Exploration of *mlo*-based resistance in vegetable crops

Zheng Zheng

Thesis committee

Promotor

Prof. dr. Richard G.F. Visser

Professor of Plant Breeding

Wageningen University

Co-promotor

Dr. Yuling Bai

Assistant professor, Laboratory of Plant Breeding

Wageningen University

Other members

Prof. dr. Bas J. Zwaan, Wageningen University

Prof. dr. Ralph Panstruga, Aachen University, Germany

Dr. ir. Bart P.H.J. Thomma, Wageningen University

Dr. Rolf T. Folkertsma, Monsanto Holland B.V., Bergschenhoek

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CONTENTS

CHAPTER 1

General Introduction

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Plant innate immunity

Plants are sessile organisms, which mean that they have to face all the challenges from different biotic and abiotic stresses including pathogens, insects, animals, salt, drought and so on in their living environments. In general, the term of “plant pathogens” means all insects, fungi, bacteria, oomycetes and viruses which invade plants. In order to survive, plants have to prepare themselves with different defense mechanisms to cope with the threats from pathogens. Plants have to mount different defense responses to prevent the invading of pathogens, which include the inducible resistant reactions as well as constitutive physical and chemical plant barriers like plant cell wall and toxic compounds. The contemporary theory of plant-pathogen interaction could be explained by an elegant model proposed by Jones and Dangl (2006): two layers of induced defense response in plants against pathogens. The first layer is formed by pattern recognition receptors (PRRs). These are plant receptors located on the cell surface that can recognize pathogen-associated molecular patterns (PAMPs). Perception of PAMPs by PRRs triggers so called PAMPs-triggered immunity (PTI) which can stop the majority of pathogens at the penetration sites. To cause a disease, pathogens have evolved effectors to suppress the PTI. This suppression is the so called effector-triggered susceptibility (ETS). In turn, plants have evolved a second layer of defense response which reacts on the effectors through plant resistance (R) proteins that recognize certain effectors, resulting in effector-triggered immunity (ETI). ETI can be overcome by newly evolved effectors that cannot be recognized by R proteins.

PAMP triggered immunity (PTI)

PAMPs are conserved in different classes of pathogens. The reason why pathogens would keep a mark to identify themselves from their hosts is that they contribute to microbial

fitness or survival (Medzhitov and Janeway, 1997; Nurnbergerand Brunner, 2002). Classical examples of PAMPs are flagellin, peptidoglycan,lipopolysaccharides (LPS), elongation factor Tu (EF-Tu) and coldshock proteins in bacteria, Pep-13, glucans and heptaglucosides in oomycetes, ergosterol and xylanase and chitin in fungi (Ayers et al., 1976; Felix et al., 1993, 1999; Dow et al., 2000; Gust et al., 2007; Erbs et al., 2008, Boller and Felix, 2009).

In plants, pattern recognition receptors (PRRs) are located at the surface of cells. Generally, the PRRs are receptor-like proteins (RLPs) with or without an intracellular kinase domain (Boller and Felix, 2009). The PRRs play a major role in detection of PAMPs and subsequently activate PTI. The most well characterized PRR is the receptor like kinase (RLK) protein FLS2. It binds to the bacterial PAMP flagellin (flg22) and forms a complex with the brassinosteroid-insensitive1 (BRI1)-associated receptor kinase 1 (BAK1). This complex actives the PTI responses against bacteria (Felix et al., 1999; Gómez-Gómez and Boller, 2000; Chinchilla et al., 2007). Other known PRRs are the EF-Tu receptor EFR that is Brassicacea specific and detects effector elf18 (Kunze et al. 2004;Zipfel et al., 2006), XA21 (identified from rice) interacting with sulfated peptides from the proteobacteria Ax21 (Lee et al., 2009), CeBiP (from rice) and CERK1 (from *Arabidopsis*) monitoring the fungal chitin (Miya et al., 2007; Wan et al., 2008; Petutschnig et al., 2010; Kaku et al., 2006), LeEIX1/2 (from tomato) monitoring the fungal xylanase EIX (Ron and Avni, 2004) and the legume NFR1/5 detecting the rhizobial Nod factor (Radutoiu et al., 2007). In addition to the perception of PAMPS effectors, the PRRs could also monitor the molecular patterns which are generated during the pathogen infection and considered as danger-associated molecular patterns (DAMPs). For example, the damaged plant cell wall fragments and compounds generated by microbial enzymes (Huffaker and Ryan, 2007; Krol et al., 2010; Yamaguchi et al., 2010).

The chitin receptors CeBiP (from rice) and CERK1 (from *Arabidopsis*) are homologs. In addition, the FLS2 receptor is also conserved between plants including tomato, tobacco and rice indicating that the PRRs are ancient proteins conserved in plant species (Gómez-Gómez and Boller, 2000; Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008; Boller and Felix, 2009, Kaku et al., 2006; Miya et al., 2007). This evolutionarily conserving evidence from both elicitors and receptors strongly indicates the co-evolution

between pathogens and their host plants.

Effector triggered immunity (ETI)

Pathogenic microbes secret effectors and deliver them into the plant cells to overcome PTI which lead to ETS. Bacteria and fungi have different effector delivery systems namely the type III secretion system (TTSS) and the haustorium delivery system, respectively. Plants counter effectors by means of developing the R proteins to recognize the presence or activity of the effectors. The majority of these R proteins are intracellular proteins with the nucleotide binding–leucine-rich repeat (NB-LRR) and the coiled-coil (CC) or toll-interleukin-1 receptor (TIR) sequence at their N-terminus (Dangl and Jones, 2001). There are also examples of cloned R genes like the *Cf* gene family which encode transmembrane RLKs (receptor like kinase) or RLPs (receptor like protein) with an extracellular LRR.

It was originally assumed that ETI is triggered by a direct interaction between R and Avr (avirulence) proteins and follow a gene-for-gene relationship (Flor, 1942). A compatible interaction between plants and pathogens is the result of the lack or absence of a matched pair of R protein and its corresponding effector. On the other hand an incompatible interaction is the outcome of the recognition between the R protein and its cognate effector. Such recognition triggers a cascade of plant defense responses and leads to localized programmed cell death (PCD), usually referred to as hypersensitive response (HR). Thus, the ETI is typically race specific and can be easily overcome by newly evolved pathogen isolates. Examples of this direct interaction between R/Avr proteins have been reported in rice/blast interaction (Pi-ta/AvrPita) and flax/rust interaction (L/AvrL567) (Jia et al., 2000; Dodds et al., 2004; Dodds et al., 2006; Dodds and Rathjen, 2010).

Later the guard hypothesis stated that R proteins do not directly interact with Avr proteins, rather they monitor the host components that are targeted by pathogen molecules (Van der Biezen and Jones, 1998). It suggests that pathogen effectors have functions to promote pathogen virulence by manipulating their host targets (guardee of the R protein) which are sensed and monitored by the R proteins (Chisholm et al., 2006; Jones and Dangl, 2006). A well characterized guardee is the RPM1 interacting protein 4 (RIN4) from *Arabidopsis* which are guarded by the R proteins RPM1 and RPS2. Interestingly, RIN4 is

the target of different pathogen effectors including AvrRpm1, AvrB and AvrRpt2, which manipulate RIN4 via either phosphorylation or degradation.

Recently, by summarizing several examples in the plant-pathogen interactions, Thomma et al (2011) proposed that there is a continuum between PTI and ETI. Their new hypothesis suggests that immune receptors in plants recognize appropriate ligands from pathogens and activate defense in plants, the amplitude of which is likely determined by the level required for effective immunity.

Plant breeding for disease resistance

A classic concept in plant breeding for disease resistance is the classification of vertical (also called qualitative) resistances and horizontal (or quantitative) resistances. According to this distinction, vertical resistances are conferred by the ETI mentioned above.

Conversely, horizontal resistances have a polygenic nature and normally confer a partial level of resistance against a broad range of pathogen races. General defense mechanisms are associated with horizontal resistances, including the metabolism of reactive oxygen species, reinforcement of the cell wall, apposition of papillae, synthesis of phytoalexins and pathogen-related (PR) proteins.

Together with the study of a high number of plant-pathogen interactions, the distinction between horizontal and vertical resistances has become weaker, although it still holds as a general rule. It is now well known that vertical and horizontal resistances share a common set of defense mechanisms (Reignault et al., 2005). In the same way, several QTLs have shown race-specificity (Geffroy et al, 2000, Calenge et al., 2004) or co-localization with qualitative loci (Bai et al., 2003).

In practical plant breeding, vertical resistances which are controlled by dominant resistant (*R*) genes are widely used in modern crop breeding activities. However, there are several disadvantages of *R* genes that have to be taken into consideration. First of all, the resistant donor species have to be able to cross with the cultivated species. This barrier had hampered the mapping and cloning of *R* genes by standard map-based-cloning strategies. Second, if the interspecific cross-ability barrier could be overcome by any of the cell biological techniques like embryo rescue, *R* gene introgression normally required extensive backcrossing to remove/introduce other un/desirable traits into the cultivated

species. Thirdly, transferring of *R* genes from one species into another does not always confer resistance in all cases. And last but not least, *R* genes confer race-specific resistance, which could be relatively easily overcome by new races of the pathogen in a short period.

As mentioned before, in order to become pathogenic on/in a plant species a microbe or other pathogen has to suppress the plants' innate immunity by exploiting effector molecules to establish ETS. Plant genes that are manipulated by pathogens and/or required for trigger susceptibility are referred to as susceptibility (*S*) genes. Disabling the function of the plant *S* genes in principle could bring resistance against diseases. Genetically, diminishing or impairment of plant *S* genes could initiate recessive resistance against corresponding diseases. This novel breeding strategy has been proposed by Eckardt (2002) and Pavan et al., (2010). One representative example is the Mildew resistance Locus O (*Mlo*) gene.

mlo-based resistance

In land plants, *Mlo* genes comprise small to medium-sized families encoding a novel type of plant-specific integral membrane proteins with as yet unknown biochemical function(s) (Devoto et al. 1999; Devoto et al. 2003). In the monocot barley and the dicot *Arabidopsis*, mutations in particular *Mlo* genes result in broad-spectrum powdery mildew resistance (Büschges et al. 1997; Consonni et al., 2006). It is thought that the respective protein isoforms modulate vesicle associated defense responses at the cell periphery and that the powdery mildew pathogen possibly exploits these proteins for successful host cell entry (Panstruga 2005). While in barley loss-of-function of a single *Mlo* gene suffices to confer full resistance, unequal genetic redundancy between three phylogenetically closely related *Mlo* co-orthologs (*AtMlo2, AtMlo6* and *AtMlo12*) results in a more complex scenario in *Arabidopsis*. Absence of *AtMlo2* confers partial powdery mildew resistance which becomes enhanced in *Atmlo2 Atmlo6* or *Atmlo2 Atmlo12* double mutants. Full resistance requires loss-of-function of all three co-orthologs, i.e. an *Atmlo2/Atmlo6/Atmlo12* triple mutant (Consonni et al. 2006). Barley *mlo* mutants have been known for more than 70 years and have been successfully employed in European barley agriculture for more than 30 years (Lyngkjaer et al. 2000), emphasizing the principal durability of *mlo*-mediated disease resistance under agricultural conditions. In case of barley, in addition to a broad range of

induced mutations (Büschges et al. 1997; Piffanelli et al. 2002; Panstruga et al. 2005), one natural allele (*mlo*-11) has been described (Piffanelli et al. 2004). This allele derives from a barley landrace, an intermediate between wild and cultivated barley, initially found in Ethiopia. It is molecularly characterized by the presence of a complex tandem repeat array, comprising *Mlo* 5´regulatory and partial coding sequences, which localize upstream of a fully intact *Mlo* wild type copy (Piffanelli et al. 2004). Experimental evidence points to a transcriptional ´read-through´ phenomenon caused by the presence of the upstream repeat units, interfering with proper transcription of the downstream *Mlo* wild type copy (Piffanelli et al. 2004). Interestingly, the *mlo*-11 allele is meiotically unstable, resulting in rare meiotic reversion events which give rise to susceptible progeny. Reversion to susceptibility was found to coincide with a loss of the repeat array and restoration of wild-type-like *Mlo* transcript and MLO protein levels (Piffanelli et al. 2004).

Signal transduction mechanisms controlling defense activation in plants are formed by interacting pathways that are dependent on the signaling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Pieterse and van Loon, 1999, 2004; Glazebrook, 2001; Shah, 2003). However, *mlo*-based resistance was largely independent from any of the three main signaling pathways mentioned above by studying the interaction of *Arabidopsis* with *Golovinomyces cichoracearum* and *Golovinomyces orontii* (Consonni et al., 2006). Resistance derived by mutations in the *Mlo* gene family requires correct functioning of other genes, required for *mlo* resistance (*Ror1*) and (*Ror2*) genes in barley (Huckelhoven et al., 2000) and syntaxin (*Pen1*), glycosyl hydrolase (*Pen2*) and putative ATP binding cassette (ABC) transporter (*Pen3*) genes in *Arabidopsis*(Collins et al., 2003, Consonni et al., 2006). Recent results showed that the tryptophan-derived metabolites including phytoalexin, camalexin and indolic glucosinolates are also essential compounds for the *Atmlo2* PM resistance (Consonni et al., 2010).

Interactions between plants and powdery mildew pathogens

The name “powdery mildew” reflects the powdery tarnish on the plant surface and the tricking spores that are the result of the non-sexual reproductive phase of the fungus. Powdery mildew is a common disease of higher plant species which is caused by obligate

biotrophic Ascomycete fungi belonging to the Erysiphales. Worldwide, approximately 500 powdery mildew species are able to colonize about 10,000 distinct plant species (Takamatsu 2004). The disease is particularly prevalent in temperate and humid climates where it frequently causes significant yield losses in agricultural production. Pathosystems, involving tomato, barley and *Arabidopsis*, are well-studied and represent experimental interactions for understanding host and non-host resistances to powdery mildews (Bai et al., 2005; Huckelhoven, 2005; Li et al., 2007; Schulze-Lefert and Vogel, 2000).

Blumeria graminis f. sp. *hordei*, is the causal agent of powdery mildew on barley. Many resistance genes have been identified in barley which arrest fungal development at different stages of the fungal infection. The *mlo*-based resistance (resulting from the loss-of-function of the *Mlo* gene) arrests fungal growth at the penetration stage via papilla formation, the *Mlg* genes stop fungal growth via a single-cell HR, and the *Mla* genes limit the fugal growth by subsequent multicell HR (Hückelhoven et al. 2000). *B. graminis* f. sp. *hordei* has been recently sequenced together with powdery mildew *Erysiphe pisi* infecting pea and *Golovinomyces orontii* infecting *Arabidopsis* (Spanu et al., 2010). Interestingly, these sequenced obligate biotrophic fungal genomes contain a massive retrotransposon proliferation and genome-size expansion. These findings demonstrated that likely the powdery mildew increases their genetic variation by gene reshuffling. In addition, a number of genes involved in the primary and secondary metabolism as well as plant cell wall depolymerization are missing in the sequenced *Blumeria graminis* f. sp.*hordei* genome. These genes are mainly involved in the process that is dispensable for biotrophy. Deletion of these genes indicated the specific life-style of biotrophic parasites. In total, 248 *Blumeria* gene candidates encoding a signal peptide (SP) but lacking a transmembrane domain have been predicted as secreted effector proteins. However, between the sequenced three powdery mildew fungi which were considered as diverged ~70 million years ago, only a very low number (less than 10 among 248 candidates) of effectors are considered as closely related, indicating that the powdery mildew fungi are evolved by species-specific adaptations.

In *Arabidopsis thaliana*, four powdery mildew species are known to establish compatible interactions including *Golovinomyces* spp. and *Oidum neolycopersici* that is also the causal agent of tomato powdery mildew disease (Xiao et al., 2001). Known

sources of resistance in *Arabidopsis* comprise induced resistance and natural resistance conferred by alleles of the *RPW8* locus. The first group includes the *enhanced disease resistance 1* (*edr1*) mutant (Frye and Innes, 1998) and *powdery mildew resistant* (*pmr*) mutants (Vogel and Somerville, 2000, Vogel et al., 2002; Vogel et al., 2004). The *RPW8* locus comprises two dominantly inherited *R*-genes, *RPW8.1* and *RPW8.2*, which control resistance to a broad range of powdery mildew species (Xiao et al., 2001). RPW8 proteins contain a predicted coiled-coil (CC) and an assumed transmembrane (TM) domain, structurally different from other R-proteins identified to date. Though *RPW8*-mediated resistance was previously reported to be effective against *O. neolycoperisci* in the Ms-0 accession, heterologous expression of *RPW8* genes in tomato and *Arabidopsis* failed to confer enhanced resistance to *O. neolycopersici* (Xiao et al., 2001). The *RPW8*-mediated resistance present in several other *Arabidopsis* accessions seems to be non-functional against *O. neolycoperisci.* Instead, at least two major loci in the accession Bay-0 appear to mediate resistance against *O. neolycopersici* (Gollner et al., 2008), demonstrating that genetic factors in *Arabidopsis* for resistance to *O. neolycoperisci* is different from that to *Golovinomyces* spp. Very likely, *O. neolycoperisci* delivers effector(s) that is/are different from the ones delivered by *Golovinomyces* spp. and is/are able to evade RPW8-mediated recognition. This is supported by the fact that no *RPW8* homologues have been identified in cultivated tomato and some wild species (Xiao, personal comm.).

Tomato powdery mildew caused by *O. neolycopersici* occurred for the first time in 1988 in The Netherlands and has spread within 10 years to all European countries and is nowadays a worldwide disease on tomato, except for Australia where another species, *O. lycopersici* occurs (Kiss et al., 2001, 2005). The origin of *O. neolycopersici* is still a mystery. It is supposed that a powdery mildew “jumped” from its host species to tomato, as documented for other pathogens (Huang et al., 2000a). The fact that *O. neolycopersici* can infect other Solanaceous plant species like eggplant and non-Solanaceous species like cucumber may imply a potential outbreak of *O. neolycopersici* in other plant species (Jankovics et al., 2008). In wild tomato relatives, many resistance genes (named *Ol* genes) have been identified and characterized. Interestingly, mechanisms associated with resistances conferred by the *Ol*-genes mimic the ones observed in the interaction between barley and *B. graminis* f. sp. *Hordei.* Single cell HR-based resistance conferred by

dominant *Ol-4* gene occurs at primary haustorium stage, resembling the *Mlg* mediated resistance (HR at primary appressorium stage). Multiple cell HR-associated resistance governed by the dominant genes of *Ol-1, Ol-3 and Ol-5* is similar to the ones conferred by *Mla12*. Finally, the papilla-associated resistance mediated by the recessive genes *ol-2* mimics the *mlo*-based resistance (Li et al., 2007).

Leveillula taurica is another powdery mildew species infecting tomato. In addition to tomato, *L. taurica* (Lév.) G. Arnaud infects a broad host range of ca. 1000 species from 74 plant families (Palti, 1988; Hirata, 1966), including the economically important crops like pepper, eggplant, onion, cucumber and cotton (Correll et al., 1987; Bubici and Cirulli, 2008; Reis et al., 2004; Vakalounakis et al., 1994). Most of the powdery mildew species like *G. cichoracearum* and *O. neolycopersici* are epiparasitic fungi which develop all fungal structures except haustorium on the surface of the host. However, *L. taurica* is easily distinguishable from them by the characteristics of endophytic mycelium and Oidiopsis-type conidiophores. Kunoh et al. (1979) illustrated that, infection hyphae of *L. taurica* grew into the intercellular spaces of the leaves and formed haustoria in the spongy and palisade parenchyma cells. The fungus is growing inside the foliar tissues until the matured spores are released from the stomata. Infected host plant leaves appeared as covered with white, powdery fungal symptoms on the abaxial surface. The chlorotic spots are present on the adaxial surface at the corresponding spots of the mildew colony. In tomato, the only resistance source to *L. taurica* is the *Lv* gene that confers resistance via HR (Chunwongse et al., 1994). In pepper, several studies have been carried out to search for resistance to *L. taurica* (Blat et al., 2005; Daubeze et al., 1995; de Souza and Cafe, 2003; Shifriss et al., 1992). So far, five Quantitative trait loci (QTLs) have been identified governing the resistance to *L. taurica* in pepper, of which the QTL *Lt-6.1* co-localizes with the tomato *Ol-1*/*Ol-3* loci and the QTL *Lt-9.1* is supposed to be orthologous to the tomato *Lv* locus (Lefebvre et al., 2003).

Scope of the Thesis

This PhD project is focused on exploration of the *mlo*-based resistance in Solanaceous crops, and aims to answer the following questions: (1) How many *Mlo* homologs are present in the Solanaceae model plant tomato? (2) Are these homologues functionally

conserved for defense modulation? (3) Can we exploit *mlo*-like resistance in other vegetable crops of the Solanaceae family? (4) What common features are shared in the *Mlo* family members of Solanaceous crops, as well as in comparison with the "mildew-effective" *Mlo* sequences in barley and *Arabidopsis*? (5) Which defense signaling pathways are involved in the *mlo*-based resistance?

In Chapter 2 of this thesis, we showed that the recessive powdery mildew resistance conferred by the *ol-2* gene in tomato is caused by the loss-of-function of a tomato *Mlo* homologue, *SlMlo1.* We demonstrated by complementation experiments using transgenic tomato lines and virus-induced gene silencing (VIGS) assays that loss of *SlMlo1* function is responsible for powdery mildew resistance conferred by *ol-2*.

In Chapter 3, we performed a genome-wide identification of the tomato *Mlo* homologs (*SlMlo*). The isolated *SlMlo*s were characterized with respect to their genomic organization, relation with *Mlo* homologs from other species, the expression in different organs and upon inoculation with the powdery mildew pathogen *Oidium neolycopersici*. Finally, an RNAi-based reverse genetic approach was followed to investigate the possibility whether *SlMlo* homologs play a role in susceptibility to powdery mildews in tomato.

In Chapter 4, we studied the infection process of *L. taurica* in pepper leaves and developed an objective scoring methodology. We attempted to monitor the development of the pathogen to time the infection process by using different microscopic techniques. To quantify the pathogen biomass, we developed a real-time PCR assay and measured the absolute and the relative *L. taurica* DNA amounts in infected plant tissues.

In Chapter 5, we focused on investigating whether loss of function in *Mlo* genes induces resistance to *L. taurica* in pepper and tomato. We showed that the loss-of-function mutant of the *SlMlo1* gene in tomato confers resistance against *L. taurica*. In pepper, two *Mlo* homologs, *CaMlo1* (Panstruga, 2005) and *CaMlo2* that was isolated in the present study, are involved in the susceptibility of pepper to *L. taurica.*

In Chapter 6, we aimed to identify defense pathways required for basal and *mlo*-based *Arabidopsis* resistance to *Oidium neolycopersici*. We demonstrate that *mlo*-based resistance requires the correct functioning of SA signaling as well as PEN-dependent and -independent pre-penetrative defense mechanisms.

In Chapter 7, by summarizing all our data, our findings of *mlo*-based resistance in the

context of plant-pathogen interaction and other functions of MLO proteins in different biological processes of the plant are discussed.

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CHAPTER 1

CHAPTER 2

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Naturally Occurring Broad-spectrum Powdery Mildew Resistance in a Central American Tomato Accession is caused by Loss of Mlo Function

Yuling Bai[1], Stefano Pavan[1,2,5], Zheng Zheng[1,5], Nana Zappel[3], Anja Reinstädler[3], Concetta Lotti[2], Claudio De Giovanni[2], Luigi Ricciardi[2], Pim Lindhout[1], Richard Visser[1], Klaus Theres[4], Ralph Panstruga[3]

[1]Laboratory of Plant Breeding, Plant Sciences Group, Wageningen University, Droevendaalsesteeg 1, 6708 PB Wageningen, The Netherlands

[2]Department of Agroforestry, Enviromental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, Bari 70126, Italy

[3]Max-Planck Institute for Plant Breeding Research, Department of Plant-Microbe Interactions, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

[4]Max-Planck Institute for Plant Breeding Research, Department of Plant Breeding and Genetics, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

[5] With equal contribution

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CHAPTER 2

ABSTRACT

The resistant cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) line LC-95, derived from an accession collected in Ecuador, harbours a naturally occurring allele (*ol-2*) that confers broad-spectrum and recessively inherited resistance to powdery mildew (*Oidium neolycopersici*). As both the genetic and phytopathological characteristics of *ol*-2-mediated resistance are reminiscent of powdery mildew immunity conferred by loss-of-function *mlo* alleles in barley and *Arabidopsis*, we initiated a candidate gene approach to clone *Ol*-2. A tomato *Mlo* gene (*SlMlo1*) with high sequence-relatedness to barley *Mlo* and *Arabidopsis AtMlo2* mapped to the chromosomal region harbouring the *Ol*-2 locus. Complementation experiments using transgenic tomato lines as well as virus-induced gene silencing (VIGS) assays suggested that loss of *SlMlo1* function is responsible for powdery mildew resistance conferred by *ol-2*. In progeny of a cross between a resistant line bearing *ol-2* and the susceptible tomato cultivar Moneymaker, a 19-bp deletion disrupting the *SlMlo1* coding region co-segregated with resistance. This polymorphism results in a frame shift and, thus, a truncated non-functional SlMLO1 protein. Our findings reveal the second example of a natural *mlo* mutant that possibly arose post-domestication, suggesting that natural *mlo* alleles might be evolutionarily short-lived due to fitness costs related to loss of *mlo* function.

INTRODUCTION

Powdery mildew is a common disease of higher plant species that is caused by obligate biotrophic Ascomycete fungi belonging to the order Erysiphales. Worldwide, approximately 500 powdery mildew species are able to colonize about 10,000 distinct plant species (Takamatsu 2004). The disease is particularly prevalent in temperate and humid climates, where it frequently causes significant yield losses in agricultural settings, including greenhouse and field tomato (*Solanum lycopersicum*) farming. Powdery mildew caused by *Oidium neolycopersici* has recently been recognized as a worldwide emerging pathogen on tomato (Jones et al. 2001). There are two known tomato powdery mildew species in the *Oidium* genus, *O. lycopersici,* occurring in Australia, and *O. neolycopersici,* in the rest of the world (Kiss et al. 2001). Several powdery mildew (*Oidium neolycopersici*, *Ol*) resistance genes have been described and mapped in tomato, of which most confer dominantly inherited, isolate-specific resistance (Bai et al. 2005). However, *ol*-2 mediates recessively inherited broad-spectrum (non-isolate-specific) resistance to *O. neolycopersici* (Bai et al. 2005; Ciccarese et al. 1998). The *ol*-2 allele originates from a wild accession of *S. lycopersicum* var. *cerasiforme* (cherry tomato), a close relative of common cultivated tomato that is thought to represent an admixture of wild and cultivated tomatoes (Ciccarese et al. 1998; Nesbitt and Tanksley 2002). The respective accession, LA-1230, was originally collected in 1970 in Ecuador (University of California Davis, Tomato Genetics Resource Center database) in a home garden and park area. Based on genetic mapping, *ol*-2 was found to reside around the centromere of tomato chromosome 4 (De Giovanni et al. 2004). Histological analysis of the host-pathogen interaction of an *ol*-2-containing line revealed an early abortion of fungal pathogenesis associated with the formation of plant cell-wall appositions (papillae) at the attempted fungal entry sites (Bai et al. 2005). Taken together, these characteristics (recessive inheritance, broad-spectrum resistance profile, early termination of fungal pathogenesis) are reminiscent of powdery mildew resistance in barley (*Hordeum vulgare*) and thale cress (*Arabidopsis thaliana*) conferred by loss-of-function *mlo* (*mildew resistance locus o*) alleles (Consonni et al. 2006; Jørgensen 1992).

In land plants, *Mlo* genes comprise small to medium-sized families encoding a novel type of plant-specific integral membrane proteins with one or more as-yet-unknown biochemical functions (Devoto et al. 1999, 2003). In the monocot barley and the dicot

Arabidopsis, mutations in particular *Mlo* genes result in broad-spectrum powdery mildew resistance (Büschges et al. 1997; Consonni et al. 2006). It is thought that the respective protein isoforms modulate vesicle-associated defence responses at the cell periphery and that the powdery mildew pathogen possibly exploits these proteins for successful host cell entry (Panstruga 2005a). While, in barley, loss-of function of a single *Mlo* gene suffices to confer full resistance, unequal genetic redundancy (Briggs et al. 2006) between three phylogenetically closely related *Mlo* co-orthologs (*AtMlo2*, *AtMlo6* and *AtMlo12*, Panstruga et al. 2005b) results in a more complex scenario in *Arabidopsis*: absence of *AtMlo2* confers partial powdery mildew resistance which becomes enhanced in *Atmlo2 Atmlo6* or *Atmlo2 Atmlo12* double mutants. Full resistance requires loss of function of all three co-orthologs, i.e., an *Atmlo2 Atmlo6 Atmlo12* triple mutant (Consonni et al. 2006). Barley *mlo* mutants have been known for more than 60 years (Freisleben and Lein 1942) and have been successfully employed in European barley agriculture for more than 25 years (Lyngkjaer et al. 2000), emphasizing the principal durability of *mlo*-mediated disease resistance under agricultural conditions.

In case of barley, in addition to a broad range of induced mutations (Büschges et al. 1997; Panstruga et al. 2005; Piffanelli et al. 2002), one natural allele (*mlo*-11) has been described (Piffanelli et al. 2004). This allele derives from a barley landrace, an intermediate between wild and cultivated barley, initially found in Ethiopia. At the molecular level, it is characterized by the presence of a complex tandem repeat array, comprising *Mlo* 5´ regulatory and partial coding sequences, which localize upstream of a fully intact *Mlo* wild type copy (Piffanelli et al. 2004). Experimental evidence points to a transcriptional ´read-through´ phenomenon caused by the presence of the upstream repeat units, interfering with proper transcription of the downstream *Mlo* wild type copy (Piffanelli et al. 2004). Interestingly, the *mlo*-11 allele is meiotically unstable, resulting in rare reversion events which give rise to susceptible progeny. Reversion to susceptibility was found to coincide with a loss of the repeat array and restoration of wild-type-like *Mlo* transcript and MLO protein levels (Piffanelli et al. 2004).

Here, we used a targeted approach based on comparative genetics to clone the tomato *Ol*-2 gene. We selected and mapped two *SlMlo* candidate genes. The chromosomal location as well as genetic gain- and loss-of-function experiments suggested that *SlMlo1* is

Ol-2. Sequence analysis revealed a short deletion in the *Ol*-2 coding region of the resistant tomato accession which co-segregated with resistance.

MATERIALS AND METHODS

Plant and fungal material

Powdery mildew susceptible *S. lycopersicum* cv. MM and SM were used as wild-type tomato lines in this study. Homozygous resistant F_3 lines R26 and R28 of a cross of the susceptible parent SM and the resistant parental line LC-95 (derived from *S. lycopersicum* var. *cerasiforme* LA-1230) served as representatives of the *ol*-2 resistance locus (De Giovanni et al. 2004). An F_2 population of a cross between MM and R28 was used to verify the co-segregation of the *ol-2* allele with resistance. *Arabidopsis Atmlo2, Atmlo6*, and *Atmlo12* single mutants as well as the respective double and triple mutants have been described before (Consonni et al. 2006). Plants were grown at 21 ^{o}C (day)/19 ^{o}C (night) with 60 to 70% RH (relative humidity). The Wageningen isolate of *O. neolycopersici* was maintained on cv. MM as described by Bai and associates (2005).

Infection assays with *O. neolycopersici*

Disease tests were performed by spraying plants with a suspension of *O. neolycopersici* conidiospores (Bai et al. 2005). The inoculum was prepared from freshly sporulating leaves of heavily infected cv. MM plants. Conidiospores were washed in tap water and were used immediately. Four week-old tomato plants were inoculated with an inoculum of 2 x 10^4 spores per milliliter, while five week-old *Arabidopsis* plants were sprayed with an inoculum of 1x 10^5 spores/ml. A DI scale of 0, 1, 2, and 3 was used to semiquantitatively assess infection phenotypes: 0 = no visible fungal sporulation, 1 = few fungal colonies, 2 = up to 30% of the leaf area covered with fungal colonies, and 3 = more than 30% of the leaf area covered with fungal colonies.

Phylogenetic analysis

For phylogenetic analysis of MLO proteins, the Phylip 3.66 software package was used

(Felsenstein 1989). First, a multiple sequence alignment (generated by CLUSTALW) was established and manually optimized. A distance matrix was calculated by PROTDIST from the multiple sequence alignment and was then transformed into a tree using the neighbor-joining method (NEIGHBOR). The resulting phylogenetic tree was visualized by TREEVIEW. For bootstrap support, SEQBOOT (1,000 replicates), PROTDIST, NEIGHBOR, and CONSENSE algorithms were sequentially applied. All programs were run with standard parameters.

SlMlo mapping

A core set of 50 tomato ILs covering overlapping segments of all 12 tomato chromosomes (Eshed and Zamir 1995) was used for mapping the *SlMlo* genes. Full-size *SlMlo1* and partial *SlMlo2* cDNAs were radioactively labelled and hybridized to filters harbouring genomic DNAs of both parental lines digested with a variety of restriction enzymes to detect polymorphisms between the two parental lines. Subsequently, filters representing genomic DNAs of the ILs cut with the selected restriction enzyme were used for the actual mapping process.

Transgenic tomato lines

Tomato EST clones cLEC80N18 (GenBank accession BI923467) and cTOC20K10 (GenBank accession BI931548) representing either incomplete or disordered *SlMlo1* cDNAs were recombined to obtain a bona fide full-length *SlMlo1* cDNA clone. The respective cDNA was shuttled via plasmid pRT101 (harboring *Cauliflower mosaic virus* 35S promoter and terminator sequences; Töpfer et al. 1987) into binary vector pPZP211 (GenBank accession number U10490; Hajdukiewicz et al. 1994), using appropriate restriction sites. *Agrobacterium*-mediated transformation of resistant line R26 and subsequent selection of transgenic lines was performed as described (Knapp et al. 1994).

VIGS

The TRV vectors, derived from the bipartite TRV virus, were previously described (Liu et al. 2002). The VIGS *SlMlo1* construct was engineered by cloning a 293-bp genomic DNA

fragment from the central part of the *SlMlo1* coding region into pTRV2. This DNA fragment, corresponding to a central part of the *SlMlo1* cDNA (bp 806 to 973; Fig. 2) harboring an intron of 94 bp, was amplified with primers 5′-gtgacggatccCGTATCTTTGGGTGCCATTT-3′ and 5′-gtgacggtaccCAGGGCGATTAAACCAGAAA-3′ (lowercase letters indicate the overhang harboring the restriction sites of *Asp*718 and *Bam*HI) from genomic tomato DNA. The PCR product was digested with *Asp*718 and *Bam*HI and was ligated into pTRV2.

For *Agrobacterium tumefaciens*–mediated virus infection, cultures of *A. tumefaciens* GV3101 containing pTRV1, empty vector control, and each of the constructs derived from pTRV2 were grown and harvested as described (van der Hoorn et al. 2000). Equal volumes of cultures harboring pTRV1 and pTRV2 were mixed and were subsequently infiltrated into the lower (adaxial) side of cotyledons of 10-day-old tomato seedlings, using a 1-ml syringe lacking a needle. Two weeks after infiltration, plants were inoculated with *O. neolycopersici*.

RNA isolation and semi-quantitative RT-PCR

To monitor *SlMlo1* transcript accumulation, total RNA was extracted using Trizol (Invitrogen Life Technologies Co., Carlsbad, CA, U.S.A.) and purified with a NucleoSpin RNA II kit (MACHEREY-NAGEL GmbH & Co. KG. Düren, Germany). cDNA were synthesized with olig$(dT)_{18}$ primers using the SuperScript III RTS first-strand cDNA synthesis Kit (Invitrogen).

For RT-PCR with MM and R28 (Fig. 5A), actin cDNA was amplified with primers 5′-GCTCCACCAGAGAGGAAATACAGT-3′ and 5′-CATACTCTGCCTTTGCAATCCA-3′. The expression of *SlMlo1* was monitored by marker cTOC20 by amplifying a transcript with primers 5′-TGTGGTTTGCTTCATCTTGC-3′ and 5′-ACCAACACTCTTGGGGACAC-3′. For RT-PCR with F_2 plants (Fig. 5B), actin cDNA was amplified with primers published by Ditt and associates (2001). The expression of *SlMlo1* was monitored by amplifying sequences flanking the 19-bp deletion in the *SlMlo1* cDNA with primers 5′-ATGGGAGGATGAAACAAAAAC-3′ and 5′-ACAATCGATCGGTAGTAGTCAGA-3′.

For analysis of *SlMlo1* expression in T1 transgenic lines (Fig. 6A and B), oligonucleotides actin1 (5′-TGAGCCTCATTTTAAGCTC-3′) and actin4 (5′-GAAATACAACTCTTCTACATA-3′) were used for the amplification of the 1.3-kb actin amplicon serving as internal reference. Transgenic *SlMlo1* was selectively amplified by oligos SlMLO2 (5′-TTCCCATCTTGCTTTCATAAG-3′, binding at the 3′ end of *SlMlo1*,

forward primer) and 35Sterm (5′-CTACTCACACATTATTCTGG-3′, binding in the *Cauliflower mosaic virus* 35S transcriptional terminator sequence present in the binary vector, reverse primer), yielding a 0.55-kb PCR fragment. Real-time PCR was performed using SYBR green chemistry (Brilliant SYBR Green qPCR core reagent kit; Stratagene, La Jolla, CA, U.S.A.) on an iQ5 Multicolor real-time PCR detection system (Bio-Red, Munched, Germany). Relative transcript levels were calculated on the basis of the ΔΔct method.

Co-segregation of the 19 bp deletion in *SlMlo1* with powdery mildew resistance

Total DNA was extracted from leaves of F_2 plants using the cetyltrimethylammonium bromide DNA isolation method (Brugmans et al. 2003) and was used for genotyping the F_2 progeny with a codominant M/*SlMlo1* marker. This PCR marker, spanning the mutant site in *ol-2*, was amplified with oligonucleotides 5′-ACCCTTAAGAAACTAGGGCAAA-3′ (forward primer) and 5′-ACCATCATGAACCCATGTCT-3′ (reverse primer) with an annealing temperature of 55°C. Product size is 197 bp for wild-type *SlMlo1* (*Ol-2* genotype) and 178 bp for the homozygous *ol-2* mutant genotype, while heterozygous individuals exhibit the presence of both amplicons.

RESULTS

We first assessed whether the tomato powdery mildew pathogen, *O. neolycopersici*, is principally amenable to *mlo*-mediated resistance. Besides tobacco and other host plants, *O. neolycopersici* has been reported to be virulent on *A. thaliana* (Xiao et al. 2001). We thus inoculated susceptible Col-0 wild type plants and the previously described set of resistant *Arabidopsis mlo* mutants with *O. neolycopersici*. In *Arabidopsis*, three phylogenetically closely related *Mlo* co-orthologs, *AtMlo2*, *AtMlo6* and *AtMlo12* (Panstruga 2005b), exhibit unequal genetic redundancy with respect to powdery mildew infection phenotypes. *Atmlo2* mutants are partially resistant to adapted powdery mildews, while *Atmlo2 Atmlo6* and *Atmlo2 Atmlo12* double mutants exhibit increased, and the *Atmlo2 Atmlo6 Atmlo12* triple mutant full resistance (Consonni et al. 2006). Reminiscent of this scenario, *Atmlo2* plants were partially resistant and its derived double (*Atmlo2 Atmlo6* and *Atmlo2 Atmlo12*) and

triple (*Atmlo2 Atmlo6 Atmlo12*) mutants showed full resistance to *O. neolycopersici* (Fig. 1, Table 1). We conclude that *O. neolycopersici*, similarly to powdery mildews colonizing barley (*Blumeria graminis* f. sp. *hordei*) or *Arabidopsis* (e.g., *Golovinomyces cichoracearum* and *Golovinomyces orontii*), is sensitive to *mlo*-based resistance.

