the necessary accuracy and produces reliable as well as rapid quantification results. But accurate quantification of nucleic acids requires a reproducible methodology and an adequate mathematical model for data analysis. This study enters into the particular topics of the relative quantification in real-time RT-PCR of a target gene transcript in comparison to a reference gene transcript. Therefore, a new mathematical model is presented. The relative expression ratio is calculated only from the real-time PCR efficiencies and the crossing point deviation of an unknown sample versus a control. This model needs no calibration curve. Control levels were included in the model to standardise each reaction run with respect to RNA integrity, sample loading and inter-PCR variations. High accuracy and reproducibility (<2.5% variation, normalised by the geometric mean of the three housekeeping genes. Averages and standard errors of *CsaMLO8* splice isoform abundance were calculated over three biological replicates per tissue, per tissue the average of the relative abundances calculated.

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# **Additional files**

Due to the size, all the additional files are available online following the links indicated.

Additional file 1: https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-015-0635-x/ MediaObjects/12870\_2015\_635\_MOESM1\_ESM.pdf Format: PDF Full length alignment of *CsaMLO8* WT, *CsaMLO8Δ72* and *CsaMLO8Δ174* coding sequences

Additional file 2: https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-015-0635-x/ MediaObjects/12870\_2015\_635\_MOESM2\_ESM.pdf Format: PDF Multiple sequence alignment of MLO proteins encoded by clade V *MLO S*-genes from different species

Additional file 3: <a href="https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-015-0635-x/">https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-015-0635-x/</a>MediaObjects/128702015635MOESM3ESM.pdfFormat: PDFRelative quantification of CsaMLO8Δ174 and CsaMLO8Δ72 transcript abundances by qRT-PCR on cDNA samples

obtained from non-inoculated (**A**) or inoculated (**B**) cucumber tissue samples. Fold changes were normalised relative to  $CsaMLO8\Delta 174$  expression. Bars represent the average fold change over three independent biological replicates. Error bars indicate standard errors of the mean.

Additionalfile4:<a href="https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-015-0635-x/">https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-015-0635-x/</a>MediaObjects/128702015635MOESM4\_ESM.pdfFormat: PDFPhotographs of 20 independent *ol-2* tomato plants transformed with either *CsaMLO8* WT or *CsaMLO8Δ174* 

Additional file 5: http://bmcplantbiol.biomedcentral.com/articles/10.1186/s12870-015-0635-x Format: XLSX Complete overview of putative LTRs and putative TEs homologous to the TE identified in *CsaMLO8* 

Additional file 6: https://static-content.springer.com/esm/art%3A10.1186%2Fs12870-015-0635-x/ MediaObjects/12870\_2015\_635\_MOESM6\_ESM.pdf Format: PDF

Multiple sequence alignment of the TE identified in CsaMLO8 and putative homologous TEs

Additional file 7: <u>http://bmcplantbiol.biomedcentral.com/articles/10.1186/s12870-015-0635-x</u> Format: XLSX

Full table of 115 resequenced accessions. The amount of reads identified is given at the overlap between *CsaMLO8* and the start of the insertion in forward (TE start-F) and reverse (TE start-R) direction, at the overlap between the end of the insertion and *CsaMLO8* in forward (TE end-F) and reverse (TE end-R) direction, and at the site of the insertion with only *CsaMLO8* sequence in forward (WT-F) and reverse (WT-R) direction.

# Chapter 7

# Discovery and characterization of a novel tomato *mlo* mutant from an EMS mutagenized Micro-Tom population

Michela Appiano, Ageeth van Tuinen, Danny Schipper, Robin Huibers, Dongli Gao, Richard GF Visser, Anne-Marie Wolters, Yuling Bai

# Abstract

In tomato, there are at least three *SIMLO* genes contributing to the powdery mildew disease caused by *Oidium neolycopersici*, namely *SIMLO1*, *SIMLO5* and *SIMLO8*. Of the three homologs, the *SIMLO1* gene plays a major role since a natural mutant allele called *ol-2* can almost completely prevent fungal penetration by forming a papilla. The *ol-2* allele contains a 19-bp deletion in the coding sequence of the *SIMLO1* gene, resulting in a premature stop codon within the second cytoplasmic loop of the predicted protein.

In this study, we describe a mutant containing a novel allele (the *m200* allele) of the tomato *SIMLO1* gene, which was discovered from an EMS mutagenized Micro-Tom population. Compared to the sequence of the *SIMLO1* gene, the *m200* allele carries a point mutation at T65A. The SNP results in a premature stop codon located in the first transmembrane domain of the complete SIMLO1 protein. The length of the predicted protein is 21 amino acids, while the SIMLO1 full-length protein is 513 amino acids. We developed a High-Resolution Melting (HRM) marker to distinguish the mutated *m200* allele from the *SIMLO1* allele in backcross populations. Tomato plants homozygous for the *m200* allele showed resistance to *Oidium neolycopersici*. The allele was recessively inherited and conferred resistance that was associated with papilla formation at fungal penetration sites of plant epidermal cells.

# Introduction

Tomato (*Solanum lycopersicum* L.) is a model crop species of high economic value with interesting developmental features such as compound leaves, fleshy fruits, and sympodial shoot branching. The amount of information currently available for the domesticated tomato is abundant. Its genome (Sato et al., 2012), transcriptome (Tomato Functional Genomics Database, http://ted.bti.cornell.edu/) and metabolome (Moco et al., 2006) are available, as well as functional genomic tools, like the RNA interference (RNAi, Xiong et al., 2005, Schijlen et al., 2007, De Jong et al., 2009), transcription activator-like effector nucleases (TALENs, Lor et al., 2014), and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 (Brooks et al., 2014; Ron et al., 2014). Tomato's amenability to Agrobacterium transformation allows the use of all these technologies.

An important aspect of the domesticated tomato is its lack of genetic diversity because of years of selection for a limited set of traits, such as fruit shape and size (Rodríguez et al., 2011). However, given the upcoming challenges for agriculture regarding climate change and food safety, it has become a prominent issue to improve tomato also for resistance or tolerance to biotic and abiotic stresses (Kissoudis et al., 2016). One way to achieve this goal is to use the diversity present in wild relatives. S. lycopersicum can be crossed with its wild relatives for the introgression of resistance traits, although crossing barriers and carry-over of undesirable traits represent substantial limitations (Bai and Lindhout, 2007). It has been a general practice in tomato breeding to use wild relatives as a donor for introgression of valuable traits present in tomato varieties. For example, almost all tomato genes conferring resistance to pathogens are derived from wild species (Barham and Winstead, 1957; Parniske et al., 1999; Chunwongse et al., 2002; Seah et al., 2004; Lanfermeijer, et al., 2005; Ji et al., 2007; Menda et al., 2014). This so-called introgression breeding is nowadays facilitated by the available genome sequences of many wild species, such as S. habrochaites, S. pennellii, S. pimpinellifolium and S. arcanum (Sato et al., 2012; Bolger et al., 2014; Aflitos et al., 2014;).

Another way to increase genetic diversity is to introduce new mutations artificially. Chemical and physical mutagenesis are frequently used for this purpose in most of the economically important crop species (Shu, 2012). Of the chemical mutagens, ethyl methane sulfonate (EMS) is very popular. EMS selectively alkylates guanine bases, which, during DNA replication, are preferably coupled with a thymine over a cytosine residue, resulting in a random point mutation. Most of these mutations (70-99%) consist of substitutions from C to T or from G to A (abbreviated as C/G to T/A) (Greene et al., 2003; Till et al., 2007).

Five EMS tomato populations were developed during the last years (Table 1), two of which used the tomato cultivar Micro-Tom (MT). In contrast to most of the cultivated tomatoes, the MT is a miniature determinate tomato cultivar, released in 1989 by J. Scott

and B. Harbaugh for home gardening purposes and introduced in the genetic research by Avraham Levi's group in 1997 (Scott & Harbaugh, 1989; Meissner et al., 1997). Because of its small size (8-10 cm when grown in 14 cm diameter pots) and short life cycle (70-90 days from sowing to fruit-ripening), MT has been compared to Arabidopsis as a model system to carry out molecular research in tomato. Recently, the Japanese mutant database, TOMATOMA became available, together with MT's genome and a wholegenome resequencing analysis of EMS-MT mutants (Saito et al., 2011; Shirasawa et al., 2016). Altogether, these features make MT a suitable cultivar for large-scale mutagenesis.

Breeders aim at finding and introducing durable resistance in cultivated crops. A known way to achieve it consists of using impaired plant susceptibility genes (*S*-genes) (Pavan et al., 2010, van Schie & Takken, 2014). The *MLO* gene is the best-characterized example of *S*-genes in several crops. Functional MLO proteins are required by adapted powdery mildew (PM) pathogens to penetrate the cell wall and cause disease (Hückelhoven, 2005). The *MLO* gene encodes a plant transmembrane protein which typically spans across the plasma membrane seven times and ends in the cytoplasm with a C-terminus domain. It is highly conserved in plant species and can be tracked back to green algae (Kusch et al., 2016). However, the biochemical function of the MLO protein is still unknown. The only indication lays in the conserved calmodulin-binding site in the C-terminus domain which was shown to interact *in vitro* with calmodulin in a Ca<sup>2+</sup> dependent manner (Kim et al., 2002).

In 1976 a natural loss-of-function barley *mlo* allele was discovered, called *mlo-11*, and since then used in the field for spring barley cultivation (Jørgensen, 1971; Jørgensen, 1992). Thanks to its durable effectiveness, barley PM disease has greatly declined in importance in countries where barley is widely cultivated, like the United Kingdom (Brown, 2015).

Each plant species contains a certain number of *MLO* paralogs. Members of clade IV and V are described as susceptibility factors towards pathogens causing the PM disease (Feechan et al., 2008; Kusch et al., 2016; Panstruga, 2005). In tomato, the *SIMLO* gene family comprises 16 homologs, of which four belong to clade V, namely *SIMLO*1, *SIMLO*3, *SIMLO5* and *SIMLO8* (Zheng et al., 2016). A naturally mutated allele of the *SIMLO*1 gene, called *ol-2*, was described in the past years (Ciccarese et al., 1998; De Giovanni et al., 2004; Pavan et al., 2008; Bai et al., 2008). The *ol-2* variant contains a 19-bp deletion in the coding sequence resulting in a premature stop codon within the second cytoplasmic loop of the predicted protein. This mutation, inherited from *S. lycopersicum* var *cerasiforme*, when in homozygous state, mediates broad-spectrum resistance to *Oidium neolycopersici*. The *ol-2* conferred resistance is characterized by the formation of papillae beneath the fungal appressoria which can significantly reduce pathogen penetration (Bai et al., 2005). In the following years, transgenic RNAi lines were developed to silence simultaneously multiple clade V-*SIMLO* homologs (Bai et al., 2005; Zheng et al., 2016). One construct, in particular, was described to silence *SIMLO1*, *SIMLO5* and *SIMLO8*. When *ol-2* plants were compared to plants of the RNAi lines, the authors noticed a higher level of resistance associated with the latter. Because of these results, it was concluded that the three *SIMLO* genes contribute to the tomato susceptibility towards PM, with *SIMLO1* having the major role (Zheng et al., 2016).

In the present study, we describe the in-house development of an EMS mutant population of the tomato cultivar MT. With its development, we aimed at finding new sources of resistance to different pathogens, including PM. In this EMS population, we discovered a new mutant defective in the *SIMLO1* gene, called *m200*. Then, we performed a comparison of the novel allele with the *ol-2* mutant as well as the RNAi line in which three clade V *SIMLO* homologues are silenced. Results and implications are further presented and discussed in the context of *mlo* mutations occurring in other plant species.

Year	Title	Tomato cultivar used	Reference
1997	A new model system for tomato genetics	Micro-Tom	Meissner et al., 1997
2004	<i>In silico</i> screening of a saturated mutation library of tomato	M82	Menda et al., 2004
6	Mutant resources for the miniature tomato (Solanum lycopersicum L.) 'Micro-Tom'	Micro-Tom	Saito et al., 2009
2009	Implementation of two high through-put technologies in a novel application: detecting point mutations in large EMS mutated plant population	Tpaadasu	<i>Gady et al.,</i> 2009
2010	A new mutant genetic resource for tomato crop improvement by TILLING technology	Red Setter	Minoia et al., 2010

# Results

#### A novel EMS *mlo* mutant (*m200*) shows resistance to powdery mildew

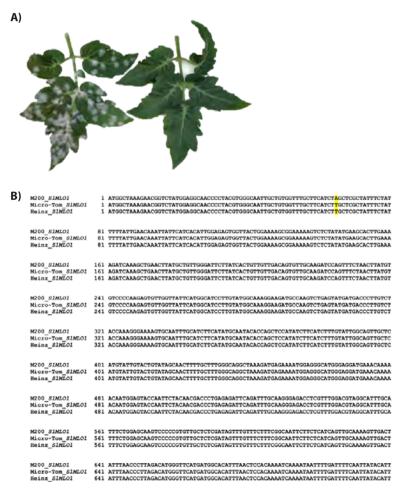
An EMS-mutagenized population of tomato cv MT was developed and phenotypically screened for resistance to the powdery mildew pathogen *O. neolycopersici* (*On*).

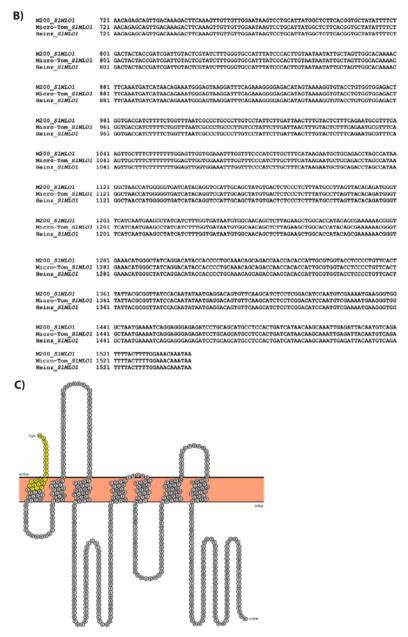
During the EMS treatment, the 1% v/v EMS concentration was mostly used to maximize the genomic variation with a minimum decrease in viability.

The  $M_1$  plants derived from the first two rounds of EMS treatment (about 1000 seeds per round) were inoculated with spores of the pathogen *On* by spray inoculation. In the first group of approximately 1000  $M_1$  plants, one plant (M200) showed no fungal sporulation, while all other plants were severely infected (Figure 1, panel A).

The  $M_1$  plants were allowed to self-pollinate and  $M_2$  seeds were collected. All the tested M200  $M_2$  plants were free of PM symptoms, and thus resistant. Except for the resistant phenotype, no other morphological differences were observed in M200  $M_1$  and  $M_2$  plants compared to wild-type MT (not subjected to the EMS treatment).

To find the causal mutation for the highly resistant phenotype of the M200 plant and its  $M_2$  progeny, we chose *SIMLO1* as the first candidate gene. We cloned the coding sequence (cds) of the *SIMLO1* gene. A SNP (T65A) was detected in the *SIMLO1* cds of the M200 plant compared to the sequence in MT and tomato cultivar Heinz (Figure 1, panel B). This point mutation results in a premature stop codon. The stop codon affects the triplet, which is translated into the amino acid leucine (L) in the full-length SIMLO1 protein of Heinz, and is located in the first transmembrane domain (Figure 1, panel C). The resulting truncated protein contains 21 aa instead of 513 aa. Using the program Protter, the truncated 21 aa protein does not contain transmembrane regions and is located in the intracellular space. This new *SIMLO1* allele was named *m200*.





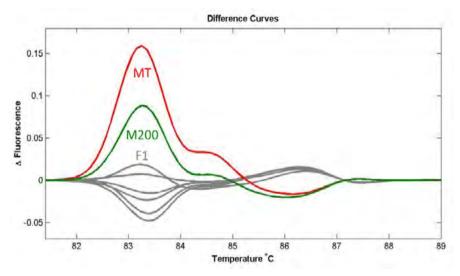
**FIGURE 1. A novel EMS** *mlo* **mutant** (*m200*) **shows resistance to powdery mildew. A**) Contrasting phenotypes of susceptible leaves of an  $M_1$  plant (on the left) and resistant leaves of the M200 plant (on the right) after *Oidium neolycopersici* inoculation. **B**) Coding sequences (cds) alignment of the *SIMLO1* gene in M200, Micro-Tom (MT), and tomato cv. Heinz. Highlighted in yellow is the base change T $\rightarrow$ A responsible for the premature stop codon in M200 plant. C) Schematic representation of the SIMLO1 protein of the cv. Heinz. The predicted *m200* protein is indicated in yellow, while the region that is absent in *m200* is shadowed in grey.

#### The resistance associated with the novel m200 allele is recessively inherited

Aiming at observing the inheritance of the m200 allele, the M200 M<sub>1</sub> plant was backcrossed to MT and crossed to the tomato cv. Moneymaker (MM). Initially, three BC<sub>1</sub> families derived from different fruits of the cross between M200 and MT were tested with On. All the BC<sub>1</sub> plants showed clear fungal sporulation, and were as susceptible as the controls, MM and MT (Supplementary Table 1).

A <u>High Resolution Melting</u> (HRM) marker was developed which could clearly distinguish the *SIMLO1* allele carried by the wild-type MT from the mutated m200 allele (Figure 2). All BC, plants were heterozygous for the m200 allele (Supplementary Table 1).

Five BC<sub>1</sub>S<sub>1</sub> families were produced and used in another disease test. The segregation ratio observed within the progenies of each backcross fitted the 3:1 (susceptible : resistant) hypothesis (Supplementary Table 2 and 3). All BC<sub>1</sub>S<sub>1</sub> resistant plants were homozygous for the *m200* allele, and all susceptible plants were either homozygous or heterozygous for the MT/MM allele (Figure 2). Overall, these results confirm that resistance to powdery mildew observed in plants carrying the *m200* allele exhibits a recessive inheritance.



**FIGURE 2. High Resolution Melting (HRM) profiles of two parental lines and their BC, population.** Red plot derived from the Micro-Tom plant -MT- (homozygous for the wild-type *SIMLO1* gene), green plot from the M200 plant (homozygous for the mutated allele *m200*) and grey plots from the six individuals of the BC, population (heterozygous) obtained after crossing M200 with Micro-Tom.

#### Papilla formation is associated with resistance in the m200 mutant

An experiment was conducted to 1) study the histological mechanism of the *m200* resistant mutant and 2) compare the level of resistance conferred by the *m200* mutant in MM background with other genotypes. The other genotypes included in this experiment

are the *ol-2* mutant in two different genetic backgrounds (MM and Super Marmande –SM) and the RNAi::*SIMLO1* line in which *SIMLO1*, *SIMLO5* and *SIMLO8* are silenced.

Symptoms were visually monitored at 10, 15 and 18 days post inoculation (dpi). The control MM plants started showing PM symptoms at 10 dpi and were heavily infected at 18 dpi (Figure 3, panel A). In contrast, no fungal sporulation was observed on all plants of the *m200* mutant, the *ol-2* mutant (in MM background) and the RNAi::*SIMLO1* line, at all time-points (Figure 3, panel B, C, D and F). For the *ol-2* mutant in SM background, no fungal sporulation was observed on the 3<sup>rd</sup> and 4<sup>th</sup> leaves at all time points (Figure 3, panel E). Occasionally, weak mycelium growth could be seen on the 1<sup>st</sup> and 2<sup>nd</sup> true leaves of the *ol-2* mutant in SM background, at 15 and 18 dpi.

At 72 hours post-inoculation (hpi) on infected MM leaves, 91% of the infection units (IU, a germinated spore) developed colonies having 1 to 6 secondary hyphae (Table 2 and Figure 3, panel A).

At the same time point in the  $m200\_BC_1S_3$ -1 family about 10% of the 97 IUs succeed in forming colonies with 1 to 4 secondary hyphae. Seventy-six of the 97 IU observed differentiated an appressorium, while 21 IUs formed only a germination tube. Of the IU that formed an appressorium, 34 were stopped by papilla formation at the fungal penetration sites and 10 IUs further developed a haustorium and formed colonies (Table 2 and Figure 3, panel B).

Of the 101 IU recorded on leaf samples of the family  $m200_BC_1S_3$ -2, all formed an appressorium. In addition, 33 IU of the 101 were stopped by papilla formation and 68 IU did not develop further than the appressorium (Table 2 and Figure 3, panel C). None of the IUs formed a haustorium.

On samples of plants carrying the *ol-2* allele in MM background, all the 90 observed IUs formed an appressorium. Of these 90 IU, 55 were stopped by a papilla, 4 developed colonies with haustorium and 2 to 4 secondary hyphae, and 31 did not develop further than the appressorium where papilla was not visible (Table 2 and Figure 3, panel D).

On samples of plants carrying the *ol-2* allele in SM background, 100 IU were counted and all produced an appressorium. Of the 100 IUs, 51 were stopped by a papilla, 11 developed into colonies where 2 to 5 secondary hyphae were visible and 38 formed only the appressorium without any visible papilla (Table 2 and Figure 3, panel E).

When comparing the IU stopped by papillae on plants carrying the *m200* allele with those carrying the *ol-2* allele (either in MM or SM), we noticed that the papillae frequency (% papilla per appresorium in Table 2) was significantly lower (Student t test, p = 0.043) in the *m200* than in the *ol-2* plants.

On samples of transgenic RNAi::*SIMLO1* plants, all the 109 IU observed formed an appressorium, of which 78 were blocked by a papilla (Figure 3, panel F), which represents

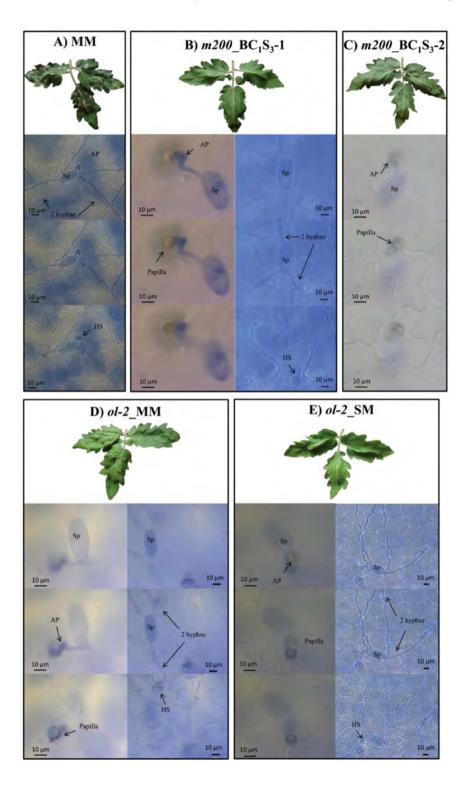
a significant increase compared to the numbers recorded on both *ol-2* (Student t test, p = 0.016) and *m200* plants (Student t test, p = 0.002) (Table 2). Six IU were able to penetrate the cell wall, producing a haustorium and differentiating 1 to 3 secondary hyphae, and 25 IU did not developed further than the appressorium.

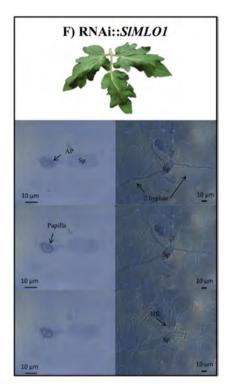
#### TABLE 2. O. neolycopersici development 72 h after the artificial inoculation.

The infection units (IU = spore producing a germination tube) were counted on each genotype along with any fungal structure and plant response.

		Numb	er of fungal stru observed	%papilla/AP	%HS/AP	
	IU	AP	papilla			
<i>m200_</i> BC <sub>1</sub> S <sub>3</sub> -1	97	76	34	10	44.74	13.2
<i>m200</i> _BC <sub>1</sub> S <sub>3</sub> -2	101	101	33	0	32.7	0
<i>ol-2</i> _MM	90	90	55	4	61.1	4.4
ol-2_SM	100	100	51	11	51	11
RNAi::SIMLO1	109	109	78	6	71.6	7.3
ММ	102	101	1	92	0.99	91.1

AP, appressorium; HS, haustorium





#### FIGURE 3. Phenotypic evaluation of the powdery mildew symptoms and development of the infection units (IU) of *Oidium neolycopersici* on six different genotypes.

In each panel photos are taken from **A**) Moneymaker (MM), **B**) and **C**) plants carrying the *m200* allele, **D**) and **E**) plants carrying the *ol-2* allele in MM and Super Marmande (SM), respectively, and in panel **F**) from a plant carrying the RNAi::*SIMLO1* construct.

