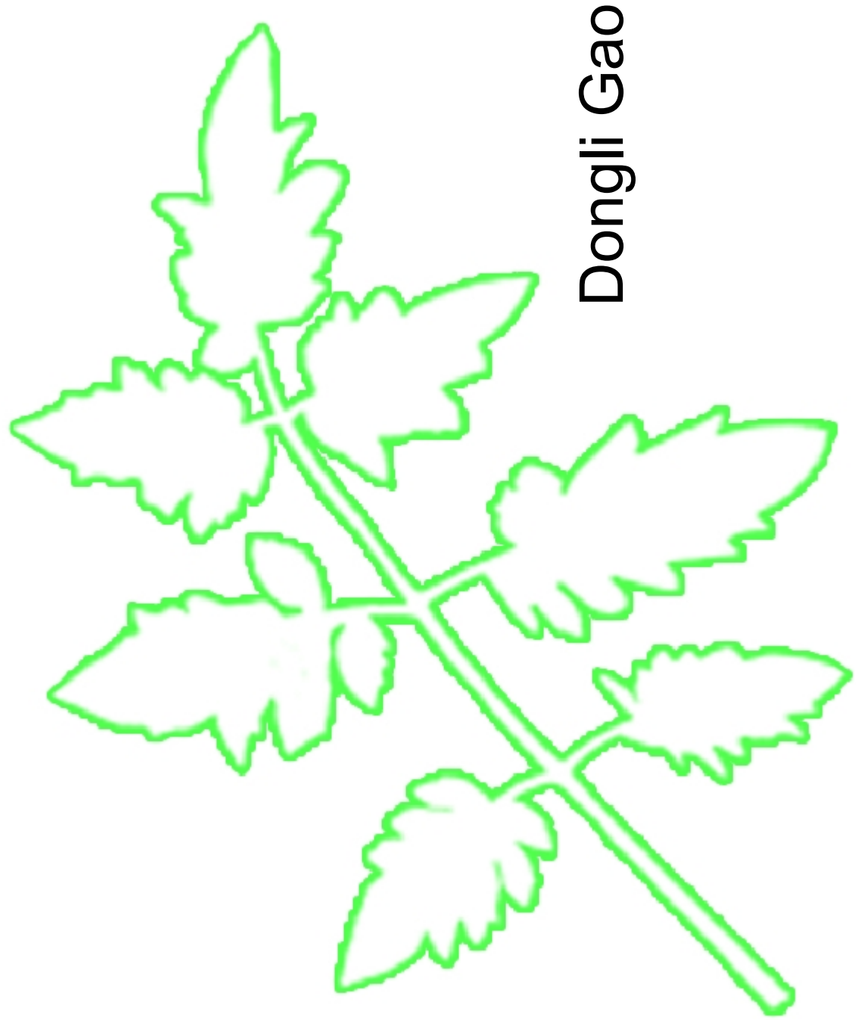
Identification of genes affecting the response of tomato and Arabidopsis upon powdery mildew infection



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Identification of genes affecting the response of tomato and Arabidopsis upon powdery mildew infection

Dongli Gao

Thesis

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Contents

Chapter 1

General introduction

Powdery mildew infection biology

Plants are continuously exposed to various pathogens and pests including viruses, bacteria, fungi, oomycetes, nematodes and insects. Based on their lifestyle, plant pathogens are generally divided into biotrophs and necrotrophs. Biotrophs feed on living tissues for growth and development, while necrotrophs derive nutrients from dead or dying tissues. Many plant pathogens use both lifestyles depending on the stage of their life cycle, and are called hemibiotrophs.

Powdery mildew fungi are excellent examples of biotrophic pathogens. The infection process of powdery mildew begins with the germination of conidia on the leaf surface (Glawe 2008) (Figure 1). The spore produces a short germ tube, which elongates and forms an appressorium. Then the appressorium produces a penetration peg to breach host cell walls by means of turgor pressure and enzymatic activity. The penetration peg extends into the host cell, invaginating the cytoplasm, and swelling to form the haustorium. The mature haustorium is a unicellular and convoluted structure and shielded from plant cytoplasm by its own extrahaustorial membrane (Micali et al. 2008). Successful establishment of the haustorial complex allows the pathogen to absorb water and nutrients from hosts to support the extracellular growth of hyphae (Voegele et al. 2001). The haustoria are thought to be sites for delivering pathogen proteins (effectors) to suppress defense responses of the plants (Whisson et al. 2007). The generation of asexual spore carriers, conidiophores, which harbour the next generation of conidia, completes the asexual powdery mildew life cycle. Typical disease symptoms caused by powdery mildew are the appearance of white spots consisting of coalesced hyphae and conidiophores on the surface of infected plant organs (predominantely leaves) (Figure 1).

Powdery mildew resistance system

Plants have evolved a suite of defense responses to resist biotrophic pathogens (Jones and Dangl 2006) (Figure 1). The primary immune response is mounted when a receptor recognizes invariant microbial structures referred to as pathogen-associated molecular patterns (PAMPs). Examples of fungal PAMPs are xylanase, cell-wall derived chitin and endopolygalacturonases (Boller and Felix 2009; Monaghan and Zipfel 2012). The corresponding receptors, Ethylene Inducing-Xylanase (EIX)2 in tomato, Chitin Elicitor Receptor Kinase (CERK)1 in Arabidopsis, Chitin Elicitor Binding Protein (CEBiP) in rice, Responsiveness to Botrytis Polygalacturonase-1 (RBPG1) in Arabidopsis have been identified (Ron and Avni 2004; Miya et al. 2007; Shimizu et al. 2010; Zhang et al. 2014). It was found that the chitin receptor CERK1 is essential for resistance against powdery mildew fungus *G. cichoracearum* in Arabidopsis (Wan et al. 2008). PAMP perception initiates signalling cascades involving Ca^{2+} fluxes, reactive oxygen species (ROS), nitric oxide (NO) as well as mitogen-activated protein kinases (MAPK), which leads to defense reactions called PAMP-triggered immunity (PTI) (Nürnberger and Lipka 2005). Specialized pathogens have evolved effectors to suppress PTI and establish effector-triggered susceptiblity (ETS). In turn plants have acquired resistance (*R*) proteins that recognize corresponding effectors resulting in a secondary immune response called effector-triggered immunity (ETI). The defense responses that are activated during PTI and ETI show substantial overlap but greater amplitude in the latter (Tsuda et al. 2008; Tsuda and Katagiri 2010). These defenses include cell wall fortification through the synthesis of

callose and lignin; the production of antimicrobial secondary metabolites and the expression of pathogenesis-related (PR) proteins (Pieterse et al. 2009). ETI is effective to specific pathogen races, and is associated with programmed cell death, a response that is referred to as hypersensitive response (HR). Race-specific resistance to powdery mildew has been reported for several genes, such as *Mla* genes in barley, *Pm3b* in wheat, *Run1* in grapevine, and *Rpp1* in rose (Jørgensen 1994; Yahiaoui et al. 2004; Donald et al 2002; Linde et al 2004). *R*-genes belong to different classes based on the presence of structural motifs (Dangl and Jones 2001; Martin et al 2003): (1) proteins with nucleotide binding site and leucine-rich repeats (NB-LRR) domains; (2) proteins containing a transmembrane (TM) domain and an extracellular LRR; (3) serine/threonine kinase proteins; (4) proteins combining LRR and TM domains with a serine/threonine kinase region. Besides there are a few *R*-proteins which do not fit in the defined classes (Fradin et al. 2009; Verlaan et al. 2013). Although in most cases *R*-genes provide race-specific resistance, they can also provide broad-spectrum resistance, such as *RPW8* in Arabidopsis, which confers resistance to a broad range of powdery mildew pathogens (Xiao et al. 2001).

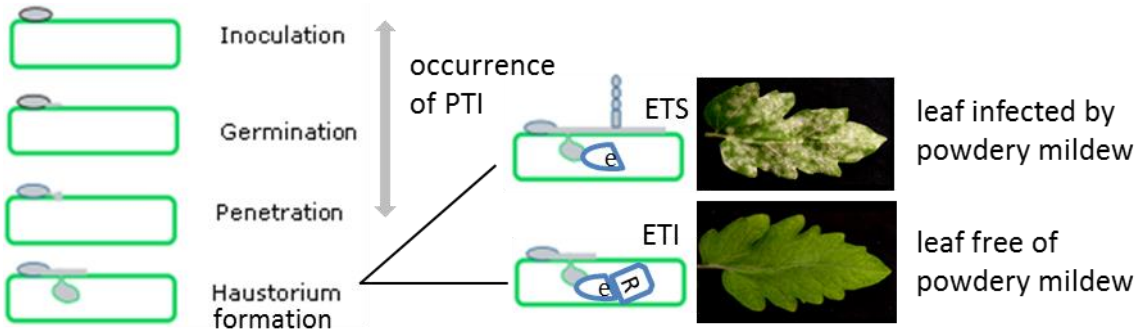

Figure 1 Overview of powdery mildew infection process and plant immunity system. PAMP-triggered immunity (PTI) can occur when the pathogen proceeds to penetrate the cell wall. However, PTI can be overcome when the pathogen secretes an effector (e) from the haustorium, making the plant susceptible (effector-triggered susceptibility or ETS). When the host contains an *R*-gene (R) that recognizes the effector, effector-triggered immunity (ETI) is established and the plant becomes resistant. (Figure is adapted from http://nature.berkeley.edu/wildermuthlab/Wildermuth_Lab_website/Research_Overview.html).

Besides *R* genes, susceptibility (*S*) genes have been identified in plants, including Arabidopsis and some crop plants. *S*-genes can be immunity-related when they exert negative control of defense, for instance, to accommodate the haustorial complex in plant cells for biotrophic pathogens. *S*-genes can also be immunity-unrelated when they serve demands of the pathogens in the process of pathogen development, accommodation and propagation (Lapin and Van den Ackerveken 2013; Hückelhoven et al. 2013). The presence of *S*-genes promotes disease susceptibility, while impairment of *S*-genes leads to loss of susceptibility and recessively inherited resistance. Disabling or interfering with host *S*-genes has the potential to provide durable resistance, as opposed to the short-lived resistance provided by the typical *R*-genes (Lapin and Van den Ackerveken 2013). In Arabidopsis, tomato and other crop species, *S*-genes have been

identified for loss of susceptibility to powdery mildew pathogens (Micali et al. 2008; Pavan et al 2010; Pavan et al 2011; Humphry et al 2011).

A given powdery mildew species can infect a narrow range of host plants, or even a particular host species. For example, *Blumeria graminis s*pecies infect cereal crops*,* and the *formae speciales (*f. sp.) *hordei* (*Bgh*) exclusively feeds on barley while f. sp. *tritici (Bgt)* infects wheat. Resistance shown by a particular plant species to all genetic variants of a non-adapted pathogen is defined as non-host resistance. In Arabidopsis, arrest of the penetration of non-adapted powdery mildew fungi is a major mechanism of non-host resistance. Three genes *PENETRATION1* (*PEN1*), *PEN2* and *PEN3* were identified, and elimination of their functions in individual mutants promoted entry and haustorium formation of *Bgh* in Arabidopsis (Lipka et al. 2005). Other components that were described to contribute to Arabidopsis pre-invasion non-host resistance against non-adapted powdery mildews include S-nitrosoglutathione reductase (GSNOR1), transcription factor ATAF1, and phopholipase Dδ (Feechan et al. 2005; Jensen et al. 2007; Pinosa et al. 2013). Although enhanced entry was observed in *pen* mutants, further fungal growth was aborted owing to the post-entry cell death response (Lipka et al. 2005). This postinvasion non-host resistance depends on Enhanced Susceptibility 1 (*EDS1*), Phytoalexin-Deficient 4 (*PAD4*) and Senescence-Associated Gene 101 (*SAG101*), which have been found to be involved in basal defense and some *R*-gene pathways (Wiermer et al. 2005). It is believed that PTI contributes to non-host resistance, evidenced by the induction of *PEN* genes by a bacterial PAMP (Lipka et al. 2008). Although HR plays a role in postinvasive non-host resistance, whether this reflects the involvement of *R*-gene-mediated ETI requires further investigation (Ellis 2006).

Plant hormone signalling plays an important role in the regulation of defense responses. The importance is usually demonstrated by the response of plants that are deficient in the hormone biosynthesis or are blocked in hormone signalling. Jasmonate (JA) and ethylene (ET) regulated defenses are efficient to deter necrotrophic pathogens. On the other hand salicylic acid (SA) synthesis is stimulated upon attack of biotrophic pathogens, and SA-dependent pathways are responsible for activation of defense-related genes (Glazebrook 2005). A global expression profiling for a SA biosynthetic mutant revealed that SA impacts processes including redox, vacuolar transport/secretion, signalling, and iron and calcium homeostasis in the Arabidopsis-powdery mildew *G. orontii* interaction (Chandran et al. 2009).

Powdery mildew *O. neolycopersici* and its hosts

The infection process mentioned above does very nicely apply to the tomato powdery mildew *Oidium neolycopersici* (*On*). Upon infection of tomato*, On* germination starts at 3-6 hours post inoculation (hpi), appressorium differentiation 6-8hpi and penetration at around 11hpi (Jones 2001). By 48 hours, extensive secondary hyphae radiate from both the primary appressorium and the conidium (Jones 2001). At 7-10 days, disease symptoms are visible to the naked eye. The isolates of *On* display considerable variability, and are able to infect the representatives of taxonomically distant groups (Lebeda et al. 2013). In this thesis, two hosts, tomato (*Solanum lycopersicum*) and *Arabidopsis thaliana*, were employed to study their interactions with *On*.

Tomato (*Solanum lycopersicum*)

Tomato (family nightshades) is an economically important vegetable crop worldwide, and the fruits can be consumed either fresh or in the form of processed products. Depending on its use, different breeding objectives are implemented such as boosting yield, sensory and nutritional quality, as well as adaptation to biotic and abiotic stresses. As for other crops, tomato improvement hinges on the existence of favourable traits in all kinds of germplam. However, cultivated tomato displays little genetic diversity resulting from its inbreeding mating system. Tomato wild species possess substantial genetic variation and have been exploited to meet the breeding challenges.

The plant group *Solanum* sect. *lycopersicon* consists of *S. lycopersicum*, which includes domesticated tomato and *S. lycopersicum* cerasiforme, and 12 wild relatives including *S. pennellii*, *S. chilense*, *S. corneliomulleri*, *S. habrochaites*, *S. huaylasense*, *S. peruvianum*, *S. arcanum*, *S. chmielewskii*, *S. neorickii*, *S. cheesmaniae*, *S. galapagense*, and *S. pimpinellifolium*. In addition, there are four tomato-like nightshades *S. juglandifolium*, *S. lycopersicoides*, *S. ochranthum*, and *S. sitiens*. Each species is adapted to prevailing environments and represents a potential source for the improvement of many important traits (Grandillo et al. 2011). For example, *S. habrochaites* is usually found at high altitude and expected to be a source of tolerance to low temperature. *S. chmielewskii* and *S. neorickii* prefer growing in moist conditions. In contrast, *S. pennellii* is adapted to hot dry environments and has long been regarded as an excellent source for tolerance to drought and numerous insects. The use of wild species as sources of traits of interest is largely influenced by crossing ability with cultivated tomato. Generally when the phylogenetic distance between the parental species is larger, the hybridization limitation is more severe (Grandillo et al. 2011). Most of the tomato wild species can be crossed with cultivated tomato, although some crosses require embryo or ovule rescue (Grandillo et al. 2011). Crosses with tomato-like nightshades have encountered more limitations, as severe reproductive barriers isolate them from the core tomato group (Smith and Peralta 2002).

Cultivated tomato belongs to the *S. lycopersicum* species. It is believed that domestication is often attributable to very few genetic loci (Gross and Olsen 2010). Koenig et al. (2013) revealed that at the transcriptional level a relatively small number of changes is associated with tomato domestication. Domesticated tomato and most wild tomato species are diploid (2n=24), and they show a strong genomic synteny (Chetelat and Ji 2007; Stack et al. 2009). The genome sequence of tomato cultivar "Heinz 1706" has been published in 2012, and the predicted genome size is 900 Mb (The Tomato Genome Consortium 2012). There are around 35000 genes arranged on 12 chromosomes, and each of the chromosomes consists of pericentric heterochromatin and euchromatin at the distal ends (The Tomato Genome Consortium 2012). Functional analysis of tomato genes is relatively easy beacuse of the well-established protocols for VIGS (virus-induced gene silencing) and stable transformation. These features including availability of the genome sequence, a rich reservoir of wild species, and the amenability to genetic manipulation reinforces the extensive use of tomato as a research subject, especially in the study of fruit development and size.

Disease resistance is recognized as an important goal for tomato breeding. Tomato wild species are a valuable source of genes conferring resistance to various pathogens and pests. At present most genes and quantitative trait loci (QTLs) for disease

resistance have been identified in the related wild species of tomato (Foolad 2007). Regarding *On* resistance, a number of loci has been identified, including *Ol-1*, *-3*, *-4*, *-5*, *-6*, *ol-2* and three QTL (Bai et al. 2003; Bai et al. 2005). *Ol-4* and *Ol-6* encode NB-LRR proteins (Seifi et al. 2011), *ol-2* is a non-functional *mlo* ortholog (Bai et al. 2008), and the identities of the remaining genes are unknown. In contrast to the wide use of wild species for traits of interest, it is rather difficult to implement high-throughput mutagenesis in tomato due to four reasons (Emmanuel and Levy 2002). In the first place, tomato needs substantial amounts of time and space to complete its life cycle. Second, although possible, tomato transformation remains a time-consuming and daunting task. Third, no well-characterized native tomato transposons are available yet for efficient insertion mutagenesis. Fourth, seeds have to be harvested from tomato fruits, which requires large-scale processing in a short period. So far no transposon-tagged or T-DNA insertion population is available in tomato to facilitate forward genetic screens, but chemical and fast-neutron mutagenesis have been implemented (Gady et al. 2012; Meissner et al. 1997; Isaacson et al. 2002).

Arabidopsis thaliana

Arabidopsis thaliana (family Brassicaceae) has been adopted as a major model plant for genetic and molecular research. This is mainly thanks to the amenability of Arabidopsis to artificial manipulation based on several features. First, Arabidopsis plants are small and have a rapid generation time. Usually within three months an entire life cycle can be completed from seed germination to mature seed production. Second, Arabidopsis is self-fertilizing, and bears thousands of seeds per plant. Third, it has a small plant genome size (125 Mb), with fewer repetitive sequences than any known higher plant. Fourth, Arabidopsis can be readily transformed without the tedious and time-consuming tissue culture. Due to these features, Arabidopsis has undergone substantial artificial mutagenesis, through which two types of mutants have been created. Disruption of gene expression gives rise to knock-out mutants (Radhamony et al. 2005), while activation tagging mutants result in promotion of gene expression (Weigel et al. 2000; Marsch-Martinez et al. 2002). Both of them have been used to screen for phenotypes of interest, and subsequently isolate the target gene so that a function can be assigned to the gene. Despite the availability of large mutant collections, the identification of gene functions by studying induced mutants is limited by the small number of genetic backgrounds analyzed. Arabidopsis strains that are generally used for mutagenesis are Landsberg *erecta* (Ler), Columbia (Col), Wassilewskija (Ws) and C24 (Page and Grossniklaus 2002). It is unlikely to detect mutant phenotypes of genes if the wild-type strain carries a natural null allele or a weak allele. This limitation can be overcome by using natural accessions, as an alternative source of (induced) mutants.

Arabidopsis is indigenous to Europe and central Asia and now is naturalized worldwide (Al-Shehbaz and O'Kane 2002). There are over 6000 accessions collected from different geographical regions (Brennan et al. 2014). By direct analysis and comparison of accessions, natural genetic variation was observed for many traits including resistance to biotic factors, tolerance to abiotic factors, developmental traits, physiological traits, biochemical traits and complex genetic mechanisms (Koornneef et al. 2004). Because some of these traits reflect adaptations to specific environments, analysis of natural variation offers an ecological and evolutionary perspective. One challenge for exploring

natural variation is that frequently such phenotypic variability is a consequence of genetic changes in multiple genes, which is in contrast to changes at a single locus for commonly studied mutants. This can be solved by application of methods of quantitative genetics that were developed and used extensively in crop plants. The general procedures are selection of two contrasting parents for the trait of interest, development of an experimental mapping population, genotyping with markers throughout the genome and phenotyping for the particular trait, Mendelizing quantitative trait loci using advanced lines, and molecular isolation of loci by association analysis between phenotypic values of the trait and genotypic classes of the polymorphic markers. In this way, many genes of agronomic interest have been uncovered, including the ones conferring disease resistance (reviewed in Koornneef et al. 2004).

Scope of the thesis

In this study we used tomato and Arabidopsis to identify genetic factors that affect or determine the outcome of interaction with tomato powdery mildew *On*. In tomato, we concentrated on *Ol-1* conferring isolate-non-specific resistance. It is of great interest to elucidate the resistance mechanism for this gene. In Arabidopsis, we screened natural accessions and activation tag lines, and aimed to identify novel genes providing resistance to powdery mildew and other pathogens.

Chapter 2 provides a comprehensive overview into the genetics and molecular mechanisms of *On* resistance in tomato. In this review, the mechanisms of powdery mildew resistance in tomato, Arabidopsis, barley and wheat are compared. Furthermore this knowledge is put in the context of our current understanding of pathogen resistance, and strategies to realize powdery mildew resistance in tomato breeding are proposed. In **chapter3** the resistance signalling pathway of *Ol-1* is dissected. *Ol-1* originates from *S. habrochaites* and conditions *On* resistance. We showed that *Ol-1*-mediated resistance requires acetolactate synthase (ALS) activity. ALS is involved in the biosynthesis of branched-chain amino acids, and is a target of commercial herbicides. Three *ALS* genes were identified in the tomato genome. Silencing of two of them simultaneously, or inhibition of the activity of ALS by herbicidal treatment compromised *Ol-1*-mediated resistance. We further proved that the requirement of ALS is specific for *Ol-1*. **Chapter 4** presents a study of natural variation of responses to *On* infection in Arabidopsis accessions. We showed that natural accessions display varying levels of resistance to *On* infection, and polygenic resistance is a major source of resistance. The genetic basis of resistance in accession C24 was dissected by QTL analysis and map-based cloning. We found that a premature stop codon occurred in the gene *EDR1* of the C24 accession used in this study (referred to as C24-W), although this mutation is not present in regular C24. Previously the *edr1* mutation induced in the background of Col-0 was reported (Frye and Innes 1998). The *edr1* mutation in C24-W occurred at a different position of the gene, thus we have identified a new *edr1* mutant. To investigate whether tomato takes advantage of a similar resistance mechanism, we generated stable transformants in which two *EDR1* homologues were suppressed individually. Disease assays showed that silencing of these two genes had no effect on the susceptibility of the transformants. **Chapter 5** describes a resistant Arabidopsis mutant, 3221, which was identified after screening a previously described activation tag collection. 3221 shows altered leaf morphology, and exhibits resistance to powdery mildew *On*, downy mildew

Hyaloperonospora arabidopsidis and green peach aphid *Myzus persicae*. All these phenotypes are caused by overexpression of a HD-Zip transcription factor *ATHB13*. In 3221, constitutive expression of *ATHB13* promotes massive changes in gene expression. Elevated expression was observed for stress inducible genes, such as *PR1*, *EDS5* and *WRKYs*, and genes likely to be involved in insect resistance. One gene, *NUDT24*, was strongly down-regulated, and we investigated whether it is a potential susceptibility gene. Disease assays showed that the *nudt24* knock-out mutant supported similar levels of fungal sporulation as the background Col-0. In **chapter 6**, the results obtained from the experimental chapters are discussed with reference to the advancement in understanding of resistance mechanisms and gene functions. Additionally, the practical use of the knowledge to achieve *O*n resistance in tomato is discussed.

Chapter 2

Genetics and molecular mechanisms of resistance to powdery mildews in tomato (*Solanum lycopersicum*) and its wild relatives

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Genetics and molecular mechanisms of resistance to powdery mildews in tomato (*Solanum lycopersicum*) and its wild relatives

Abstract
Powdery mildews (PMs) cause disease in a wide range of plant species including important crops. Taking tomato as an example, here we review findings on the genetic basis and mechanisms of plant resistance to PMs. First, we present a summary of our research on tomato resistance to two PM species, with the focus on *Oidium neolycopersici*. We discuss the genetics of resistance to this pathogen in tomato. Then, we compare different forms of resistance mediated by different resistance genes based on molecular and cytological data. Also, we provide a comparison between these resistance genes in tomato with those in barley, Arabidopsis and wheat, in order to present a model for the genetic basis of resistance to PMs in plants. We try to accommodate these resistance mechanisms in the current model of plant innate immunity. At the end we discuss possibilities to translate these findings to practical approaches in breeding for resistance to PMs in crops.

Keywords Tomato, Powdery mildew, Resistance genes, Susceptibility genes, Resistance breeding

Introduction

Powdery mildews (PMs) are obligate biotrophic fungal pathogens that establish long-lasting interactions with their living host tissues by forming haustoria in plant cells. There are approximately 700 PM species capable of colonizing about 10,000 plant species (Braun and Cook 2012). These fungal pathogens produce discernible symptoms consisting of white colonies of mycelia on the surface of aerial green organs and sometimes on fruits upon heavy infection (Jones et al. 2001). The interaction of PMs with tomato, barley and Arabidopsis are well studied and, therefore, these pathosystems provide experimental models for understanding host and nonhost resistance to PMs (Bai et al. 2005; Hückelhoven 2005; Li et al. 2007; Schulze-Lefert and Vogel 2000).

Three PM species can infect tomato (*Solanum lycopersicum*), namely *Oidium neolycopersici, O. lycopersici* and *Leveillula taurica.* Upon the outbreak of *O. neolycopersici* in Europe in the late 1980s, all tomato cultivars turned out to be susceptible to this pathogen and this disease had to be controlled by using fungicides in greenhouse tomato production in Northwest Europe (Huang et al. 2000a). Requested and also supported by Dutch vegetable seed companies, we started our research on searching for resistance genes against this pathogen in 1996.

We have identified five dominant resistance genes (*Ol*-genes) from wild tomato species and introgressed them into cultivated tomatoes*,* and cloned one recessive gene (*ol-2)* that confers *mlo*-based broad-spectrum resistance (Bai et al. 2005, 2008). In addition, we have mapped and introgressed three quantitative trait loci (QTLs) conferring different levels of resistance to *O. neolycopersici.* After many years, we have been able to set up tomato as the third well-characterized plant system, after barley and Arabidopsis, to study the interaction between plants and obligate PMs.

Plant innate immunity relies on a set of specialized receptors, so called pattern-recognition receptors (PRRs), which recognize microbe-associated molecules (Ausubel 2005). There are two groups of PRRs in plant cells: PAMP-receptors and resistance (*R*) proteins (in the literature PRR is sometimes used only to describe PAMP-receptors). PAMP-receptors are plant molecules that can perceive pathogen-associated molecular patterns (PAMPs), which are evolutionary conserved pathogen-derived molecules (*i.e.*, chitin in fungi and flagellins in bacteria). *R*-proteins are localized in the plasma membrane (like Cf-2 and Xa21 proteins) or, more frequent, in the intracellular area. The most common *R*-proteins are the NBS-LRR (nucleotide-binding site-leucine rich repeats) proteins. Based on these two types of receptors, plant innate immune system has been divided into two distinct processes in a model known as Zig-Zag model (Jones and Dangl 2006). According to this model, perception of PAMPs by PAMP-receptors results in PAMP-triggered immunity (PTI), while *R*-proteins perceive pathogen effectors (directly or indirectly) (Dangl and Jones 2001) and thereby, activate effector-triggered immunity (ETI) (Jones and Dangl 2006).