Fig. 1. *Oidium neolycopersici* requires *AtMlo2* function for pathogenesis on *Arabidopsis thaliana*. Exemplary infection phenotypes of rosette leaves of 7-week old *A. thaliana* wild type (Col-0) plants and T-DNA insertion mutants (*Atmlo2*, *Atmlo6*, *Atmlo12* single, double and triple mutants) inoculated with *O. neolycopersici*. The photograph was taken 15 days post inoculation. The experiment was repeated once with similar results.

Table 1. Infection phenotypes of *Arabidopsis mlo* mutants upon challenge with *Oidium neolycopersici.*

	Disease index (DI)[a]		
Genotype	**8dpi[b]**	**13dpi**	**15dpi**
Col-0	1.6±0.5[c]	2.8±0.4	2.8±0.4
Atmlo2	0.0	1.0±0.0	1.0±0.0
Atmlo6	0.0	2.0±0.0	1.5±0.0
Atmlo12	0.0	2.0±0.0	2.00.0
Atmlo2 Atmlo6	0.0	0.0	0.0
Atmlo2 Atmlo12	0.0	0.0	0.0
Atmlo6 Atmlo12	0.0	2.0±0.0	1.5±0.5
Atmlo2 Atmlo6 Atmlo12	0.0	0.0	0.0

[a]To semiquantitatively assess infection phenotypes, the following DI scale was used: 0 = no visible fungal sporulation, 1= few fungal colonies, 2 = up to 30% of the leaf area covered with fungal colonies, and 3 = more than 30% of the leaf area covered with fungal colonies.
[b]dpi = days post-inoculation.
[c]*DI mean*± standard deviation of five plants per genotype are presented.

Given the fact that *O. neolycopersici* requires host *Mlo* function for pathogenesis and based on the genetic and phytopathological similarities between *ol*-2-mediated powdery mildew resistance and the well-characterized loss-of-function *mlo* mutants in barley and *Arabidopsis*, we initiated a candidate approach to clone *Ol*-2. We inspected the publicly available tomato expressed sequence tag (EST) collections by BLAST analysis for cDNAs with a significant sequence relatedness to *AtMlo2*-encoded transcripts. We identified two EST contigs comprising twelve and three EST sequences, respectively, which correspond to cDNAs of two distinct genes, which we designated *SlMlo1* and *SlMlo2* (Fig. 2A). While multiple overlapping EST sequences enabled the inference of a conceptual full-length *SlMlo1* cDNA sequence (National Center for Biotechnology Information [NCBI] UniGene database, tomato Les.746), *SlMlo2* was only represented as a partial cDNA covering the 3´ end of the coding sequence (Fig. 2). Tissue origin of the ESTs indicates that *SlMlo1* is ubiquitously expressed (leaf, callus, flower), while expression of *SlMlo2* appears to be predominant in tomato breaker fruits (data not shown).

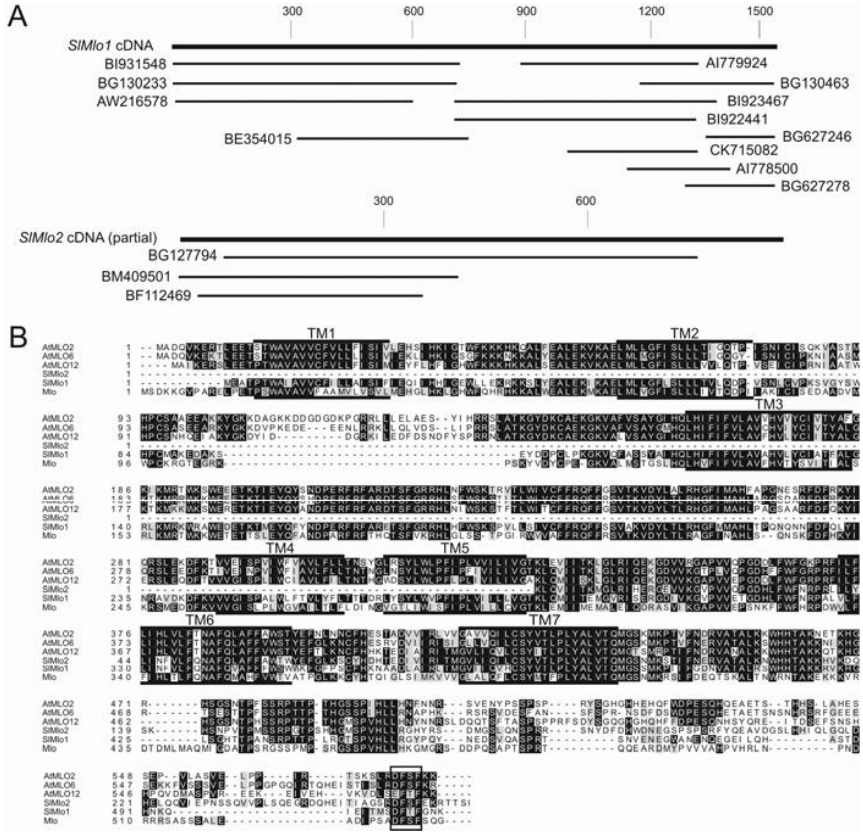

Fig. 2. ***SlMlo1*** **and** ***SlMlo2*** **encode polypeptides that are highly sequence related to barley MLO,** ***Arabidopsis*** **AtMLO2, AtMLO6, and AtMLO12. A**, The scheme represents the coverage of *SlMlo1* (top bold line) and *SlMlo2* (bottom bold line) cDNAs by expressed sequence tag (EST) sequences (thin lines) as revealed by BLASTN analysis using the *SlMlo1* or *SlMlo2* cDNA contig as query sequences, respectively. Designations next to the EST-signifying lines specify GenBank accession numbers. Scales above cDNAs indicate length in nucleotides. Note the different scales for *SlMlo1* and *SlMlo2*. **B**, Multiple amino-acid sequence alignment of barley MLO, tomato SlMLO1 and SlMLO2 as well as *Arabidopsis* AtMLO2, AtMLO6, and AtMLO12 polypeptide sequences. The alignment was generated by CLUSTALW using the default parameter. Positions of the seven transmembrane regions (TM1 through TM7) inferred from the experimentally determined topology of barley MLO (Devoto et al. 1999) are indicated by bars above and below the sequences. The C-terminal D/E-F-S/T-F tetra-peptide sequence, one of several motifs characteristic of barley MLO orthologs (Panstruga 2005b), is boxed.

We designed oligonucleotide primers flanking the deduced sequence contigs and performed reverse transcription polymerase chain reaction (RT-PCR) on RNA obtained from wild-type tomato leaves to verify the predicted *SlMlo1* and *SlMlo2* cDNA sequences. This confirmed the presence of either transcript in tomato leaves and validated the

nucleotide sequences of the respective EST-derived cDNA contigs (data not shown). Phylogenetic analysis comprising all *Arabidopsis* MLO protein sequences as well as SlMLO1 and SlMLO2 revealed that the two deduced tomato proteins cluster in the same clade as AtMLO2, AtMLO6, and AtMLO12 (Fig. 3).

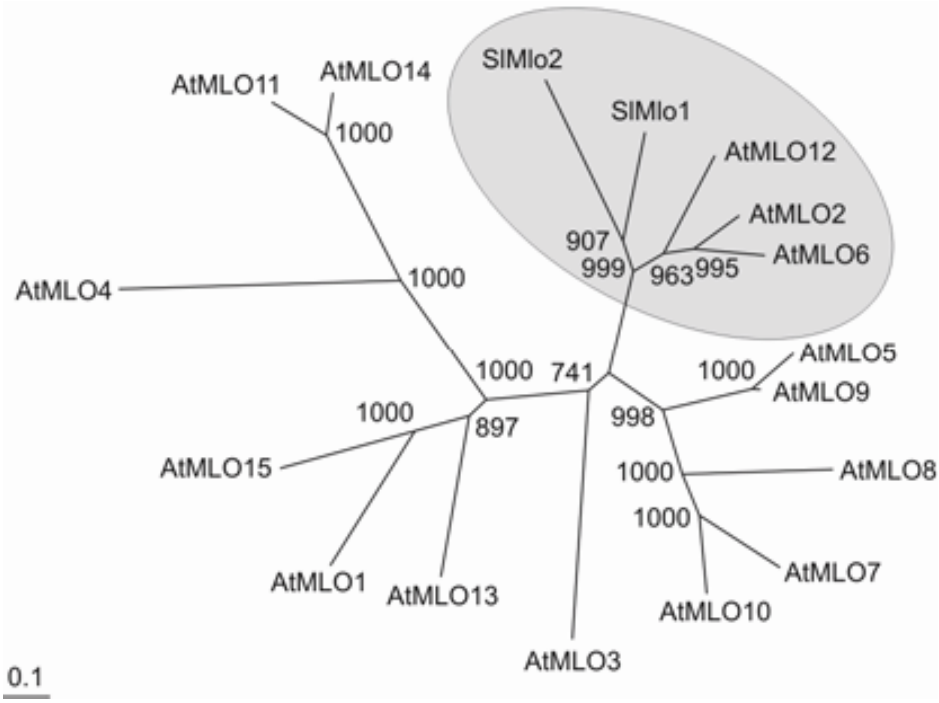

Fig. 3. A neighbor-joining phylogenetic tree of *Arabidopsis* and selected tomato MLO proteins. SlMLO1 and SlMLO2 are co-orthologs of AtMLO2, AtMLO6, and AtMLO12. The unrooted radial tree comprises all 15 *Arabidopsis* MLO paralogs (AtMLO1 to AtMLO15; Devoto et al. 2003) as well as SlMLO1 and SlMLO2 and was established on the basis of an optimized multiple-sequence aligment using the PHYLIP software package. Numbers above nodes indicate bootstrap values (based on 1,000 replicates) that support the respective branch. The scale (left bottom corner) indicates the number of amino-acid exchanges per site. The circled clade shaded in light gray harbors potential tomato (SlMLO1 and SlMLO2) and *Arabidopsis* (AtMLO2, AtMLO6, and AtMLO12) co-orthologs.

Based on this finding and the presence of a shared distinctive polypeptide motif at the C-terminus of the encoded polypeptides (Fig. 2B), which is considered to be diagnostic of an orthologous phylogenetic relationship (Panstruga 2005b), *SlMlo1* and *SlMlo2* likely represent co-orthologs of *AtMlo2*, *AtMlo6*, and *AtMlo12*.

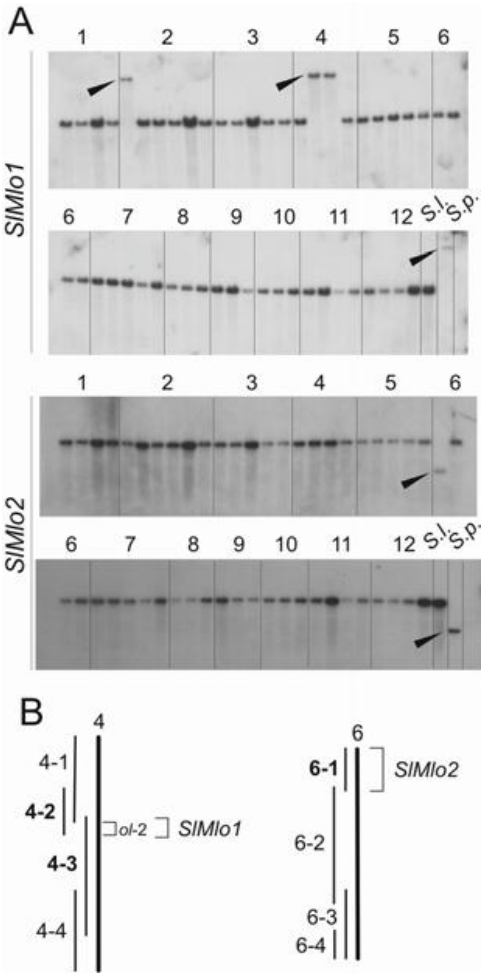

Fig. 4. Introgression line(IL)-based mapping of *SlMlo1* and *SlMlo2*. A, Gel blot hybridization of labelled *SlMlo1* (top panels) or *SlMlo2* (bottom panels) cDNA fragments on filters comprising restriction-digested genomic DNAs of a core set of *Solanum lycopersicum* x *Solanum pennelliii* ILs and respective parental lines. Genomic DNAs were either digested with restriction enzyme *Bgl*II (*SlMlo1*) or *Eco* RV (*SlMlo2*). Parental polymorphisms in *S. pennellii* and ILs showing the particular polymorphism are highlighted by black arrowheads. Numbers above gel sections indicate the lines representing the respective tomato chromosome. *S.l.* = *Solanum lycopersicum*; *S.p.* = *Solanum pennellii* (parental lines). Note the additional *SlMlo1* polymorphism in line 2-1 (upper panel). Occurrence of two overlapping chromosomal segments of chromosome 4 (lines 4-2 and 4-3) and absence of a second hybridizing band in any lane of this blot suggests that this polymorphism is most likely due to presence of an ectopic genomic fragment of chromosome 4 in line 2-1 (an impurity of the ILs; Bonnema et al. 2002). **B,** Scheme depicting the interpretation of the IL-based mapping shown in A. Solid thick lines represent tomato chromosome 4 (left) and 6 (right). Thin lines designate chromosomal segments covered by the respective ILs, bold numbers highlighting segments harbouring a sequence polymorphism of *SlMlo1* or *SlMlo2*. The previously determined map position of *ol*-2 (De Giovanni et al. 2004) as well as the deduced intervals harbouring *SlMlo1* and *SlMlo2* are indicated.

A collection of *S. lycopersicum* × *S. pennellii* introgression lines (IL; Eshed and Zamir 1995) provides an efficient tool for the rapid mapping of genes to tomato chromosomal segments. Using a core set of 50 IL covering each of the 12 tomato chromosomes with overlapping introgressed segments of *S. pennellii*, we determined a likely localization of *SlMlo1* at the center of chromosome 4 in a small region that also harbors the *ol*-2-containing interval (De Giovanni et al. 2004), while *SlMlo2* was found to reside on top of chromosome 6 (Fig. 4). Since *ol-2* was previously unequivocally found to be a single recessive locus located on tomato chromosome 4 (De Giovanni et al. 2004), we focused in the following on *SlMlo1* as a candidate for *Ol*-2 and excluded *SlMlo2* from further analysis.

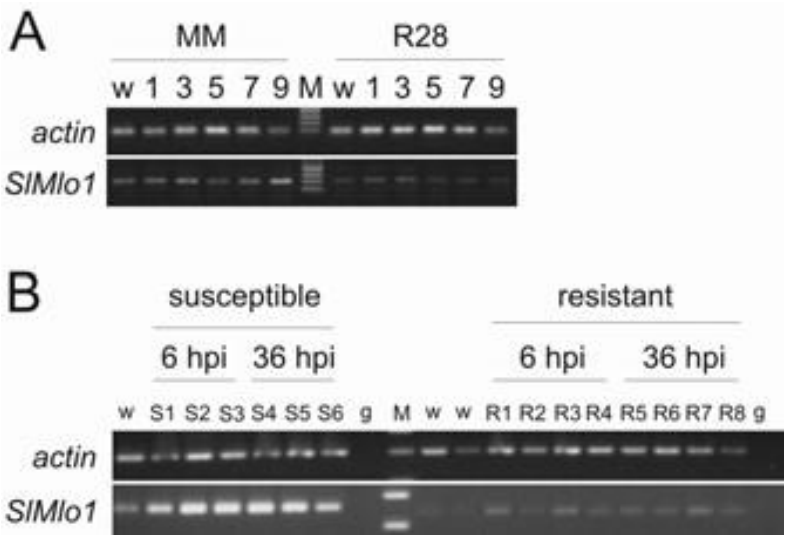

Fig. 5. *SlMlo1* transcript accumulation upon powdery mildew challenge. A, Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of *SlMlo1* and *actin* transcript levels in susceptible wild-type tomato (cv. Moneymaker [MM]) and the powdery mildew resistant F3 line R28. RNAs were extracted from leaves of either unchallenged (w =water control) or powdery mildew–challenged plants at 1, 3, 5, 7, and 9 days after inoculation with *Oidium neolycopersici*. Lane 'M' indicates DNA size marker. **B**, Semiquantitative RT-PCR analysis of *SlMlo1* and *actin* transcript levels in homozygous susceptible (*Ol-2/Ol-2* genotype; lines S1 to S6) and resistant (*ol-2/ol-2* genotype; lines R1 to R8) F2 progeny of a cross between cv. MM and the powdery mildew–resistant F3 line R28. RNAs were extracted from leaves of either unchallenged or powdery mildew–challenged plants at 6 and 36 h after inoculation with *O. neolycopersici*. Lane g = amplification using genomic DNA as template, lane M = DNA size marker. Note the slight size difference (owing to the 19-bp deletion) between *SlMlo1* cDNAs derived from susceptible (*Ol-2*) or resistant (*ol-2*) lines. Absence of products derived from genomic DNA as template likely is due to either the presence of multiple introns in the respective genomic sequences, the potential location of primer binding sites on exon or intron junctions, or both.

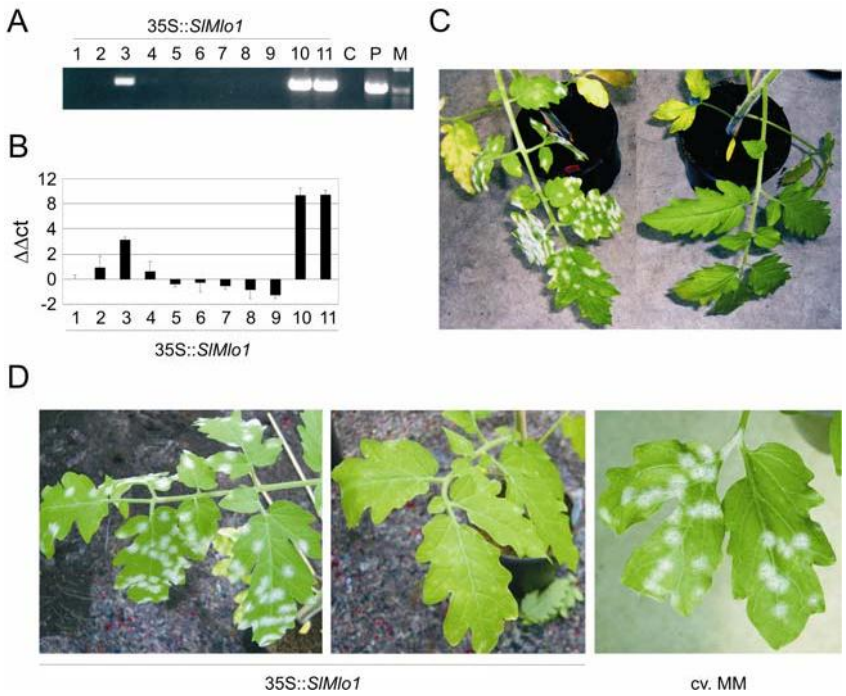

Fig. 6. Complementation of *ol*-2–mediated resistance in transgenic tomato lines overexpressing *SlMlo1*. A, Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis (25 cycles) of eleven (1 to 11) transgenic T1 lines expressing 35S::*SlMlo1* derived from the powdery mildew resistant line R26. RT-PCR reactions were performed using the *SlMlo1* transgene-specific oligonucleotide combination *SlMlo2*/35S term. Note that the panel has been composed of various sections of a larger photograph of the respective agarose gel. C = water instead of template negative control, P = plasmid positive control, and M = size marker. **B**, Quantitative real-time PCR analysis of 35S::*SlMlo1* transgene expression. Real-time PCR reactions were performed with the *SlMlo1* transgene-specific oligonucleotide combination SlMlo2/35Sterm (*SlMlo1* expression) and the *actin*-specific primer combination actin1/actin4 (*actin* expression). Relative expression levels based on the ΔΔct values (average ± standard deviation of four replicates) are indicated for the 11 transgenic lines in relation to line 1. Note that ΔΔct values are logarithmic indicators of differences in transcript accumulation. A second experiment yielded similar results. **C**, Exemplary infection phenotypes upon powdery mildew (*Oidium neolycopersici*) challenge of T2 progeny of a transgenic line. Depicted are two descendants of T1 line 11 (described above), one being susceptible (left, DI = 3) and one being largely resistant (right, DI = 0). The photograph was taken 17 days post-inoculation. **D**, Close-up views of powdery mildew–infected leaves of susceptible (left panel) and resistant (middle panel) progeny of a susceptible transgenic line (T2 descendants of T1 line 11 expressing 35S::*SlMlo1*) and the susceptible tomato wild-type control (cv. Moneymaker [MM]; right panel). Photographs were taken 17 days post-inoculation. Note that infection phenotypes of the susceptible transgenic line and cv. MM are comparable (DI = 3).

We performed semiquantitative RT-PCR analyses in a long-term timecourse experiment following powdery mildew challenge, to study *SlMlo1* expression in susceptible

Ol-2 wild-type and resistant *ol*-2 (line R28) mutant plants. We observed moderately reduced *SlMlo1* transcript accumulation in line R28 as compared with wild-type plants at all tested time points (Fig. 5A). Similar results were obtained upon comparison of *SlMlo1* transcript levels at early timepoints following *O. neolycopersici* inoculation in homozygous susceptible (*Ol-2*/*Ol-2* genotype) and resistant (*ol-2*/*ol-2* genotype) F_2 progeny of a cross between the susceptible cv. Moneymaker (MM) and the resistant line R28 (Fig. 5B). Consistent with previous findings in barley (Piffanelli et al. 2002), both *Ol*-2 and *ol*-2 genotypes exhibited a noticeable increase in *SlMlo1* transcript levels at early timepoints following powdery mildew challenge (Fig. 5B).

Table 2. Segregating analysis of transgenic tomato lines.

T_1 generation	**T_2 generation**		
Line	Susceptible[a]	Resistant[a]	χ^2 (3:1)[b]
2	0	22	n.a.
4	0	16	n.a.
5	0	16	n.a.
8	0	12	n.a
9	0	11	n.a
10	7	5	*P*>0.5
11	12	4	*P*>0.9

[a]Susceptible plants with disease index (DI) score of 2 or 3 and resistant plants with a DI of 0 or 1 (0 = no visible fungal sporulation, 1= few fungal colonies, 2 = up to 30% of the leaf area covered with fungal colonies, and 3 = more than 30% of the leaf area covered with fungal colonies.
[b]n.a. = not applicable.

To directly assess a potential role of *SlMlo1* in conferring powdery mildew susceptibility in wild-type tomato, we initiated both genetic gain- and loss-of-function experiments. We reasoned that, if loss of *SlMlo1* function was responsible for resistance conferred by the *ol*-2 gene, then strong constitutive expression of a *SlMlo1* transgene should restore susceptibility in resistant plants bearing *ol*-2. Vice versa, *SlMlo1* gene silencing in susceptible tomato wild-type plants should result in enhanced powdery mildew resistance. For this and the following experiments, we used a resistant F_3 line, R26, of a cross between the susceptible cv. Super Marmande (SM) and the original resistant line *S.*

lycopersicum var. *cerasiforme* LC-95 (De Giovanni 2004). We generated a binary vector harbouring *SlMlo1* under control of the strong constitutive *Cauliflower mosaic virus* 35S promoter and transformed tomato line R26 with the respective construct. Upon selection on kanamycin, we obtained 11 independent transgenic lines. We performed semi-quantitative as well as real-time RT-PCR, using RNA extracted from leaf material of T_1 plants to assess expression levels of the transgene. Both types of analysis revealed that transgenic lines 3, 10, and 11 expressed the highest levels of wild-type *SlMlo1* cDNA (Fig. 6A and B). We challenged selfed progeny (T_2 plants) of the individual transgenic lines with *O. neolycopersici* and observed restoration of macroscopically visible powdery mildew growth among the offspring of the two lines (lines 10 and 11) that, beforehand, exhibited strongest transgene expression in the T_1 generation (Fig. 6C and D; T_1 seeds of transgenic lines 1, 3, 6, and 7 did not germinate, preventing analysis of the respective T_2 progeny). Powdery mildew infection phenotypes of susceptible segregants derived from transgenic lines 10 and 11 were comparable to the fully susceptible cv. MM (disease index [DI] score = 3). PCR fingerprint analysis employing codominant markers TOM316 and U3-2*Hha*I, closely linked to *ol*-2, confirmed the identity of the parental line (R26) used for transformation and excluded the possibility of a seed contamination (data not shown). In total, 28 T_2 plants (12 from line 10 and 16 from line 11) were tested, and susceptibility to *O. neolycopersici* segregated in both these T_2 generations in an approximately 3:1 (susceptible/resistant) ratio, suggesting the presence of single-transgene loci in both of the transgenic lines (Table 2). Occurrence of susceptible T_2 plants and segregation in the T_2 progeny indicates that restored susceptibility is a heritable genetic trait upon transgenic expression of *SlMlo1.*

Fig. 7. VIGS of *SlMlo1* confers enhanced powdery mildew resistance in wild-type tomato. Leaves of wild-type tomato (cv. MoneyMaker) were infiltrated with agrobacteria harbouring either a T-DNA with *Tobacco rattle virus* (TRV) carrying no insert (right) or a T-DNA with a TRV derivative harbouring a *SlMlo1* fragment (left). At 17 days after bacterial inoculation, leaves were challenged with *Oidium neolycopersici*. The photograph was taken 13 days after fungal inoculation.

For genetic loss-of-function experiments, we engineered a transgenic *Tobacco rattle virus* (TRV) harboring a *SlMlo1* fragment for virus-induced gene silencing (VIGS) experiments. Upon inoculation of the modified virus (TRV-*SlMlo1*) on susceptible wild-type tomato plants of *S. lycopersicum* cv. MM and subsequent challenge with *O. neolycopersici*, we observed a dramatic reduction of macroscopically visible powdery mildew colonies as compared with inoculation with wild-type TRV (Fig. 7). Two independent VIGS assays yielded comparable results. Taken together with the complementation experiments described above, this finding suggests that, indeed, a loss of *SlMlo1* function is responsible for powdery mildew resistance conferred by the recessively inherited *ol-2* gene.

In principle, lack of the *SlMlo1* transcript or alterations in the encoded cDNA or polypeptide sequence could lead to the observed disease-resistance phenotype. In barley, all induced mutants analyzed to date were found to confer changes in the *Mlo* coding sequence (Büschges et al. 1997; Panstruga et al. 2005; Piffanelli et al. 2002), while the sole known natural *mlo* allele, *mlo*-11, is characterized by severely perturbed *Mlo* transcript levels (Piffanelli et al. 2004). Since RT-PCR analysis revealed *SlMlo1* transcript accumulation in both *Ol-2* and *ol-2* genotypes (discussed above), we analyzed *SlMlo1*

cDNA sequences of tomato wild-type plants (cv. SM) and the resistant progeny (F_3 line R26) of *S. lycopersicum* var. *cerasiforme* LA-1230. While the cDNA sequence of SM was identical to the wild-type *SlMlo1* reference sequence (GenBank accession number AY967408; NCBI UniGene tomato Les.746), we found a 19-bp deletion in the cDNA of line R26. This sequence alteration leads to a frameshift in the coding region, predicted to result in premature translational termination in the second cytoplasmic loop of SlMLO1 and, thus, a severely truncated protein (Fig. 8). We found the same DNA sequence polymorphism in the genomic sequence of the independent resistant F_3 line R28 (data not shown). Notably, apart from this difference, the nucleotide sequences of the *SlMlo1* open reading frames of the susceptible SM and the resistant R26 lines were identical (data not shown). We conclude that the resistant natural accession is expected to encode a *SlMlo1* null allele conditioned by a single mutational event in the *SlMlo1* coding region.

We designed oligonucleotide primers flanking the 19-bp deletion in the *SlMlo1* cDNA and analyzed the *SlMlo1* genotype in an F_2 population segregating for *ol-2*-mediated powdery mildew resistance. As judged from PCR data, presence of the 19-bp deletion fully correlated with resistance (n = 106 analyzed F_2 individuals), further substantiating that *SlMlo1* is *Ol-2* (Fig. 9).

DISCUSSION

We have shown here that the recessively inherited *ol*-2-mediated powdery mildew resistance originally identified in a natural accession (LA-1230) of *Solanum lycopersicum* var. *cerasiforme* collected in Ecuador is mediated by loss of *SlMlo1* function. The chain of evidence consists of a common map position (Fig. 4B), genetic gain- and loss-of-function experiments (Figs. 6 and 7), as well as co-segregation of a small (19 bp) deletion in the *SlMlo1* coding region with the resistant phenotype (Figs. 8 and 9). Taken together, these findings indicate that the resistant mutant derived from the cherry tomato accession LA-1230 encodes a *SlMlo1* null allele, caused by a small nucleotide polymorphism in the coding region of the gene.

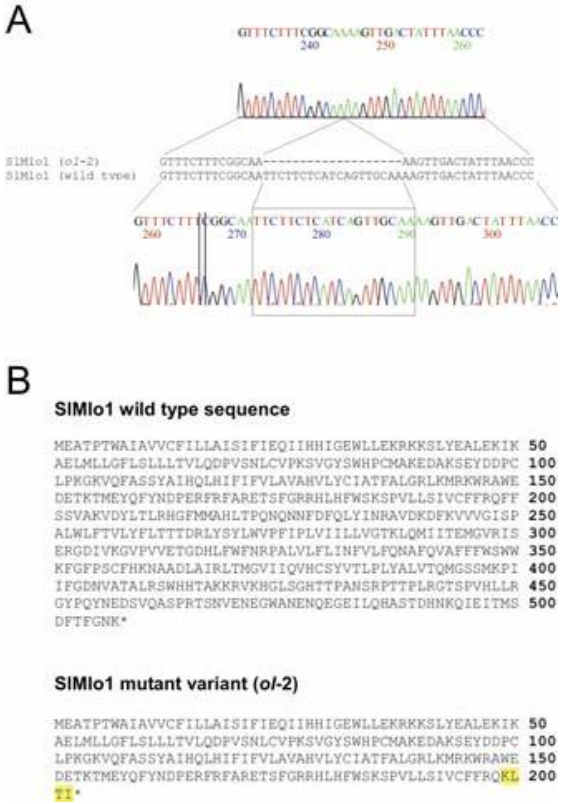

Fig. 8. The *ol*-2 allele represents a *SlMlo1* mutant variant with a 19-bp deletion in the coding region. A, Segment of sequence trace files obtained by direct sequencing of a full-size *SlMlo1* reverse transcription-polymerase chain reaction product using either RNA from leaves of resistant line R26 or RNA from wild-type tomato (cv. Super Marmande) as source material. Corresponding sequence alignments of *SlMlo1* wild-type cDNA and the mutant variant emphasize the 19-bp deletion in the *SlMlo1* coding region of *ol*-2 plants (boxed in the *SlMlo1* wild-type sequence). **B**, Consequence of the 19-bp deletion on the conceptual SlMLO1 amino-acid sequence. Upper panel, predicted wild-type SlMLO1 amino-acid sequence; lower panel, predicted mutant SlMLO1 amino-acid sequence. The last four amino acids of the mutant protein variant (highlighted in yellow) are due to the frameshift and are not present in the SlMLO1 wild-type polypeptide.

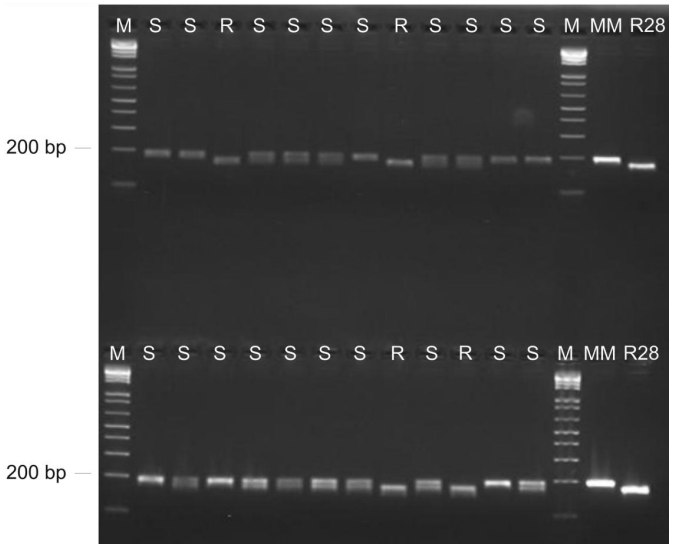

Fig. 9. The 19bp polymorphism in *SlMlo1* co-segregates with powdery mildew resistance. F_2 progeny of a cross of wild-type tomato (cv. MM) and the resistant line R28 were analyzed for *O. neolycopersici* infection phenotypes and the respective genotype of the co-dominant M/SlMLO1 marker. PCR products representing the marker were amplified from genomic DNA as a template and resolved on a 2.5% agarose gel. Like R28, resistant (R) F_2 plants are homozygous for the mutant *ol-2* allele while susceptible (S) F_2 plants are either homozygous for the wild-*Ol-2* allele (like cv. MM) or heterozygous (indicated by the presence of a double band). M=DNA size marker.

In tomato, currently available ESTs indicate the presence of at least two paralogs that likely represent co-orthologs of *AtMlo2*, *AtMlo6* and *AtMlo12* in *Arabidopsis* (Figs. 2 and 3; Panstruga 2005b). Interestingly, unlike in *Arabidopsis*, loss-of-function of a single gene (*SlMlo1*) in tomato suffices to convey virtually full powdery mildew resistance (Bai et al. 2005). In *Arabidopsis*, *AtMlo2*, *AtMlo6* and *AtMlo12* exhibit unequal genetic redundancy (Briggs et al. 2006), and a mutation in the gene with the major ´susceptibility conferring´ activity, *AtMlo2*, results merely in partial powdery mildew resistance (Consonni et al. 2006). This indicates that the functional specialization of MLO isoforms evolved differently in *Arabidopsis* and tomato. In barley, like in tomato, a mutation in a single *Mlo* gene is sufficient to confer full powdery mildew resistance. However, unlike as in tomato, *Mlo* is the only expressed gene copy of this (co-)ortholog cluster in barley. A closely sequence-related paralog, *HvMlo2* (GenBank accession number Z95496) appears to represent a non-expressed pseudogene (R. Panstruga, *unpublished findings*). In sum, it seems that the genetic complexity and functional specialization within the *Mlo* co-ortholog cluster related to

powdery mildew susceptibility significantly differs between various monocot and dicot plant species. With respect to plant breeding aspects, this implies that the time and effort required for obtaining powdery mildew resistant *mlo* mutants by either forward or reverse genetics or transgenic gene silencing approaches may also vary considerably between plant species. Since in addition to cherry tomato, the *ol*-2 allele also confers resistance in the genetic background of cultivated round tomatoes (*S. lycopersicum*, e.g. cv. MM or SM), *ol*-2 might be useful for breeding in different types of tomatoes. Given the unusual durability of *mlo*-mediated powdery mildew resistance in barley (Lyngkjaer et al. 2000), the *ol*-2 allele promises to represent indeed a precious resource for future tomato breeding, the 19 bp deletion being a valuable polymorphism for marker-assisted breeding. Interestingly, resistant tomato lines lack any obvious pleiotropic phenotype(s) such as early senescence-like leaf chlorosis of unchallenged plants, which has been reported to occur in both barley and *Arabidopsis mlo* mutants (data not shown; Consonni et al. 2006; Piffanelli et al. 2002). However, the extent of expression of pleiotropic effects in barley and *Arabidopsis* is considerably dependent on environmental conditions (Consonni et al. 2006; Jørgensen et al. 1992), leaving the possibility that lack of obvious pleiotropic phenotypes in *ol*-2 lines is based on the particular growth settings used in this study.

We observed moderately lower *SlMlo1* transcript levels in *ol-2* as compared to *Ol-2* genotypes, both in unchallenged as well as in powdery mildew–inoculated plants (Fig. 5A and B). Given that *ol-2* likely is a null allele, one may hypothesize that the SlMLO1 protein exerts direct or indirect positive feedback regulation on the transcription of its own gene. Evidence for such a positive regulatory feedback loop has, however, neither been found in barley nor *Arabidopsis mlo* mutants (Piffanelli et al. 2002; C. Consonni and R. Panstruga, unpublished data). Unless this was a tomato-specific phenomenon, it is thus more likely that the observed difference in *SlMlo1* transcript levels is due to intrinsic genetic variation of tomato (sub)species, e.g., within the *SlMlo1* promoter region. Alternatively, the reduced transcript levels might result from one or more second-site mutational events in *SlMlo1* regulatory sequences following the inactivation of *SlMlo1* function by the 19-bp deletion in the coding region. The current unavailability of *SlMlo1* genomic sequences covering the 5′ regulatory region prevents testing these hypotheses at present. Intriguingly, despite lower *SlMlo1* transcript levels, the *ol-2* genotype retained rapid pathogen-responsiveness of

SlMlo1 transcript accumulation following powdery mildew challenge (Fig. 5B).

The *ol*-2 allele is the second example, in addition to barley *mlo*-11, of a naturally occurring powdery mildew-resistant *mlo* mutant and one of few examples, besides the *Fer* gene controlling iron uptake (Ling et al. 2002) and the *Cnr* fruit-ripening locus (Manning et al. 2006), of a cloned natural tomato mutant gene. Identification of a second natural *Mlo* loss-of-function allele indicates that this type of mutant might be more prevalent in nature than previously anticipated. In pea, recessively inherited broad-spectrum *er-1* powdery mildew resistance is another candidate for natural *mlo*-based immunity (Fondevilla et al. 2006). Likewise, collections of novel barley accessions from various regions of the Old World represent further aspirants for natural *mlo* mutants (Czembor and Czembor 2002). It remains, however, to be seen whether *mlo-11* and *ol-2* signify the tip of the natural *mlo* mutant iceberg or whether they represent the rare exceptions.

The *mlo*-11 allele, originating from a barley landrace collected in Ethiopia, is characterized by the presence of a complex tandem-repeat array (Piffanelli et al. 2004). Resistance in *mlo*-11 is principally reversible due to a meiotic instability of the respective polymorphism. Based on this reversibility, it has been speculated that the barley *mlo-11* allele might represent an adaptive balanced polymorphism which could dampen the adverse effects of *mlo* mutants and thus be advantageous in natural barley populations facing varying pathogen pressure (Piffanelli et al. 2004). Unlike barley *mlo*-11, the mutational event in *ol*-2 tomato is rather simple, considered to be essentially meiotically stable and is supposed to represent the result of a fortuitous incident during meiotic replication or recombination. The likely presence of this mutational event in a heterozygous state in the *S. lycopersicum* var. *cerasiforme* accession LA-1230 (Ciccarese et al. 1998) may indicate that *ol*-2 is also maintained as a beneficial balanced polymorphism in natural tomato populations. Interestingly, the natural epigenetic mutation found at the *Cnr* fruit ripening locus in the tomato hybrid cv. Liberto has also been reported to be subject to rare somatic reversion events and is thus also reversible (Manning et al. 2006).