On the top part of each panel, photos of leaves are depicted collected 18 days after the pathogen inoculation. Below that, photos of two IU/genotype are shown, except for the MM and BC<sub>1</sub>S<sub>3</sub>-2 carrying the *m200* allele where only one IU is shown. Each photo is taken with different focus to observe all the fungal structures, from the most superficial to the deepest ones.

Sp = spore, AP = appressorium; HS = haustorium; 2 hyphae = secondary hyphae.

#### Discussion

Powdery mildew disease can be a problem in greenhouses and field tomato cultivations. The humidity that forms at the leaf surface when cold nights change to warm days or when plants are grown in crowded locations without sufficient air circulation is enough to ignite an infection (Horst, 2013). The availability of resistant cultivars is, therefore, essential to control this disease in a sustainable way. The resistance can be achieved in several ways, each of them with pros and cons. One of the ways consists of inducing mutations artificially with chemical mutagens.

In this work we describe the set-up of an EMS mutant population of the tomato cv. MT with which we aimed at finding new sources of resistance to various diseases. Here we focused, in particular, on finding sources of resistance to the PM disease caused by *O. neolycoperisici*.

#### Is the m200 mutation a real product of the EMS mutagenesis?

A PM disease test was performed on the M<sub>1</sub> plants initially obtained with the intention of finding dominant mutations. We spotted the M200 mutant in the first disease test, and the sequence analysis showed that the resistance was due to a nonsense mutation in the coding region of the *SIMLO1* gene (Figure 1, panel B). This mutation results in a stop codon at

T65 (Figure 1, panel C). This location differs from the stop codon identified in the *ol-2* allele, located in the second intracellular loop.

It is unexpected that a recessive mutation occurred homozygously in an  $M_1$  plant since the probability of having a mutation on both alleles has been shown to be extremely low (Oladosu et al., 2016).

In order to verify whether any natural impaired *SIMLO1* allele is already present in MT, we blasted the full-length nucleotide sequence of the Heinz *SIMLO1* gene to the MT database MiBASE (http://www.kazusa.or.jp/jsol/microtom/indexe.html). Two transcripts with 100% identity were found, the LEFL1037DE09 and the LEFL1063CA10, both shorter than the full-length *SIMLO1* (623 bp and 610 bp, respectively), indicating that the transcript of MT *SIMLO1* is not complete. However, we could further clone the full-length sequence by PCR amplification of mRNA derived from MT plants. A multi-alignment composed of the two partial EST sequences retrieved by blasting, the sequenced MT *SIMLO1* transcript and the known Heinz *SIMLO1* did not reveal any mutation (Supplementary Fig. 1). Thus, these findings indicate that the *SIMLO1* gene in MT does not differ from the one in other cultivated tomatoes, like Heinz and MM. This is to be expected since MT originated from two cultivated tomatoes (Scott and Harbaugh, 1989).

In addition, we searched for any predicted mutations of the *SIMLO1* gene among the 115 tomato accessions, recently sequenced, using the haploSmasher online server (http://www. plantbreeding.wur.nl/hs/) (Aflitos et al., 2014). The output of this analysis also revealed that there are no predicted natural mutations at the T65 where the *m200* SNP occurs.

It is also important to notice that the mutation detected is not typically produced by the EMS mutagen. The latter notoriously favors transitions, e.g. purine replaced by purine  $A \leftrightarrow G$ , and pyrimidine replaced by pyrimidine  $C \leftrightarrow T$  (indicated as  $G/C \rightarrow A/T$ , Sega, 1984; Griffiths et al., 1999). In Arabidopsis, almost all the EMS mutations so far described correspond to a G/C to A/T transitions (Greene et al., 2003). We have also investigated the available literature regarding EMS *mlo*-alleles and found 16 in wheat and 11 in barley (Table 3, Reinstädler et al., 2010; Acevedo-Garcia et al., 2016). Recently, two additional *mlo*-alleles have been detected in petunia EMS mutants (Jiang et al., 2016). In all cases, except three, the mutagenized treatment produced the expected base substitutions ( $G/C \rightarrow A/T$ ). In contrast, the barley mutants *mlo*-13, *mlo*-26 and *mlo*-30 are characterized by transversions (purine replaced by a pyrimidine, and vice-versa) T  $\leftrightarrow$  A, as observed in *m200*. In *mlo*-13 and *mlo*-26, the transversion caused two missense mutations, V30E and L270H respectively, which in both cases lead to the loss-of-function of the protein. In *mlo*-30, the mutation occurred in intron sequences which affected transcript splicing (Piffanelli et al., 2002) and resulted in one transcript containing an 18-nucleotide deletion of exon 12 and another containing the entire unspliced intron 11.

Therefore, although not common, the mutation observed in the M200 mutant is not an exception.

The fact that the mutation occurred homozygously in an  $M_1$  plant, can also lead to the hypothesis that it spontaneously occurred. Spontaneous mutations in Arabidopsis are known to take place at a rate of  $10^{-7}$  to  $10^{-8}$  bp/generation (Kovalchuk et al., 2000; Bashir et al., 2014). Anyway, it is reported that the large majority of spontaneous mutations are G/C-A/T transitions (Ossowski et al., 2009).

In tomato, the *ol-2* is a natural mutation originated from the wild accession LA-1230 of *S. lycopersicum var. cerasiforme* (cherry tomato) caused by a 19-bp deletion in corresponding of the 7<sup>th</sup> exon which determines a truncated protein (Ciccarese et al., 1998; Bai et al., 2008). The molecular reasons behind this deletion were not investigated, although it would be interesting to know what kind of mutation triggered the deletion and if it is also one of the most frequent spontaneous transitions.

Plant	Gene name	Allele	SNP		Effect on	Defense	
species		name	WT	Mutant transcript/protein		Reference	
Petunia	PhMLO1	n.a.	G	А	S130L	Jiang et al., 2016	
Petunia	PHIVILOT	n.a.	С	Т	G176E	Jiang et al., 2016	
		mlo-5	А	G	M1I		
		mlo-6	G	А	aberrant splicing variants		
		mlo-7	G	А	G226D		
		mlo-8	Α	G	M1V		
		mlo-9	С	Т	R10W		
Barley	HvMlo1	mlo-13	т	Α	V30E	Reinstdler et al., 2010	
		mlo-16	G	А	aberrant splicing variants		
		mlo-17	С	Т	S31F		
		mlo-26	т	Α	L270H		
		mlo-27	G	А	G318E		
		mlo-30	Α	т	aberrant splicing variants		
	TaMLO	TaMLO-A1	С	Т	P325L		
		TaMLO-A1	С	Т	A354V		
		TaMLO-B1	G	А	G296E		
		TaMLO-B1	С	Т	T297I		
		TaMLO-B1	С	Т	R313W		
		TaMLO-B1	G	А	S315N		
		TaMLO-B1	G	А	G319R		
		TaMLO-B1	G	А	A320T	Acevedo-Garcia et al	
Wheat		TaMLO-B1	С	Т	T345M	2016	
		TaMLO-D1	G	А	V316T		
		TaMLO-D1	G	А	G319R		
		TaMLO-D1	G	А	A320T		
		TaMLO-D1	С	Т	P321S		
		TaMLO-D1	G	А	V323I		
		TaMLO-D1	С	Т	P335L		
		TaMLO-D1	C	Т	T345M		

#### TABLE 3. EMS *mlo* null alleles reported in literature.

Mutations corresponding to base transition are indicated by bold characters.

#### Is the level of *mlo*-based resistance influenced by the position of the mutation?

We initially assumed that the cause of the higher resistance of the m200 plant is the severe truncation of this allele because m200 plants are always free of any PM symptoms while ol-2 plants (at least in SM background) can occasionally show faint mycelium on 1<sup>st</sup> and 2<sup>nd</sup> leaves.

After reviewing the available literature on barley *mlo*-mutants, we found three interesting cases, namely *mlo-13*, *mlo-17* and *mlo-32* (Supplementary Fig. 2). The first two were obtained from cv Plena after EMS treatment; the last was obtained from cv Prudentia with sodium azaide (NaN<sub>3</sub>) (Büschges et al., 1997; Molina-Cano et al., 2003; Panstruga et al., 2005). All three mutants carry mutations leading to a stop codon in the first transmembrane of the HvMlo protein, which corresponds to the same domain where the *m200* mutation is found. No difference in level of resistance was observed between *mlo-13* or *mlo-17* and *mlo-32*. They were all indicated as complete resistant mutants.

Moreover, another barley mutant, the *mlo-43*, was found to carry a stop codon in the second intracellular domain, the same as the nonsense mutation identified in tomato *ol-2* mutant (Reinstädler et al., 2010). The *mlo-43* is a mutant of the cv Bonus and it was also described as completely resistant (Lundqvist et al., 1991).

A mutant of the same cultivar, *mlo-36*, was described to contain a nonsense mutation at W357, in the sixth transmembrane domain (Supplementary Fig. 2, Lundqvist et al., 1991; Reinstädler et al., 2010). Both mutants were only phenotypically scored, and considered highly resistant, with *mlo-36* even annotated as immune (Lundqvist et al., 1991).

We have not found other more recent evaluations of barley impaired alleles due to premature protein truncation. The reason is that it was shown that defective protein variants would probably not pass the quality test of the ERAD machinery (Endoplasmic Reticulum-Associated protein Degradation, Müller et al., 2005). The ER-localized quality control system monitors and validates proper folding and modification of proteins, among which the membrane proteins. Though it is currently largely unknown which signatures classify malformed membrane proteins, Muller et al. 2005 and Reinstadler et al. 2010 hypothesized that the second cytoplasmic loop and the transmembrane regions are the major quality determinant of the *HvMlo* protein variants. Premature truncations heavily affect protein folding, therefore, mutants containing amino acid substitutions were preferred to truncated *mlo* alleles in studies addressed at evaluating the biological activity of the *Mlo* variant.

If this holds true, the extremely truncated m200 protein, as well as the ol-2 variant, should be subjected to a dramatic reduction in accumulation. Thus, both variants should lead to a similar level of resistance, if compared in the same background.

A verification of this hypothesis in tomato would require more advanced crosses using as a recurrent parent the cv MM to observe the contribution to the resistance of each impaired *mlo* alleles in the same background.

Alternatively, it is possible to exploit the results described in Appiano et al., 2015 to select predicted amino acid positions that, being under negative selection, can represent targets of protein loss-of-function. Using one of the recent genome editing methods, mutants with predicted protein truncations at different level of the SIMLO1 protein could be systematically obtained. In this way, we would have a larger panel of MM mutants to understand the influence of several degrees of truncation on the resistance level.

# **Materials and methods**

#### **Development of the Micro-Tom EMS populations**

Approximately 1000 seeds ( $M_0$ ) of the tomato cultivar Micro-Tom (MT), obtained from the Beekenkamp Plants B.V. company (The Netherlands), were pre-soaked in distilled water for 8 hours and treated overnight with two concentrations of an EMS (ethyl methane sulfonate) solution, 0.5% (v/v) and 1% (v/v), respectively.

In total, five rounds of EMS treatment were performed. In the first round, two different concentrations (0.5% v/v and 1% v/v) of EMS were tested. Several studies showed that the 1% concentration yielded almost 2-fold more mutations per genome than other concentrations, like 0.5% or 0.75%, without affecting too much the rate of viability (Minoia et al., 2010; Saito et al., 2011). Therefore, in the following rounds only the 1% v/v dilution was used.

The obtained  $M_1$  seeds were then thoroughly washed with distilled water, dried, sown in the greenhouse and grown under standard agricultural practice. Three-weeks-old seedlings were transplanted individually to 14 cm pots and grown until 5 to 10 fruits per plant could be harvested. The  $M_2$  seeds, collected from these fruits, were surface sterilized in 2% (v/v) of HCl, following by air drying.

#### Disease tests set-up and evaluation of the symptoms

The approximately 1000 M<sub>1</sub> plants of the first round of EMS treatment were inoculated by spraying a fresh suspension of *Oidium neolycopersici* (*On*-Wageningen isolate) spores, after being transplanted into individual pots.

The suspension was made by rinsing heavily sporulating leaves of the cultivar MM with tap water and adjusting this suspension to a concentration of  $2 \times 10^4$  spores per milliliter. The *On* isolate was maintained on the cv MM as previously described by (Bai et al., 2005).

Ten to fifteen days after the inoculation, the  $M_1$  plants were visually inspected. To each plant, a score was given based on a disease index (DI) varying from 0 to 3, where 0 indicates that no fungal sporulation is visible and three that fungal colonies cover most of the surface of the inoculated leaves, as in the cv. MM.

One  $M_1$  plant showing resistance to powdery mildew (M200) was crossed with Moneymaker (MM) and backcrossed to MT to obtain  $BC_1$  seeds which were harvested from each fruit and kept separately. Three  $BC_1$  deriving from the cross M200 x MT were tested with powdery mildew and all the plants of each family kept for self-pollination and seed production. Two of the three corresponding progenies ( $BC_1S_1$ ) were further tested with powdery mildew and selected for seed production if showing a resistant phenotype.

The BC<sub>1</sub> plants deriving from the cross M200 x MM, selected for the loss of the dwarf and determinate growth characteristics of cultivar Micro-Tom, were allowed to self-pollinate. Their progenies (BC<sub>1</sub>S<sub>1</sub>), tested with powdery mildew, were selected if, next to being MM-like in their appearance, were homozygous for the *m200* allele.

The disease test and the visual inspection of further generations were performed as for the M<sub>1</sub> plants.

# Cloning and sequencing of the *SIMLO1* coding sequence from the mutagenized resistant Micro-Tom plant M200

Leaves of the M200 plant and two MT plants (not subjected to the EMS treatment) were collected after the powdery mildew test and total RNA was isolated with the RNeasy<sup>®</sup> plant mini kit (Qiagen) according to manufacturer's instructions. The concentration of the total RNA was measured using the Nanodrop. Approximately 1 µg of RNA was treated with DNase (Invitrogen) to remove any DNA contamination. This treated RNA was used in a one-step PCR with the SuperScript<sup>®</sup> III (Invitrogen) and the specific primers for the *SIMLO1* gene used in Zheng et al., 2016 (sequences in supplementary Table 4). The amplified PCR products were run on a 1% agarose gel. The bands with the desired product size (1743 bp) were excised from the gel and the products recovered using the QIAquick gel extraction kit (Qiagen). The eluted PCR products were sequenced and the obtained sequences aligned with the known *SIMLO1* coding sequence (cds) of Heinz (Solyc04g049090) using the package MegAlign of the software DNASTAR<sup>\*</sup> Lasergene8. The predicted protein derived from the *SIMLO1* sequence cloned from the M200 plant was analyzed using the TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/) and the PROTTER web-tool to predicted sequence features and visualize the protein (Omasits et al., 2014).

#### Development of an HRM marker for detection of the mutation in the SIMLO1 gene

In order to follow the segregation of the SNP associated with the *m200* allele in BC<sub>1</sub> and BC<sub>1</sub>S<sub>1</sub> progenies of the backcrosses and crosses between the M200 plant x MT and M200 plant x MM, the DNA of each plant was isolated using 2% CTAB in a protocol adapted for a 96-well plate (Doyle, 1987). The quantity and integrity of genomic DNA were determined using the Nanodrop and running 1  $\mu$ l of the isolated DNA on an agarose gel (1%), respectively.

Primers amplifying a DNA fragment of 225 bp containing the mutation site were designed for a high-resolution melting assay (HRM). The sequences of these primers are reported in the Supplementary Table 4.

PCR amplifications were carried out in a 10  $\mu$ L reaction mixture containing 10 ng of genomic DNA, 2  $\mu$ L of 5X PCR buffer, 0.4  $\mu$ L of 5 mM dNTPs, 0.5 U Phire<sup>TM</sup> Hot Start II DNA Polymerase (ThermoFisher), 0.25  $\mu$ M of forward and reverse primer (10 mM each) and 1  $\mu$ L of LC Green<sup>Plus</sup> (Idaho technology inc., Salt Lake City, Utah, USA). The amplification included an initial denaturation at 98°C for 30 s, followed by 41 cycles of 98°C for 5 s, 60 °C for 5 s and 72 °C for 15 s, and finishing with a final elongation at 72 °C for 30 s.

The HRM genotyping was performed on a Light Scanner instrument (HR96 model, Idaho technology Inc., Salt Lake City, Utah, USA) with continuous melting curve acquisition (10 acquisitions per °C) during a 0.1 °C/s ramp from 40 to 95 °C.

Data were retrieved and analyzed using the Light Scanner software followed by manual curation of the obtained genotype calls.

#### Experimental set-up for histological study

In order to study the resistance mechanism of the *m200* allele, an experiment for microscopic analysis was set up.

Eight BC<sub>1</sub>S<sub>3</sub> plants carrying the *m200* allele derived from two backcrosses M200 x MM were chosen for this experiment. We also included three plants of two BC<sub>3</sub>S<sub>2</sub> lines derived from a cross between a resistant plant homozygous for the *ol-2* allele and MM (Ciccarese et al., 1998; Zheng et al., 2016). Moreover, we added three resistant F<sub>4</sub> plants also carrying the *ol-2* allele derived from the self-pollination of the cross between the original line LC-95 of *S. lycopersicum* var. cerasiforme and the cv Super Marmande (SM). For simplicity during the description of Figures and Tables, we refer to the first *ol-2* genotype as *ol-2\_MM* and the second as *ol-2\_SM*. Furthermore, three transgenic plants of a T<sub>2</sub> family carrying the RNAi construct able to silence *SIMLO1*, *SIMLO5*, and *SIMLO8* as described in Zheng et al., 2016 were selected. As susceptible control, three MM plants were included in this experiment. The transgenic plants carrying the RNAi construct were selected by standard

PCR performed on DNA isolated with the 2% CTAB method (described above) from all the germinated seedlings, using two primer pairs, one targeting the NPTII gene and the other the 35S promoter. Primer pair sequences are reported in Supplementary Table 4.

The powdery mildew disease assay was performed on four-weeks-old plants as described in the previous paragraph, but using a higher concentration of *On* spores equal to  $3 \times 10^5$ conidia / ml. From the sowing to the sampling, plants were grown in a climate-controlled compartment with 16 h light/ 21°C, 8 h dark/ 19°C and 60% - 70% relative humidity.

Four samples were collected 72 h post inoculation, bleached in a 1:3 (v/v) acetic acid/ ethanol solution, stained 48 h later by boiling in 0.005% trypan blue in lactophenol : ethanol (1:2 v/v) solution for 3-5 min and finally cleared in a nearly saturated aqueous solution of chloral hydrate (5:2 w/v). We defined an infection unit (IU) as a spore with a germination tube. About 100 IU per genotype were randomly counted at 65X magnification using a Zeiss Axiophot bright field microscope. For each IU, the presence of appressorium, haustorium, papilla, and the number of secondary hyphae was recorded. For some IU, photos were taken using the 100x magnification coupled with the differential interface contrast (DIC) technique at different focus to be able to observe all of the fungal structures eventually developed.

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# **Supplementary Tables**

**SUPPLEMENTARY TABLE 1.** Genotyping and phenotyping of three  $BC_1$  populations derived from the cross between the resistant M200 plant and Micro-Tom (M200 x MT). High resolution Melting (HRM) profiles correspond to the ones reported in Figure 2, panel A.

				HRM profiles	
M200 x MT	N susceptible plants	N resistant plants	Wild-type allele	heterozygous	m200 allele
BC <sub>1</sub> _4	12	0	0	12	0
BC <sub>1</sub> _5	3	0	0	3	0
BC <sub>1</sub> _6	4	0	0	4	0
total	19	0	0	19	0

N = number

**SUPPLEMENTARY TABLE 2**. Genotyping and phenotyping of four progenies ( $BC_1S_1$ ) derived from two each of the three crosses M200 x MT listed in Supplementary Table 1.

			Expected	3S:1R		HRM profiles	
M200 x MT	N susceptible plants	N resistant plants	X²	р	Wild-type allele	heterozygous	m200 allele
BC <sub>1</sub> S <sub>1</sub> _4-1	10	6			2	8	6
BC <sub>1</sub> S <sub>1</sub> _4-2	13	4			4	9	4
BC <sub>1</sub> S <sub>1</sub> _4-4	13	6			3	10	6
BC <sub>1</sub> S <sub>1</sub> _4-10	12	7	2.07	0.15	7	4	7
BC <sub>1</sub> S <sub>1</sub> _6-1	14	4			4	10	4
BC <sub>1</sub> S <sub>1</sub> _6-6	11	7			4	7	7
BC <sub>1</sub> S <sub>1</sub> _6-7	17	2			4	13	2
BC,S,_6-9	11	8	0.45	0.50	2	9	8

S = susceptible, R = resistant, N = number

**Supplementary Table 3.** Genotyping and phenotyping of the progenies  $(BC_1S_1)$  of three crosses between the resistant M200 plant and the tomato cv Moneymaker (M200 x MM).