The study of plant-pathogen interactions involves communication between two living organisms, and thus, requires knowledge from both sides. Although we have characterized the mechanisms by which tomato respond to PM infection, the mechanism of *O. neolycopersici* pathogenicity is still largely unknown. One reason for this knowledge gap is that this obligate fungus needs to be maintained and propagated on tomato plants and, like other obligate PMs, is not easily amenable to molecular analysis (Bardin et al. 2007). Moreover, its sexual stage has not been reported so far, and this hampers genetic

studies on this fungus (Lebeda et al. 2014). One way to compensate this shortage of knowledge is to explore the discovered mechanisms of pathogenicity in other PM species. Till now, a few fungal effectors have been cloned including two effectors from *Blumeria graminis* f. sp. *hordei* (*Bgh*), the causal agent of barley PM disease (Kamoun 2007). Thanks to recent advances in next generation sequencing technologies, the genomes of three PM species (*Bgh* infecting barley, *Erysiphe pisi* infecting pea and *Golovinomyces orontii* infecting Arabidopsis) have been sequenced and a pile of information of putative effectors in these PMs is now available (Spanu et al. 2010).

In this review, we first summarize the genetics, specificity and (molecular) mechanisms of tomato resistance to powdery mildews, with the focus on the *Ol*-genes and QTLs identified for resistance to *O. neolycopersici*. We then compare the genetics and mechanisms of tomato defense against *O. neolycopersici* with that in barley and Arabidopsis in response to their adapted PM species, in order to understand common mechanisms, if any, by which plants defend themselves against PMs. Further, we discuss the resistance to different PMs in the context of PTI and ETI. Finally, we present our thoughts on potential approaches for achieving durable resistance to PMs in crops.

Powdery mildews infecting tomato

Oidium lycopersici and *O. neolycopersici*

The first report on tomato PM dates back to the late 19th century when *O. lycopersici* was found in Australia (Cooke and Massee 1888). After almost a century an epidemic of tomato PM occurred in the Netherlands and spread within the next 10 years to all European countries. *O. lycopersici* was initially assumed to be the cause for this epidemic, however, later studies discovered that the causal agent is *O. neolycopersici* (Jones et al. 2000, 2001; Kiss et al. 2001). Now it is believed that *O. neolycopersici* is present worldwide, except in Australia where *O. lycopersici* is the causal agent for PM disease in tomato (Kiss et al. 2001, 2005). Although there is not a consensus on the host range of *O. neolycopersici* (Jones et al. 2001; Lebeda et al. 2014), there is some evidence suggesting that this pathogen is adapted to plant species from 13 plant families (Whipps et al. 1998; Jankovics et al. 2008). For further information of *O. lycopersici* and *O. neolycopersici*, please see the comprehensive review by Lebeda et al. (2014).

Leveillula taurica

Another PM fungus that can infect tomato is *Leveillula taurica* (Lév.) Arnaud (asexual state *Oidiopsis taurica* (Lév.) Salmon). Morphologically, *L. taurica* can be easily distinguished from *O. neolycopersici*. The mycelia of *L. taurica* grow through mesophyll and are visible on the abaxial side of the leaf, while *O. neolycopersici* grows mainly on the adaxial side and does not penetrate into the mesophyll (Lindhout et al. 1994).

L. taurica is an important pathogen of tomato in hot and dry tropical to sub-tropical zones, and in glasshouses (Blancard 2012). It can also infect pepper, eggplant, cucumber, onion, cotton and other crops, as well as many wild plant species (Braun and Cook 2012). In total, more than 1000 species belonging to 74 botanical families are hosts for *L. taurica* (Palti 1988). Molecular analyses revealed that *L. taurica* is actually an aggregate species consisting of several biological lineages, for which the exact host range is not known (Khodaparast et al. 2001, 2007, 2012).

While *O. neolycopersici* is epiparasitic, as most other powdery mildew species are, *L. taurica* grows endophytically. In tomato, after germination of conidia on the leaf surface the hyphae enter the leaf directly by perforating the cuticle, and subsequently develop an intercellular mycelium in the mesophyll tissue. Infection hyphae grow into the spongy and sometimes the palisade parenchyma tissues. Penetration pegs penetrate the host's cell wall followed by the development of haustoria (Palti 1988). After a latency period of approximately 20 days conidiophores emerge from stomata, mainly on the abaxial leaf surface, and produce spearheaded terminal conidia (Blancard 2012). Usually, at this stage bright yellow spots are visible on the adaxial leaf surface and become necrotic later. Eventually, the complete leaf may turn yellow, shrivel and dry up, but it remains attached to the plant. At this stage fruits are exposed to destructive sun scorch, resulting in economic losses (Palti 1988). It is worthwhile to note that the infection process of *L. taurica* in tomato is different from that in pepper (Zheng et al. 2013a).

The genetics and mechanisms of resistance to powdery mildews in tomato (*Solanum lycoperisum*) and its wild relatives

Cultivated tomato has limited variability, largely because of artificial selection during domestication and development of modern cultivars. To improve disease resistance and agronomic traits, tomato wild germplasm is a useful resource (Bai and Lindhout 2007).

Resistance to *L. taurica*

Tomato cultivars differ greatly in their susceptibility to *L. taurica* (Palti 1988). Resistant accessions of *S. lycopersicum* var. *cerasiforme* and wild tomato species *S. chilense*, *S. habrochaites* and *S. peruvianum* have been reported (Palti 1988; Hernandes and Stamova 1990). The dominant resistance gene *Lv* from *S. chilense* accession LA1969 (Yordanov et al. 1975; Stamova and Yordanov 1987, 1990) is effective against *L. taurica*, but not against *O. neolycopersici* (unpublished data). This gene, which is mapped on chromosome 12, confers resistance via inducing hypersensitive response (HR) (Chungwongse et al. 1994, 1997). The *Lv* gene has been the only gene in tomato germplasm for resistance to *L. taurica*. The recessive *ol-2* gene (Ciccarese et al. 1998; Bai et al. 2008) identified in *S. lycopersicum* var. *cerasiforme* confers incomplete resistance to *L. taurica* (Zheng et al. 2013b).

Resistance to *O. neolycopersici*

O. neolycopersici was only distinguished from *O. lycopersici* in 2001 by Kiss et al. (2001). As far as we know no specific resistance genes for *O. lycopersici* have been reported. Although resistance sources against *O. lycopersici* were published, they proved to be resistances against *O. neolycopersici*. Thus, there is no report on resistance to *O. lycopersici* and it is unknown whether the identified resistance sources to *O. neolycopersici* are also effective to *O. lycopersici*.

Mapped loci for resistance to *O. neolycopersici* in tomato

Whereas no effective sources of resistance to *O. neolycopersici* have been found in tomato cultivars released by the end of 20th century, several resistant accessions have been discovered in wild tomato species (Lebeda et al. 2014). Till now, nine loci have been mapped which confer resistance to *O. neolycopersici* (Figure 1). *Ol-1*, identified

from *S. habrochaites* G1.1560 (Van der Beek et al. 1994), has been mapped on the long arm of tomato chromosome 6 (Bai et al. 2005). *ol-2* is a recessive resistance gene found in *S. lycopersicum* var. *cerasiforme* LA1230 and is located on chromosome 4 (Ciccarese et al. 1998). Cloning of this gene revealed that *ol-2* is a homologue of the barley *Mlo* gene (Bai et al. 2008). *Ol-3,* introgressed from *S. habrochaites* G1.1290, is located in the same chromosomal region as *Ol-1.* There is some evidence suggesting that *Ol-1* and *Ol-3* are allelic variants (Huang et al. 2000b; Bai et al. 2005). *Ol-4,* originating from *S. peruvianum* LA2172, is located on the short arm of chromosome 6 (Bai et al. 2004). *Ol-5*, introgressed from *S. habrochaites* PI247087, is closely linked to *Ol-1* and *Ol-3* on the long arm of chromosome 6 (Bai et al. 2005). *Ol-6*, which was found in an advanced breeding line with unknown origin, is mapped in the same position as *Ol-4* (Bai et al. 2005). Very likely, *Ol-4* and *Ol-6* are allelic variants. On the short arm of tomato chromosome 6, a cluster of disease resistance (*R*) genes has evolved from the *Mi-1* gene that confers resistance to root-knot nematodes (*Meloidogyne* spp.), potato aphids (*Macrosiphum eluphorbiae*), and whiteflies (*Bemisia tabaci* and *B. tabaci* biotype B) (Milligan et al. 1998; Rossi et al. 1998; Nombela et al. 2003). Silencing *Mi-1* homologues in tomato lines carrying *Ol-4* and *Ol-6* compromised the resistance to *O. neolycopersici* in those lines, demonstrating that *Ol-4* and *Ol-6* are *Mi-1* homologues (Seifi et al. 2011). In addition to these monogenic resistance genes, three quantitative trait loci (*Ol-qtls*) were identified in *S. neorickii* G1.1601 (Bai et al. 2003). *Ol-qtl1* was mapped on chromosome 6 in a chromosomal region where *Ol-1, Ol-3* and *Ol-5* are located. *Ol-qtl2* and *Ol-qtl3* were mapped on chromosome 12 in the vicinity of the *Lv* gene (Bai et al. 2003). *Ol-qtl1* and *Ol-qtl2* were further fine-mapped using advanced populations, while the presence of *Ol-qtl3* needs to be further confirmed (Figure 1) (Faino et al. 2012).

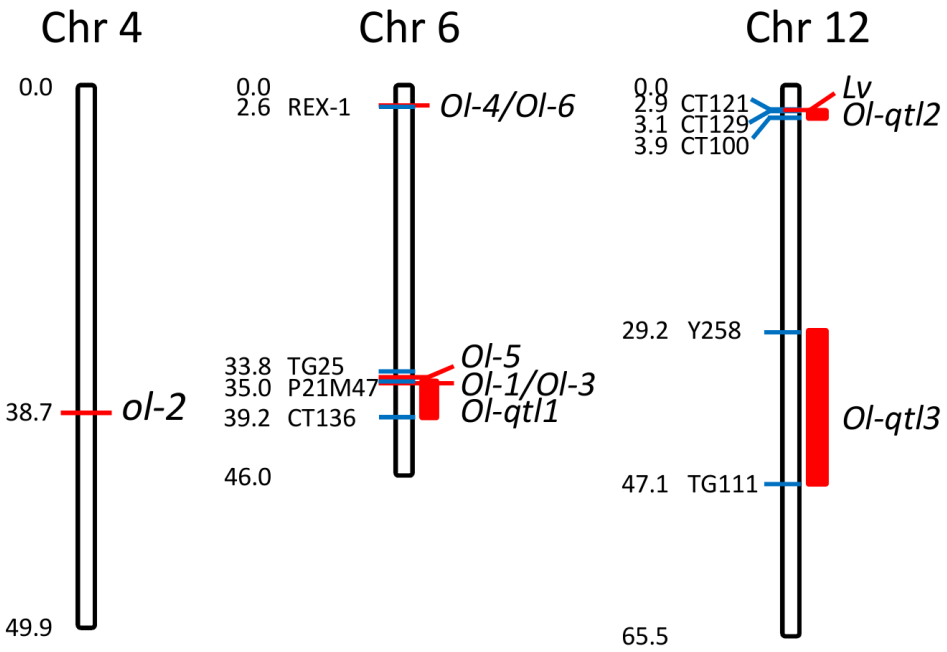

Figure 1 Physical map of tomato chromosomes 4, 6 and 12 showing the positions of markers on the left (blue lines) and powdery mildew resistance genes and QTL regions on the right (red lines and boxes). Positions are indicated in Mega basepairs (Mbp), based on the Heinz SL2.40 tomato genome sequence (http://solgenomics.net).

Mechanisms associated with resistance conferred by the *Ol*-genes and *Ol-qtls*

The resistance mechanisms associated with *Ol*-genes and *Ol-qtls* have been studied by using a unique set of nearly isogenic lines (NILs), which harbour an introgression carrying the particular *Ol*-gene/QTL in the genetic background of *S. lycopersicum* cv. Moneymaker (MM) (Bai et al. 2005; Li et al. 2012). These NILs have been compared for their response to *O. neolycopersici* based on histological and biochemical events, changes in gene expression pattern, and fluctuation in phytohormone pathways during infection with *O. neolycopersici* (Bai et al. 2005; Li et al. 2007, 2012; Seifi 2011).

Histological characteristics of resistance conferred by different *Ol* genes

Plant cell death is one of the resistance mechanisms against biotrophic pathogens by delimiting pathogen progress on plant tissue. HR is a form of cell death triggered typically upon recognition of pathogen avirulence (Avr) proteins by plant R proteins (Nimchuk et al. 2003). HR has been reported to be associated with resistance conferred by the dominant *Ol*-genes (Huang et al. 1998; Bai et al. 2005). Particularly, two different forms of HR have been observed in the tomato response to *O. neolycopersici*. Single-cell HR (Huang et al. 1998; Bai et al. 2005), also defined as fast HR (Li et al. 2007) happens in the presence of *Ol-4* and *Ol-6*. These two *Ol*-genes are homologous to the *Mi-1* gene encoding an NBS-LRR protein (Seifi et al. 2011). This type of HR occurs in all intruded epidermal cells in which primary haustoria are formed, resulting in a complete stop of fungal growth (Bai et al. 2005). On the other hand, multiple-cell HR (Huang et al. 1998; Bai et al., 2005), also described as slow HR (Li et al. 2007) occurs in tomato plants carrying *Ol-1, Ol-3* and *Ol-5*. Interestingly, these three *Ol*-genes originate from different accessions of *S. habrochaites* and cluster together on the long arm of tomato chromosome 6 (Figure 1) (Bai et al. 2003). Since such type of HR occurs only in about 30% of infected cells, fungal colonization is not prevented completely and thus leads to an incomplete resistance.

The recessive gene, *ol-2,* which is a homologue of the barley *MLO* gene, mediates resistance by formation of papillae, *i.e.*, cell wall appositions of callose and other constituents at plant-PM interaction sites (Bai et al. 2003, 2008). Papillae are formed before and/or immediately after the formation of primary haustoria, resulting in early stop of fungal growth and leading to a complete resistance. Interestingly, the resistance in NILs carrying *Ol-qtls* is associated with HR and papilla formation, though cell death is predominant (Li et al. 2012). Three types of HR have been described, micro-HR (similar to fast HR), particle-HR (similar to slow HR) and micro/particle HR. The last one has not been observed in NILs carrying dominant *Ol*-genes. The three QTLs jointly confer a very high level of resistance.

Biochemical characteristics of resistance conferred by different *Ol* genes

Accumulation of reactive oxygen species (ROS, *e.g.*, H_2O_2) upon pathogen attack is one of the earliest events that occur in host cells, a phenomenon known as oxidative burst (OB) (Lamb and Dixon 1997). The apoplastic OB occurs rapidly due to the function of membrane enzymes, NADPH oxidases, peroxidases, amine oxidases, and oxalate oxidases (Hückelhoven 2007). In compatible interactions there is a weak induction of OB, however, in incompatible interactions a second OB with higher magnitude occurs (Lamb and Dixon 1997). The ROS produced in OB are antimicrobial agents. In addition, H_2O_2

contributes to cell wall fortification, induces cell death, and acts as a diffusible signal for induction of systemic defense response (Lamb and Dixon 1997; Torres et al. 2006). The role of H_2O_2 in the cell wall fortification is both in cross-linking of the cell wall proteins and also in serving as a substrate in cell wall apposition (papilla formation) (Hückelhoven 2007).

Accumulation of H_2O_2, occurrence of cell death, and deposition of callose in tomato in response to *O. neolycopersici* has been monitored (Mlíčková et al. 2004; Tománková et al. 2006). In comparison with susceptible tomatoes (*S. lycopersicum*), the level of H_2O_2 increased significantly in resistant wild species, *S. habrochaites* and *S. chmielewskii* in which HR occurred (Mlíčková et al., 2004; Tománková et al. 2006). We have also studied H_2O_2 accumulation and callose deposition in different NILs at different time-points after infection with *O. neolycopersici* (Li et al. 2007, 2012). Our results showed that both HR and papilla formation in tomato attacked by *O. neolycopersici* are associated with H_2O_2 and callose accumulation (Li et al. 2007, 2012). In the susceptible MM and a NIL carrying the *ol-2* gene (NIL-*ol-2*), H_2O_2 accumulation in epidermal cells is almost absent. In contrast, in NILs carrying *Ol-1* (NIL-*Ol-1*), *Ol-4* (NIL-*Ol-4*) and *Ol-qtls* (NIL-*Ol-qtls*), H_2O_2 accumulates in every cell that underwent cell death, consistent with the results of previous works (Mlíčková et al. 2004; Tománková et al. 2006). In cells undergoing HR, callose deposition was also observed. At the first interaction sites (where primary appressoria are formed), both H_2O_2 accumulation and callose deposition was more abundant in NIL-*ol-2* and NIL-*Ol-1* compared with other lines, however, only in NIL-*ol-2* the deposited callose formed papillae. In the latest stage of infection (41 hours post inoculation) in MM and NIL-*Ol-1* callose deposition was observed at about 60% of the first interaction sites, where the fungus penetrates the epidermal cells, indicating that the timing of callose deposition is relevant for the outcome of resistance/susceptibility in the interaction of tomato and *O. neolycopersici* (Li et al. 2007, 2012).

Reprogramming of gene expression associated with different *Ol* genes

In order to compare the resistance mechanism mediated by monogenic *Ol* genes and *Ol-qtls*, we studied the transcript profiles by cDNA-AFLP (Li et al. 2006, 2007) and microarray analysis (unpublished data).

cDNA-AFLP profiling clarified that the majority of the up-regulated differentially expressed-transcript derived fragments (DE-TDFs) are common in MM, NIL-*Ol-1* and NIL-*Ol-qtls*, with differences in timing of expression for certain DE-TDFs (Li et al. 2006, 2007, 2012). This similarity is likely due to the fact that slow HR is involved in the resistance mediated by *Ol-1* and *Ol-qtls*, resulting in a similar pattern of fungal growth in MM and NILs carrying *Ol-1* and *Ol-qtls* (Li et al. 2007, 2012). Most of these sequenced inducible transcripts showed homology to genes with functions in defense responses, implying that *Ol-1*- and *Ol-qtls*-mediated responses likely employ overlapping components of defense pathways occurring in basal immunity, however the timing and magnitude of responses may determine the interaction outcome (Li et al. 2006, 2012). Though the resistance mediated by *ol-2* is associated with papilla formation, distinct from HR, more than 50% of the DE-TDFs that were induced in NIL-*Ol-1* also showed up-regulation in NIL-*ol-2* (Li et al. 2007). This unexpected result may be explained by the fact that papilla formation occurred only in about 40% of the *ol-2* epidermal cells attacked by primary appressoria (Bai et al. 2005). In contrast, NIL-*Ol-4* showed a highly divergent set of DE-TDFs

compared with the ones from NIL-*Ol-1*. For example, more than 70% of the DE-TDFs that were up-regulated in NIL-*Ol-1* were not detected in NIL-*Ol-4* (Li et al. 2007).

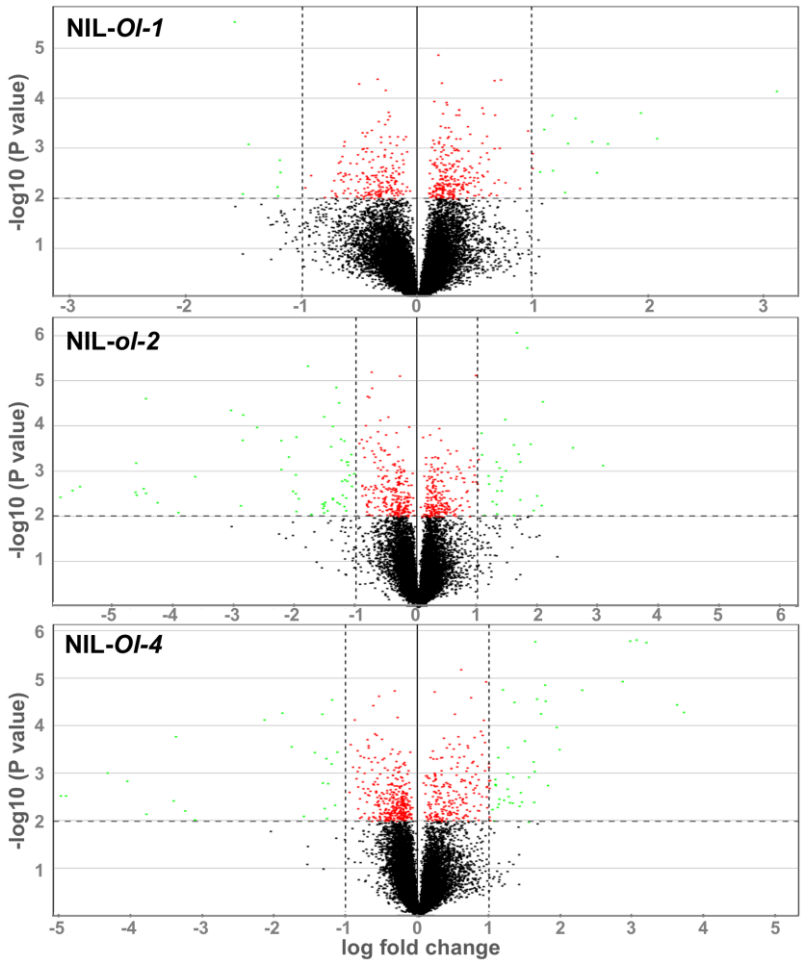

Figure 2 Volcano plot representing the differences in fold change in gene expression in different NILs compare to MM, challenged with *O. neolycopersici*. Total RNA from MM, NIL-*Ol-1*, NIL-*ol-2*, and NIL-*Ol-4* leaf tissue collected at 1, 5, or 7 days after inoculation with *Oidium neolycopersici* or spraying with water was isolated. This RNA was hybridized to the tomato Syngenta Affymetrix array and the data were normalized by RMA (robust multi-array) method. The MeV free software (www.tm4.org/**mev**) was used to analyse the data. In each plot, the X axis shows differences in fold change in the gene expression between each NIL and MM, and the Y axis shows the probability (log *p* value) of the differences. Horizontal dashed line determines the threshold 2 for probability ($p = 0.01$) of significance and the vertical dashed lines set the threshold 1 for difference in fold change of gene expression. The green dots show genes which expression level is at least 1 fold different in a NIL compare to MM, with $p < 0.01$. The positive values on the X axis indicate higher expression in NILs compare to MM, and negative values indicate lower expression in NILs compare to MM.

Microarray analysis was performed using RNA extracted from PM-inoculated and mock-inoculated leaf samples at 1, 5 and 7 days post inoculation (dpi) on the tomato Syngenta Affymetrix array (unpublished data). Of the 22,000 genes on the array, the expression of about 250 genes was different at least between two of the samples. Interestingly, these genes are mainly differentially expressed between NILs and MM,

regardless of the pathogen infection. In NIL-*ol-2* and NIL-*Ol-4* the number of genes showing significant differential expression compared to MM (fold change above 2, $p<0.01$) is higher than that in NIL-*Ol-1* (Figure 2). The narrower range of differentially expressed genes in NIL-*Ol-1* suggested that compared to NIL-*ol-2* and NIL-*Ol-4* the response of NIL-*Ol-1* upon PM attack is more similar to MM, which is in agreement with the results obtained from cDNA-AFLP analysis (Li et al. 2006, 2007).

The fact that our microarray study revealed only differences in constitutive gene expression in different genotypes, but not upon fungal inoculation within the genotype, may be due to the sampling method. We collected entire infected leaves and isolated RNA for microarray analysis. It is worth mentioning that PM only infects the epidermal cell layer and, therefore, it is expected that molecular events associated with the infection occur in this cell layer. Micro-dissection of the epidermal cell has been shown to be an effective approach to get a better understanding of gene expression reprogramming upon PM infection. In the Arabidopsis - *G. orontii* pathosystem, the epidermal cell layer was first microdissected by using laser and then the gene expression pattern was studied in the collected cells (Chandran et al. 2010). This elegant experiment revealed involvement of new genes, including 67 transcription factors, in response to PMs that have not been discovered before by whole tissue analysis. Interestingly, one of these transcription factors, known as MYB3R4, induces endoreduplication in the infected cells, probably to increase the metabolism of the plant cell in the favour of the pathogen (Chandran et al. 2010).

In barley, genes have been identified that are required for the resistance mediated by certain *Mla* genes (e.g. *Rar1*, *Rar2*), as well as for *mlo* (*Ror1* and *Ror2*) (Freialdenhoven et al. 1994, 1996; Hückelhoven et al. 2001). In tomato, silencing a putative *glutathione S-transferase* (*GST*) compromised the resistance conferred by the *Ol-1* gene (Pei et al. 2011). We are performing functional analysis of genes showing differential expression between MM and the NILs and expect to find different genes essential for specific *Ol* genes.

RNA silencing in PM resistance

There is overwhelming evidence implicating plant RNA silencing pathways in plant defense responses to viruses, bacteria, oomycetes, and fungi (reviewed by Katiyar-Agarwal and Jin 2010; Ruiz-Ferrer and Voinnet 2009; Seo et al. 2013). To be cost effective, defense responses need to be suppressed during normal conditions and to be rapidly activated upon pathogen attack. Endogenous gene silencing is suggested to be one of the mechanisms for this rapid "off" and "on" regulation (Jin 2008). In agreement with this idea, recently a miR482/2118 superfamily was discovered in tomato that silences numerous *NBS-LRR* genes, and upon pathogen infections this silencing mechanism is suppressed (Shivaprasad et al. 2012).

RNA silencing (also called RNA interference, RNAi) is the most common antiviral mechanism in plants, and thus, viruses interfere with their host's RNA silencing pathways (reviewed by Voinnet 2005). Such an ability had not been reported for other pathogens, until recently that Qiao and co-workers demonstrated that several effectors of *Phytophtora sojae* supress the RNA silencing in plants by inhibiting the biogenesis of small RNAs (Qiao et al. 2013).

It has been shown that expression of RNAi constructs for *Blumeria*'s effectors (*Avra10* and *AvraK1*) and *Glucanosyltransferase* genes in barley results in a reduction in fungal development (Nowara et al. 2010). Whether this host-induced gene silencing (HIGS) degrades fungal transcripts inside the pathogen or inside the plant cells is not certain yet, but the fact that some of the silenced genes function inside the pathogen and also there is no evidence for secretion of *Avra10* and *AvraK1* transcripts inside epidermal cells, favours the scenario that silencing occurs inside the pathogen (Nowara et al. 2010). This phenomenon may suggest the involvement of RNA silencing in plant response to PMs.

Interestingly, we also have data suggesting that *O. neolycopersici* suppresses tomato RNA silencing pathways in order to establish the pathogenicity. We discovered that the expression of a regulator of gene silencing is strongly induced in tomato plants infected with *O. neolycopersici* (Seifi 2011). From the microarray dataset mentioned before we found a subset of genes that were highly up-regulated in the early stages of infection in the compatible interaction compared to the incompatible interactions (unpublished data). One of these genes is a calmodulin-like regulator of gene silencing (known as rgs-CaM; GeneBank accession: AY642285). An ortholog of this gene in tobacco is induced in response to tobacco mosaic virus (Anandalakshmi et al. 2000; Nakahara et al. 2012). We verified the expression of this gene in our NILs as well as in MM, and results clearly showed that this gene is indeed induced drastically in MM (compatible interaction) in the early time-points (Figure 3).