The *SlMlo1* null mutant allele present in *ol-2* genotypes is presumably free of any evolutionary constraints. This circumstance would allow for the accumulation of further nucleotide alterations in the defective *SlMlo1* gene over time. Absence of any additional sequence variation—besides the 19-bp deletion—in the *SlMlo1* coding region thus points to

a recent origin of the *ol-2* allele. The domestication history of tomato has not yet been fully resolved and is still controversially discussed (Nesbitt and Tanksley 2002; Peralta et al. in press). It was, however, recently proposed that *S. lycopersicum* var. *cerasiforme* represents an admixture of wild and cultivated tomatoes (Nesbitt and Tanksley 2002). Since *ol-2* was originally identified in a Central American accession of *S. lycopersicum* var. *cerasiforme*, it seems that, reminiscent of barley *mlo-11* (Piffanelli et al. 2004), the mutation in *ol-2* originates from a domestication intermediate of a presently cultivated plant species. In addition, as in the case of *mlo-11*, the *ol-2* mutational event possibly occurred after or coincident with the domestication of wild tomatoes. In the case of barley *mlo-11*, it has been speculated that the emergence of the natural allele might have compensated for the erosion of the natural genetic variation (including race-specific disease resistance) following barley domestication (Piffanelli et al. 2004). Occurrence of a second natural *mlo* allele derived from a domestication intermediate and of supposedly recent origin principally supports this hypothesis. Alternatively, owing to the known adverse effects of mutations in *mlo* genes, such as spontaneous callose deposition and early leaf senescence (Consonni et al. 2006; Piffanelli et al. 2002), *mlo* mutants might have a long-term selective disadvantage in natural settings, resulting in a selective sweep that prevents the discovery of evolutionary older *mlo* mutants that harbor additional DNA footprints. It appears that only the discovery and molecular analysis of more ancient *mlo* mutants will allow discrimination between these two possibilities.

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CHAPTER 3

Genome-wide Study of The Tomato *Mlo* Gene Family and its Functional Characterization in Response to The Powdery Mildew Fungus *Oidium neolycopersici*

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Genome-wide Study of the Tomato Mlo Gene Family and its Functional Characterization in Response to The Powdery Mildew Fungus Oidium neolycopersici

Zheng Zheng[1], Michela Appiano [1, 2], Stefano Pavan[2], Robin Huibers[1], Annelies Loonen[1], Richard Visser[1] and Yuling Bai[1,*]

[1]Wageningen UR Plant Breeding, Wageningen University & Research Centre, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

[2] Department of Agroforestry, Enviromental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, 70126 Bari, Italy

*Corresponding author: Yuling Bai, Email: bai.yuling@wur.nl

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CHAPTER 3

ABSTRACT

The *Mlo* gene family encodes for plant-specific seven transmembrane domains proteins which are likely to act in the transduction of calcium-dependent stimuli. It is now clear that some members of the *Mlo* family act as susceptibility factors for fungi causing the powdery mildew disease. Other *Mlo* homologs have been shown to regulate the physiological processes of root thigmomorphogenesis and pollen tube reception by the female gametophyte. However, the role of most *Mlo* genes remains unknown. In this study, we took advantage of the recent developments in tomato genomics to perform a genome-wide study of the tomato *Mlo* gene family (*SlMlo* family). 16 *SlMlo* homologs were identified and characterized in relation to their structure and polymorphism, genomic organization, phylogenetic relationship and expression profile in axenic conditions and upon inoculation with the powdery mildew fungus *Oidium neolycopersici*. Results obtained from RNAi-based silencing experiments showed that *SlMlo1* is the only one out of four closely related *SlMlo* homologs to play a major role in susceptibility to *O. neolycopersici*. The outcome of this study provides useful information to retrieve *Mlo* susceptibility genes across cultivated species, since we show that *SlMlo1* is univocally identified by the combination of comparative and expression analyses. Moreover, it gives genetic and genomic information useful for the reconstruction of evolution on the *SlMlo* gene family and further investigations to assign biological functions to (*Sl*)*Mlo* homologs.

INTRODUCTION

The *Mlo* gene family encodes for seven transmembrane domains proteins in plants, topologically related to metazoan G-protein coupled receptors (GPCRs) (Devoto et al., 2003), and containing a calmodulin-binding domain which is likely involved in sensing calcium influxes into cells. Previous genome-wide studies have identified 15 *Mlo* homologs (*AtMlo*) in *Arabidopsis* and 17 *Mlo* homologs (*VvMlo*) in grapevine (Devoto et al., 2003; Feechan et al., 2008).

Specific homologs of the *Mlo* family act as susceptibility genes towards epiphytic fungi causing the powdery mildew disease, since their inactivation, through loss-of-function mutations or silencing, has been associated to increased resistance (*mlo* resistance) (Pavan et al., 2010). Three *Mlo* susceptibility genes have been identified in *Arabidopsis* (*AtMlo2*, *AtMlo6* and *AtMlo12*) (Consonni et al., 2006; Bai et al., 2008), whereas single *Mlo* susceptibility genes have been identified in barley (*HvMlo*), tomato (*SlMlo1*), and pea (*PsMlo1*) (Buschges et al., 1997; Bai et al., 2008; Pavan et al., 2010; Humphry et al., 2011). Dicot MLO isoforms involved in powdery mildew susceptibility have been shown to cluster in the same phylogenetic clade in the comparative trees of the MLO protein family (Pavan, 2011).

Recent studies in *Arabidopsis* have indicated that *Mlo* genes play a role in different biological processes. *AtMlo4* and *AtMlo11* together control 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. The biological roles of other *Mlo* homologs are still elusive.

The tomato genome sequencing project started in 2004 and recently culminated with the release of full genome sequence of the processing tomato cultivar Heinz 1706 (The Tomato Genome Consortium 2012, http://solgenomics.net/). In this study, we exploited tomato sequence information to identify tomato *Mlo* homologs (*SlMlo*), which were then characterized with respect to their genomic organization, relation with *Mlo* homologs from other species and expression in different organs and upon inoculation with the powdery mildew pathogen *Oidium neolycopersici*. Finally, an RNAi-based reverse genetic approach was followed to investigate the possibility that *SlMlo* homologs closely related to *SlMlo1*

could play a role in susceptibility to *O. neolycopersici*.

MATERIALS AND METHODS

Plant material, fungal material and inoculation

The tomato cultivar Moneymaker (MM) and the Wageningen isolate of *O. neolycopersici* were used in this study (Bai et al., 2008). For inoculation, a suspension of *O. neolycopersici* conidia (1.25 x 10^4 conidia/ml or 2 x 10^5 conidia/ml, as specified below) was prepared, by rinsing freshly sporulating leaves of infected tomato plants in water. Inoculations were performed on one month-old plants.

Identification and cloning of the *SlMlo* gene family

Predicted tomato MLO protein sequences were identified in the Sol Genomics Network (SGN) database by using the BLASTP and TBLASTN algorithms with *Arabidopsis* AtMLO protein sequences as a query. Chromosomal localization, sequences of the corresponding genes and intron/exon boundaries were inferred by annotations from the International Tomato Annotation Group (ITAG). In order to clone and sequence the *SlMlo* gene family from the cultivar MM, total RNA from leaves, roots, flowers and ripened fruits were isolated (RNeasy plant mini kit, Qiagen). Two primer pairs designed to specifically amplify sequences from individual *SlMlo* homologs were designed by the Primer3 software (Rozen and Skaletsky, 2000) and used for one-step RT-PCR (SuperScript® III One-Step RT-PCR System, Invitrogen) (Table 1). Corresponding amplicons were cloned into the pGEM®-T Easy vector (Promega) and recombinant plasmids were sequenced by using universal T7 and SP6 primers (Greenomics™). In order to reveal gene structures and polymorphisms, *SlMlo* sequences identified by cloning were aligned with *SlMlo* sequences identified *in silico* and the corresponding genomic regions.

Table 1. Primer pairs used to amplify and clone *SlMlo* homologs. Primer pairs A and B were used to amplify fragments at the 5' and the 3' end of each gene, respectively. The successful (√) or unsuccessful (−) amplification from four different tissues (leaf, root, flower and ripened fruit) is indicated.

Homolog	Product size	primer pair A (5'—3')	Primer pair B (5'—3')	LEAF	FLOWER	ROOT	FRUIT
SlMlo1	925 bp	TTGACATTTCCCCTTCTTCTTA	TATTGCTAGTTGGCACAAAACT	√	√	√	−
	753 bp	CCCTTTCTGAAATCCTTACTCC	TACAAAATCATTGCCATTTGAA				
SlMlo2	800 bp	GAATCAACATAGTGTTTTTCATCTT	CGGTTTCTAGGATTTGGTAAAA	√	√	√	−
	931 bp	TCTAATTTTGTGACAGATCCAAAG	TCCTTTACAGTCATAACAGCTTTG				
SlMlo3	1089 bp	TCTGAGAAACAAGTTTTGAGTGA	AATAACAAAAATGGGGCTAAGG	√	−	√	√
	824 bp	AACCACAGGTACCCCTTTTACT	GAATATAGTATTAATGTGCTCCTTGTT				
SlMlo4	1016 bp	GCTTTCTCTGCATCTGAAAGTT	TTCCAAAAGTATATCAAAAGGTCAT	√	−	−	−
	954 bp	AGATGTCAAGAGCCATTCTTGT	GAAGAAGCAAGCCACTATTCAG				
SlMlo5	852 bp	CTCTCACAAATTTCCCTTATCATTA	CAAGAAAAGAATTTTGATTTTCAAT	√	√	−	√
	916 bp	TAACCTTATTCCCACAACATCTTTA	ATTTCAAAGGTAAACACGTACAAGA				
SlMlo6	402 bp	TTCTGTTTCAAGAAGATGATGTAAA	TGCCATTAACTTATGATTTCCA	√	√	√	−
	311 bp	CAATATCACGAAATTCTTCCTC	TGTCTAAGGTTGTGGATTCTGT				
SlMlo7	596 bp	CATTTGTGAAGAAGAAGAAGAGTTT	TTCTTTAAGCAATTTTATGGTTCTG	√	−	−	−
	971 bp	CACGAATCATGTACTTGTGAAAAT	GAATATGGTTCCTCAGAAGAGTTG				
SlMlo8	978 bp	TCTCATTAATTTCAATTTACGCTACG	TCCAGAGAGATTTCGATTTGC	√	−	−	−
	998 bp	TAACGGAATGAACGGTAGCC	AAGTGACTTTGCCACTCATGATA				
SlMlo9	730 bp	CATATTTGCCTAATCCATGAAA	TTGCATCACCTTCATGTTTTTA	√	−	√	−
	1077 bp	GCTCCTCCAAATACAATAGTCAA	TGGTATACTGGATCATACCTAACTTTT				
SlMlo10	857 bp	TGATACATCTGAAGCTGAAATACTG	TTTTTCAAGTCTGTCAGTAAGTCAG	√	√	√	√
	1050 bp	CCTCTTGATGTACTTTTGGAAGTTA	ACATTGATATGACTTGAGGGAAAAG				
SlMlo11	511 bp	TGTCTTTAATTCATCAAGACATGC	CTTCTAGTAGGAACAAAATTGGAG	√	√	−	√
	850 bp	ACCTCTTGAGCCAATTCTGTTA	TTTAAAGAATAATGTATTGTTCATCCT				
SlMlo12	972 bp	TTCCCTTTTTCCCTCACTGAA	TTTCAGGTCAGTTAGGAAAGCTG	−	−	−	−
	982 bp	AGGTGCTAAATGGACCGAGA	GATGACACAGCCAATCTCCA				
SlMlo13	833 bp	GAGATGAAGATCCCATTTGGT	GAAGGGTCCACTTATTGGATTAC	√	√	√	√
	853 bp	GTCGTAATAGTATCTTCGGTTTTCC	ACAGAATAAATACTGTGTAGCATGA				
SlMlo14	810 bp	TTTGAAGTTCTGGTAGATTAGTTGG	ACTACTCGTGGGTACAAAACTGC	√	−	−	−
	780 bp	GTAATTCCAGCACTCTCCAATG	GAAGTATCAACACCAACTTCACTTC				
SlMlo15	910 bp	AATAAAGAGAGATCTTTACCCCTGA	TGAGGCAATTTTATGGATCAGTAT	√	−	−	−
	750 bp	CATGCCGGAGAGTTAAGTAGTCT	GAAGTTTCAAAAGATGGTGAAATA				
SlMlo16	940 bp	TCGTCTGTGATTCATTCAACCT	GATCCCTCAGCTTTGGATCA	√	−	−	−
	946 bp	GGCCACAATTGTCTCCAACT	CCTACCAAACAACCCCAAGT				

Comparative analysis

Protein sequences of the SlMLO homologs predicted by the SGN database were used, together with those of the 15 *Arabidopsis* AtMLO homologs, pea PsMLO1, barley HvMLO, rice OsMLO1, maize ZmMLO1 and grapevine VvMLO14 which has been shown the only MLO protein phylogenetically grouped into the monocotyledon MLO clade (Feechan et al.,

2008), as dataset in the CLC sequence viewer software (www.clcbio.com) for ClustalW alignment and the obtainment of an UPGMA-based comparative tree (bootstrap value was set equal to 100).

Expression analysis in different tissues

Approximately equal amounts of leaf, root, flower or ripened fruit tissues from five MM plants were pooled and used for RNA isolation as above described. cDNAs were synthesized by using the SuperScript III first-strand synthesis kit (Invitrogen) using the oligo$(dT)_{20}$ primer. Specific primer pairs for each of the 16 *SlMlo* homologs, amplifying fragments ranging from 70 bp to 230 bp, were designed as described above (Table 2). The amplification of single fragments of the expected size for each homolog was verified by agarose gel electrophoresis. The following primer pairs, *L33*for (5'GGGAAGAGGCTGGGATACATC3')/*L33*rev (5'AGGAGGCAAATTGGACTTGAAC3') and *EF*for (5'ACAGGCGTTCAGGTAAGGAA3')/*EF*rev (5'GAGGGTATTCAGCAAAGGTCTC3'), respectively amplifying fragments from genes encoding the ribosomal protein L33 and the elongation factor α1, were used for normalization of the expression of *SlMlo* homologs. cDNAs were diluted 10-fold and used in real-time qPCR with a Bio-Rad CFX96^{TM} thermal cycler. The thermal cycling conditions used were 95 ^{o}C for 1 min, followed by 40 cycles of: 95 ^{o}C for 15s, 60 ^{o}C for 1min and 72 ^{o}C for 30s, followed by a melt cycle of 0.5 ^{o}C increment per min from 65 to 95^{o} C. Comparable amplification efficiencies between target and reference genes were determined using the LinRegPCR software (Karlen et al., 2007). Data analysis was performed according to the ΔΔCt method (Livak and Schmittgen, 2001). Three technical replicates for each sample were performed, and Student's t-tests were applied in order to assess significant differences between the treatments.

Expression analysis of the *SlMlo* gene family in response to *O. neolycopersici*

Tissue samples from the third leaf of one month-old tomato plants were collected immediately before fungal inoculation (2 x 10^5 conidia/ml) and at two time points after inoculation (6 and 10 hours). RNA isolation, cDNA synthesis and real time qPCR quantification of the 16 *SlMlo* transcripts were performed as described above. Four

biological replicates and two technical replicates were used in this experiment.

Table 2. Primer pairs used to assess expression of the *SlMlo* gene family upon inoculation with *Oidium. neolycopersici*.

***Mlo* homolog**	**pPCR size**	**Fw primer sequence (5'--3')**	**Rev primer sequence (5'--3')**
*SlMlo*1	128 bp	CTTTGGGCAGGCTAAAGATG	AATGCCTACGTCCAAACGAG
*SlMlo*2	145 bp	AGCTGTCAATGGCTCAAACC	TGGAGTTGAGTTGGTCGTTC
*SlMlo*3	93 bp	TCAGTCCCCCAATATGGTTC	TCAAGGGGATGAATGGTAGC
*SlMlo*4	144 bp	AGATGGGCTCCAACATGAAG	AGGACCTTGGACTTGCATTG
SlMlo5	91 bp	GCTTTCTTTGCTTGGAGCTG	CCCCCATGGAAAGTGTTATG
SlMlo6	95 bp	TACATGGATGGCGACACAAG	CGCCATGGAGTCTAATGATG
SlMlo7	128 bp	ATACGTCAACGGAAGCACTG	CCATACCCACAAATCGATCC
SlMlo8	108 bp	ATTGCAAGTACGTGGCATCC	ATCGTCAGCCGTGAAAAGTC
SlMlo9	70 bp	GGGCTGAGAAGGTGAAAAAG	ACCGTCATTTGAACGGGTAG
SlMlo10	136 bp	AACAACACCGATGACTCGTG	AGGAGCAGGCTTCACAAATG
SlMlo11	93 bp	ATGCCCTATTCAAGGCACTC	TTATTGCCCCTTGAGACACC
SlMlo12	230 bp	AGACACCTGAGGCTGGAGAA	TTCCCACTCCTTCCACTCAC
SlMlo13	83 bp	CTTGTGGGGATTGAAGCAAC	GCACAAGAACCCCTGAAATC
SlMlo14	84 bp	CTCTGCGCAAGGGTTTTATC	CTTCTTCCATCGAGCGAATC
SlMlo15	128 bp	TAACTCTCCGGCATGGATTC	CCAAATCCATGGGCTAATTG
SlMlo16	119 bp	GAGCTGGCCTTCTTTGTTTG	CACTTCTGTGTGCCAATGCT

Generation of RNAi silencing lines

Four primer pairs were designed to amplify and clone fragments suitable for specifically silencing of *SlMlo1, SlMlo3, SlMlo5* and *SlMlo8.* Fragments were cloned into the gateway-compatible vector pENTR D-TOPO (Invitrogen) (Table 3) and subsequently recombined into pHELLSGATE12 (Invitrogen). Corresponding recombinant plasmids were transferred into the AGL1-virG strain of *Agrobacterium tumefaciens* by electroporation and plated on medium containing appropriate antibiotics.

The transformation procedure for tomato was carried out similarly to that described by McCormick et al. (1986). In brief, MM 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 and submerged in *A. tumefaciens* suspension. Infected cotyledonary explants were placed abaxially, on a GCF10 medium (4.3 g/l MS basal salt mixture, 8g/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^{-1} acetosyringone at 25^{o}C for 48h. Then, they were transferred to GCF10 medium, to

which 100 mg/ml timentin and 50 mg/ml kanamycin were added, and subcultured onto fresh medium every 3 weeks until shoot buds were observed. These were excised from the callus and transferred to GCF11 medium (4.3 g/l MS basal salt mixture, 8g/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, 8g/l agar, 30 g/L sucrose, 112mg/L vitamin B5, 50 mg/ml kanamycin, pH 5.8). Once roots were developed, the plantlets were transferred to rockwool and grown under greenhouse conditions. Silencing levels were determined on both T_1 and T_2 plants by real-time qPCR.

Table 3. Primer pairs used to prepare RNAi constructs

Mlo homolog	PCR size	Fw primer sequence (5'--3')	Rev primer sequence (5'--3')
SlMlo1	216 bp	CGTATCTTTGGGTGCCATTT	CAGGGCGATTAAACCAGAAA
SlMlo3	225 bp	AGAGTAGCAACAGCATTGAAGAAT	GTTGTCCCAATGATCAAAATC
SlMlo5	226 bp	ATACGGACATGGCATCAGAC	CATGACTTCCATCATCAAAGATT
SlMlo8	219 bp	CATTGAGAAAATGGCACCATAG	ATGGTGAGCCCTCAATATCC

Disease quantification on silenced lines

T_2 lines originating from T_1 plants were inoculated with *O. neolycopersici*. The 3rd and 4th leaves were sampled two weeks post inoculation and plant and fungal DNA ratios were determined by qPCR as a measure of fungal growth. DNA was extracted by using the DNeasy DNA extraction kit (Qiagen, Germany) and 20 ng of DNA was used for qPCR. Primers used to quantify *On* DNA were F (5'CGCCAAAGACCTAACCAAAA3') and R (5'AGCCAAGAGATCCGTTGTTG3'), designed on *On*-specific internal transcribed spacer sequences (GenBank accession number EU047564.1). Primers *EF*for/*EF*rev were used for quantifying tomato DNA.

RESULTS

In silico characterization and sequencing of the tomato *SlMlo* gene family

To characterize the *Mlo* gene family in tomato we searched the tomato genome sequence (available at http://solgenomics.net/) for homologs of the *Arabidopsis Mlo* gene family

(Devoto et al., 2003). In total we identified 16 homologs to which we refer here as *SlMlo1* to *SlMlo16* and those we listed in Table 4 together with the gene names given by the International Tomato Annotation Group (ITAG). In order to keep consistency with the ITAG preditction, the *SlMlo2* indentified in Chapter 2 is hereafter referred to as *SlMlo3*. Fig. 1 shows the chromosomal localisation of all identified members. All 16 *SlMlo* genes could be PCR amplified from cDNA derived from leaves, roots, flowers and ripened fruits from Moneymaker plants, demonstrating that all members are transcript in this cultivar. We cloned all 16 *SlMlo* homologs from MM derived cDNA, except for *SlMlo12* (Table 1). Sequencing revealed several polymorphisms, among which large insertions/deletions, and an additional splicing variant for *SlMlo1*, *SlMlo8* and *SlMlo13* (Fig. 2).

Table 4. Features of the *SlMlo* gene family as inferred by the Sol Genomics Network Database.

SGN locus name	*Mlo* name	Chromosome	ORF length (aa)	Introns	Number of ESTs spanning the locus
Solyc04g049090	*SlMlo1*	4	507	14	14
Solyc08g015870	*SlMlo2*	8	504	13	28
Solyc06g010030	*SlMlo3*	6	591	14	6
Solyc00g007200	*SlMlo4*	n.a.	554	14	2
Solyc03g095650	*SlMlo5*	3	517	14	2
Solyc02g082430	*SlMlo6*	2	553	14	9
Solyc09g018830	*SlMlo7*	9	270	10	2
Solyc11g069220	*SlMlo8*	11	506	13	n.a.
Solyc06g082820	*SlMlo9*	6	511	13	3
Solyc02g083720	*SlMlo10*	2	533	14	1
Solyc01g102520	*SlMlo11*	1	475	13	1
Solyc08g067760	*SlMlo12*	8	532	14	2
Solyc10g044510	*SlMlo13*	10	558	14	n.a.
Solyc07g063260	*SlMlo14*	7	563	14	1
Solyc02g077570	*SlMlo15*	2	375	10	n.a.
Solyc06g010010	*SlMlo16*	*6*	*477*	*14*	*n.a.*

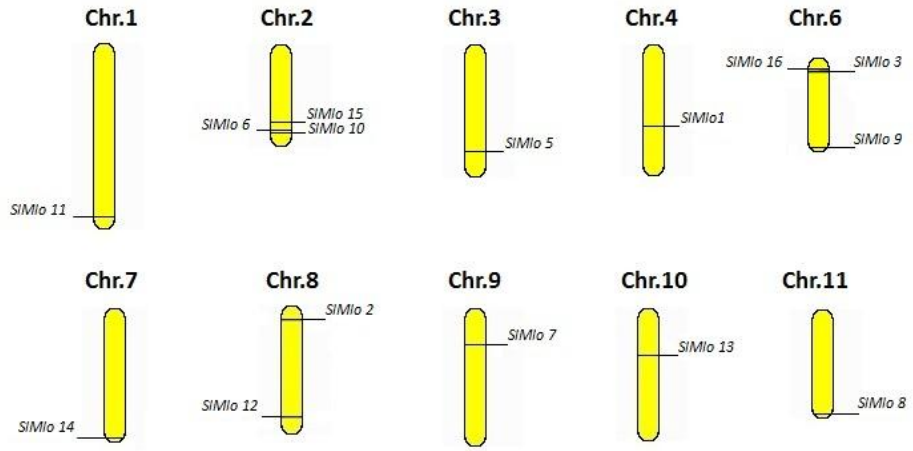

Fig. 1. Genomic distribution of 15 out of 16 homologs of the *SlMlo* gene family. The *SlMlo4* could not be assigned to any chromosome.

Phylogenetic analysis of the tomato SlMLO protein family

The obtained MM cDNA sequences, were used to construct a phylogenetic tree of all SlMLO isoforms in relation to MLO family members of other plant species (including *Arabidopsis*, tomato, pea, barley, rice, maize and grapevine). The whole dataset used in this study contained 36 MLO homologs, which formed six different clades. Similar phylogenetic relationships were obtained by substituting the SlMLO sequences retrieved in the SGN database with the ones obtained by *de novo* sequencing (data not shown).

Five clades contained both tomato and *Arabidopsis* homologs, whereas one had monocot MLO homologs and the grapevine MLO homolog VvMLO14 which (Fig. 3). No clade could be assigned for SlMLO7 and SlMLO15, which thus form their own clade. SlMLO8, SlMLO5 and SlMLO3 were found to cluster in clade V, containing all the known dicot MLO homologs involved in powdery mildew susceptibility (AtMLO2, AtMLO6, AtMLO12, PsMLO1 and SlMLO1). SlMLO4, SlMLO6, and SlMLO12 grouped in clade III together with AtMLO7, which was shown to regulate *Arabidopsis* pollen tube reception by the synergid cells (Kessler et al., 2010). SlMLO10, SlMLO13 and SlMLO14 were the closest tomato homologs to the root thigmomorphogenesis regulating proteins AtMLO4 and AtMLO11 in clade I (Chen et al., 2009).

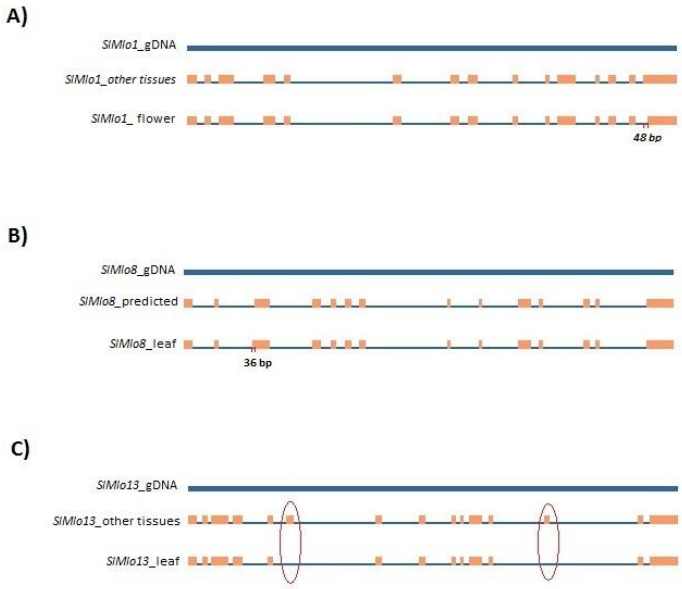

Fig. 2. Schematic representation of the three splicing variants identified in this study. Blue thick lines represent genomic DNAs; blue thin lines and thick orange lines represent respective introns and exons as inferred from cDNA sequences derived from the indicated tissues. A) a 48bp deletion in the last exon of the *SlMlo1* transcripts in flower. B) a 36 bp insertion occuring in the *SlMlo8* transcripts in leaf. C) two missing exons (cycled) in the *SlMlo13* transcript in leaf.

Expression profiles of *SlMlo* homologs upon powdery mildew challenge

In un-inoculated tomato plants, expression levels were found to vary considerably between individual *SlMlo* homologs and, for the same homolog, between the tissues analyzed in this study (leaf, root, flower and mature fruit, Fig. 4). It was not possible to assign clade-specific expression pattern in the tested plant tissues.

In addition, we investigated the expression profiles of the *SlMlo* gene family members in response to *O. neolycopersici*. As shown in Fig. 5, *SlMlo1* transcripts showed considerable increase 6 and 10 hours after *O. neolycopersici* challenge. No other *SlMlo* homolog in clade V (*SlMlo3*, *SlMlo5* and *SlMlo8*) showed pathogen-dependent up-regulation. Rather, *SlMlo3* exhibited a slight, but significant down-regulation both at 6 and 10 hpi. Up-regulation was also observed for *SlMlo* homologs outside clade V. For example, the expression of *SlMlo4* and *SlMlo14* 10 hours after inoculation was ~4-fold and ~3-fold higher than that of control plants, respectively.

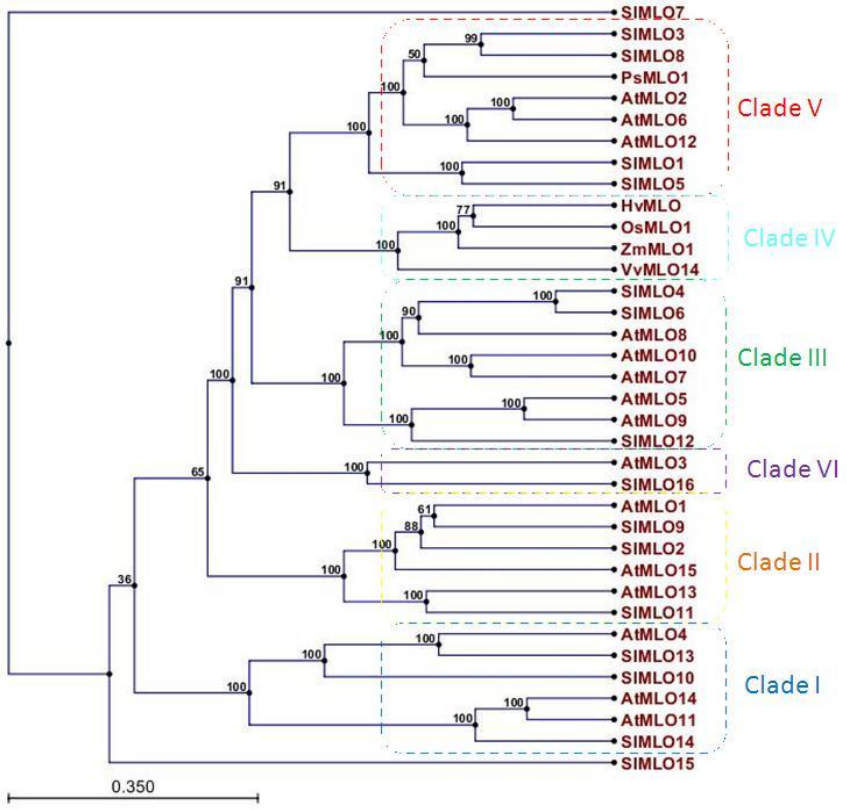

Fig. 3. UPGMA-based comparative analysis of MLO isoforms from *Arabidopsis* (At), tomato (Sl), pea (Ps) barley (Hv), rice (Os), maize (Zm) and grapevine (Vv). Numbers on each node represent bootstrap based on 100 replicates.

Functional characterization of *SlMlo* homologs for powdery mildew invasion

Four *SlMlo* members (including *SlMlo1*) group in clade V, that contains all dicot *Mlo* genes so far known to be involved in powdery mildew susceptibility. To investigate whether *SlMlo3*, *SlMlo5* and *SlMlo8* are as well involved in powdery mildew susceptibility we generated RNAi constructs specifically targeting these genes. As a control, we silenced *SlMlo1* which is known to be involved in powdery mildew susceptibility (Bai et al., 2008). T1 plants showing a good (>2 fold) level of silencing were selected and multiple cuttings were generated that were challenged with *O. neolycopersici.* Good correlation between silence effect and resistance was observed (Fig. 6). T_2 progenies corresponding to the selected T_1 plants were subjected to inoculation with *O. neolycopersici*. As expected, T_2 progenies

derived from *SlMlo1* silenced T_1 plants were segregating for powdery mildew resistance (Fig. 7). In contrast, T_2 progenies of *SlMlo3, SlMlo5* and *SlMlo8* silenced plants showed normal levels of *O. neolycopersici* sporulation visually. Although quantification of disease severity on these lines using real-time qPCR revealed slightly significant difference among plants within one line, no co-segregation between silencing level and resistance could be revealed (data not shown).

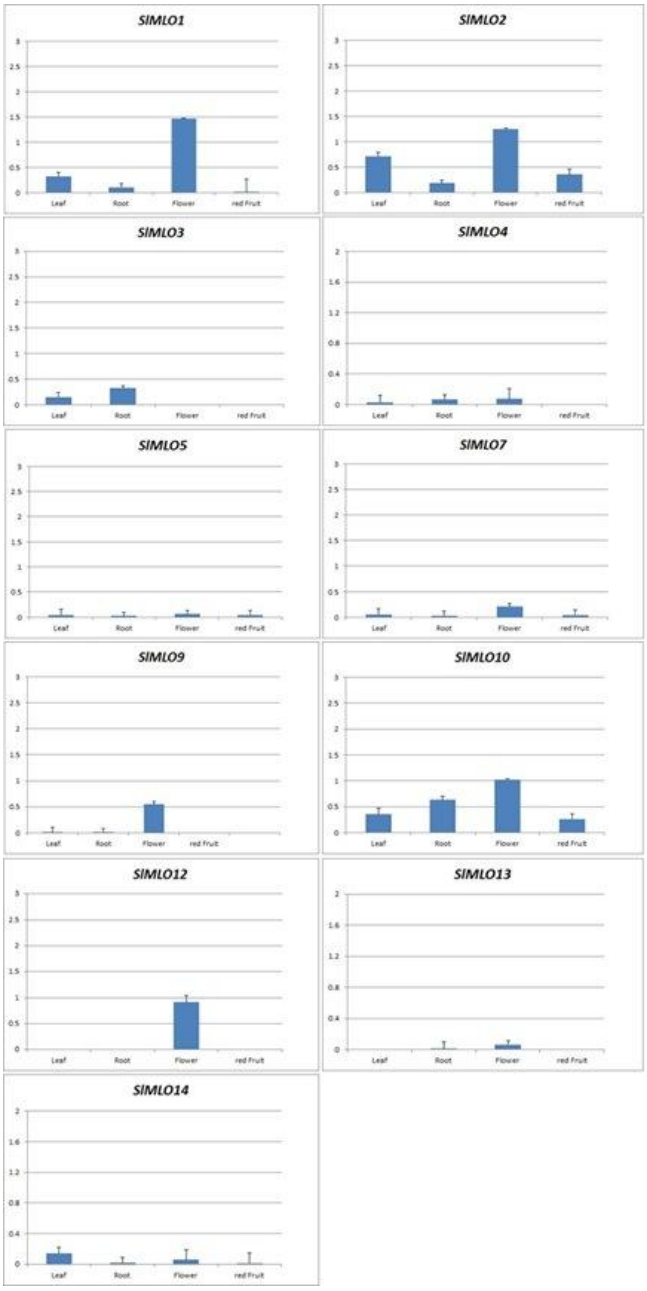

Fig. 4. Quantitative analysis of the expression profile of *SlMlo* homologs in four different tissues (leaf, root, flower and ripened fruit). The expression of *SlMlo6*, *SlMlo8*, *SlMlo11*, *SlMlo15*, *SlMlo16* was not shown in the figure due to a very low level of the expression. Transcript levels are normalized against the housekeeping gene *Elongation factor α1*. Means are calculated from three technical replicates from a cDNA pool of five biological replicates. Bars show standard errors. Consistent results were obtained by using another housekeeping gene *L33*.

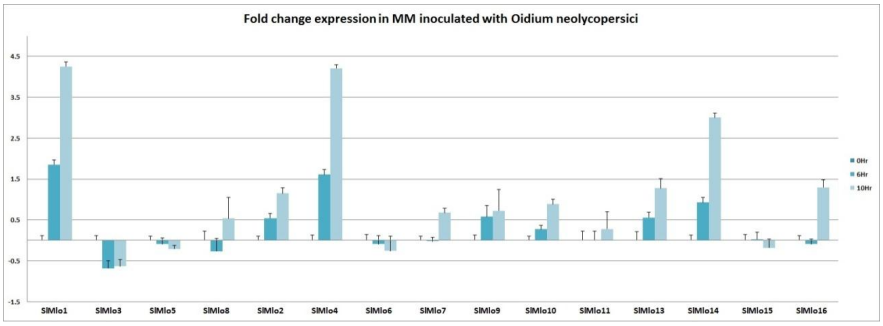

Fig. 5. Quantitative analysis of the *SlMlo* gene family expression profile in response to *Oidium neolycopersici* inoculation. Samples were collected at 0, 6 and 10 hr after inoculation. Transcript levels of each *SlMlo* homolog were normalized against the transcription level of the housekeeping gene *L33*. Consistent results were obtained by using the housekeeping gene *Elongation factor α1 for* normalization. Bars show standard errors based on four biological replicates.

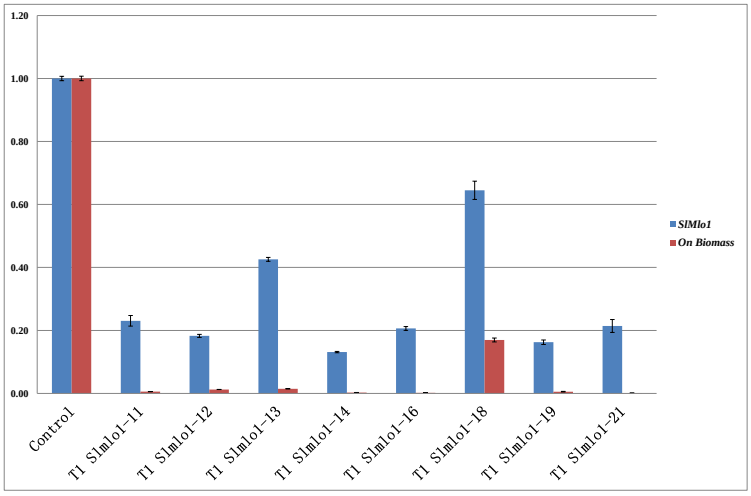

Fig. 6: Relative quantification of the *SlMlo*1 expression (in blue) and relative quantification of fungal biomass (in red) of tomatoT1 plants in which *SlMlo1* is silenced via RNAi. Results were normalized with the housekeeping gene *elongation factor α1*. Bars show standard errors based on three technical replicates.

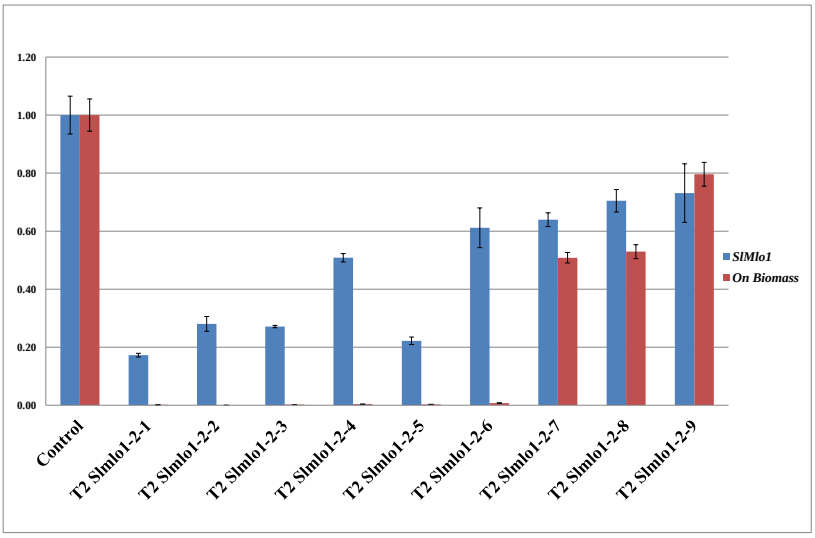

Fig. 7: Quantification of the *SlMlo*1 expression (in blue) and relative quantification of fungal biomass (in red) of T2 progeny derived from *SlMlo1* silenced T1 plants, showing segregating of powdery mildew resistance. Results were normalized with the housekeeping gene *elongation factor α1.* Bars show standard errors based on three technical replicates.

DISCUSSION

Genomic organization and evolution of the *SlMlo* gene family

In this study, we followed an *in silico* approach to assign 16 homologs to the tomato *Mlo* gene family *SlMlo*. Similar numbers of *Mlo* homologs in the genome of *Arabidopsis* and grapevine were identified, which contains 15 and 17 members respectively (Devoto et al., 2003; Feechan et al., 2008). It suggests that a similar number of *Mlo* homologs are likely to be retrieved in future genome-wide investigations involving diploid dicot species. Most of the SlMLO proteins were found to group in five phylogenetic clades together with other dicot MLO homologs, including the complete *Arabidopsis* AtMLO family. In contrast, SlMLO7 and SlMLO15 formed two divergent lineages. Interestingly, sequences of the *SlMlo7* and *SlMlo15* genes from two different tomato cultivars (Heinz1706, on which the SGN database is based, and MM, used for re-sequencing in this study) resulted to have abnormal features, shorter ORF and reduced exon number with respect to other *Mlo* homologs (Table 4). This suggests that these two homologs may be pseudogenes, being generally not functional, and thus are likely not present in *Arabidopsis.* As expected, none

of the tomato SlMLO homologs could be assigned to a MLO phylogenetic clade previously shown to contain monocot MLO homologs (clade IV) and, as the only exception, for the grapevine MLO homolog VvMLO14.