			Expecte	d 3S:1R	н	RM marker profile	s
M200 x MM	N susceptible plants	N resistant plants	X²	р	Wild-type allele	heterozygous	m200 allele
BC <sub>1</sub> S <sub>1</sub> _1	25	14	2.47	0.12	9	16	14
BC <sub>1</sub> S <sub>1</sub> _2	27	11	0.32	0.57	11	16	11
BC <sub>1</sub> S <sub>1</sub> _3	25	14	2.47	0.12	5	19	14

S = susceptible, R = resistant

#### SUPPLEMENTARY TABLE 4. Primer pairs used in this study

Primer name	Fw primer (5'3')	Rev primer (5'3')
SIMLO1_full-length	TTGACATTTCCCCTTCTTCTTA	TACAAAATCATTGCCATTTGAA
HRM_marker	TGGCTAAAGCACGGTCTA	CTGGATCTTGCAACACTGTCA
35S_promoter	GCTCCTACAAATGCCATCA	GATAGTGGGATTGTGCGTCA
NPTII_marker	TCGGCTATGACTGGGCACAAC	AAGAAGGCGATAGAAGGCGA
SIEf1a	ACAGGCGTTCAGGTAAGGAA	GAGGGTATTCAGCAAAGGTCTC
On_ITS	CGCCAAAGACCTAACCAAAA	AGCCAAGAGATCCGTTGTTG

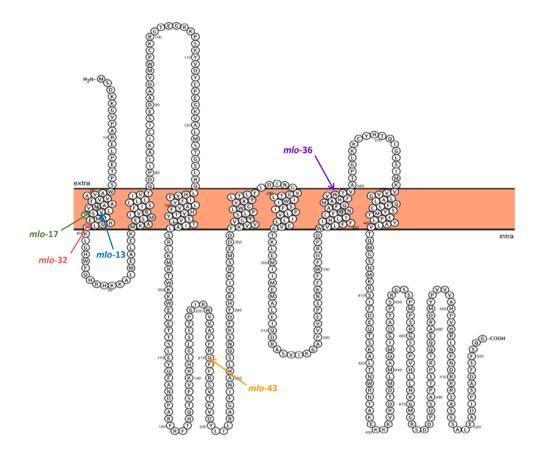
### **Supplementary Figures**

SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	1 1 1 1	GACTTATTGTTTGAAACTTGCAACTACAATTCTTCTTTATCATCATTTGACATTTCCCCTTCTTCTTATTCCTATTTTA
SlML01_MT SlML01_Heinz LEFL1063CA10 LEFL1037DE09	1 1 81	
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	1 1 161	ATGGCTAAAGAACGGTCTATGGAGGCAACCCCTACGTGGGCAATTGCTGTGGTTTGCTTCAT
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	63 63 56 241	CTTGCTCGCTATTTCTATTTTATTGAACAAATTATTCATCACAATTGGAGAGTGGTTACTGGAAAAGCGGAAAAAGTCTC CTTGCTCGCTATTTCTATTTTATTGAACAAATTATTCATCACAATTGGAGAGTGGTTACTGGAAAAGCGGAAAAAGTCTC
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	143 143 136 321	TATATGAAGCACTTGAAAAGATCAAAGCTGAACTTATGCTGTGGGATTCTTATCACTGTTGACAGTGTTGCAAGAT TATATGAAGCACTTGAAAAGATCAAAGCTGAACTTATGCTGTTGGGATTCTTATCACTGTTGTGACAGTGTTGCAAGAT
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	223 223 216 401	CCAGTTTCTAACTTATGTGTCCCCAAGAGTGTTGGTTATTCATGGCATCCTTGTATGGCAAAGGAAGATGCCAAGTCTGA CCAGTTTCTAACTTATGTGTCCCCAAGAGTGTTGGTTATTCATGGCATCCTTGTATGGCAAAGGAAGATGCCAAGTCTGA
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	303 303 296 481	GTATGATGACCCTTGTCTACCAAAGGGAAAAGTGCAATTTGCATCTTCATATGCAATACACCAGCTCCATATCTTCATCT GTATGATGACCCTTGTCTACCAAAGGGAAAAGTGCAATTTGCATCTTCATATGCAATACACCAGCTCCATATCTTCATCT
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	383 383 376 561	TTGTATTGGCAGTTGCTCATGTATTGTACTGTATAGCAACTTTTGCTTTGGGCAGGCTAAAGATGAGAAAATGGAGGGCA TTGTATTGGCAGTTGCTCATGTATTGTACTGTATAGCAACTTTTGCTTTGGGCAGGCTAAAGATGAGAAAATGGAGGGCA
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	463 463 456	TGGGAGGATGAAACAAAAACAATGGAGTACCAATTCTACAACGACCCTGAGAGATTCAGATTTGCAAGGGAGACCTCGTT
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	543 543 536	TGGACGTAGGCATTTGCATTTCTGGAGCAAGTCCCCCGTGTTGCTCTCGATAGTTTGTTT
SlMLO1_MT SlMLO1_Heinz LEFL1063CA10 LEFL1037DE09	623 623 616	CACTTEC AAAAGTTGACTATTTAACCCTTAGACATGGGTTCATGATGGCACATTTAACTCCACAAAATCAAAATAATTTT
	703	GATTTTCAATTATACATTAACAGAGCAGTTGACAAAGACTTCAAAGTTGTTGTTGGAATAAGTCCTGCATTATGGCTCTT GATTTTCAATTATACATTAACAGAGCAGTTGACAAAGACTTCAAAGTTGTTGTTGGAATAAGTCCTGCATTATGGCTCTT 
SlML01_MT SlML01_Heinz LEFL1063CA10 LEFL1037DE09	783	CACGGTGCTATATTTTCTGACTACTACCGATCGATTGTACTCGTATCTTTGGGTGCCATTTATCCCACTTGTAATAATAT CACGGTGCTATATTTTCTGACTACTACCGATCGATTGTACTCGTATCTTTGGGTGCCATTTATCCCACTTGTAATAATAT

**SUPPLEMENTARY FIGURE 1**.

 9 GTACCTGTGGTGGAGACTGGTGACCATCTTTTCTGGTTTAATCGCCCTGCCCTTGTCCTATTCTTGATTAACTTTGTACT 9 GTACCTGTGGTGGAGACTGGTGACCATCTTTTCTGGTTTAATCGCCCTGCCCTTGTCCTATTCTTGATTAACTTTGTACT 
CTTTCAGAATGCGTTTCAAGTTGCTTTCTTTTTTGGAGTTGGTGGAAATTTGGTTTCCCATCTTGCTTTCATAAGAATG CTTTCAGAATGCGTTTCAAGTTGCTTTCTTTTTTTGGAGTTGGTGGAAATTTGGTTTCCCATCTTGCTTTCATAAGAATG
CTGCAGACCTAGCCATAAGGCTAACCATGGGGGGTGATCATACAGGTCCATTGCAGCTATGTGACTCTCCCCTCTTATGCC CTGCAGACCTAGCCATAAGGCTAACCATGGGGGTGATCATACAGGTCCATTGCAGCTATGTGACTCTCCCCTCTTTATGCC
TTAGTTACACAGATGGGTTCATCAATGAAGCCTATCATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGGCACCA TTAGTTACACAGATGGGTTCATCAATGAAGCCTATCATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGGCACCA
 TACAGCGAAAAAACGGGTGAAACATGGGCTATCAGGACATACCACCCTGCAAACAGCAGACCAACCA
GTACCTCCCCTGTTCACTTATTACGCGGTTATCCACAATATAATGAGGACAGTGTTCAAGCATCCCCGGACATCCAAT GTACCTCCCCTGTTCACTTATTACGCGGTTATCCACAATATAATGAGGACAGTGTTCAAGCATCTCCTCGGACATCCAAT
GTCGAAAATGAAGGGTGGGCTAATGAAAATCAGGAGGGGAGAGATCCTGCAGGATGCCTCCACTGATCATAACAAGCAAAT GTCGAAAATGAAGGGTGGGCTAATGAAAATCAGGAGGGAG
I GAGATTACAATGTCAGATTTTACTTTTGGAAACAAATAA I GAGATTACAATGTCAGATTTTACTTTTGGAAACAAATAA

**SUPPLEMENTARY FIGURE 1**. Nucleotide alignment of the *SIMLO1* sequence experimentally obtained from the tomato cv Micro-Tom (MT), the one from the cv Heinz as in the SGN database (Solyc04g49090) and the two EST sequences obtained by blasting the Heinz *SIMLO1* sequence to the MiBASE database of Micro-Tom.



**SUPPLEMENTARY FIGURE 2. Schematic representation of the complete barley HvMlo protein**. The orange bar represents the plant membrane. Arrows indicate the amino acids that in their corresponding *mlo*-mutants are mutated into stop codons.

# Chapter 8

## **General discussion**

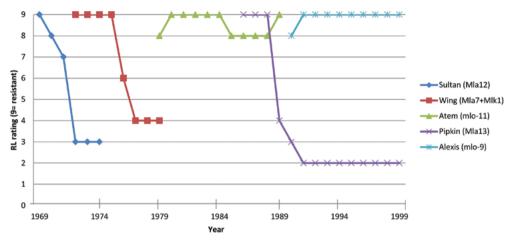
Plant diseases reduce global crop yields by 10–16% each year [1]. This constraint represents a tremendous challenge which has to be overcome if global food production should meet the demands of a growing world population by 2050.

Among plant pathogens, a small minority including powdery mildews (PM) can infect a broad range of plant species. More than 400 fungal PM species can infect thousands of plant species, causing a disease known for its whitish powdery appearance [2]. In addition, climate change is influencing the number of host species and the geographic distribution of this disease [3, 4]. For example, PM disease on tomato caused by *Oidium neolycopersici* has been reported for the first time in 2015 in South Africa, which till 2008 was still an unexplored area of the world for PMs [2, 5]. Moreover, in 2011, the same pathogen was first identified in Taiwan on leaves of papaya (*Carica papaya* L.) [6]

So, how to halt its progression? There are several methods that can contribute to the control of the PM disease, from chemical and biological control to good farm management. However, my interest lies in the use of genetic resistance that ensures a durable effectiveness in crops affected by this disease.

Barley cultivars resistant to the PM pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*) are available since 1969 in the United Kingdom [7]. Most of the race-specific resistance to *Bgh* is conferred by the dominant resistant (R-) genes at the *Mla* locus. Thirty-one *Mla* alleles with race-specific resistances have been identified, numbered from number 1 to 31 [8]. In addition, five other resistance loci have been mapped: *Mlat, MlGa, Mlk, Mlnn* and *Mlra* [9]. The gene *Mlk-1* was previously shown to be identical to *Mla-4* [10]. Resistant cultivars based on the deployment of one or more allelic variants of the *Mla* gene, like Sultan, Wing, and Pipkin, have had a useful lifespan of just a few years (Figure 1).

On the other hand, the cultivars Atem and Alexis, introduced in the market in the 1980's, maintained a high PM-resistance level during the years (Figure 1). Both cultivars display the so-called *mlo*-based resistance, which, next to being durable, is recessive and broad-spectrum. It is characterized at the cellular level by the timely deposition of papillae at the attempted fungal penetration sites, preventing colonization [11]. The resistance has been associated with defective alleles of the barley *HvMlo* gene (*Mildew resistance Locus Q*) and has for a long time been considered to be exclusive for this cereal [12]. However, more recent studies have shown that *mlo*-resistance is highly conserved as it occurs in many other plant species, among which Arabidopsis, pea, pepper, apple, cucumber and wheat [13-18], [19]. In tomato, a resistant cherry-type line, LC-95, harbors a natural loss-of-function allele of the tomato *SIMLO1* gene, called *ol-2*, which is characterized by a 19-bp deletion in the coding sequence [20].



**FIGURE 1. Resistance to barley powdery mildew disease in five barley cultivars deploying different resistance genes.** Rate of resistance is according to the AHDB (Agriculture and Horticulture Development Board) Cereal and Oilseed Recommended Lists (RL) from 1968 to 1999. The cvs Sultan and Pipkin were the first ones to employ the R-genes *Mla12* and *Mla13*, respectively. The cv Wing was the first harbouring a combination of R-genes, *Mla7 and Mlk1*. Atem and Alexis were the first cvs carrying loss-of-function alleles of the *HvMlo* gene, *mlo-11* and *mlo-9*, respectively. This figure is a modified version of the one reported in Brown (2015).

The *MLO* gene represents probably the best-characterized example of a class of plant genes known to facilitate the infection and provide compatibility with the pathogen. These are named susceptibility genes (S-genes) [21, 22]. In each plant species, *MLO* genes occur in multiple copies constituting a gene family [23]. So far, all known plant *MLO* genes, that act as S-genes towards the PM pathogens, cluster in two specific phylogenetic clades, clade IV for monocot and clade V for dicot [24-27]. At the moment there is a body of experimental evidence showing that identification and inactivation of those genes is an effective breeding strategy to introduce resistance to PM in multiple cultivated species [21].

This thesis focused on the study of *mlo*-based resistance in Solanaceae and Cucurbitaceae crops. These families include several crops of agronomic importance, like tomato, eggplant, tobacco and cucumber. The results of the individual chapters represent a contribution to the current understanding of the role of *MLO* genes in PM susceptibility. From these findings, guidelines can be derived to aid and improve breeding activities in order to introduce the *mlo*-based resistance in new crop species suffering from the PM disease. These are shown in Figure 2 and discussed below.

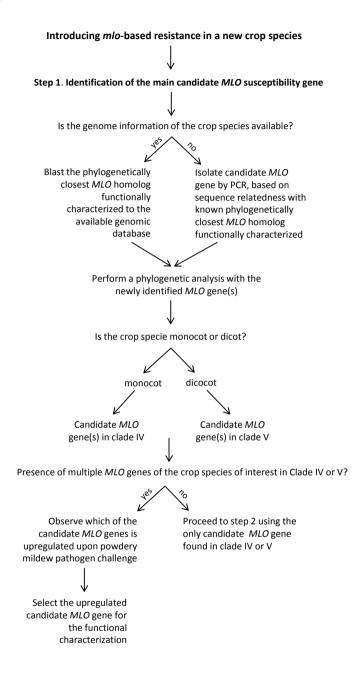


FIGURE 2.

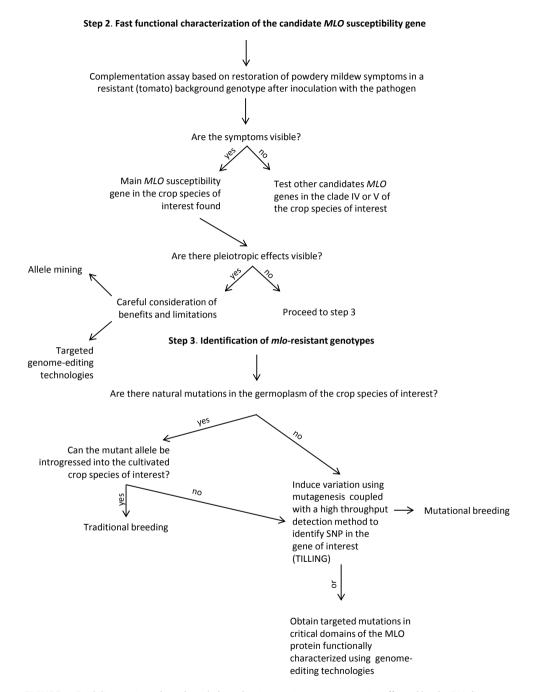


FIGURE 2. Guidelines to introduce the mlo-based resistance in new crop species affected by the PM disease.

# How to find the main candidate *MLO* susceptibility gene(s) in the species of interest?

#### 1. Identification of the main candidate MLO gene

The genome sequence of the crop of interest (or of a closely-related plant species) and the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) are important resources to achieve this aim [28-32].

In Chapter 2, we used the available genomic information at the SOL genomic database (https://solgenomics.net/) to identify all the homologs of *SIMLO1* in tomato, which was previously characterized as PM-susceptibility factor in this specie [20].

In Chapter 4, we isolated candidate *MLO* genes involved in PM susceptibility of three important solanaceous crops, namely tobacco, eggplant, and potato. At the time we were working on this chapter, only the genomes of potato and tobacco were published, while the one of eggplant was not available yet [33, 34]. Nevertheless, due to the close phylogenetic distance of these plant species with tomato, a combined approach based on database search (of the available genomes) and PCR amplification allowed the identification of tobacco *NtMLO1*, eggplant *SmMLO1*, and potato *StMLO1*.

When the genome sequence of the crop species of interest is not yet available, a possible approach is to obtain first genomic or EST libraries. Feechan et al (2008) [25] prepared a grape genomic library based on a partial *Mbol* restriction digest and screened it with radiolabelled cDNA fragments of Arabidopsis *MLO* genes. In rose, Kaufmann et al. (2012) [35] produced two ESTs libraries of untreated and PM-inoculated leaf samples, against which they blasted Arabidopsis *MLO* genes.

However, since the number of available plant genome sequences is increasing, there may be genomic information available for a closely related plant species. It is feasible, then, to apply a homology-based cloning approach as shown in chapter 4 for eggplant *SmMLO1*, and for pepper *CaMLO2* by Zheng et al. (2013) [18].

The next step is to establish which of the identified homologs cluster together with characterized *MLO* susceptibility genes. It is known that, following a phylogenetic analysis, dicot candidate *MLO* susceptibility genes group in clade V, while, monocot *MLO* susceptibility genes are clustered in clade IV [26].

In Chapter 4, for example, we observed that five *NtMLO* and two *StMLO* genes, among which the newly identified homologs, cluster in clade V together with *SIMLO1* and *CaMLO2*. Similarly, in Chapter 2, four tomato *SIMLO* homologs grouped in clade V.

When there are multiple *MLO* homologs, as in the above examples, how to choose the best candidate *MLO* susceptibility gene in the crop species of interest?

Consonni et al. (2006) showed that *Arabidopsis thaliana AtMLO2, AtMLO6* and *AtMLO12* genes are functionally redundant, but *AtMLO2* plays a major role in Arabidopsis susceptibility to the PM pathogen *Golovinomyces orontii*. Other examples followed, like the two pepper clade V-*MLO* homologs, *CaMLO1* and *CaMLO2* in Zheng et al. (2013) [18] and a more recent article published by Pessina et al. (2016) [19]. The latter showed that, of the four clade V-*MLO* genes in apple, *MdMLO19* is the main gene responsible for the susceptibility to the PM pathogen *Podosphaera leucotricha*.

Because of the functional redundancy, the first criterion for selection is based on the highest level of conservation of the candidate clade V-*MLO* genes with the closest related characterized *MLO* gene. For instance, the phylogenetic tree presented in Figure 1 of Chapter 4 showed that two of the five *NtMLO* genes are very homologous with each other and with *SIMLO1* and *CaMLO2*. By PCR we isolated only *NtMLO1* and our results provided evidence of its role as PM susceptibility gene. However, a recent paper showed that also a functional *NtMLO2* is required for a successful PM infection of transgenic tobacco plants [36]. All together, these results show that high similarity to proven *MLO*-like susceptibility genes is a valuable criterion for selection.

In case none of the candidates can be excluded based on sequence relatedness, transcript abundance of each gene should be assessed upon PM infection. It was shown that the expression of *MLO* susceptibility genes is triggered few hours after PM inoculation, particularly between 4 and 10 hrs [18, 25, 31, 32, 37]. In Chapter 2, we showed that among the four *SIMLO* homologs, only the expression of *SIMLO1* is significantly increased at 6 and 10 hrs. In fact, loss-of-function of this gene can almost completely prevent PM penetration [38]. In contrast, if the other three homologs are silenced individually, the susceptibility level of the background genotype in which they were tested (Moneymaker) does not change (Chapter 2).

The induction of gene expression upon powdery mildew infection should be considered as an absolute selection criterion for clade IV or clade V *MLO* genes. In Chapter 2 we observed that two *SIMLO* homologs outside clade V, *SIMLO4* and *SIMLO14*, are up-regulated after PM inoculation. Preliminary results, which were not included in Chapter 2, indicated that *SIMLO14* does not act as PM susceptibility factor in tomato. Similarly, in apple, a recent paper excluded clade VII- *MdMLO18* as susceptibility gene, although its expression was induced by the PM pathogen [19].

#### 2. Fast approach to functionally validate the candidate MLO susceptibility gene

Once a candidate gene has been identified, it is important to verify that it acts as a PM susceptibility factor in a given plant species. This is of interest for the application of the *mlo*-resistance in breeding activities and basic research on plant-pathogen interactions.

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Commonly *MLO* genes are validated using a resistant Arabidopsis mutant (*Atmlo2/6/12*) or a resistant barley mutant (*mlo-11*) in assays involving overexpression of heterologous dicot and monocot *MLO* candidates, respectively [14, 19, 39-42]. If PM symptoms occur, the tested *MLO* gene is a susceptibility factor because it is able to rescue the function of the impaired *Atmlo* genes or *Hvmlo* gene, depending on the genotype used.

The results reported in Chapter 5 indicate that the resistant tomato genotype *ol-2*, containing an impaired allele of the *SIMLO1* gene, can be used to quickly validate the function of a candidate *MLO* gene. We have developed a routine tomato transformation protocol in our lab which is reliable in testing both monocot and dicot *MLO* genes. Moreover, the examples of successful complementation of closely related solanaceous homologs of *SIMLO1* as well as more distant *MLO* genes (Chapter 4, 5 and 6), indicate that a high degree of sequence conservation is not required in order to complement the loss-of-function of a susceptibility *MLO* gene, as was previously hypothesized by Panstruga (2005).

The implication of this finding is that the function of the *MLO* susceptibility genes is extremely conserved, which raises questions about its core biochemical activity. Once these questions are answered it will be possible to understand the reason for which a plant species has maintained, during evolution, a protein that is hijacked by PMs. More insights on this topic are described further in this chapter.

Another suitable option to verify the function of a candidate *MLO* gene consists of knockingdown its expression, in a transient or stable way. The virus-induced gene silencing (VIGS) technology offers an easy and quick alternative to the generation of stable transgenic plants, especially for species in which the latter is not achievable. This approach, for example, has been followed in barley, wheat, peach and pepper [18, 43-45]. Despite its advantages, this technology has several limitations inherent to the uneven silencing of the gene throughout an inoculated plant, the high variation between plants and experiments and the possible alteration of plant development, especially height and leaf morphology [46]. These limitations affect the observation of the phenotype upon silencing of the candidate *MLO* gene. For these reasons, the use of the stable silencing RNA interference (RNAi) is preferred over VIGS application.

Moreover, although the silencing approach has the advantage of using the native plantpathogen interaction, it may not be the best method to identify minor *MLO* genes involved in PM susceptibility. For example, in Chapter 2, the involvement of clade V-*SIMLO3* gene could not be verified because no change in PM susceptibility was observed in RNAi silenced transgenic plants. Moreover, the minor effect of *SIMLO5* and *SIMLO8* was noticed only when comparing plants carrying the RNAi::*SIMLO1* construct and *ol-2* plants.

Novel genetic techniques to modify genes have been developed in recent years [47]. These include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases

(TALENs), and the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) system [48-50]. These techniques allow genome editing by adding, removing or replacing DNA at specific locations. Therefore, they are widely used to study gene function. Recently, Wang et al. (2014) used the TALEN genome editing technology to generate transgenic winter wheat plants containing simultaneous knock-out lesions in the three *TaMLO* homoeologues. Their results suggest that all three genes contribute to the response of wheat to PM infection. In addition, they used the CRISPR/Cas9 technology to generate mutations (small deletions and one bp insertion) in the *TaMLO-A1* allele, demonstrating for the first time the application of this novel system on *MLO* genes. Similarly to TALENs, CRISPR/Cas9 is highly specific for the target sequence but much easier to handle. Both are particularly useful in polyploid outcrossing crops for which it is difficult to obtain homozygous mutants.

One careful consideration regarding the occurrence of pleiotropic effects is needed after verifying the involvement of the *MLO* gene in the crop species of interest. If deleterious phenotypes are visible, it is important to consider whether or not introducing the *mlo*-resistance in a new crop.

Impaired *mlo* alleles produced severe pleiotropic effects in barley, consisting of spontaneous necrosis and premature leaf senescence, but also in Arabidopsis, where spontaneous callose deposition was observed [37, 51, 52]. Moreover, both barley and Arabidopsis mutants were more susceptible to certain hemibiotrophic and necrotrophic pathogens which indicate that MLO proteins are exploited by a plethora of microorganisms [51, 53-55].

In Arabidopsis, it was shown that the pleiotropic effects can be uncoupled from *mlo*-based resistance by applying second-site mutations in the NahG gene to reduce the higher SA level observed in single and double mutants compared to wild-type plants [51]. However, since SA is involved in response to other pathogens, further analyses are needed to assess the possibilities of application in an agronomically beneficial manner.

A very recent article showed the identification of a new natural variant of the barley *mlo-11* allele, called *mlo-11(cnv2)*, found in the Ethiopian barley landrace Eth295 [56]. Developmental stage influences the resistance provided by this novel allele. The resistance obtained is partial, although effective. However, most importantly, the landrace carrying the novel variant lacks the pleiotropic effects typical of the barley mutants. The gene expression of the two alleles was compared and causally related to the phenotypic differences observed between the landrace Eth295 and the cv Westminster, carrying the *mlo-11* allele. Therefore, they suggest the use of "temperate" *MLO* mutations to fine tune the balance between expression, pleiotropic effects and resistance levels.

No pleiotropic effects were observed in tomato silenced plants (Chapter 2) nor in stable silenced apple plantlets, while a reduction in plant size was shown in pepper silencing

*CaMLO2* gene [18, 19]. Taken together, these findings indicate that pleiotropic effects vary among plant species upon knockdown or knockout of the susceptibility *MLO* genes. This means that there is a large space for the possible utilization of the *mlo*-resistance in breeding programs, without the limitations connected to the pleiotropic effects, and for a suitable compromise between level of resistance and pleiotropic effects.

#### 3. Identification of mlo-resistant genotypes...

At this point of the pipeline, the correct target *MLO* gene has been found, functionally validated, and its utilization considered potentially beneficial for the studied crop species.

In Europe, the deployment of transgenic plants (including those resulting from genome editing approaches) in plant breeding and agriculture is still socially and politically debated. Therefore, currently, non-transgenic approaches are favored in the selection of new varieties.

#### a)...through natural variation

The first non-transgenic strategy to identify *mlo*-resistant genotypes consists of searching for variation within the natural germplasm of the crop species of interest.

In Table 1, an overview of all the *mlo*-mutant alleles described in literature is presented, among which natural alleles are found in pea (*er-1*, *er-2*, *er-3*, *er-4* and *er-6*), rose, tomato (*ol-2*), apple, cucumber, tobacco and barley (*mlo-11* and *mlo-11(cnv2)*) [13, 15, 20, 35, 36, 56, 57].

Nowadays, with the huge amount of sequence information derived from sequencing projects of cultivated vegetable and fruit crops, like tomato, cucumber and apple, allele mining is another promising approach to identify novel natural *MLO* alleles useful for the development of new cultivars [58, 59] (http://www.fruitbreedomics.com). In Chapter 6, we exploited the genomic information of the 115 cucumber accessions to search for the occurrence of the transposable element found in the cucumber susceptibility gene *CsaMLO8*. By doing so, we gathered information regarding the natural variation of the *CsaMLO8* gene and found out that it probably derived from the wild cucumber species, *C. sativus* var *hardwickii*, from which it was introgressed in cultivated cucumbers.

Therefore, if an impaired natural allele of the *MLO* gene of interest is found, and the accession or wild species can be crossed, a long program aimed at inserting the desired allele in the crop under investigation starts.