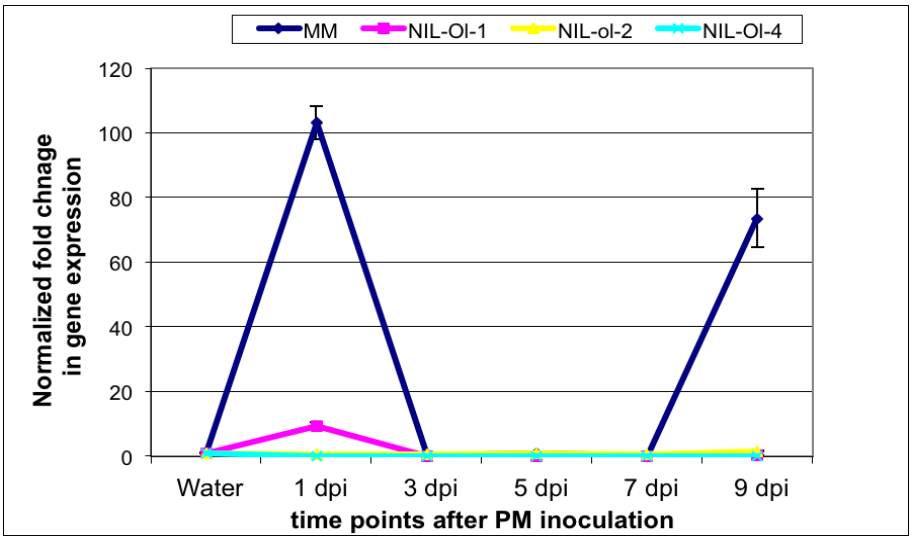

Figure 3 The expression pattern of a regulator of RNA silencing in different tomato interactions with *Oidium neolycopersici*. MM is the susceptible line to *O. neolycopersici*, and is the background for near isogenic lines (NILs), harbouring different resistance genes to PM. NIL-OI-4 and NIL-ol-2 show a high level of resistance to *O. neolycopersici*, while NIL-OI-1 is partially resistant. Error bars show standard deviation (Adapted from Seifi 2011).

This suggests that probably *O. neolycopersici* manipulates the tomato RNA silencing machinery in MM in order to establish a compatible interaction. However, in incompatible interactions, when resistance genes are present, this interference is significantly decreased, proportional to the strength of the corresponding resistance genes. We are currently investigating this interesting gene in more details.

Phytohormone pathways involved in resistance conferred by *Ol*-gene/*Ol-qtls*

Plant hormone signalling pathways are an important part of downstream pathways in immunity responses. Ample evidence has shown that salicylic acid (SA), jasmonic acid (JA), ethylene (ET), auxin, abscisic acid (ABA), and gibberellic acid (GA), cytokinin and brassinosteroid signalling pathways play a role in defense (Grant and Jones 2009; Bari and Jones 2009). In general, SA and JA are believed to be signalling molecules in defense against biotrophic and necrotrophic pathogens, respectively (Glazebrook 2005). The SA pathway is well-documented as an essential component in ETI, PTI and systemic acquired resistance (Vlot et al. 2009). JA in the presence of low levels of ET is only able to trigger a response to herbivores and wounding, while in combination with high ET levels, it triggers responses to necrotrophs as well (Grant and Jones 2009). ABA is mainly considered as a negative regulator of plant immunity (Mauch-Mani and Mauch 2005), probably because of its antagonistic interaction with the ET-JA signalling pathways (Anderson et al. 2004). SA, JA and ET pathways are considered as the backbone of phytohormone networks in the plant immune system, with which auxin, ABA, and GA pathways interact (Pieterse et al. 2009).

In tomato responses to *O. neolycopersici*, only the involvement of phytohormones in basal defense has been studied to some extent. Results suggested that the SA pathway has no role in basal defense (Achuo et al. 2004; Lebeda et al. 2014), but ABA-deficiency or ET-insensitivity enhances basal resistance in tomato against biotrophs including *O. neolycopersici* (Achuo et al. 2006; Lund et al. 1998).

We did a different study and compared the hormonal pathways in different tomato-PM interactions using the NILs carrying the *Ol*-genes and *Ol-qtls* (Seifi 2011). An early significant induction in the SA pathway was observed in NIL-*Ol-4* (Li et al. 2007; Seifi 2011). The *Ol-4* gene is a homologue of the *Mi-1* gene, and triggers accumulation of H_2O_2 and induction of HR at 1 dpi upon PM infection (Li et al. 2007). Given the important role of SA in HR induction (Vlot et al. 2009; Love et al. 2008), the early induction in SA pathway in NIL-*Ol-4* is expected. Interestingly, SA is required for the *Mi-1*-mediated resistance to potato aphids, but not to nematodes (Li et al. 2006; Mantelin et al. 2013). These results highlight the diverse modes of hormone signalling pathways in resistance conferred by *Mi-1* homologues. Although we provide evidence suggesting that the SA pathway plays a role in *Ol-4* mediated resistance to *O. neolycopersici*, further confirmations are required to reach a more definite conclusion, for instance by testing the *Ol-4* function in a mutant deficient in the SA pathway. In contrast to the SA pathway, JA, ABA and ET pathways in NIL-*Ol-4* showed the same trend as in the susceptible genotype MM. Accordingly, disruptions of the ET, JA and ABA pathways had no effect on *Ol-4*-mediated resistance (Seifi 2011).

In NIL-*Ol-1* and NIL-*Ol-qtls*, ET pathway induction started from 7 dpi and reached a maximum level at 9 dpi in NIL-*Ol-1* (Seifi 2011). In contrast, the other NILs showed the same pattern as that observed in MM. Further, ET-insensitivity compromises the PM resistance in these two NILs. Late induction of the SA pathway was also observed in NIL-*Ol-1* and NIL-*Ol-qtls*, which is distinguishable from the induction in other lines. The involvement of the SA pathway in resistance conferred by *Ol-1* and *Ol-qtls* needs to be further studied.

In MM and the NILs, marker genes for JA and ABA pathways showed a constant level of expression in the period of infection followed by an induction during late stages of infection with the highest rate in MM (Seifi 2011). Late accumulation of ABA and JA in compatible interactions of tomato with other pathogens has also been reported by others (O'Donnell et al. 2003; De Torres-Zabala et al. 2007; Fan et al. 2009), which suggests that this accumulation is the result of disease establishment and stress rather than a defense response. Surprisingly, ABA-deficiency compromised resistance mediated by both *ol-2* and *Ol-qtls*. ABA induces callose deposition (Flors et al. 2005; Flors et al. 2008), which is the main mechanism of resistance mediated by *ol-2* (Bai et al. 2008) and is also triggered by *Ol-qtls* (Li et al. 2007). Thus, we assume that a basal level of induction of the ABA pathway is required for the process of callose deposition that contributes to the resistance mediated by *ol-2* and *Ol-qtls*.

In addition to ABA, JA-deficiency also compromised *ol-2*-mediated resistance. The resistance conferred by the recessive *ol-2* gene is due to the loss-of-function of MLO (Bai et al. 2008), a transmembrane protein accumulating at attempted fungal penetration sites in plasma membrane microdomains (Bhat et al. 2005). In barley, Arabidopsis and tomato, loss-of-function mutation in *Mlo* homologues results in resistance to different PM species, demonstrating that MLO represents a conserved plant host cell protein required in PM pathogenesis (Consonni et al. 2006). In Arabidopsis, *mlo*-based resistance to *Golovinomyces* spp. is largely independent from SA, JA and ET pathways. However, our data showed that the SA pathway was induced at 1 dpi in NIL-*ol-2* and that impairment of ABA and JA pathways compromised *ol-2*-mediated resistance. Thus, molecular mechanisms underlying the *mlo*-mediated resistance in tomato and Arabidopsis are not completely the same. Considering that the JA pathway is involved in regulating programmed cell death (PCD; Reinbothe et al. 2009), and that MLO protein is a negative regulator of PCD (Shirasu and Schulze-Lefert 2000), the involvement of the JA pathway in *ol-2*-mediated resistance is conceivable. It is intriguing how SA, JA and ABA signalling pathways are coordinated in *ol-2*-mediated resistance that is associated with cell wall apposition but not with PCD.

Specificity of the resistance conferred by the *Ol*-genes and *Ol-qtls*

O. neolycopersici is a highly polyphagous fungus (Jones et al. 2001) and the presence of different races has been reported in different parts of the world (Lebeda et al. 2014). Using our NILs, we have shown that resistance conferred by the *Ol-4* and *Ol-6* genes can be overcome by the isolate from Czech Republic and one of the two Japanese isolates (KTP-02) (Bai et al. 2005; Seifi et al. 2012). The resistance conferred by other *Ol*-genes and *Ol-qtls* remain effective to all the tested isolates (Bai et al. 2005; Li et al. 2012).

Resistance to PMs in different crops

An overview of resistance to *O. neolycopersici* in tomato

In summary, resistance to *O. neolycopersici* identified so far in tomato can be classified into four categories based on the genetics, mechanisms and specificities of the resistance conferred by the *Ol*-genes and *Ol-qtls* (Table 1).

Table 1 Genetic basis and mechanisms associated with *Ol*-genes and *Ol-qtls* conferring resistance to tomato powdery mildew

Gene	Origin	Chromosome location[1]	Genetic basis	Specificity of resistance[2]	Resistance level	Resistance mechanism
ol-2	*S. lycopersum* var. *cerasiforme* LA1230	Chr. 4: 38.7 Mbp	Recessive	All tested isolates	High	Papillae
Ol-4	*S. peruvianum* LA2172	Chr. 6: 2.5 Mbp	Dominant	All tested isolates except On-Cz & KTP-02	Immune	Fast HR (single-cell)
Ol-6	unknown	Chr. 6: 2.5 Mbp	Dominant	All tested isolates except On-Cz & KTP-02	Very high	Fast HR (single-cell)
Ol-1	*S. habrochaites* G1.1560	Chr. 6: 34 Mbp	Dominant	All tested isolates	Incomplete	Slow HR (multi-cell HR)
Ol-3	*S. habrochaites* G1.1290	Chr. 6: 34 Mbp	Dominant	All tested isolates	Incomplete	Slow HR (multi-cell HR)
Ol-5	*S. habrochaites* PI247087	Chr. 6: 34 Mbp	Dominant	All tested isolates	Incomplete	Slow HR (multi-cell HR)
Ol-qtl1	*S. neorickii* G1.1601	Chr. 6: 35-39 Mbp	QTL		Intermediate	
Ol-qtl2	*S. neorickii* G1.1601	Chr. 12: 3 Mbp	QTL		Unknown	
Ol-qtl3	*S. neorickii* G1.1601	Chr. 12: 29-47 Mbp	QTL		Unknown	
Ol-qtl2,3	*S. neorickii* G1.1601	Chr. 12	QTL		High	
Ol-qtl1,2,3	*S. neorickii* G1.1601	Chr. 6+12	QTL	All tested isolates	Very high	HR (fast & slow) + Papillae
Lv	*S. chilense* LA1969	Chr. 12: 3 Mbp	Dominant	Susceptible to *Oidium* isolate On-Ne	High	HR (single-cell)

[1] Position based on *S. lycopersicum* 'Heinz' sequence

[2] Tested isolates described in Bai et al. (2005), Kashimoto et al. (2003), Li et al. (2012) and Seifi et al. (2012).

The comparison of these different forms of resistance based on the histological characteristics, trend of phytohormone pathways, and level of resistance is illustrated in Figure 4.

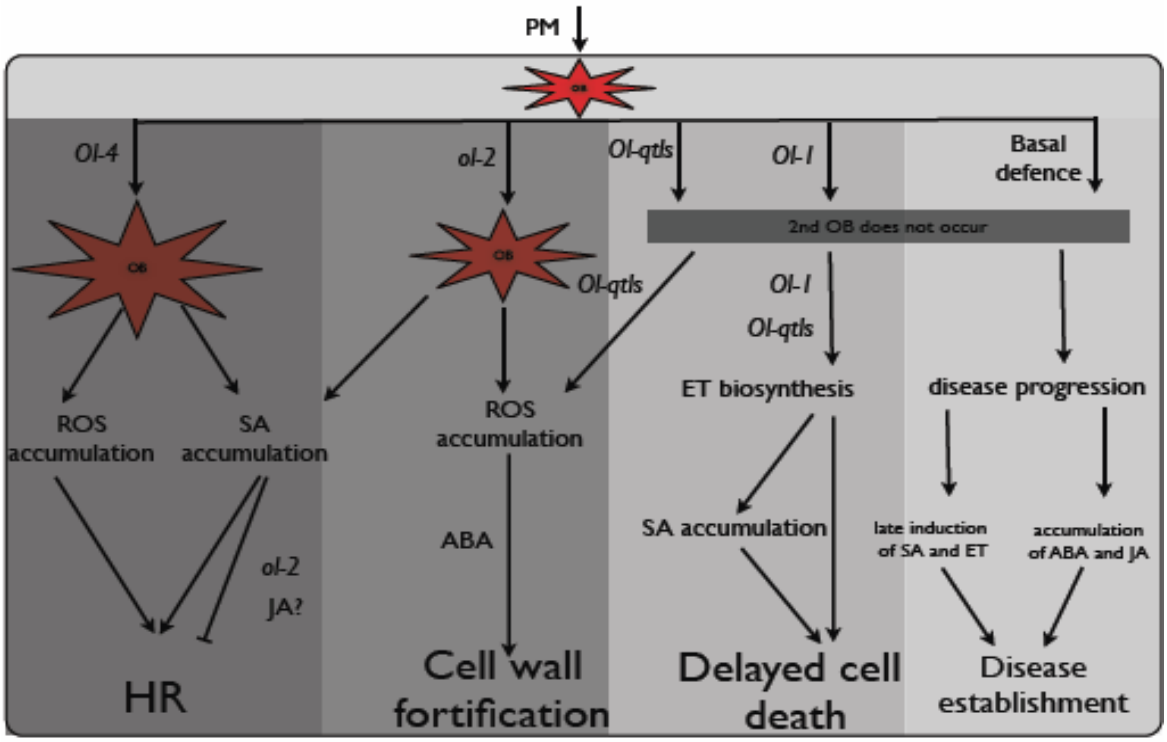

Figure 4 A proposed model for different tomato responses to PM. Upon infection of a tomato epidermal cell by PM, an oxidative burst (OB) occurs in this cell, regardless of the identity of the cell (resistant or susceptible). In the presence of *Ol-4* or *ol-2*, this initial OB is exaggerated and results in a second and stronger OB, which accumulates reactive oxygen species (ROS) and triggers SA pathway. The magnitude of this OB exaggerated by *Ol-4* is strong enough to promote HR. However, OB exaggeration by *ol-2* is relatively weaker and also this gene, probably in coordination with JA pathway, has the ability to block the pathways which would result in HR. Instead, *ol-2* triggers recruitment of ROS produced upon OB for reinforcement of the cell wall. This pathway probably requires ABA. The second OB probably does not occur in the presence of *Ol-qtls*, *Ol-1* and MM (basal defense). Instead, *Ol-qtls* and *Ol-1* lead to DCD by triggering ET accumulation, probably by triggering SA pathway or in collaboration with this pathway. *Ol-qtls* also triggers callose deposition in an ABA-dependent manner. In the absence of these *Ol* resistance genes, *i.e*, in the basal defense of Moneymaker, neither strong early induction in SA pathway and ROS accumulation, nor late induction of ET pathway occurs, resulting in the establishment of PM. In this picture the intensity of the grey colour represents the level of resistance, which is the highest in the presence of *Ol-4* and gradually decreases to basal resistance (Adapted from Seifi 2011).

The first category is the incomplete and broad-spectrum resistance that is controlled by dominant genes (*Ol-1*, *Ol-3* and *Ol-5*). All the three genes originated from *S. habrochaites* accessions cluster on the long arm of chromosome 6. Histologically, slow HR is associated with the resistance conferred by these genes (Bai et al. 2005; Li et al. 2007). The ET pathway plays a role in the *Ol-1*-mediated resistance. Though in NIL-*Ol-1* fungal growth pattern is similar to that in susceptible MM, slow HR in NIL-*Ol-1* is effective enough to prevent further pathogen progress leading to incomplete and broad-spectrum resistance. Also, similar molecular events are observed in NIL-*Ol-1* and MM (Li et al.

2006, 2007). Thus we suggest that *Ol-1,* and likely *Ol-3* and *Ol-5,* encode enhancers of basal defense, which induce delayed cell death in the later stages of pathogen infection.

The second category is the complete and race-specific resistance conferred by dominant *Ol-4* and *Ol-6* genes, which are derived from *S. peruvianum* and an unknown genetic resource, respectively. These genes encode CC(coiled-coil)-NBS-LRR proteins (Seifi et al. 2011) and induce fast HR in the very early stages of pathogen attack (Bai et al. 2005; Bai et al. 2005; Li et al. 2007). This HR prevents further fungal development and the pathogen can hardly produce any secondary haustoria (Li et al. 2007), resulting in complete resistance. As expected for HR-mediated resistance, the SA pathway is induced in NIL-*Ol-4* at early time-points after pathogen infection (Seifi 2011).

The third category is the recessive and broad-spectrum resistance controlled by the recessive *ol-2* gene and associated with papilla formation (Bai et al. 2008), with involvement of ABA and JA pathways (Seifi 2011).

The fourth category is polygenic and broad-spectrum resistance that is governed by three QTLs identified in *S. neorickii* G1.1601 (Bai et al. 2003; Faino et al. 2012) and associated with a combination of HR and papilla formation (Li et al. 2012). ET and ABA pathways likely contribute to this type of resistance (Seifi 2011).

Comparison of PM resistance in tomato and barley

In the well-studied barley and barley powdery mildew (*Bgh*) pathosystem, many resistance genes have been characterized (Schulze-Lefert and Vogel 2000). Based on genetics and histological characteristics, these genes can be classified briefly into three groups. The first one is the recessive resistance conferred by loss-of-function alleles of the *Mlo* gene (*e.g.*, *mlo-5*), which arrests fungal development at the penetration stage while the attacked cells stay alive. The second one is represented by a subset of dominant *Mla* (*e.g. Mla1, Mla6* and *Mla13*) and *Mlg* genes, conferring complete resistance at the penetration stage by inducing a single-cell HR reaction. The third one includes a subset of dominant *Mla* genes (*e.g.*, *Mla7, Mla10* and *Mla12*), which confer incomplete resistance by inducing multi-cell HR to stop fungal growth after penetration (Hückelhoven et al. 2000).

Though there are differences between barley resistance to *Bgh* and tomato resistance to *O. neolycopersici*, similarities are obviously present (Li et al. 2007): the recessive *mlo*-based resistance (*mlo-5* and *ol-2*), fast HR-associated resistance governed by the dominant genes *Ol-4* (HR at primary haustorium stage) and *Mlg* (HR at primary appressorium stage), and slow HR-associated resistance by the dominant genes of *Ol-1* and *Mla12*.

In barley, the complex *Mla* locus (located in a chromosomal interval of ~250 kb) contains 8 CC-NBS-LRR genes (Table 2), of which more than 30 alleles are known to confer race-specific resistance to *Bgh* (Jørgensen and Wolfe 1994; Wei et al. 2002; Seeholzer et al. 2010*).* Similarly, the *Ol-1, Ol-3* and *Ol-5* genes are clustered in a short chromosomal region (Bai et al. 2005). The resistance conferred by *Mla* genes (*i.e.*, *Mla6* and *Mla12*) is SA independent (Hückelhoven et al. 1999). The *Ol-1* gene most probably does not encode a NBS-LRR protein (Seifi 2011), while the *Ol-4* and *Ol-6* are shown to be homologues of the *Mi-1* gene, thus encoding a CC-NBS-LRR protein (Seifi et al. 2011). The resistance conferred by *Ol-4/Ol-6* is race-specific and likely SA-dependent.

Table 2 Cloned powdery mildew resistance genes in plant species other than tomato

Gene	Plant species	Gene identity	Powdery mildew species	Specificity	Mechanism/pathway	Reference
mlo	barley	transmembrane (TM) domains	*Blumeria graminis* f. sp. *hordei*	broad spectrum	papilla formation	Jørgensen 1992
MLA1	barley	CC-NB-LRR	*Blumeria graminis* f. sp. *hordei*	race-specific	single-cell HR	Zhou et al. 2001
MLA6	barley	CC-NB-LRR	*Blumeria graminis* f. sp. *hordei*	race-specific	single-cell HR	Halterman et al. 2001
MLA7	barley	CC-NB-LRR	*Blumeria graminis* f. sp. *hordei*	race-specific	multi-cell HR	Halterman & Wise 2004
MLA10	barley	CC-NB-LRR	*Blumeria graminis* f. sp. *hordei*	race-specific	multi-cell HR	Halterman & Wise 2004
MLA12	barley	CC-NB-LRR	*Blumeria graminis* f. sp. *hordei*	race-specific	multi-cell HR	Shen et al. 2003
MLA13	barley	CC-NB-LRR	*Blumeria graminis* f. sp. *hordei*	race-specific	single-cell HR	Halterman et al. 2003
MLA genes	barley	CC-NB-LRR	*Blumeria graminis* f. sp. *hordei*	race-specific	HR	Seeholzer et al. 2010
Lr34/Pm38	wheat	ABC transporter	*Blumeria graminis* f. sp. *tritici* *Blumeria graminis* f. sp. *hordei*	broad spectrum	leaf tip necrosis/ senescence	Krattinger et al. 2009 Risk et al. 2013
Pm3b	wheat	CC-NB-LRR	*Blumeria graminis* f. sp. *tritici*	race-specific	single-cell HR	Yahiaoui et al. 2004
Pm21	wheat	serine/threonine protein kinase	*Blumeria graminis* f. sp. *tritici*	broad spectrum	H_2O_2, HR	Cao et al. 2011
Atmlo2, -6, -12	Arabidopsis	transmembrane (TM) domains	*Golovinomyces* spp.	broad spectrum	SA	Consonni et al. 2006
RPW8	Arabidopsis	CC & TM domains	*G. cichoracearum, G. orontii, G. cruciferarum, Oidium neolycopersici*	broad spectrum	HR, SA	Xiao et al. 2001, 2005
ssi2	Arabidopsis	stearoyl-ACP desaturase	*G. cichoracearum*	broad spectrum	SA, PR genes	Kachroo et al. 2001; Song et al. 2013
edr1	Arabidopsis	MAP kinase kinase kinase	*G. cichoracearum*	broad spectrum	SA	Frye and Innes 1998; Frye et al. 2001
pmr2 (=Atmlo2)	Arabidopsis	transmembrane (TM) domains	*Golovinomyces* spp.	broad spectrum	SA	Consonni et al. 2006
pmr4	Arabidopsis	callose synthase	*G. cichoracearum*	broad spectrum	SA	Nishimura et al. 2003
pmr5	Arabidopsis	unknown function	*G. cichoracearum,G. orontii*	broad spectrum	cell walls enriched in pectin	Vogel et al. 2004
pmr6	Arabidopsis	pectate lyase-like	*G. cichoracearum*		unknown	Vogel et al. 2002
er1	pea	transmembrane (TM) domains	*Erysiphe pisi*	broad spectrum	papilla formation	Pavan et al. 2011

The *Mlo* gene encodes a 65 KDa membrane protein with seven transmembrane domains. Loss of function of this gene results in a broad-spectrum resistance to barley powdery mildew (Table 1 and 2) (Büschges et al. 1997). In tomato the *ol-2* gene is a loss-of-function allele of the tomato *Mlo* ortholog (*SlMlo1*) (Bai et al. 2008).

Comparison of PM resistance in tomato and Arabidopsis

In *Arabidopsis thaliana*, four powdery mildew species are known to establish compatible interactions including *Golovinomyces* spp. and *O. neolycopersici* (Xiao et al. 2001). Known sources of resistance in Arabidopsis comprise natural resistance conferred by alleles of the *RPW8* locus and mutation-induced resistance. The *RPW8* locus comprises two dominantly inherited *R*-genes, *RPW8.1* and *RPW8.2*, which control resistance to a broad range of PM species (Xiao et al. 2001). RPW8 proteins contain a predicted coiled-coil and a transmembrane (TM) domain, structurally different from other R proteins identified to date (Table 2). Though *RPW8*-mediated resistance was previously reported to be effective against *O. neolycopersici* in the Ms-0 accession, heterologous expression of *RPW8* genes in tomato failed to confer enhanced resistance to *O. neolycopersici* (Xiao et al. 2003). The *RPW8*-mediated resistance present in several other Arabidopsis accessions seems to be non-functional against *O. neolycopersici.* Instead, at least two other major loci in the accession Bay-0 appear to mediate such a resistance (Göllner et al. 2008), demonstrating that genetic factors in Arabidopsis for resistance to *O. neolycopersici* are different from those to *Golovinomyces* spp. Very likely, *O. neolycopersici* 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 (Personal communication, Dr. S. Xiao, Institute of Bioscience and Biotechnology Research, The University of Maryland, USA).

The induced resistance via loss-of-function mutations is represented by *powdery mildew resistant* (*pmr*) mutants (*pmr1* to *pmr6*) (Vogel and Somerville 2000; Vogel et al. 2002, 2004). Four *Pmr* genes have been cloned and they are involved in different cellular activities (Table 2). The *pmr2* turned out to be an *mlo*-mutant (*Atmlo2*); *Pmr4* encodes a callose synthase, *Pmr5* belongs to a large family of plant-specific genes with unknown function and *Pmr6* encodes a putative pectate lyase. In Arabidopsis, unequal genetic redundancy between three phylogenetically closely related *Mlo* orthologs (*AtMlo2, AtMlo6* and *AtMlo12*) is observed. Absence of *AtMlo2* confers partial PM resistance, which is 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 is completely resistant (Consonni et al. 2006). The *Atmlo2*-conferred resistance to *Golovinomyces* spp. is largely independent of the SA signalling pathway (Consonni et al. 2006). However, we found that *Atmlo2* resistance to *O. neolycopersici* 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 (Zheng 2012). 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 activation might be an important feature of *Atmlo2* resistance to *O. neolycopersici*.

Cloned genes for resistance to PMs in other plant species

We tried to summarize all the cloned genes for PM resistance in different plant species, including the ones discussed above (Table 2). In wheat there are 59 resistance genes mapped in 43 different loci, conferring resistance to PM caused by *B. graminis* f. sp. *tritici* (He et al. 2009). Up to now, three of these genes, *Pm3b, Pm21* and *Lr34/Pm38*, have been cloned. *Pm3b*, which confers race-specific resistance, encodes a CC-NBS-LRR protein (Yahiaoui et al. 2004). *Lr34/Pm38* encodes an ABC transporter and confers race-non-specific resistance (Krattinger et al. 2009). *Pm21* encodes a serine/threonine protein kinase, which is present in membrane, cytosol and nucleus of epidermal cells. *Pm21* confers durable and broad-spectrum resistance, which is associated with HR (Cao et al. 2011).

Pathogenomics: powdery mildew effectors

From the genome sequences of three PM species, *Bgh*, *G. orontii*, and *E. pisi*, it is revealed that PMs have a large, expanded genome up to four times larger than other Ascomycete species (Spanu et al. 2010). Strikingly, the number of protein-coding genes is lower than that in filamentous fungi, but a large portion of the genome consists of transposable elements (TEs); for instance 67% of the genome in *B. graminis* (Spanu et al. 2010). It is proposed that the low number of protein-coding genes in the genomes of biotrophic fungi explains their obligate nature, and massive proliferation of TEs might be a mechanism to increase genetic variation (Spanu 2012).

Till now, only two PM effectors, namely *Avra10* and *Avrk1*, have been cloned. These genes were isolated by map-based cloning from *Bgh*, and their products are recognized by barley *R*-proteins MLA10 and MLK1, respectively (Ridout et al. 2006). With the available PM genomes, plenty of genes encoding for putative effectors, *i.e.*, genes encoding for proteins that have a secretion signal and have no match in organisms outside the PMs, have been identified. Following this algorithm, 248 candidate secreted effector protein (CSEP) were found, most of them highly expressed in haustoria (Spanu 2012). Later by searching for homologues of these CSEPs in the *Bgh* genome, the number of effector candidates increased to 491 (Pedersen et al. 2012).