Most *SlMlo* homologs were found to be scattered throughout the tomato genome, thus suggesting that segmental duplication events have been a major source for the evolution of the *SlMlo* gene family. Exception were represented by two physical gene clusters, one containing *SlMlo6*, *SlMlo10* and *SlMlo15* on chromosome 2 and the other containing *SlMlo3, SlMlo9* and *SlMlo16* on chromosome 6. The fact that physically close *SlMlo* homologs are not phylogenetically related (Fig. 3) suggests that they have arisen from ancient tandem duplication events.

Co-functioning between *Mlo* homologs in several biological processes

Database searches for tomato expressed sequences, RT-PCR and real-time qPCR of the *SlMlo* gene family members confirmed the expression of all the 16 *SlMlo* homologs. Often, it was possible to detect a high level of transcription of the same *SlMlo* homolog in more than one of the four tissues under study (leaf, root, flower and ripened fruit). This is in line with findings of a previous study of Chen et al. (2006), on the expression pattern of the *Arabidopsis AtMlo* gene family in several tissues. Overall, this evidence indicates that: a) different *Mlo* homologs may have synergistic or antagonistic roles in regulating the same biological process; b) as the same *Mlo* homolog expressed in different tissues, suggested that the one *Mlo* homolog may be involved in different biological processes. By the fact that, 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 thigmomorphogenesis (Consonni et al., 2006; Chen et al., 2009). A yet unidentified additional biological function could be hypothesized for *SlMlo1*, previously shown to act as a susceptibility gene towards *O. neolycopersici* (Pavan et al., 2010; Chapter 2 of this thesis). Indeed, this gene was found to exhibit its stronger expression level in tomato flowers and moderate expression in roots, two tissues which are less or not attacked by the fungus, respectively. Moreover, additional biological roles for *SlMlo1* would explain why this gene has not been excluded during evolution, despite promoting pathogenesis.

In this study, we showed that silencing of tomato *SlMlo3*, *SlMlo5* and *SlMlo8*, that phylogenetically related to all the dicot *Mlo* homologs shown to act as powdery mildew susceptibility genes (including *SlMlo1*), did not result in a significant reduction of disease symptoms caused by *O. neolycopersici*. Moreover, we provided evidence that *SlMlo3*, *SlMlo5* and *SlMlo8*, in contrast to *SlMlo1*, do not increase their expression upon pathogen challenge. If a similar scenario holds true for other plant-pathogen interactions, these results suggest that phylogenetic and expression studies can together unambiguously identify, across cultivated species, the *Mlo* homologs which play a major role in powdery mildew susceptibility. This might be of great interest for plant breeding, since powdery mildew is one of the most widespread fungal diseases worldwide and the inactivation of *Mlo* susceptibility genes, through targeted mutagenesis or gene silencing, has been repeatedly indicated as a valid strategy in breeding for resistance to powdery mildews in different plant species (Pavan et al., 2010 and 2011). In order to keep accordance with the sequence annotations from the International Tomato Annotation Group (ITAG) for this homolog the *SlMlo2* partial sequence described in Chapter 2 of this thesis was renamed as *SlMlo3*. In Chapter 2 of this thesis, we demonstrated that this *Mlo* homolog only represented as a partial EST covering the 3' end of the coding sequence (Fig. 2, Chapter 2 of this thesis). Tissue origin of the ESTs indicates that its expression appears to be predominant in tomato breaker fruits. In this study, we found that *SlMlo3* was expressed in the leaf and root but not in flower and ripened fruit (Fig. 4). It is very likely that the ESTs used in Chapter 2 are obtained from a specific EST library made from the fruit pericarp of breaker fruit.

It is intriguing result that *SlMlo4* and *SlMlo14,* not belonging to the clade containing MLO homologs involved in powdery mildew susceptibility, showed up regulation upon *O. neolycopersici* infection (Fig. 5). SlMLO4 is closely related to AtMLO4 and AtMLO11 which are involved in the root thigmomorphogenesis (Chen et al., 2009), while SlMLO14 to AtMLO7 involved in the pollen tube reception (Kessler et al., 2010). In Arabidopisis, mutation of AtMLO4, AtMLO7 and AtMLO11 did not result in powdery mildew resistance (Consonni et al.; 2006). Thus, we expect that silencing of these two *Mlo* homlogs will likely not lead to resistance against powdery mildew. RNAi experiments are being carried out to verify our hypothesis. Based on their sequence relatedness with *Arabidopsis* AtMLO proteins of known function, it is logical to argue that one or more of the tomato *SlMlo*

homologs in clade III and clade I could regulate respectively the processes of root thigmomorphogenesis and pollen tube reception in tomato. Functional studies similar to the ones reported here for *SlMlo* homologs in clade V is on-going and might test these hypotheses. Importantly, once that the *SlMlo* gene family has been characterized, the same kind of studies could assign new functions to MLO proteins, which might have been unnoticed by the phenotypic observation of *Arabidopsis Mlo* mutants.

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CHAPTER 3

CHAPTER 4

Detection and Quantification of *Leveillula taurica* Growth in Pepper Leaves

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Zheng Zheng[1, 5], Teruo Nonomura[2, 5], Károly Bóka[3], Yoshinori Matsuda[2], Richard Visser[1], Hideyoshi Toyoda[2], Levente Kiss[4] and Yuling Bai[1,*]

[1] Wageningen UR Plant Breeding, Wageningen University & Research Centre, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands
[2] Laboratory of Phytoprotection Science and Technology, Faculty of Agriculture, Kinki University, Nara 631-8505, Japan
[3] Department of Plant Anatomy, Eötvös Loránd University, H-1117 Budapest, Pázmány Péter sétány 1/C, Hungary
[4] Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, H-1525 Budapest, P.O. Box 102, Hungary
[5] With equal contribution

*Corresponding author: Yuling Bai, Email: bai.yuling@wur.nl

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ABSTRACT

Leveillula taurica is an obligate fungal pathogen that causes powdery mildew disease on a broad range of plants including important crops such as pepper, tomato, eggplant, onion, cotton and so on. The early stage of this disease is difficult to diagnose and the disease can easily spread unobserved for example in pepper and tomato production fields and greenhouses. The objective of this study was to develop a detection and quantification method of *L. taurica* biomass in pepper leaves with special regard to the early stages of infection. We monitored the development of the disease to time the infection process on the leaf surface as well as inside the pepper leaves. The initial and the final steps of the infection taking place on the leaf surface were consecutively observed using a dissecting microscope and a scanning electron microscope. The development of the intercellular mycelium in the mesophyll was followed by light and transmission electron microscopy. A pair of *L. taurica*-specific primers was designed based on the internal transcribed spacer (ITS) sequence of *L. taurica* and used in real-time PCR assay to quantify the fungal DNA during infection. The specificity of this assay was confirmed by testing the primer pair with DNA from host plants and also from another powdery mildew species, *Oidium neolycopersici* infecting tomato. A standard curve was obtained for absolute quantification of *L. taurica* biomass. In addition, we tested a relative quantification method by using a plant gene as reference and the obtained results were compared with the visual disease index scoring. The real-time PCR assay for *L. taurica* provides a valuable tool for detection and quantification of this pathogen in breeding activities as well in plant-microbe interaction studies.

INTRODUCTION

Powdery mildew is a serious fungal threat to agricultural production. The endoparasitic powdery mildew fungus *Leveillula taurica* (Lév.) G. Arnaud (anamorph: *Oidiopsis taurica* (Lév.) Salmon) is an important pathogen of pepper, tomato, eggplant, onion, cotton, and other crops and it has also been recorded on many wild plant species (Braun U., 2012). This pathogen represents a challenge from many perspectives. Firstly, the early stages of infection are difficult to diagnose, thus the disease can rapidly spread in both field and greenhouse crop production. Secondly, the species delimitation in the genus *Leveillula* is problematic and the binomial '*L. taurica*' clearly refers to a species complex that includes several biological species (Khodaparast et al., 2001; Khodaparast et al., 2007; Khodaparast et al., 2012). Moreover, the exact host ranges of the different *L. taurica* lineages recognized by phylogenetic studies are still not known (Khodaparast et al., 2001).

Most powdery mildew species are epiparasitic because all their structures except haustoria are developed on the host plant surfaces. In contrast, *Leveillula* and the other genera in tribe *Phyllactinieae*, namely *Phyllactinia* and *Pleochaeta* develop a partly endophytic mycelium and are endotrophic because their haustoria are produced in the mesophyll cells. Kunoh et al. (1979) illustrated that in *L. taurica* the germinated conidia are attached to the leaf surface by 'adhesion bodies' that differ from appressoria produced by epiphytic species because they do not initiate infection hyphae that penetrate the epidermal cells. Instead, infection hyphae of *L. taurica* enter the host plant through stomata and develop an intercellular mycelium in the spongy and palisade parenchyma tissue with haustoria in some of its cells. Later on conidiophores emerge through stomata, mainly on the abaxial leaf surface (Fig. 1B and C), producing primary, and numerous secondary, conidia that differ in their morphology (Braun U., 2012). At that stage hyphae are also produced mainly on the abaxial leaf surfaces. On the adaxial surface of infected leaves, chlorotic spots are usually visible indicating the development of mildew colonies underneath the spots (Fig. 1A).

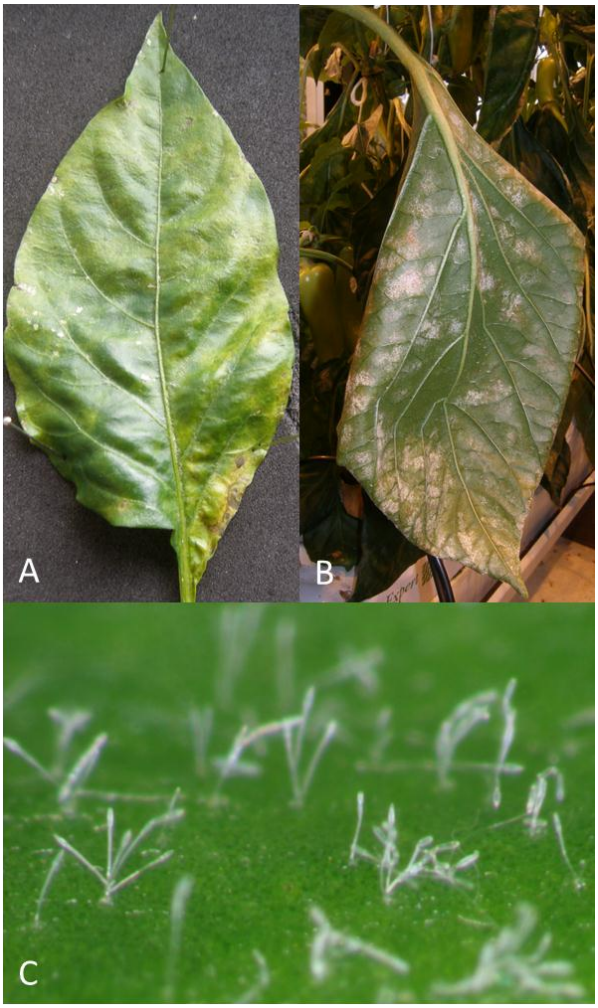

Fig. 1. (**A**) Disease symptom (yellowish spots) on adaxial, and (**B**) white fungal colonies on abaxial leaf surface of a susceptible pepper cultivar in production greenhouse. (**C**) Mature *Leveillula taurica* conidiophores emerging from stomata on the abaxial leaf surface.

For studies on plant-microbe interactions and in practical breeding programs for disease resistance, detection, monitoring and quantification of the pathogen are crucial steps. Depending on the characteristics of the pathogen, there are numerous methods to quantify the amount of pathogen present in the host plants. Such methods include visual inspection, digital image analysis, histological analysis, light microscopic methods, immunological techniques, biochemical quantification of microorganism compounds and nucleic acid-based technologies (Tisserant et al., 1993; Laurans and Pilate, 1999; Boyle et al., 2005; Bock et al., 2008). The simplest way to evaluate the level of disease in plants is based on the visual evaluation of symptoms using disease index (DI) values. This method is fast, easy and normally does not require any extra equipment. However, the visual inspection has drawbacks. Firstly, the disease symptoms have to be visible on the plant tissues. Secondly, the evaluation is subjective. Thirdly, it is hard to distinguish the small differences between intermediates. To overcome the drawbacks, other methods, like ELISA and measurements of pathogen specific components like chitin and ergosterol, have been developed (Pacovsky and Bethlenfalvay, 1982; Harrison et al., 1990;

Gardner et al., 1993; Newton and Reglinski, 1993). However, the use of these methods in daily research and breeding activities is also limited by factors like the specificity of detection probes, laborious process and expensive materials. Another technique, real-time PCR assay, can quantify PCR amplicons by measuring the fluorescent signals. Due to its rapidity, sensitivity, reproducibility and high specificity, it has quickly become a popular method for pathogen detection and quantification (Vincelli and Tisserat, 2008).

So far, the disease scoring system for *L. taurica* is mainly based on visual inspection of leaves. By scoring symptoms, Shifriss et al. (1992) included the infected plants in three groups: symptomless, susceptible and intermediate. Others like Souza et al. (2003) counted the latency period, days from inoculation till the appearance of *L. taurica* conidia on plants. To date, the most accurate disease scoring method for *L. taurica* is the synthetic disease index described by Daubèze et al. (1995), which has been used for a QTL mapping study (Lefebvre et al., 2003). This synthetic disease index sums up two parameters in order to represent a good indication of resistance. One parameter is the disease symptom represented by the proportion of infected leaves per plant (PrF) with a scale from 0 to 5 (0 = no infected leaves to 5 = the whole foliage infected). The other one is the sporulation intensity (SpF) with again a 0-5 scale (Daubeze et al., 1995; Lefebvre et al., 2003). However, the main difficulty of all these scoring methods is how to evaluate the size and/or the density of individual mildew colonies.

The main goals of the present work were to follow the infection process of *L. taurica* in pepper leaves and to develop an objective scoring methodology. We attempted to monitor the development of the pathogen to time the infection process by using different microscopic techniques, such as a high-fidelity digital microscope and a scanning electron microscopy (SEM) for the study of the infection steps on the leaf surface and light and transmission electron microscopy (TEM) for steps taking place inside the leaves. To quantify the pathogen biomass, we developed a real-time PCR assay and measured the absolute *L. taurica* DNA amount and the relative *L. taurica* DNA to plant DNA in infected plant tissues.

CHAPTER 4

MATERIALS AND METHODS

Disease assay

To develop a disease scoring system, and also for the real-time PCR studies, the powdery mildew pathogen *L. taurica* was collected from infected pepper plants in a greenhouse in The Netherlands and maintained on susceptible pepper plants, cultivar Maor. The inoculation was performed on six-week old plants. Conidia of *L. taurica* were washed off with water from heavily infected pepper leaves. Inoculum was adjusted to a final concentration of 2.5 x 10^4 conidia/ml, and sprayed onto the abaxial surface of the leaves. Inoculated plants were kept for 24 h in plastic cages in the greenhouse with 100% relative humidity at 21°C. Then, the temperature and humidity were adjusted to 21°C (day)/19°C (night) and relative humidity 70% in the greenhouse.

To perform light microscopy and TEM studies, the pathogen was maintained on potted pepper plants, cv. Cibere F1, in an experimental greenhouse in Hungary. The environmental factors and the inoculation method were similar to those applied in the Dutch greenhouse. Infected leaves with disease indexes determined as described below were used in these studies.

To carry out the digital microscopic analysis, pepper (*Capsicum annum* L. cv. Kyounami) was used in this study in Japan. Seeds were placed on wet filter papers in petri dishes, and then germinated for three-four days in a growth chamber under continuous illumination (4,000lux) at 25 ± 2°C. 14 days-old seedlings (one-two folded leaves) were used for inoculation of *L. taurica* conidia. For maintaining of the pathogen, inoculum was made and sprayed onto leaves of 14-days-old seedlings of pepper according to the method of Bai et al. (2003). Inoculated seedlings were placed for one month in a growth chamber with 70-80% relative humidity at 20 ± 1°C under continuous illumination of 4,000 lux. Newly produced conidia were used as inoculum for the maintenance as described earlier.

Macroscopic disease scoring

We modified the disease scoring method of Daubeze et al. (Daubeze et al., 1995) based on the scoring method described by Bai et al. (Bai et al., 2003) for *O. neolycopersici*. Disease index (DI) was used to score the sporulation intensity of *L. taurica* on each infected leaf.

This DI system was based on a scale of 0 to 5; 0 = no visible sporulation, 1 = restricted chlorotic spots on the adaxial leaf surface with weak or no sporulation on the corresponding abaxial leaf areas, 2 = several isolated sporulation spots on the abaxial leaf area, 3 = numerous sporulation spots covering up to 40-50% of the abaxial leaf area, 4 = numerous coalescent sporulation spots covering up to 75% of the abaxial leaf area and 5 = the whole abaxial surface of the leaf, and also parts of the adaxial leaf surface, covered with dense sporulation. The third, fourth and fifth true leaves were scored for each plant.

Light microscopy

To study the epiphytic fungal structures on the abaxial leaf surfaces as well as the penetration process through stomata and the production of haustoria in the mesophyll cells, samples were prepared in the following ways for light microscopy: (i) Leaf segments of approximately 1 x 3 cm in size were collected from powdery mildew-infected pepper plants in a greenhouse and fixed in acetic acid/ethanol (1:3), followed by staining with 0.03% trypan blue in lactophenol/ethanol (1:3) as described by Bai et al. (2003). (ii) Leaf segments collected in the same way were decolorised in a boiling alcoholic lactophenol solution for 1-2 min, and stained with aniline blue, according to Sameshima et al. (2004). (iii) Leaves collected from powdery mildew-free pepper plants, cv. Cibere F1, grown in isolation in a climate chamber, were placed with their abaxial surfaces up in glass Petri dishes of 9 cm diameter, on polystyrene balls floated on sterile water. The abaxial leaf surfaces were inoculated with *L. taurica* conidia, the plates were closed and kept at 25^{o}C at continuous light for 48, 72 and 96 h. At each time interval, small pieces were cut out of the inoculated leaves where germinated conidia were observed under a dissecting microscope, macerated in a droplet of water or boiled in lactic acid on a microscope slide, occasionally stained with cotton blue in lactophenol, and examined under a light microscope.

Digital microscopy

Conidia were collected from conidiophores developed on infected leaves and transferred to the abaxial leaf surfaces (one conidium per leaf), using a glass needle installed with micromanipulator and a high-fidelity digital microscope KH-2700 (Hirox, Tokyo, Japan). Separate inoculation was conducted five times. To show the conidial germination and the

penetration process in an animated way, the conidia were photographed on the abaxial surface of a pepper leaf at 0.5-1 h intervals for 48 hr using a CCD-camera of the digital microscope. Photographs were treated using an image processing software (Adobe Photoshop ver.5) (Adobe Systems, CA, USA), and 110 image-analyzed photographic data were fed into to a Windows live movie maker software (Microsoft, WA, USA). The photographic data collected at 3-4 h and at 18-30 h after inoculation were analyzed for conidial germination and penetration, respectively.

Scanning electron microscopy

Pepper leaves infected with *L. taurica* were fixed in 0.1 M phosphate buffer (0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4) containing 2.5% glutaraldehyde at pH 7.4 for 1 day, and postfixed in 1% osmium tetroxide (Nacalai tesque, Kyoto, Japan) in the same buffer for 2 h. The fixed leaves were dehydrated through graded ethanol series (50-99.8%) and then soaked in t-butyl alcohol (Nacalai tesque, Kyoto, Japan) for 20 min at room temperature. The samples were frozen at 4^{o}C for 2 h and dried using a freeze-drying device JFD-300 (JEOL, Tokyo, Japan). The dried leaves were coated with platinum-palladium metal particles using an ion sputtering device JFD-1100E (JEOL, Tokyo, Japan) and observed with a scanning electron microscope JSM-6510LA (JEOL, Tokyo, Japan).

Transmission electron microscopy

Pepper leaf segments infected with *L. taurica*, approx. 5 x 5 mm in size, were fixed in 2% glutaraldehyde for 3 h and in 1% osmium tetroxide for 2 h (both fixatives were dissolved in 0.1 M K-Na-phosphate buffer at pH 7.2) and embedded in Durcupan resin after dehydration in an ethanol series ended with propylene oxide. Semi-thin sections (1 μm) for light microscopy were prepared with a Microm HM 360 microtome (Microm, Walldorf, Germany) and stained with toluidine blue. Ultra-thin sections (70 nm) were cut with a Reichert Ultracut E ultramicrotome (Leica Microsystems, Vienna, Austria) mounted on uncoated Cu/Pd grids (Polysciences, Warrington, USA), and stained with 5% uranyl acetate dissolved in methanol for 4 min and lead citrate for 6 min. Sections were viewed with a Hitachi 7100 TEM (Hitachi, Tokyo, Japan) at an accelerating voltage of 75 kV and a JEOL JEM 1011 TEM (JEOL Ltd., Tokyo, Japan) at 60 kV.

Primers used for real-time PCR

ITS sequences of *L. taurica* infecting pepper were retrieved from GenBank and specific primers were designed by using the online software: Primer3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The forward primer LV-F (5'AGCCGACTAGGCTTGGTCTT3') and the reverse primer LV-R (5'GCGGGTATCCCTACCTGATT3') amplified a 208 base pairs (bp) fragment. The house-keeping gene *CaActin*, as a plant reference gene, was amplified from pepper leaves using the forward primer CaActin-F (5'ATCCCTCCACCTCTTCACTCTC3') and the reverse primer CaActin-R (5'GCCTTAACCATTCCTGTTCCATTATC3') as described by Silvar et al. (2008).

DNA isolation

L. taurica conidia, as free of any contamination as possible, were collected in a glass vial with a vacuum pump-operated cyclone spore collector (Viljanen-Rollinson et al., 1998) from heavily infected pepper plants in the greenhouse of Wageningen University. Conidia were disrupted by grinding them in liquid nitrogen. DNA was isolated using the DNeasy Kit including the RNAse treatment (Qiagen, Germany). The experiment was repeated by using DNA isolated from an independent experiment.

Powdery mildew infected pepper leaves which were assigned to different DI classes by visual inspection scoring were collected and ground in liquid nitrogen. DNA was isolated using the DNeasy Kit (Qiagen, Germany) and DNA quality was checked by NanoDrop® ND-1000, UV–Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA). The concentration of each sample was adjusted to 15 ng/µl.

Real-time PCR

Real-time PCR was performed by using the SYBR® Green dye on Bio-Rad iCycleriQ® machine. PCR reactions were made by mixing 5 µl of iQ SYBR Green Supermix (Bio-Rad) with 0.3 µl of each primer (10 µM), and 1 µl of DNA template (15 ng/µl). Each PCR reaction was done in triplicates in a total volume of 10 µl. PCR reactions were done with the following program: one cycle at 95°C (3 min), 40 cycles of denaturation at 95°C (15 s),

primer annealing and extension at 60°C (60 s) and one cycle at 95°C (60 s). Finally, melting curve analysis was performed from 65 to 95°C (0.5°C per cycle of 61 s). The pepper gene *CaActin* was used as a reference to normalize the DNA proportion of plant by ΔΔCt methods (Livak and Schmittgen, 2001).

RESULTS

Infection steps of *L. taurica* on pepper leaf surfaces

Conidia germinated and formed primary adhesion bodies at the ends of the first germ tubes 3 to 4 h following inoculation of the abaxial pepper leaf surfaces (Fig. 2A, Supplementary video). From primary adhesion bodies, infection hyphae emerged which either quickly penetrated the leaf tissues through stomata starting from 6 h (Fig. 2B) or continued to grow on the leaf surface, sometimes even through the nearest stoma, branched (Fig. 2C) and produced secondary (or hyphal) adhesion bodies (Fig. 2D) before penetrating the leaf through a stoma. The primary and the hyphal adhesion bodies were clearly distinguished from each other based on their distinct shapes. Those hyphae that did not branch on the leaf surface following their emergence from primary adhesion bodies entered the leaf tissues through stomata within 24 h following inoculation, while the tips of the branched hyphae entered stomata at different time intervals, for example from 30 to 48 h after inoculation as shown in the Supplementary video. The pathogen projected hyphae and conidiophores from stomata 14 to 16 days after inoculation (Fig. 2E). The hyphae vigorously elongated, and formed many secondary adhesion bodies on the leaf surface (Fig. 2F). Mature conidia produced on conidiophores became the sources of subsequent infections (Fig. 1).

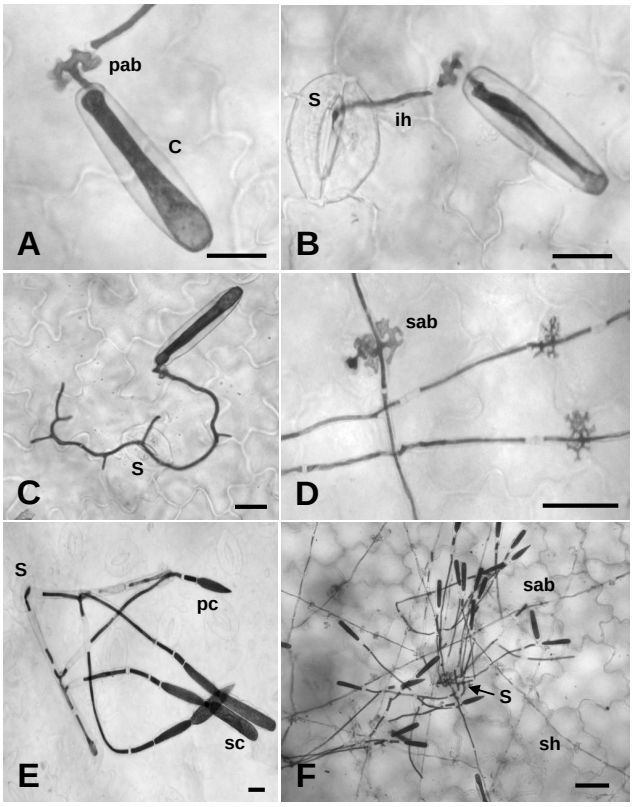

Fig. 2. Infection steps of *Leveillula taurica* on the surface of pepper leaves. (**A**) Germinated conidia (c) produce lobed primary adhesion bodies (pab). (**B**) These initiate infection hyphae (ih) that penetrate the leaves through stomata (s). (**C**) Before entering the nearest stoma (s), these hyphae sometimes continue their growth epiphytically and branch on the abaxial leaf surfaces. (**D**) The branched superficial hyphae produce secondary adhesion bodies (sab). (**E**) Conidiophores start to emerge from stomata (s) approximately 14-20 days following inoculation. Primary conidia (pc) produced on conidiophores are lanceolate while secondary conidia (sc) are ellipsoid to cylindrical. (**F**) Superficial hyphae (sh) appear from conidiophores emerged from stomata (s), continue their growth epiphytically, attach to the leaf surfaces with secondary adhesion bodies (sab) and produce new conidiophores with primary and secondary conidia. Bars equal 20 µm on Fig. 2A-E and 100 µm on Fig. 2F.

Infection steps inside pepper leaves

The pathogen did not produce haustoria in epidermal cells since neither semi-thin sections observed using light microscopy (Fig. 3) nor the TEM study revealed haustoria in the epidermis. The absence of penetration pegs underneath primary and secondary adhesion bodies has also suggested the lack of haustoria in those cells. In this study, the SEM-photographs (Figs. 4A and 4B) of 48 h-secondary adhesion body were given, because the previous works (Homma et al., 1980; Kunoh et al., 1979) reported no penetration peg underneath primary adhesion bodies.

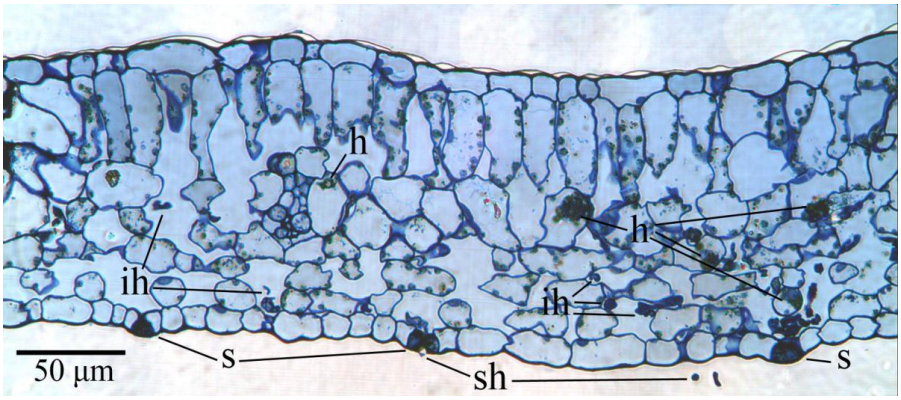

Fig. 3. Semi-thin section of a pepper leaf infected with *Leveillula taurica* and stained with toluidine blue. Intercellular hyphae (ih) are present and haustoria (h) are present in the spongy parenchyma cells. Superficial hyphae (sh) are also seen on the adaxial leaf surface, sometimes in close vicinity of stomata (s).

After entering the leaf tissues through stomata, the powdery mildew hyphae continued their growth intercellularly in the spongy parenchyma tissue, without swelling in the substomatal cavities (Fig. 3 and 5A). Intercellular hyphae were detected even in the veins, among the cells of the vascular tissues (Fig. 5B). The septal pores with woronin bodies attached to the septa with thin filamentous structures were also observed in the intercellular hyphae (Supplemental Figures 1-3). Mesophyll cells were entered by penetration pegs arising from intercellular hyphae (Fig. 5C). These were always surrounded by papillae consisting of thinner electron-dense and much thicker electron-lucent, callose-like materials (Fig. 5C+D and Supplemental Figure 4). Penetration pegs continued their intracellular development and gave rise to haustoria. In none of the observed plant cells, papillae arrested the development of haustoria. The fully developed haustorium consisted of a central body and several lobes embedded in a haustorial matrix (Fig. 6A) and surrounded by an extrahaustorial membrane (Fig. 6B). Large spherical structures, up to 1-1.2 µm diameter, resembling lipid bodies, were seen inside the central bodies and lobes of haustoria, in the haustorial matrices, and also outside the extrahaustorial membrane, in the cytoplasm of the invaded parenchyma cells (Fig. 6C). Such voluminous structures did not appear in the uninfected plant cells. Chloroplast architecture was also altered in the infected parenchyma cells: the grana were organized in larger structures and the chloroplasts contained large, up to 0.8-1 µm diameter, plastoglobuli (Fig. 6D and

Supplemental Figure 5). The nucleus of some of the infected plant cells exhibited a lobed shape (Fig. 6D). Both the altered chloroplast structure and the lobed nucleus shape are characteristic of plant cells exposed to various stress factors (Sasek et al., 2012). The first haustoria were observed in the mesophyll cells of leaf macerates 72 to 96 h following inoculations. At this stage the infected leaves were completely symptomless although the pathogen has already been established in the mesophyll.

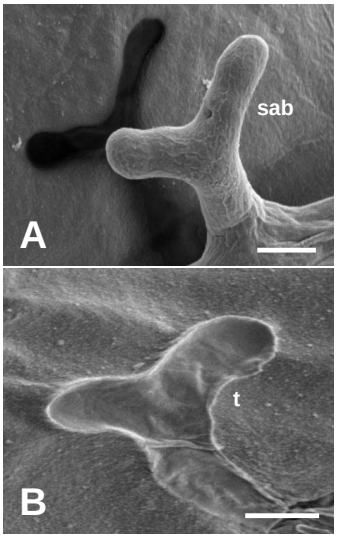

Fig. 4. Scanning electron micrographs showing the tracks of secondary adhesion bodies produced by superficial hyphae of *Leveillula taurica* on abaxial surfaces of pepper leaves. (**A**) A secondary adhesion body (sab) lifted up from the leaf epidermis by micromanipulation. (**B**) The track (t) of a secondary adhesion body removed by micromanipulation. Note the absence of any penetration hole in the track. Thirty-six leaves were used to analyze tracks underneath the secondary adhesion bodies. Bars equal 2 µm.

DNA standard curve for *L. taurica*

The LV-F/LV-R primer pair developed in this work amplified a specific fragment of the ITS region of *L. taurica*. To verify the specificity of these primers, DNA was isolated from *O. neolycopersici* conidia as well as from powdery mildew-free pepper leaves. These two DNA samples were used as templates in a real-time PCR with LV-F and LV-R primers. No amplicons were detected from plant DNA samples and on agarose gel after 40 cycles of amplification, furthermore the melting curve analysis of the PCR products did not retrieve any unexpected amplicons. Thus, the developed LV-F/LV-R primer pair was suitable for the diagnosis and quantification of *L. taurica* from pepper leaves.

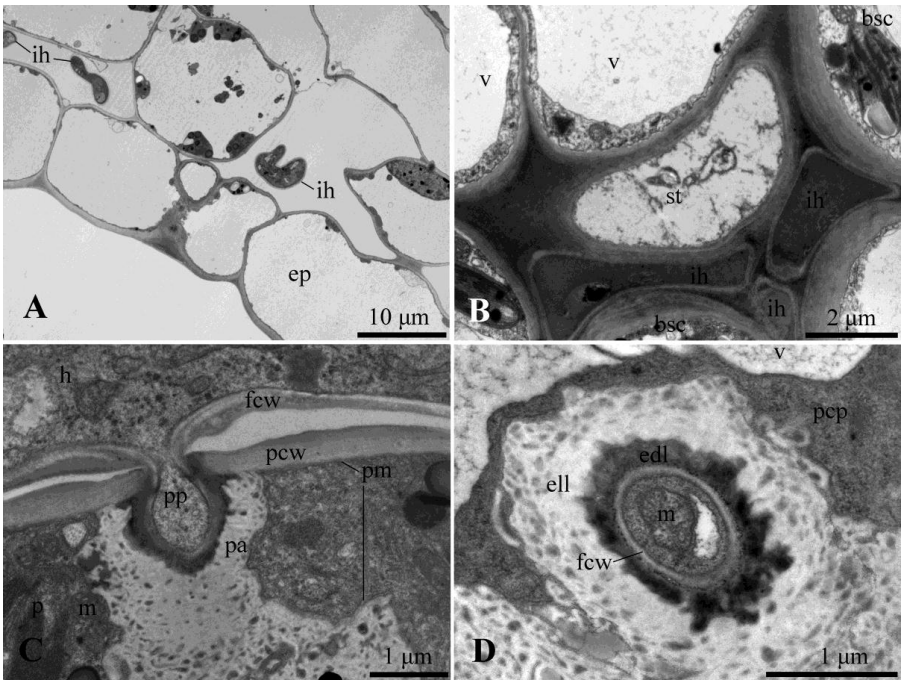

Fig. 5. Transmission electron micrographs of *Leveillula taurica* hyphae inside pepper leaves. (**A**) An early stage of infection with intercellular hyphae (ih) in a substomatal cavity and also in the regular intercellular spaces below the epidermis (ep). (**B**) Intercellular hyphae (ih) in a leaf vein, among sieve tube (st) and vascular bundle sheath cells (bsc); v = vacuole. (**C**) Almost longitudinal section through a penetration peg (pp) emerged from an intercellular hypha (h) in the mesophyll and surrounded by a papilla (pa); fcw = fungal cell wall; m = mitochondrion; p = plastid; pcw = plant cell wall; pm = plasma membrane. See also Supplemental Figure 4 for more details. (**D**) Transverse section through a penetration peg (pp) surrounded by a papilla consisting of an electron dense layer (edl) and an electron lucent layer (ell); pcp = plant cytoplasm; m = mitochondrion; v = vacuole.

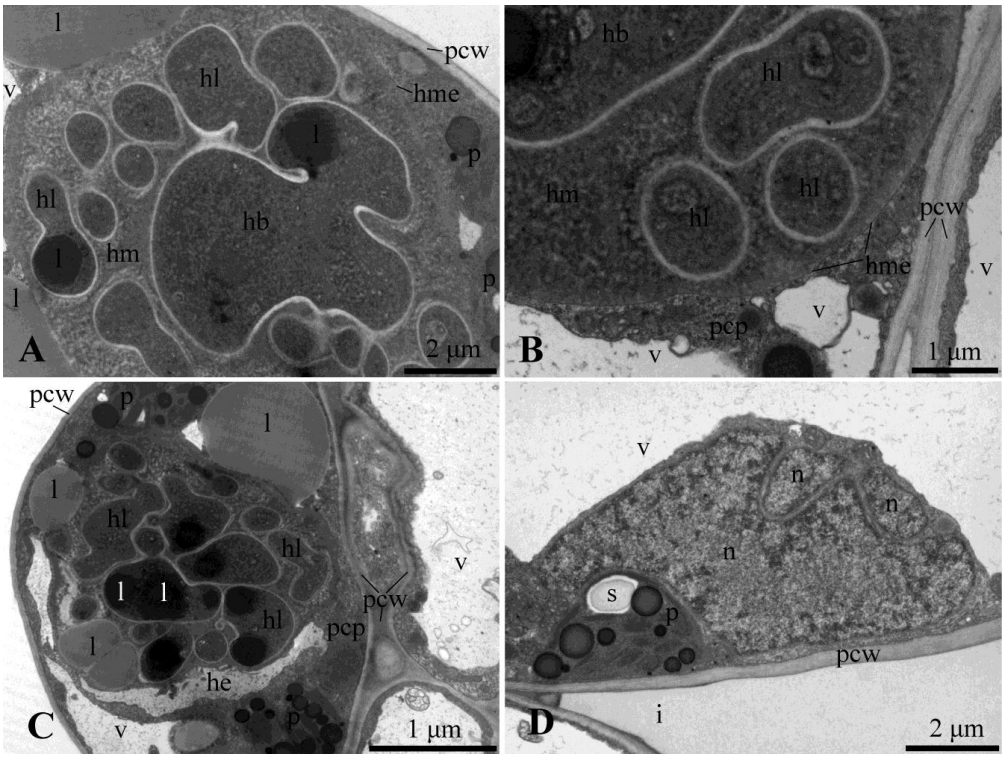

Fig. 6. Transmission electron micrographs showing haustoria of *Leveillula taurica* and the ultrastructure of the infected mesophyll cells. (**A**) A haustorium consisting of a haustorial body (hb) and several haustorial lobes (hl) embedded in a haustorial matrix (hm) and surrounded by an extrahaustorial membrane (hme). Note lipid bodies (l) inside the haustorium and also in the plant cytoplasm; p = plastid; pcw = plant cell wall. (**B**) Details of a haustorium developed close to the plant cell wall (pcw). Note the structural differences between the haustorial matrix (hm) and the plant cytoplasm (pcp); hb = haustorial body; hl = haustorial lobe; hme = extrahaustorial membrane; v = vacuole. (**C**) Lipid deposition inside and outside a haustorium in an infected pepper cell. Lipid bodies (l) are present in the plant cytoplasm (pcp) as well as in the haustorial lobes (hl) and the haustorial matrix; he = part of the haustorial encasement; pcw = plant cell wall; v = vacuole. (**D**) Parts of a multilobed nucleus (n) and a plastid (p) with starch (s) and numerous plastoglobuli in a cell of the infected pepper leaf. See also Supplemental Figure 5 for more details on plastid ultrastructure; i = intercellular space; pcw = plant cell wall; v = vacuole.