Plant species	MLO gene	Allele name	Amino-acid exchange	Location	Plant accession	Origin	Pleitropic effects?	Reference
		er1-1	S227→ stop	2IC	JI 1559	natural origin		
		er 1-2	aberrant splicing variants	n.a.	JI 2302	natural origin		
		er 1-3	G288 → stop	TM4	JI210	natural origin		Humphry et al. 2011
Pea		er1-4	ΔA91 (in frame shift)	miss 1EXTRA &TM1	JI 1951	natural origin		
(Pisum sativum)	PSMLUT	er 1-5	W190 → stop	2IC	ROI3/02	DES (diethyl sulfate)	ou	Pavan et al. 2011
		er 1-6	L353P	TM6	JI 2480	natural origin		Sun et al., 2016
		n.a.	Q226 →stop	2IC	S(er1mut1)	ENU (N-ethyl-N- nitrosourea)		Santo et al. 2013
		n.a.	312Y → stop	TM5	F(er1mut2)	ENU		
Petunia		n.a.	S130L	TM3	Petunia x hybrida Mitchell dihaploid	EMS/TILLING	2	3100 lo to soci
(Petunia x hybrida)		n.a.	G176E	2IC	Petunia x hybrida Mitchell dihaploid	EMS/TILLING		
Rose	2017 A 41 0 4	n.a.	V519 →stop	C-terminal	R.multiflora or R. hybrida	natural origin		
(Rosa multiflora)	RIINILO4	n.a.	T532 →stop	C-terminal	R.multiflora or R. hybrida	natural origin	n.a.	Naurmann et al. 2012
Tomato	CIMIO	m200	l21 →stop	TM1	S. lycopersicum cv Micro- Tom	EMS	2	chapter 7
lycopersicum)	JINICOL	ol-2	Q198 →stop	2IC	S.lycopersicum var carasiforme (LA1230)	natural origin	2	Bai et al. 2008
Apple (Malus domestica)	610TWPW	n.a.	E405 → stop	3EXTRA	McIntosh, Priscilla, Jonathan	natural origin	ои	Pessina et al. (submitted)
			V170G	TM3	n.a.	natural origin	n.a.	
	CsaMLO1	n.a.	V472I	C-terminal	n.a.	natural origin	n.a.	berg et al. (In
-			V557I	C-terminal	n.a.	natural origin	n.a.	preparation)
Cucumber			K178stop	2IC	R077, R078, R079, 115, 930	natural origin	n.a.	1 i ot al 2016 h
		5	K487stop	C-terminal	PI197088-R	natural origin	n.a.	
	COMMENO		Δ174	3IC, part of		natural origin	n.a.	Berg et al. 2015

TABLE 1. Overview of the *mlo*-mutants described in this thesis and in the literature.

Plant species	MLO gene	Allele name	Amino-acid exchange	Location	Plant accession	Origin	Pleitropic effects?	Reference
		n.a.	Q198R	2IC	Peit havana SR1	PCR error during cloning	n.a.	Appiano et al. 2015
Tobacco (Nicotiana	N†MLO 1	n.a.	6 aberrant splicing variants	Jic	Коћићи	natural origin (N. tomentosiformis)	2	3016 le to curmiinE
tabacum)	NtML02	n.a.	2 aberrant splicing variants		nanaoy	natural origin (N. sylvestris)	2	rujililula et al. 2010
		n.a.	N76L	TM2	CGN0524	natural origin (Ethiopian landrace)		
		mlo-12	F240L	2IC	Elgina	NMU (N-Nitroso-N- methylurea)		
		mlo-28	T222I	2IC	Nadja	NaN3 (sodium azaide)		
		mlo-1	W162R	2IC	Haisa	X-rays		
		mlo-6	3 aberrant splicing variants	n.a.	Carlsberg II	EMS		
		mlo-44	1 aberrant splicing variant	n.a.	Bonus	NaN3		
		mlo-16	1 aberrant splicing variant	n.a.	Alsa	EMS		
		mlo-30	2 aberrant splicing variants	C terminal	Alsa	EMS		
		mlo-11	aberrant splicing variants	n.a.	Ethiopian landrace	natural origin		
		mlo-2	A349T	TM6	Vollkorn	X-rays		
Barley	CIWM	mlo-34	W423 →stop	C-terminal, CaMBD	Kristina	EHOES (Ethyl hydroxyethanesulfonate)	30//	Reindstadler et al.
(Hordeum vulgare)		mlo-36	W357 → stop	TM6	Bonus	iso-PMS (isopropyl- methane-sulfonate)	6	2010
		mlo-39	Q351 → stop	TM6	Bonus	iso-PMS		
		mlo-43	Q210 → stop	2IC	Bonus	NaN3		
		mlo-32	E35 → stop	TM1	Prudentia	NaN3		
		mlo-31	$G276 \rightarrow stop$	TM5	Ursula	NaN3		
		mlo-35	H231L	2IC	Kristina	iso-PMS		
		mlo-37	S71F	1 EXTRA	Bonus	iso-PMS		
		mlo-38	G318R	3IC	Kristina/Bonus	iso-PMS/NaN3		
		mlo-40	G264D	TM4	Bonus	NaN3		
		mlo-41	R209K	2IC	Bonus	NaN3		
		mlo-42	S187L	2IC	Bonus	X-rays		
		mlo-5	M1I	N-terminus	Carlsberg II	EMS		
		mlo-8	M1V	N-terminus	Carlsberg II	EMS		

pare         mode         constant         co	Plant MLO	.0 Allele	Amino-acid	Location	Plant	Origin	Pleitropic	Reference
Image         Reserve         And         A			Conen de	JL		EAAC		
mio-9R10WN-terminusDiamantEMSmio-13V30ETM1PlenaEMSmio-17S131ETM1PlenaEMSmio-17G318E31CPlenaEMSmio-28L270HTM4PlenaEMSmio-29F334L31CPlenaEMSmio-21G318E31CPlenaEMSmio-23A306T31CVirulaEMSmio-33fameshift P396TM7Materia HedaEMSmio-30fameshift P320TM7Materia HedaG-3NSmio-31fameshift P320ZICFomaY-3NSmio-31deletion F182 and T18321CFomaG-3NSmio-31MIS2M or W163E21Cn.a.G-3NSnaU152M or W163E21Cn.a.Site direct mutagenesisnaU152M or W163E21Cn.a.Site direct mutagenesisnaU123N21Cn.a.Site direct mutagenesisnaU231NTM4n.a.Site direct mutagenesisnaU231NTM4n.a.Site direct mutagenesisnaF332A31Cn.a.Site direct mutagenesisnaF332A31Cn.a.Site direct mutagenesisnaF333A31Cn.a.Site direct mutagenesisnaF333A31Cn.a.Site direct mutagenesisnaF333A31Cn.a.Site direct mutagenesisnaF333A31C<		1-0IUI	0770D	717		EIVIS		
m0-13         V30E         TMI         Plena         EMS           m0-17         531F         TMI         Plena         EMS           m0-26         L270H         TMI         Plena         EMS           m0-27         531F         TMI         Plena         EMS           m0-27         531F         31C         Plena         EMS           m0-28         7030F         31C         Plena         EMS           m0-31         frame shift P396         TM7         Maltrai Heda         EMS           m0-35         frame shift P396         TM7         Maltrai Heda         EMS           m0-36         frame shift P396         TM7         Maltrai Heda         EMS           m0-37         detetorift P182         21C         Nau3         Site direct muspensis           m0-31         21C         n.a.         Site direct muspensis           n.a.         V162A or W163E         21C         n.a.         Site direct muspensis           n.a.         V162A or W163E         21C         n.a.         Site direct muspensis           n.a.         V162A or W163E         21C         n.a.         Site direct muspensis           n.a.         V1307         31C		mlo-9	R1 0W	N-terminus	Diamant	EMS		
m0-17         S31F         TM1         Plena         EMS           m0-26         L270H         TM4         Plena         EMS           m0-26         L270H         TM4         Plena         EMS           m0-27         G318E         31C         Usula         EMS           m0-28         A301         31C         Usula         EMS           m0-29         Farae shift P396         TM7         Materia Heda         EMS           m0-3         Farae shift P396         TM7         Materia Heda         G-ays           m0-3         S107         21C         Ursula         S14 direct mutagenesis           m0-10         deletion F182 and T183         21C         n.a.         S14 direct mutagenesis           m0-11         M159R         21C         n.a.         S14 direct mutagenesis           na<		mlo-13	V30E	TM1	Plena	EMS		
Image         Loot         Mate         Mease         M		mlo-17	S31F	TM1	Plena	EMS		
m(o-27         G318E         31C         Plena         EMS           m(o-29         P334L         31C         Sutan5         NaN3           m(o-29         P334L         31C         Ursula         NaN3           m(o-33         frame shift/y159         1M7         Materia Heda         9-rays           m(o-10         defetion F183 and T133         21C         Foma         7-rays           m(o-10         defetion F183         21C         n.a.         9-rays           m(o-10         defetion F183         21C         n.a.         9-rays           n.a.         V15SR         21C         n.a.         9-rays           n.a.         V162A or W163E         21C         n.a.         9-rays           n.a.         V162A or W163E         21C         n.a.         9-rays           n.a.         V163R         21C         n.a.         9-rays           n.a.         V163R         21C         n.a.         9-rays           n.a.         V163R         21C         n.a.         9-rays         9-rays           n.a.         V164R         21C         n.a.         9-rays         9-rays           n.a.         V164R         n.a.		mlo-26	L270H	TM4	Plena	EMS		
Info-29         P334L         3IC         Sutan5         Na3           Info-33         A306T         3IC         Ursula         Na3           Info-33         frame shift P396         TW7         Ursula         Na3           Info-3         frame shift P396         TW7         Materia Heca         9-rays           Info-3         frame shift W159         2IC         Foma         9-rays           Info-10         deeton F182 and T183         2IC         n.a.         8-rays           Info-10         deeton F182 and T183         2IC         n.a.         8-rays           Info-10         deeton F182 and T183         2IC         n.a.         8-rays           Indo         W162A or W163E         2IC         n.a.         8-rays           Indo         W162A or W163E         2IC         n.a.         8-rays           Indo         W162A or W163E         2IC         n.a.         8-rays           Indo         W162A         M164         n.a.         8-rays           Indo         W162A         W163         M164         R-rays           Indo         W162A         W163         M164         R-rays           Indo         W12A         W164		mlo-27	G318E	ЗІС	Plena	EMS		
m(o-33         A306T         31C         Ursula         Nal3           m(o-3         frame shift P396         TM7         Materia Heda         g-rays           m(o-4         frame shift P396         TM7         Materia Heda         g-rays           m(o-4         frame shift W159         21C         Foma         g-rays           m(o-4         frame shift W159         21C         Foma         g-rays           m(o-4         delton F182 and T183         21C         n.a.         g-rays           m(o-4         delton F182 and T183         21C         n.a.         g-rays           n.a.         W155R         21C         n.a.         g-rays           n.a.         W155R         21C         n.a.         g-rays           n.a.         W155R         21C         n.a.         g-rays           n.a.         S187F         1.a.         S1E direct mutagenesis </td <td></td> <td>mlo-29</td> <td>P334L</td> <td>3IC</td> <td>Sultan5</td> <td>NaN3</td> <td></td> <td></td>		mlo-29	P334L	3IC	Sultan5	NaN3		
mlo-3         frame shift P396         TM7         Materia Heda         9-rays           mlo-10         deletion F182 and T183         2IC         Foma         X-rays           mlo-10         deletion F182 and T183         2IC         Foma         9-rays           mlo-10         deletion F182 and T183         2IC         Foma         9-rays           mlo-10         deletion F182 and T183         2IC         na.         9-rays           mlo-10         deletion F182 and T183         2IC         na.         9-rays           na         W159R         2IC         na.         9-rays           na         S187F         2IC         na.         9-rays           na         J37A         3IC         na.         9-rays           na         P320A         3IC         na.         9-rays           na         P323A         3IC         na.         9-rays           na         P323A         3IC         na.         9-rays		mlo-33	A306T	ЗІС	Ursula	NaN3		
mlo-4frame shift W1592ICFomaX-raysmlo-10deletion F182 and T1832ICFoma $g-ays$ n.a.W159R2ICn.a.site direct mutagenesisn.a.W162A or W163E2ICn.a.site direct mutagenesisn.a.W162A or W163E2ICn.a.site direct mutagenesisn.a.E163R2ICn.a.site direct mutagenesisn.a.S187F2ICn.a.site direct mutagenesisn.a.D2192ICn.a.site direct mutagenesisn.a.D2192ICn.a.site direct mutagenesisn.a.P320A3ICn.a.site direct mutagenesisn.a.P320A3ICn.a.site direct mutagenesisn.a.P320A3ICn.a.site direct mutagenesisn.a.P320A3ICn.a.site direct mutagenesisn.a.P320A3ICn.a.site direct mutagenesisn.a.F329A3ICn.a.site direct mutagenesisn.a.F329A3ICn.a.site direct mutagenesisn.a.F333A3ICn.a.site direct mutagenesisn.a.F333A3ICn.a.site direct mutagenesisn.a.F333A3ICn.a.site direct mutagenesisn.a.F333A3ICn.a.site direct mutagenesisn.a.F333A3ICn.a.site direct mutagenesisn.a.F333A3ICn.a.site direc		mlo-3	frame shift P396	TM7	Malteria Heda	g-rays		
mlo-10         deletion F182 and T183         21C         Foma         g-rays           n.a.         W159R         21C         n.a.         site direct mutagenesis           n.a.         W162A or W163E         21C         n.a.         site direct mutagenesis           n.a.         W162A or W163E         21C         n.a.         site direct mutagenesis           n.a.         E163R         21C         n.a.         site direct mutagenesis           n.a.         D219         21C         n.a.         site direct mutagenesis           n.a.         P320A         31C         n.a.         site direct mutagenesis           n.a.         P320A         31C         n.a.         site direct mutagenesis           n.a.         W330A         31C         n.a.         <		mlo-4	frame shift W159	2IC	Foma	X-rays		
n.a.         W159R         2IC         n.a.         site direct mutagenesis           n.a.         W162A or W163E         2IC         n.a.         site direct mutagenesis           n.a.         E163R         2IC         n.a.         site direct mutagenesis           n.a.         S187F         2IC         n.a.         site direct mutagenesis           n.a.         D219         2IC         n.a.         site direct mutagenesis           n.a.         P32AA         3IC         n.a.         site direct mutagenesis           n.a.         P32AA         3IC         n.a.         site direct mutagenesis           n.a.         F33AA         3IC         n.a.         site d		mlo-10	deletion F182 and T183	2IC	Foma	g-rays		
n.a.W162A or W163E21Cn.a.site direct mutagenesisn.a.E163R21Cn.a.site direct mutagenesisn.a.5187F21Cn.a.site direct mutagenesisn.a.021921Cn.a.site direct mutagenesisn.a.D251NTM4n.a.site direct mutagenesisn.a.D251NTM4n.a.site direct mutagenesisn.a.D251NTM4n.a.site direct mutagenesisn.a.D251NTM4n.a.site direct mutagenesisn.a.P32AA31Cn.a.site direct mutagenesisn.a.P32AA31Cn.a.site direct mutagenesisn.a.F32AA31Cn.a.site direct mutagenesisn.a.F32AA31Cn.a.site direct mutagenesisn.a.F33A31Cn.a.site direct mutagenesisn.a.F33A17KAn.a.site direct mutagenesisn.a.F34A17KAn.a.site direct mutagenesis<		n.a.	W159R	2IC	n.a.	site direct mutagenesis		
hMMLOn.a.E163R21Cn.a.site direct mutagenesishMMLOn.a.5187F21Cn.a.site direct mutagenesisn.a.D21921Cn.a.site direct mutagenesisn.a.D251NTM4n.a.site direct mutagenesisn.a.L307A31Cn.a.site direct mutagenesisn.a.P320A31Cn.a.site direct mutagenesisn.a.P324A31Cn.a.site direct mutagenesisn.a.P324A31Cn.a.site direct mutagenesisn.a.P324A31Cn.a.site direct mutagenesisn.a.F330A31Cn.a.site direct mutagenesisn.a.F331A31Cn.a.site direct mutagenesisn.a.F331A31Cn.a.site direct mutagenesisn.a.F331A31Cn.a.site direct mutagenesisn.a.F331A31Cn.a.site direct mutagenesisn.a.F331A31Cn.a.site direct mutagenesisn.a.F331A31Cn.a.site direct mutagenesisn.a.F333A31Cn.a.site direct mutagenesisn.a.F333A31Cn.a.site direct mutagenesisn.a.F333A31Cn.a.site direct mutagenesisn.a.F333A117RAn.a.site direct mutagenesisn.a.F334127RAn.a.site direct mutagenesisn.a.F335GTM7n.a. <td></td> <td>n.a.</td> <td>W162A or W163E</td> <td>2IC</td> <td>n.a.</td> <td>site direct mutagenesis</td> <td></td> <td></td>		n.a.	W162A or W163E	2IC	n.a.	site direct mutagenesis		
HMILDn.a. $5187F$ $2IC$ n.a.ite direct mutagenesisn.a. $D219$ $2IC$ n.a.ite direct mutagenesisn.a. $D2251N$ $TM4$ n.a.ite direct mutagenesisn.a. $D237N$ $3IC$ n.a.ite direct mutagenesisn.a. $P32AA$ $3IC$ n.a.ite direct mutagenesisn.a. $P32AA$ $3IC$ n.a.ite direct mutagenesisn.a. $P32AA$ $3IC$ n.a.ite direct mutagenesisn.a. $F329A$ $3IC$ n.a.ite direct mutagenesisn.a. $F329A$ $3IC$ n.a.ite direct mutagenesisn.a. $F324A$ $3IC$ n.a.ite direct mutagenesisn.a. $C367A$ $3IC$ n.a.ite direct mutagenesisn.a. $C367A$ $3IC$ n.a.ite direct mutagenesisn.a. $C367A$ $1ICN2$ $1.a.$ ite direct mutagenesisn.a. $C367A$ $1EXTRA$ $1.a.$ ite direct mutagenesisn.a. $C367A$ $1EXTRA$ $1.a.$ ite direct mutagenesisn.a. $C367A$ $1EXTRA$ $1.a.$ ite direct mutagenes	-	n.a.	E163R	2IC	n.a.	site direct mutagenesis		
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D251NTM4n.a.site direct mutagenesisL307A3lCn.a.site direct mutagenesisP32A3lCn.a.site direct mutagenesisP32A3lCn.a.site direct mutagenesisP32A3lCn.a.site direct mutagenesisP32A3lCn.a.site direct mutagenesisP32A3lCn.a.site direct mutagenesisF33A3lCn.a.site direct mutagenesisF331A3lCn.a.site direct mutagenesisF331A3lCn.a.site direct mutagenesisF331A3lCn.a.site direct mutagenesisF331A3lCn.a.site direct mutagenesisF331A3lCn.a.site direct mutagenesisC367A3ETRAn.a.site direct mutagenesisC367A1EXTRAn.a.site direct mutagenesisParant Splicing variantsn.a.site direct mutagenesisaberrant splicing variantsn.a.site direct mutagenesisAbornant splicing variantsn.a.site direct mutagenesis </td <td>hiniacani vaigare)</td> <td>n.a.</td> <td>D219</td> <td>2IC</td> <td>n.a.</td> <td>site direct mutagenesis</td> <td></td> <td>0.07</td>	hiniacani vaigare)	n.a.	D219	2IC	n.a.	site direct mutagenesis		0.07
L307A         3IC         n.a.         site direct mutagenesis           P320A         3IC         n.a.         site direct mutagenesis           P324A         3IC         n.a.         site direct mutagenesis           P324A         3IC         n.a.         site direct mutagenesis           P324A         3IC         n.a.         site direct mutagenesis           W330A         3IC         n.a.         site direct mutagenesis           W331A         3IC         n.a.         site direct mutagenesis           F331A         3IC         n.a.         site direct mutagenesis           R333A         1EXTRA         n.a.         site direct mutagenesis           C36A         1EXTRA         n.a.         site direct mutagenesis           C36A         1EXTRA         n.a.         site direct mutagenesis           C114A         1EXTRA         n.a.         site direct mutagenesis           P395G         TM7         n.a.         site direct mutagenesis           <		n.a.	D251N	TM4	n.a.	site direct mutagenesis		
P320A         3IC         n.a.         site direct mutagenesis           P324A         3IC         n.a.         site direct mutagenesis           P324A         3IC         n.a.         site direct mutagenesis           F329A         3IC         n.a.         site direct mutagenesis           W330A         3IC         n.a.         site direct mutagenesis           F331A         3IC         n.a.         site direct mutagenesis           R333A         3IC         n.a.         site direct mutagenesis           C367A         3EXTRA         n.a.         site direct mutagenesis           C36A         1EXTRA         n.a.         site direct mutagenesis           C36A         1EXTRA         n.a.         site direct mutagenesis           C36A         1EXTRA         n.a.         site direct mutagenesis           C114A         1EXTRA         n.a.         site direct mutagenesis           P395G         TM/7         n.a.         site direct mutagenesis		n.a.	L307A	3IC	n.a.	site direct mutagenesis		
P324A         3IC         n.a.         site direct mutagenesis           F329A         3IC         n.a.         site direct mutagenesis           W330A         3IC         n.a.         site direct mutagenesis           F331A         3IC         n.a.         site direct mutagenesis           R333A         3IC         n.a.         site direct mutagenesis           R333A         1EXTRA         n.a.         site direct mutagenesis           C36A         1EXTRA         n.a.         site direct mutagenesis           C114A         1EXTRA         n.a.         site direct mutagenesis           C114A         1EXTRA         n.a.         site direct mutagenesis           P395G         TM7         n.a.         site direct mutagenesis           aberrant splicing variants         n.a.         th.a.         site direct mutagenesis		n.a.	P320A	3IC	n.a.	site direct mutagenesis		
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C86A     1EXTRA     n.a.     site direct mutagenesis       C114A     1EXTRA     n.a.     site direct mutagenesis       P395G     TM7     n.a.     site direct mutagenesis       aberrant splicing variants     n.a.     Ethiopian landrace     natural origin		n.a.	C98A	<b>1EXTRA</b>	n.a.	site direct mutagenesis		
C114A     1EXTRA     n.a.     site direct mutagenesis       P395G     TM7     n.a.     site direct mutagenesis       aberrant splicing variants     n.a.     Ethiopian landrace     natural origin		n.a.	C86A	<b>1EXTRA</b>	n.a.	site direct mutagenesis		
P395G TM7 n.a. site direct mutagenesis aberrant splicing variants n.a. Ethiopian landrace natural origin		n.a.	C114A	1EXTRA	n.a.	site direct mutagenesis		
aberrant splicing variants n.a. Ethiopian landrace natural origin		n.a.	P395G	TM7	n.a.	site direct mutagenesis		
		mlo- 11(cnv2)	aberrant splicing variants	n.a.	Ethiopian landrace	natural origin	ou	Ge at al. 2016

species	MLO gene	Allele name	Amino-acid exchange	Location	Plant accession	Origin	Pleitropic effects?	Reference
Arabidopsis thaliana AtMLO2	4tMLO2	Atmlo2-8 (pmr2-2)	G66R	TM2	n.a.	EMS	yes	Consonni et al. 2006
76	TaMLO-A1		P325L	3IC		EMS/TILLING		
Tc	TaMLO-A1		A354V	TM6		EMS/TILLING		
27	TaMLO-B1		G296E	TM5		EMS/TILLING		
76	TaMLO-B1		T297I	3IC		EMS/TILLING		
76	TaMLO-B1		R313W	3IC		EMS/TILLING		
76	TaMLO-B1		S315N	3IC		EMS/TILLING		
27	TaMLO-B1		G319R	3IC		EMS/TILLING		
	TaMLO-B1		A320T	3IC	Ū	EMS/TILLING		Acevedo-Garcia et al.
	TaMLO-B1		T345M	TM6	Ladenza	EMS/TILLING	ou	2016
(Triticum aestivum) Ta	TaMLO-D1		V316T	3IC		EMS/TILLING		
Ta	TaMLO-D1		G319R	3IC		EMS/TILLING		
Ta	TaMLO-D1		A320T	3IC		<b>EMS/TILLING</b>		
Ta	TaMLO-D1		P321S	3IC		<b>EMS/TILLING</b>		
Ta	TaMLO-D1		V323I	3IC		EMS/TILLING		
Та	TaMLO-D1		P335L	3IC		<b>EMS/TILLING</b>		
Ta	TaMLO-D1		T345M	TM6		EMS/TILLING		
Тс	TaMLO-A1	a	aberrant splicing variants and T78K	2IC	n.a.	CRISPR-Cas9		Wang et al. 2014

#### b) ...through artificial mutations

#### i) ... obtained with mutagens

However, the problem of many cultivated species is the lack of genetic diversity. For this reason, mutagenesis programs aimed at obtaining new mutants became very popular from the 1950's onwards. In 1995, the number of commercial mutant varieties reached 484 [60]. The mutagens used are grouped into two broad categories, namely chemical and physical mutagens [61]. Among the most commonly used physical mutagens there are X- and  $\gamma$ -rays, also called ionizing radiations because of the impact they have [60]. Chemical mutagens frequently used are alkylating agents, like EMS, MNU, ENU, azide, hydroxylamine, nitrous acid, acridines and base analogues. The use of chemical mutagens is preferred when point mutations are desired, while the physical mutagens usually induce larger deletion, like chromosomal aberration or rearrangements [60].