Interestingly, these CSEP-encoding genes are highly conserved between different isolates of *Bgh* (Hacquard et al. 2013) but not among other sequenced PMs (Spanu 2012). For instance, only 16 out of 491 *CSEPs* form *B. graminis* are conserved in *G. orontii* and *E. pisi* (Pedersen et al. 2012). The lack of conservation in putative effector genes in different PMs suggests that evolution of effectors is highly dependent on species-specific adaptation. Since the *CSEPs* varied among different PMs, the genome information of the three aforementioned PMs is not very useful to fish out putative effectors in other PMs like *O. neolycopersici*, based on homology.

The *CSEPs* of *Bgh* are not or are barely expressed in germinating spores, but successive waves of massive expression of these genes was detected during and after penetration to barley epidermis (Hacquard, et al. 2013). In incompatible interactions (*e.g.*, presence of *Mla1* gene) the *CSEPs* expression decreases following a transcriptional reprogramming in barley epidermal cells and at the onset of cell death in those cells, suggesting a defense mechanism by which host suppresses production or secretion of pathogen's effectors (Hacquard et al. 2013). Functional analyses of 50 of these *CSEPs* showed that silencing of eight of them, that are similar to glucosyltransferases,

metalloproteases, and microbial secreted ribonucleases, inside the pathogen compromised disease development (Pliego et al. 2013). The ribonuclease-like effectors probably interfere with programed cell death in the host cells and, therefore, help establishment of pathogenicity (Pliego et al. 2013). In another study, it was shown that one of the CSEPs (CSEP0055) interacts with apoplastic pathogenesis-related proteins of barley, including PR17, and thereby suppresses the host defense (Zhang et al. 2012).

With the available PM genomes, the RNAseq approach is very appealing to identify effectors in PM's transcriptome. For example, mRNA extracted from the fugal haustoria of *G. orontii* was sequenced (Weßling et al. 2012). In this study, authors identified 70 CSEPs of which 19 are among the top 50 expressed secreted proteins during the interaction with Arabidopsis (Weßling et al. 2012). We have taken a similar approach to analyse haustorial transcriptome of *O. neolycopersici* with the aim to identify putative effectors of this pathogen.

A retrospect of Zig-Zag model based on plant-PM interactions

The Zig-Zag model is proposed based on biotrophic interactions (Jones and Dangl 2006), hence mechanisms of plant interactions with PMs should fit well in this model. Here we discuss different forms of plant resistances to PMs in the frame of the Zig-Zag model. The aim is to summarize and organize all the mechanisms of resistance to PMs, and also to validate this model to explain plant-PM interactions.

ETI: race-specific resistance mediated by *R*-genes

Breeding for resistance has been focused on introducing *R*-genes that encode proteins, which recognize specific pathogen effector proteins leading to ETI (host resistance). This type of host resistance is frequently broken as new pathogen races constantly appear, which forms a bottleneck for durable resistance breeding. The resistance mediated by genes like *Ol-4*, *Ol-6*, *Pm3b*, *Mla1* and *Mla10* fits well with the criteria of ETI, since these genes encode NBS-LRR proteins, confer race-specific resistance, and induce HR response. For few of this type of genes, the interacting pathogen effector is also identified and further verifies that these genes perceive a pathogen's effector and trigger ETI. For example barley MLA10 recognizes AVR_{A10} effector from *Bgh* (Ridout et al. 2006).

PTI: race-non-specific spectrum resistance conferred by PAMP-receptors

Among the genes that have been discussed above, some do not encode NBS-LRR proteins and confer race non-specific resistance. For example, *Pm21* induces strong HR to prevent formation of primary haustoria, encodes a serine/threonine kinase protein and confers a broad–spectrum resistance. Although genes like *Pto*, *FLS2*, *Xa21*, *PBS1*, *Rpg5*, and *Yr36* that confer resistance to different pathogens in different plants, also encode proteins with a serine/threonine kinase domain, however, the homology between these genes and *Pm21* is lower that 40% (Cao et al. 2011). Similarly, the *Ol-1* gene and *Ol-qtls* confer race non-specific resistance by mediating slow HR. We have fine-mapped *Ol-1* locus to an 73 Kb interval, in which there is no *NBS-LRR* gene, and the annotation of the candidate genes (10 genes) suggests that they are involved in metabolic pathways, and not in known defense responses (Seifi 2011). Our preliminary data showed that the *Ol-qtl2* does not belong to *NBS-LRR* gene family either (unpublished data). PTI is defined by a set of PAMP-receptors that recognize PAMPs, leading to activation of a range of basal

defense mechanisms contributing to resistance with a broad spectrum. At this stage, we cannot rule out the possibility that any of these genes encode for a PAMP-receptor, but the predictions of intracellular localization of the proteins encoded by these genes, and lack of similarity of them with known PAMP receptors, suggests a very low probability for this scenario.

Besides the *Pm21* gene, the *Lr34/Pm38* gene conferring partial resistance to leaf rust, stripe rust and stem rust, also confers resistance to PM in wheat (Spielmeyer et al. 2008). This gene encodes an ATP-binding cassette (ABC)-transporter, located in the plasma membrane (Krattinger et al. 2009). Biochemical and cytological studies showed that *Lr34*-mediated resistance is not based on ROS accumulation, callose deposition and HR induction (Rubiales and Niks 1995; Risk et al. 2012). The molecular mechanism of *Lr34*-mediated resistance is not well understood, but there is evidence suggesting that it is similar to the mechanism induced in response to abiotic stresses and possibly is related to metabolic pathways (Hulbert et al. 2007; Bolton et al. 2008). Interestingly, ectopic expression of *Lr34* in barley resulted in resistance against barley leaf rust and barley PM, implying that the substrate and mechanisms of LR34 transporter are conserved between barley and wheat, and thus, promises the possibility of using this valuable gene in cereal breeding for durable resistance (Risk et al. 2013).

The Zig-Zag model formulates the process of arms races between the host and the pathogen and provides a simple and useful model for pathogen-host coevolution studies. However, it is an oversimplification of reality on the post-perception pathways leading to immunity responses. Firstly, this model is based on a clear distinction between PAMPs and effectors in pathogens, and their distinct receptors in the host cells. Recent studies revealed that this distinction is not easy to make in most cases (Thomma et al. 2011). For example, bacterial flagellin and lipopolysaccharide (LPS) are considered as PAMPs, however modifications in these molecules influence bacterial virulence, too (Taguchi et al. 2006, 2010; Naito et al. 2008; Newman et al. 2007), and thus they resemble bacterial effectors. On the other hand, it is not always true that effectors are perceived by R proteins; there is evidence of perception of apoplastic effectors by PAMP-receptors (de Jonge et al. 2010; Win et al. 2012). In addition, as the above mentioned non-*R*-genes *Pm21* and *Lr34* encode neither *R*-proteins nor PAMP-receptors, can we call this kind of resistance PTI? Secondly, the Zig-Zag model boils down the complex innate immune system into two forms of responses, PTI and ETI, merely based on the type of the pathogen receptors, assuming that the post-perception processes are similar in PTI and ETI and only their magnitude is different (Jones and Dangl 2006; Tsuda and Katagiri 2010). Recently, this difference in the magnitude of induction of downstream pathways between ETI and PTI has been questioned. Based on evidence from different pathosystems it seems that a more realistic view is that depending on specific interactions and even environmental conditions, both ETI and PTI could trigger strong or weak responses (Thomma et al. 2011). Last but not least, Lebeda et al. (2014) demonstrated that the nonhost resistance to PMs is also associated with HR, a hallmark of resistance conferred by *R*-genes. Therefore, a clear distinction between PTI and ETI is difficult to claim (Thomma et al. 2011).

Nonhost-like resistance conferred by editing plant *S*-genes

Based on the Zig-Zag model, pathogens have to suppress PTI in order to overcome nonhost resistance, for which pathogen effectors and their host targets play a central role. The absence of certain host-factors (also known as effector targets) encoded by plant susceptibility genes (*S*-genes) (Eckardt, 2002) enable plants to escape the defense suppression and thus to maintain their nonhost status (Figure 5, middle panel). One example is the *Xa13* gene encoding a host factor targeted by the TAL effector of *Xanthomonas oryzae* pv *oryzae*. Natural mutant *xa13* alleles results in resistance to bacteria strains that use the PthXo1 effector (Yuan et al. 2009).

Though there is no evidence showing that the MLO protein is targeted by PM effectors, the *Mlo* gene is one of the well-characterized *S*-gene examples, which negatively regulates the two *Pen* genes involving in pathways for nonhost resistance to PMs. In barley, Arabidopsis, tomato, pepper, and pea, loss-of-function mutations in *Mlo* result in efficient pre-invasion resistance to adapted PMs, and orthologs of this gene have been found in the genome of wheat, rice, maize, and grapevine (reviewed by Zheng 2012).

In addition to the *mlo* and *pmr* mutants, a considerable number of *S*-genes have been identified in Arabidopsis (reviewed by Pavan et al. 2010); *e.g.*, *downy mildew resistance* (*dmr1 to 6*) mutants (Van Damme et al. 2005). Based on studies on effector-triggerred susceptibility and by looking from a different point of view into host and nonhost resistance (Figure 5), we proposed in 2010 a novel breeding strategy: disabling plant *S*-genes to achieve nonhost-like resistance (Pavan et al. 2010). We are currently verifying whether silencing tomato orthologs of these Arabidopsis *S*-genes leads to resistance to *O. neolycopersici*. Our results till now showed that (1) Arabidopsis *dmr1* and *pmr4* mutants are resistant to *O. neolycopersici* and, (2) silencing *SlDmr1* and *SlPmr4* in tomato results also in resistance to *O. neolycopersici* (Huibers et al. 2013). Together with the tomato *ol-2* mutant (*Slmlo1*), our results demonstrate that orthologs of Arabidopsis *S*-genes are present in crops and disruption in their *S*-gene function leads to resistance to different pathogens.

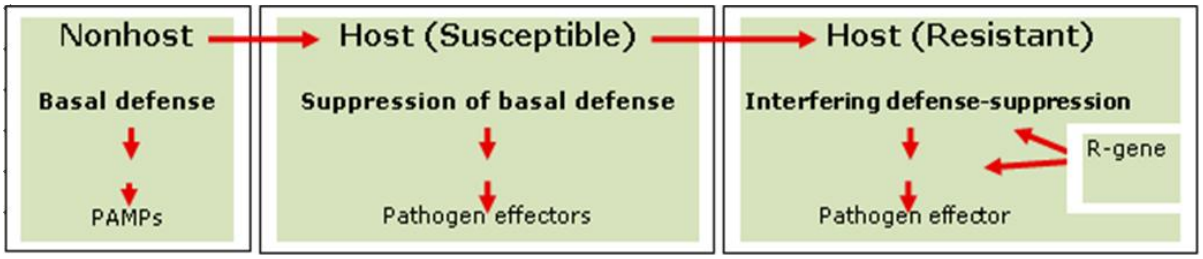

Figure 5 Plant innate immunity: the Zig-Zag model from a breeding point of view. Left panel: perception of PAMPs (pathogen-associated molecular patterns) by PAMP-receptors leads to PAMP-triggered immunity (PTI), which contributes to nonhost (basal) resistance. Middle panel: plant host factors targeted by pathogen effector resulting in effector-triggered susceptibility (ETS). When this host factor (effector target) cannot be exploited by pathogen effectors, plants will maintain their nonhost status. Right panel: perception of pathogen effectors by plant resistance (R) proteins (directly or indirectly) leading to effector-triggered immunity (ETI). When multiple R-genes are present in a plant for the same pathogen species, the combined ETI will lead theoretically to nonhost resistance.

Outlook on breeding perspectives

Nonhost resistance is defined as a resistance in all genotypes of a plant species to all genotypes of a pathogen species. Thus, nonhost resistance is a durable and a common type of plant resistance to potential pathogens and therefore a valuable trait that can be potentially exploited to control adapted pathogens. However, little is known about the genetic factors and molecular mechanisms associated with nonhost resistance. The use of mutants in Arabidopsis has advanced our understanding on mechanisms of nonhost resistance to PMs. Three genes known as *Pen1*, *Pen2*, *Pen3* genes, encoding a syntaxin protein, a glycosyl hydrolase, and an ABC-transporter, respectively (Collins et al. 2003; Consonni et al. 2006) have been found to be associated with nonhost resistance to PMs (Lipka et al. 2005, 2010). In the review paper by Niks and Marcel (2009), it is suggested that the molecular basis of nonhost resistance is very similar to that in basal defense.

Two models of nonhost resistance are adapted to the above described Zig-Zag concept (Schweizer 2007). According to the first postulation nonhost resistance is simply because of inadaptability of a certain pathogen to a certain plant species. In other words, the pathogen has not evolved enough to have effectors to suppress PTI (Figure 5, left panel). The second model anticipates the presence of stacks of multiple *R*-genes that leads to durable resistance by functional redundancy in simultaneously recognizing a number of pathogen effectors (multiple ETI, Figure 5, right panel). In the future, the identified components for PTI (PAMPs and their receptors) and ETI (*R*-genes, effectors and their host targets) will be interconnected to unravel the genetics and molecular mechanisms behind disease resistance/susceptibility in order to understand how a plant can or cannot be exploited by a pathogen as a host (Figure 5). For example, what are the features of effector targets, which can be exploited by adapted pathogens to turn a plant species from a nonhost into a host? In other words, which effectors are used by pathogens to make a "jump" from one host to another? The study of plant *S*-genes and pathogen effectoromics will give fundamental new insights in how pathogens act to suppress plant immunity to promote diseases. As demonstrated above, the knowledge obtained is essential and crucial to open up ways for novel breeding strategies that lead to durable resistance to combat present and future diseases.

From a breeding point of view, nonhost resistance is ideal but difficult to achieve. Since current data suggest that molecular basis of nonhost resistance is very similar to those in PTI (Niks and Marcel 2009), the identification of genes contributing to PTI would contribute to resistance with the feature for nonhost resistance. In practical programs of resistance breeding, introgression of individual dominant *R*-genes from wild species to cultivated crops still plays a major role. In tomato, the resistance gene *Ol-4* can be a perfect candidate for complete resistance to *O. neolycopersici*, nematodes and aphids (Seifi et al. 2011, 2012), being aware that this resistance is race-specific. Dominant resistance is highly effective; however, its race-specific nature makes it vulnerable by the diversity in the genetic pool of the pathogen. In most cases, resistance conferred by *R*-genes can be overcome by pathogens resulting in outbreaks of large epidemics, which 'burst' the once 'booming' cultivars. Repeated boom-and-burst cycles in agriculture continuously force breeders to introduce cultivars with new resistance genes.

Theoretically, pyramiding genes with different specificities and mechanisms can lead to broad-spectrum and durable resistance. For example, the *Ol-1/Ol-3/Ol-5* gene confers incomplete broad-spectrum resistance, thus the combination of these individual genes with *Ol-4* will theoretically result in a complete broad-spectrum resistance. For a

successful resistance genes pyramiding several aspects need to be considered including chromosomal location of resistance genes, their specificity, and their resistance pathways.

The chromosomal location of the resistance genes to be pyramid is an important factor. The race-specificities are often conferred by alleles of the same locus (genes located in the same chromosomal position). For example, the *Mla* locus in barley represents a very "creative" locus that encodes more than 30 different resistance specificities (*Mla-1* to *Mla-32*) against barley PM; therefore, accumulation of these *Mla* alleles in one genotype is impossible by classical breeding. The allelic variants can only be combined in one F1 hybrid in heterozygous status via crossing parental lines with each homozygous for one of these alleles; similar to what is suggested for *Ty-1* and *Ty-3* resistance genes in tomato (Verlaan et al. 2011). In many cases, *R*-genes tend to be clustered. An example is the *Mi-1* gene cluster on tomato chromosome 6, which contains two *Cf* genes (*Cf-2* and *Cf-5*) conferring resistance to *Cladosporium fulvum*, the *Mi-1* gene, as well as *Ol-4* and *Ol-6* (Seifi et al. 2011). The clustering of these resistance genes renders considerable challenges to plant breeders to introgress and pyramid these genes in one breeding line, especially when suppression of recombination is present in such a cluster (Verlaan et al. 2011). Luckily, some *R*-gene clusters are actually natural pyramids of resistance genes to different pathogens; for instance we have shown that the closely linked or the same *Mi-1* homologues in NIL-*Ol-4* confer resistance to PM, nematodes and aphids (Seifi et al. 2011).

To achieve durable resistance, the combined genes should have complimentary race-specificities. Also, the downstream pathways involved in the resistance of the pyramided genes need to be in parallel and not antagonistic. Otherwise, the cross-talk and interaction of defense pathways may result in negative interaction between resistances conferred by the combined resistance genes. For instance, resistance mediated by *Ol-1* and *Ol-4* comes from induction of two different forms of PCD with different hormonal pathways involved. There is a risk that in plants containing both *Ol-1* and *Ol-4* genes, the PCD-triggering pathways antagonize each other.

In addition to intogressing resistance genes from wild species, we demonstrated that silencing *S*-genes leads to nonhost-like resistance. Since *S*-genes encode proteins that are manipulated by the pathogens, and most probably this manipulation is through effectors, an alternative approach to accelerate discovery of *S*-genes is to look for effector's targets (Gawehns et al. 2013). The main problem associated with the *S*-gene breeding strategy is to find *S*-genes for which loss-of function has no pleiotropic effect on plant growth and development, while it diminishes their function as the susceptibility factor. There are promising results from targeted engineering of *S*-genes, in order to disturb only the *S*-gene function. One successful example is the mutations induced via TALEN-based disruption in the rice bacterial blight *S*-gene *Os11N3* that interfere with *S*-gene function but not with the developmental function of *Os11N3* (Li et al. 2012).

An emerging breeding method for pathogen resistance is based on the importance of plant RNA silencing pathways to silence PM's genes. HIGS phenomenon was first observed in barley- *Bgh* interaction (Nowara et al. 2010) and now the idea has provided a method to investigate the role of the pathogen's genes (Pliego et al. 2013). It also suggests a potential approach to engineering plants for resistance to PMs (Nowara et al. 2010).

Crop production is affected by both biotic and abiotic stress factors, thus the best-selling cultivars are those with stable high quality and high yield even under a combination of environmental stresses. In this review, we have focused on PM resistance without taking into account the influence of abiotic stresses. However, it has been demonstrated that environmental conditions influence the outcome of plant-PM interactions (Reviewed by Lebeda et al. 2014). Using our NILs, we are currently investigating to what extent pathways for resistance to PM and tolerance to abiotic stresses are shared, and how abiotic stresses might modify resistance responses mediated by different *Ol* genes and QTLs. Understanding these responses will enable fine–tuning of breeding efforts towards breeding plants with enhanced PM resistance that can exhibit high level of resistance and maintain their performance under conditions of combined stress.

Chapter 3

Down-regulation of *acetolactate synthase* compromises *Ol-1*-mediated resistance to powdery mildew in tomato

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Down-regulation of *acetolactate synthase* compromises *Ol-1*- mediated resistance to powdery mildew in tomato

Abstract
In a cDNA-AFLP analysis comparing transcript levels between powdery mildew (O*idium neolycopersici*)-susceptible tomato cultivar Moneymaker (MM) and near isogenic lines (NILs) carrying resistance gene *Ol-1* or *Ol-4*, a transcript-derived fragment (TDF) M11E69-195 was found to be present in NIL-*Ol-1* but absent in MM and NIL-*Ol-4*. This TDF shows homology to *acetolactate synthase* (*ALS*). ALS is a key enzyme in the biosynthesis of branched-chain amino acids (BCAAs) valine, leucine and isoleucine, and it is also a target of commercial herbicides. Three *ALS* homologs *ALS1*, *ALS2*, *ALS3* were identified in the tomato genome sequence. *ALS1* and *ALS2* show high similarity, whereas *ALS3* is more divergent. Transient silencing of both *ALS1* and *ALS2* in NIL-*Ol-1* by virus-induced gene silencing (VIGS) resulted in chlorotic leaf areas that showed increased susceptibility to *O. neolycopersici* (*On*). VIGS results were confirmed by stable transformation of NIL-*Ol-1* using an RNAi construct targeting both *ALS1* and *ALS2*. In contrast, silencing of the three *ALS* genes individually by RNAi constructs did not compromise the resistance of NIL-*Ol-1*. Application of the herbicide chlorsulfuron to NIL-*Ol-1* mimicked the VIGS phenotype and caused loss of its resistance to *On*. Susceptible MM and *On*-resistant line NIL-*Ol-4* carrying a nucleotide binding site and leucine rich repeat (NB-LRR) resistance gene were also treated with chlorsulfuron. Neither the susceptibility of MM nor the resistance of NIL-*Ol-4* was affected. The specific involvement of ALS in *Ol-1*-mediated resistance suggested that ALS-induced change in amino acid homeostasis is important for resistance conferred by *Ol-1*.

Keywords Acetolactate synthase, *Oidium neolycopersici*, Resistance, *Solanum lycoperisum,* Amino acid homeostasis

Introduction

In their natural environment plants are constantly attacked by a variety of pathogens. Nevertheless, plants can detect and evade most infection attempts through constitutive and inducible immune responses. The inducible responses consist of two layers (Jones and Dangl 2006). The first layer is triggered by multifarious pathogen-associated molecular patterns (PAMPs). The perception of PAMPs by plant pattern recognition receptors (PRRs) stimulates a number of cellular events, which include production of reactive oxygen species, activation of mitogen-activated kinases, enhanced expression of defense genes and production of antimicrobial compounds (Schwessinger and Zipfel 2008; Stotz et al. 2013). The second layer of inducible responses is activated by variable pathogen-specific effectors. Recognition of effectors by the plant is mostly mediated by a class of resistance proteins which contain nucleotide binding site and leucine rich repeat (NB-LRR) domains. The regulation and execution of both inducible responses involve hormone signalling pathways (Bari and Jones 2009).

Emerging evidence illustrates that defense pathways are not only regulated by classical hormones, but also amino acid metabolic pathways constitute an important part of the plant immune system (Zeier 2013). Besides the fact that some amino acids serve as precursors of antimicrobial compounds (e.g. glucosinolates) (Sønderby 2010), amino acid homeostasis is pivotal for the outcome of plant-microbe interactions. A dominant nematode resistance gene in soybean encodes a serine hydroxymethyltransferase (SHMT), which plays a key role in one-carbon folate metabolism (Liu et al. 2012). The SHMT allele in the resistant genotype encodes an isoform of the enzyme with altered kinetic properties compared with the isoform in susceptible genotypes. This altered SHMT enzyme is likely associated with perturbation of the folate pathway resulting in nutritional deficiency for nematodes. Overexpression of a pepper *asparagine synthetase* in Arabidopsis enhanced the resistance to bacterial and oomycete pathogens, which was correlated with increased asparagine levels (Hwang et al. 2011). Arabidopsis recessive downy mildew-resistant (*dmr1*) mutants defective in homoserine kinase were found to be resistant to the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) (van Damme et al. 2009). The resistance was homoserine-induced, and independent of known signalling pathways. Suppression of the ortholog *SlDMR1* in tomato resulted in elevated resistance to powdery mildew *Oidium neolycopersici* (Huibers et al. 2013). Resistance to *Hpa* was also obtained in Arabidopsis *rar1*-suppressor (*rsp*) mutants, in which the level of threonine (Thr) was highly elevated (Stuttmann et al. 2011). The *rsp1* mutant carries a mutation in the aspartate kinase2 gene, which catalyzes the first step in the aspartate-derived amino acid pathway. The *rsp2* mutant contains a loss-of-function allele of dihydrodipicolinate synthase2 , which is the key enzyme in lysine biosynthesis. Disruption of an amino acid transporter *LHT1* (*lysine histidine transporter 1*) confers a broad spectrum disease resistance in Arabidopsis plants, likely as a result of deficiency of glutamine (Liu et al. 2010).

Oidium neolycopersici (*On*) is an important biotrophic fungal disease for greenhouse crops. Unlike most powdery mildews that are host specific, *On* can infect a wide range of hosts, including species of the *Solanaceae* and *Cucurbitaceae* families (Whipps et al. 1998). A favourable strategy to control the disease consists of exploration of resistant alleles from wild species and introgression of these alleles into cultivated species to develop resistant cultivars. In tomato nine loci conferring resistance to *On*

have been identified (Bai et a. 2003, 2005). One of them - *Ol-1* - originates from *Solanum habrochaites* G1.1560 (Lindout et al. 1994), and confers incomplete resistance associated with slow hypersensitive response (HR) (Li et al. 2007). It is located on chromosome 6 and has been fine-mapped to a region encompassing six predicted genes, based on the sequence of tomato cultivar Heinz 1706 (Seifi 2011, unpublished results). None of the six genes encodes a protein with NB-LRR domains. Unravelling the identity of *Ol-1* has not been successful yet, because silencing of the predicted candidate genes individually did not attenuate the resistance level of the near-isogenic line carrying *Ol-1* (NIL-*Ol-1*) (unpublished results). Another resistance gene - *Ol-4* – which has been introgressed from *S. peruvianum* LA2172 confers complete resistance to *On* with fast HR (Li et al. 2007). It has been mapped to the *Mi-1* gene cluster on chromosome 6 (Bai et al. 2005). Disease tests showed that NIL-*Ol-4* was resistant to root-knot nematodes, indicating the presence of a functional *Mi-1* homolog encoding a NB-LRR type protein. Furthermore, silencing of *Mi-1* homologs in NIL-*Ol-4* compromised the resistance to both *On* and root-knot nematodes, showing that *Ol-4* is a *Mi-1* homolog (Seifi et al. 2011).

In a previous study designed to elucidate the pathways of *On* resistance, a cDNA-AFLP approach was used to identify transcript-derived fragments (TDF) showing differential presence or intensity in resistant tomato NILs relative to susceptible Moneymaker (MM) after mock-inoculation or inoculation with powdery mildew *On* (Li et al. 2006, 2007). A BLAST analysis of the sequences of a number of differentially expressed TDFs was performed using the Sol Genomics Network (SGN) database to identify unigene sequences showing highest homology to each TDF. Subsequently, Tobacco Rattle Virus (TRV)-based Virus-Induced Gene Silencing (VIGS) constructs were generated targeting the unigenes. Then, VIGS was performed in the genotypes in which the TDF was detected to analyse whether silencing of targeted genes altered *On* resistance. In this way, it was shown that a putative glutathione *S*-transferase gene is required for *Ol-1*-mediated resistance against *On* (Pei et al. 2011).

In the present study we focused on another of these differentially expressed TDF (M11E69-195) and analysed its involvement in *On* resistance. M11E69-195 was specifically present in NIL-*Ol-1* but absent in MM and NIL-*Ol-4* (Li et al. 2006, 2007). The sequence of this TDF showed homology to *acetolactate synthase* (*ALS*). ALS (EC 2.2.1.6) is more frequently referred to as acetohydroxyacid synthase (AHAS) (Duggleby and Pang 2000) in other studies. In this study, we describe it as ALS based on the annotation in the SGN database. ALS catalyzes the first step in the production of the branched-chain amino acids (BCAAs) valine, leucine and isoleucine (McCourt and Duggleby 2006). It is extensively studied since it is a target of commercially successful herbicides. Different herbicide molecules can block substrate access to the active site of the ALS enzyme (Duggleby et al. 2008). Here, we report the involvement of *ALS* in *Ol-1*-mediated resistance to powdery mildew in tomato.