In a real-time PCR plotting assay, a standard curve could be constructed to express the equivalent relation between the cycle numbers and the concentration of a given substance in the template. For pathogen quantification, a standard curve is normally generated by plotting the cycle numbers of a pathogen DNA dilution series against the known concentration of the pathogen. In this study, a standard curve for *L. taurica* was generated

by amplifying a dilution series of *L. taurica* genomic DNA. Starting from 5,800 pg DNA, dilutions were done in six steps with 10-fold dilution per step. A standard curve was obtained by plotting threshold cycle (Ct) (in Y axis) against genomic DNA concentration of *L. taurica* (in X axis) (Fig. 7). A linear correlation (R^2=0.9978) was obtained between the Ct values and the *L. taurica* DNA concentration. The melting curve analysis of the PCR samples retrieved only a single peak. Additionally, a primer pair specific for the pepper house-keeping gene *CaActin* was used to check whether the *L. taurica* DNA is free of pepper DNA contamination. With the *L. taurica* DNA samples, no PCR products were detected after 40 cycles of amplification using the primer pair for *CaActin*. Thus, there was no pepper DNA contamination in the collected *L. taurica* DNA samples used in this study. The experiments were repeated and the obtained standard curves supported the stability of this assay.

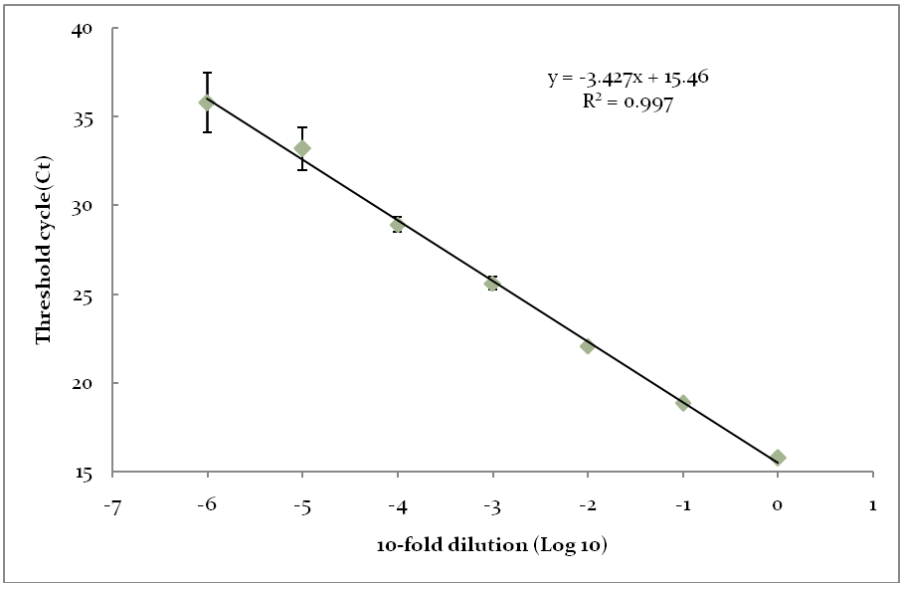

Fig. 7. Standard curve made by plotting threshold cycle (Ct) against a 10-time dilution series of genomic DNA, starting with 5800 picogram of *Leveillula taurica* DNA. The efficiency of the curve is 95.8%, the correlation coefficient 0.9978 and a slope of −3.4277. The experiment was repeated by using DNA isolated from an independent experiment.

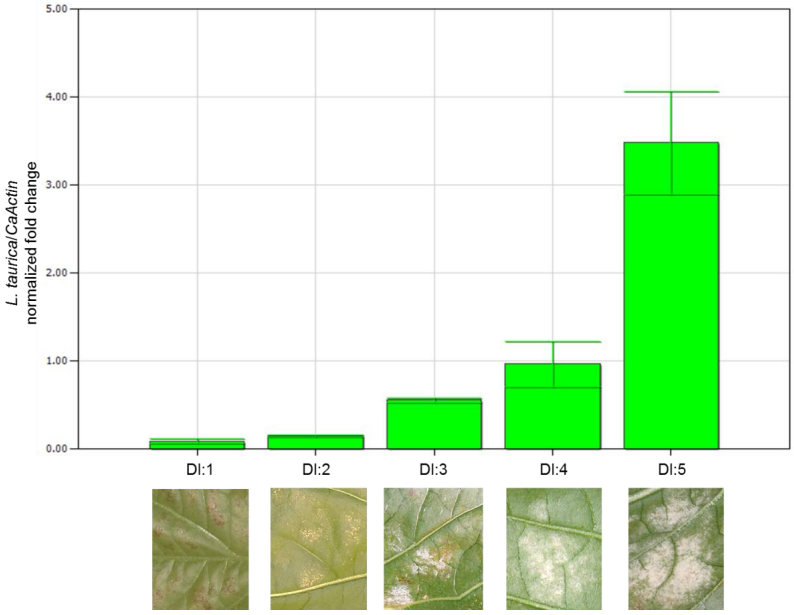

Fig. 8. Detection and quantification of *Leveillula taurica* DNA in pepper cultivars. Five cultivars were assigned with different disease indexes (DI) and the mean DI score of three plants per cultivar is presented. To test the relative quantification methods, two infected leaves per plant were pooled for DNA isolation and two technical replications for qRT-PCR were used. Results were normalized by ΔΔCt method with *CaActin* as the reference gene. Y axis represents the fold changes between fungal DNA and host plant DNA. Error bars indicate the standard deviation. The experiment was repeated three times by taking different leaf samples of the same DI classes from the same plants as biological controls.

Detection and quantification of *L. taurica* mycelium in pepper plants

Although the absolute amount of the pathogen in the host tissues was determined by using the DNA standard curve of *L. taurica* mycelium, results were not normalized with the amount of plant tissues used during the isolation of fungal DNA. Therefore, it was necessary to quantify the relative amount of pathogen DNA in infected plant tissues by measuring both fungal and plant DNA in the same samples. To do so, we used a plant gene *CaActin* as reference to normalize the *L. taurica* DNA concentration in the infected sample. Five pepper cultivars were selected which showed different levels of susceptibility against *L. taurica* in the greenhouse. First, the DI values were determined for each cultivar. As a second step, DNA was isolated from *L. taurica*-infected leaves. Two real-time PCR reactions were performed with the same DNA sample by using primers specific to plant and fungal DNA. The quantities of *L. taurica* DNA in different samples were normalized by plant

DNA using ΔΔCt methods (Livak and Schmittgen, 2001) (Fig. 8). A good correlation was obtained between real-time PCR and the DI values; pepper plants displaying mild mildew symptoms contained a lower fungal DNA amount and *vice versa* (Fig. 8). This relative quantification method is useful to distinguish small differences in mycelial biomass that is proportional with disease severity

DISCUSSION

So far only a few histological studies have been carried out to follow the infection process of *L. taurica.* Actually, these earlier studies focused on morphological characteristics of infection structures of the pathogen; germ tube (Clerk and Ayesu-Offei 1967; Homma et al., 1980), primary (Homma et al., 1980; Kunoh et al., 1979) and secondary adhesion bodies (Homma et al., 1980), conidiophores (Thomson and Jones 1981). To our knowledge, however, none of these works timed the development of the powdery mildew mycelium on the surface and inside the infected pepper leaves. In the present study, we first followed the infection process starting from conidial germination on the abaxial leaf surface. A consecutive observation of this process under a stereomicroscope led to a full description of the first steps of the infection (Supplementary video). Light microscopy and a TEM study of the infected leaf tissues confirmed the earlier findings regarding the lack of *L. taurica* haustoria in the epidermal cells, the sole targets of the haustoria of those powdery mildew species which, in contrast to *L. taurica*, produce hyphae only on the surfaces of their host plants. In fact, *L. taurica* also develops haustoria in a single cell type only, namely the cells of the spongy and palisade parenchyma. These were produced as early as 3 to 4 days post inoculations. Our results supported the TEM work by Kunoh et al. (1979) and, in addition, we have shown that conidial germination is followed by the development of branched hyphae on the abaxial leaf surfaces, besides the production of primary (infection) hyphae which quickly enter the leaves through stomata. We have also distinguished primary and secondary adhesion bodies on these epiphytic hyphae and have shown the presence of *L. taurica* intercellular hyphae in the vascular tissues of the leaves. Recently, Micali et al. (2011) demonstrated that papillae produced in the epidermal cells of *Arabidopsis thaliana* infected with the epiphytic powdery mildew species *Golovinomyces orontii* contain callose

(β-1,3-glucan). As the papillae examined in this work (Fig. 5C, 5D and Supplemental Figure 4) were morphologically similar to those seen in the *Arabidopsis-Golovinomyces* interaction, we suppose the electron-lucent materials observed around the penetration pegs are made of callose, as well. Also, similar to the *Arabidopsis-Golovinomyces* interaction, membranous and vesicular structures were observed in the callose layer surrounding penetration pegs (Supplemental Figure 4). Callose encasement of haustoria was observed, as well (Fig. 6C) but it was not as prominent as in the case of *Arabidopsis* epidermal cells invaded by *G. orontii* haustoria (Micali et al., 2011).

The TEM study has also revealed structural changes in the organelles of pepper cells containing *L. taurica* haustoria. The multilobed nucleus (Fig. 6D) and the presence of enlarged and flattened grana in the chloroplasts of the infected cells (Supplemental Figure 5) have already been described as the results of various biotrophic plant-microbe interactions (Gáborjányi et al., 2006; Sasek et al., 2012).

Due to the long latency period of the *L. taurica* infection, which may last for more than two weeks following conidial germination on the abaxial leaf surfaces, the early diagnosis of the pathogen is almost impossible before mildew colonies become visible as white patches. Even with symptoms, it is also difficult to evaluate the disease severity in practice like during resistance breeding programs. In this study, we developed two real-time PCR assays for quantification of *L. taurica* mycelium using ITS sequences. These sequences were recently proposed as official DNA barcodes for the fungal kingdom (Schoch et al., 2012) in spite of some limitations (Kiss, 2012). At present, real-time PCR identification, detection and quantification of pathogens is widely used in medical diagnosis, plant-microbe interaction studies and breeding programs (McCartney et al., 2003; Boyle et al., 2005). With the standard curve (Fig. 7) developed in this study, absolute quantification of the pathogen DNA can be obtained. On the other hand, the relative quantification method developed in this work allows the measurement of the ratio between fungal DNA and host plant DNA in the same sample (Fig. 8) similar to other works (Winton et al., 2002; Gachon and Saindrenan, 2004).

By testing a set of *L. taurica*-infected pepper plants showing different levels of resistance, the correlation between the DI values and amount of fungal DNA confirmed the sensitivity and robustness of the relative quantification method (Fig. 8). The fact that no

amplicon could be detected in the reactions when using DNA of uninfected plants and other fungal powdery mildews demonstrates the specificity of both assays.

In most cases, genomic DNA was used as template in the PCR assay. There is evidence that the DNA content could vary in different pathogen organs. Therefore, several studies had been carried out to use RNA as template in the PCR assay (Voegele and Schmid, 2011). However, using RNA as template also presents challenges. For example, genes change their expression levels under different growing circumstances or during different pathogen developmental stages. Also we showed that the *L. taurica* DNA isolated from infected leaves can be correctly measured using this standard curve and the relative quantification of *L. taurica* DNA accorded with their DI scores. Thus, using *L. taurica* DNA for quantification is reliable. One important note is that, when applying the detection method involving the absolute amount of *L. taurica* DNA in the host tissues, we used plant leaves of similar size for DNA isolation. If the plant tissue cannot be controlled for similar size or amount, the relative quantification method is more recommended for disease evaluation than using the *L. taurica* DNA standard curve.

In conclusion, the real-time PCR assays described in this study were shown to be quantitative, sensitive and reproducible. Low amounts of *L. taurica* DNA could be quantified in the DNA sample of infected plants (Fig. 8). The relative quantification assay has the potential to be utilized as a convenient tool in breeding for resistance to powdery mildew disease and in studying host/*L. taurica* interaction.

ACKNOWLEDGEMENTS

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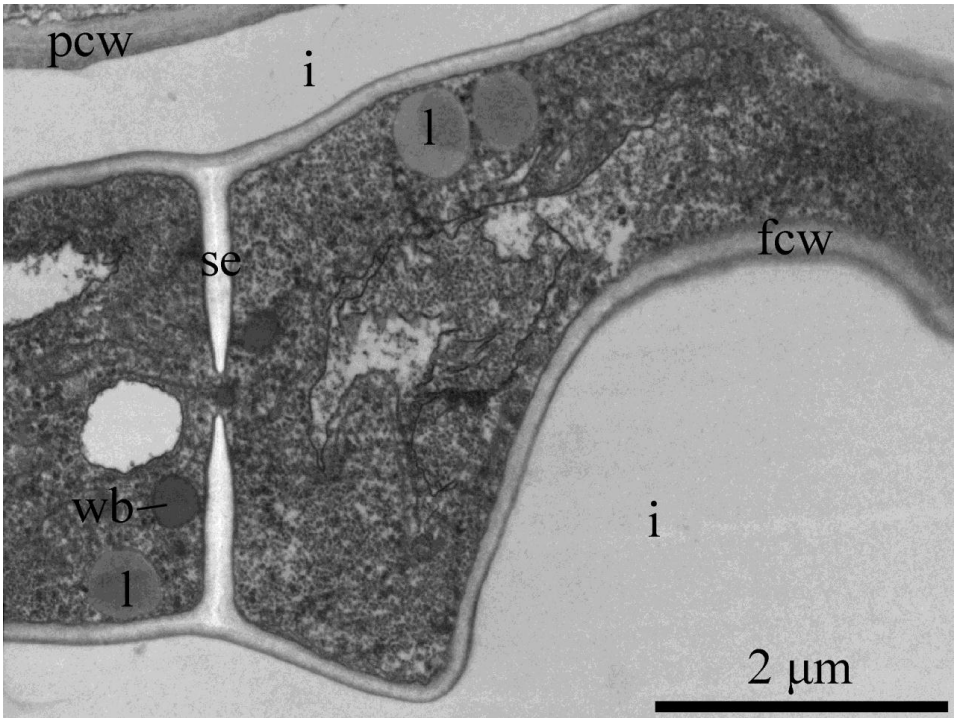
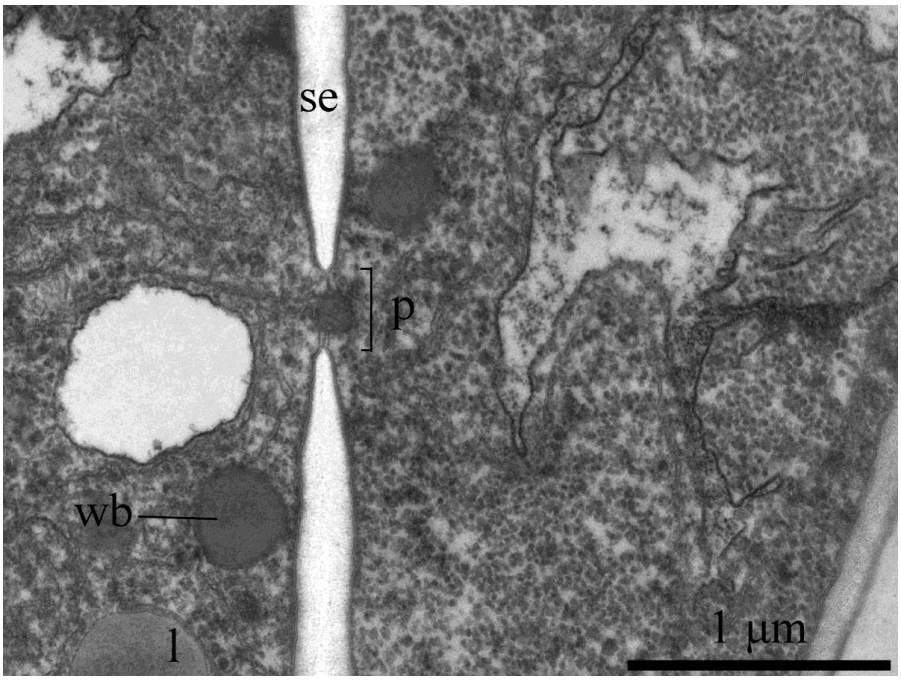
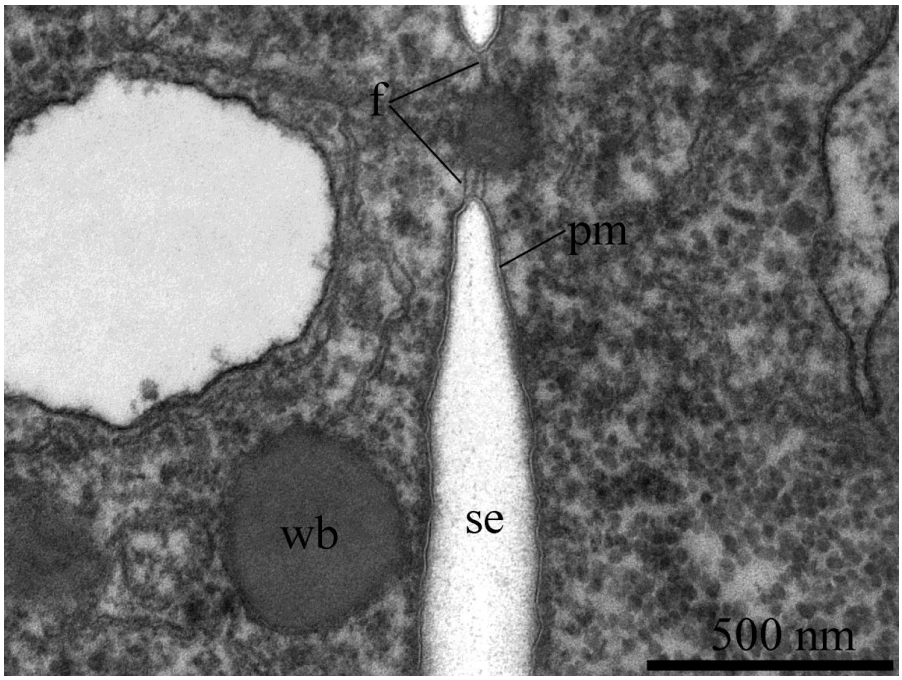


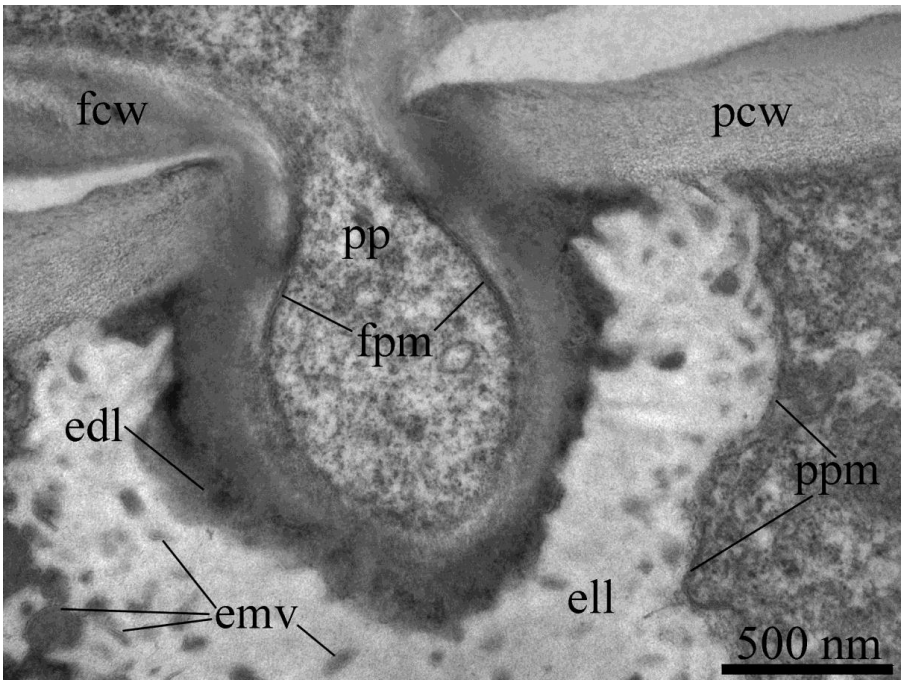
Supplemental Fig. 1. A hypha of *Leveillula taurica* in the intercellular space (i) of the spongy parenchyma of a pepper leaf. Note the septum (se) and a Woronin body (wb) in the hypha; fcw = fungal cell wall; l = spherical body resembling lipid body; pcw = plant cell wall.



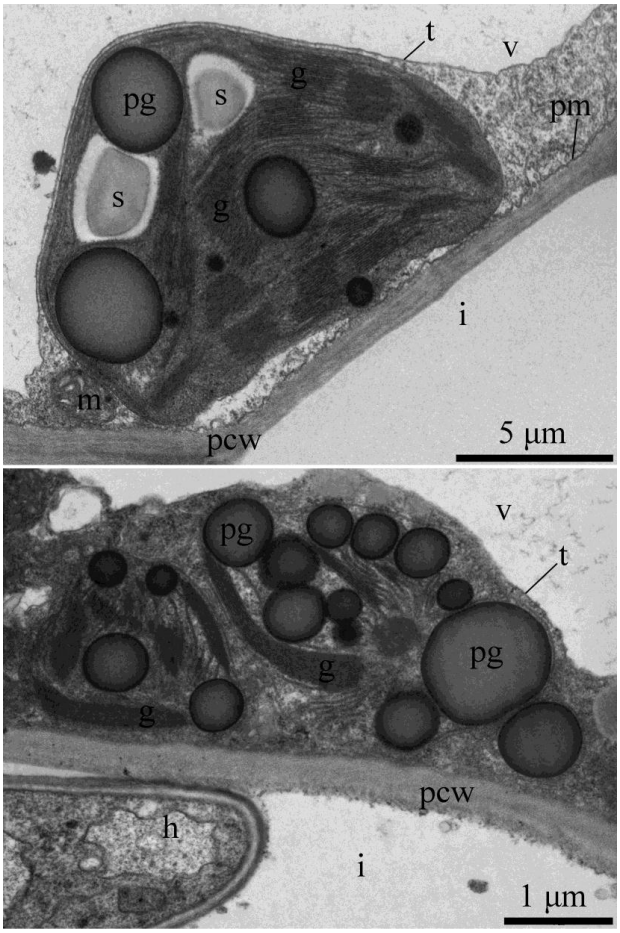
Supplemental Fig. 2. Details of a septal pore (p) shown in Suppl. Fig. 1; l = spherical body resembling lipid body; se = septum; wb = Woronin body.



Supplemental Fig. 3. Filaments (f) attached to the septum (se) and an electron dense spherical body in the middle of the septal pore shown in Suppl. Figs. 1 and 2; pm = plasma membrane; wb = Woronin body.



Supplemental Fig. 4. Details of a penetration peg (pp) of *Leveillula taurica* in a pepper cell. The papilla around the peg consists of an electron dense layer (edl) and an electron lucent layer (ell) containing several embedded membranous and vesicular structures (emv). See also Figs. 5C and 5D; fcw = fungal cell wall; ppm = plant plasma membrane; pcw = plant cell wall.



Supplemental Fig. 5. Differences in the ultrastructure of chloroplasts in uninfected pepper cells and cells infected with *Leveillula taurica*. Note the more or less regular shape of grana (g) in an uninfected cell (top) vs. the enlarged and flattened grana of an infected cell (bottom). The high number of large plastoglobuli (pg) makes these plastids similar to gerontoplasts; h = intercellular hypha of *L. taurica*; i = intercellular space; m = mitochondrion; pcw = plant cell wall; pm = plant plasma membrane; s = starch; t = tonoplast; v = vacuole.

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CHAPTER 4

CHAPTER 5

Loss of Function in *Mlo* Orthologs Reduces Susceptibility of Pepper and Tomato to Powdery Mildew Disease caused by *Leveillula taurica*

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Loss of Function in Mlo Orthologs Reduces Susceptibility of Pepper and Tomato to Powdery Mildew Disease caused by Leveillula taurica

Zheng Zheng[1], Teruo Nonomura[2], Yoshinori Matsuda[2], Hideyoshi Toyoda[2], Richard G.F. Visser[1]and Yuling Bai[1*]

[1] Wageningen UR Plant Breeding, Wageningen University & Research Centre, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

[2] Laboratory of Phytoprotection Science and Technology, Faculty of Agriculture, Kinki University, Nara 631-8505, Japan

[*]Corresponding author: Dr. Yuling Bai; Email: bai.yuling@wur.nl

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CHAPTER 5

ABSTRACT

Powdery mildew disease caused by *Leveillula taurica* is a serious fungal threat to greenhouse pepper production. The correct functioning of plant *Mlo* genes has been found to be required for powdery mildew pathogenesis in *Arabidopsis*, tomato and pea. In tomato, a loss-of-function mutation in the *SlMlo1* gene results in resistance to powdery mildew disease caused by *Oidium neolycopersici*. The aim of this study was to investigate whether loss-of-function in *Mlo* genes induces resistance to *L. taurica* in pepper and tomato. Firstly, the tomato *SlMlo* mutant was inoculated with *L. taurica* and it proved to be less susceptible compared to the susceptible control, *Solanum lycopersicum* cv. Moneymaker. Secondly, one pepper *Mlo* gene, *CaMlo2* was isolated by applying a homology-based cloning approach. Phylogenetic analysis showed that *CaMlo2* is more homologous to *SlMlo1* than the previously identified *CaMlo1* gene. Compared to *CaMlo1,* the expression of *CaMlo2* was higher and was more up-regulated upon *L. taurica* infection. However, functional analysis via virus-induced gene silencing demonstrated that knocking down the expression of either *CaMlo1* or *CaMlo2* resulted in reduced susceptibility of pepper to *L. taurica*.

INTRODUCTION

Powdery mildews are conspicuous plant fungal pathogens that comprise approximately 500 species and infect more than 1500 plant genera (Braun, 1987). In Europe, the largest application of fungicide is for controlling powdery mildew diseases in agricultural and horticultural production (Hewitt, 1998). The powdery mildew pathogen *Leveillula taurica* (Lév.) G. Arnaud is a serious fungal threat to pepper as well as tomato production. Heavy epidemics of powdery mildew disease could cause a significant yield loss up to 2 to 4 kg/m^2 in greenhouse pepper production (Cerkauskas and Buonassisi, 2003).

Beside repeated application of fungicides, powdery mildew diseases could be controlled by using resistant cultivars. In practice, breeding for resisitance is mainly done by introgressing the resistance trait from wild species into the cultivated crop. In tomato, the *Lv* gene is the only resistance (R) gene identifed so far conferring resistance to *L. taurica*. The *Lv* gene was found in a wild tomato accession of *Solanum chilense* and mapped on chromosome 12 (Chunwongse et al., 1994). In the *Capsicum* genus, several studies have been carried out to search for resistance to *L. taurica* (Blat et al., 2005; Daubeze et al., 1995; de Souza and Cafe, 2003; Shifriss et al., 1992). So far, five Quantitative trait loci (QTLs) have been identified governing the resistance to *L. taurica* in pepper, with one of the QTLs namely *Lt-9.1* co-linearizing with the tomato *Lv* locus (Lefebvre et al., 2003).

In principle, all resistance resources discovered from the wild accessions could be promising materials for isolation of the potential *R*-genes and be used in resistance breeding. However, there are several weaknesses of using *R*-genes. First of all, the interspecific crossability barrier could restrict introgressing an *R*-gene from the resistant donor to cultivated species (Fu et al., 2009). Even if the resistant donor can be easily crossed with the cultivated species, extensive backcrossing is required to remove/introduce other un/desirable traits. Secondly, transferring *R*-genes from one species into another does not guarantee that resistance conferred by the *R*-genes is retained in the receptor species in all cases. The function of an *R*-gene sometimes requires additional gene(s) in signaling pathways and/or metabolites (Bent, 1996; Xiao et al., 2003). Thirdly, *R*-genes confer race-specific resistance, which could easily be overcome by new races of the pathogen in a short period.

CHAPTER 5

Complementary to introgression of *R*-genes, a novel breeding strategy has been proposed, which is to disable plant susceptibility genes (*S*-genes) (Eckardt, 2002; Pavan et al., 2010). In order to infect a plant species a pathogen should be able to suppress the plant's innate immunity by exploiting effector molecules to establish effector-triggered susceptibility. Plant genes, which are required for triggering susceptibility to pathogens and play a negative role in defense responses, are referred to as *S*-genes. Impairment of the function of plant *S*-genes results in recessive resistance. One representive example is the *Mildew resistance Locus O* (*Mlo*) gene identified in barley. Loss-of-function mutants of the barley *Mlo* gene give resistance to powdery mildew (*Blumeria graminis* f. sp. *hordei*) and have been used in European barley cultivation for more than 30 years (Humphry et al., 2006). In addition to barley, mutations in *Mlo* orthologues result in recessively inherited resistance in *Arabidopsis* (Consonni et al., 2006), tomato (Bai et al., 2008) and pea (Humphry et al., 2011; Pavan et al., 2011). So far, no natural powdery mildew isolates could break down the *mlo*-based resistance, which thus represents a broad-spectrum and possibly durable resistance and has become a successful example of using *S*-genes in crop protection.

The *Mlo* genes encode a class of plant-specific proteins anchored in the plasma membrane by seven transmembrane domains (Buschges et al., 1997; Devoto et al., 1999). When the MLO protein is lacking, powdery mildew fungi fail to enter their host. It is a well-studied pre-penetration resistance conferred by cell wall appositions (Consonni et al., 2006; Huckelhoven et al., 2001; Huckelhoven et al., 2000; Thordal-Christensen et al., 1997). To date, 15, 17, 7, 9, 12 *Mlo* paralogs have been reported in the genomes of *Arabidopsis*, grape, wheat, maize and rice, respectively (Consonni et al., 2006; Feechan et al., 2008; Devoto et al., 2003; Sasanuma et al., 2010; Liu and Zhu, 2008). Of these paralogs, members of a specific phylogenetic clade play a role in powdery mildew susceptibility. These members are characterized by the presence of a tetra-peptide (D/E-F-S/T-F) motif in the cytoplasmic region of the protein (Bai et al., 2008; Feechan et al., 2008; Panstruga, 2005; Reinstadler et al., 2010) and their response to powdery mildew infection in the early time points (Bai et al., 2008; Chen et al., 2006; Piffanelli et al., 2002).

In tomato, we have demonstrated that the recessive *ol-2* gene conferring resistance to the powdery mildew pathogen *Oidium neolycopersici* contains a 19-bp deletion in the

coding region of the tomato *Mlo* ortholog, *SlMlo1* (Bai et al., 2008; Pavan et al., 2008). Another powdery mildew, *L. taurica,* can also infect tomato. Compared to *O. neolycopersici* which penetrates tomato leaves via epidermal cells, *L. taurica* penetrates the leaf through stomata. The aim of this study was to verify in pepper and tomato whether loss of *Mlo* function gives rise to resistance against *L. taurica*. In pepper, one *Mlo* gene has been identified so far. This *CaMlo1* gene is considered to be a mildew-effective *Mlo* ortholog to *SlMlo1* (Humphry et al., 2011; Panstruga, 2005). In the present work, we show that a loss-of-function mutant of the *SlMlo1* gene in tomato confers resistance against *L. taurica*. In pepper, two *Mlo* homologues, *CaMlo1* (Panstruga, 2005) and *CaMlo2* that is isolated in the present study, are involved in the susceptibility of pepper to *L. taurica.*

MATERIALS AND METHODS

Plant materials

Four tomato genotypes were used: a breeding line *ol-2*, a homozygous T3 *ol-2* line (35S::*SlMlo1*), *Solanum lycopersicum* cv. Moneymaker (MM) and cv. Laurica. The *ol-2* line carries the mutated allele (a 19-bp deletion in the coding sequence) of *SlMlo1* and is resistant to *O. neolycopersici* (Bai et al., 2008). Homozygous T3 *ol-2* line is the selfed progeny (T_2 plants) of a transgenic *ol-2* line over-expressing the *SlMlo1* gene under the 35S promoter (35S::*SlMlo1*) and fully susceptible to *O. neolycopersici* (Bai et al., 2008). MM carrying the natural *SlMlo1* allele was used as the susceptible control. cv. Laurica was used as the resistant control which carries the wildtype *SlMlo1* allele and the *Lv* gene derived from *S. chilense* LA1969.

Three pepper genotypes, cv. Maor and two anonymous cultivars A and B, were used which are all susceptible to *L. taurica*. The doubled-haploid line HV-12 (kindly provided by Dr.Alain Palloix, INRA, France) was used as the resistant control.

Disease assay

L. taurica was obtained in the greenhouse of a seeds company from The Netherlands and maintained on susceptible pepper plants in a climate chamber at Wageningen University, The Netherlands. The inoculation was performed on six weeks-old plants by a spray

method. Conidiospores of *L. taurica* were washed off from heavily infected pepper leaves with water. Inoculum was adjusted to a final concentration of 2.5 x 10^4 conidia/mL. The abaxial surface of plant leaves was spray-inoculated. After the inoculation, plants were kept for 24 h in plastic cages with 100% relative humidity at 21°C and subsequently with a temperature of 21°C (day) / 19°C (night) and relative humidity of 65%.

For scoring *L. taurica* symptoms, the disease index (DI) method described by Zheng *et al*. (2012, Chapter 4 of this thesis) was used. In addition, numbers of fungal colonies on the 4^{th}, 5^{th} and 6^{th} true leaves were counted. In order to minimize the bias caused by the subjective DI scoring, a real-time PCR method was used to quantify the fungal biomass (Chapter 4 of this thesis). ITS sequences of *L. taurica* infecting pepper were retrieved from GenBank and specific primers (5'AGCCGACTAGGCTTGGTCTT3') and (5' GCGGGTATCCCTACCTGATT3') were designed by using the online software: Primer3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The DNA was isolated from infected leaves. House-keeping genes *CaActin* with forward primer *CaActin*-F (5'ATCCCTCCACCTCTTCACTCTC3') and reverse primer *CaActin*-R (5'GCCTTAACCATTCCTGTTCCATTATC3') for pepper (Silvar et al., 2008); as well as the *Elongation factor* with forward primer *EF*-F (5'GGAACTTGAGAAGGAGCCTAAG3') and reverse primer *EF*-R (5'CAACACCAACAGCAACAGTCT3') for tomato (Lovdal and Lillo, 2009) were used as reference to normalize the plant DNA proportion by $\Delta\Delta$Ct methods (Livak and Schmittgen, 2001).

RNA/DNA isolation, RACE and gene expression analysis

The Rapid amplification of cDNA ends (RACE) reaction was conducted using the GeneRacer™ kit (Invitrogen). For obtaining the 5' ends Untranslated Regions (UTR) of the *CaMlo2*, primer 5'R (5' TCTCTACAGTAATGAACTCGAGACAAA3') were used together with the GeneRacer™ 5' primer (5'CGACTGGAGCACGAGGACACTGA3'). For the 3' ends UTR, primer 3'F (5' CGTGGGAATAAGTCCAGCAT3') were used together with the GeneRacer™ 3' primer (5' GCTGTCAACGATACGCTACGTAACG3'). For gene expression analysis, the 3^{rd}, 4^{th} and 5^{th} leaves were collected from three individual plants at time points: 0 hpi (hours post inoculation), 1 hpi, 3 hpi, 5 hpi, 7 hpi, 21 hpi, 25 hpi, 30 hpi, 47 hpi, 72 hpi, 96 hpi and 21 dpi (days post inoculation) after *L. taurica* inoculation. Quantitative real-time

PCR was performed by using the SYBR® Green dye on Bio-Rad iCycleriQ® machine (Bio-Rad). For gene expression analysis of *CaMlo1* the forward primer *CaMlo1-ge-F* (5'CAAACATCATTCAAATCCAGCAACACCA3') and the reverse primer *CaMlo1-ge-R* (5'AATTTGATGCATATGGGACGGCGAAGAC3') were used; and for *CaMlo2* the forward primer *CaMlo2-ge-F* (5'CTGGCACAATACAGCGAAAA3') and the reverse primer *CaMlo2-ge-R* (5'TTCATTAGCCCAGCCTTCAT3') were used in the real-time PCR. Gene expression levels at different time points were normalized by ΔΔCt methods with a house-keeping gene mentioned above as reference. RNA and DNA were isolated by using the RNeasy Kit and DNeasy Kit (Qiagen, Germany) respectively according to manufacturer's recommendations.

Virus Induced Gene silencing (VIGS) in Pepper

The VIGS experiments were performed as described by Liu *et al*. (2002). Primer pairs (Supplementary Table 1) were designed to amplify fragments suitable for specifically silencing of *CaMlo1* and *CaMlo2* by using the online software: Primer3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Target fragments of *CaMlo1* were amplified and cloned into the pGEM®-T Easy Vector (Promega), positive plasmids were digested with *Eco*RI and target fragments were excised from the gel. The pTRV2 (pYL156) vectors (Liu *et al*. 2002) were digested with *Eco*RI and dephosphorylation were performed by using *Thermosensitive Alkaline Phosphatase* (TSAP) (Promega). Target fragments of *CaMlo1* were ligated by T4 ligase into the linearized pTRV2 vectors. Positive clones were confirmed by sequencing. Target fragments of *CaMlo2* were cloned into the gateway-compatible vector pENTR D-TOPO (Invitrogen) and subsequently recombined into pTRV2-gateway VIGS vector. The pTRV2 vectors carrying the target gene fragment were transformed into *Agrobacterium* strain GV3101 by electroporation. A 100-mL culture of *Agrobacterium* containing the target vectors was grown overnight at 28°C in YEP (yeast extract/ bactopeptone) medium with antibiotics (50 mg/mL kanamycin and 50 mg/mL rifampicin). The cells were resuspended into infiltration medium MMA (150 mM acetosyringone, 10 mM MgCl and 10 mM MES, pH 5.7) with OD600 = 2. Cultures were kept at room temperature for 1 to 6 hours before agroinfiltration. *Agrobacterium* strains containing the pTRV1 vector and pTRV2 were mixed at a 1:1 ratio and co-infiltrated into two

weeks-old cotyledons of pepper. Infiltrated plants were grown at 22°C with a 16-h-light/8-h-dark photoperiod cycle. Three weeks after agroinfiltration, plants were inoculated with *L. taurica*. Powdery mildew symptoms were observed three weeks after inoculation. Tested plants were scored with DI and numbers of mildew colonies were counted on the 4th, 5th and 6th leaves. After colony counting, these three leaves were collected for DNA isolation and fungal quantification with qRT-PCR.

Consecutive digital micrographs by a dissecting microscope

Conidia were collected from conidiophores using a probe of an electrostatic spore collector and were transferred to particular sites of test leaves (Nonomura et al., 2009). Conidial growth on host epidermal cells were photographed at 0.5-1 hour intervals after inoculation using a CCD-camera of a high-fidelity digital microscope KH-2700 (Hirox, Tokyo, Japan). Photographs were treated using an image processing software (Adobe Photoshop ver.5) (Adobe Systems, CA, USA), and 110 image-analyzed photographic data were input to a Windows live movie maker software (Microsoft, WA, USA) to present the animated data of the conidial development.

RESULTS

L. taurica is sensitive to *mlo*-based resistance in tomato

Previously, we reported that loss-of-function in the *SlMlo1* gene in tomato causes resistance against the powdery mildew pathogen *O. neolycopersici* (Bai et al., 2008). In order to investigate whether *L. taurica* was principally amenable to *mlo*-based resistance, tomato lines carrying different alleles of the *SlMlo1* gene were challenged with *L. taurica*. Disease symptoms were macroscopically scored by DI and pathogen quantification in the infected leaves was performed by qRT-PCR. Plants of the breeding line *ol-2* carrying the mutant allele of *SlMlo1* showed less symptoms and a significant decrease in fungal DNA amount compared to plants of the susceptible control MM (Fig. 1). In contrast, plants of the *SlMlo1* over-expression line were more susceptible and showed a marked increase in fungal DNA amount compared to MM plants. It could be inferred that *SlMlo1* over-expression enhances the susceptibility to *L. taurica* in tomato and that loss-of-function

in *SlMlo1* results in partial resistance against *L. taurica*. The resistant control cv. Laurica, harbouring the *Lv* gene, showed no fungal sporulation and a clear HR (hypersensitive response) phenotype upon *L. taurica* infection. No HR could be seen macroscopically in plants of the breeding line *ol-2*, indicating that the resistance in these plants is likely not conferred by the *Lv* gene (Fig. 1). These data collectively indicate that in tomato *L. taurica* is sensitive to *mlo*-based resistance.