In Chapter 7, we describe the development of an EMS mutant population of tomato cv Micro-Tom in which a PM resistant plant was discovered. The resistance observed is of the *mlo*-type because associated with the loss-of-function of the *SIMLO1* gene.

Normally, the identification of individuals with a target mutation takes time because it involves the screening of large populations. TILLING (Targeting Induced Local Lesions IN Genomes) is a powerful approach that integrates chemical mutagenesis with a high throughput detection method to identify single nucleotide mutations in a specific region of a gene of interest [62]. Moreover, although until recently no TILLING-derived crop variety has been released commercially, they represent a great advantage for plant breeding (especially in Europe) since these varieties will be considered non-transgenic [63]. A very recent paper showed the application of this technology to generate hexaploid bread wheat lines with enhanced resistance to the PM disease without affecting growth and development [64].

Looking at all the *mlo*-mutant alleles in Table 1, the highest number is found in barley (33), followed by wheat (16), petunia (2), pea (3) and tomato (1) [64-67], [68] and chapter 7].

These mutants are obtained either with ionizing radiations or with chemical agents, the latter being the predominant method. EMS (ethyl methane sulfonate) is by far the chemical mutagen most commonly used since twenty-nine *mlo*-mutants were obtained with it. Eight mutants were obtained with NaN<sub>3</sub> (sodium azide), four with iso-PMS, and two with ENU (Table 1). Ionizing radiation, particularly X- and  $\gamma$ -rays were used only in barley to obtain four and two mutants, respectively (Table 1). The type of mutations consists of missense mutations, where a single codon is altered to encode a different amino acid, nonsense mutations, where a premature stop codon is introduced producing an incomplete protein product, and aberrant splicing variants, translated in truncated proteins or missing certain domains.

It was observed in Humphry et al. (2011) that the majority of barley mutants is characterized by single amino acid substitutions, while, in pea, *mlo*-mutants derive from more dramatic

changes of the PsMLO1 protein. This observation still holds true, considering the enlarged panel of *mlo*-mutants, like tobacco, cucumber, rose and tomato. This might be an indication of different evolutionary strategies of these plant species.

The large majority of the mutations of Table 1 are found in the second (21) and third (23) cytoplasmic domains, which have already been identified as relevant regions for the MLO proteins acting as PM-susceptibility factors (see also Figure 3) [69]. Transmembrane (TM) regions are additional sites of loss-of-function mutations in 24 cases, with the predominant occurrence in the sixth transmembrane (7), indicating that TM domains harbor important sites for protein conformational changes.

		I	II
AtMLO2	1 MA-DOVKERTLEETSTWAV	AVVCFVLLFISIVL <mark>E</mark> HSIHKIGTWFKKKHKQALFEALEKV	KAELMLLGFISLLLTIGOT
AtML06		avvcfvlllisivi <mark>e</mark> klihkigswfkkknkkalyealekv	
AtML012		AVVCFVLLFISIMI <mark>E</mark> YFLHFIGHWFKKKHKKALSEALEKV	
PsML01	1 MAEEGVKERT <mark>L</mark> EE <mark>TPTW</mark> AV	AVVCLVLLAVSILI <mark>E</mark> HIIHVIGKWLKKRNKNALYEALEKI	KG <mark>ELM</mark> LL <mark>G</mark> FI <mark>S</mark> LLLTVFQD
MtML01		AVVCFVLLAISIVI <mark>E</mark> HIIHAIGKWFKKKNKNALYEALEKV	
LjMLO1		AVVCFVMLAISIII <mark>E</mark> HGIEAIEKWLEKRHKKALHEAVEKI	
CaMLO2		avvcfillaisixi <mark>e</mark> qimhhlgewllkkhkkplyealeki	
SIML01		AVVCF <mark>I</mark> LLAISIFI <mark>E</mark> QIIHHIGEWLLEKRKKSLYEALEKI	
NtMLO1		AAVCFILLAISIFI <mark>E</mark> QIIHHLGEWLLKKHKKPLYEALEKI	
SmMLO1		AVVCFILLAISIFI <mark>E</mark> QIIHHLGEWLLEKHKKPLHEALEKI	
CsaMLO8 CsaMLO1		AVVCFFLVVISIFI <mark>E</mark> HVIHLTGKWLEKRHKPALVEALEKV AVVCFVLLVISIFI <mark>E</mark> YSLHLIGHWLKKRHKRALFEALEKI	
MdMLO19		AVVCFVLLVISIFIEISLALIGAWLKKRAKRALFEALEKI AVVCFVLVLISILIEYFIHLIGKWLKKRNKRALYEALEKI	
TaMLO Bla		ALVFAVMIIVSVLL <mark>E</mark> HALHKLGHWFHKRHKNALAEALEKI	
TaMLO A1b		ALVFAVMIIVSVLL <mark>E</mark> HALHKLGHWFHKRHKNALAEALEKM	
TaMLO1-D1		ALVFAVMIIVSVLL <mark>E</mark> HALHKLGHWFHKRHKNALAEALEKI	
HVMLO		avvfaamvl <mark>vs</mark> vlm <mark>e</mark> hglhklghwfqhrhkkalwealekm	
OsMLO2		AVVCAVIVLVSVAM <mark>E</mark> HGLHKLGHWFHKREKKAMGEALEKI	
			_
AtMLO2		AEEAKKYGKKDAGKKDDGDGDKPGRRLLLELAESYIH	
AtML06		SEEARKYGKKDVPKEDEEENLRRKLLQLVDSLIF	
AtML012		HQEIAKYGKDYIDDGRKILEDFDSNDFYSF	
PsMLO1		SNTKAKAKSDESLDYKTNNDRKLLEYFDPIF	
MtMLO1 LjMLO1		PKTKT-ASNDENSESE-NHDRKLLEYFDPNF SEKKKGPEGY	
CaMLO1		DEDVK	
SIMLO1		KEDAK	
NtML01		DEAAK	
SmMLO1		 QEDDKP	
CsaML08		- RAKTGVKVAKNSRLRLLEFLDPDYGS	
CsaML01		EREDEMNKEVEKSVEHLGLNRRRLLHLLGNGESFR	
MdMLO19		KQEVKSDKNEDKSSVSDDNARRRLLSALDSSGGG	
TaMLO_B1a		-PPGSVKSKY	
TaMLO_A1b	81 PI-SGICISQKAASIMRPCKV	-EPGSVKSKY	KDYYCAKEGK
TaMLO1-D1	81 PI-SGICISEKAASIMRPCSL	PPGSVKSKYKD-Y	YCAKKGK-
HVMLO		GTEGRKPSKY	
OsMLO2		GQDIVKGLKGKKDHRRRLLWYTGEEESHR	RSLAGAAGEDI <mark>C</mark> AQSGK-
AtMLO2	152 VAFV <mark>S</mark> AYGIHQ <mark>L</mark> HIFIFVLAV	/ <mark>H</mark> VVYCIVTYAFGKIKMRT <mark>W</mark> KS <mark>WE</mark> EETKTIEYQYSNDPER	FRFARDTSFGRRHLNFWSK
AtML06	149 VAFV <mark>S</mark> AYGMHQ <mark>L</mark> HIFIFVLAV	C <mark>H</mark> VIYCIVTYALGKTKMRR <mark>W</mark> KK <mark>WE</mark> EETKTIEYQYSHDPER	FRFARDTSFGRRHLSFWSK
AtML012		F <mark>H</mark> VLYCIITYALGKTKMKK <mark>W</mark> KS <mark>WE</mark> RETKTIEYQYANDPER	
PsML01		F <mark>H</mark> ILQCIITLTLGRIKMRK <mark>W</mark> KT <mark>WE</mark> DETRTVEYQFYNDPER	
MtML01		F <mark>H</mark> ILQCIITLALGRFKMRR <mark>W</mark> KK <mark>WE</mark> DETRTVEYQFYNDPER	
LjMLO1		F <mark>H</mark> ILQCITTLALGRTRMAM <mark>W</mark> KK <mark>WE</mark> EETKTLEHQFDNDPER	
CaMLO2		A <mark>H</mark> VLYCIATFALGRLKMRK <b>W</b> RA <mark>WE</mark> DETKTIEYQFYNDPER	
SIMLO1		A <mark>H</mark> VLYCIATFALGRLKMRK <mark>W</mark> RA <mark>WE</mark> DETKTMEYQFYNDPER	
NtMLO1		A <mark>H</mark> VLYSIATFALGRLKMRKWRAWEEETKTIEYQFYNDPER	
SmMLO1 CsaMLO8		A <b>H</b> VLYCIATFALGRLKMRK <mark>WRAME</mark> DETKTIEYQFYNDPER F <b>H</b> VLYCIITLAFGRT <mark>K</mark> MSK <mark>WKAME</mark> DETKTIEYQYYNDPAR	
CsaML08 CsaML01		FHVLICTITLAFGKI <b>K</b> MSKWKAWEDEIKTIEIQIINDFAF FHVLYCVLTYALARAKMRS <mark>W</mark> KT <mark>WE</mark> KETKTAEYQFSHDPER	
MdMLO19		FHVLICVLIIALARARMKSWRINGELEIKIAEIOFSHDFER FHVLYCITTLVLGRAKMRKWKTWELEIKIAAYQFSHDPER	
TaMLO Bla		FHVETCITTEVEGRAGMARTMEDETRIATOFS DIE F FHVTYSVIIMALSRLKMRTWKKWETETASLEYQFANDPAR	
TaMLO A1b		FHVTYSVIIMALSRLKMRT <mark>W</mark> KK <mark>WE</mark> TETASLEYQFANDPAR	
TaMLO1-D1		F <b>HV</b> TYSVIIMALSRLKMRT <mark>W</mark> KK <mark>WE</mark> TETXSLEYQFANDPAR	
HVMLO	119 VALM <mark>S</mark> TGSLHQ <mark>L</mark> HVFI <mark>F</mark> VLAV	F <mark>H</mark> VTYSVITIALSRLKMRT <mark>W</mark> KK <mark>WE</mark> TETTSLEYQFANDPAR	FR <b>FT</b> HQT <mark>S</mark> FVKRHLGL-SS
OsMLO2	144 VALM <mark>S</mark> SGGMHQ <mark>LHIFIFVLA</mark> V	F <mark>HVTYCVITMALG</mark> RLKMKK <mark>W</mark> KK <mark>WE</mark> LETNSLEYQFANDPSR	RFRFTHQTSFVKRHLGL-SS