Results

Down-regulation of two *ALS* genes simultaneously compromises *Ol-1*-mediated resistance to powdery mildew *On*

In the cDNA-AFLP study by Li et al. (2006, 2007) TDF fragment M11E69-195 (No. 71 in Appendix 1 in 2006; No. 24 in Appendix 1 in 2007) was observed to be present in *On*-resistant NIL-*Ol-1*, but absent in *On*-susceptible MM and *On*-resistant NIL-*Ol-4*. BLAST

analysis of the sequence of this 195-bp TDF fragment was initially performed using the SGN database before the tomato genome sequence became publicly available. Highest homology was obtained for unigene SGN-U196237, a *Capsicum annuum* acetolactate synthase (*ALS*) gene (Supplemental figure 1A). Primers were designed based on the U196237 sequence, and a 287-bp PCR product obtained using NIL-*Ol-1* cDNA as template was cloned into VIGS vector TRV2, resulting in vector TRV-U196237 (Figure 1). Infiltration of TRV-U196237 into NIL-*Ol-1* induced morphological changes, including short stature and curled leaves with chlorotic areas (Figure 2A). Subsequently, VIGS plants were inoculated with *On*. Quantification of fungal biomass showed that there was a significant (3-fold) increase on plants infiltrated with TRV-U196237 compared to plants infiltrated with the empty vector (TRV-EV) (Figure 2B).

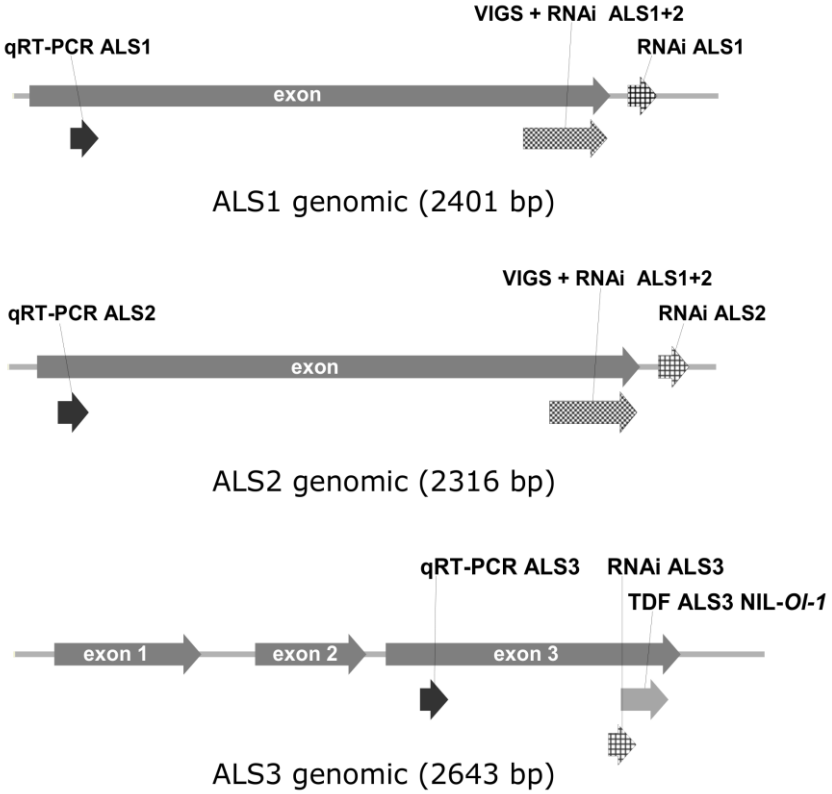


Figure 1 Schematic representation of the genomic sequences of tomato *acetolactate synthase* genes *ALS1*, *ALS2* and *ALS3*. PCR fragments used in VIGS and RNAi constructs are indicated, as well as gene-specific fragments amplified in qRT-PCR analyses for quantification of gene expression. The fragment indicated as 'VIGS + RNAi ALS1+2' was present in TRV-U196237 and also used for stable transformation using an RNAi construct. TDF M11E69-195 from NIL-*Ol-1* showed the highest level of homology with exon 3 of *ALS3*.

After the tomato genome sequence became accessible a new BLAST analysis of the sequence present in the VIGS vector was performed. This resulted in the identification of three putative *ALS* genes in tomato named *ALS1* (*Solyc03g044330*), *ALS2* (*Solyc07g061940*) and *ALS3* (*Solyc06g059880*) (Supplemental figure 1B). The latter one, although present on chromosome 6, does not reside in the *Ol-1* region (Seifi 2011). ALS1 and ALS2 predicted proteins are 94% identical at the amino acid level, while ALS3 is quite different from ALS1 and ALS2 (75% and 78% identity with ALS1 and ALS2,

respectively) (Supplemental figure 1C). The *ALS1* and *ALS2* genes are predicted to contain one exon, whereas *ALS3* is predicted to contain three exons (Figure 1). Alignment of the TDF sequence (derived from the NIL-*Ol-1* line) with the three annotated *ALS* genes showed that the TDF was probably derived from the *ALS3* ortholog in *S. habrochaites (*Supplemental figure 1A). However, alignment of the cloned fragment in the VIGS construct with the three annotated *ALS* genes resulted in highest homology to *ALS2*. This discrepancy can be explained, because for construction of the VIGS vector primers were designed based on the SGN unigene showing highest homology to the TDF, but no unigene based on EST sequences from *ALS3* was present in the SGN database. The alignment suggested that the VIGS vector targeted both *ALS1* and *ALS2*, but not *ALS3,* based on the assumption that an identical sequence of at least 21 nucleotides is necessary for efficient silencing.

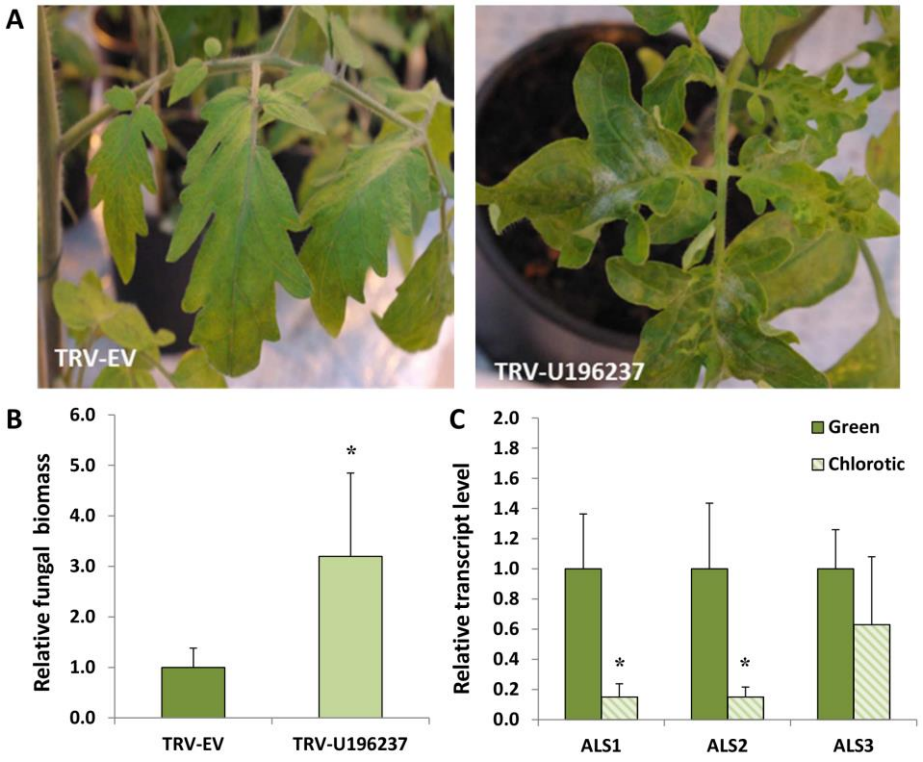


Figure 2 Down-regulation of two *ALS* genes simultaneously via VIGS compromises *Ol-1*-mediated resistance. (A), Effects of VIGS on growth and *On* infection of NIL-*Ol-1* plants infiltrated with empty vector as the control (TRV-EV) and TRV-U196237. (B), Quantification of fungal biomass of TRV-EV plants and TRV-U196237 plants. Values were normalized relative to *elongation factor 1α* (*EF*), and calibrated to levels in TRV-EV plants. Error bars represent standard deviation of five TRV-EV and ten TRV-U196237 plants. For each plant DNA was extracted from pooled 3rd and 4th leaves. (C), Transcript levels of *ALS1*, *ALS2*, and *ALS3* in green and chlorotic areas of TRV-U196237 leaves. Values were normalized relative to *EF*, and calibrated to levels in green area. Error bars represent standard deviation of five biological replicates. Asterisks indicate significant difference from the control according to independent-samples t-test (P <0.05).

To validate the specificity of silencing, transcript levels of *ALS1*, *ALS2* and *ALS3* in NIL-*Ol-1* plants subjected to VIGS were measured by qRT-PCR using RNA isolated after pooling the third and fourth whole leaves of each plant. In this experimental set-up

expression levels of the three *ALS* genes were not significantly reduced in TRV-U196237-infiltrated plants compared with TRV-EV-infiltrated plants (data not shown), although the alteration in leaf morphology indicated a VIGS effect. However, we noticed that fungal colony growth was stronger on the chlorotic areas of the leaves than on the green areas. Therefore, transcript levels of *ALS1*, *ALS2* and *ALS3* were compared between leaf samples collected from excised green and chlorotic areas of TRV-U196237-infiltrated plants. The expression levels of *ALS1* and *ALS2* were significantly lower in chlorotic areas in comparison with green areas, while expression of *ALS3* was also somewhat decreased in chlorotic areas, but not significantly (Figure 2C). This indicated that the fragment present in the TRV-U196237 VIGS construct specifically silenced *ALS1* and *ALS2*, but not *ALS3*.

To confirm the results obtained with VIGS, we generated stable transformants of NIL-*Ol-1* in which both *ALS1* and *ALS2* were silenced by an RNAi construct (RNAi-ALS1+2) containing an inverted repeat of the same sequence as present in the TRV-U196237 vector. We expected that when *ALS1* and *ALS2* were efficiently silenced by the RNAi construct, the transformants would show the same visible phenotype as in VIGS, *i.e.* smaller plants with chlorotic leaves. Nine primary transformants (T1) were selfed to produce T2 families. For each T2 family ten plants were phenotypically examined. One T2 family (216) showed clear segregation for the phenotypic traits mentioned above (reduced stature and chlorotic leaves) (Figure 3A). The altered phenotype co-segregated with the presence of the silencing construct, as indicated by amplification of the 35S promoter (Figure 3A). After inoculation with *On* the T2 plants with an altered phenotype showed increased sporulation when compared with the untransformed NIL-*Ol-1* plants (Figure 3B), although full susceptibility as in cultivar MM was not reached. Fungal growth and transcript levels of *ALS* genes were quantified in NIL-*Ol-1* plants and in T2 plants with altered phenotype. The results showed that the *On* fungal biomass for these T2 plants was significantly increased compared with that for NIL-*Ol-1* (Figure 3C). As expected, the expression levels of both *ALS1* and *ALS2* were significantly suppressed in the RNAi-ALS1+2 T2 plants, whereas *ALS3* expression was not significantly reduced (Figure 3D).

In addition to the production of stable transformants in which both *ALS1* and *ALS2* were silenced simultaneously, stable NIL-*Ol-1* transformants were produced in which the three *ALS* genes were silenced individually to evaluate their involvement in *Ol-1*-mediated resistance. No cross-silencing was observed (Supplental figure 2). Transformed T1 plants were selfed to obtain T2 families. One T2 family for *ALS1*, three for *ALS2*, and two for *ALS3* were obtained. The NPTII-containing, and thus transgenic, T2 plants were selected by PCR analysis. The transgenic T2 progeny showed a significant reduction of expression of the targeted *ALS* gene (Figure 3F). Silencing of the three *ALS* genes individually did not lead to morphological alteration, and fungal abundance was not enhanced compared to that in untransformed NIL-*Ol-1* plants (Figure 3E). The fact that suppression of individual *ALS* genes did not compromise *Ol-1*-mediated resistance, but suppression of at least two *ALS* genes compromised the resistance indicated that the function of *ALS* genes is likely overlapping.

We did not try to generate a construct targeting all three genes simultaneously, because no continuous stretch of at least 21 identical nucleotides is present when aligning the complete coding sequences of the three *ALS* genes (supplemental figure 1B).

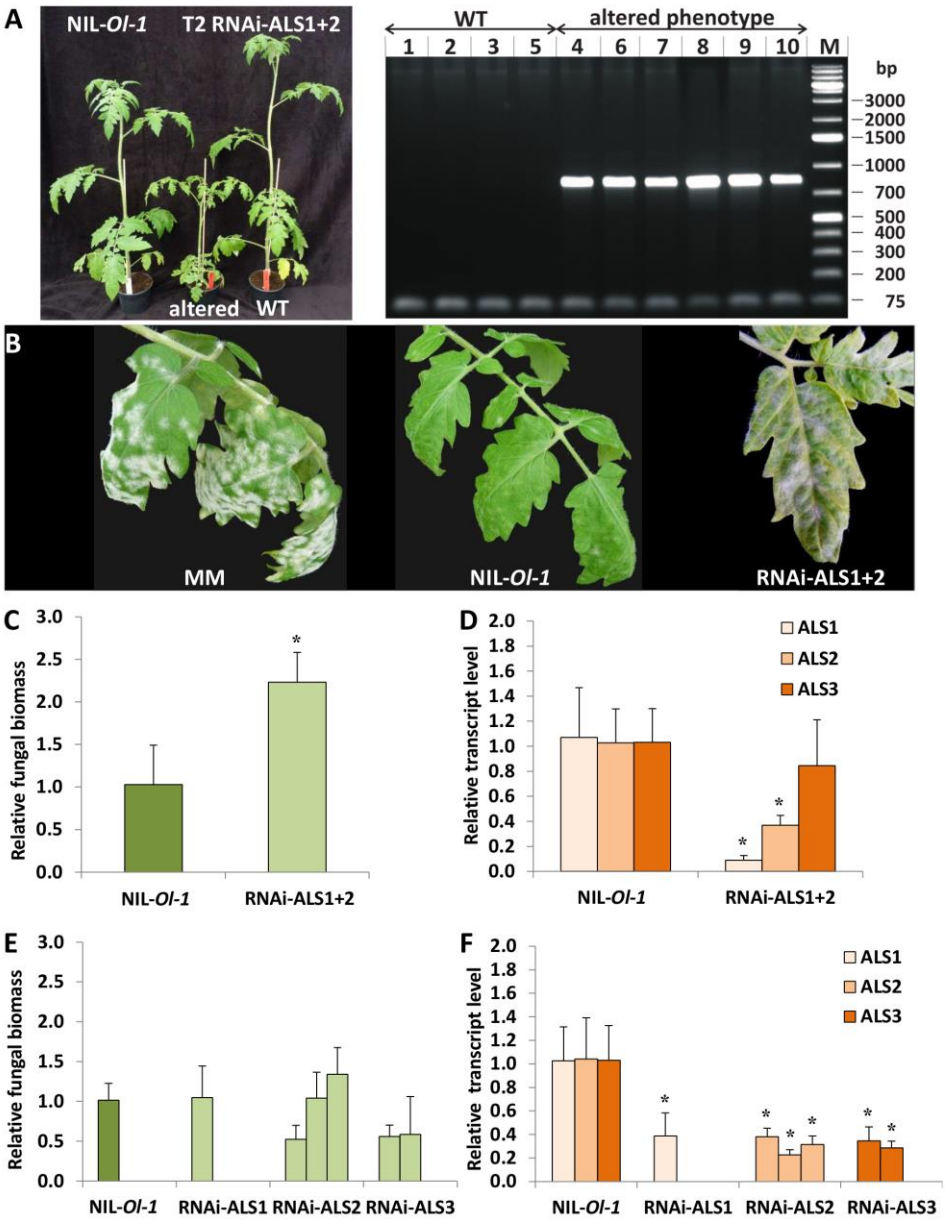


Figure 3 Assessment of resistance in RNAi transformants of NIL-*Ol-1*. (A), Segregation for phenotypic traits in T2 plants obtained after selfing RNAi-ALS1+2 transformant 216, compared with the NIL-*Ol-1* untransformed plant. PCR analysis of the 35S promoter of the silencing construct showed co-segregation of altered phenotype with presence of the expected PCR product. (B) Fungal symptoms on MM, NIL-*Ol-1* and NIL-*Ol-1* transgenic plant with the ALS1+2 RNAi construct (T2 plant). (C), Fungal biomass and (D), transcript levels of *ALS1*, *ALS2*, and *ALS3* in RNAi-ALS1+2 T2 plants of family 216 showing an altered phenotype, compared with that in NIL-*Ol-1* plants. (E), Fungal biomass and (F), transcript levels of *ALS1*, *ALS2*, and *ALS3* in NPTII-containing T2 plants in which three ALS genes were targeted individually by RNAi (RNAi-ALS1, RNAi-ALS2, and RNAi-ALS3). For each plant RNA was isolated from pooled 3rd and 4th leaves. Values were normalized relative to *EF*, and calibrated to the levels in untransformed NIL-*Ol-1* plants. Error bars represent standard deviation of three biological replicates. Asterisks indicate significant difference from the controls according to independent-samples t-test and one way analysis of variance (P <0.05).

ALS is specifically involved in *Ol-1*-mediated resistance

Acetolactate synthase is a well-known target for commercial herbicides, which block binding of substrates to the active site of the ALS enzyme (Duggleby et al. 2008). We employed this system to determine whether ALS was generally involved in powdery mildew resistance or specifically involved in *Ol-1*-mediated resistance. The herbicide chlorsulfuron was used as the ALS inhibitor. First, we studied the effect of herbicide treatment in NIL-*Ol-1* plants. As chlorsulfuron was dissolved in acetone, we included plants to which only acetone was applied as well as plants to which water was applied as controls. The herbicide application caused inhibition of shoot growth and overall chlorosis of the plant (Figure 4A). Quantification of fungal DNA showed that a significant increase of fungal biomass was attributable to herbicide treatment, as compared with the acetone control (Figure 4A, 4B).

As powdery mildew fungi depend on living tissue for nutrient uptake, we wondered whether the probable perturbation of amino acid homeostasis due to silencing of *ALS* could be exploited by the pathogen, and in turn influence the basal defense. To address this question we treated susceptible tomato MM with chlorsulfuron. If ALS is important for basal defence against powdery mildew, one would anticipate an increase of sporulation. After herbicide treatment we observed morphological changes in MM plants, which were similar to those in NIL-*OL-1* plants. However, fungal biomass in chlorsulfuron-treated MM plants was similar to fungal biomass in water- and acetone-treated MM, suggesting that ALS was not involved in basal defence (Figure 4B). Chlorsulfuron was also applied to NIL-*Ol-4* plants to determine whether ALS is generally involved in powdery mildew resistance signalling pathways. Quantification of fungal biomass showed that herbicide-treated NIL-*Ol-4* plants retained a similar resistance level to powdery mildew as the control NIL-*Ol-4* plants, suggesting that ALS is dispensable for resistance conferred by *Ol-4*, encoding a NB-LRR type protein (Figure 4B).

Similar to the results obtained with the VIGS and RNAi plants, we observed that, although NIL-*Ol-1* plants in which ALS function was impaired showed increased susceptibility to *On*, full susceptibility as in cultivar MM was not reached. This is shown in Figure 4C, in which fungal biomass in NIL-*Ol-1* and NIL-*Ol-4* plants is calibrated to the level in water-treated MM.

Expression of *ALS* genes upon powdery mildew attack in NIL-*Ol-1* and MM

To investigate the response of three *ALS* genes under powdery mildew attack, their transcript levels were measured in NIL-*Ol-1* and MM. Expression of *ALS1* and *ALS2* was detected in both genotypes, and they were not induced by powdery mildew inoculation in either genotype (Figure 5). ANOVA analysis indicated that there was no significant difference in expression levels of *ALS1* and *ALS2* between NIL-*Ol-1* and MM. *ALS3* expression was only detected in NIL-*Ol-1*, while in MM it may be either weakly expressed below detection level, or not expressed at all (Figure 5). We could exclude the possibility that primers for quantifying *ALS3* expression were not suitable for MM because a PCR product of the expected size was obtained using genomic DNA as template. Further, RNA-seq data of *ALS3* (*Solyc06g059880*) from tomato cultivar Heinz (Tomato Functional Genomics Database. http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi.) confirm that *ALS3* is not expressed in tomato leaves (Supplemental figure 3).

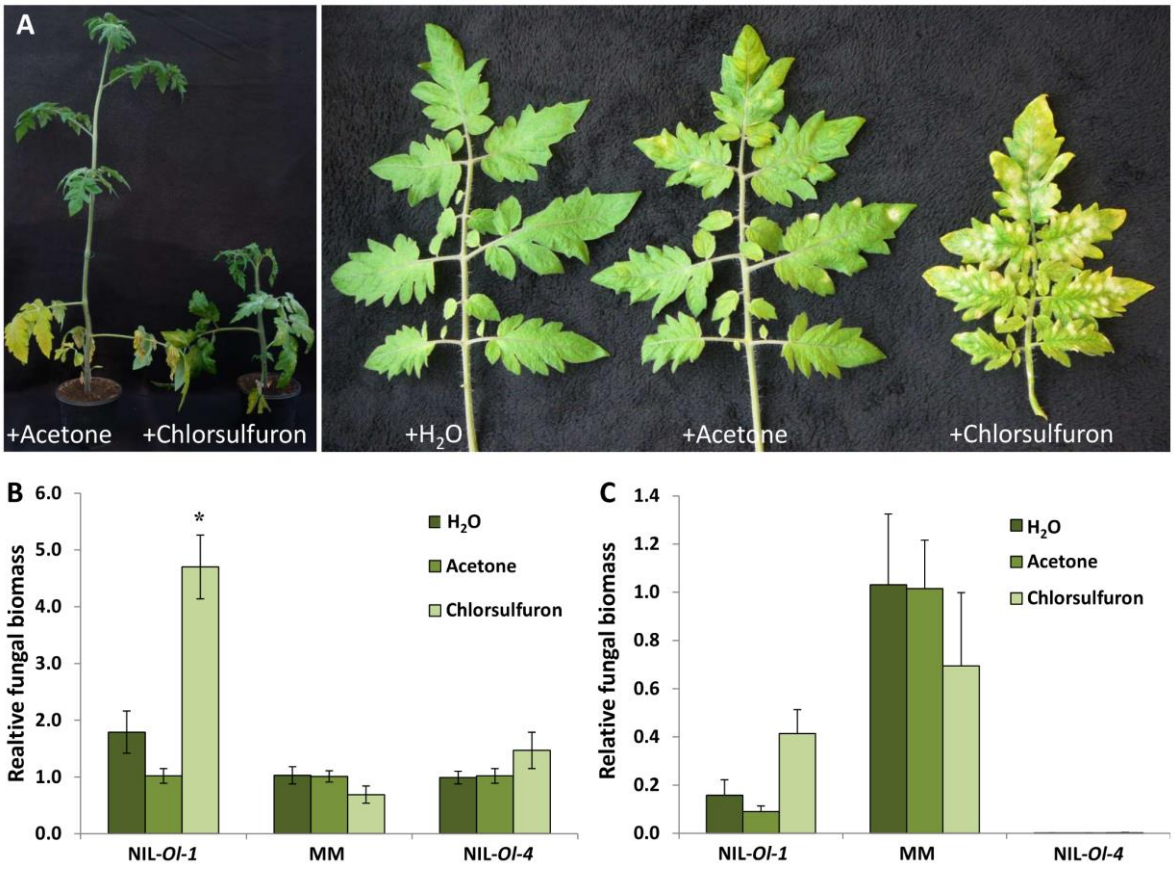


Figure 4 ALS is specifically involved in *Ol-1*-mediated resistance against *On*. (A), Phenotype of NIL-*Ol-1* plants in soil to which acetone (+ Acetone) or chlorsulfuron dissolved in acetone (+ Chlorsulfuron) has been added, and close-up of *On* development on the leaves. (B), Relative fungal biomass on NIL-*Ol-1*, MM and NIL-*Ol-4* plants grown in soil to which water (H_2O), acetone or chlorsulfuron has been added. For each plant DNA was extracted from pooled 3rd and 4th leaves. Values are normalized relative to *EF*, and calibrated to level on plants grown in soil with acetone. Error bars represent three biological replicates for H_2O and acetone treatments respectively, and five or more replicates for chlorsulfuron treatment. Two independent experiments were performed with similar results, and data from one experiment are presented. Asterisk indicates significant difference from the controls according to one way analysis of variance (P <0.05). (C), Relative *On* fungal biomass on NIL-*Ol-1*, MM and NIL-*Ol-4* plants as in Panel B, but calibrated to the level on water-treated MM plants.

Discussion

In a screen of differentially expressed transcripts showing a difference in presence or intensity when comparing powdery mildew-resistant NILs with susceptible MM, TDF M11E69-195 was identified (Li et al. 2006, 2007), which shows homology to *acetolactate synthase*. This TDF was specifically present in NIL-*Ol-1*, but absent in both MM and NIL-*Ol-4* (Li et al. 2006, 2007). By targeting acetolactate synthases via VIGS, RNAi and herbicide application, we demonstrated that ALS activity was specifically important for *Ol-1*-based resistance (Figure 2B, 3C and 4B). ALS does not seem to be involved in basal defense as indicated by unchanged susceptibility after herbicide treatment of MM, nor required for resistance controlled by NB-LRR-type resistance genes as indicated by the results from herbicide treatment of NIL-*Ol-4* (Figure 4B).

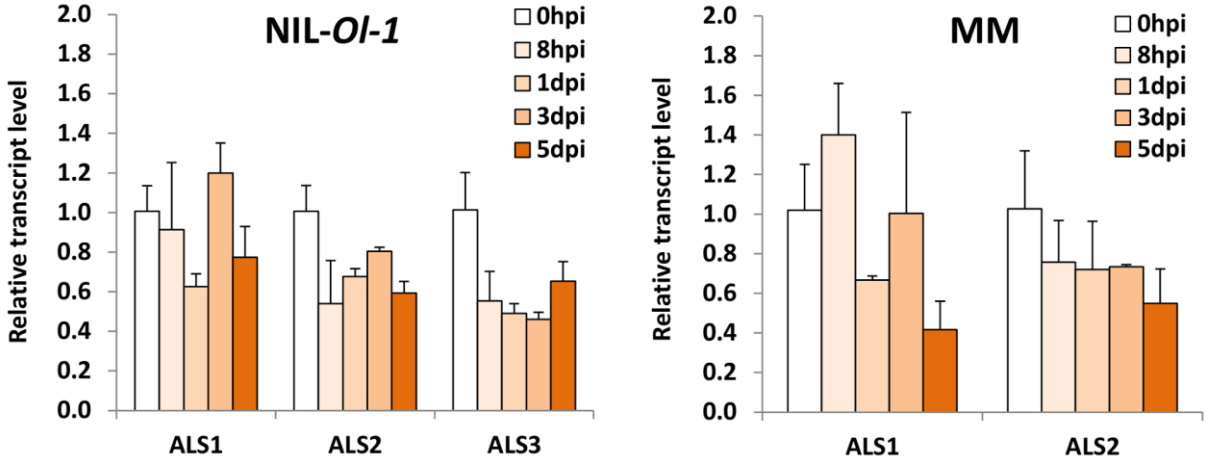


Figure 5 Expression profiles of three *ALS* genes in NIL-*Ol-1* and MM upon powdery mildew attack. Samples were harvested at 0 hpi (prior to inoculation), 8 hpi (hours post inoculation), 1 dpi (days post inoculation), 3 dpi and 5 dpi. For each biological replicate the 3rd and 4th leaves were pooled from three plants. Values are normalized relative to *EF*, and expression level at each time point after inoculation were calibrated to levels of counterpart plants without inoculation. Error bars represent standard deviation of three biological replicates. To test whether expressions of *ALS1* and *ALS2* were different between NL-*Ol-1* and MM, two-way between groups ANOVA was used.