Fig. 1. Tomato leaves infected by *Leveillula taurica.* Upper panel: from left to right: the most susceptible genotype (breeding line *ol-2* with over-expression of *SlMlo1* (*ol-2*::*SlMlo1*), the susceptible control cv. Moneymaker (MM) carrying the wildtype *SlMlo1* allele), intermediate-resistant genotype (the breeding line *ol-2* carrying the *SlMlo1* mutant allele) and the resistant control cv. Laurica (LV) carrying the *Lv* gene for hypersensitive response-based resistance. Pictures were taken four weeks post fungal inoculation. Lower panel: Fungal DNA quantification by real-time PCR. The *L. taurica* DNA is normalized by a plant reference gene elongation factor (*Ef*) with ΔΔCt method. The relative pathogen bio-mass in MM plants is set as 1. In total, three leaves (4th, 5th and 6th) were pooled together from three plants per genotype. Error bar indicates the S.D of the mean for three PCR replicates.

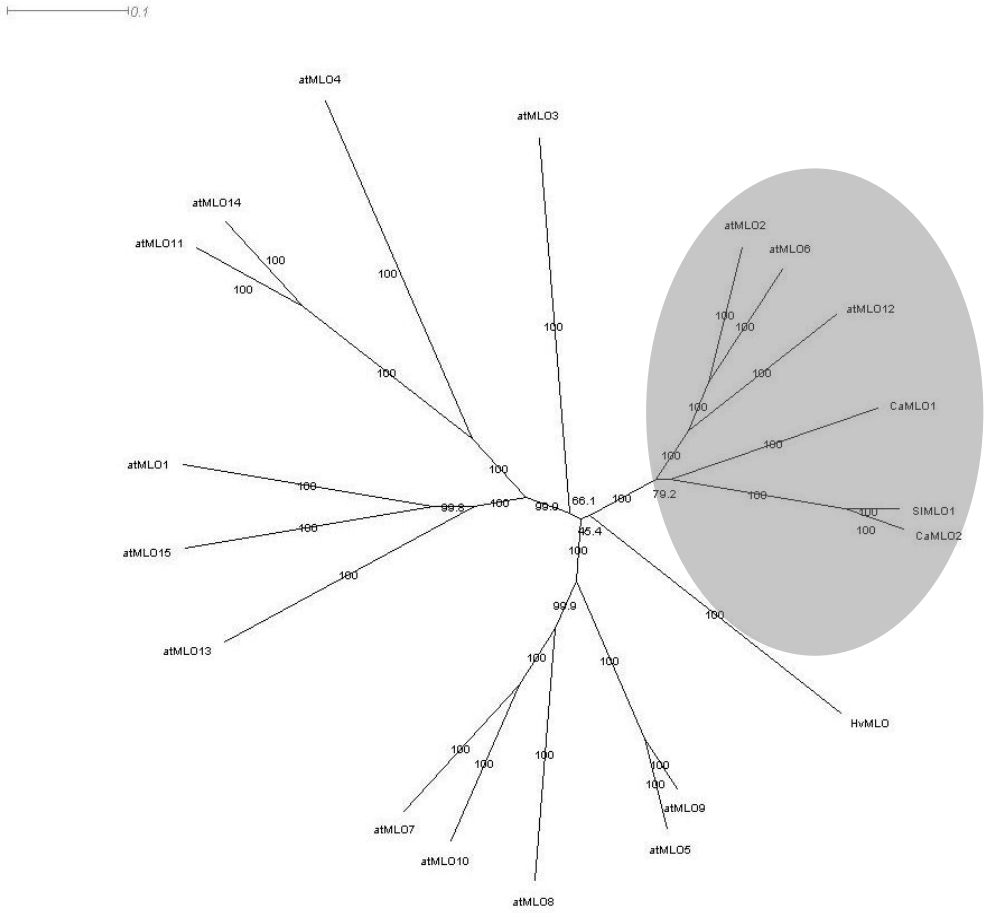
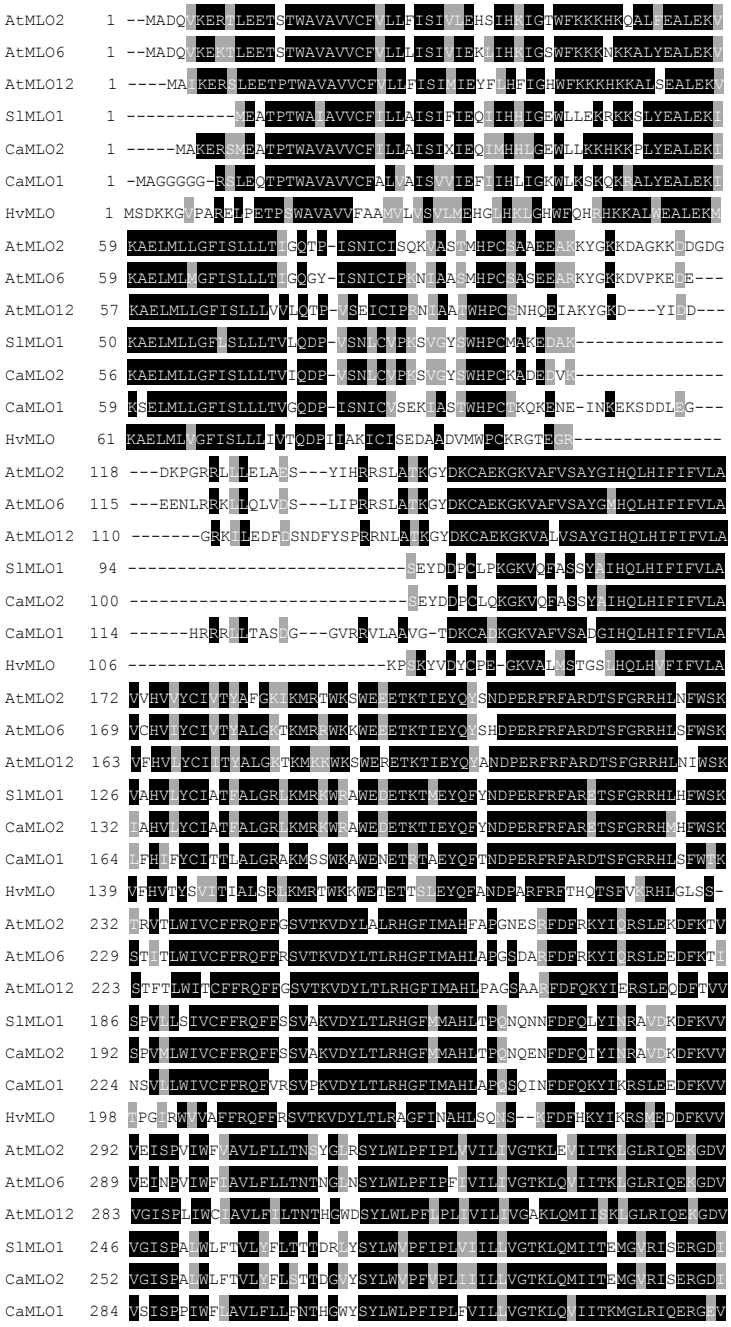


Fig 2. A neighbour-joining phylogenetic tree of MLO proteins, showing that CaMLO1 and CaMLO2 are grouped together with AtMLO2, AtMLO6, AtMLO12 and SlMLO1 (grey-marked circle). The un-rooted radial tree comprises all 15 *Arabidopsis* MLO paralogs (Devoto et al., 2003), SlMLO1 of tomato as well as CaMLO1 and CaMLO2. The tree was established on the basis of an optimized multiple-sequence alignment using the PHYLIP software package. Numbers above nodes indicate bootstrap values (based on 100 replicates) that support the respective branch. The scale (up corner on the left) indicates the number of amino-acid exchanges per site.

Isolation of the *CaMlo2* gene in pepper

The sensitivity of *L. taurica* to *mlo*-based resistance in tomato promotes the possibility to use loss-of-function mutations in pepper *Mlo* ortholog(s) for resistance to *L. taurica.* So far, *CaMlo1* is the only identified *Mlo* homologue in pepper and considered as the ortholog of *SlMlo1* (Humphry et al., 2011; Panstruga, 2005). By BLAST analysis, we inspected the

publicly available pepper expressed sequence tag (EST) collections from the SOL Genomics Network (SGN) and identified one EST sequence (SGN-U202700) that is distinct from *CaMlo1*.



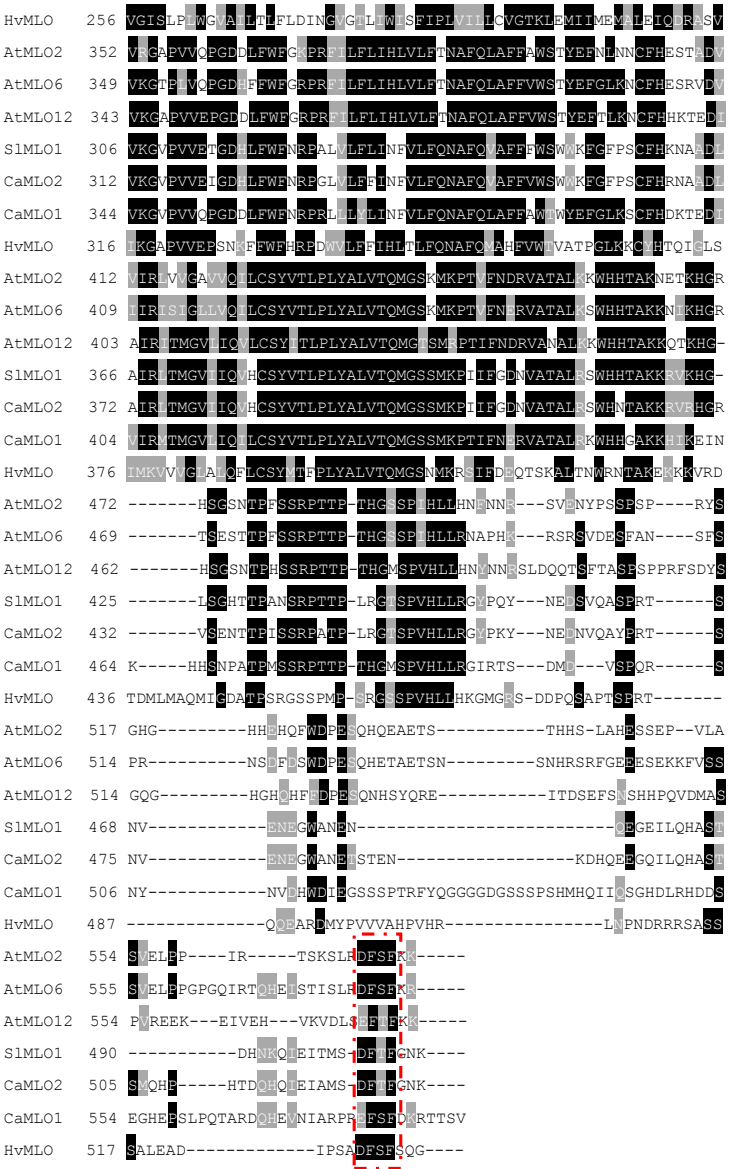


Fig. 2. Multiple amino acid sequence alignment of pepper CaMLO1 and CaMLO2, barley HvMLO, tomato SlMLO1, as well as *Arabidopsis* AtMLO2, AtMLO6, and AtMLO12. The alignment was generated by CLUSTALW using default parameters. Grey boxes indicate similar amino acids and black boxes indicate identical amino acids. Beside the conserved transmembrane regions, the C-terminal D/E-F-S/T-F tetra-peptide sequence, one of several motifs characteristic of barley *Mlo* orthologs (Panstruga, 2005) is boxed. For this tetra-peptide sequence, CaMLO2 contains D-F-T-F which is identical to SlMLO1.

Full-length cDNA sequence of this EST was obtained by RACE, which was designated as *CaMlo2.* Phylogenetic analysis comprising all *Arabidopsis* as well as barley, tomato and

pepper MLO protein sequences (AtMLO1-15, HvMLO, SlMLO1 and CaMLO1) revealed that the newly isolated CaMLO2 is closer to SlMLO1 than CaMLO1(Fig. 2). The presence of a shared distinctive polypeptide motif at the C-terminus of the encoded polypeptides (Fig. 3, boxed region), which is considered to be diagnostic of an orthologous phylogenetic relationship (Panstruga 2005), points to the fact that *CaMlo2* likely represents the pepper ortholog of *SlMlo1*.

Accumulation of *CaMlo* transcripts upon *L. taurica* infection

Previously, we showed that the expression of *SlMlo1* is induced in tomato at early time points upon challenge with *O. neolycopersici*. Data obtained by Piffanelli et al. (2002) also demonstrated similar expression changes of the barley *Mlo* gene upon powdery mildew infection. In order to verify whether *CaMlo* genes are responsive to powdery mildew infection in pepper, we performed semi-quantitative RT-PCR and qRT-PCR to study the expression of *CaMlo* genes in pepper plants which are susceptible to *L. taurica*. Results of the semi-quantitative RT-PCR showed an obvious induction of *CaMlo2* transcript at time points of five and 25 h post inoculation (hpi) and 21 days post inoculation (dpi) (Fig. 4).

Fig. 4. Expression of *CaMlo1* and *CaMlo2* in pepper upon *Leveillula taurica* infection measured by Semi quantitative and real-time RT-PCR. Upper panel: leaf samples were collected from (in a left to right order) 1. Susceptible cultivar A, 2. Susceptible cultivar B, 3. Maor, 4. HV-12, 5. Maor, 6. Susceptible cultivar A, 7. HV-12, 8. Susceptible cultivar B. Samples from different time points were separated by 1Kb marker (M). In total, three leaves per plant were collected and pooled for RNA isolation, two plants per genotype were tested. Two different primer sets have been used and showed similar trends for expression.

In order to correlate the expression with the infection process, we consecutively tracked the infection progress of *L. taurica* on the pepper leaves with a digital microscope (supplementary video) and performed qRT-PCR with more detailed time points (Fig. 5-A). Based on the infection process of *L. taurica*, we could cluster time points into four stages. The first stage corresponds to the period from 0 to 3 hpi. In this stage fungal spores landed

on the leaf surface and started the germination, defined as fungal germination stage (Fig. 5-B1). During this stage both *CaMlo* genes showed a very low level of expression and the expression of *CaMlo1* was higher than that of *CaMlo2*. The second stage includes four time points from 4 to 25 hpi. During this period, most spores formed a primary adhesion body (around 5 hpi), primary hyphae and secondary adhesion bodies (after 7 hpi). The adhesion bodies helped the fungus fix itself on the leaf surface and primary and secondary (branched) hyphae grew into the stomata (Fig. 5-B2). This stage is defined as stomata penetration stage in this study. During this stage, the expression of *CaMlo2* was obviously induced at 5 hpi (the highest) and 25 hpi; and the expression of *CaMlo1* was slightly induced at 21 hpi and 25 hpi. The third stage, defined as fungal growing stage, includes four time points from 30 to 96 hpi. During this stage, intercellular growth (including haustorium formation in mesophyll cells) of *L. taurica* took place (Fig. 5-B3) and the expression of *CaMlo1* was slightly induced (Fig. 5-A). The last time point is 21 dpi, when *L. taurica* finished the whole life cycle and released the next generation spores (Fig. 5-B4). Both *CaMlo1* and *CaMlo2* showed the highest expression at this time point. This stage is a mixture of spores of all the stages. These data showed that the newly isolated *CaMlo2* gene is responsive to *L. taurica* at the stomata penetration stage and *CaMlo1* at the intercellular-growth stage. The gene expression levels were measured with two different primer pairs located in different regions of each gene and similar results were obtained.

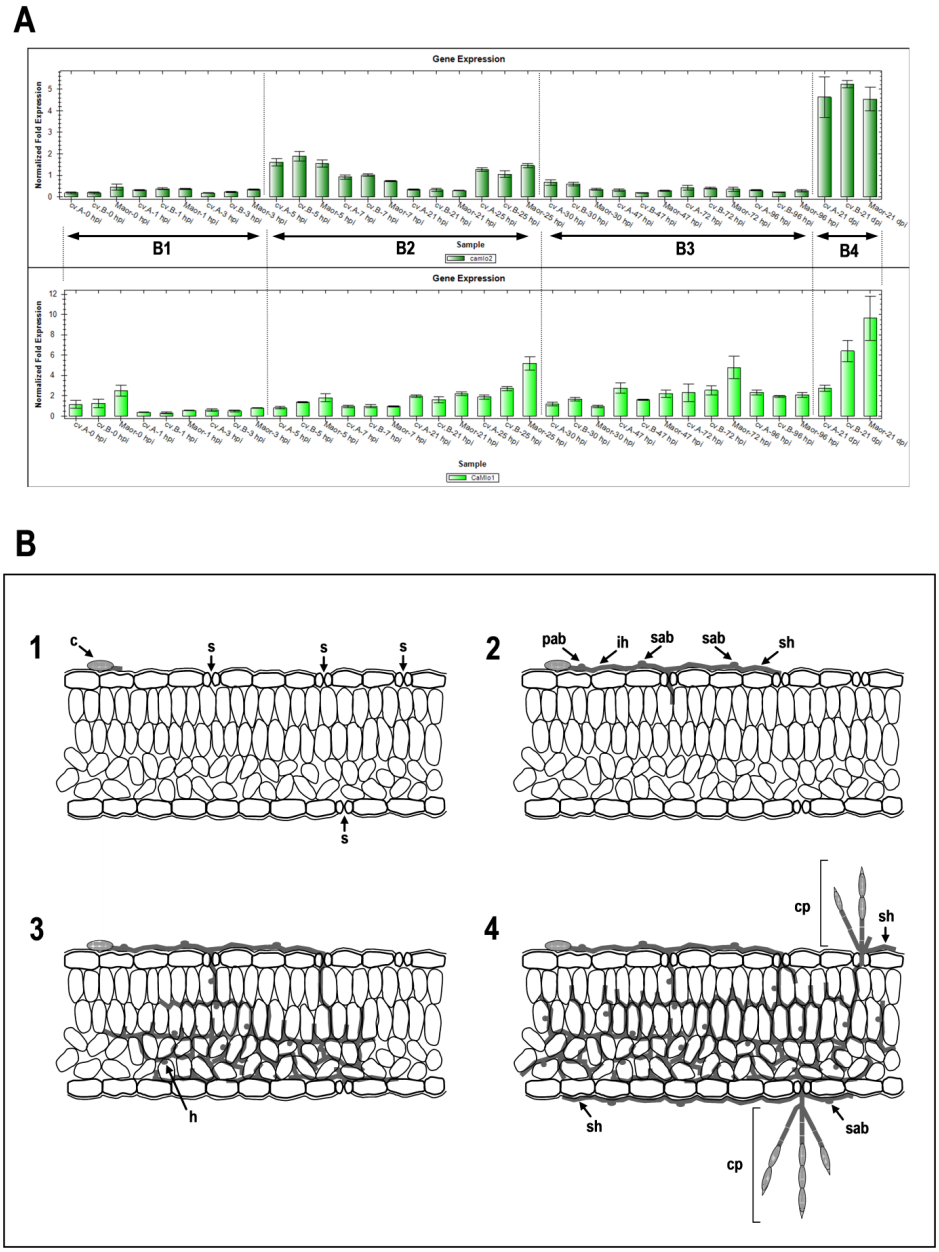


Fig. 5. A. The expression profile of the *CaMlo* genes measured by qRT-PCR in pepper leaves upon *Leveillula taurica* infection. The bars indicate normalized gene expression by ΔΔCt methods with the reference gene *CaActin*. Samples were taken from three whole pepper leaves (the 3rd, 4th and 5th leaf) upon *L. taurica* infection at 0 hpi, 1 hpi, 3 hpi, 5 hpi, 7 hpi, 21 hpi, 25 hpi, 30 hpi, 47 hpi, 72 hpi, 96 hpi and 21 dpi. Means are calculated from triplicate technical Real-time PCR measurements within three biological replicates. Error bars

show standard errors. B. Infection process of endophytic powdery mildew *Leveillula taurica* on surface of pepper leaves. 1) a conidium (c) germinated and a primary adhesion body (pab) formed at the tip of germ tube. 2) primary (infection) hyphae (ih) growing into the stomata (s) and secondary adhesion bodies (sab) formed on the secondary hyphae (sh). 3) The pathogen growing in the intercellular space and haustria (h) formed in mesophyll cells. 4) Conidiophores (cp) projected from stomata three weeks after inoculation and superficial hyphae (sh) elongating on both sides of the leaves for a new round of infection. Abbreviations: c, conidium; pab, primary adhesion body; ih, infection hyphae; h, haustorium; sab, secondary adhesion body; sh, secondary hyphae; cp, conidiophore; s

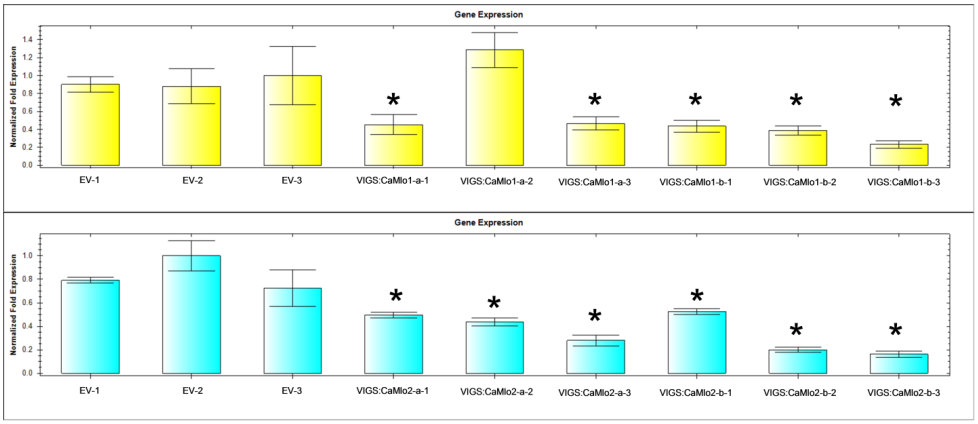


Fig. 6. Quantification of target cDNA in *CaMlo*-silenced and empty-vectors silenced pepper plants (Cultivar Maor is used as example, upper panel: *CaMlo1* silenced plants and lower panel: *CaMlo2* silenced plants). Results were normalized by ΔΔCt method with *CaActin* as the reference gene. tRNA were isolated before *Leveillula taurica* inoculation from pooled 4th, 5th and 6th infected leaves per plant. In total, three plants per VIGS vector were tested to check the silencing effect before fungal inoculation. Normalized fold expression shows the amount of target cDNA in the tested leaves. One of the EV control plants is set as control. (*) indicated significant difference ($P < 0.05$) from control EV plants.

Silencing of *CaMlo* genes in pepper by VIGS

In order to evaluate the potential role of the *CaMlo* genes in conferring powdery mildew susceptibility in pepper, we performed functional analysis via VIGS. In total, four constructs were generated for the *CaMlo1* and *CaMlo2* gene (two constructs per gene). The TRV2 empty vector (EV) was used as control. All pepper plants infiltrated with EV constructs showed symptoms indicating that the TRV-based VIGS system does not influence the susceptibility of pepper to *L. taurica.* Before fungal inoculation, expressions of the targeted genes were verified via qRT-PCR and results indicated that the target genes were silenced in most of the *CaMlo1* and *CaMlo2*-silenced plants (Fig. 6). A significant decrease in visible fungal colonies was observed on *CaMlo1* and *CaMlo2*-silenced plants (Fig. 7). Via

qRT-PCR, we measured the biomass of *L. taurica* in the infected leaves. The *CaMlo1* and *CaMlo2*-silenced plants showed decreased amounts of fungal biomass compared with EV control plants (Fig. 7). The strongest reduction was observed in the *CaMlo2*-silenced plants, showing that the newly isolated *CaMlo2* gene is likely the pepper ortholog of *SlMlo1* and *AtMlo2*. Two independent VIGS assays were performed for *CaMlo2* and three for *CaMlo1,* with similar results. It is worth to note that, in the VIGS experiments, eight out of 10 *CaMlo2*-silenced plants showed a remarkable decrease in size when compared with *CaMlo1*-silenced and EV control plants (Fig. 8).

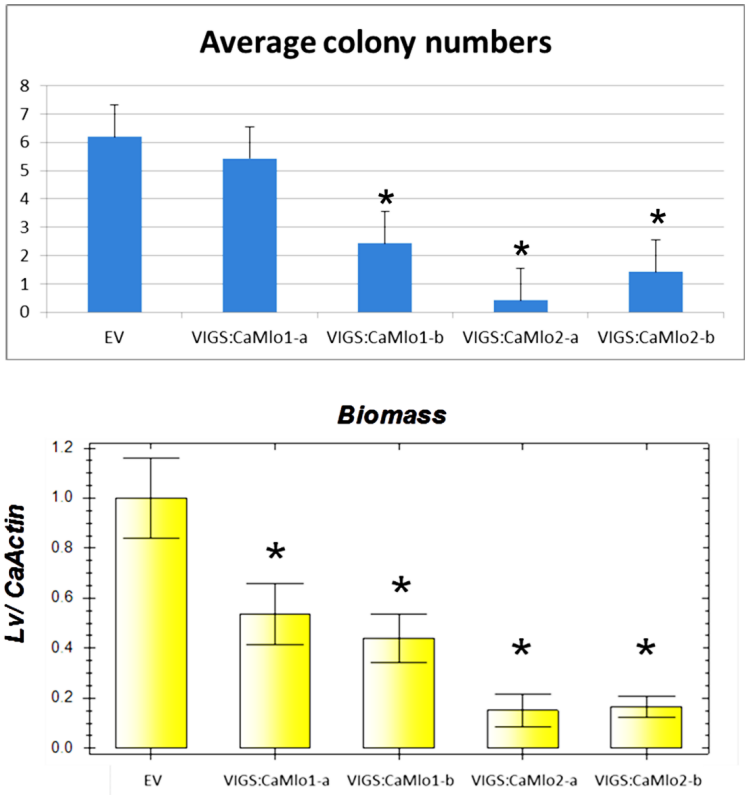


Fig. 7. Upper panel: *Leveillula taurica* colony numbers on pepper plants infiltrated with *CaMlo*-silencing (VIGS:CaMlo) and empty (EV) vectors. Cultivar Maor was used and colony numbers were counted on the 4th, 5th and 6th leaves. In total, seven plants per VIGS vector were scored and mean values are presented. Error bars indicate the standard deviation. The difference between VIGS vectors and EV control was compared by two-sample t-test. Except for the vector CaMlo1-a, all silenced plants showed significant difference ($P < 0.05$) (*) from EV. Lower panel: Quantification of *Leveillula taurica* DNA in pepper plants infiltrated with *CaMlo*-silencing and empty-vectors. Cultivar Maor is used. Results were normalized by ΔΔCt method with *CaActin* as the reference gene. DNA were isolated from pooled 4th, 5th and 6th infected leaves per plant. In total, seven biological replicates (silenced pepper plants) were tested for each VIGS vector. Normalized fold expression shows the amount of *L.taurica* DNA in the tested leaves. The value of EV control plants is set as 1. All fungal biomass contained in silenced plants showed significant difference ($P < 0.05$) (*) from EV plants.

Fig. 8. Plants in which the *CaMlo2* gene is silenced (left) show obvious size decrease compared with plants where *CaMlo1* is silenced (right).

DISCUSSION

Previous studies documented that *Mlo* homologues are present in several plant species (e.g barley, *Arabidopsis*, rice, maize, wheat and grapevine) and mutations in certain *Mlo* homologues (e.g. barley, *Arabidopsis*, pea and tomato) result in resistance to different powdery mildew species (Consonni et al., 2006; Devoto et al., 2003; Feechan et al., 2008; Liu and Zhu, 2008; Sasanuma et al., 2010). In this study, we showed that a loss-of-function mutation in the tomato *SlMlo1* gene reduces susceptibility of tomato to *L. taurica*. In pepper, we demonstrated that there are at least two *CaMlo* homologs present and responsive to the infection of *L. taurica,* and that silencing either *CaMlo1* or *CaMlo2* reduces the susceptibility of pepper to *L. taurica*.

Studies in tomato, barley and *Arabidopsis* have documented that mildew-effective *Mlo* gene(s) respond to fungal penetration at a very early time point (Bai et al., 2008; Buschges et al., 1997; Consonni et al., 2006; Humphry et al., 2011; Jorgensen, 1992; Piffanelli et al., 2002). Though both *CaMlo1* and *CaMlo2* transcripts accumulated during *L. taurica* infection, *CaMlo2* transcripts showed a significant up-regulation at the time point of 5 hpi (Fig. 4 and Fig. 5), corresponding to the stage of first adhesion body formation (supplementary video). Our results are in agreement with the observation that the barley *Mlo* transcripts increased at an early time point (6 hpi) (Piffanelli et al., 2002). Via VIGS, we demonstrated that silencing of both *CaMlo* genes caused a significant decrease of pepper susceptibility to *L.*

taurica, indicating that both of them are required for *L. taurica* infection (Fig. 7).

In barley, pea and tomato, the mutation of a single *Mlo* gene suffices to confer full resistance against the adapted powdery mildews, *B. graminis* f. sp. *hordei, E. pisi* and *O. neolycopersici*, respectively. In *Arabidopsis*, full penetration resistance to the adapted powdery mildew species *G. cichoracearum* and *G. orontii* is observed upon simultaneous loss-of-function of three *Mlo* homologues (*AtMlo2, AtMlo6* and *AtMlo12*), whereas *AtMlo2* single mutants are characterized by a dramatic reduction of fungal penetration and a very low level of fungal sporulation (Consonni et al., 2006). Since *CaMlo2* is more homologous to *SlMlo1* than the *CaMlo1* gene, it is very likely that the *CaMlo2* represents *AtMlo2*, while *CaMlo1* represents *AtMlo6* or *AtMlo12* of the *Arabidopsis Mlo* family.

There are several reports showing that phylogenetically related MLO members are involved in controlling one trait. For example, the *Arabidopsis* mildew-effective *AtMlo2*, *AtMlo6* and *AtMlo12* genes (Consonni et al., 2006) and root development-related *AtMlo4* and *AtMlo11* genes (Chen et al., 2009). Previously, we have demonstrated in tomato that *SlMlo1* is required for successful infection of *O. neolycopersici*. In this study, we showed that *SlMlo1* is also required for *L. taurica* infection in tomato (Fig. 1) and loss-of-function of the *SlMlo1* gene could confer resistance against *L. taurica*. However, mutation in the *SlMlo1* gene could not confer full resistance against *L. taurica.* In tomato, many *Mlo*-like genes have been identified and probably several tomato *Mlo* genes are involved in the interaction with *L. taurica.* The difference between these two powdery mildew species is that *L. taurica* penetrates the plants from the stomata, while *O. neolycopersici* penetrates from epidermal cells. Our data on gene expression and fungal infection process suggest that the *CaMlo2* gene is involved in the early penetration stage of *L. taurica* and the *CaMlo1* gene is mainly involved in the intracellular fungal growing stage. Likely, for full resistance to *L. taurica* in pepper and tomato, mutant alleles of more than one *Mlo* homologue are needed. Recently, the tomato genome sequence has become available from The Tomato Genome Consortium (http://solgenomics.net/organism/Solanum_lycopersicum/genome) (2012). Together with the already known *Mlo* data, this source provides us the chance to explore the *Mlo* family in tomato. Future work is to investigate the functional analysis of mildew-effective *SlMlo* paralogs in the interaction of tomato and *L. taurica*. Since pepper is recalcitrant to transformation, we are transforming the *CaMlo2* gene into the tomato *mlo*

mutant in order to obtain complementation. Meanwhile, we are carrying out an allele mining approach to search for natural mutations in *CaMlo* genes using publicly available germplasms.

In general, resistances against plant diseases are achieved by introduction of *R*-genes into susceptible plant genotypes. With the recently proposed new *S*-genes breeding strategy, resistance could be achieved by impairment of susceptibility factors that evolved in the disease development (Eckardt, 2002; Pavan et al., 2010). A sound example of using *S*-genes to acquire resistance is the *Mlo* gene mutation used in the European barley cultivars. Our results strongly indicate that the *CaMlo* genes could be a valuable *S*-gene resource for pepper breeding against *L. taurica*. However, pleiotropic effects of the achieved resistance as observed when silencing *CaMlo2* should also be considered for the application of mutant alleles of *S*-genes (Fig. 8).

During the preparation of this manuscript, another study was released online showing the isolation of the *CaMlo2* gene and demonstrating that the *CaMlo2* is involved in the processes of cell death in pepper (Kim and Hwang, 2012). Since the newly isolated *CaMlo2* sequence (JN896629) from Kim and Hwang (2012) has not yet been released from NCBI, we performed the sequence alignment of the two *CaMlo2* coding sequence (CDS) manually. Several single nucleotide polymorphisms (SNPs) and one small insertion between the two *CaMlo2* sequences (Supplemental Fig. 1) were uncovered. With phylogenetic analysis, the *CaMlo2* isolated by Kim and Hwang (2012) is also grouped in the same clade (data not shown) as the *CaMlo2* gene in this study (Fig. 2), showing that the two *CaMlo2* sequences are alleles of the same pepper *Mlo* ortholog. The results of Kim and Hwang (2012) are in agreement with ours demonstrating that CaMLO2 is a negative regulator of pepper defense response. Together, the data support the conclusion that silencing of the *CaMlo2* gene in pepper lead resistance to *L. taurica* and *Xanthomonas campestris* pv. *Vesicatoria*.

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Supplementary Table 1. Primer pairs used to prepare VIGS constructs

Primer name	**Target size**	**Fw primer sequence (5'--3')**	**Rev primer sequence (5'--3')**
VIGS:CaMlo1-a	493 bp*	CGGTTGCCGTAGTTTGTTTT	TGGAATGAATGGTAGCCACA
VIGS:CaMlo1-b	667 bp**	TGTGGCTACCATTCATTCCA	GGCTCGTGACCTTCTGAGTC
VIGS:CaMlo2-a	202 bp	caccTGTCCCCAAAAGTGTTGGTT	TAACCTGCCCAAAGCAAAAG
VIGS:CaMlo2-b	239 bp	caccCGTGGGAATAAGTCCAGCAT	CAGGGCGATTAAACCAGAAA

* The PCR fragments of *CaMlo1-a* should be 896 bp. Due to the fact that the amplified PCR product contains an *Eco*RI enzyme digestion site at 403 bp, the target fragment in the pTRV2 vector of *VIGS:CaMlo1-a* is 493bp.

** The PCR fragments of *CaMlo1-b* should be 748 bp. Due to the fact that the amplified sequence contains an *Eco*RI enzyme digestion site at 81 bp, the target fragment in the pTRV2 vector of *VIGS:CaMlo1-b* is 667 bp.

Supplemental Figure 1. Sequence alignment of the two newly isolated *CaMlo2* genes. The alignment was generated by CLUSTALW using default parameters. *CaMlo2*_WUR indicates the CDS sequence isolated from this study and the *CaMlo2*_Kr indicates the CDS identified in the study of Kim and Hwang (2012) (sequence were manually input according their Figure S1).