		IV
AtMLO2	232	
AtML06	229	STITLWIVCFFRQFFRSVTKVDYLTLRHCFIMAHLAPGSDARFDFRKYIQRSLEEDFKTIVEINPVIWFIAVLFLLTNTN
AtML012	223	STFTLWITCFFROFFGSVTKVDYLTLRHGFIMAHLPAGSAARFDFQKYIERSLEQDFTVVVGISPLIWCLAVLFILTNTH
PsML01	227	SFILLWIVSFFRQFGGSISRV <mark>UY</mark> MALRHGFIMAHLPPGHDAQFDFQKYISRSIEEDFKVVVGISPTIWLFTVLFLLTNTH
MtML01	225	SPISLWIVCFFRQFFGSISRVDYLALRHGFIMAHLAPGNDAEFDFQKYISRSLEKDFKVVVGISPTIWFFAVLFLLTNTH
LjMLO1	198	SPISLWIVS <mark>FFRQF</mark> YG <mark>S</mark> VDKV <mark>DY</mark> MVLRHGFIIAHLAPGSESKFD <mark>FQKYISR</mark> SVDEDEKVVVGISPTVWFFAVLILLINTH
CaMLO2 S1MLO1	192	SPVMLWIVCFFRQFFSSVAKVDYLTLRHGFMMAHLTPQNQENFDFQIYINRAVDKDFKVVVGISPALWLFTVLYFLSTTD
NtML01	186 186	SPVLLSIVCFFRQFFSSVAKVDYLTLRHGFMAHLTPQNQNNFDFQLYINRAVDKDEKVVVGISPALWLFTVLYFLTTTD SPVLLWIVCFFRQFFSSVAKVDYLTLRHGFMAHLTPQNQENFDFQIYINRAVEKDEKFVVEISPALWLFTVLYFLTTTN
SmML01	192	SFVLENFVCFFNQFFSSVARVDIELENNGFMANETFQNQENEDFQIIINNAVERDENFVDISFALMEFIVEIFEITEN SPLLLWIVCFFRQFFSSVARVDYLTLRHGFMMAHLTPENQKNFDFQIYINNAVDRDFKVVVGISPALWLFTVLYFLTTTD
CsaML08	222	TPISLWIVCFFRQFFGSVTKVDYMTLRHGFIVAHLAPGSEVKFDFHKYIXRSLEDDFKVVVGISPAMWLFAVLFILTNTN
CsaML01	230	NPALMWIVCFFROFVRSVPKVDYLTLRHGFIMAHLAPQSHTQFDFQKYINRSLEEDFKVVVGISPPIWFFAVLFLLSNTH
MdMLO19	229	SPISLWIVCFFRQFVRSVPKVDYLTLRHGFIAAHLAPQSQTKFDFQKYINRSLEEDFKVVVGISPTIWLFAVLILMSNTH
TaMLO_B1a	199	
TaMLO_A1b	199	TPGVRWVVA <mark>FFRQFFRS</mark> VTKV <mark>DY</mark> LIL <mark>RAGF</mark> INAHLSQNSKFD <mark>F</mark> HK <mark>Y</mark> IK <mark>R</mark> SMEDD <b>F</b> KVVVGISLPLWAVAILTLFLDID
TaMLOI-D1 HvMLO	199 198	TPGIRWVVAFFROFFRSVTKVDYLTLRAGFINAHLSHNSKFDFHKYIKRSMEDDFKVVVGISLPLWCVAILTLFLDID TPGIRWVVAFFROFFRSVTKVDYLTLRAGFINAHLSQNSKFDFHKYIKRSMEDDFKVVVGISLPLWGVAILTLFLDIN
OsMLO2	223	
0011202	220	
		V VI
AtMLO2	312	GLRSYL <mark>M</mark> LPFI <b>PL</b> VVILIV <mark>G</mark> TKLEVIITKLGLRIQEKGDVVRGAPVV_PGDDL <mark>FWP</mark> GK <mark>P</mark> RFILFLIHLVL <mark>FTNAF</mark> QLAFF
AtML06		GLNSYLWLPFIPFIVILIVCTKLQVIITKLGLRIQEKGDVVKGTPLVOPGDHFFWPGRPRFILFLIHLVLFTNAFQLAFF
AtML012	303	GWDSYL <mark>W</mark> LPFL <mark>PL</mark> IVILIV <b>G</b> AKL <mark>OMIISKLGLRIQEKGDVVKGAPVVEPGDDL<mark>FWF</mark>GR<mark>P</mark>RFI<mark>L</mark>FLIHLVLFTNAFQLAFF</mark>
PsML01		GWYSY <mark>YW</mark> LPFL <mark>P</mark> LIVILLV <mark>G</mark> AKLQMTITKMGLRIQDRGEVIKGAPVVEPGDHL <mark>FWE</mark> NR <mark>P</mark> HLL <mark>L</mark> FTIHLVLFQNAFQLAFF
MtML01		GWYSSY <mark>M</mark> LPFL <mark>P</mark> LIIILLV <mark>GAKL</mark> QMIITKMGLRIQDRGEVIKGAPVVEPGDHL <mark>FWF</mark> NS <mark>P</mark> NLLFIIHLVLFQNAFQLAFF
LjMLO1 CaMLO2	278	GWHSYLMLPFIPLIIILLVGTKLQMIITNMGLKIQERGDVIKGAPLVEPGDDLFWFNRPRLILSLVHLVLFQNAFQLAFF GVYSYLMVPFVPLIIILLVGTKLQMIITEMGVRISERGDIVKGVPVVEIGDHLFWFNRPGLVLFFINFVLFQNAF
SIML01		RUSSILWYFY YN HITTHLYGIRHOMITHEMAYRISERODIYRGYFY VEIDDHLFWERREGLUFFIRWYLFORDU GWFF RLYSYL <mark>W</mark> YPFIPLVIILLY <mark>GTKL</mark> QMIITEMGYRISERGDIYRGYPYVEIGDHL <mark>FWE</mark> RR <mark>P</mark> ALVL <mark>F</mark> LINFYL <mark>F</mark> QNAFQVAFF
NtML01		GLYSYLWYFIILWYFIHLYGTHLEMIIAEMGVRISKRGDIVRGYFYYDIGDHLFWFNRPGFYLFLINFYLFQNAFQVAFF
SmMLO1		GLYSYLWVPFVPLIIILLVGTKLQMIITEMGVRISERGDIVKGVPVVETGDHLFWFNRPGLVLFLINFVLFQNAFQVAFF
CsaMLO8		GWYSYL <mark>W</mark> LPFISLIIILLV <mark>GTKL</mark> HVIITHMGLTIQERGHVVKGVPVVOPRDDL <mark>FWF</mark> GR <mark>P</mark> QLI <mark>F</mark> LIHFVL <mark>FMNAF</mark> QLAFF
CsaML01		GWRAYL <mark>M</mark> LPFI <mark>P</mark> LIILLLI <mark>G</mark> TKLQVIITKMALRIQERGEVVKGVPVVEPGDDL <mark>FWP</mark> NR <mark>P</mark> RLI <mark>L</mark> YLINFVLFQNAFQVAFF
MdMLO19		GSRSYLWLPFVPLVMILVVGTKLQVIITKMGLKLSERGEVVRGTPLVEPGDHLFWPNNPRLLUVIIHFVLFQNAFALAFF
TaMLO_B1a TaMLO A1b	277	GIGTLTWISFIPIVILLCVCTKLEMIIMEMALEIQDRASVIKGAPVVEPSNKFFWFHRPDWVLFFIHLTLFQNAFQMAHF GIGTLTWVSFIPLIILLCVCTKLEMIIMEMALEIQDRSSVIKGAPVVEPSNKFFWFHRPDWVLFFIHLTLFQNAFQMAHF
TaMLO D1		GIGTLTWISFIPLVILLCVGTKLEMIIMEMALEIODRASVIKGAPVVEPSNKFFWEHRPDWVLFFIHLTLFONAFOMAHF
HVMLO		GVGTLIWISFIPLVILLCVGTKLEMIIMEMALEIQDRASVIKGAPVVEPSNKFFWFHRPDWVLFFIHLTLFONAFQMAHF
OsMLO2	301	GFGTLIWISFVPLVILVLVGTKLEMVIMEMAQEIQDRATVIKGAPVVEPSNKYFWPNRPDWVLFFIHLILFQNAFQMAHF
		VII
AtMLO2	392	A <mark>N</mark> STYEFNLNNCFHESTADVVIRLVVGAVVQILCSYV <mark>TLPL</mark> YALVTQ <mark>MG</mark> SKMKPTVFNDRVATALKKW
AtML06	389	A <mark>N</mark> STYEFNLNN <mark>C</mark> FHESTADVVIRLVVGAVVQILGSYV <mark>TLPL</mark> YALVTO <mark>MS</mark> SKMKPTVFNDRVATALKKW V <mark>N</mark> STYEFGLKN <mark>C</mark> FHESRVDVIIRISIGLLVQILGSYV <mark>TLPL</mark> YALVTOMSSKMKPTVFNERVATALKSW
AtMLO6 AtMLO12	389 383	A <mark>N</mark> STYEFNLNN <mark>C</mark> FHESTADVVIRLVVGAVVQILGSYV <mark>TLPL</mark> YALVTQ <mark>MG</mark> SKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRVDVIIRISIGLLVQILGSYVTLPLYALVTQMGSKMKPTVFNDEVATALKSW VNSTYEFTLKNCFHHKTEDIAIRITMGVLIQVLGSYITLPLYALVTQMGTSMNPTIFNDRVANALKKW
AtMLO6 AtMLO12 PsMLO1	389 383 387	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILCSYVTLPLYALVTCMCSKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRVDVIIRISIGLLVGILSYVTLPLYALVTOMCSKMKPTVFNDRVATALKSW VNSTYEFILKNCFHHKTEDIAIRITMGVLIQVLCSYITLPLYALVTOMCTSMPTIFNDRVANALKKW ARSTYEFSITSCFHKTTADSVIRITVGVVIQTLSYVTLPLYALVTCMCSMKFTFFNDRVATALKNW
AtMLO6 AtMLO12 PsMLO1 MtMLO1	389 383 387 385	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILCSYVTLPLYALVTCMGSKMKPTVFNDRVATALKKW VMSTYEFGLKNCFHESRVDVIIRISIGLLVQILCSYVTLPLYALVTQMGSKMKPTVFNERVATALKSW VMSTYEFTLKNCFHHKTEDIAIRITMGVLLQVLCSYTTLPLYALVTQMGSTM.PTIFNDRVANALKKW ARSTYEFSITSCFHKTTADVVIRVSVGILLQFLCSYVTLPLYALVTQMGSTMKPTIFNDRVATALKNW SMSTYEFSINSCFHKTTADVVIRVSVGILQFLCSYVTLPLYALVTQMGSTMKPTIFNDRLATALKNW
AtMLO6 AtMLO12 PsMLO1	389 383 387 385 358	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILCSYVTLPLYALVTCMCSKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRVDVIIRISIGLLVGILSYVTLPLYALVTOMCSKMKPTVFNDRVATALKSW VNSTYEFILKNCFHHKTEDIAIRITMGVLIQVLCSYITLPLYALVTOMCTSMPTIFNDRVANALKKW ARSTYEFSITSCFHKTTADSVIRITVGVVIQTLSYVTLPLYALVTCMCSMKFTFFNDRVATALKNW
AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1	389 383 387 385 358 358	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILCSYVTLPLYALVTCMGSKMKPTVFNDRVATALKKW VMSTYEFGLKNCFHESRVDVIRISIGLLVQILCSYVTLPLYALVTCMGSKMKPTVFNERVATALKSW VMSTYEFJLKNCFHHKTEDIAIRITMGVLQVLCSYITLPLYALVTCMGSKMKPTVFNERVATALKSW ANSTYEFSITSCFHKTTADVIRITVGVVIQILSYVTLPLYALVTCMGSTMKPTIFNERVATALKSW SMSTYEFSITSCFHKTTADNVIRVSVGILLGFLSYVTLPLYALVTCMGSTMKPTIFNERVATALKSW VMSWKFGFSCFHKSTADVVIRITLGVVTQVLCSYVTLPLYALVTCMGSTMKPTIFNERVATALKSW VMSWWKFGFPSCFHKNAADLAIRLTMGVIQVHCSYVTLPLYALVTCMGSSMKPIFGDNVATALKSW
AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1 CaMLO2 S1MLO1 NtMLO1	389 383 387 385 358 358 352 346 346	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILSYVTLPTYALVTQMGSKMKPTVFNDRVATALKKW VNSTYEFILKNCFHESRVDVIRLSIGLVQILSYVTLPTYALVTQMGSKMKPTVFNDRVATALKSW VNSTYEFILKNCFHKTEDIALTIMGVIQULSYTLPTYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSITSCFHKTTADSVIRITVGVVIQTLSYVTLPTYALVTQMGSTMKPTIFNDRVATALKSW SNSTYE-FSINSCFHKTTADSVIRITVGVVIQTLSYVTLPTYALVTQMGSTMKPTIFNDRVATALKSW NNSAYDFKINSCFHKTTADNVIRVSVGILUGFLSYVTLPTYALVTQMGSTMKPTIFNDRVATALKSW VNSWWKFGFPSCFHKNAADLAIRLTMGVIQVLSYVTLPTYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHKNAADLAIRLTMGVIQVHSYVTLPTYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHKNAADLAIRLTMGVIQVHSYVTLPTYALVTQMGSSMKPIIFGDNVATALRSW
AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 SlMLO1 NtMLO1 SmMLO1	389 383 387 385 358 352 346 346 352	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRVDVIRISIGLVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSW VNSTYEFILKNCFHHKTEDIAIRITMGVIQILGSYITLPIYALVTQMGSTMFPTIFNDRVATALKSW ARSTYEFSINSCFHKTTADSVIRITVGVIQILGSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSINSCFHKTTADSVIRITVGVIQILGYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW ANSACDNDFKINSCFHKTTADNVIRVSVGILIGFLSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW VNSWWKFGFPSCFHKNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSMKPIIFGDNVATALKSW VNSWWKFGFPSCFHKNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALKSW VNSWWKFGFPSCFHKNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHKNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHKNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHKNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHKNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW
AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 SIMLO1 NtMLO1 SmMLO1 CsaMLO8	389 383 387 385 358 352 346 346 352 324	ARSTYEFOLNNCFHESTADVVIRLVVGAVVQILCSYVTLPLYALVTCMGSKMKPTVFNDRVATALKKW VNSTYEFOLKNCFHESRVVVIRISIGLVQILCSYVTLPLYALVTCMGSKMKPTVFNDRVATALKSW VNSTYEFILKNCFHHKTEDIAIRITMGVLIQULCSYITLPLYALVTCMGSTMKPTIFNDRVATALKSW SNSTYEFSITSCFHRTTADNVIRVSVGILLQFLCSYVTLPLYALVTCMGSTMKPTIFNDRVATALKSW SNSTCNDFKINSCFHRSTADVVIRUTLGVVTQVLCSYVTLPLYALVTCMGSTM.PTIFNDRVATALKSW VNSWFGFSCFHRSTADVVIRUTLGVVTQVLCSYVTLPLYALVTCMGSTMKPTIFNDRVATALKSW VNSWWKFGFPSCFHKNAADLAIRLTMGVIQVHCSYVTLPLYALVTCMGSSMKPIIFGDNVATALKSW VNSWWKFGFPSCFHKNAADLAIRLTMGVIQVHCSYVTLPLYALVTCMGSSMKPIIFGDNVATALKSW VNSWWKFGFPSCFHKNAADLAIRLTMGVIQVHCSYVTLPLYALVTCMGSSMKPIIFGDNVATALKSW VNSWWKFGFPSCFHKNAADLAIRLTMGVIQVHCSYVTLPLYALVTCMGSSMKPIIFGDNVATALKSW VNSW
AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 SlMLO1 NtMLO1 SmMLO1 CsaMLO8 CsaMLO1	389 383 387 385 358 352 346 346 352 324 390	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKW VNSTYEF7LKNCFHESRVDVIRISIGLVQILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSW VNSTYEF7LKNCFHEKTEDIAITIMGVIQILSYTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSITSCFHKTTADSVIRITVGVIQILSYTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYE-FSINSCFHKTTADSVIRIVGVIQILSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSACDDFKINSCFHKTTADSVIRIVGVIQILSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW VNSW
AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 SIMLO1 NtMLO1 SmMLO1 CsaMLO8	389 383 387 385 358 352 346 346 352 324 390	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRVDVIRISIGLVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSW VNSTYEFSITSCFHHTTADSVIRITVGVIQILGSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSITSCFHHTTADSVIRITVGVIQILGSYVTLPIYALVTQMGSTMKPTIFNDRVATALKKW ANSAYEFSINSCFHHTTADSVIRITVGVIQILGSYVTLPIYALVTQMGSTMKPTIFNDRVATALKKW ANSAWKFGFPSCFHHTADDVIRITUGVTQVLGSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW VNSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSMKPIIFGDNVATALKSW VNSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW VNSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW NSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW NSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW NSWWKFGFPSCFHHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMKPIIFGDNVATALRSW NSWWKFGFPSCFHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMPFIIFGDNVATALRSW NSWWKFGFPSCFHNAADLAIRITMGVIIQVHSYVTLPIYALVTQMGSSMPFIIFGDNVATALRSW ANTTYAFKWMGCFHORVEDIVIRISMGVIQVISVTLPIYALVTQMGSSMFFIIFDNVATALRSW ANTTYAFKWMGCFHORVEDIVIRISMGVIQVISVTLPIYALVTQMGSSMFFIIFNDRVATALRSW ANTTYAFKWMCCFHORVEDIVIRISMGVIQVISVTLPIYALVTQMSSTMKPTIFNDRVATALRSW ANTTYA
AtMLO6 AtMLO12 PsMLO1 LjMLO1 CaMLO2 SlMLO1 NtMLO1 SmMLO1 CsaMLO8 CsaMLO1 MdMLO19	389 383 387 385 358 352 346 352 324 390 389 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPLYALVTQMGSKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRDVVIRISIGLVQILGSYVTLPLYALVTQMGSKMKPTVFNDRVATALKSW VNSTYEFSINSCFHKTADSVIRITVGVIQILGSYVTLPLYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSINSCFHKTADSVIRITVGVIQILGSYVTLPLYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSINSCFHKTADDVIRUSVGILIGFLSYVTLPLYALVTQMGSTMKPTIFNDRVATALKSW SNSTWKFGFPSCFHKTADDVIRUSVGILIGFLSYVTLPLYALVTQMGSTMKPTIFNDRVATALKSW SNSTWKFGFPSCFHKNADLAIRITMGVIIQVHSYVTLPLYALVTQMGSTMKPTIFODVATALKSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVHSYVTLPLYALVTQMGSSMKPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVHSYVTLPLYALVTQMGSSMKPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVHSYVTLPLYALVTQMGSSMKPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVHSYVTLPLYALVTQMGSSMKPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVHSYVTLPLYALVTQMGSSMKPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVHSVTLPLYALVTQMGSSMKPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVISVTLPLYALVTQMGSSMKPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVISVTLPLYALVTQMGSSMPIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVISVTLPLYALVTQMGSSMPFIIFGDNVATALRSW SNSWWKFGFPSCFHKNADLAIRITMGVIIQVISVTLPLYALVTQMGSSMFFIIFGDNVATALRSW ANTYYAFKWMGCFHQRVEDIVIRISMGVIQVISVTLPLYALVTQMGSSMFFIIFDNVATALRSW ANTYYAFKWMGCFHQRVEDIVIRISMGVIQVISVTLPLYALVTQMSSMFFIIFNDRVATALRSW ANTYYAFKWMGCFHQRVEDIVIRISMGVIQVISVTLPLYALVTQMSSTMFPTIFNDRVATALRSW ANTYYA
AtMLO6 AtMLO12 PSMLO1 MtMLO1 CAMLO2 SIMLO1 NtMLO1 SiMLO1 CsaMLO8 CsaMLO1 MdMLO19 TaMLO_A1b TaMLO_D1	389 383 387 358 352 346 352 324 390 389 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRDVVIRISIGLVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSW VNSTYEFSINSCFHKTEDIAIRITMGVIQULGSYITLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSINSCFHKTEDIAIRITMGVIQULGSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSINSCFHKTEDNVIRUSVGILLGFLSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNST
AtML06 AtML012 PsML01 LjML01 CaML02 SlML01 NtML01 SsML01 CsaML08 CsaML01 CsaML08 TaML0_19 TaML0_191 TaML0_11 TaML0_11 HvML0	389 383 387 385 358 352 346 352 324 390 389 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILCSYVTLPIYALVTCMCSKMKPTVFNDRVATALKKW VNSTYEFILKNCFHESRVVIIRISIGLLVQILCSYVTLPIYALVTCMCSKMKPTVFNDRVATALKSW VNSTYEFILKNCFHHKTEDIAIRITMGVIQULCSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW SMSTYEFSITSCFHKTTADNVIRTVSVGILLQFLCSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW SMSTCNDFKINSCFHRSTADVVIRLTLGVVTQULSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW VNSWCNDFKINSCFHRSTADVVIRLTLGVVTQULSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW VNSW
AtMLO6 AtMLO12 PSMLO1 MtMLO1 CAMLO2 SIMLO1 NtMLO1 SiMLO1 CsaMLO8 CsaMLO1 MdMLO19 TaMLO_A1b TaMLO_D1	389 383 387 385 358 352 346 352 324 390 389 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKW VNSTYEFGLKNCFHESRDVVIRISIGLVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSW VNSTYEFSINSCFHKTEDIAIRITMGVIQULGSYITLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSINSCFHKTEDIAIRITMGVIQULGSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNSTYEFSINSCFHKTEDNVIRUSVGILLGFLSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSW SNST
AtML06 AtML012 PsML01 LjML01 CaML02 SlML01 NtML01 SsML01 CsaML08 CsaML01 CsaML08 TaML0_19 TaML0_191 TaML0_11 TaML0_11 HvML0	389 383 387 385 358 352 346 352 324 390 389 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILCSYVTLPIYALVTCMCSKMKPTVFNDRVATALKKW VNSTYEFILKNCFHESRVVIIRISIGLLVQILCSYVTLPIYALVTCMCSKMKPTVFNDRVATALKSW VNSTYEFILKNCFHHKTEDIAIRITMGVIQULCSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW SMSTYEFSITSCFHKTTADNVIRTVSVGILLQFLCSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW SMSTCNDFKINSCFHRSTADVVIRLTLGVVTQULSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW VNSWCNDFKINSCFHRSTADVVIRLTLGVVTQULSYVTLPIYALVTCMCSTMKPTIFNDRVATALKSW VNSW
AtMLO6 AtMLO12 PSMLO1 MtMLO1 LjMLO1 CAMLO2 SIMLO1 NtMLO1 SIMLO1 CSAMLO8 CSAMLO8 CSAMLO1 MdMLO19 TAMLO_B1A TAMLO_A1b TAMLO_D1 HvMLO OSMLO2	389 383 387 358 352 346 342 324 390 389 357 357 357 356 381	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRDVIIRISIGLVQILGSYTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSTYEFILKNCFHHKTEDIAIRITMGVIQUGSYTLPIYALVTQMGSTMFPTIFNDRVATALKSM NSTYESINSCFHKTADSVIRITVGVIQILSYVTLPIYALVTQMGSTMFPTIFNDRVATALKSM NSTYESINSCFHKTADSVIRITVGVIQILSYVTLPIYALVTQMGSTMFPTIFNDRVATALKSM NSSTWEFSINSCFHKTADDVIRUSVGILIGFLSYVTLPIYALVTQMGSTMFPTIFNDRVATALKSM NSSTWEFSINSCFHKTADDVIRUSVGILIGFLSYVTLPIYALVTQMGSTMFPTIFNDRVATALKSM NSSWEFSINSCFHKTADDVIRUSVGILIGFLSYVTLPIYALVTQMGSTMFPTIFNDRVATALKSM NSSWEFSIPSCFHKNADLAIRITMGVIIQVESYVTLPIYALVTQMGSMKPIFGDNVATALRSM NSSWEFSIPSCFHKNADLAIRITMGVIIQVESYVTLPIYALVTQMGSMKPIFGDNVATALRSM NSSWEFSIPSCFHKNADLAIRITMGVIIQVESYVTLPIYALVTQMGSMKPIFGDNVATALRSM NSSM
AtML06 AtML012 PsML01 MtML01 CaML02 SIML01 SIML01 SiML01 SiML01 CsaML08 CsaML01 TaML019 TaML019 TaML02 HvML07 OsML02 AtML02	389 383 387 385 358 358 358 358 346 352 324 390 387 357 357 356 381 460	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKM VNSTYEFGLKNCFHESRVVIIRISIGLVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHEXTADSVIRITVGVIQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM SNSTYEFSINSCFHEXTADSVIRITVGVIQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM SNSTYEFSINSCFHEXTADNVIRUSVGILIGFLSYVTLPIYALVTQMGSKMKPTFNDRVATALKSM SNST
AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1 CaMLO2 S1MLO1 NtMLO1 SmMLO1 CsaMLO3 TaMLO_B1a TaMLO_D1 HvMLO OSMLO2 AtMLO2 AtMLO2 AtMLO6	389 383 387 358 358 346 346 346 352 324 390 389 357 357 357 356 381 460 457	ARSTYEFNLNNCFHESTADVVIRLVVGAVVGILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESROVVIRISIGLVGILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSTYEFSITSCFHKTADSVIRITMGVLQILSYTTLPIYALVTQMGSKMKPTVFNDRVATALKSM NSTYEFSITSCFHKTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM NSTYEFSITSCFHKTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM NSSAWEFGFPSCFHKTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSWWKFGFPSCFHKNADLAIRLTMGVIQVLSYVTLPIYALVTQMGSKMKPIFGDNVATALRSM VNSW
AtML06 AtML012 PsML01 MtML01 CaML02 SIML01 SIML01 SiML01 SiML01 CsaML08 CsaML01 TaML019 TaML019 TaML02 D1 HvML07 OsML02 AtML02	389 383 387 385 352 346 352 324 352 324 352 324 357 357 357 357 357 357 351 460 457 451	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRDVVIRITSIGLVQILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSTYEFILKNCFHHKTEDIAIRITMGVIQUGVISYITLPIYALVTQMGSTMKPTIFNDRVATALKSM NSTYESITSCFHHYTADSVIRITVGVIQILSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSM SNSTYEFSINSCFHHYTADSVIRITVGVIQULSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSM NSTWEFSINSCFHHYTADSVIRITVGVIQULSYVTLPIYALVTQMGSTMKPTIFNDRVATALKSM NSS
AtMLO6 AtMLO12 PSMLO1 MtMLO1 LjMLO1 CAMLO2 SIMLO1 NtMLO1 SiMLO1 CSAMLO3 CSAMLO3 CSAMLO3 TAMLO_B1A TAMLO_D1 HVMLO OSMLO2 AtMLO2 AtMLO6 AtMLO6 AtMLO12	389 383 387 385 358 352 346 352 324 352 324 357 357 357 357 357 357 351 460 457 451 455	ARSTYEFNLNNCFHESTADVVIRLVVGAVVGILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESROVVIRISIGLVGILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSTYEFSITSCFHKTADSVIRITMGVLQILSYTTLPIYALVTQMGSKMKPTVFNDRVATALKSM NSTYEFSITSCFHKTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM NSTYEFSITSCFHKTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM NSSAWEFGFPSCFHKTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSWWKFGFPSCFHKNADLAIRLTMGVIQVLSYVTLPIYALVTQMGSKMKPIFGDNVATALRSM VNSW
AtMLO6 AtMLO12 PSMLO1 MtMLO1 CAMLO2 SIMLO1 NtMLO1 SsMLO1 CsAMLO3 CsAMLO3 MdMLO19 TAMLO_B1a TAMLO_B1a TAMLO_D1 HvMLO OSMLO2 AtMLO2 AtMLO6 AtMLO6 AtMLO12 PSMLO1	389 383 387 385 352 324 352 324 390 389 357 357 357 357 357 357 351 460 457 451 455 453	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILESYVTLPIYALVTQMSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRUDVIRISIGLVQILSYTLPIYALVTQMSKMKPTVFNDRVATALKSM VNSTYEFILKNCFHHKTEDIAIRITMGVIQULSYTLPIYALVTQMSSKMKPTVFNDRVATALKSM NSTYESISTSCFHKTTADSVIRITVGVIQILSYVTLPIYALVTQMSSTMKPTIFNDRVATALKSM NSTYESISTSCFHKTTADSVIRITVGVIQILSYVTLPIYALVTQMSSTMKPTIFNDRVATALKSM NSSWKFGFPSCFHKTADDVIRUSVGILIGFLSYVTLPIYALVTQMSSTMKPTIFNDRVATALKSM NSSWKFGFPSCFHKNADLAIRITMGVIIQVESYVTLPIYALVTQMSSTMKPTIFNDRVATALKSM NSSWKFGFPSCFHKNADLAIRITMGVIIQVESYVTLPIYALVTQMSSMKPIIFGDNVATALRSM NSSWKFGFPSCFHKNADLAIRITMGVIIQVESYVTLPIYALVTQMSSMKPIIFGDNVATALRSM NSSWKFGFPSCFHKNADLAIRITMGVIIQVESYVTLPIYALVTQMSSMKPIIFGDNVATALRSM NSS
AtML06 AtML012 PSML01 MtML01 CGML02 SIML01 SIML01 SIML01 SIML01 CSAML08 CSAML08 TAML09 TAML09 TAML09 TAML00 SIML02 AtML02 AtML02 AtML02 AtML02 AtML012 PSML01 MtML01 LjML01 CGML02	389 383 387 358 352 324 352 324 390 357 357 357 357 357 357 357 357 453 440 457 451 455 453 420	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRDVVIRISIGLVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHESRDVVIRISUGLVQILGSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM NSTYEFSINSCFHENTADSVIRITVGVIQTLGSYVTLPIYALVTQMGSKMKPTFNDRVATALKSM NSTYEFSINSCFHENTADNVIRUSVGILLGFLSYVTLPIYALVTQMGSKMKPTFNDRVATALKSM NSS
AtMLO6 AtMLO12 PSMLO1 MtMLO1 LjMLO1 CaMLO2 SIMLO1 NtMLO1 SmMLO1 CsaMLO8 CsaMLO8 CsaMLO8 CsaMLO1 MdMLO19 TAMLO_A1b TAMLO_A1b TAMLO_A1b TAMLO_D1 HvMLO OSMLO2 AtMLO2 AtMLO2 AtMLO12 PSMLO1 MtMLO1 LjMLO1 LjMLO1	389 383 387 355 358 352 324 350 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKKM VNSTYEFOLKNCFHESROVVIRISIGLVQILSYVTLPIYALVTQMGSKMKPTVFNDRVATALKSM VNSTYESITSCFHKTTADSVIRITMGVLQVLSYTTLPIYALVTQMGSKMKPTVFNDRVATALKSM SNSTYESITSCFHKTTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTFFNDRVATALKSM SNSTYEFSINSCFHKTTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTFFNDRVATALKSM SNSTYEFSINSCFHKTTADSVIRITVGVIQTLSYVTLPIYALVTQMGSKMKPTFFNDRVATALKSM SNST
AtMLO6 AtMLO12 PSMLO1 MtMLO1 CAMLO2 SIMLO1 NtMLO1 SSMMLO1 CSAMLO3 CSAMLO1 CSAMLO1 TAMLO_B1a TAMLO_A1b TAMLO_D1 HVMLO OSMLO2 AtMLO2 AtMLO2 AtMLO2 AtMLO1 ZSMLO1 CAMLO1 SIMLO1	389 383 387 352 324 352 324 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILESYVTLPIYALVTQMSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRUDVIRITSIGLVQILSYTLPIYALVTQMSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHHYTADSVIRITMGVLQVLSYTLPIYALVTQMSTMKPTFINDRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQTLSYVTLPIYALVTQMSTMKPTFINDRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQTLSYVTLPIYALVTQMSTMKPTFINDRVATALKSM SNSTYEFSINSCFHHYTADSVIRITVGVIQTLSYVTLPIYALVTQMSTMKPTFINDRVATALKSM SNST
AtML06 AtML012 PsML01 MtML01 LjML01 CaML02 SIML01 SmML01 CsaML08 CsaML01 CsaML08 TaML0_B1a TaML0_D1 HvML07 OsML02 AtML02 AtML02 AtML02 AtML012 PsML01 MtML01 LjML01 CaML02 SIML01 NtML01	389 383 387 352 324 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRUDVIRISIGLVGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKSM VNSTYEFSITSCFHKTTADSVIRITVGVUGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKSM VNSTYEFSITSCFHKTTADSVIRITVGVUGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKSM SNSTYEFSITSCFHKTTADSVIRITVGVUGILSYVTLPIYALVTCMGSKMKPTFNDRVATALKSM SNSTYEFSITSCFHKTTADSVIRITVGVUGILSYVTLPIYALVTCMGSKMKPTFNDRVATALKSM SNSTYEFSITSCFHKTTADSVIRITVGVUGILSYVTLPIYALVTCMGSKMKPTFNDRVATALKSM SNS
AtMLO6 AtMLO12 PSMLO1 MtMLO1 CAMLO2 SIMLO1 NtMLO1 SSMMLO1 CSAMLO3 CSAMLO1 CSAMLO1 TAMLO_B1a TAMLO_A1b TAMLO_D1 HVMLO OSMLO2 AtMLO2 AtMLO2 AtMLO2 AtMLO1 ZSMLO1 CAMLO1 SIMLO1	389 383 387 352 358 352 324 346 352 324 324 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILESYVTLPIYALVTQMSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRUDVIRITSIGLVQILSYTLPIYALVTQMSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHHYTADSVIRITMGVLQVLSYTLPIYALVTQMSTMKPTFINDRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQTLSYVTLPIYALVTQMSTMKPTFINDRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQTLSYVTLPIYALVTQMSTMKPTFINDRVATALKSM SNSTYEFSINSCFHHYTADSVIRITVGVIQTLSYVTLPIYALVTQMSTMKPTFINDRVATALKSM SNST
AtML06 AtML012 PSML01 MtML01 LjML01 CaML02 SIML01 NtML01 SmML01 CsaML08 CsaML08 CsaML01 MdML019 TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML02 AtML02 AtML02 AtML02 AtML02 SIML01 NtML01 SiML01 CsaML03 SiML01 CsaML01 SiML019	389 383 385 358 352 358 352 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKKW VNSTYEFILKNCFHESROVIIRISIGLVGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKSW VNSTYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKRPTFNDRVATALKSW VNSTYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKRPTFNDRVATALKSW SNSTYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKPTFNDRVATALKSW SNSYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKPTFNDRVATALKSW VNSW
AtMLO6 AtMLO12 PSMLO1 MtMLO1 LjMLO1 CaMLO2 SIMLO1 NtMLO1 SIMLO1 SIMLO1 CSAMLO8 CSAMLO8 CSAMLO1 MdMLO19 TAMLO_B1A TAMLO_D1 HvMLO OSMLO2 AtMLO6 AtMLO12 PSMLO1 MtMLO1 LjMLO1 LjMLO1 CSAMLO8 SIMLO1 NtMLO1 SIMLO1 CSAMLO8 CSAMLO8 CSAMLO1 MdMLO19 TAMLO_B1A	389 383 352 346 352 324 390 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESRDVVIRLVVGAVVQILSYVTLPIYALVTQMSKMKPTVFNDRVATALKKM VNSTYEFOLKNCFHESRDVVIRLVGILSYVTLPIYALVTQMSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHKTEDIAIRITMGVLQVLSYTTLPIYALVTQMSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHKTEDIAIRITMGVLQVLSYTTLPIYALVTQMSSTMKPTIFNDRVATALKSM SNSTYEFSINSCFHKTEDIAIRITMGVLQULSYVTLPIYALVTQMSSTMKPTIFNDRVATALKSM SNSTYEFSINSCFHKTEDIAVIRVSVGILLQFLSYVTLPIYALVTQMSSTMKPTIFNDRVATALKSM SNST
AtML06 AtML012 PSML01 MtML01 C3ML01 SIML01 NtML01 SiML01 SiML01 C3ML01 G3ML01 G3ML01 TAML0_B1a TAML0_D1 HtML05 AtML02 AtML02 AtML06 AtML012 PSML01 C3ML01 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 C3ML02 SiML01 SiML01 C3ML02 SiML01 SiML01 SiML01 SiML01 SiML01 SiML01 SiML01 SiML01 SiML01 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML02 SiML03 SiML03 SiML03 SiML03 SiML03 SiML04 Si	389 383 352 358 352 352 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILESYVTLPIYALVTQMSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRUDVIRITSIGLVQILSYTLPIYALVTQMSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHHYTADSVIRITMGVIQULSYTTLPIYALVTQMSTMKPTFINBRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQULSYTTLPIYALVTQMSTMKPTFINBRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQULSYVTLPIYALVTQMSTMKPTFINBRLATALKKM ANSACDNDFKINSCFHRYTADNVIRVSVGILIGFLSYVTLPIYALVTQMSTMKPTFINBRLATALKKM ANSA
AtML06 AtML012 PSML01 MtML01 LjML01 CaML02 SIML01 NtML01 SmML01 CsaML08 CsaML08 CsaML01 MdML019 TaML0_A1b TaML0_A1b TaML0_A1b TaML02 AtML02 AtML02 AtML02 AtML02 AtML02 SIML01 NtML01 SIML01 CsaML03 SIML01 SiML01 SiML019 TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b TaML0_A1b	389 383 352 3466 352 324 390 357 357 357 357 357 357 357 357 357 357	ARSTYEFNLNNCFHESTADVVIRLVVGAVVGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKKW VNSTYEFOLKNCFHESRDVIIRISIGLVGILSYVTLPIYALVTCMGSKMKPTVFNDRVATALKSW VNSTYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKRPTFNDRVATALKSW NSTYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKRPTFNDRVATALKSW SNSTYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKRPTFNDRVATALKSW SNSTYEFSITSCFHKTTADSVIRITVGVIGTLSYVTLPIYALVTCMGSMKRPTFNDRVATALKSW SNS
AtML06 AtML012 PSML01 MtML01 C3ML01 SIML01 NtML01 SiML01 SiML01 C3ML01 G3ML01 G3ML01 TAML0_B1a TAML0_D1 HtML05 AtML02 AtML02 AtML06 AtML012 PSML01 C3ML01 SiML01 C3ML02 SiML01 TAML0_B1a TAML0_B1ba TAML0_B1ba TAML0_B1ba TAML0_B1ba	3893383353535353535353535353535353535353	ARSTYEFNLNNCFHESTADVVIRLVVGAVVQILESYVTLPIYALVTQMSKMKPTVFNDRVATALKKM VNSTYEFILKNCFHESRUDVIRITSIGLVQILSYTLPIYALVTQMSKMKPTVFNDRVATALKSM VNSTYEFSINSCFHHYTADSVIRITMGVIQULSYTTLPIYALVTQMSTMKPTFINBRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQULSYTTLPIYALVTQMSTMKPTFINBRVATALKSM SNSTYESINSCFHHYTADSVIRITVGVIQULSYVTLPIYALVTQMSTMKPTFINBRLATALKKM ANSACDNDFKINSCFHRYTADNVIRVSVGILIGFLSYVTLPIYALVTQMSTMKPTFINBRLATALKKM ANSA

FIGURE 3.