Possible involvement of *ALS3* in *Ol-1*-mediated resistance to *On*

The fact that TDF M11E69-195 was observed in NIL-*Ol-1*, but absent in both MM and NIL-*Ol-4* could be caused solely by the presence of nucleotide polymorphisms between MM and NIL-*Ol-1*. However, we have shown that the corresponding *ALS3* gene is truly differentially expressed, as *ALS3* transcript was observed in leaves from NIL-*Ol-1* but not in MM leaves (Figure 5). The sequence of M11E69-195 from NIL-*Ol-1* showed higher similarity to *ALS3* than to *ALS1* and *ALS2* from *S. lycopersicum* (Supplemental figure 1A). *ALS3* (*Solyc06g059880*) is located on the long arm of chromosome 6, but not in the *Ol-1* region. *ALS1* and *ALS2* are located on chromosomes 3 and 7, respectively. As NIL-*Ol-1* only contains (part of) chromosome 6 of *S. habrochaites* G1.1560 whereas all other chromosomes are from *S. lycopersicum* MM, we expected that the *ALS1* and *ALS2* genes from NIL-*Ol-1* were identical to those from MM. This was indeed observed after sequencing complete *ALS1* and *ALS2* cDNAs from NIL-*Ol-1* (data not shown). In contrast, sequencing of the complete *ALS3* cDNA from NIL-*Ol-1* revealed a number of SNPs and indels in NIL-*Ol-1* compared to the predicted sequence from tomato cultivar Heinz in the SGN database (Supplemental figure 1B). NIL-*Ol-4*, containing an introgression of part of chromosome 6 from *S. peruvianum* accession LA2172 (Seifi 2011), was expected to contain *ALS1* and *ALS2* sequences identical to those from MM, whereas the *ALS3* sequence from NIL-*Ol-4* differed from both MM and NIL-*Ol-1*(data not shown).

The VIGS and RNAi constructs targeted *ALS1* and *ALS2*, but not *ALS3* (Figure 2C, 3D). As *ALS1* and *ALS2* in all three genotypes are identical, but the effect of silencing is specific for NIL-*Ol-1,* we wonder whether *ALS3* plays a role in resistance to tomato powdery mildew conferred by *Ol-1*. Although *ALS3* is homologous to acetolactate synthase genes whose function has been proven, the exact function of the ALS3 protein

is unknown. In plants ALS is a heteromultimer, consisting of catalytic and regulatory subunits (Duggleby et al. 2008; Binders 2010; Chen et al. 2010). All three tomato proteins ALS1, ALS2 and ALS3 are homologous to known catalytic subunits, such as the SuRA and SuRB proteins of *Nicotiana tabacum* (Chaleff and Bascomb 1987). In Solanaceous species from which genome sequences are available three *ALS* genes coding for catalytic subunits are present. In contrast, Arabidopsis only contains one *ALS* gene encoding the catalytic subunit, *i.e. At3g48560*.

In MM and NIL-*Ol-4* only *ALS1* and *ALS2* are expressed in leaves, while *ALS3* is not. Similarly, the orthologs of *ALS3* in *S. pimpinellifolium* and *S. tuberosum* are not expressed in leaves (Supplemental figure 3). NIL-*Ol-1* is exceptional, as in this genotype *ALS3* is expressed in leaves, together with *ALS1* and *ALS2*, and therefore ALS3 may be incorporated in the ALS holoenzyme. Possibly, the presence of different catalytic subunits in the ALS holoenzyme confers different functionalities or substrate specificities. Although silencing of only *ALS3* in the NIL-*Ol-1* background did not result in increased susceptibility to *On* (Figure 3E), this does not exclude the possibility that *ALS3* is involved in resistance. The obtained transformants showed significant silencing of *ALS3* (Figure 3F), but no complete silencing comparable to a knock-out mutation was achieved. Additional experiments are needed to elucidate the function of *ALS3* in leaves of NIL-*Ol-1*, for example expression of the *S. habrochaites ALS3* gene in MM background.

Involvement of amino acid homeostasis caused by altered ALS activity in *Ol-1*-mediated resistance

Although acetolactate synthase is a known target of several herbicides, it is unclear how herbicide-binding affects the amino acid metabolism in plants. Scheel and Casida (1985) found that chlorsulfuron treatment of soybean suspension cultures caused a decrease of the valine and leucine contents, but had no effect on other amino acids. They showed that growth inhibition by chlorsulfuron was alleviated by supplying exogenous valine or leucine, or a combination of valine, leucine and isoleucine. Consistent with a reduction of BCAAs caused by an ALS-affecting herbicide, Ray (1984) observed that addition of valine and isoleucine to excised pea root cultures reversed herbicide-induced growth inhibition. Growth retardation can also result from ALS feedback inhibition by individual end products. Chen et al. (2010) showed that addition of valine or leucine to the growth medium inhibited root growth of Arabidopsis seedlings, whereas addition of isoleucine had no effect. When a combination of valine + isoleucine, or leucine + isoleucine was added to the medium root growth inhibition was less pronounced, suggesting isoleucine counteracted the inhibitory effect of valine and leucine. Royuela et al. (1991) detected an increase in the relative proportion of some amino acids other than BCAAs in chlorsulfuron-treated wheat and maize. Höfgen et al. (1995) silenced *ALS* in potato by antisense inhibition, resulting in a decrease of ALS activity of up to 85%. Strong silencing of *ALS* resulted in severe growth retardation and stunting, and leaf chlorosis. Similar phenotypic alterations were obtained by treatment with an imidazolinone herbicide. Measurement of amino acids showed an accumulation of total free amino acids as well as perturbed composition in antisense and herbicide-treated plants. Unexpectedly, instead of decreased levels, elevated amounts of amino acids including valine, leucine and isoleucine were observed, especially in older sink leaves.

Recently, another example of a link between herbicide resistance, increased amino acid levels, and resistance to fungal species was reported. Patent US8383887 (Fakhoury and Lightfoot 2013) discloses that corn plants expressing the bacterial *gdhA* gene (NADPH-dependent glutamate dehydrogenase) are resistant to aflatoxin accumulation following *Aspergillus* infection. Furthermore, corn and tobacco plants transformed with the *gdhA* gene are resistant to root rot following *Fusarium virguliforme* infection. Previously, it has been shown that tobacco plants transformed with the *gdhA* gene show an increased level of resistance to the herbicide glufosinate (Nolte et al. 2004) and that total free amino acids were increased in these plants (Ameziane et al. 2000; Mungur et al. 2005).

Taken together, the effect of ALS inhibition on levels of individual amino acids is difficult to predict, as it seems to depend on the level of residual ALS activity in different tissues, and the feedback-inhibition effect of (combinations) of individual amino acids. Despite this, we investigated whether BCAAs contents influence powdery mildew susceptibility or resistance in tomato cultivar MM and NIL-*Ol-1* by exogenously applying leucine, isoleucine and valine (Huibers et al. 2013, Supplemental figure4). Homoserine and threonine were also included in the experiment, because they were found to affect plant immunity, and threonine is the precursor of isoleucine. If a higher level of BCAA contributes to *Ol-1*-mediated resistance, we expected to gain powdery mildew resistance to some degree in MM with elevated BCAAs levels. Quantification of fungal DNA showed that only exogenous application of homoserine significantly reduced the susceptibility of MM and increased resistance of NIL-*Ol-1* to *On*, whereas application of the other amino acids did not alter the responses of MM and NIL-*Ol-1* to *On* (Huibers et al. 2013, Supplemental figure4). We also did not observe the growth retardation which can be caused by individual end products, possibly because the concentration was not sufficiently high to cause this. The results suggested that instead of an elevated level, a reduced level of BCAAs or changed compositions of individual amino acids may play a role in *Ol-1*-mediated powdery mildew resistance. Amino acid deprivation is known to activate defences in Arabidopsis. For instance, the accumulation of camalexin, a pathogen-inducible antimicrobial phytoalexin was induced by BCAAs starvation (Zhao et al. 1998). An alternative hypothesis is the involvement of an amino acid-derived signal(s) in defense signalling pathways, as suggested for Arabidopsis genes *ALD1* and *AGD2* encoding aminotransferases (Song et al. 2004). Furthermore, studies on plant hormone conjugates showed that jasmonate (JA) can conjugate BCAAs (Sembdner et al. 1994) and, in particular, JA-isoleucine is the main bioactive form of the hormone (Fonseca et al. 2009). In addition, altered expression of an enzyme involved in conjugation affects salicylic acid (SA)-mediated disease resistance (Park et al. 2007).

In the case of *Ol-1*, perturbation of amino acid balance by silencing of *ALS* or herbicide treatment may impair the integrity of the signalling network, leading to the loss of resistance conferred by *Ol-1*. The unknown identity of *Ol-1* makes it harder to understand the link between *Ol-1*-mediated resistance and amino acid homeostasis. Cloning of *Ol-1,* determination of amino acid homeostasis, and dissection of changes in hormone signalling pathways will aid in understanding the requirement of ALS activity for *Ol-1*-based resistance and shed light on the interaction of amino acid metabolism and plant immunity.

Materials and Methods

Plant materials, fungal isolate and inoculation

All the near isogenic lines have been described previously (Bai et al. 2005). They were obtained by crossing wild tomato species containing the resistance gene(s) with *S. lycopersicum* cultivar MM, three backcrosses with MM, followed by two selfings (BC3S2 plants). *On* isolate Netherlands was maintained on susceptible MM plants in a growth chamber at 21/19 ºC (day/night). Fungal spores were washed off from heavily infected tomato leaves with tap water and diluted to a concentration of 2.5 $x10^4$ spores per mL. The inoculum was evenly sprayed on 3 to 4 weeks-old plants.

Virus-induced gene silencing (VIGS)

VIGS was performed using the TRV-based vector system (Liu et al. 2002). Primers for the TRV2 construct targeting SGN-U196237 were Fw-U196237-caccCAATGGGAGGATCGGTTCTA and Rv-U196237-ATCTCCCATCACCCTCTGT. A 290–bp fragment was amplified from cDNA of NIL-*Ol-1* plants, and subsequently cloned into pENTR/D-TOPO vector (Invitrogen). After verification of the sequence the fragment was introduced into the pTRV2-attR1-attR2 vector via LR recombination. The resulting TRV-U196237 vector was transformed into *Agrobacterium* strain GV3101. To establish VIGS, cotyledons of 10-days-old plants were agroinfiltrated with a mixture of TRV1 and TRV-U196237 (combined in a 1: 1 ratio). As a control, a mixture of TRV1 and empty TRV2 vector (TRV-EV) was used. Three weeks after agroinfiltration the plants were sprayed with *On* inoculum. Three independent experiments were performed. For two experiments disease symptoms were visually scored 21 dpi by counting the number of fungal colonies on the leaves. For one experiment silencing level and fungal growth were quantified by qRT-PCR, using RNA from five plants for TRV-EV and 10 plants for TRV-U196237.

Generation of stable silenced lines

To suppress both *ALS1* and *ALS2* by RNAi, the same fragment as used in VIGS construct TRV-U196237 was introduced into the pHellsgate8 vector (Helliwell et al. 2002). For the purpose of targeting *ALS1* and *ALS2* separately, primers were designed based on the 3' UTR sequences. For *ALS1*: Fw-*ALS1*-caccGCCAAAAGTGTTCGATTTGT and Rv-*ALS1*-AGTGAACATAAATACCAAGTAGAAGAT. For ALS2: Fw-*ALS2*-caccTGTTTACTTAAAAGTTTTTC ATTGTG and Rv-*ALS2*-TTAGTCATACTAAATAGAGCTCCAAA. To suppress *ALS3*, primers were designed based on the sequences in coding region: Fw-*ALS3*-caccTTATCTTGGAAATCCTTCTAACAA and Rv-*ALS3*-TTCTTATGAATCACTTGAGCA. Fragments amplified with abovementioned primers were introduced into pHellsgate8 vector and finally transformed into *Agrobacterium* strain AGL1+virG. For generation of silenced lines the protocol described by Huibers et al. (2013) was used. Primary transformants (T1) were selfed to produce T2 families. For each segregating T2 family, CaMV 35S promoter primers Xho-Fw-TGCTGACCCACAGATGGTTA and 35S2-GATAGTGGGATTGTGCGTCA (Hurst et al. 1999) amplifying a 756-bp fragment, or NPTII primers Fw-NPTII-TTCCCCTCGGTATCCAATTA and Rv-NPTII-GATTGTCTGTTGTGCCCAGT amplifying a 170-bp fragment from the pHellsgate8 T-DNA, were used to select transgenic T2 plants.

Herbicide application

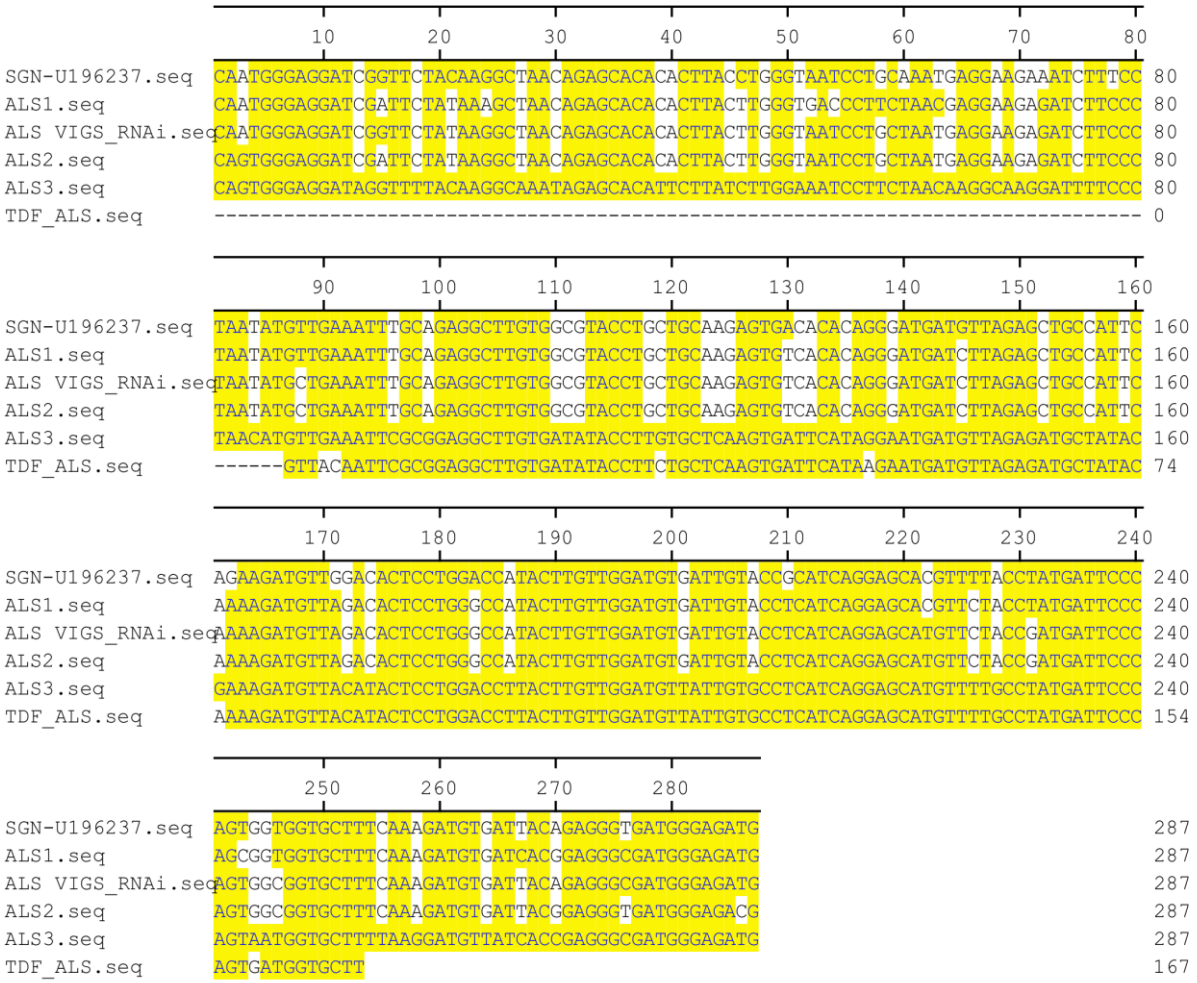
Chlorsulfuron was purchased from Aldrich-Sigma (PS-1065), and the powder was dissolved in acetone (0.2 mg/ml). The herbicide solution was applied to 30-day-old plants of MM and NIL-*Ol-1* growing in ø14 cm pots in normal potting soil. Before application, watering was suspended for two days to ensure that the solution could be absorbed completely. As controls, water and acetone were applied. The chlorsulfuron solution, acetone and water were added to the soil with a pipette (8 ml per pot). After this, the plants were challenged with powdery mildew *On* at the same day of herbicide application. Per genotype 15 plants were treated with chlorsulfuron, and five plants with water or acetone.

Quantitative reverse transcription PCR (qRT-PCR) and data analysis

For quantification of fungal biomass, DNA or RNA extracted from tomato leaves was used. For quantification of transcript levels, RNA was used. DNA was isolated with DNeasy plant mini kit (Qiagen). Total RNA was extracted from leaflets using the RNeasy kit (Qiagen). After removal of DNA with DNase I (Invitrogen), 1 µg total RNA was used for cDNA synthesis using SuperScript II Reverse Transcriptase kit (Invitrogen). Quantitative real-time PCR was conducted using the iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). The PCR amplification consisted of an initial denaturation step of 3 min at 95°C, followed by denaturation for 15 sec at 95°C, annealing and extension for 1 min at 60°C for 39 cycles, then a final melt step from 65°C to 95°C ramp with 0.5°C increments per cycle to monitor specificity. Primers used for fungal quantification were Fw-*On*-CGCCAAAGACCTAACCAAAA and Rv-*On*-AGCCAAGAGATCCGTTGTTG. Primers for tomato *EF* were Fw-*EF*-GGAACTTGAGAAGGAGCCTAAG and Rv-*EF*-CAACACCAACAGCAACAGTCT (Løvdal and Lillo 2009). For detection of relative transcript levels of the *ALS* genes primers were Fw-*ALS1*-CGCTCAACATAATCGTCGTG and Rv-*ALS1*-ACGGGAAACGAATGTTTCAG for *ALS1*; Fw-*ALS2*-CCCTTCTTCCCAAATCTACCT and Rv-*ALS2*-TTGAAACAGTGAAACGGCTATG for *ALS2*; Fw-*ALS3*-TTTGCTGCTAGCATTTGGAG and Rv-*ALS3*- GGAGTCGATATCAATGTGAACAA for *ALS3*. For the time-course experiment in which the expression of three *ALS* genes was monitored after inoculation with *On*, the same set of primers was used as for detection of relative transcript levels of each *ALS* gene after silencing. For analysis of the relative expression level and fungal biomass the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (Livak and Schmittgen 2001) was used. Data were statistically examined using independent-samples t-test, one-way analysis of variance or two-way between groups ANOVA based on Post-hoc comparisons using Tukey's HSD test (P <0.05). All analyses were performed using SPSS Statistics 20 following the instructions of SPSS Survival Manual 4th edition (Pallant 2010).

Supplemental figure 1 Sequence alignments. (A), Sequence alignment of TDF M11E69-195 and *ALS* PCR fragment used in VIGS and RNAi constructs ALS1+2 (both from NIL-*Ol-1*) with the corresponding part of unigene SGN-U196237 from *Capsicum annuum*, and of *ALS1*, *ALS2* and *ALS3* transcripts from tomato cultivar Heinz. Nucleotides identical with the *ALS3* sequence are highlighted. (B), Sequence alignment of *ALS1*, *ALS2* and *ALS3* coding sequences (CDS) of tomato cultivar Heinz, and the *ALS3* CDS of NIL-*Ol-1*. Nucleotides identical with the Heinz *ALS3* sequence are highlighted. (C), Sequence alignment of protein sequences from tomato cultivar Heinz ALS1, ALS2 and ALS3 with ALS3 from NIL-*Ol-1*. Amino acids identical with the ALS3 sequence are highlighted.

Supplemental figure 1A



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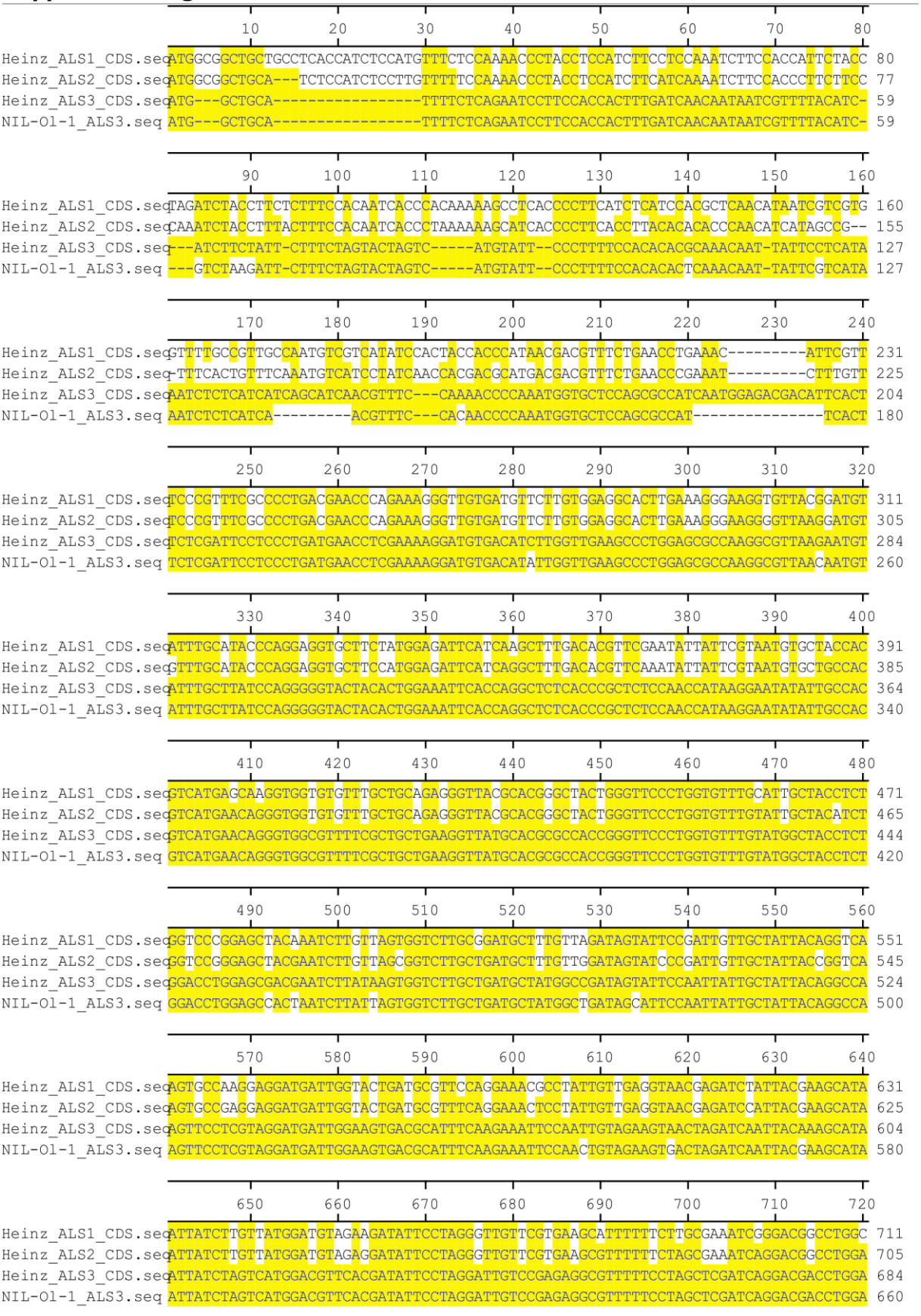