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CaMlo2_WUR      ATGGAGGCAACCCCTACGTGGGCGGTTGCCGTGGTTTGCTTCATCTTGCTGGCTATTTCC 60
CaMlo2_Kr       ATGGAGGCAACCCCTACGTGGGCGGTTGCCGTGGTTTGCTTCATCTTGCTGGCTATTTCC 60
                ************************************************************

CaMlo2_WUR      ATTTGTATTGAACAAATTATGCATCACCTTGGAGAGTGGTTGTTGAAAAAACACAAAAAG 120
CaMlo2_Kr       ATTTTTATTGAACAAATTATGCATCACCTTGGAGAGTGGTTGTTGAAAAAACACAAAAAG 120
                **** ********************************************************

CaMlo2_WUR      CCTCTATACGAAGCACTTGAAAAGATCAAAGCAGAGCTTATGTTGTTGGGATTC-CGGCA 179
CaMlo2_Kr       CCTCTATACGAAGCACTTGAAAAGATCAAAGCAGAGCTTATGTTGTTGGGATTCATATCA 180
                ***************************************************     **

CaMlo2_WUR      CGG--GTTGACAGTGATACAAGACCCAGTTTCTAACTTATGTGTCCCCAAAAGTGTTGGT 237
CaMlo2_Kr       TTGTTGTTGACAGTGATACAAGACCCAGTTTCTAACTTATGTGTCCCCAAAAGTGTTGGT 240
                  *  ********************************************************

CaMlo2_WUR      TATTCCTGGCATCCTTGTAAGGCAGATGAAGATGTCAAGTCTGAGTATGATGACCCTTGT 297
CaMlo2_Kr       TATTCCTGGCATCCTTGTAAGGCAGATGAAGATGTCAAGTCTGAGTATGATGACCCTTGT 300
                ************************************************************

CaMlo2_WUR      TTACAAAAGGGAAAAGTTCAATTTGCATCTTCATATGCAATACACCAGCTCCATATCTTC 357
CaMlo2_Kr       TTACAAAAGGGAAAAGTTCAATTTGCATCTTCATATGCAATACACCAGCTCCATATCTTC 360
                ************************************************************

CaMlo2_WUR      ATCTTTGTGTTGGCAATTGCGCATGTTTTGTACTGTATAGCAACTTTTGCTTTGGGCAGG 417
CaMlo2_Kr       ATCTTTGTGTTGGCAATTGCGCATGTTTTGTACTGTATAGCAACTTTTGCTTTGGGGAGG 420
                ************************************************************ ***

CaMlo2_WUR      TTAAAGATGAGAAAATGGAGGGCCTGGGAGGATGAAACAAAAACAATTGAGTACCAATTC 477
CaMlo2_Kr       TTAAAGATGAGAAAATGGAGGGCCTGGGAGGATGAAACAAAAACAATTGAGTACCAATTC 480
                ************************************************************

CaMlo2_WUR      TATAACGATCCTGAGAGGTTTAGGTTTGCAAGGGAGACCTCATTTGGACGTAGGCATATG 537
CaMlo2_Kr       TATAACGACCCTGAGAGGTTTAGGTTTGCAAGGGAGACCTCATTTGGACGTAGGCATATG 540
                ******** ***************************************************

CaMlo2_WUR      CATTTTTGGAGCAAGTCGCCGGTGATGCTCTGGATAGTTTGTTTCTTCAGGCAATTCTTT 597
CaMlo2_Kr       CATTTTTGGAGCAAGTCGCCGGTGATGCTCTGGATAGTTTGTTTCTTCAGGCAATTCTTT 600
                ************************************************************

CaMlo2_WUR      TCATCAGTAGCAAAAGTTGACTATTTAACCCTTAGACATGGGTTCATGATGGCACATTTA 657
CaMlo2_Kr       TCATCAGTAGCAAAAGTTGACTATTTAACCCTTAGACATGGGTTCATGATGGCACATTTA 660
                ************************************************************

CaMlo2_WUR      ACACCACAGAATCAAGAGAACTTTGATTTTCAAATATACATTAATAGAGCAGTTGACAAA 717
CaMlo2_Kr       ACACCACAGAATCAAGAGAACTTTGATTTTCAAATATACATTAATAGAGCAGTTGACAAA 720
                ************************************************************

CaMlo2_WUR      GATTTCAAAGTTGTCGTGGGAATAAGTCCAGCATTATGGCTCTTCACGGTATTATATTTT 777
CaMlo2_Kr       GATTTCAAAGTTGTCGTGGGAATAAGTCCAGCATTATGGCTCTTCACGGTATTATATTTT 780
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CaMlo2_WUR      CTATCCACCACCGATGGAGTTTACTCGTATCTTTGGGTTCCATTTGTCCCACTCATTATA 837
CaMlo2_Kr       CTATCCACCACCGATGGAGTTTACTCGTATCTTTGGGTTCCATTTGTCCCACTCATTATA 840
                ************************************************************
CaMlo2_WUR      ATATTGTTGGTTGGGACAAAACTTCAAATGATCATAACAGAAATGGGGGTTAGAATTTCA 897
CaMlo2_Kr       ATATTGTTGGTTGGGACAAAACTTCAAATGATCATAACAGAAATGGGGGTTAGAATTTCA 900
                ************************************************************
CaMlo2_WUR      GAAAGGGGAGACATAGTGAAAGGTGTACCAATGGTGGAGATCGGTGACCATCTTTTCTGG 957
CaMlo2_Kr       GAAAGGGGAGACATAGTGAAAGGTGTACCAGTGGTGGAGATCGGTGACCATCTTTTCTGG 960
                ******************************* ****************************
CaMlo2_WUR      TTTAATCGCCCTGGCCTTGTGCTTTTCTTCATTAACTTTGTCCTCTTTCAGAATGCGTTT 1017
CaMlo2_Kr       TTTAATCGCCCTGGCCTTGTGCTTTTCTTCATTAACTTTGTCCTCTTTCAGAATGCGTTT 1020
                ************************************************************
CaMlo2_WUR      CAAGTTGCTTTCTTTGTTTGGAGTTGGTGGAAATTTGGTTTTCCATCCTGCTTTCATAGA 1077
CaMlo2_Kr       CAAGTTGCTTTCTTTGTTTGGAGTTGGTGGAAATTTGGTTTTCCATCCTGCTTTCATAGA 1080
                ************************************************************
CaMlo2_WUR      AATGCTGCAGACCTAGCCATTAGGCTAACCATGGGAGTAATCATACAAGTCCATTGCAGC 1137
CaMlo2_Kr       AATGCTGCAGACCTAGCCATTAGGCTAACCATGGGAGTAATCATACAAGTCCATTGCAGC 1140
                ************************************************************
CaMlo2_WUR      TATGTAACTCTCCCTCTATATGCCTTAGTTACTCAGATGGGTTCATCAATGAAGCCTATC 1197
CaMlo2_Kr       TATGTAACTCTCCCTCTATATGCCTTAGTTACTCAGATGGGTTCATCAATGAAGCCTATC 1200
                ************************************************************
CaMlo2_WUR      ATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGGCACAATACAGCGAAAAAGCAG 1257
CaMlo2_Kr       ATCTTTGGTGATAATGTGGCAACAGCTCTTAGAAGCTGGCACAATACAGCGAAAAAGCGG 1260
                ************************************************************ *
CaMlo2_WUR      GTGAGACATGGGCGGGTATCAGAAAACACCACTCCGATATCTAGCAGACCGGCCACACCA 1317
CaMlo2_Kr       GTGAGACATGGGCGGGTATCAGAAAACACCACTCCGATATCTAGCAGACCGGCCACACCA 1320
                ************************************************************
CaMlo2_WUR      TTGCGTGGTACCTCCCCAGTTCACTTGCTACGTGGCTACCCAAAATATAACGAGGACAAT 1377
CaMlo2_Kr       TTGCGTGGTACCTCCCCAGTTCACTTGCTACGTGGCTACCCAAAATATAACGAGGACAAT 1380
                ************************************************************
CaMlo2_WUR      GTTCAAGCATATCCTCGAACATCGAATGTAGAAAATGAAGGCTGGGCTAATGAAACATCC 1437
CaMlo2_Kr       GTTCAAGCATATCCTCGAACATCGAATGTAGAAAATGAAGGCTGGGCTAATGAAACATCC 1440
                ************************************************************
CaMlo2_WUR      ACTGAGAATAAAGATCATCAGGAGGAGGGACAAATCCTGCAGCATGCCTCCACTTCTATG 1497
CaMlo2_Kr       ACTGAGAATAAAGATCATCAGGAGGAGGGACAAATCCTGCAGCATGCCTCCACTTCTATG 1500
                ************************************************************
CaMlo2_WUR      CAACATCCGCATACTGATCAACATCAAATTGAGATTGCAATGTCAGATTTTACTTTTGGA 1557
CaMlo2_Kr       CAACATCCGCATACTGATCAACATCAAATTGAGATTGCAATGTCAGATTTTACTTTTGGA 1560
                ************************************************************
CaMlo2_WUR      AACAAATAG 1566
CaMlo2_Kr       AACAAATAG 1569
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CHAPTER 6

Involvement of *Pen* Genes and Salicylic Acid Signalling in *Arabidopsis* Basal and *mlo*-based Resistance to the Powdery Mildew Fungus *Oidium neolycopersici*

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Chapter 6

Involvement of Pen Genes and Salicylic Acid Signalling in Arabidopsis Basal and mlo-based Resistance to the Powdery Mildew Fungus Oidium neolycopersici

Zheng Zheng[1], Stefano Pavan[2], Robin Huibers[1], Zhe Yan[1], Richard Visser[1] and Yuling Bai[1,*]

[1] Wageningen UR Plant Breeding, Wageningen University & Research Centre, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

[2] Department of Agroforestry, Enviromental Biology and Chemistry, Section of Genetics and Plant Breeding, University of Bari, Via Amendola 165/A, 70126 Bari, Italy

*Corresponding author: Yuling Bai, Email: bai.yuling@wur.nl

To be submitted

CHAPTER 6

ABSTRACT

Oidium neolycopersici (*On*) is the powdery mildew fungal species naturally attacking tomato. However, under experimental conditions it can also accomplish a successful lifecycle on *Arabidopsis*. In this work we studied the interactions between several *Arabidopsis* mutants and *On*, in order to identify pathways involved in basal resistance, limiting pathogen spread on susceptible genotypes, and in resistant genotypes associated to the loss of function of members of the *AtMlo* gene family. Results obtained were in part overlapping with those obtained in previous investigations focusing on the interactions between *Arabidopsis* and the virulent powdery mildew fungi *Golovinomyces orontii* and *Golovinomyces cichoracearum*. One important peculiarity was found, as the impairment of the defense response mediated by the hormone salicylic acid compromised resistance associated to the loss of function of *Atmlo2*. The newly generated quadruple mutants *Atmlo2/6/12/pen1* and *Atmlo2/6/12/pen2* and the quintuple mutant *Atmlo2/6/12/pen1/pen2* were also assayed in this study following *On* challenge, in order to determine the role of *Pen* genes in full penetration resistance exhibited by *Atmlo2/6/12* mutants. Since all of these mutants are still immune to pathogen penetration, we demonstrate for the first time that *mlo*-based penetration resistance in *Arabidopsis* also relies on unknown defence mechanisms independent from *Pen* genes.

INTRODUCTION

Powdery mildew (PM), caused by obligate biotrophic fungi belonging to the ascomycete order of Erysiphales, is one of the most widespread plant diseases worldwide and causes significant losses in many crop species. The biological cycle of PM fungi includes germination of asexual (conidia) or sexual spores (ascospores), penetration into plant cells, development of colonies and spore production.

In order to establish pathogenesis, PM fungi have to circumvent plant defence layers acting before and after fungal penetration (Lipka et al., 2005). The *Arabidopsis* genes *Pen1, Pen2* and *Pen3*, respectively encoding a syntaxin, a glycosyl hydrolase and an ATP binding cassette (ABC) transporter, have been found to play an important role in pre-penetrative defence mechanisms (Lipka et al., 2005; Stein et al., 2006). PEN1 proteins, together with vesicle-associated membrane proteins (VAMP) VAMP721 or VAMP722 and with synaptosomal-associated protein 33 (SNAP33), form plasma membrane ternary complexes involved in the polarized secretion of a toxic cargo at fungal attempted penetration sites (Lipka et al., 2007). PEN2 and PEN3 proteins are likely to act synergistically in another defence pathway, in which PEN2 produces a toxic substrate which is then transported, directly or after further modification, to the apoplast through PEN3 (Stein et al., 2006).

Post-penetration defence mechanisms to PM fungi have been associated to the rise of salicylic acid (SA), which is known to activate defence signalling pathways affecting biotrophic pathogens. In contrast, jasmonic acid (JA) and ethylene (ET), two other plant hormones usually involved in responses to necrotrophic pathogens and herbivores, have been repeatedly shown to be dispensable for PM resistance (Glazebrook, 2005).

The *Mlo* gene family encodes for plant seven transmembrane domain calmodulin-binding proteins, topologically reminiscent of metazoan G-protein coupled receptors (GPCR) (Kim et al., 2002; Devoto et al., 2003). Specific *Mlo* homologs have been demonstrated to act as susceptibility factors for powdery mildew fungi, as their loss-of-function mutations result in recessively inherited disease resistance (*mlo* resistance) (Pavan et al., 2010). So far, *mlo* resistance has been reported to occur in barley, *Arabidopsis*, tomato and pea (Buschges et al., 1997; Consonni et al., 2006; Bai et al., 2008; Humphry et al., 2011; Pavan et al., 2011). The defence mechanism of *mlo* resistance is

pre-penetrative and associated to the formation of cell wall appositions (termed papillae) at the plant-pathogen interface (Thordal-Christensen et al., 1997; Huckelhoven et al., 2000; Bai et al., 2005). In barley, pea and tomato, the loss-of-function mutations of a single *Mlo* orthologue suffices to confer full resistance to the adapted PM species *Blumeria graminis* f. sp*. hordei* (*Bgh*)*, Erysiphe pisi* (*Ep*) and *Oidium neolycopersici* (*On*), respectively (Buschges et al., 1997; Consonni et al., 2006; Bai et al., 2008; Humphry et al., 2011; Pavan et al., 2011). In *Arabidopsis*, full penetration resistance to the adapted PM species *Golovinomyces cichoracearum* (*Gc*) and *Golovinomyces orontii* (*Go*) requires the simultaneous loss-of-function of three *Mlo* co-orthologues (*AtMlo2, AtMlo6 and AtMlo12*), whereas *Atmlo2* single mutants are characterized by a dramatic reduction of fungal penetration and a macroscopically resistant phenotype (Consonni et al., 2006).

Some studies have indicated that functional MLO proteins may be involved in the negative regulation of PEN1- and PEN2/PEN3-dependent penetration resistance pathways at the cell periphery (Keinath et al., 2010). Indeed, the penetration ratio of *Gc* and *Go* is significantly increased in *Arabidopsis Atmlo2* mutants when *Pen* genes are mutated (Consonni et al., 2006). Furthermore, the loss of function of the *Pen1* ortholog *Ror2* in barley breaks *mlo* resistance (Collins et al., 2003). The MLO-based regulation of PEN1 is supported by experiments with MLO-YFP fusion proteins, showing physical interaction between MLO and PEN1 orthologues in both barley and *Arabidopsis* (Schulze-Lefert, 2004; Bhat et al., 2005; Panstruga, 2005).

Analyses using double mutants have shown that *Arabidopsis Atmlo2* resistance to *Go* and *Gc* and barley *mlo* resistance to *Bgh* are largely independent from functional SA, JA and ET signalling pathways (Huckelhoven et al., 1999; Consonni et al., 2006)..

On is a PM species causing disease on tomato (Jones et al., 2001). Though it can also grow on several other plant species, including *Arabidopsis* (Xiao et al., 2001; Xiao et al., 2003), there is so far no report of natural *On* infection on other species than tomato (Jankovics et al., 2008). In previous studies, we optimized the inoculation procedure required for *On* to accomplish a successful lifecycle on the *Arabidopsis* ecotype Col-0. Moreover, we found that mutations of *Arabidopsis Atmlo2*, *Atmlo6* and *Atmlo12* genes affect the interaction with *On* similarly to the interactions between *Arabidopsis* and the PM species *Gc* and *Go* (Bai et al., 2008). Here, we aimed to identify defence pathways required

for basal and *mlo*-based *Arabidopsis* resistance to *On*. Importantly, we demonstrate that *mlo* resistance requires the correct functioning of SA signaling as well as PEN-dependent and -independent pre-penetrative defence mechanisms

MATERIALS AND METHODS

Plant material

The *Arabidopsis* ecotype Col-0 and the loss-of-function mutant lines *pen1, pen2, pen3, Atmlo2/pen1, Atmlo2/pen2, Atmlo2/pen3, Atmlo2*/*ein2, Atmlo2*/*jar1*, *Atmlo2*/*eds5, Atmlo2*/*npr1*, *Atmlo2*/*pad4*, *Atmlo2*/*sid2*, *Atmlo2/NahG* and *Atmlo2/6/12,* previously used by Consonni et al. (2006) to study the interaction between *Arabidopsis* and the powdery mildew pathogens *Gc* and *Go,* were used (Table1). In addition, the single mutants *eds5, npr1, pad4* and the newly generated *Atmlo2/6/12/pen1* and *Atmlo2/6/12/pen2* quadruple mutants and *Atmlo2/6/12/pen1/pen2* quintuple mutant were also included in this study, which were kindly provided by Dr. Ralph Panstruga, (Max-Planck-Institut für Züchtungsforschung, Department of Plant Microbe Interactions, Köln, Germany).

Disease assays

The Wageningen *On* isolate was used in this study (Bai et al., 2005). Inoculation was carried out on four weeks-old plants by spraying a suspension of conidia at the concentration of $1.25x10^5$ conidia/ml. Inoculated plants were grown in a greenhouse compartment at 20±2°C and 70±15% relative humidity, under short day conditions. Disease symptoms were evaluated according to a Disease Index (DI) described by Bai et al. (2008) ranging from 0 to 4 according to the leaf area covered with fungal colonies: 0 = no visible fungal sporulation, 1 = a few fungal colonies, 2 = up to 30% of the leaf area covered with fungal colonies, and 3 = more than 30% of the leaf area covered with fungal colonies. 4 = more than 50% of the leaf area covered with fungal colonies. In addition, disease severity on Col-0, *Atmlo2*, *pen1*, *pen2*, *pen3*, *Atmlo2/pen1*, *Atmlo2/pen2*, *Atmlo2/pen3, Atmlo2/6/12/pen1, Atmlo2/6/12/pen2* and *Atmlo2/6/12/pen1/pen2* genotypes was also estimated by real-time qPCR quantification of *On* biomass, at 15 days post inoculation. Plant and fungal DNA was extracted using the DNeasy DNA extraction kit (Qiagen,

Germany). The following *On*-specific primer pair, F(CGCCAAAGACCTAACCAAAA) and R(AGCCAAGAGATCCGTTGTTG), was designed based on the internal transcribed spacer (ITS) sequences (GenBank accession number EU047564.1). The primer pair F(CATACATACAATTCACTAACCAAAA) and R(TGGATCTCCTTAATAGTTTAAAAGG), was used to detect and quantify *Arabidopsis* DNA.

Quantification of fungal penetration rate

Two days after *On* inoculation, fungal structures on leaf epidermal cells were stained with trypan blue and visualized under the optical microscope for multiple biological replicates, Penetration success rate was inferred by the proportion of germinated spores producing secondary hyphae. For each genotype tested, more than 100 infection units (one infection unit representing a geminated *On* spore) were examined.

RESULTS

Requirement of *Pen* genes in *Arabidopsis* basal defense to *On*

To study the involvement of PEN-dependent pathways in *Arabidopsis* basal defense to *On*, we first examined *On* entry rates on the susceptible genotype Col-0 and its respective mutants impaired for any of the *Pen* genes. Plants of Col-0 and those of *pen1* and *pen3* mutants showed a comparable *On* penetration rate, approximately 70% of the germinated spores. In contrast, plants of the *pen2* mutant demonstrated a significant higher penetration rate of about 90% (Fig. 1), suggesting that PEN2-dependent mechanisms restrict penetration of *On* and contribute to basal defense of *Arabidopsis* to *On*. We next quantified the amount of disease at 15 days post inoculation on the same genotypes. In line with the penetration levels inferred by histological analyses, plants of the *pen2* and *pen3* mutants exhibited, respectively, a higher and similar disease index compared to Col-0 plants. Although the pathogen entry rate was comparable between Col-0 and *pen1* plants, fungal colonization was much lower on the latter (Table 1). Results of disease index-based scoring were confirmed by quantifying relative fungal/plant DNA ratios by real-time qPCR (Table 1 and Fig. 2). Together the results indicate that *pen1* limits post-entry growth of *On* in *Arabidopsis*.

Table 1 Disease index (DI) of tested mutants. DI is ranging from 0 to 4 according to the percentage of the leaf area covered with fungal colonies. SD are the standard deviations of the observations made for each genotype

Genotype	Disease index	
	Average	SD
Col-0	2.03	0.39
Atmlo2	0.46	0.24
Atmlo2/6/12	0	0
pen1	1.5	0.41
pen2	2.9	0.39
pen3	2	0.43
eds5	2.43	0.33
npr1	2.5	0.55
pad4	3.39	0.29
Atmlo2/NahG	1.89	0.4
Atmlo2/eds5	1.57	0.61
Atmlo2/pad4	2.07	0.26
Atmlo2/jar1	0.46	0.13
Atmlo2/ein2	0.5	0.2
Atmlo2/npr1	1.04	0.38
Atmlo2/sid2	1.64	0.49
Atmlo2/pen1	0.15	0.24
Atmlo2/pen2	2.06	0.52
Atmlo2/pen3	0.06	0.17
Atmlo2/6/12/pen1	0	0
Atmlo2/6/12/pen2	0	0
Atmlo2/6/12/pen1/pen2	0	0

Pen-genes are required for *mlo*-based resistance to *On*

In order to verify whether *Pen* genes play a role in *Atmlo2*-based resistance to *On,* fungal penetration and colonization rates were quantified using the *Atmlo2* single mutant and the double mutants *Atmlo2/pen1, Atmlo2/pen2* and *Atmlo2/pen3*. Compared to *Atmlo2*, plants of all double mutants showed an increased *On* penetration rate with plants of the *Atmlo2/pen2* mutant displaying the highest level of pathogen entry (Fig. 1). Clearly, this shows that mutations in all three *Pen* genes compromise *On* penetration resistance mediated by *Atmlo2*. Macroscopically, only *Atmlo2*/*pen2* plants showed significantly higher disease symptoms (Table 1). *On* colonization on *Atmlo2/pen1* and *Atmlo2/pen3* mutants was even less compared to *Atmlo2* plants, suggesting that *pen1* and *pen3* enhance post-entry defence mechanisms restricting *On* growth.

Since *On* resistance in the *Atmlo2* genotype is partial, we next aimed to verify whether any of the *Pen* genes could influence *Arabidopsis mlo*-based complete resistance, as shown in the *Atmlo2/6/12* triple mutant (Consonni et al., 2006). The quadruple mutants *Atmlo2/6/12/pen1* and *Atmlo2/6/12/pen2* and the quintuple mutant *Atmlo2/6/12/pen1/pen2* were tested, together with *Atmlo2/6/12* plants, for their responses to *On.* No *On* penetration was observed for any of the genotypes tested (Fig. 1) and consequently, no disease symptoms could be observed. This indicates that a PEN-independent defense mechanism restricts penetration of *On* in *Atmlo2/6/12*.

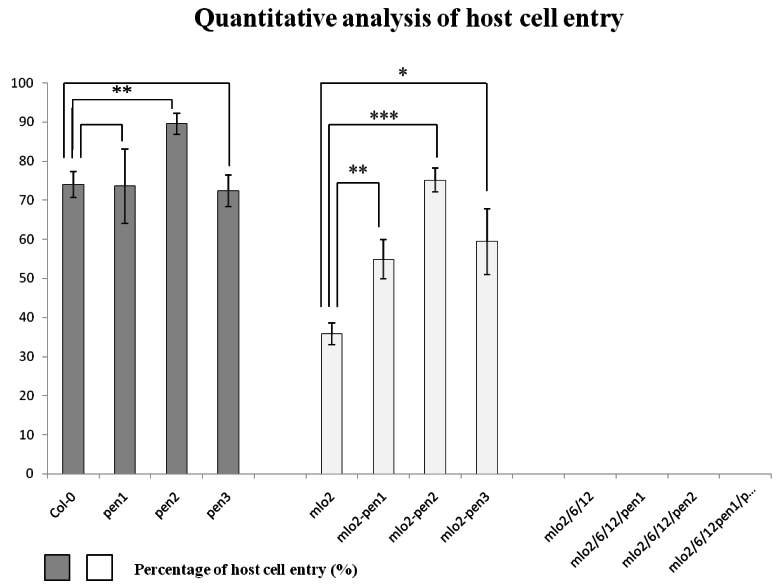

Fig.1. Quantitative analysis of *Oidium neolycopersici* penetration rate in epidermal cells of the Arabidosis ecotype Col-0 and several *Arabidopsis* mutants 48 h after inoculation. The penetration rate was inferred by histological observations, based on the relative amount of germinated spores differentiating secondary hyphae. Bars represent standard deviations based on three biological replicates. Asterisks point to pairwise significant differences based on Student's t-test: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

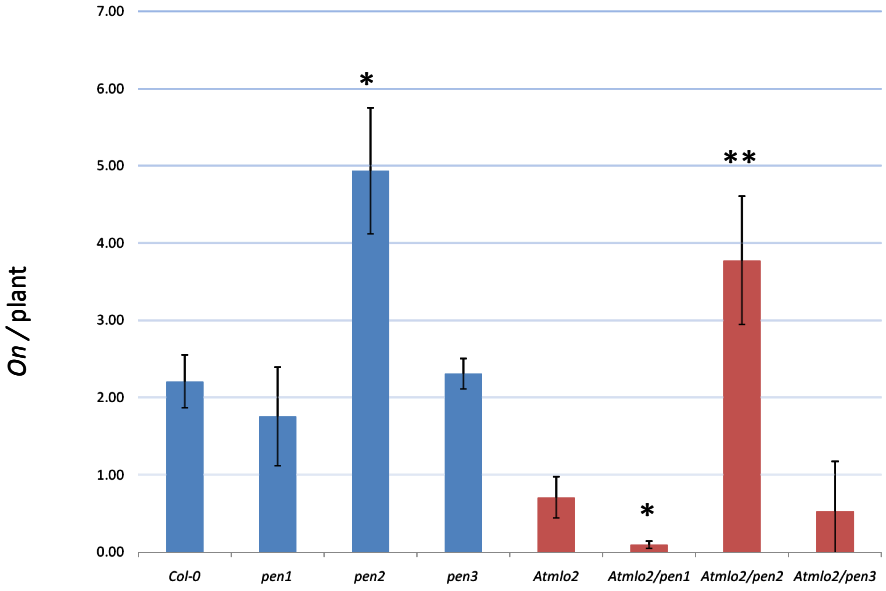

Fig. 2. Quantitative analysis of *Oidium neolycopersici* growth on leaves of the *Arabidopsis* wild-type genotype Col-0 and several *Arabidopsis* mutants. Samples were collected 15 days after artificial inoculation. Fungal biomass was quantified by qPCR, using a primer pair specific for an *Arabidopsis* for normalization. Bars represent standard deviations from three biological replicates. Asterisks on the blue bars represent pairwise significant differences from the genotype Col-0; asterisks on the red bars represent significant differences from the mutant *Atmlo2*. * = $p < 0.05$; **= $p < 0.01$

Hormonal signaling pathways in basal defense and *Atmlo2*-mediated resistance to *On*

To investigate the role of hormonal signalling pathways on basal resistance of *Arabidopsis* to *On,* we first compared *On* growth in Col-0 and the single mutants *npr1*, *eds5* and *pad4*, which are impaired for key steps of the SA-mediated defence signalling pathway. All of these mutants displayed increased disease symptoms compared to Col-0 at 15 dpi (DI: 2.5, 2.43 and 3.39, respectively (Table 1), indicating a major role for SA signalling in limiting *On* colonization.

We next investigated whether impairment of any of the SA, JA or ET signalling pathways could interfere with resistance to *On* mediated by *Atmlo2*. Double mutants *Atmlo2*/*eds5*, *Atmlo2/npr1, Atmlo2/pad4* and *Atmlo2/sid2*, as well as *Atmlo2* mutants overexpressing the salicylate hydroxylase gene *NahG*, showed a dramatic increase in disease symptoms at 15 dpi compared to Col-0 plants, indicating that *Atmlo2* mediated

resistance requires a functional SA pathway (Table 1 and Fig. 3). In contrast, impairment of the JA and ET signalling pathways in *Atmlo2* (*Atmlo2*/*jar1* and *Atmlo2*/*ein2*) did not result in increased disease symptoms, suggesting that *Atmlo2* mediated resistance does not require functional JA nor ET signalling pathways (Table 1).

Fig. 3. Phenotypic reponse to *Oidium neolycopersici* of the *Arabidopsis* mutants *Atmlo2*, *Atmlo2*/*eds5*, *Atmlo2/npr1*, *Atmlo2/pad4* and *Atmlo2/sid2,* and the *Arabidopsis* line *Atmlo2/NahG,* overexpressing the salicylate hydroxylase gene in the *Atmlo2* genetic background. Photos were taken 15 days after artificial inoculation with the pathogen

DISCUSSION

In this study, we assessed the role of different defence pathways in basal and *mlo*-mediated resistance in *Arabidopsis* to the tomato powdery mildew fungus *On*. For the basal defence, we found a positive contribution of *Pen2*, since fungal penetration and colonization were significantly increased in *pen2* compared to the wild-type. *Pen1* likely functions in restricting post-entry growth of *On* in *Arabidopsis*, since *Pen1* plants had a significant lower disease index than Col-0 plants (Table 1), albeit a similar penetration rate (Fig. 1). For the *mlo*-based resistance in *Atmlo2* plants, our results show that penetration defence mechanisms associated to this form of immunity rely on all the three *Pen* genes, since *Atmlo2/pen1, Atmlo2/pen2* and *Atmlo2/pen3* double mutants are characterized by a dramatic increase of fungal penetration with respect to *Atmlo2* mutantsThese results are largely overlapping with those showed by Consonni et al. (2006), who studied the response

of the same set of genotypes to *Gc* and *Go*. In both Col-0 and *Atmlo2* genetic backgrounds, *pen2* plants showed higher *On* penetration rates compared to *pen3* plants. This suggests that the hydrolytic activity of PEN2 is not only coupled to the PEN3-mediated transport across the membrane, but might be also involved in additional defence responses. In agreement with our data, recent findings (Hiruma et al., 2010) suggest that a PEN2-dependent and PEN3-independent mechanism terminates entry attempts of anthracnose pathogens.

Notably, the quadruple mutants *Atmlo2/6/12/pen1* and *Atmlo2/6/12/pen2* and the quintuple mutant *Atmlo2/6/12/pen1/pen2* mutant still resulted in immunity to *On*. This clearly indicates that, in these genotypes, *PEN1*- and *PEN2/PEN3*-independent defence responses suffice to completely prevent fungal entry. Conversely, complete penetration resistance observed in *mlo* resistance of barley is broken by mutation of *Pen* genes (Peterhansel et al., 1997). Therefore, our findings suggest that *PEN*-independent pre-penetrative defence in *mlo* resistance might be a prerogative of *Arabidopsis*. Evidence for an *Arabidopsis*-specific defence pathway involved in *mlo*-resistance comes from the findings by Consonni et al. (2010), showing that the impairment of the biosynthesis of indolic metabolites such as camalexin, which only occurs in the taxonomic order of Brassicales, breaks *Atmlo2* resistance to *Go*.

Penetration resistance to powdery mildew fungi is conferred by localized cell wall apposition, termed papilla formation which relies on timely reorganization of pre-synthesised membrane material. A very recent finding (Nielsen et al., 2012) showed that the secretion of membrane material does not require PEN1 function. Rather, the focal accumulation of syntaxin PEN1 requires a BFA-sensitive ADP ribosylation factor-GTP exchange factor (ARF-GEF), GNOM. Mutations in PEN1 delay papillae formation, but do not result in a maximum penetration level. Rather, full penetration resistance in *Arabidopsis* to powdery mildew *Bgh* relies on the correct function of GNOM.

Evidence that *On* colonization on the *pen1* mutant is lower than the one on its background genotype Col-0, and the one on *Atmlo2/pen1* and *Atmlo2/pen3* mutants is lower than the one on *Atmlo2* mutants, indicate that PEN1 and PEN3 act as negative regulators of post-penetration defence mechanisms limiting *On* colonization. These results can be explained by the findings of Zhang et al. (2007) and Stein et al. (2006) that PEN1

and PEN3 are negative regulators of the SA signalling pathway, thus establishing a negative cross-talk between pre- and post-penetrative defence. In addition, the PEN3 mutation is characterized by extensive chlorosis, presumably due to the accumulation of a toxic compound derived from the enzymatic activity of PEN2 (Stein et al., 2006), which is likely to affect the biotrophic lifestyle of *On* and limit its sporulation.

Susceptibility of Col-0 to *On* was exacerbated by the loss of function of three key genes involved in the SA defence signalling pathway (*NPR1*, *EDS5* and *PAD4*). This is in accordance with the results of a previous study using the same set of mutants and the powdery mildew species *Gc and Go* (Reuber et al., 1998) and validates the notion that SA signalling is important for basal resistance to biotrophic pathogens (Glazebrook, 2005).

We found that *Atmlo2* resistance to *On* is broken by the impairment of SA signalling in *Atmlo2/eds5, Atmlo2/npr1, Atmlo2/pad4* and *Atmlo2/sid2* double mutants and in the *Atmlo2/NahG* line, resulting in extremely susceptible phenotypes. Aberrant high levels of SA have been associated to *Arabidopsis Atmlo2* mutants and the early senescence phenotype of *Atmlo2* mutants is suppressed by the impairment of SA signalling (Consonni et al., 2006; Yoshimoto et al., 2009). Together, these findings indicate that *AtMlo2* might also function as a negative regulator of the SA pathway and that SA up-regulation might be an important feature of *Atmlo2* resistance to *On*. However, studies using the same set of mutants used by us and the powdery mildew species *Gc* and *Go* lead to the opposite conclusion that *Atmlo2* resistance to these powdery mildew species is largely independent from SA signalling (Consonni et al., 2006).

Since *Atmlo2* mutant still allows a consistent rate of *On* penetration, we believe that the requirement for SA signalling derives from the plants need of an effective post-penetration defence layer. The use of a lower dose of fungal inoculum, resulting in moderate fungal entry, could explain the above mentioned findings of Consonni et al. (2006). An alternative intriguing hypothesis would be that, with respect to *On*, *Gc* and *Go* have differentiated effectors enabling a more efficient interference with the *Arabidopsis* SA defence pathway. This last scenario would indeed reflect the fact that, unlike *Gc* and *Go*, *On* is not thought to have co-evolved with *Arabidopsis* and is likely to have made a host jump in order to be pathogenic on this species (Schulze-Lefert and Panstruga, 2011). In addition, further support to this hypothesis comes from the evidence that a very few

effectors seem to be conserved among PM species with different host specificities (Spanu et al., 2010). Finally, the *Arabidopsis* gene *RPW8* confers broad-spectrum powdery mildew resistance, but fails to confer resistance to the *On* isolate used in this study (Gollner et al., 2008) and experiments of heterologous expression of *Arabidopsis RPW8* genes could not confer resistance to *On* in transformed tomato (Xiao et al., 2003). Taking everything into account, it could be that mechanisms underlying *Arabidopsis* resistance towards *On* are at least partly different to those towards *Gc* and *Go*.

The higher level of susceptibility displayed by *pad4* mutants with respect to *eds5* and *npr1* mutants, and of *Atmlo2/pad4* mutants with respect to *Atmlo2/eds5*, *Atmlo2/npr1*, *Atmlo2/sid2* and *Atmlo2/NahG* mutants is in accordance with a body of evidence demonstrating the involvement of the lipase-like PAD4 protein not only in SA signalling, but also in SA-independent defence mechanisms (Bartsch *et al.*, 2006; Louis *et al.*, 2012; Yoshioka *et al.*, 2006). Therefore, it is likely that, in both basal and *Atmlo2*-based *Arabidopsis* resistance to *On*, *PAD4*-dependent and SA-independent mechanisms are also involved in the defence response.

CHAPTER 6

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CHAPTER 7

General Discussion

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It is widely accepted that the embryophytes (land plants) are coevolving with microorganisms since their first appearance on land approximately 450 million years ago. Some of the microorganisms have even facilitated the formation of modern plants. For example, the chloroplast, an important plant organelle that conducts photosynthesis, is assumed to be originated from cyanobacteria through endosymbiosis. Thus, microorganisms are an essential cohort of plants during evolution. Therefore, the ability to detect, differentiate and monitor interacting microorganisms is very important to plants. There are different forms of interactions between plants and microorganisms, which can be divided into epiphytic, symbiotic, and pathogenic. Consequently, plants have evolved and developed a surveillance system to distinguish different groups of microbes in order to mount different responses, including defense responses leading to resistance.

In practice, resistance breeding is making use of plant defense responses against pathogens. It could reduce the loss of crop production and minimize the use of chemicals like fungicides and pesticides. Breeding for resistance can often be achieved by introgression of resistance genes (*R*-gene) from wild-relatives of crops. However, it is rarely durable since race-specific resistance conferred by *R*-genes asserts selective pressure on pathogen populations promoting the appearance of new races rendering the introgressed *R*-gene ineffective. Pyramiding of *R*-genes has been proposed as a solution to this problem, but has so far not actively been deployed in varieties although from natural populations results are known which would suggest that this might be an effective form of resistance. Based on studies on effector-triggered susceptibility and by looking from a different point of view into host and non-host resistance, a new breeding strategy was proposed by disabling plant susceptibility (*S)* genes to achieve durable and broad-spectrum resistance in crops (Pavan et al. 2010). Several natural loss-of-function alleles of *S*-genes are known in agriculture that support the idea that impairment of specific host genes results in durable disease resistance, such as barley *mlo* mutants (Lyngkjaer et al., 2000) and the rice *pi21* mutant allele (loss-of-function of a proline-containing protein) for resistance to rice blast

throughout a century of cultivation (Fukuoka et al., 2009). With increasing interest in the research topic on suppression of plant immunity, a considerable amount of potential *S*-genes has been identified in *Arabidopsis* (e.g. reviewed by Pavan et al., 2010). However, it is largely unknown whether orthologs of these *Arabidopsis S*-genes in crop species exist which are functional to corresponding crop pathogens. Thus, the challenge in crop breeding is how to exploit *S*-genes, complementary to *R*-genes, to achieve durable and broad-spectrum resistance.

So far, the best-characterized example on durable and broad-spectrum resistance by disabling *S*-genes is the *mlo*-based resistance to powdery mildew species in barley. The respective MLO protein isoforms modulate vesicle-associated defense responses at the cell periphery and the powdery mildew pathogen possibly exploits these proteins for successful host cell entry (Panstruga, 2005). In barley, mutations in a particular *Mlo* gene result in broad-spectrum powdery mildew resistance (Büschges *et al.,* 1997). Barley *mlo* mutants have been known for more than 60 years (Freisleben and Lein, 1942) and have been successfully employed in European barley agriculture for more than 35 years (Lyngkjaer *et al.,* 2000), emphasizing the principal durability of *mlo*-mediated disease resistance under agricultural conditions. Taking *Mlo* as the target gene, this thesis shows that *Mlo* orthologs are present in tomato and pepper acting as *S*-genes for two different powdery mildew species, namely *Oidium neolycopersici* and *Leveillula taurica*. Thus, this study presents a proof-of-concept research demonstrating the potential use of orthologs of *Arabidopsis S*-genes in resistance breeding in vegetable crops.

Presence and expression of the Mlo gene family in plant species

Mlo (Mildew resistance Locus O) belongs to a gene family which encodes the plant-specific seven-transmembrane domain protein with unknown biochemical functions (Buschges et al., 1997; Devoto et al., 1999 and reviewed by Hückelhoven and Panstruga 2011). The structural features of these proteins are similar to the G-protein-coupled receptors (GPCRs) in metazoan and mutagenesis studies on the phylogenetic related members showed the involvement of *Mlo* family members in different biological processes of plants (Devoto et al., 1999). Since the first *Mlo* member was demonstrated of having a crucial role in modulating

defense responses (Buschges et al., 1997; Piffanelli et al., 2002), *Mlo* homologues (paralogs and co-orthologs in other species) are still referred as members of the *Mlo* family.

The first *Mlo* gene (*HvMlo1)* was identified in barley which is the only expressed gene copy of this (co-)ortholog cluster. A closely sequence-related paralog, *HvMlo2* (GenBank accession number Z95496) appears to represent a non-expressed pseudo gene. The transcription level of *HvMlo1* exhibited a noticeable increase at early time points (6 hours post inoculation) upon powdery mildew challenge in both the natural (*HvMlo1*) and mutant (*Hvmlo1*) genotypes. In other monocotyledonous plant species like *Oryza sativa*, *Triticum aestivum, Zea mays* and *Sorghum bicolor*, existence of *Mlo* homologues has also been shown (Devoto et al., 2003; Liu and Zhu, 2008; Sasanuma et al., 2010; and Singh et al. 2012).

The *Mlo* gene family is well characterized in the model plant *Arabidopsis thaliana*. There are 15 *AtMlo* homologs (Devoto et al., 2003) in *Arabidopsis*, of which, four clades (with analysis of the AtMLO family only) have been revealed by phylogenetic analysis (Chen et al. 2006). All *AtMlo* genes exhibited altered expression patterns in different tissues as well as in response to various cues. However, none of the 15 *AtMlo* genes showed identical expression profiles between different clade members indicating their distinct functions in biological processes. Nevertheless, overlapping expression patterns between *AtMlos* in the same phylogenetic clade were observed. For example, the AtMLOs in Clade IV (AtMLO2, AtMLO6 and AtMLO12) showed abundant expression in leaf tissues, and appeared to be the most responsive AtMLO members under biotic stresses including biotrophic fungal pathogens *Golovinomyces cichoracearum* and *Golovinomyces orontii,* the hemibiotrophic oomycete pathogen *Phytophtora infestans*, the necrotrophic fungal pathogen *Botrytis cinerea* and the bacterial pathogen *Pseudomonas syringae* (Chen et al. 2006). These mildew effective AtMLO (Clade IV in Chen et al. 2006) proteins are involved in modulating plant defense to powdery mildew responses (Consonni et al., 2006). AtMLO4 and AtMLO11 in Clade I (Chen et al. 2006) were shown to be predominantly expressed in the epidermal cells of the root meristematic zone and implicated in an Auxin dependent root thigmomorphogenesis (Chen et al., 2009). AtMLO7 in Clade III (Chen et al. 2006) was proven to be co-involved with the receptor-like kinase FERONIA in pollen tube reception (Kessler et al., 2010). The biological functions of the other AtMLOs are largely unknown.

In grape (*Vitis vinifera*) 17 *Mlo* homologs (*VvMlo*) have been identified. In total, six phylogenetic distinct clades were assembled by an analysis of the 17 VvMLO proteins with the whole MLO protein family from *Arabidopsis*, and selected MLO proteins from barley, rice, maize and tomato (Feechan et al., 2008). Interestingly, the VvMLO10 and VvMLO11 were grouped together with the AtMLO3 and formed the additional Clade VI. The AtMLO3 was considered as a single divergent lineage in the analysis of *Arabidopsis* MLO with those from the monocots (Devoto et al., 2003). Furthermore, comparative analysis retrieved four grapevine MLO homologs (VvMLO3, VvMLO4, VvMLO13 and VvMLO17) that are closely related to the *Arabidopsis* mildew effective MLOs (AtMLO2, AtMLO6 and AtMLO12). The *VvMlo3, VvMlo4* and *VvMlo17* showed up-regulation upon the challenge of grape powdery mildew fungus *Uncinula necator*, thus, have been considered as associated to powdery mildew susceptibility (Feechan et al. 2008).