At.MLO2	520	HHEHQFWDPESQHQEAETSTH-HS-LAHESSEPVLASVELPPIRTSKSLRDFSFKK
AtML02		DFDSWDPESOHETAETSNSNH-RSRFGEEESEKKFVSSSVELPPGPGOIRTOHEISTISLRDFSFKR
AtML012		HGHQHFFDPESQNEI HGHQHFFDPESQNHSYOREITDSEFSNSHHPQVDMASPVREEKEIVEHVKVDLSEFTFKK
PsML012		DYKNEOWDIEGEGPTSLRNDOTGOHE-IOIAGVESFSSTELPVRIR-HESTSGSKDFSFEKRH
Mt.MLO1		NYENEOWDVEGGGSTSPRNNOTVASE-IEIPIVESFSTELEVKIK-HEIGTTSSSKDFSFEKKH
LjMLO1		NIENEQWIVEGGGSISFRNNQIVASE-IEIFIVESFSIIELFVSVR-HEIGIISSSKDFSFERKH NYETEQWYLEPNSPSNHTRGHDQTLQMQVLGSSATEFSPAEVH-HEI-TPIGLPEFSFDKAP
CaMLO2		NVENEGWANETSTENKDHOEEGOILOHASTSMOHPHTDOHOIEIAMSDFTFGNK-
SIML01		NVENEGWANEISIENKDRQEEGQILQHASISMQHFHIDQHQI-EIAMSDFIFGNK-
NT.MLO1		NVENEGWANENNQIEIIMSDFIFGNK- NAENEGWANEIPTSPRRQIENIKDDDHOEGEIHASSSVHOVEIAMSEFIFGNK-
SmMLO1		NVENEGWANEIFNVENEGWANEIF
CsaML08		NVENEGWANEISHAHNQQIEIIMSDFIFGNK- LSDAEPDRWEELPPSSHHSRAPHHDNHODOOEOSETIIREOEMTVOGPSSSETGSITRPARPHOEITRTP-SDFSFAKX-
CsaML08 CsaML01		
		SPSPSRHVDGSSSSQPHVEMGGYEKDPVESSSSQVDPVQPSRNRNQHEIHIGGPKDFSFDR
MdMLO19		FHHQDNLTWSQQGTNMEGQKEEISAHGPNAESNALGAYGSIIQHEIQIHSAALTFEK
TaMLO_B1a		SPRAMEEARDMYPVVVAHPVHRLNPADRRRSVSSSALDVDIPSADFSFS-Q-
TaMLO_A1b		SPRTMEEARDMYPVVVAHPVHRLNPADRRRSVSSSALDADIPSADFSFS-Q-
TaMLO_D1		PRTMEEARDMYPVVVAHPVHRLNPADRRRSVSSSALDADIPSADFSFS-Q-
HVMLO		SPRTQQEARDMYPVVVAHPVHRLNPNDRRRSASSSALEADIPSADFSFS-Q-
OSML02		
0011202	500	SPGFAGEARDMYPVPVAPVVRPHGFNRTDP-DKRRAASSSAIQVDIADSDFSFSVQ-
AtMLO2	573	KKKKK
	573 583	K R
AtMLO2	573 583 576	K 
AtMLO2 AtMLO6	573 583 576 568	K 
AtMLO2 AtMLO6 AtMLO12 PsMLO1 MtMLO1	573 583 576 568 568	K 
AtMLO2 AtMLO6 AtMLO12 PsMLO1	573 583 576 568 568 545	K 
AtMLO2 AtMLO6 AtMLO12 PsMLO1 MtMLO1	573 583 576 568 568 545 527	K 
AtMLO2 AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1	573 583 576 568 568 545 527 516	K
AtMLO2 AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1 CaMLO2	573 583 576 568 568 545 527 516 580	
AtMLO2 AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1 CaMLO2 CsaMLO8	573 583 576 568 568 545 527 516 580 506	
AtMLO2 AtMLO6 AtMLO12 PsMLO1 MtMLO1 LjMLO1 CaMLO2 CsaMLO8 CsaMLO1	573 583 576 568 545 527 516 580 506 516	
AtMLO2 AtMLO2 AtMLO12 PsMLO1 LjMLO1 CaMLO2 CsaMLO2 CsaMLO1 SIMLO1 NtMLO1 SmMLO1	573 583 576 568 545 527 516 580 506 516 522	
AtMLO2 AtMLO2 AtMLO1 PsMLO1 MtMLO1 LjMLO1 CaMLO2 CsaMLO3 S1MLO1 S1MLO1 NtMLO1 SmMLO1 MdMLO19	573 583 576 568 545 527 516 580 506 516 522 586	
AtMLO2 AtMLO2 AtMLO12 PSMLO1 JJMLO1 CaMLO2 CsaMLO3 CsaMLO3 SIMLO1 NtMLO1 NtMLO1 SmMLO1 SMMLO19 TAMLO_BIa	573 583 576 568 545 527 516 580 506 516 522 586 522 586 533	
AtMLO2 AtMLO2 AtMLO2 PSMLO1 LjMLO1 CaMLO2 CaMLO2 CaMLO2 CaMLO1 SIMLO1 SIMLO1 SIMLO1 SIMLO1 MdMLO19 TaMLO_B1a TaMLO_A1b	573 583 576 568 568 545 527 516 580 506 516 522 586 533 533	
AtMLO2 AtMLO2 AtMLO12 PSMLO1 MtMLO1 LjMLO1 CaMLO2 CsaMLO2 CsaMLO1 SIMLO1 NtMLO1 SIMLO1 NtMLO19 TaMLO_A1b TaMLO_A1b TaMLO_A1b	573 583 576 568 545 527 516 520 506 516 522 586 533 533 534	
AtMLO2 AtMLO2 AtMLO2 PSMLO1 LjMLO1 CaMLO2 CaMLO2 CaMLO2 CaMLO1 SIMLO1 SIMLO1 SIMLO1 SIMLO1 MdMLO19 TaMLO_B1a TaMLO_A1b	573 583 576 568 545 527 516 580 506 516 522 580 506 512 583 533 533 534 532	

**FIGURE 3.** Protein alignment of functionally characterized MLO sequences of Arabidopsis (At), pea (Ps), barrel clover (Mt), Lotus japonicas (Lj), pepper (Ca), cucumber (Csa), tomato (Sl), tobacco (Nt), eggplant (Sm), apple (Md), wheat (Ta), barley (Hv) and rice (Os). Highlighted in green and in light blue are the conserved amino acids among the whole MLO family indicated by Kush et al. 2016 [26] and by Elliott et al. 2005 [69], respectively. Amino acids highlighted in gray refer to the ones reported to be under negative selection by Appiano et al. 2015 [24, chapter 5). Letters displayed in green, light blue or gray indicate amino acids identified in *mlo*-mutants for each of three categories above described. Letters in red bold indicate the position of the transmembrane domains which have been numbered with romans numbers.

#### *ii)* ... or through targeted genome editing

Several studies have addressed the characterization of relevant functional regions of the MLO protein [26, 40, 70]. With the increasing number of MLO sequences being identified in various plant species, multiple alignments point out the occurrence of highly conserved residues that are predicted to have an important functional/structural role (Figure 3). Also, in Chapter 5 we identified 130 codons under negative selection that are translated into amino acids predicted to be conserved during evolution (Figure 3). We also highlighted amino acids specific for monocot and dicot MLO proteins which do not seem to influence the interaction with PM pathogens. We combined the information of important predicted amino acids with the actual mutations found in natural and artificial mutants in the alignment of Figure 3.

Any novel S-MLO protein characterized in a certain crop species can be added to this alignment to pinpoint potential loss-of-function sites. If artificial or natural mutants are

not available, the information of Table 1 and Figure 3 can be usefully coupled with the editing technologies presented above to obtain loss-of-function mutations, especially within the protein domains highlighted in the previous paragraph.

In case it was previously verified that knocked-down *MLO* alleles are not coupled with undesired pleiotropic effect in the species under investigation, it is convenient to decide upon the artificial induction of severe mutations (i.e. nonsense) in order to ensure a severe resistant phenotype.

On the other hand, when loss of the susceptibility function needs to be adjusted in such a way that pleiotropic effects are avoided, missense mutations that do not produce drastic amino acid changes are preferable to nonsense mutations.

Missense mutations can be grouped in two categories: synonymous and non-synonymous coding changes. The first do not impact protein structure and function, while the latter might do. To evaluate the impact of non-synonymous changes, the physicochemical environment in which the amino acid exists, the structural context of the amino acid and the functional context of the amino acid within the protein should be taken into consideration [71].

The cellular location of the MLO proteins can be divided in three levels: intracellular, extracellular and transmembrane. The structural context is more difficult to determine as the crystal structure of the MLO proteins is not known because they are insoluble. The crystal structure of one GPCR (G-protein-coupled receptor), bovine rhodopsin, has been determined [72]. Since MLO proteins are structurally reminiscent of GPCRs, the rhodopsin structure could be taken into account as a model, even if with some caution because MLO function seems to be independent of G-proteins [73-75].

The final step is to evaluate the annotated features of an amino acid variant and the level of conservation of the amino acid in an alignment. Finally, protein function is the key to understanding the consequences of amino acids substitution. Since this is not known yet for MLO proteins, it becomes crucial to rely on conservation of the amino acid position and knowledge of actual loss-of-function site (through mutants).

For example, in barley three mutant alleles (V76L, T222I, and F240L) are called weak alleles because they do not confer full resistance since they still allow about 30% of PM penetration rate [37, 67]. Nevertheless, PM progression is slowed down in these lines (penetration success on susceptible wild type *MLO* genotypes ranges between 50-80%) and the use of chemicals to control the disease can be reduced. These three amino-acids are either conserved within the alignment or substituted with favorable ones (Figure 3). The latter consists of replacements with (predicted) neutral amino acids, which partially impair protein function.

More examples of weak alleles were obtained through site direct mutagenesis in the study of Muller et al. (2004) [76] and Reinstädler et al. (2010) [67] showing how single amino acid replacement influences the functionality of the MLO protein.

The advent of the CRISPR/Cas9 technology promises the possibility to introduce any DNA modification, among which precise site mutations. Few studies have so far addressed this application, but its feasibility has been recently demonstrated in yeast (*Saccharomyces cerevisiae*) [77].

The CRISPR/Cas9 technique could also be used to modulate the *MLO* expression by targeting the promoter of the susceptibility *MLO* gene. It is known that its expression is induced upon PM infection and required for PM successful colonization [37, 78]. Although it is not known if MLO is directly or indirectly targeted by PM elicitors/effectors, there must be some element(s) in the promoter of the *MLO* gene that are responsive to them, triggering the expression. Since prediction of responsive element(s) is difficult, promoter studies should be conducted first. These involve cloning both the full-length promoter and truncated promoter fragments in front of e.g. a GUS or GFP gene, transforming them into tomato or Arabidopsis, and studying which region is required for induction of expression by powdery mildew infection. Once the region is known, the effect of amino acids changes can be analyzed by CRISPR/Cas9 so that induction of *MLO* expression by PM pathogen is prevented, without interfering with expression levels required for normal development.

One successful application of this concept was achieved in rice using TALENs to disrupt the effector binding site of the bacterial blight, *Xanthomonas oryzae* pv. *oryzae*, located in the promoter region of the *SWEET14* gene [115].

Ultimately, CRISPR/Cas9 could also be used for epigenome editing [79]. In the work of Ge et al. (2016) [56], it was shown that *HvMLO* expression is DNA methylation-dependent. Moreover, they associated the partial resistance level of the Ethiopian landrace carrying the *mlo-11(cnv2)* allele to its intermediate level of expression (and methylation) compared to wild-type barley and full resistant cv Westminster. Therefore, by modulating the methylation of the *MLO* gene promoter, it should be possible to knockdown its expression to a degree that would not interfere with any other possible developmental function but would effectively stop PM penetration.

## Towards the discovery of the function of the MLO protein: direction for future research

Since MLO proteins are exploited by PM pathogens to cause the disease, a question still torturing MLO researchers is why plants have them.

In the recent paper of Kush et al. (2016) [26], MLO proteins were traced back to red and green algae, which finding implies a fundamental ancestral role of the MLO proteins.

As mentioned in the introduction, not all the MLO proteins act as susceptibility genes, but some have been associated with root aberrant phenotypes and others with female infertility. The MLO proteins involved in root response to mechanical stimuli group in clade I, which seems to be the most ancient embryophyte MLO clade [80]. It is tempting, then, to imagine that their ancestral role was to mediate root interaction with symbiotic organisms, like mycorrhizal fungi, important for land colonization. However, it was described that also species that do not produce true roots, like the moss *Physcomitrella* possess clade I MLOs [26].

It is known that HvMlo protein is able to remodel actin cytoskeleton upon PM contact with the cells [81, 82]. The cytoskeleton is a key factor in several processes including cell division and elongation, vesicle and organelle trafficking, adhesion and motility, and establishing polarity [83-85]. Cell polarity is essential for a wide range of biological processes in both unicellular and multicellular organisms [86]. Set-up of plant defense responses against pathogens, pollen tube reception, and root tip growth are processes that involve cell polarization and comprise characterized functions of MLO proteins described in Arabidopsis. Consequently, it is reasonable to imagine that the ancestral MLO proteins in the unicellular organisms were involved in cell polarity, for division, elongation, adhesion and motility. Then, when, the first embryophytes colonized the land, MLO proteins helped the emergence of roots. Later, with the development of gymnosperms, MLO proteins were involved in fertilization for seed development. One MLO-like sequence of the clade II (for pollen tube reception) is indeed present in the gymnosperm *Picea abies* [26].

It seems that to unravel the mystery of the core biochemical function of the MLO protein, results of researches on the cellular components of pollen tube reception, root development and PM resistance mechanisms will have to be synergistically combined to gain important insights. For example, the interaction between Receptor-like Kinases (RLK) and MLO proteins was highlighted in studies on pollen tube reception and in experiments showing genes co-expressed with *MLO* during pathogen defense [87-89]. A functional connection between them, though, still has to be proven.

Our expertise concerns the PM resistance side. Therefore the set–up of suitable experiments could ultimately benefit this scientific discussion. Moreover, we mainly focus on using tomato, instead of Arabidopsis, as model species to carry out molecular research, because of the immediate translation of the results to other cultivated (Solanaceous) crops.

As a starting point, it would be important to determine the expression of the tomato *MLO* genes in different conditions (both environmental and biotic stresses).

It is known that expression of barley *HvMlo* gene is induced upon inoculation with several pathogens, including its adapted PM *Blumeria graminis* f.sp. *hordei*, the non-adapted PM *Blumeria graminis* f.sp. *tritici* and the rice blast fungus, *Magnaporte grisea*, and upon leaf wounding and application of the herbicide paraquat [37]. Also in Arabidopsis, the expression of clade V-*AtMLO* genes is affected by biotic and/or abiotic stresses [78]. The tested biotic stresses comprised the adapted PM *Erysiphe cichoracearum*, *Golovinomyces orontii*, the hemibiotrophic oomycete pathogen *Phytophthora infestans*, the necrotrophic fungal pathogen *Botrytis cinerea*, and the bacterial pathogen *Pseudomonas syringae*. Among the abiotic stresses, Chen and collaborators tested cold, wounding, salt and osmotic stress. Barley *mlo*-mutants are more susceptible to *P. infestans*. Both pathogens are hemibiotroph and able to induce the expression of *MLOs* genes in their respective hosts [14, 53].

In tomato, we have so far verified the involvement of *SIMLO1* in susceptibility towards the adapted PM pathogen *Oidium neolycopersici* and the other PM infecting tomato *Leveillula taurica* ([18]; Chapter 2). Thanks to the material we have generated, the expression and involvement of the other clade V-*MLO* genes could be tested with *L. taurica*, but also with pathogens having different infection styles. Provisory results show that expression of both wild-type *SIMLO1* and impaired *ol-2* allele are induced upon inoculation with adapted and non-adapted PM fungi like observed for barley *HvMlo*. However, contrary to the findings of Piffanelli et al. (2002) [37], in our experiment the wild-type *SIMLO1* gene had a stronger induction than the *ol-2* allele towards both PM fungi. It would be interesting to add in these experiments the novel EMS mutant allele, *m200* (Chapter 7), to verify its expression and eventually correlate it with the resistant phenotype since it was hypothesized that the stronger the mutation, the more severe the phenotype [56].

With respect to abiotic stresses, the expression of *SIMLO1* was also observed to be induced upon imposition of a mild level of salinity [90].

Together, all these studies confirm the involvement of the *MLO* genes in various biological processes. Nevertheless, expanding the knowledge of what triggers their expressions can point towards those processes that obviously share similar molecular components. For this aim, *mlo*-mutants also have a central importance because they can assist the discovery of genes differentially expressed in wild-type and loss-of-function genotypes.

In barley, for example, the deployment of cDNA arrays already showed differential gene expression upon PM inoculation in *mlo*-mutants compared to a susceptible genotype [91, 92]. The plant membrane/signaling protein interaction network database is another tool that can serve as a resource for gene discovery (http://biodb.lumc.edu/mind/, Jones et al., 2014).

Finally, in order to identify proteins interacting with MLOs, a complementary approach should be followed. The complication here is that the MLO protein is embedded in the plant membrane, so approaches like co-immunoprecipitation will most probably not work. Traditionally, the yeast two-hybrid approach represents the method of choice to unravel protein interaction partners. A recent paper showed its suitability also for cell-wall proteins [93]. Nonetheless, since yeast two-hybrid screens are well known to produce false-positive results, verification of individual interaction partners by other approaches, preferentially *in planta*, is required. The fluorescence resonance energy transfer (FRET) microscopy, for example, allows monitoring protein-protein interactions *in vivo* and in real-time [94]. Since its introduction, the number of publications containing FRET as a keyword increased rapidly and continued to do so till now [95]. This technique was also very elegantly used in Bhat et al. (2005) [94] to show the dynamics of the interaction between barley HvMlo proteins (wild-type and impaired) with calmodulin (CaM) upon *B. graminis* f.sp. *hordei* inoculation.

The implementation of non-invasive imaging technologies, like FRET microscopy, with a large set of *mlo*-mutants, may be a step forward towards the characterization of the molecular mechanics of *MLO*-dependent fungal entry.

#### Bird's-eye view of the SNARE proteins involved in plant-microbe interaction

Eukaryotic cells are divided in several intracellular membranous compartments connected by vesicular traffic [96]. After vesicles bud off from a membrane, they must fuse with the correct target membrane. SNARE proteins (soluble <u>N</u>-ethylmaleimide-sensitive factor <u>a</u>ttachment protein <u>re</u>ceptor) act as mediators of vesicle membrane fusion with specific organelles. Phylogenetic studies on SNAREs of animals, plants, protists, and fungi distinguished four main branches, based on specific motif profiles: Qa-, Qb-, Qc and R-SNARE [97, 98].

When a non-adapted pathogen is detected, pre-invasive defenses at the cell periphery are engaged as the first layer of protection. Members of the Qa- clade, also called syntaxins, are involved in this layer of plant immunity. Well-described syntaxins involved in PM resistance are found in this clade, such as Arabidopsis PEN1 (AtSYP121) and AtSYP122, barley ROR2, and grapevine VvPEN1 [99-101].

PEN1 and AtSYP122 have overlapping functions in growth and development because *pen1 syp122* double mutant plants are severely dwarfed and necrotic, but not the respective single mutants [102]. PEN1 has also a function in plant immunity because *pen1* mutants allow enhanced entry of non-adapted pathogens like *Blumeria graminis* f.sp. *hordei* (*Bgh*) and *Erysiphe pisi* into leaf epidermal cells [99]. The mutant *syp122*, on the other hand, shows only a slight increase (10%) of *Bgh* penetration, which was not reproducible [94, 102].

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Additionally, PEN1 is required for *Atmlo2*-based resistance since *Atmlo2 pen1* double mutants show elevated levels of *G. cichoracearum* (*Go*) cell entry [14]. However, following penetration, pathogen development is restricted by the increase of salicylic acid levels. This phenomenon is also observed in Arabidopsis double mutants of SYP42 and SYP43, two other Qa-SNARE proteins. The *syp42 syp43* mutants exhibit pleiotropic developmental phenotypes (semi-dwarfism, short roots and a large number of lateral roots), reduced penetration resistance to non-adapted PM and extensive chlorosis upon infection with *Go* [103]. However, the level of susceptibility to this pathogen is macroscopically not altered. The chlorosis is dependent on the accumulation of high SA levels in response to powdery mildew challenge.

In Chapter 3 of this thesis, we identified in tomato two members of the Qa-SNARE group, designated *SIPEN1a* and *SIPEN1b*. Using the RNAi approach, both homologs were individually silenced in a tomato line harboring the impaired *ol-2* allele; the transgenic plants obtained were challenged with the adapted PM *O. neolycopersici* (*On*) and non-adapted *Bgh*. We deduced that *SIPEN1a* has a major role in the *mlo*-based resistance in tomato because its silencing causes a significant increase of *On* growth. In addition, it is involved in non-host resistance because of the enhanced entry of *Bgh* in epidermal cells of silenced plants. The role of *SIPEN1b* gene could not be confirmed. Thus, we hypothesized that the difference in critical amino acids is responsible for the functional specialization. Protein alignment indicated that there are three non-synonymous substitutions in functional and non-functional tomato and Arabidopsis syntaxins that can play a role in plant defense specialization.

This explanation was also proposed for SYP122 and PEN1 [104]. To verify this hypothesis, the authors obtained a chimeric syntaxin by swapping the first 175 amino acids of the PEN1 N-terminal domain with the corresponding sequence of SYP122. The construct was then used to transform *pen1* and *pen1 syp122* mutants. They did not observe any difference in the level of *Bgh* entry rate, nor a rescue of the dwarfed double mutant phenotype, deducing that interplay between N- and C-terminal regions is critical for the functionality of the respective proteins. If domain swap experiments are not useful in this context, a genome editing approach could target the sharp amino acid differences between *SIPEN1a* and *SIPEN1b* and verify the original hypothesis.

Interestingly, Arabidopsis PEN1 is largely accumulated in the lateral root cap [105]. The root cap is the section of tissue at the tip of a plant root, also called calyptra. It is involved in gravity perception in plants and possibly in communication with the soil microbiota [106]. Among the microbiota, root nodule-forming rhizobacteria and arbuscular mycorrhizal (AM) fungi are important beneficial microorganisms. The latter can colonize most land plants with few exceptions, such as Arabidopsis. It is, therefore, unusual that PEN1 is largely accumulated in the lateral root cap.

There are other Qa- syntaxins expressed mostly in roots, such as the SYP123, SYP132, SYP31, and SYP32. Of these syntaxins, the SYP132 of the model legume *Medicago truncatula*, a homolog of AtSYP132, was recently shown to be localized not only to the plasma membrane surrounding the infection of the rhizobacteria but also abundantly to the membrane of the nitrogen-fixing nodule, called symbiosome. This finding suggests that MtSYP132 is involved in symbiosome formation [107, 108]. Intriguingly, this contrasts with the plasma membrane syntaxin SYP132 of *Nicotiana benthamiana* that is thought to be involved in plant resistance against pathogenic bacteria *Pseudomonas syringae* [109]. In another model legume, *Lotus japonicus*, Qa-syntaxin SYP32-1, a homolog of AtSYP32, was shown to be required for differentiation of nodule tissues [110].