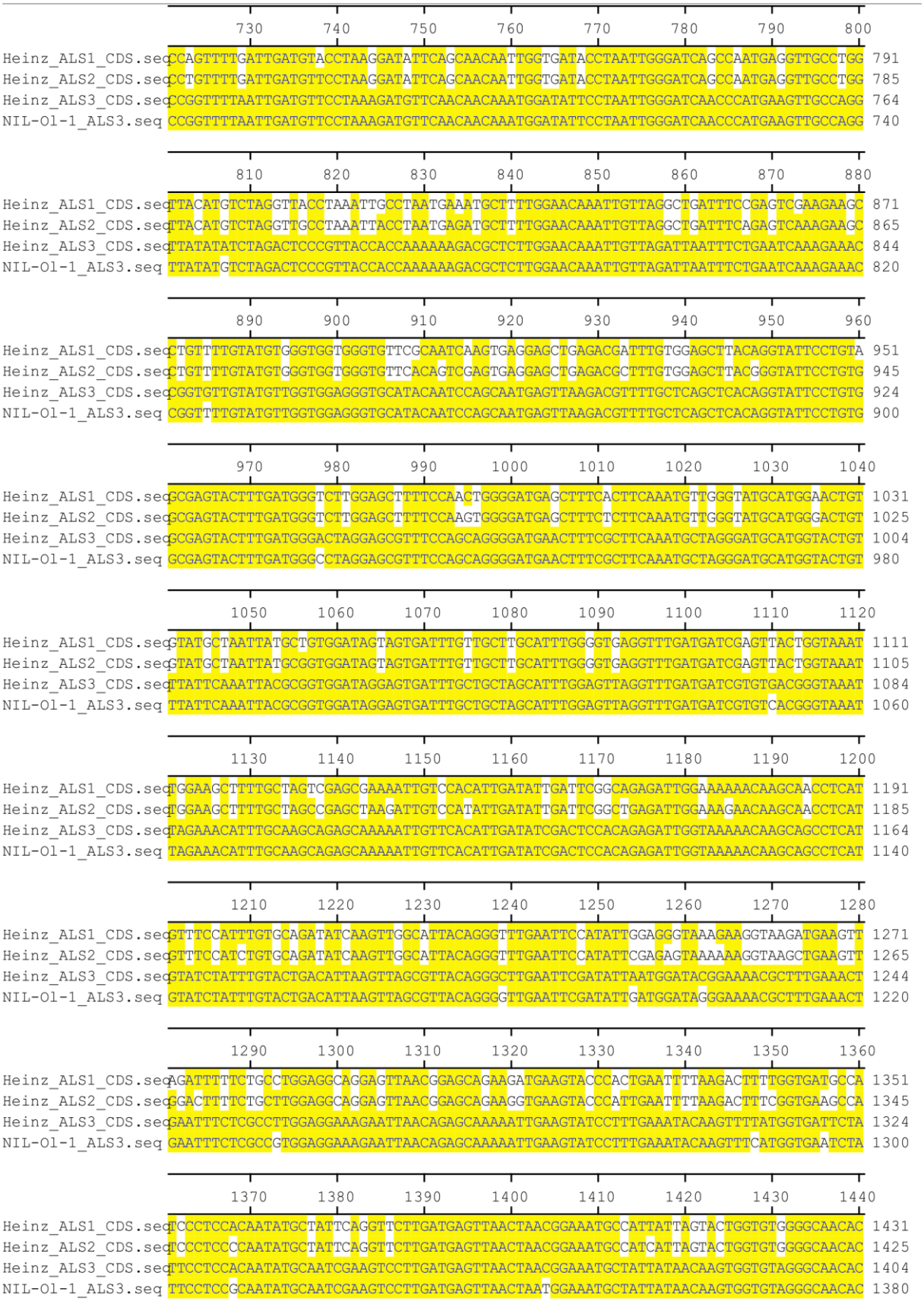
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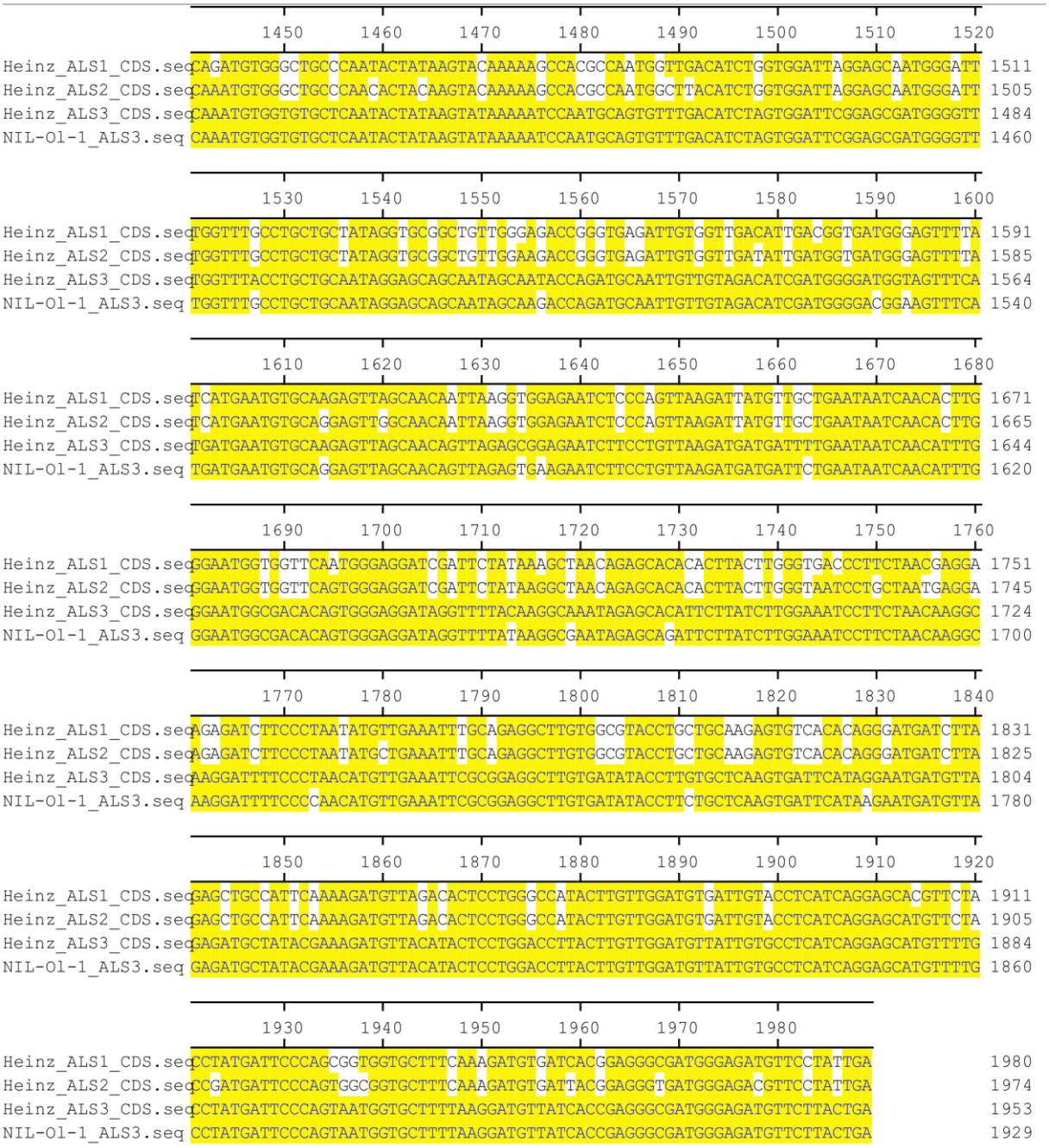
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1530 1540 1550 1560 1570 1580 1590 1600
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Heinz_ALS2_CDS.seq TGGTTTGCCTGCTGCTATAGGTGCGGCTGTTGGAAGACCGGGTGAGATTGTGGTTGATATTGATGGTGATGGGAGTTTTA 1585
Heinz_ALS3_CDS.seq TGGTTTACCTGCTGCAATAGGAGCAGCAATAGCAATACCAGATGCAATTGTTGTAGACATCGATGGGGATGGTAGTTTCA 1564
NIL-Ol-1_ALS3.seq TGGTTTGCCTGCTGCAATAGGAGCAGCAATAGCAAGACCAGATGCAATTGTTGTAGACATCGATGGGGACGGAAGTTTCA 1540
1610 1620 1630 1640 1650 1660 1670 1680
Heinz_ALS1_CDS.seq TCATGAATGTGCAAGAGTTAGCAACAATTAAGGTGGAGAATCTCCCAGTTAAGATTATGTTGCTGAATAATCAACACTTG 1671
Heinz_ALS2_CDS.seq TCATGAATGTGCAGGAGTTGGCAACAATTAAGGTGGAGAATCTCCCAGTTAAGATTATGTTGCTGAATAATCAACACTTG 1665
Heinz_ALS3_CDS.seq TGATGAATGTGCAAGAGTTAGCAACAGTTAGAGCGGAGAATCTTCCTGTTAAGATGATGATTTTGAATAATCAACATTTG 1644
NIL-Ol-1_ALS3.seq TGATGAATGTGCAGGAGTTAGCAACAGTTAGAGTGAAGAATCTTCCTGTTAAGATGATGATTCTGAATAATCAACATTTG 1620
1690 1700 1710 1720 1730 1740 1750 1760
Heinz_ALS1_CDS.seq GGAATGGTGGTTCAATGGGAGGATCGATTCTATAAAGCTAACAGAGCACACACTTACTTGGGTGACCCTTCTAACGAGGA 1751
Heinz_ALS2_CDS.seq GGAATGGTGGTTCAGTGGGAGGATCGATTCTATAAGGCTAACAGAGCACACACTTACTTGGGTAATCCTGCTAATGAGGA 1745
Heinz_ALS3_CDS.seq GGAATGGCGACACAGTGGGAGGATAGGTTTTACAAGGCAAATAGAGCACATTCTTATCTTGGAAATCCTTCTAACAAGGC 1724
NIL-Ol-1_ALS3.seq GGAATGGCGACACAGTGGGAGGATAGGTTTTATAAGGCGAATAGAGCAGATTCTTATCTTGGAAATCCTTCTAACAAGGC 1700
1770 1780 1790 1800 1810 1820 1830 1840
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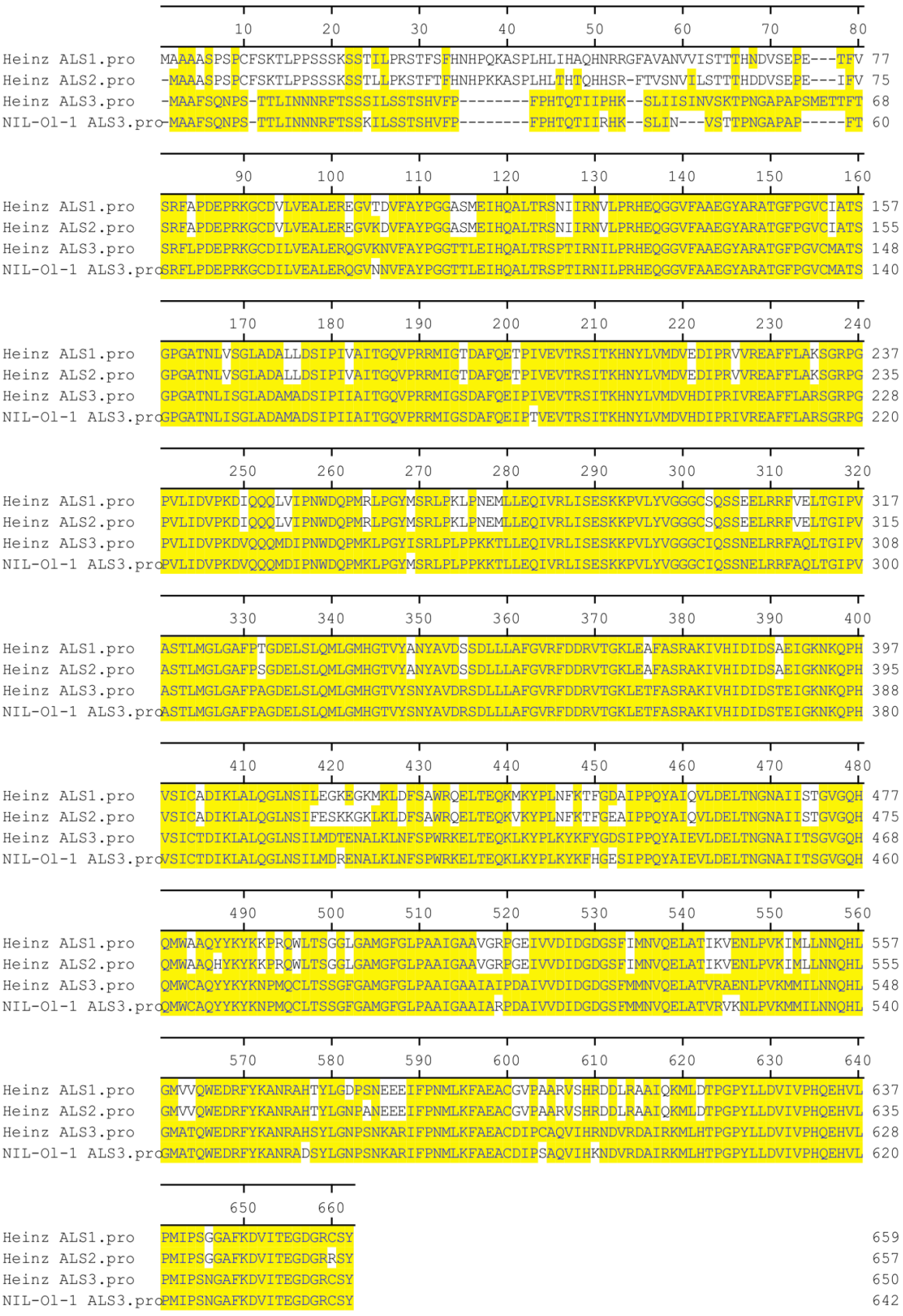
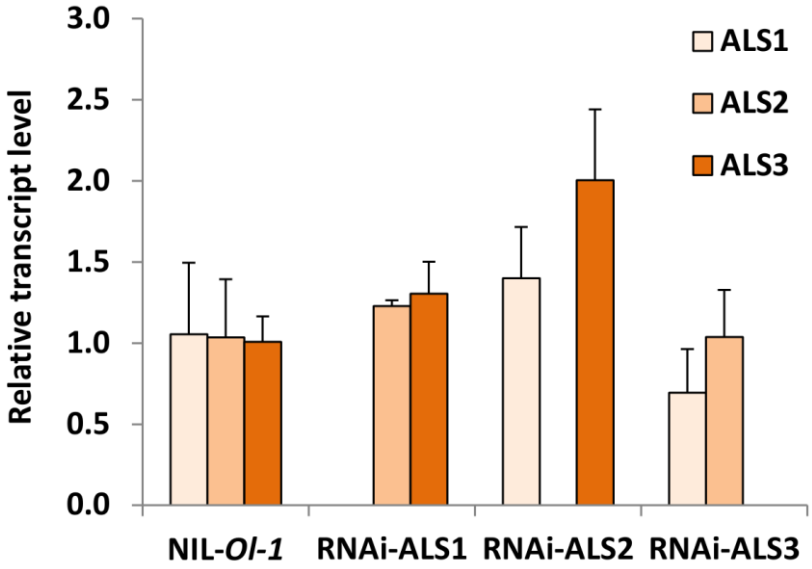
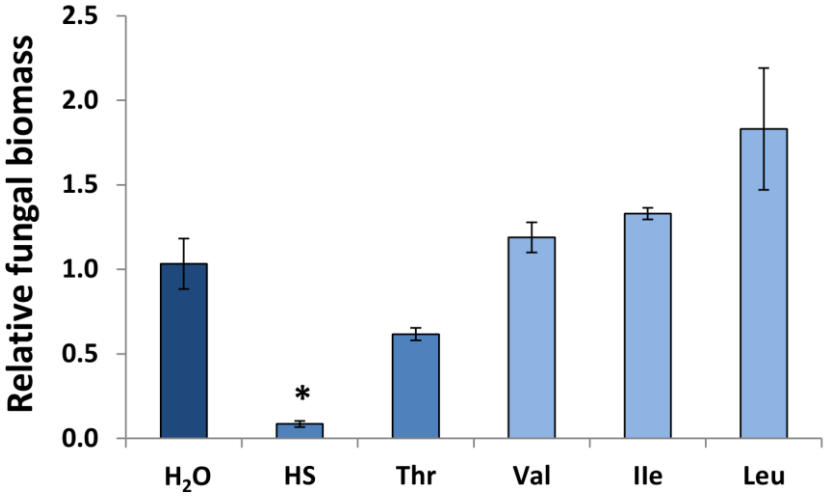
Heinz_ALS2_CDS.seq AGAGATCTTCCCTAATATGCTGAAATTTGCAGAGGCTTGTGGCGTACCTGCTGCAAGAGTGTCACACAGGGATGATCTTA 1825
Heinz_ALS3_CDS.seq AAGGATTTTCCCTAACATGTTGAAATTCGCGGAGGCTTGTGATATACCTTGTGCTCAAGTGATTCATAGGAATGATGTTA 1804
NIL-Ol-1_ALS3.seq AAGGATTTTCCCCAACATGTTGAAATTCGCGGAGGCTTGTGATATACCTTCTGCTCAAGTGATTCATAAGAATGATGTTA 1780
1850 1860 1870 1880 1890 1900 1910 1920
Heinz_ALS1_CDS.seq GAGCTGCCATTCAAAAGATGTTAGACACTCCTGGGCCATACTTGTTGGATGTGATTGTACCTCATCAGGAGCACGTTCTA 1911
Heinz_ALS2_CDS.seq GAGCTGCCATTCAAAAGATGTTAGACACTCCTGGGCCATACTTGTTGGATGTGATTGTACCTCATCAGGAGCATGTTCTA 1905
Heinz_ALS3_CDS.seq GAGATGCTATACGAAAGATGTTACATACTCCTGGACCTTACTTGTTGGATGTTATTGTGCCTCATCAGGAGCATGTTTTG 1884
NIL-Ol-1_ALS3.seq GAGATGCTATACGAAAGATGTTACATACTCCTGGACCTTACTTGTTGGATGTTATTGTGCCTCATCAGGAGCATGTTTTG 1860
1930 1940 1950 1960 1970 1980
Heinz_ALS1_CDS.seq CCTATGATTCCCAGCGGTGGTGCTTTCAAAGATGTGATCACGGAGGGCGATGGGAGATGTTCCTATTGA 1980
Heinz_ALS2_CDS.seq CCGATGATTCCCAGTGGCGGTGCTTTCAAAGATGTGATTACGGAGGGTGATGGGAGACGTTCCTATTGA 1974
Heinz_ALS3_CDS.seq CCTATGATTCCCAGTAATGGTGCTTTTAAGGATGTTATCACCGAGGGCGATGGGAGATGTTCTTACTGA 1953
NIL-Ol-1_ALS3.seq CCTATGATTCCCAGTAATGGTGCTTTTAAGGATGTTATCACCGAGGGCGATGGGAGATGTTCTTACTGA 1929

Supplemental figure 1C





Supplemental figure 2 Absence of cross-silencing by RNAi constructs targeting individual ALS genes. Cross-silencing was not detected in three representative silenced lines in each of which a specific *ALS* gene was targeted by RNAi (RNAi-ALS1, 2 and 3). Values were normalized relative to *EF*, and calibrated to the levels in untransformed NIL-*Ol-1* plants. Error bars represent standard deviation of three biological replicates.



Supplemental figure 4 Effect of exogenous application of amino acids on fungal growth. Amino acids homoserine (HS), threonine (Thr), or branched-chain amino acids valine (Val), isoleucine (Ile) or leucine (Leu) were applied as described in Huibers et al. (2013). Data indicate the mean of three biological replicates with error bars representing the standard deviation. The asterisk indicates significant difference from the H_2O control according to one way analysis of variance (P <0.05).

Tomato FPKM values, RNA-seq data from Solanaceae Genomics Resource, MSU (file tomato_RNA_v2.4_FPKMs, from Robin Buell)

					Heinz leaf		Heinz Root	
gene_id_v2.4		chr	Left	Right	SRR404309 [Heinz Leaf]	SRR404310 [Heinz Leaf]	SRR404311 [Heinz Root]	SRR404312 [Heinz Root]
Solyc03g044330.1.1	ALS1	ch03	12823168	12825148	32.53	10.84	28.71	12.77
Solyc07g061940.2.1	ALS2	ch07	62037697	62039615	43.58	28.83	61.50	30.82
Solyc06g059880.2.1	ALS3	ch06	34171357	34173892	0	0	12.82	11.89

Tomato RPKM values, RNA-seq data from TFGD (file D004_RPKM.xlsx, http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi)

gene		chr	Left	Right	Heinz_leaf	S. pimp leaf	Heinz_root	
Solyc03g044330	ALS1	chr03	12823168	12825148	51.01	120.62	51.92	in tomato: only
Solyc07g061940	ALS2	chr07	62037697	62039615	67.88	55.48	95.16	considerable ALS3
Solyc06g059880	ALS3	chr06	34171357	34173892	0	0	14.29	expression in roots

Potato FPKM values, RNA-seq data from PGSC (file DM_RH_RNA-Seq_FPKM_summary_for_DM_v4.03.xlsx, http://potato.plantbiology.msu.edu/pgsc_download.shtml)

transcript_id	gene	chr	start	stop	BV_L [DM Leaves]	S2 [RH Leaf]	S7 [RH Young Tuber]	in potato: only
PGSC0003DMT400084507	ALS1 (Sotub10g015690)	chr03	8175606	8177917	195.29	201.92	67.95	considerable expression
PGSC0003DMT400018236	ALS2 (Sotub07g025380)	chr07	52142533	52144824	91.84	229.86	54.30	of ALS3 in stolons/young
PGSC0003DMT400033897	ALS3 (Sotub06g018760)	chr06	41503197	41506962	0	0.17	23.37	tubers

Supplemental figure 3 RNA-seq data of *ALS1*, *ALS2* and *ALS3* genes. Expression level of *ALS1*, *ALS2* and *ALS3* genes from tomato, *S. pimpinellifolium* and potato in leaves and root (for tomato) or tuber (for potato) derived from RNA-seq data, and indicated as Fragments per Kilobase of exon per Million fragments mapped (FPKM) or Reads per Kilobase of transcripts per Million mapped (RPKM) values.

Chapter 4

Natural loss-of-function mutation of *EDR1* conferring resistance to tomato powdery mildew in *Arabidopsis thaliana* accession C24

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Natural loss-of-function mutation of *EDR1* conferring resistance to tomato powdery mildew in *Arabidopsis thaliana* accession C24

Abstract
To screen for potentially novel types of resistance to tomato powdery mildew *Oidium neolycopersici*, a disease assay was performed on 123 *Arabidopsis thaliana* accessions. Forty accessions were fully resistant, and one of them, C24, was analysed in detail. By quantitative trait locus (QTL) analysis of a F_2 population two QTL associated with resistance were identified in C24. Fine-mapping of QTL-1 on chromosome 1 delimited the region to an interval of 58 kb encompassing 15 candidate genes. One of these was *Enhanced Disease Resistance 1* (*EDR1*). Evaluation of the previously obtained *edr1* mutant of Arabidopsis accession Col-0, which was identified because of its resistance to powdery mildew *G. cichoracearum*, showed that it also displayed resistance to *O. neolycopersici*. Sequencing of *EDR1* in our C24 germplasm (referred to as C24-W) revealed two missing nucleotides in the second exon of *EDR1* resulting in a premature stop codon. Remarkably, C24 obtained from other laboratories do not contain the *EDR1* mutation. To verify the identity of C24-W, a DNA region containing a SNP unique to C24 was sequenced showing that C24-W contains the C24-specific nucleotide. C24-W showed enhanced resistance to *O. neolycopersici* compared with the C24 not containing *edr1* mutation. Furthermore, C24-W displayed a dwarf phenotype, which was not associated with the mutation in *EDR1* nor caused by differential accumulation of pathogenesis-related genes. In conclusion we identified a natural *edr1* mutant in the background of C24.

Keywords Arabidopsis accessions, C24, natural mutaion, *EDR1*, tomato powdery mildew

Introduction

Powdery mildews are able to colonize a wide variety of plant species including Arabidopsis, and many economically important crops such as wheat, barley and tomato. Resistance to powdery mildews can be manifested through the action of dominantly or semi-dominantly inherited resistance genes (*R*-genes). The most abundant dominant *R*-genes encode proteins containing nucleotide binding site and leucine rich repeat (NB-LRR) domains, such as *Pm3b* in wheat (Yahiaoui et al. 2004), *MLA* alleles in barley (Seeholzer et al. 2010) and *Ol-4* in tomato (Seifi et al. 2011). Due to specific recognition of a matching pathogen-encoded avirulence protein by the cognate *R*-gene product, *R*-gene-mediated resistance is usually race- or isolate-specific (Ellis et al. 2000). In Arabidopsis the only dominant *R*-gene characterized to date conferring resistance to powdery mildews is *RPW8* which is structurally different from common *R*-genes and imparts resistance to several isolates of powdery mildew (Xiao et al. 2001).

Another form of powdery mildew resistance is governed by recessively inherited genes conferring race-non-specific resistance. Based on the resistance mechanism they can be generally classified into three groups. Resistance in the first group is based on loss of function of negative regulators of immune responses. An example is the *edr1* mutation in Arabidopsis resulting in resistance to powdery mildew *G. cichoracearum* and bacteria pathogen *Pseudomonas syringae* (Frye and Innes 1998). *EDR1* encodes a putative MAPKK kinase, and is considered to be a negative regulator because *edr1* resistance is caused by activation of multiple defense responses, including increased defense gene expression and accelerated cell death response at the site of infection (Frye et al. 1998; Frye et al. 2001). Xiao et al. (2005) showed that *EDR1* negatively regulates *RPW8*. The resistance phenotype of *edr1* depends on the salicylic acid (SA) signalling pathway, because double mutants combining *edr1* with mutations that block SA defense responses or reduce SA production reverted to susceptibility for powdery mildew (Frye et al. 2001).

The second group is defined by loss of a host susceptibility factor required for pathogen growth. In a screen for Arabidopsis mutants showing resistance to powdery mildew *G. cichoracearum* independent of constitutive expression of *PR1* or formation of lesions, the *pmr6* mutant was identified (Vogel et al. 2002). *PMR6* encodes a putative pectate lyase and the loss-of-function mutation causes altered cell wall composition (Vogel et al. 2002). *pmr6*-mediated resistance is independent of known defense responses, because mutations in genes encoding components of SA or jasmonate/ethylene pathways do not alter *pmr6* resistance status (Vogel et al. 2002). Furthermore, *pmr6* controls resistance to two powdery mildew species but retains full susceptibility to unrelated pathogens such as bacterium and oomycete species, suggesting PMR6 may be a true powdery mildew compatibility factor (Vogel et al. 2002; Micali et al. 2008). Therefore, *pmr6* likely confers resistance as a result of loss of susceptibility factor rather than activation of known host defense responses.

In the third group, well-defined signalling pathways are not engaged while resistance to unrelated pathogens is displayed. An example is *dmr1*, which mediates resistance to both downy mildew *Hyaloperonospora arabidopsidis* and powdery mildew *O. neolycopersici* (van Damme et al. 2009; Huibers et al. 2013). *DMR1* encodes a homoserine kinase, and its impairment results in accumulation of homoserine, which is responsible for the resistance to downy mildew. *dmr1*-mediated resistance to downy

mildew might trigger a novel defense pathway because exogenous application of homoserine still induces resistance in the single mutant impaired in immune responses or double mutants combining *pmr4* (defective in the production of pathogen-induced callose) with mutations that impairs SA-signalling pathways.

O. neolycopersici (*On*) is a powdery mildew species causing worldwide disease on tomato. Resistance genes have been identified in wild tomato species, including 6 monogenic genes comprising five dominant (*Ol-1*, *-3*, *-4*, *-5*, *-6*) and one recessive (*ol-2*) loci, and 3 polygenic resistance QTL (Bai *et al.*, 2003; Bai *et al.*, 2005). However, to date only the identities of *ol-2*, *Ol-4* and *Ol-6* have been (partially) revealed (Bai et al. 2008; Seifi et al. 2011). *ol-2* was shown to encode a non-functional MLO protein which causes resistance as a result of enhanced cell death response and the deposition of a callose-rich barrier (papilla) at the site of invasion. Hence, MLO is considered to be a negative regulator. *Ol-4* and *Ol-6* are likely *Mi-1* homologues which encode NBS-LRR type *R*-proteins. They provide effective protection against three unrelated pests, i.e. powdery mildew, nematodes and aphids. However, neither gene has been cloned to date.

By studying 23 *Arabidopsis thaliana* accessions, Göllner et al. (2008) showed that *RPW8* (located on chromosome III) and polygenic resistance are major sources of resistance to powdery mildew *G. orontii*. In the same study, seven of these accessions were challenged with *On.* Intriguingly Sha, which contains *RPW8*, was resistant to three powdery mildew species but susceptible to *On*, implying that *RPW8* is not effective against *On*. Furthermore, heterologous expression of *RPW8* genes in tomato did not result in resistance to *On* (Xiao et al. 2003). These data indicate that genetic factors for *On* resistance in Arabidopsis are different from the ones involved in resistance to *G. orontii*.

In this study we employed the *O. neolycopersici*-Arabidopsis pathosystem to (1) determine the mode of inheritance of *On* resistance in natural accessions, (2) identify novel Arabidopsis genes conferring resistance to tomato powdery mildew. We were mainly interested in recessive genes, because these are less likely to be NB-LRR type *R*-genes and may confer race-non-specific resistance. Ultimately, our goal is to silence or induce mutations in tomato orthologs of Arabidopsis resistance genes to achieve *O. neolycopersici* resistance in tomato. Here we describe the map-based cloning of a recessive resistance locus in Arabidopsis, which turned out to be a natural mutation in *EDR1* gene.

Results

Genetic analysis of *On* resistance in Arabidopsis accessions

To explore natural variation for *On* resistance, 123 accessions (5 plants per accession) were inoculated with *On* spores and evaluated based on a disease index (DI) score ranging from 0 to 3. In total, 40 accessions were fully resistant to *On* (DI=0), whereas the others showed varying levels of susceptibility from low to high (Supplemental table 1). To determine the genetic mode of resistance, 19 resistant accessions were crossed with susceptible Col-0 or Sha. The F_1 plants (5 plants per cross) from 18 crosses displayed a susceptible phenotype (DI>0) (Supplemental table 2). To assess whether the resistance is mediated by a single gene or more than one gene, a $\chi 2$ test was performed on respective F_2 generations (Supplemental table 2). Segregation ratios (resistant : susceptible plants, or resistant : intermediate : susceptible plants) following a single

gene pattern were observed in four accessions. For the remaining 15 accessions the segregation ratios were not compatible with a single-gene hypothesis ($P < 0.05$), suggesting that resistance to *On* in Arabidopsis is mostly polygenic.