The tomato genome sequence was recently published by the International Tomato Genome Sequencing Consortium (TGSC) (http://solgenomics.net/). In the tomato genome, 16 tomato *Mlo* homologs (*SlMlo*) could be identified (**Chapter 3)**. Some of the *SlMlo* homologs (e.g. *SlMlo4, SlMlo7, SlMlo8, SlMlo14, SlMlo15* and *SlMlo16*) showed a tissue-specific expression, indicating probably a specific function of those MLO proteins in different biological processes. These *SlMlos* were grouped in different clusters based on a phylogenetic analysis by aligning protein sequences of *SlMlos* with the MLO members of other plant species including the whole *Arabidopsis* MLO protein family. In addition to the presence of SlMLO members in the five clades corresponding to the *Arabidopsis* clades, Clade VI was identified containing SLMLOs which were grouped together with the AtMLO3 protein that was considered as a single divergent lineage in the analysis of *Arabidopsis* MLO with those from the monocots (Devoto et al., 2003) (Fig. 3 in **Chapter 3**). Additionally, the expression profiles of the *SlMlo* gene family members were analysed in response to the tomato powdery mildew *O. neolycopersici*. *SlMlo1* transcripts showed considerable increase at 6 and 10 hours after *O. neolycopersici* challenge. In contrast, no other *SlMlo* homolog in Clade V (*SlMlo3*, *SlMlo5* and *SlMlo8*) showed up-regulation upon fungal infection. Surprisingly and contrast to expectation based on sequence homologies, the expression of *SlMlo4* and *SlMlo14* was also up-regulated at 10 hours after inoculation of *O. neolycopersici*.

With the available genome sequences of many plant species, such as cucumber, apple and etc., *Mlo* like sequences are being identified. Towards understanding the biological functions of the *Mlo* gene family, their expression patterns are being analysed in different plant species (Chen et al., 2006, Feechan et al., 2008; Winterhagen et al., 2008; Dry et al., 2010 and **Chapter 3** in this thesis). Molecular phylogeny and evolution of the plant specific *Mlo* family are being gradually uncovered.

Mlo homologs functioning as plant susceptibility genes

In barley, the MLO protein was identified as a plant susceptibility factor manipulated by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* to cause disease (Büschges et al., 1997). The naturally occurring and mutation-induced recessive alleles of the *Mlo* locus confer a broad spectrum resistance against all known isolates of barley powdery mildew identified so far. The resistance conferred by the loss-of-function mutants in the *Mlo* gene prevents the mildew fungus from penetrating host cells, and thus prevents fungal haustorium formation (Peterhansel et al., 1997; Piffanelli et al., 2002). The later identification of natural and mutation-induced *mlo*-mutants in *Arabidopsis*, tomato and pea for resistance to different powdery mildews demonstrates a conserved requirement for MLO proteins in powdery mildew pathogenesis in plants (Bai et al. 2008, **Chapter 2** in this thesis; Consonni et al., 2006; Humphry et al. 2011; Pavan et al., 2011). In breeding practices, these findings imply the success in applying *mlo*-based resistance to combat powdery mildew disease in a wide range of crop species either by mining the natural variation in wild crossable species and/or by using conventional mutagenesis to generate mutations in the gene (Pavan et al. 2010).

In barley, pea and tomato, mutation in one *Mlo* gene is sufficient to confer full resistance to powdery mildews (Jørgensen 1994; Büschges et al., 1997; Humphry et al. 2011; Pavan et al., 2011; Bai et al., 2008, **Chapter 2** of this thesis). On the other hand in *Arabidopsis*, deletion of three *Mlo* homologues (*AtMlo2, AtMlo6* and *AtMlo12*) is required for full powdery mildew resistance (Consonni et al., 2006). Phylogenetic analysis of *AtMlo* homologues has identified a special mildew effective clade, in which *AtMlo2, AtMlo6* and *AtMlo12* are grouped. This clade is labeled as having the *Mlo* gene members acting as susceptibility

factors manipulated by the powdery mildews. In tomato, the mildew effective clade has four *SlMlo* homologues, (*SlMlo1*, *SlMlo3*, *SlMlo5* and *SlMlo8*), of which loss-of-function in *SlMlo1* resulted in resistance to *O. neolycopersici* (**Chapter 2**). Silencing *SlMlo3*, *SlMlo5* or *SlMlo8* did not result in a significant reduction of disease symptoms caused by *O. neolycopersici,* while, in *Arabidopsis* loss of function of individual *AtMlo* homologs (*AtMlo2, AtMlo6* and *AtMlo12*) in the mildew effective clade led to different levels of resistance against *G. orontii* and *G. cichoracearum* (Consonni et al., 2006;) and *O. neolycopersici* (**Chapter 2** of this thesis). These findings imply that the involvement of *Mlo* homologs in the susceptibility to powdery mildews is dependent on the plant species.

The expression of *Mlo* homologues in the mildew effective clade of different plant species has been shown to be induced upon powdery mildew infection. In barley, *Mlo* transcripts have been demonstrated to be increased at an early time point of the interaction (6 hours post inoculation) (Piffanelli et al., 2002). In tomato, *SlMlo1* transcripts showed considerable increase at 6 and 10 hours after *O. neolycopersici* challenge (**Chapter 2** and **Chapter 3**). However, no other *SlMlo* homologs in Clade V (*SlMlo3*, *SlMlo5* and *SlMlo8*) showed pathogen-dependent up-regulation. This result suggested that *SlMlo3*, *SlMlo5* and *SlMlo8* may after all not be involved in the powdery mildew susceptibility of *O. neolycopersici* although they are closely related to the mildew effective *Mlo* homologs. Intriguingly, *SlMlo4* and *SlMlo14* showed up regulation upon *O. neolycopersici* infection. These two homologs are not in the clade containing MLO homologs involved in powdery mildew susceptibility. SlMLO4 is closely related to AtMLO4 and AtMLO11 which are in Clade III and are involved in the root thigmomorphogenesis. SlMLO14 is clustered with AtMLO7 in Clade I which is involved in the pollen tube reception. Furthermore, mutations in AtMLO4, AtMLO7 and AtMLO11 in *Arabidopsis* did not result in powdery mildew resistance (Consonni et al. 2006). Thus, it is logical to argue that silencing of these two *Mlo* homologs would likely not lead to resistance against tomato powdery mildew. However with the new data on the up regulation of these two homologs upon infection with the pathogen it will be very useful to create RNAi lines in which these two *SlMlo* homologues (and in view of these results also as many of the others) are silenced. Experiments are being carried out to observe whether altered phenotypes of these RNAi lines can be obtained and we hope to verify the involvement of *SlMlo4* and *SlMlo14* in susceptibility to powdery mildew. If these

two *SlMlo* homologues are powdery mildew effective, the claim on mildew effective clade *Mlo* homologues will be challenged. In view of these results it is however clear that the assumption that members of the same clade retain a similar biological function is no longer valid.

O. neolycopersici is epiparasitic and develops all structures except haustoria on the host surfaces. In contrast, *Leveillula taurica,* another powdery mildew species infecting both tomato and pepper, is endotrophic because fungal haustoria are produced in the mesophyll cells. So far *mlo*-based resistance is only demonstrated to confer resistance to epiparasitic mildews in barley, *Arabidopsis*, tomato and pea. Interestingly, the tomato *Slmlo1* mutant, which had full resistance to *O. neolycopersici* (Bai et al., 2008; **Chapter 2** in this thesis), showed incomplete resistance to *L. taurica* (**Chapter 5**). Currently, there are at least two *CaMlo* (*CaMlo1* and *CaMlo2*) present in the pepper genome. Interestingly, knocking down the expression of either *CaMlo1* or *CaMlo2* resulted in reduced susceptibility of pepper to *L. taurica* (**Chapter 5**), indicating that more than one *Mlo* homologue are involved in the susceptibility to endotrophic powdery mildew, *L. taurica*. Though silencing of *SlMlo3*, *SlMlo5* or *SlMlo8* in tomato did not give resistance to *O. neolycopersici*, the possibility cannot be ruled out that these homologues are involved in the susceptibility to *L. taurica*. This is something which should be tested in the future.

Comparative analysis revealed a close homology between *CaMlo1* and *SlMlo3*, suggesting the possible involvement of *SlMlo3* in susceptibility to *L. taurica*. Thus, the *SlMl*o homologues (*SlMlo3, SlMlo5* and *SlMlo8*) that are phylogenically related to *SlMlo1* should be the first targets to be investigated in order to see whether silencing of these three homologs (RNAi lines generated in **Chapter 3**) will reduce the susceptibility of tomato against *L. taurica*. Thus, for the same plant species, full resistance to different powdery mildew species likely requires mutations in different numbers of *Mlo* homologs. Thus, the specificity or redundancy of plant MLO functions in powdery mildew interaction in different pathosystems requires attention. Hopefully, there is always one *Mlo* homolog having a major effort, such as the *AtMlo2* in *Arabidopsis*, which will simplify the use of *mlo*-based resistance in breeding practices.

A recently study has indicated the identification of the *CaMlo2* gene in pepper (Kim and Hwang, 2012) which represents a different allele of the *CaMlo2* gene isolated in **Chapter 5.**

Their results demonstrated that the newly isolated *CaMlo2* gene is involved in cell death response as well as formation of the reactive oxygen species (ROS). In addition, the *CaMlo2* was distinctly induced by the hemibiotrophic bacterial pathogen *Xanthomonas campestris* pv. *Vesicatoria*, the oomycete pathogen *Phytophthora capsici*, exogenous salicylic acid (SA), methyl viologen (MV), NaCl and drought stress treatment. Silencing of the *CaMlo2* could induce the resistance against *Xanthomonas campestris* pv. *Vesicatoria* in pepper, while, overexpression of *CaMlo2* in *Arabidopsis* resulted in enhanced susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 and *Hyaloperonospora arabidopsidis*. The results of Kim and Hwang (2012) are in agreement with our results demonstrating that CaMLO2 is a negative regulator of defense response in pepper and also show that *mlo*-based resistance is not restricted to powdery mildews.

Role of MLO in plant immunity and its dependent signalling pathway

Plant immune responses can be separated into two layers: PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). The suppression of plant defense is a key step for pathogens to establish susceptibility, for which pathogens exploit their effectors to interact with host targets (Jones and Dangl, 2006). Plant MLO protein is known to contribute to powdery mildew susceptibility, but it is not clear yet whether MLO is the host protein targeted by powdery mildew effectors. In barley, the *Mlo* transcripts increased in abundance at the time point of six hours post inoculation (6 hpi) (Piffanelli et al., 2002). This time point represents the recognition and penetration stage of epiparasitic powdery mildew. In combination with a consecutive observation on the infection process of *L. taurica* under a stereomicroscope (**Chapter 4**), it was demonstrated that the up-regulation of *Mlo* upon mildew challenge is before the formation of *L. taurica* haustoria in pepper plants (**Chapter 5**). It takes a longer time (at least 72 hpi) from the first contact of the pathogen with the host till the establishment of haustoria (**Chapter 5**). This special feature of *L. taurica* enabled us to clarify whether the powdery mildew is manipulating the *Mlo* by haustorium delivered effectors. According to the timing of the infection process of *L. taurica*, we investigated the expression of two pepper *Mlo* genes with respect to the different infection stages (**Chapter 5)**. In conformity with the findings in barley (Piffanelli et al.2002), our results showed that

CaMlo2 was up regulated at the penetration stage of *L. taurica* when no haustoria are formed yet. Thus, it is unlikely that the expression of *CaMlo2* is induced by *L. taurica* effectors. The causal agent of powdery mildew on barley *B. graminis* f. sp. *hordei* has been sequenced and released together with the initial sequencing results of pea powdery mildew *E. pisi* and *Arabidopsis* powdery mildew *G. orontii* (Spanu et al., 2010). In addition, a transcriptome analysis of *O. neolycopersici* haustorium is on-going (L. Faino, Z. Zheng, A. Seifi and Y. Bai; Unpublished results). The discovery of pathogen factors involved in pathogenesis will shed light on the mechanisms associated with MLO-mediated susceptibility to powdery mildews.

Signal transduction mechanisms controlling defense activation in plants are formed by interacting pathways that are, in most cases, dependent on the signaling molecules salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Pieterse and van Loon, 1999; Glazebrook, 2001; Shah, 2003; Pieterse and Van Loon, 2004). However, *mlo*-based resistance (*mlo* represents mutation in *HvMlo1* and *AtMlo2)* was largely independent of any of the three main signaling pathways mentioned above by studying the interaction of *Arabidopsis* with *G. cichoracearum* and *G. orontii* (Consonni et al., 2006). In addition, *mlo*-based resistance requires correct functioning of specific (*Ror1* and *Ror2*) genes in barley (Huckelhoven et al., 2000) and syntaxin (*Pen1*), glycosyl hydrolase (*Pen2*) and putative ATP binding cassette (ABC) transporter (*Pen3*) genes in *Arabidopsis* (Collins et al., 2003; Consonni et al., 2006). Moreover, recent results showed that the tryptophan-derived metabolites including phytoalexin camalexin which only occur in the taxonomic order of Brassicales and indolic glucosinolates are also essential compounds for the *Atmlo2* powdery mildew resistance (Consonni et al., 2010). In **Chapter 6** of this thesis, we tested *O. neolycopersici* on a set of *Arabidopsis* mutants which have genetic impairment of pre-invasive or post-invasive immune responses, including the *Atmlo2* mutants with impaired SA, JA and ET as well as *Pen1* and *Pen2*/*Pen3* pathways. Strikingly, *Atmlo2* mutants, with impairment of SA signaling, resulted in susceptible phenotypes (Fig. 3 in **Chapter 6**). Notably, the quadruple mutants *Atmlo2/6/12/pen1* and *Atmlo2/6/12/pen2* and the quintuple mutant *Atmlo2/6/12/pen1/pen2* were immune to fungal penetration, demonstrating for the first time that *mlo*-based penetration resistance in *Arabidopsis* relies on unknown defence mechanisms independent from *Pen* genes.

In order to expand the host range, the microbes have to make a so called host-jump by overcoming different barriers of the plant defense mechanisms. Abolishing PTI and ETI could sufficiently change the status of *Arabidopsis* from non-host to a host for non-adapted powdery mildew pathogens (Lipka et al., 2005 Schulze-Lefert and Panstruga 2011). The PEN1 protein has been demonstrated to hold opposing functions in penetration resistance and post cell entry defense signaling pathways. *Pen1* is essential for the SNARE machinery, controlling vesicle trafficking and bulk transport of cargos in cells (Bock et al., 2001). It is possibly also involved in secretion of components for the formation of cell wall appositions. A recent study demonstrated that PEN1 can negatively regulate SA accumulation and the programmed cell death (PCD) (Zhang et al., 2007). Another example is the dual function of the callose synthase GSL5/PMR4 protein which has been shown to be a negative regulator of SA-dependent defense responses. Loss of function of the GSL5/PMR4 could result in resistance against powdery mildew fungus (Jacobs et al., 2003; Nishimura et al., 2003; Micali et al., 2008).

Here we propose a model in which plants have a surveillance system which contains two layers of alarm or so called defence triggering parts (Fig. 1). The first is based on membrane-localized receptors that guard the apoplastic space which can sense the colonization progress and position by observing PAMPs. It will then start the performance of pre-penetration resistance in order to stop the pathogens outside the plant cell wall. Genes involved by this defense response in this layer normally have another dual-function which is to negatively regulate the second layer of defense response. The second layer is based on presumably intracellular pattern recognition receptors that monitor the cytoplasm which can sense the colonization progress and position by observing pathogen factors (or organ cues) by monitoring the intracellular space and perform the post-penetration resistance (HR). By this two parts surveillance system plants can detect the pathogens inter/intra cellular. According to the position plants will trigger the corresponding resistance mechanism. To use the right weapons in the right battle field and situation plants control the two layers of defence by differently expression of *Pen1* and *Pmr4* like genes which have dual-opposing functions. *Mlo* could play a similar role which serves as a sentry of the cell wall which positively promotes penetration of the pollen tube, powdery mildew and possibly other symbiotic microbes or plants. In order to make the proposed biological process effective,

the dual functions of the MLO have to be a negative regulator of both PTI and ETI (Fig. 1).

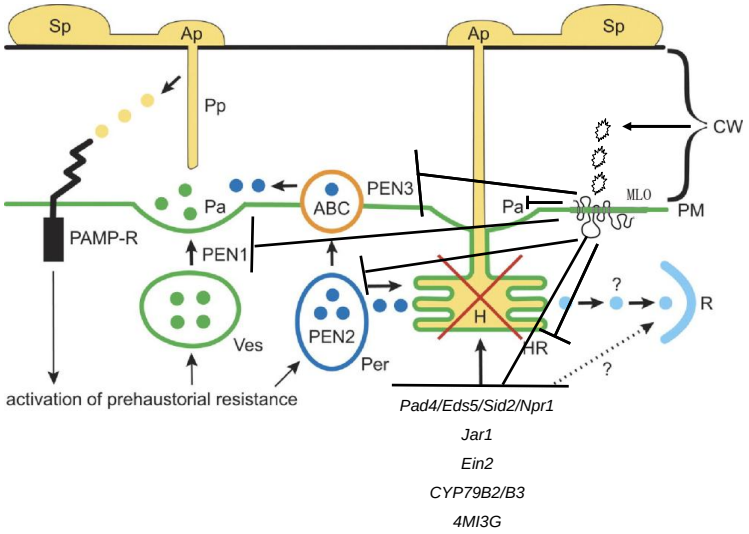

Fig. 1. A hypothetical model for MLO-mediated plant defence adapted according to (Ellis, 2006). When infecting the host, powdery mildew spores (Sp) produce the penetration peg (Pp), form the appressorium (Ap),which penetrate plant cell wall (CW) and produce the haustorium (H) from which fungal effector molecules (light blue circles) are delivered to/and through the plasma membrane (PM). The powdery mildew fungus possibly manipulates the MLO protein which likely functions as a sentry of the cell wall or DAMPs by unknown molecules (white cloud like cycles) that negatively regulates defense response pathways involving indole glucosinolate (4MI3G), jasmonates (*Jar1*), ethylene (*Ein2*), salicylic acid (*Pad4/Eds5/Sid2/Npr1*), monooxygenases (CYP79B2/3) and hypersensitive Response (HR). DAMPs include the *PEN1*-mediated vesicle (Ves) secretory pathway, papillae (Pa) formation, biosynthesis of Trp-derived secondary metabolites, postulated toxin(s) (dark blue circles) that are synthesized in peroxisomes (Per) in a *PEN2*-mediated pathway and delivered by a *PEN3*-encoded ABC transporter.

The functional redundancy of *AtMlo* genes provides us an exciting system to study the *mlo*-based resistance. As mentioned above the *Atmlo2* mutants confer partial pre-penetration resistance, leading to a very low pathogen sporulation. This partial pre-penetration resistance allows certain amounts of fungal spores making successful entries into plant cell and developing the disease symptoms. The *Atmlo2* partial penetration provides us the only opportunity so far for studying the function of MLO after fungal cell entry. *Atmlo2* and *Atmlo2/6/12* triple mutant open a door to understand the MLO function in both pre- and post-penetration defense response. In addition, *O. neolycopersici* is a powdery mildew fungal species that was not thought to have co-evolved with *Arabidopsis*

(Chapter 6). It has very likely just made its host jump to become pathogenic on *Arabidopsis* (Schulze-Lefert and Panstruga, 2011), since natural occurrence of *O. neolycopersici* has to date only been demonstrated on tomato and *Arabidopsis* is a host for *O. neolycopersici* only with artificial infection under experimental conditions (**Chapter 6**). The availability of mutants in *Arabidopsis* can be exploited to follow a reverse genetic approach to study the interaction with *O. neolycopersici*. In order to discover the *mlo* resistance dependent pathways, non-targeting mutagenesis study should be carried out to abolish the resistance.

Functions of Mlo homologues in plants

By comparing features of *Mlo* like genes of several monocot species, the dicot *Arabidopsis* and the moss *Ceratodon purpureus*, it is assumed that the origin of the *Mlo* gene family can be tracked back at least to the early evolutionary stages of land plant development (Devoto et al., 2003). The ancient presence of plant *Mlo* gene families implies their vital function for plant development. In addition to being a negative regulator of plant defence to powdery mildews, functions have been discovered for other members of MLO proteins. AtMLO4 and AtMLO11 were implicated in an Auxin dependent root thigmomorphogenesis (Chen et al., 2009). AtMLO7 was proven to be co-involved with the receptor-like kinase FERONIA in pollen tube reception (Kessler et al., 2010). However, the biological functions of the other AtMLOs are largely unknown. Barley and *Arabidopsis mlo*-mutants showed some pleiotropic effect in older plants and under certain conditions, suggesting that mildew effective clade MLOs may be involved in other biological functions as well (Pavan et al. 2010). In support of this assumption is the fact that silencing of *CaMlo2* also resulted in small pepper plants (**Chapter 5**). However, the tomato *Slmlo1* mutant did not show an abnormal plant phenotype. Thus, the functions of the *Mlo* gene family need to be further characterized.

A remarkable study was performed by Humphry et al., (2011) to test whether in the symbiosis interaction also MLO is required for invasion of plant cells. In that study, rhizobial bacteria and arbuscular mycorrhizal fungus were used to inoculate the *PsMlo1* mutant *er1* plant which shows resistance to pea powdery mildew. However, their results showed unaltered colonization of the arbuscular mycorrhizal fungus and nitrogen-fixing rhizobial

bacteria. Nevertheless, there are 17, 7, 9, 12 *Mlo* paralogs reported in the grape, wheat, maize and rice genome respectively (Devoto et al., 2003; Consonni et al., 2006; Feechan et al., 2008; Liu and Zhu, 2008; Sasanuma et al., 2010). We have identified 16 *Mlo* paralogs in the tomato genome (**Chapter 3**). Thus an elegant experiment could be by using a set of different *mlo* paralogs in a legume plant species to test whether in a symbiotic interaction that plant could benefit from the symbionts, which, not like pathogenic interaction for example with powdery mildews pathogen, also require correct MLO functioning for invasion of plant cells.

With increasing interest in the research topic on suppression of plant immunity, a considerable amount of potential susceptibility (*S*) genes have been identified in *Arabidopsis*. However, it is largely unknown whether orthologs of *Arabidopsis S*-genes in crop species exist and are functional to corresponding crop pathogens. Taking *Mlo* as the target gene, this study shows that there are functional orthologs present in tomato and pepper, demonstrating the potential use of *S*-genes identified in *Arabidopsis* in breeding crops with durable resistance. This thesis presents a proof-of-concept research: how to make use orthologs of *Arabidopsis S*-genes in resistance breeding in crop species, which presents new insights into functional *Mlo* orthologs in tomato and pepper. In addition, it delivers new findings on mechanisms associated with *mlo*-based resistance. For example, we demonstrated for the first time that *mlo*-based penetration resistance in *Arabidopsis* relies on unknown defense mechanisms independent from *Pen* genes. Also, the qPCR assay for fungal biomass quantification represents a convenient tool in scoring powdery mildew disease and in studying plant and *L. taurica* interaction. Thus, the research in this thesis provides a solid basis for studying plant pathogen interaction with powdery mildew in the future and facilitates the breeding of cultivars resistant to *O. neolycopersici* and *L. taurica*.

CHAPTER 7

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SUMMARY

Powdery mildew is a common seen disease of plants which is caused by obligate biotrophic Ascomycete fungi belonging to the Erysiphales. It represents one of the most important threats for the cultivation of many crops in both open field and greenhouses. The disease is particularly prevalent in temperate and humid climates where it causes significant yield losses in agricultural production. The recessively inherited durable and broad spectrum *mlo*-based resistance against powdery mildew disease has been exploited for more than 40 years in spring barley production in Europe. The aim of this thesis is to investigate the potential use of *mlo*-based resistance in Solaneaceous crops.

In this thesis we used two powdery mildew species *Oidium neolycopersici* and *Leveillula taurica*. *O. neolycopersici* is an epiphytic fungus which develops all fungal structures except haustorium on the surface of the host; however, *L. taurica* is endotrophic fungus which grows inside the host tissues and infects both tomato and pepper (*Capsicum annuum*). In order to gain a better understanding of the differences in infection process between *L. taurica* and *O. neolycopersici,* we made an effort to monitore the development of *L. taurica* by using different microscopic techniques as described in **Chapter 4**. Different infection stages of *L. taurica* were defined by continuously observation with a high-fidelity digital microscope. In addition, we developed a detection and quantification method of *L. taurica* biomass in plant leaves by real-time PCR assay. A standard curve was obtained for absolute quantification of *L. taurica* biomass. The real-time PCR assay for *L. taurica* provides a valuable tool for detection and quantification of this pathogen in breeding activities as well in plant-microbe interaction studies.

The resistant cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) line LC-95, derived from an accession collected in Ecuador, harbours a natural allele (*ol*-2) that confers broad-spectrum and recessively inherited resistance to *O. neolycopersici*. In **Chapter 2**, we initiated a candidate gene approach and cloned the *SlMlo1* gene from both the resistant and susceptible genotypes. Complementation experiments using transgenic tomato lines and virus-induced gene silencing (VIGS) assays suggested that loss of *SlMlo1* function is responsible for powdery mildew resistance conferred by *ol-2*. In progeny of a cross between a resistant line bearing *ol-2* and the susceptible tomato cultivar (cv.) Moneymaker (MM), a 19 bp deletion disrupting the Sl*Mlo1* coding region co-segregated with resistance.

This polymorphism results in a frame shift and thus a truncated non-functional SlMLO1 protein.

In **Chapter 3** of this thesis, we exploited the tomato genome sequence to discover the *Mlo* homologs (*SlMlo*) by using the *Arabidopsis Mlo* sequences. In total, 16 *SlMlo* homologs were identified and characterized in relation to their structure, polymorphism, genomic organization, phylogenetic relationship, expression profile in different tissues and upon inoculation with the powdery mildew fungus *O. neolycopersici*. In addition, RNAi-based silencing experiments were carried out. Results showed that *SlMlo1* is the only one out of four closely related *SlMlo* homologs from the mildew effective clade to play a major role in susceptibility to *O. neolycopersici*. The outcome of this study provides useful information to retrieve *Mlo* susceptibility genes across cultivated species. Moreover, it gives genetic and genomic information useful for the reconstruction of evolution on the *SlMlo* gene family and further investigations to assign biological functions to (*Sl*)*Mlo* homologs.

Subsequently in **Chapter 5**, we focused on investigating whether loss of function in *Mlo* genes induces resistance to *L. taurica* in pepper and tomato. We showed that loss-of-function mutant of the *SlMlo1* gene in tomato confers resistance against *L. taurica*. In pepper, there is one *Mlo* homolog (*CaMlo1*) was previously identified and considered to be involved in powdery mildew susceptibility. By homology-based cloning approach one new *CaMlo* gene namely *CaMlo2* was isolated. Phylogenetic analysis showed that *CaMlo2* is more homologous to *SlMlo1* than the *CaMlo1* gene. Compared to *CaMlo1,* the expression of *CaMlo2* was higher and up-regulated upon *L. taurica* infection. Functional analysis via VIGS demonstrated that knocking down the expression of either *CaMlo1* or *CaMlo2* resulted in reduced susceptibility of pepper to *L. taurica*.

Signal transduction mechanisms controlling defense response in plants. In order to identify pathways involved in basal and *mlo*-based resistance, we studied in **Chapter 6** the interactions between *O. neolycopersici* and several pre- and post-penetration signaling pathway (including PEN1, PEN2/PEN3, SA, JA and ET) deficient *Arabidopsis* mutants. We demonstrated that *Atmlo2/6/12*-based penetration resistance in *Arabidopsis* relies on unknown defense mechanisms independent from *Pen* genes and the *Atmlo2*-based resistance is likely to rely on correct functioning of SA signaling against *O. neolycopersici*. The results from this thesis show that for different plants species *mlo*-based resistance is a

fact but the redundancy of gene functions and dependent signaling pathway may differs per plant/ powdery mildew species.

Summary

SAMENVATTING

Meeldauw is een algemeen voorkomende ziekte bij veel planten die veroorzaakt wordt door obligate biotrofe Ascomyceet schimmels. Deze schimmels behoren wereldwijd tot een van de meest gevaarlijke ziekten die de groei en cultivatie van veel verschillende gewassen in zowel kas als veld bedreigen. De ziekte is vooral een groot probleem in gematigde en vochtige klimaten waar de opbrengst verliezen als gevolg van deze ziekte enorm kunnen zijn. Er bestaat een recessieve en zogenaamde breedspectrum resistentie (*mlo* genaamd) die al meer dan 40 jaar met succes wordt toegepast in de teelt van gerst in Europa. Het doel van de in dit proefschrift beschreven onderzoek was om het mogelijke gebruik van met gerst vergelijkbare *mlo*-gebaseerde resistentie in groenten van de nachtschadeachtige planten (Solanaceae) te bepalen.

In dit proefschrift zijn de resultaten van experimenten met twee verschillende meeldauw veroorzakende schimmels (*Oidium neolycopersici* en *Leveillula taurica)* beschreven. *O. neolycopersici* is een epifytische schimmel die alle schimmel structuren behalve haustorium vorming op de gastplant ontwikkeld, terwijl *L. taurica* een endotrofe schimmel is die aan de binnenkant van het plantenweefsel groeit van zowel tomaat als paprika. Om een beter begrip te krijgen van de verschillen in infectieproces van de twee schimmels werd de ontwikkeling van *L. taurica* met verschillende microscopische technieken gevolgd (**Hoofdstuk 4**). Verschillende infectiestadia van *L. taurica* konden onderscheiden worden door het continue monitoren met een digitale microscoop. Daarnaast werd een detectie en kwantificerings methode ontwikkeld om de hoeveelheid biomassa van *L. taurica* in bladeren te kunnen bepalen middels een moleculaire assay (real-time PCR). Deze methode is een belangrijk en betrouwbaar hulpmiddel om de aanwezigheid én de concentratie van *L. taurica* te bepalen in allerlei veredelingsgerelateerde activiteiten alsmede voor plant-pathogeen interactie studies.

De resistente cherry tomaat (*Solanum lycopersicum* var. *cerasiforme*) lijn LC-95, afkomstig van een wilde accessie verzameld in Ecuador, draagt een natuurlijk allel (*ol*-2) dat breed-spectrum en recessief overervende resistentie tegen *O. neolycopersici* geeft. In **Hoofdstuk 2**, werd een kandidaat-gen benadering gebruikt om het *SlMlo1* gen uit zowel resistente als vatbare genotypen te kloneren. Complementatie experimenten met transgene tomaat lijnen en ‘virus-induced gene silencing’ (VIGS) toetsen suggereerden dat

verlies van de *SlMlo1* functie de reden is voor de meeldauw resistentie veroorzaakt door *ol-2*. In nakomelingen van een kruising tussen een resistente lijn met *ol-2* en de vatbare tomaten cultivar (cv.) Moneymaker (MM), co-segregeerde een 19 bp deletie in de coderende sequentie van Sl*Mlo1* met de resistentie. Deze deletie resulteert in een frame shift en derhalve een niet compleet en dus niet functioneel SlMLO1 eiwit.

In **Hoofdstuk 3** van dit proefschrift staat beschreven hoe de tomaten genoom sequentie gebruikt werd om *Mlo* homologen (*SlMlo*) te vinden gebruik makend van Arabidopsis *Mlo* sequenties. In totaal konden er 16 *SlMlo* homologen geïdentificeerd en gekarakteriseerd worden gebruikmakend van hun structuur, polymorfismen onderling, genomische organisatie, fylogenetische verwantschap, expressie patroon in verschillende weefsels en na inoculatie met de schimmel *O. neolycopersici*. Daarnaast werden er RNAi (gene silencing) experimenten uitgevoerd. Resultaten lieten zien dat *SlMlo1* de enige van vier zeer nauw gerelateerde *SlMlo* homologen van de meeldauw effectieve tak is die een belangrijke rol speelt bij de vatbaarheid voor *O. neolycopersici*. De resultaten geven bruikbare informatie over de mogelijkheid om ook in andere gewassen *Mlo*-achtige vatbaarheids genen te vinden.

In **Hoofdstuk 5** tenslotte werd onderzocht of verlies van functie van *Mlo* genen resistentie tegen *L. taurica* in paprika en tomaat kan geven. Dit bleek inderdaad het geval te zijn in tomaat (verlies van functie van het *SlMlo1* gen geeft resistentie tegen *L. taurica)*. In paprika was eerder een *Mlo* homoloog (*CaMlo1*) beschreven die mogelijk betrokken was bij meeldauw resistentie. Via homologie gebaseerde klonering kon naast dit al bekende gen een nieuw gen, *CaMlo2,* geïsoleerd worden. Fylogenetische analyse toonde aan dat *CaMlo2* meer homoloog is met *SlMlo1* dan het *CaMlo1* gen. De expressie van *CaMlo2* was sterker en ‘up-regulated’ na infectie met *L. taurica*. Functionele analyse via VIGS liet zien dat uitschakelen van de expressie van zowel *CaMlo1* of *CaMlo2* resulteerde in verlaagde vatbaarheid van paprika tegen *L. taurica*.

Signaal transductie mechanismen bepalen de afweer reactie tegen pathogenen in planten. Om te bepalen welke signaal transductie routes een rol spelen in de basale en *mlo*-gebaseerde resistentie werden in **Hoofdstuk 6** de interacties tussen *O. neolycopersici* en verschillende pre- en post-penetratie signaal transductie route (onder andere PEN1, PEN2/PEN3, SA, JA and ET) deficiente *Arabidopsis* mutanten bestudeerd. De driedubbel

gemuteerde *Atmlo2/6/12*-penetratie resistentie in *Arabidopsis* blijkt gebaseerd te zijn op een onbekend afweer mechanisme dat onafhankelijk van de *Pen* genen functioneert. De *Atmlo2* resistentie berust hoogstwaarschijnlijk op het correct functioneren van het op salicylzuur reagerende signaal transductie mechanisme tegen *O. neolycopersici*. De resultaten beschreven in dit proefschrift laten zien dat voor verschillende plantensoorten *mlo*-gebaseerde resistentie een feit is, maar ook dat de overmaat van gen functies van de verschillende homologen en hun interactie met verschillende signaal transductie routes kunnen verschillen afhankelijk van plantensoort en meeldauw soort.

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Acknowledgments

ABOUT THE AUTHOR

Zheng Zheng was born in Xuchang (China) on April 7th 1982. He entered the joint program between China Agricultural University and Larenstein University of Professional Education in 2001 and obtained his BSc degree in Plant Biotechnology in 2005. He continued his MSc study in Wageningen University in 2005 and obtained his MSc degree in Plant Science with specialization of Plant Breeding in 2007. Research presented in this thesis is the result of his PhD research on Exploration of *mlo*-based resistance in vegetable crops, which is partly funded by the Wageningen UR Plant Breeding and the Technological Top Institute Green Genetics (TTI-GG).

He is currently working as a Junior Researcher in the Institute of Vegetables and Flowers at Chinese Academy of Agricultural Sciences (IVF-CAAS). After his defence for the doctorate, he will return to IVF-CAAS to continue his career in tomato research.

CONTACT INFORMATION

Zheng Zheng

Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences

Address: Zhongguancun Nandajie NO.12, Haidian District, Beijing 100081, China

Tel: 86-10-82109530

Fax:86-10-62174123

E-mail: zhengzheng@caas.net.cn / zheng.zheng@live.com

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Zheng Zheng
Date: 4 December 2012
Group: Plant Breeding, Wageningen University & Research Centre, The Netherlands

1) Start-up phase	date
► **First presentation of your project**	
Exploration of *mlo* -based resistance in vegetable crops	Nov 27, 2007
► **Writing or rewriting a project proposal**	
Exploration of *mlo* -based resistance in vegetable crops	2008
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7,5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Day, Wageningen	Sep 13, 2007
EPS PhD Student Day, Leiden	Feb 26, 2009
EPS PhD Student Day, Utrecht	Jun 01, 2010
► **EPS theme symposia**	
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Utrecht	Jan 23, 2009
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Utrecht	Jan 15, 2010
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Amsterdam	Feb 03, 2011
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Experimental Plant Sciences, Lunteren	Apr 07-08, 2008
NWO-ALW Experimental Plant Sciences, Lunteren	Apr 06-07, 2009
NWO-ALW Experimental Plant Sciences, Lunteren	Apr 19-20, 2010
NWO-ALW Experimental Plant Sciences, Lunteren	Apr 04-05, 2011
► **Seminars (series), workshops and symposia**	
PBR Research Day, 4x times	2008-2011
Seminar Jian-Kang Zhu, Mechanism and function of active DNA demethylation in arabidopsis	Nov 03, 2008
Seminar Pamela J. Hines, Science from an editor's view	Nov 06, 2008
Seminar Isgouhi Kaloshian, Tomato innate immunity to root-knot nematodes and aphids	May 14, 2009
Seminar J.D.H. Keatinge, AVRDC and WUR need to fight the battle against poverty & malnutrition together	Jun 18, 2009
Plant Science Seminar, Harro Bouwmeester (Plant Physiology) & Ton Bisseling (Molecular Biology)	Sep 08, 2009
Plant Science Seminar, Fred van Eeuwijk (Biometris) & Pierre de Wit (Phytopathology)	Nov 10, 2009
Illumina Agrigenomics Seminar	Mar 03, 2010
EPS Seminar Paul Birch, Trying to understand susceptibility and exploit resistance in potato-Phytophthora infestans interaction	May 20, 2010
Seminar David Baulcombe, Mobile RNA silencing in plants	Sep 27, 2010
► **Seminar plus**	
► **International symposia and congresses**	
XIII International Congress on Molecular Plant-Microbe Interaction, Sorrento, Italy	Jul 21-27, 2007
Genomics Momentum 2007, Amsterdam, The Netherlands	Nov 28, 2007
XVIth EUCARPIA tomato Meeting, Wageningen, The Netherlands	May 12-15, 2008
SOL 2008 Cologne, Germany	Oct 12-16, 2008
XIV International Congress on Molecular Plant-Microbe Interaction, Quebec, Canada	Jul 19-23, 2009
SOL 2010 Dundee, UK	Sep 05-09, 2010
From plant biology to crop biotechnolgy, Suzhou, China	Oct 25-29, 2010
► **Presentations**	
Oral Presentations to TTI-GG Breeding Companies (per half year)	2009-2011
Poster Presentation XIII International Congress on Molecular Plant-Microbe Interaction, Sorrento, Italy	Jul 21-27, 2007
Poster Presentation XIV International Congress on Molecular Plant-Microbe Interaction, Quebec, Canada	Jul 19-23, 2009
Poster Presentation on PBR Research Day	Feb 08,2010
Poster Presentation on SOL 2010	Sep 05-09, 2010
Poster Presentation on TTI GG Networking Event	Sep 22, 2010
Oral Presentation on TTI GG Networking Event	Sep 21, 2011
► **IAB interview**	Dec 05, 2008
► **Excursions**	
Subtotal Scientific Exposure	*29,0 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
EPS Summer School - On the Evolution of Plant-Microbe Interaction	Jun 18-20, 2008
EPS Summer School - Environmental Signaling	Aug 24-26, 2009
Bioinformatics - A User's Approach	Mar 15-19, 2010
► **Journal club**	
Plant Breeding Journal club, Monday morning	2007-2011
Resistance Group weekly seminars, Tuesday morning	2007-2011
► **Individual research training**	
Subtotal In-Depth Studies	*6,3 credits**

4) Personal development	date
► **Skill training courses**	
Mobilising your Scientific Network	Jun 03 &10, 2010
Workshop: Search the digital library efficiently	Jul 13, 2010
Moral Dilemmas in Daily Scientific Practices	Feb 15-16, & 18, 2011
How to write a world-class paper	Apr 19, 2011
► **Organisation of PhD students day, course or conference**	
Organisation of international food tasting evening in Plantbreeding department	Jun 24, 2010
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**45.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

Cover: Zheng Zheng and Zheng Yao
Artistic representation of *Leveillula taurica* conidia penetrates host through plant stoma.