Another SNARE involved in plant-microbe interaction belongs to the Qc-clade, namely Arabidopsis SYP71. This protein was reported to be essential for successful turnip mosaic virus (TuMV) infection by mediating the fusion of the TuMV-induced vesicles with chloroplasts [111]. Its homolog in wheat, TaSYP71, is involved in resistance to the wheat stripe rust caused by *P. striiformis* f.sp. *tritici* [112]. Moreover, in *Lotus japonicus*, LjSYP71 is required for effective symbiotic nitrogen fixation [113].

These findings indicate that SNAREs are involved in general plant-microbes (damaging or beneficial) interactions. However, of the Qa-SNARE/syntaxin clade, only the Arabidopsis PEN1 (SYP121) and its homologs in barley, grapevine and tomato are so far shown to be involved in non-host as well as *mlo*-based resistance to PMs. To complete vesicle fusion, PEN1 will then form a tetrameric complex with the Qb,c-SNARE type protein, called SNAP-33, and an R-SNARE/vesicle-associated membrane protein, VAMP722 [110]. This mechanism ultimately leads to the formation of a papilla that stops PMs at the entry sites [14, 38, 39, 114].

The research in this thesis has shed light on a number of questions and has delivered new starting points for follow up research; among them, how pathogens hijack plant proteins and what are the mechanisms that allow the pathogens to use these proteins to their own advantage.

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Summary Samenvatting Acknowledgements About the author List of publications

### Summary

Powdery mildew (PM) is a worldwide-occurring plant disease caused by ascomycete fungi of the order Erysiphales. A conspicuous number of plant species are susceptible to this disease, the occurrence of which is increasing due to the influence of climate change. Symptoms are easy to recognize by the powdery whitish fungal structures growing on the surface of plant organs. Severe infections cause significant losses in crops, such as tomato, cucumber and wheat, as well as in ornamentals, like rose and petunia. Accordingly, breeding crops with a robust immunity to this disease is of great economic importance.

A significant step in this direction was the discovery of *mlo* (mildew locus o) mutant alleles of the barley *HvMlo* gene, which are responsible for the non-race specific resistance to the barley PM pathogen, *Blumeria graminis* f.sp. *hordei* (*Bgh*). During the years, this recessively inherited resistance was observed to be durable, contrary to the short life-span of resistances conferred by dominant resistance (R-) genes used in barley breeding programs. Studies on the histological mechanisms of the *mlo*-based resistance showed that the PM pathogen was stopped during penetration of the cell wall by the formation of a papilla. This structure prevents the formation of the feeding structure of the pathogen, called a haustorium.

After sequencing many plant genomes, we are discovering that *MLO* genes are not only typical of this cereal, but are ubiquitously present in higher plant species in multiple copies per species, forming a gene family. The impairment of some members of a number of ever increasing plant species lead to broad-spectrum resistance towards their adapted PM pathogens. For example, in tomato the *ol-2* gene, naturally harbored by the cherry tomato *Solanum lycopersicum* var. *cerasiforme*, represents the loss-of-function allele of the *SIMLO1* gene, conferring resistance to the PM pathogen *Oidium neolycopersici* (*On*). Consequently, the use of *mlo* mutants represents a suitable alternative to the classical use of R-genes in breeding programs.

In **Chapter 2**, we describe the *in silico* identification of the complete tomato *SIMLO* gene family using the available information in the SOL genomic network database. In total, 16 tomato *SIMLO* members were cloned from leaf, root, flower and fruit of the susceptible tomato cv. Moneymaker to confirm the sequences retrieved from the database and to verify their actual expression in these tissues. We observed the presence of various types of splicing variants, although their possible functional meaning has not been investigated. Motif analyses of each of the translated protein sequences and phylogenetic studies highlighted, on one hand, amino acid stretches that characterize the whole MLO family, and, on the other hand, stretches conserved in MLO homologs that are phylogenetically related. Following a gene expression study upon *On* inoculation, we identified members of the *SIMLO1*, none of the three newly identified homologs in clade V, thus phylogenetically close to *SIMLO1*, are induced. Interestingly, two homologs, each found in different clades, are upregulated similarly to

*SIMLO1*. Using an RNAi approach, we silenced the additional clade V-*SIMLO* homologs, namely *SIMLO3*, *SIMLO5* and *SIMLO8*, to investigate their possible role in PM resistance. We observed that none of these homologs if individually silenced, leads to PM resistance. However, if *SIMLO5* and *SIMLO8* are silenced together with *SIMLO1*, a significantly higher level of resistance is achieved compared to plants carrying the *ol-2* allele. The role of *SIMLO3* could not be verified. We, therefore, concluded that there are three *SIMLO* genes in tomato unevenly contributing to the PM disease, of which *SIMLO1* has a major role.

**Chapter 3** focuses on the components of the tomato *mlo*-based resistance. In Arabidopsis, it is known that four members of the SNARE protein family, involved in membrane fusion, are involved in *mlo*-based resistance. In this chapter, we focused on the identification of tomato homologs of the Arabidopsis syntaxin PEN1 (AtSYP121). Among the group of syntaxins identified in tomato, two were closely related to each other and also to *AtPEN1*, denominated *SIPEN1a* and *SIPEN1b*. Another Arabidopsis syntaxin that shows a high level of homology with *PEN1*, called *SYP122*, was also found to group together with the newly identified *SIPEN1* genes. However, the role of *SYP122* in plant immunity was not shown in literature. After obtaining individual silencing RNAi constructs, we transformed the resistant *ol-2* line, and we challenged the obtained transformants with the adapted PM *On*, and the non-adapted *Bgh*. Interestingly, we observed a significant *On* growth and an enhanced *Bgh* cell entry only in *SIPEN1a* silenced plants but not in *SIPEN1b* silenced ones. We performed a protein alignment of tomato and Arabidopsis functional and non-functional PEN sequences. The presence of three differently conserved non-synonymous amino-acid substitutions is hypothesised to be responsible for the specialization in plant immune function.

In **Chapter 4** and **Chapter 5**, we build up a body of evidence pointing to the fact that the function of the *MLO* susceptibility genes is highly conserved between monocot and dicot plant species.

In **Chapter 4** we started by identifying and functionally characterizing two new *MLO* genes of Solanaceous crops affected by the PM disease, tobacco (*Nicotiana tabacum*) and eggplant (*Solanum melongena*). We named them *NtMLO1* and *SmMLO1* in the respective species, as they are the closest homologs to tomato *SIMLO1*. By overexpressing these genes in the resistant *ol-2* line, we obtained transgenic plants that were susceptible to the PM pathogen *On*. This finding demonstrates that both heterologous MLO proteins can rescue the function of the impaired *ol-2* allele in tomato. In addition, we found in tobacco NtMLO1 an amino acid (Q198) of critical importance for the susceptibility function of this protein.

In **Chapter 5**, we used the same approach adopted in Chapter 4 to show that other MLO proteins of more distant dicot species, like pea PsMLO1, can rescue the loss-of-function of the tomato *ol-2* allele. And finally, we stretched this concept also to monocot MLO proteins, using barley HvMlo. While performing these experiments, we could verify that the function of the monocot and dicot susceptibility MLO proteins does not rely on the presence of class-

specific conservation. The latter can be the reason for the phylogenetic divergence, placing monocot MLO proteins in clade IV and dicot MLO proteins in clade V of the phylogenetic MLO tree. However, functional conservation might depend on crucial shared amino acids of clade IV and V MLO proteins. Therefore, we also conducted a codon-based evolutionary analysis that resulted in the identification of 130 codons under negative selection, thus strongly maintained during evolution.

In **Chapter 6** we introduce the PM disease in cucumber caused by *Podosphaera xanthii* (*Px*). We cloned the candidate susceptibility gene for PM in cucumber, *CsaMLO8*, from susceptible and resistant genotypes. The latter was described as an advanced cucumber breeding line characterized by hypocotyl resistance. In this line, we found the presence of aberrant splicing variants of the *CsaMLO8* mRNA due to the insertion in its corresponding genomic region of a Class LTR retrotransposon. Heterologous expression of the wild-type cucumber allele in the tomato *ol-2* line restored its PM susceptibility, while the heterologous expression of the advanced cucumber breeding line is due to the disruption of the coding region of this gene. We also showed that the expression of *CsaMLO8* in the susceptible genotype is induced by *Px* in hypocotyl tissue, but not in cotyledon or leaf. Finally, by examination of the resequencing data of a collection of 115 cucumber accessions, we found the presence of the TE-containing allele in 31 of them among which a wild cucumber accession that might have been used in breeding programs to obtain resistance to the PM disease in cucumber.

In **Chapter 7** a novel loss-of-function allele of the *SIMLO1* gene is described, designated *m200*. This allele was found in a resistant plant (M200) from a mutagenized tomato Micro-Tom (MT) population obtained with the chemical mutagen ethyl methanesulfonate (EMS). The *m200* mutation corresponds to a nucleotide transversion ( $T \rightarrow A$ ) which results in a premature stop codon. The length of the predicted SIMLO1 protein in the M200 plant is only 21 amino acids, thus much shorter than the predicted protein of the previously described *ol-2* allele, consisting of 200 amino acids. Thanks to the development of a High-Resolution Melting (HRM) marker designed to detect the *m200* mutation, we observed that this allele confers recessively inherited resistance in backcross populations of the resistant M200 plant with MT and Moneymaker. Histological study showed that the rate of *On* penetration in epidermal cells of *m200* plants with the one of plants carrying the *ol-2* allele and the transgenic plants in which multiple *SIMLO* homologs were silenced, generated in Chapter 2.

Ultimately, in **Chapter 8** the results of the previous chapters are discussed in the context of 1) practical applications in breeding programs aimed at introducing the *mlo*-based resistance in new crops, 2) possible research aimed at unraveling the function of the MLO protein and 3) the role of other SNARE proteins.

## Samenvatting

Echte meeldauw is een wereldwijd voorkomende plantziekte, veroorzaakt door schimmels uit de stam Ascomycota, de orde Erysiphales. Een opvallend aantal plantsoorten is vatbaar voor deze ziekte, en het aantal uitbraken van deze ziekte neemt verder toe door klimaatverandering. Symptomen van de ziekte zijn makkelijk te herkennen door de witte, poederachtige schimmelstructuren die op de buitenkant van de organen van de geïnfecteerde plant groeien. Heftige infecties leiden tot aanzienlijke verliezen in zowel voedingsgewassen zoals tomaat, komkommer en tarwe, maar ook in siergewassen als roos en petunia. Daarom is het van groot economisch belang om gewassen zo te veredelen dat ze een robuuste immuniteit tegen deze ziekte verwerven.

Een belangrijke stap in deze richting was de ontdekking van *mlo* (meeldauw locus o) mutant allelen van het gen *HvMlo* in gerst, die leiden tot niet ras-specifieke weerstand tegen *Blumeria graminis* f.sp. *hordei* (*Bgh*), de veroorzaker van meeldauw in gerst. In de loop der jaren werd ontdekt dat deze recessief overervende resistentie duurzaam bleek, in tegenstelling tot de korte levensspanne van meeldauwresistentie veroorzaket door dominant overervende resistentie (R-) genen die in veredelingsprogramma's van gerst gebruikt werden. Studies naar de histologische mechanismen van op *mlo* gebaseerde resistentie wezen uit dat de infectie werd gestopt tijdens de penetratie van de celwand van de plant, door het vormen van een papilla. Deze structuur voorkomt het vormen van een haustorium, het orgaan van de ziekteverwekker waarmee de schimmel voedingsstoffen op kan nemen.

Nu het genoom van veel plantsoorten in kaart is gebracht, ontdekken we dat *MLO* genen niet iets typisch van dit graangewas zijn, maar alomtegenwoordig aanwezig zijn in hogere planten, met meerdere kopieën per plantsoort die samen een genfamilie vormen. Beschadigde versies van sommige genen in deze familie leiden in een toenemend aantal plantsoorten tot weerstand tegen hun respectievelijke meeldauwpathogenen. Het *ol-2* gen in tomaat, dat van nature voorkomt in kerstomaat (*Solanum lycopersicum* var. *cerasiforme*), vertegenwoordigt bijvoorbeeld een verlies-van-functie allel van het gen *SIMLO1*, en geeft hierdoor weerstand tegen de meeldauwpathogeen *Oidium neolycopersici* (*On*). Derhalve is het gebruik van *mlo* mutanten een geschikt alternatief voor de klassieke R-genen in veredelingsprogramma's.

In **Hoofdstuk 2** beschrijven we de *in silico* identificatie van de complete tomaat *SIMLO* genfamilie, waarbij we gebruik maken van de beschikbare informatie in de "SOL genomic network database". We kloneerden in totaal 16 tomaat *SIMLO* genen uit bladeren, wortels, bloemen en vruchten van de vatbare tomaat cultivar "Moneymaker", om de sequenties uit de databank te bevestigen, en te controleren of deze genen inderdaad tot expressie komen in deze weefsels. We observeerden de aanwezigheid van verscheidene typen van alternatieve splicing, mogelijke functionele gevolgen hiervan hebben we echter niet

bestudeerd. Analyses van motieven in de getranslateerde eiwitsequenties en fylogenetisch onderzoek markeerden enerzijds groepen van aminozuren die karakteristiek zijn voor de gehele MLO familie, maar anderzijds ook groepen van aminozuren die specifiek geconserveerd zijn tussen MLO homologen die fylogenetisch nauw aan elkaar verwant zijn. Naar aanleiding van onderzoek naar genexpressie in weefsels geïnoculeerd met On identificeerden we leden van de SIMLO familie waarvan de genexpressie enkele uren na de inoculatie opgereguleerd werd. Behalve SIMLO1 werd geen van de drie nieuwe homologen in klade V, die dus fylogenetisch nauw aan SIMLO1 verwant zijn, geïnduceerd. Interessant genoeg werden wel twee andere homologen, in verschillende klades, geïnduceerd op vergelijkbaar niveau met SIMLO1. Met een RNAi aanpak hebben we de transcriptie van de andere klade V-SIMLO homologen (SIMLO3, SIMLO5 en SIMLO8) stilgelegd, om hun mogelijke rol in meeldauwresistentie te onderzoeken. We observeerden dat bij geen van deze homologen het stilleggen van de transcriptie tot meeldauwresistentie leid, mits één homoloog tegelijk stilgelegd werd. Als daarentegen de transcriptie van zowel SIMLO5, SIMLO8 als SIMLO1 tegelijkertijd stilgelegd wordt kan een merkbaar hoger niveau van resistentie bereikt worden dan dat in planten met het ol-2 allel. Een rol van SIMLO3 kon niet worden geverifieerd. We concludeerden daarom dat er drie SIMLO genen, op ongelijke wijze, bijdragen aan de meeldauwziekte, waarvan SIMLO1 de belangrijkste rol heeft.

De focus van **Hoofdstuk 3** ligt op de componenten van op *mlo* gebaseerde resistentie in tomaat. Het is bekend dat in Arabidopsis vier leden van de SNARE eiwitfamilie, die betrokken zijn bij fusie van membranen, betrokken zijn bij op *mlo* gebaseerde resistentie. In dit hoofdstuk hebben we de focus gelegd op de identificatie van tomaat-homologen van de Arabidopsis syntaxin PEN1 (AtSYP121). In de groep van geïdentificeerde tomaat syntaxins waren er twee nauw verwant aan elkaar en aan AtPEN1, die we daarom SIPEN1a en SIPEN1b noemden. Een andere Arabidopsis syntaxin die een hoge mate van homologie liet zien met PEN1, SYP122 genaamd, bleek ook samen te vallen met de groep van nieuw geïdentificeerde SIPEN1 genen. Een rol van SYP122 in immuniteit was echter niet bekend uit de literatuur. Nadat we individuele RNAi constructen verkregen hadden transformeerden we de resistente *ol-2* lijn en testen de verkregen transformanten met de aangepaste meeldauwpathogeen On en de niet-aangepaste Bgh. Interessant genoeg observeerden we een significante groei van On en een verhoogde celwandpenetratie van Bgh in planten waarin expressie van SIPEN1a verzwakt was, maar niet in planten waarin de expressie van SIPEN1b verzwakt was. We voerden een eiwit-alignering uit op zowel functionele als niet-functionele PEN eiwitten in Arabidopsis en tomaat. De aanwezigheid van drie verschillende geconserveerde niet-synonieme aminozuur substituties is volgens onze hypothese verantwoordelijk voor de specialisatie in immuunsysteem functie.

In **Hoofdstuk 4** en **Hoofdstuk 5** bouwen we aan bewijsmateriaal dat er op duidt dat de functie van *MLO* vatbaarheidsgenen in grote mate geconserveerd is tussen eenzaadlobbige en tweezaadlobbige plantsoorten.

In **Hoofdstuk 4** beginnen we met het identificeren en functioneel karakteriseren van twee nieuwe *MLO* genen in gewassen in de familie *Solanaceae* die vatbaar zijn voor meeldauwziekten, tabak (*Nicotiana tabacum*) en aubergine (*Solanum melongena*). We noemen deze genen *NtMLO1* en *SmMLO1* in de respectievelijke plantensoorten, omdat ze de nauwste verwanten zijn van het tomaat gen *SlMLO1*. Door deze genen tot over expressie te brengen in de resistente *ol-2* lijn verkregen we transgene planten die vatbaar waren voor de meeldauwpathogeen *On*. Deze bevinding laat zien dat beide heterologe MLO eiwitten de functie van het beschadigde *ol-2* allel in tomaat kunnen herstellen. In aanvulling hierop ontdekten we dat in het tabak eiwit NtMLO1 een aminozuur (Q198) van vitaal belang was voor de vatbaarheidsfunctie van dit eiwit.

In **Hoofdstuk 5** gebruikten we dezelfde aanpak als in Hoofdstuk 4 om te laten zien dat andere MLO eiwitten van minder aan tomaat verwante tweezaadlobbige plantsoorten, zoals erwt (*Pisum sativum*) PsMLO1, het verlies van functie van het tomaat *ol-2* allel kunnen herstellen. Tenslotte strekken we dit concept ook uit naar eenzaadlobbige MLO eiwitten, gebruik makend van gerst HvMlo. Door middel van deze experimenten konden we nagaan dat de functie van eenzaadlobbige en tweezaadlobbige MLO eiwitten betrokken bij vatbaarheid niet berust op de aanwezigheid van klasse-specifieke conservatie. Dit laatste kan de reden zijn voor de fylogenetische divergentie, die eenzaadlobbige MLO eiwitten in klade IV plaatst en tweezaadlobbige MLO eiwitten in klade V van de fylogenetische MLO boom. Echter, functionele conservatie zou af kunnen hangen van cruciale gedeelde aminozuren in klade IV en V MLO eiwitten. Daarom voerden we ook een codon-gebaseerde evolutionaire analyse uit die resulteerde in de identificatie van 130 codons onder negatieve selectie, die dus sterk bewaard bleven tijdens de evolutie.

In Hoofdstuk 6 introduceren we de meeldauwziekte in komkommer, veroorzaakt door Podosphaera xanthii (Px). We kloonden het kandidaat vatbaarheidsgen voor meeldauw in komkommer, CsaMLO8, uit vatbare en resistente genotypes. De tweede werd beschreven als een geavanceerde veredelingslijn gekarakteriseerd door hypocotyl-specifieke resistentie. In deze lijn vonden we de aanwezigheid van afwijkende splicingvarianten van het CsaMLO8 mRNA, veroorzaakt door de insertie van een klasse LTR retrotransposon in de corresponderende genomische regio. Heterologe expressie van het wild-type komkommer allel in de tomaat ol-2 lijn herstelde de meeldauwvatbaarheid, terwijl heterologe expressie van de gewijzigde eiwitvariant dat niet deed. Deze bevinding bevestigd dat de resistentie van de geavanceerde komkommer veredelingslijn komt door de ontwrichting van de coderende regio van dit gen. We lieten ook zien dat de expressie van CsaMLO8 in het vatbare genotype wordt geïnduceerd door Px in hypocotylweefsel, maar niet in cotyl of blad. Tenslotte ontdekten we, door resequencingdata van een collectie van 115 komkommeraccessies te bestuderen, dat het TE-bevattende allel in 31 van deze lijnen voorkomt, onder welke een wilde komkommerlijn die wellicht in het verleden gebruikt is in veredelingsprogramma's om resistentie tegen meeldauw in komkommer te verwerven.

#### **260** | Samenvatting

In **Hoofdstuk 7** beschrijven we een nieuw verlies-van-functie allel van het *SIMLO1* gen, die we *m200* noemen. Dit allel is gevonden in een resistente plant (M200) uit een gemutageniseerde tomaat Micro-Tom (MT) populatie, verkregen met de chemische mutagen ethyl methaansulfonaat (EMS). De *m200* mutatie is een nucleotide transversie ( $T \rightarrow A$ ) die leidt tot een premature stop codon. De lengte van het verwachte SIMLO1 eiwit in de M200 plant is slechts 21 aminozuren, dus veel korter dan het verwachte eiwit gecodeerd door het eerder beschreven *ol-2* allel, dat uit 200 aminozuren bestaat. Dankzij de ontwikkeling van een Hoge-Resolutie Smeltcurve Analyse (HRM) merker, ontworpen om de *m200* te detecteren, observeerden we dat dit allel recessief overervende resistentie geeft in terugkruisingspopulaties van de resistentie M200 plant met MT en Moneymaker. Histologisch onderzoek laat zien dat de resistentie van de *m200* mutant geassocieerd is met papillaformatie. Tenslotte vergeleken we de verhouding tussen de *On* penetratie in epidermale cellen van *m200* planten met die in planten met het *ol-2* allel, en de transgene planten waarin de expressie van meerdere *SIMLO* homologen stilgelegd is, gemaakt in Hoofdstuk 2.

Uiteindelijk bediscussiëren we de resultaten van de voorgaande hoofdstukken in **Hoofdstuk 8** in de context van 1) praktische toepassingen in veredelingsprogramma's met als doel op *mlo*-gebaseerde resistentie in nieuwe gewassen te introduceren, 2) mogelijk toekomstig onderzoek met als doel de functie van het MLO eiwit te ontrafelen, en 3) de rol van andere SNARE eiwitten.

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Michela

## About the author

Michela Appiano was born in Foggia (Italy) on October 2<sup>nd</sup>, 1984. She obtained her Bachelor and Master studies in Agricultural Science in 2006 and 2009, respectively, in Foggia.

During her Bachelor study she was fascinated by the course of Molecular Biology and chose to do an experimental thesis on the characterization of different varieties of artichoke through SSR marker.



During her Master she won a fellowship granted by the International Program on Postharvest Technology, which arose thanks to the collaboration of several research groups in Europe and United States. In California, she was welcomed by the group of - now emeritus - Prof. Michael Reid of UC Davis. Here she investigated the changes in gene expression during senescence and aging of a number of cut flower species.

Doing her Master thesis in this group inspired her to pursue an academic career, starting by doing a PhD. In order to decide where to do a PhD she accepted a fellowship immediately after her MSc degree at the School of Advanced Study Sant'Anna, in Pisa (Italy). Here she performed biochemical and molecular analyses on lignin biosynthesis in Gerbera cut stem. However, in January 2010 she moved back to south Italy and accepted the PhD position in Bari (Italy) within the group of prof. Luigi Ricciardi. The researcher of the group, Dr Stefano Pavan, was working on the topic of plant-powdery mildew interaction, which she found very interesting to investigate. Thus Michela started exploring the world of the *MLO* susceptibility genes involved in this disease.

After the first year of her PhD she went to the Laboratory of Plant Breeding in Wageningen (The Netherlands) thanks to an ongoing collaboration between her Italian group and the chair of this department, Prof. Richard Visser. She spent the two following years as guest PhD under the supervision of Dr. Yuling Bai, leader of the group breeding for resistance in Solanaceae. In April 2013 she obtained her Italian Doctorate title.

It became clear to her that working in this multicultural, inspiring environment was a challenge she wanted to accept to deepen her scientific skills. Therefore, she continued working in the same group as PhD student of Wageningen University. The results of her project aimed at understanding the resistance based on impaired *mlo* genes are described in this thesis.

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- Zheng† Z., <u>Appiano† M.</u>, Pavan S., Bracuto V., Ricciardi L., Visser R.G.F., Wolters A.M., Bai Y. (2016) Genome-wide study of the tomato *SIMLO* gene family and its functional characterization in response to the powdery mildew fungus *Oidium neolycopersici*. Frontiers in Plant Science, 7:380.
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