Fine-mapping of QTL-1 controlling resistance to *On* in C24

C24 is one of the accessions exhibiting absolute resistance. It was crossed with susceptible accession Sha to generate a mapping population. The F_1 plants were susceptible (Figure 1A), and the segregation ratio of F_2 plants suggested the involvement of more than one resistance gene (Supplemental table 2). Preliminary quantitative trait locus (QTL) analysis of 96 F_2 plants with 21 Indel markers (Supplemental table 3) covering all five chromosomes resulted in the identification of two QTL with logarithm of odds (LOD) score higher than 2.5 (Figure 1B). QTL-1 was located on chromosome 1 and acted in a recessive manner, as only plants homozygous for the C24 allele in this region were resistant (Figure 1C). QTL-2 on chromosome 2 acted in a semi-dominant manner (Figure 1C). To separate the effects of the two QTL we selected single F_2 plants showing a heterozygous genotype at one QTL locus and homozygous for the Sha allele at the other locus, and selfed these to produce F_3 progeny. Analysis of F_3 progeny showed a tight correlation between phenotype and genotype for QTL-1 (Supplemental figure 1A); plants with the C24 genotype were fully resistant or only slightly infected, whereas plants with a heterozygous or Sha genotype supported a high level of fungal sporulation. For QTL-2, plants with the C24 genotype and heterozygous plants showed full resistance, except for two heterozygous plants (Supplemental figure 1B). Disease index for plants with the Sha genotype fluctuated between 0 and 2 (Supplemental figure 1B). Considering that QTL-1 confers full resistance and shows a tight correlation between genotype and phenotype, we focussed our attention on cloning of QTL-1.

To fine-map QTL-1, two flanking markers 159 and 162 (Supplemental table 4) were used to screen 136 F_3 plants derived from a single F_2 plant. Two recombinants REC1 and REC2 were obtained (Figure 1D) and examination of their responses to *On* narrowed down QTL-1 region between markers 30 and 38. Then recombinants were sought between these two markers by analysing 3552 F_3 plants. Five informative recombinants (REC3-REC7) were obtained (Figure 1D), and disease assays were performed using either F_3 recombinants, or their F_4 progenies, or both. By combining the genotypic and phenotypic data the QTL-1 interval was reduced to a 58-kb chromosomal region between markers SNP1 and SNP50 (chr. 1 nucleotides 2,754,401-2,811,983; Figure 1D).

A natural mutation in *EDR1* confers resistance to *On*

The 58-kb interval between markers SNP1 and SNP50 encompasses 15 candidate genes. Interestingly, one of these is *EDR1* (At1g08720). Previously, an induced mutation in *edr1* allele was obtained in the background of Col-0 (Frye and Innes 1998). The induced mutation caused a premature stop codon in the fourth exon of EDR1. The *edr1* mutant was shown to be resistant to powdery mildew *G. cichoracearum*. To investigate whether *EDR1* is a good candidate for QTL-1 we challenged the *edr1* mutant obtained from Frye and Innes (1998) with tomato powdery mildew *On*. Col-0 showed clear symptoms of infection by *On*, *edr1* was free of symptoms (Figure 2A). Quantification of fungal DNA indicated an approximately 20-fold decrease of fungal biomass on the *edr1* mutant compared with Col-0 (Figure 2B).

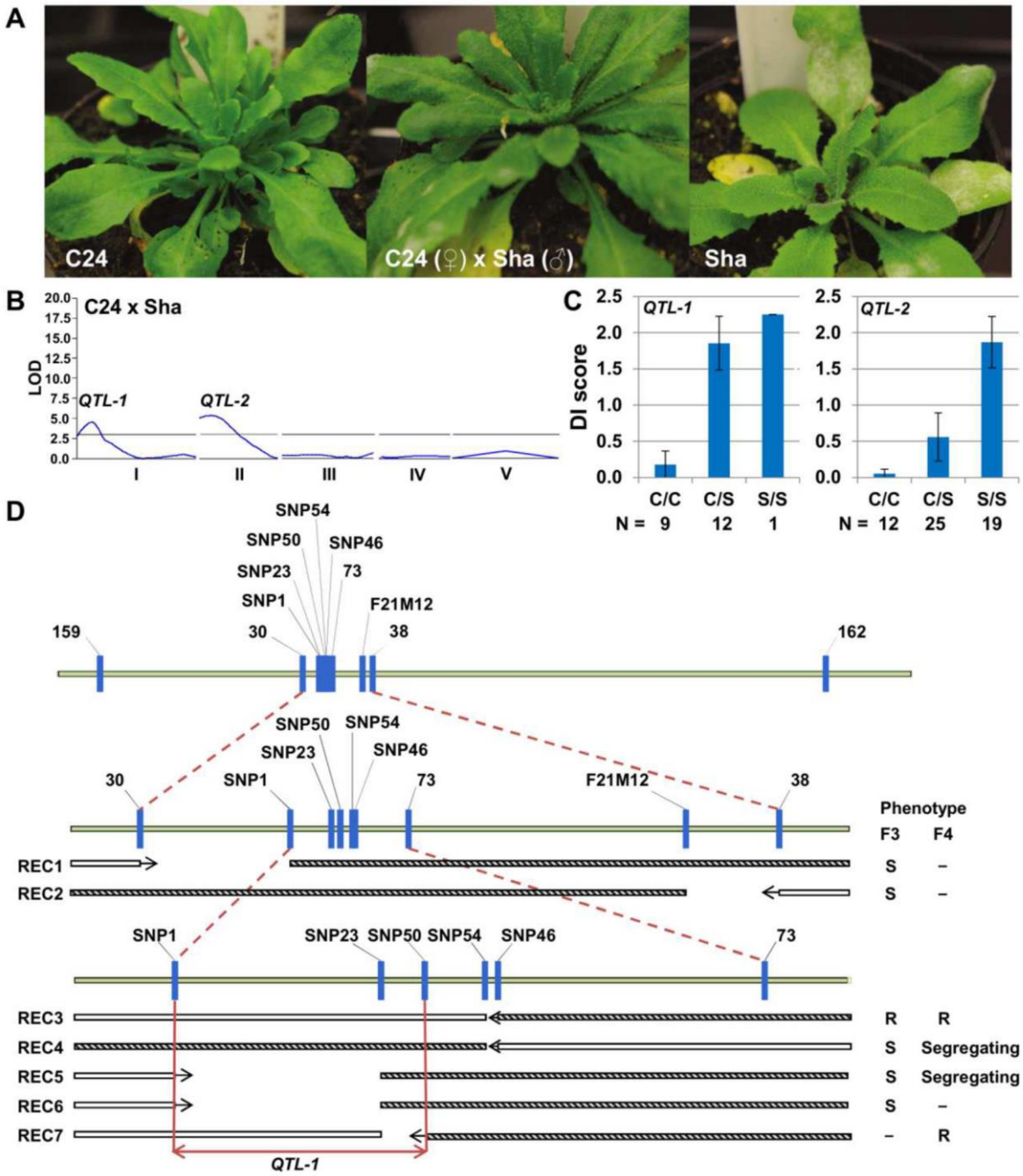


Figure 1 QTL analysis of *On* resistance and fine-mapping of QTL-1 on chromosome 1 in C24 x Sha. A, Symptoms of *On* infection on C24, F_1 C24 x Sha, and Sha. B, Two QTL located on chromosome 1 and 2 were identified. C, Disease index (DI) after *On* infection. Class C/C is homozygous for the C24 allele, C/S is heterozygous, and S/S is homozygous for the Sha allele. For each class, disease index (DI) value is the average score of F_2 plants with the designated genotype for marker 159 flanking QTL-1 on chromosome 1, or for marker 515 flanking QTL-2 on chromosome 2. D, Markers used for fine-mapping of QTL-1 (Supplemental table 4) are indicated. The distance between markers is proportional to the physical distance. White bars represent regions homozygous for the C24 allele, while shaded bars represent heterozygous regions. The space in between white and shaded bars denotes a crossover event between two flanking markers for each recombinant. The arrows point towards the interval in which QTL-1 resides. For each rcombinant (REC) the phenotype of F_3 and/or F_4 populations is indicated. S, susceptible; R, resistant.

Figure 2 The *edr1* mutation causes resistance to tomato powdery mildew *On*. A, fungal growth on Col-0 plant and *edr1* mutant. B, Fungal biomass quantification. Values were normalized relative to *act2*, and calibrated to levels on *edr1* mutants. Error bars represent standard deviation of three biological replicates, and for each replicate rosette leaves were collected. Asterisk indicates significant difference from the control according to independent-samples t-test (P <0.05). A representative of two experiments is presented.

These results showed that *EDR1* is a good candidate for QTL-1. Examination of protein sequences of *EDR1* from C24 (accession no. EF470629) and Col-0 (accession no. AF305913) in the NCBI database revealed an amino acid difference (V395E) in the fourth exon in a non-conserved region of the protein. To investigate whether this difference is associated with resistance, we sequenced the coding regions of *EDR1* in parental lines C24 and Sha. Surprisingly, a premature stop codon in the second exon of *EDR1* was produced in C24 as a result of loss of two nucleotides (GT) compared with the Sha allele (Figure 3A, genomic sequence chromosome 1, position 2,775,090-2,775,091). *EDR1* is located between markers SNP1 and SNP23 (Figure 1C). Recombinants REC3 and REC7 were homozygous for the C24 allele of both markers, and they were both resistant to *On*. Therefore, we expected them to contain the *edr1* mutation. Sequencing results confirmed that REC3 and REC7 indeed carried the *edr1* mutation (data not shown), indicating that *edr1* mutation is correlated with resistance to *On*.

Because the deletion of dinucleotide GT (nucleotides 1033-1034) was not present in *EDR1* (accession no. EF470629) of C24, we suspected that our C24 germplasm (referred to as C24-W) might be different from other C24 sources. Therefore, part of the second exon of *EDR1* was sequenced from plants of the stock (C24-stock) from which we obtained C24-W and two other C24 sources (referred as C24-H and C24-U, see Materials and Methods). Sequencing results showed that none of these C24 sources carried the mutation (data not shown). One possibility is that the accession we used was not C24. To exclude this possibility we screened C24-W, C24-H, C24-U, C24-stock, DNA of the parental plants C24 and Sha used for crossing and for developing all the markers for mapping in the F2 population, and additionally Col-0, with 12 Indel markers from all five chromosomes of Arabidopsis (Supplemental table 5). Genotyping data indicated that C24-W was not different from the other sources of C24 (Supplemental figure 2). As these data may not be conclusive we searched for a C24-specific DNA signature. A SNP of MIR164A unique to C24 was found by analyzing 96 Arabidopsis accessions; the C on chromosome 2 at position 19,520,846 is substituted by T only in C24 (Todesco et al 2012). PCR products containing the MIR164A SNP were obtained from genomic DNA of

several sources of C24 and also Col-0. Sequencing results showed that all C24 sources, including C24-W, carried T instead of C at this position, while Col-0 contained the expected T (Supplemental figure 3). Thus, we confirmed that C24-W is truly of C24 lineage.

We chose C24-W and C24-U for further analysis. A notable difference was that C24-W plants were smaller than C24-U (Figure 3B). A reduced plant size is observed in a number of Arabidopsis mutants which constitutively accumulate high levels of SA (Lu et al 2003). As elevated level of SA induces expression of pathogenesis-related (*PR)* genes, the expression of *PR1* and *PR2* in C24-W and C24-U were compared. Results showed that transcript levels of *PR1* and *PR2* were not significantly increased in C24-W compared with C24-U (Figure 3C), suggesting that the smaller size of C24-W is not caused by accumulation of SA. Since C24-U does not carry the *edr1* mutation, it was expected to be less resistant to *On* than C24-W, which was confirmed by quantification of fungal biomass (Figure 3D).

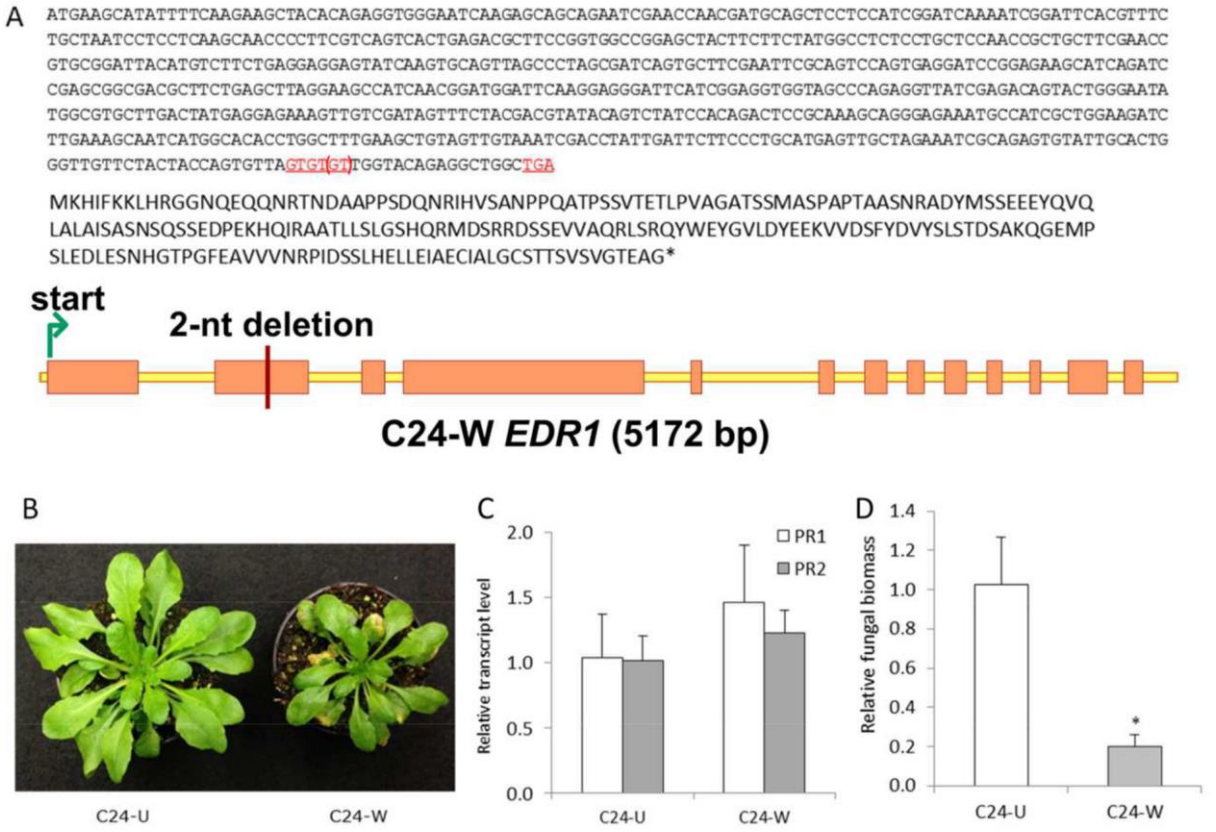


Figure 3 Characterization of C24-W. A, Mutation in *EDR1* of C24-W. Upper panel shows that among three GT repeats (red, underlined) one repeat was missing in C24-W, leading to the occurrence of premature stop codon TGA (red, underlined). Middle panel shows the resultant protein sequence when one GT repeat was missing. Lower panel depicts the position of the 2-nucleotides (nt) deletion in *EDR1* of C24-W. B, Size differences of C24-U and C24-W plants. C, Relative level of expression of defense genes *PR1* and *PR2*. D, Quantification of fungal biomass in C24-W and C24-U. Values were normalized relative to *act2*, and calibrated to levels in C24-U plants. Error bars represent standard deviation of three biological replicates, and for each replicate rosette leaves were collected. Asterisks indicate significant difference from the control according to independent-samples t-test (P <0.05). A representative of two experiments is presented.

Suppression of putative homologues of *EDR1* in tomato

The aim of our study was to identify genes conferring broad spectrum resistance to powdery mildews in Arabidopsis, and subsequently investigate whether putative orthologous genes in tomato also confer resistance to powdery mildew. With the protein sequence of Arabidopsis EDR1 as a query, multiple genes showing a relatively high level of homology were found in the tomato genome database SGN (Sol Genomics Network). We chose the first two genes *Solyc01g097980* (*Solyc01g*) and *Solyc06g068980* (*Solyc06g*) to investigate their involvement in resistance. The protein sequences encoded by *Solyc01g* and *Solyc06g* show 56% and 45% identity with the Arabidopsis EDR1 protein, respectively, while they show 42% identity with each other. The protein encoded by *Solyc01g* (accession no. AJ005077) probably is an EDR1-like MAPKKK protein, because it is more similar to Arabidopsis EDR1 protein than to any of the five Arabidopsis EDR1 paralogs (Frye et al. 2001). Furthermore, the kinase domains of the *Solyc01g*-encoded protein and Arabidopsis EDR1 show 86% identity (Frye et al. 2001). Tomato cultivar Moneymaker (MM) was transformed with RNAi silencing constructs, and several primary transformants (RNAi-*Solyc01g* and RNAi-*Solyc06g*) were obtained. These were selfed to produce T2 progeny. One T2 family for *Solyc01g* and three for *Solyc06g* were obtained. Nine plants harbouring the NPTII resistance gene from each T2 family were challenged with *On*. All of them supported abundant powdery mildew sporulation as the untransformed control, as judged by visual inspection. Subsequently, three plants from each T2 family were analysed for expression of the targeted *EDR1* homologues, and fungal biomass was quantified. Although significantly reduced expression of *Solyc01g* and *Solyc06g* was detected in the RNAi-*Solyc01g* and RNAi-*Solyc06g* lines respectively (Figure 4A), the level of fungal growth in the transgenic lines was comparable to the level in MM (Figure 4B), suggesting that silencing of these two genes separately did not result in resistance against tomato powdery mildew.

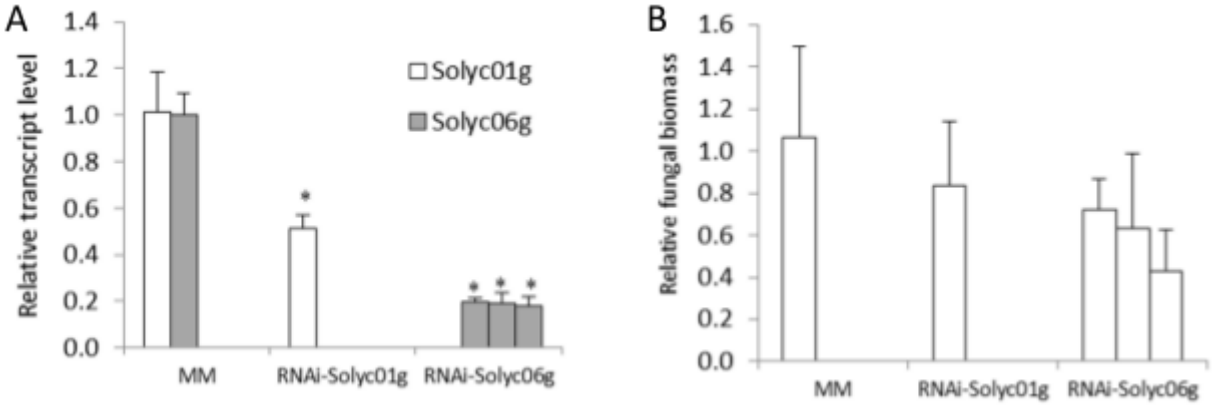


Figure 4 Suppression of putative homologues of *EDR1* in tomato did not affect the susceptibility level of MM. A, relative expression of *Solyc01g097980* (*Solyc01g*) and *Solyc06g068980* (*Solyc06g*) in silenced lines. B, relative fungal growth in silenced lines and MM. Values were normalized relative to *Elongation Factor 1α* (EF), calibrated to levels in untransformed MM plants. Error bars represent standard deviation of three biological replicates, and for each replicate 3rd and 4th leaves were pooled. Asterisks indicate significant difference from the control according to one way analysis of variance (P <0.05).

Discussion

The reference species *Arabidopsis thaliana* displays abundant genetic variation among wild accessions (Alonso-Blanco and Koornneef 2000), which was illustrated by our results obtained after challenging 123 accessions with virulent tomato powdery mildew *On*. With the inoculum dosage routinely used, 40 accessions showed complete resistance (Supplemental table 1). Segregation analysis of 19 crosses in F1 and F2 (Supplemental table 2) indicated that polygenic resistance to *On* is more common than monogenic resistance. This observation was also made in a study of resistance to powdery mildew in multiple Arabidopsis accessions (Göllner et al. 2008). Both observations support the notion that polygenic resistance seems more manifest in interactions of powdery mildews with Arabidopsis than with barley, and also over-represented compared to other Arabidopsis plant-pathogen interactions (Schulze-Lefert and Vogel 2000).

In this study we identified a natural mutation of *EDR1* in C24 background, resulting from deletion of two nucleotides from a dinucleotide repeat array $(GT)_3$. Eukaryotic genomes contain strings of DNA in which a single base or a small number of bases is repeated (microsatellites). Rearrangement can occur within repeated sequences, resulting in repeat addition and deletion (Flavell 1986). This is probably caused by slippage during DNA replication (Ellegren 2004). AC/GT repeats are scarce in plants in comparison with mammalian genomes (Lagercrantz et al. 1993). Examination of dinucleotide repeats in Arabidopsis confirmed that AC/GT is least abundant (Morgante et al. 2002; Marriage et al. 2009). Marriage et al. (2009) estimated the mutation rate of dinucleotide repeats in Arabidopsis, and revealed that the majority of mutations are gains or losses of a single repeat, where the AC/GT motif is the least mutable. The mutation rate is positively affected by repeat length across motifs, but the AC/GT motif does not fit this general trend. Although meiotic and mitotic errors cannot be distinguished in the study, they suggested that meiotic errors are more likely contributing to the mutation rate. Our observation that C24-W contains a dinucleotide deletion from a microsatellite sequence, whereas all other sources of C24 do not contain this mutation suggests that the mutation is a recent event.

It is notable that C24-W carrying the *edr1* mutation exhibits reduced stature, while the *edr1* mutant in Col-0 background does not. Reduced stature of C24-W is not caused by differential expression of *PR* genes (Figure 3C), nor is it associated with the *edr1* mutation because progenies only segregating for QTL-1 did not show dwarfing. In contrast, progenies only segregating for QTL-2 showed size differences, which was not closely correlated with resistance (data not shown). However, the QTL-2 region has not been fine-mapped yet, and this prevents us to unravel the mechanism underlying the dwarf phenotype.

Accession C24 shows broad-spectrum resistance to several unrelated pathogen species. C24 exhibited resistance to three species of powdery mildew, i.e. *G. orontii*, *G. cichoracearum* and *G. cruciferarum* (Göllner et al. 2008). Mapping of the gene(s) underlying this resistance had been unsuccessful (Göllner et al. 2008). In addition, C24 provided downy mildew isolate-specific resistance and dominant resistance against bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (Lapin et al. 2012). Furthermore, C24 conferred effective resistance against cucumber mosaic virus mediated by a CC-NBS-LRR-type protein RCY1 (Takahashi et al. 2002). Therefore, C24 seems to be an example of natural pyramiding of different resistance loci.

Here, we showed that C24 without the *edr1* mutation was less resistant to tomato powdery mildew *O. neolycopersici* than C24-W carrying the mutation (Figure 3D), but compared with Col-0 it was less susceptible as judged by visual inspection. This might be explained by elevated levels of salicylic acid, hydrogen peroxide and expression of SA-mediated defense-related genes such as *PR1* in C24 (Lisec et al. 2008; Bechtold et al. 2010). However, these inherent traits are not necessarily contributing to pathogen resistance, because Col-0 introgression lines containing resistance QTLs to downy mildew *Hpa* did not show enhanced expression of *PR1* compared with susceptible Col-0 (Lapin et al. 2012).

The paradigm examples of naturally occurring loss-of-function mutations conferring resistance to powdery mildew are *mlo* orthologs in barley (*mlo11*; Jørgensen, 1992), tomato (*ol-2*; Bai et al. 2008), and pea (*er1*; Pavan et al. 2011 and Humphry et al. 2011). For these species single gene mutations are sufficient to achieve full resistance. However, in Arabidopsis silencing of three *MLO* genes (*AtMLO2*, *AtMLO6* and *AtMLO12*) is required to obtain full resistance against powdery mildew. This indicates that although the role of *mlo* mutation promoting resistance to different powdery mildew species is conserved, the mechanism in Arabidopsis may not be representative for the situation in other plant species. We observed that although the Arabidopsis *edr1* mutant conferred full resistance to tomato powdery mildew, silencing of two putative tomato homologues of *EDR1* separately did not decrease fungal sporulation (Figure 4). There are three possibilities to explain this phenomenon. First, in the RNAi transformants the tomato *EDR1* homologues still retained a low level of expression, which may be sufficient to produce enough protein for sustained functionality. It is essential to obtain null alleles of the putative *EDR1* orthologs in tomato to clarify their roles. Second, the tomato *EDR1* homologues may show redundancy, and silencing of more than one gene may be necessary to obtain resistant plants. Third, although these two genes rank top in terms of similarity to Arabidopsis EDR1, they may not be true functional orthologs.

In summary, we identified a natural mutation of *EDR1* in Arabidopsis accession C24-W conferring full resistance to tomato powdery mildew *On*. We plan to investigate whether C24-W shows resistance to additional pathogens. Furthermore, it will be of interest to reveal the overall differences between C24-W and C24 sources not having the *edr1* mutation, which may improve the understanding of the molecular mechanism of *edr1*-mediated resistance and the complex resistance in C24 in general. Additionally, we plan to study allelic effects of the two *edr1* mutations by comparing resistance mechanisms in C24-W and Col-0-*edr1*.

Materials and Methods

Plant growth conditions and pathogen inoculation

All the *Arabidopsis thaliana* accessions were obtained from the Max Planck Institute in Köln, Germany. C24-H was obtained from Hanzi He of Plant Physiology of Wageningen University and C24-U from Dr. Guido van den Ackerveken of Plant-Microbe Interactions of Utrecht University. The plants were grown in soil substrate in a growth chamber at the day/night cycle of 16h/8h with 21ºC/18ºC day/night temperature. The relative humidity was kept at 70% and the light intensity was 100 W/m2. The Netherlands isolate of *On* was maintained on susceptible tomato cultivar MM plants. Fungal spores were washed off

from infected MM leaves with water and diluted to a concentration of 2.5 x 10^5 spores per mL for inoculation of Arabidopsis, or 2.5 x 10^4 spores per mL for inoculation of tomato. Approximately 30-day-old plants were inoculated by spraying spores on the leaves. Disease index was recorded 8-14 days after *On* inoculation, ranging from 0 to 3: 0, no sporulation; 1 slight and 2 moderate sporulation; 3, abundant sporulation.

QTL mapping and recombinants screening

To locate resistance loci, Joinmap 4 (Van Ooijen 2006) and MapQTL 6 (van Ooijen 2009) were used with default settings. For recombinant screening, DNA was extracted using the protocol described by Kasajima et al. (2004). For development of Indel markers, primers were designed based on the flanking sites of known insertion and deletion polymorphisms between Col-0 and Ler, as obtained from the Cereon database administered by Monsanto (Jander et al. 2002). For development of SNP markers, the known SNPs between C24 and Col-0 available from 1001 genome database (http://www.1001genomes.org) were examined with Lightscanner™ to determine whether the SNP was applicable to distinguish C24 from Sha.

Generation of stable silenced lines

To suppress tomato genes *Solyc01g097980* and *Solyc06g068980* individually, fragments with a length of 150-300 bp were amplified from MM cDNA using primers Fw-caccTCAGGTGCAGCGTTGGCTGAG and Rv-TGCCCTTTGCCACATCAAGGG for *Solyc01g097980*, and primers Fw-caccAGTGGATGGCCCCAGAAGTGCTG and Rv-ACGGTGCTGAAACCCCACAGCG for *Solyc06g068980*. The fragments were recombined into pENTR/D-TOPO vector (Invitrogen) and sequenced. Subsequently, the fragment was introduced into the pHellsgate8 vector (Helliwell et al. 2002) and finally transformed into *Agrobacterium* strain AGL1+virG. For transformation of tomato cultivar MM the same protocol as described by Huibers et al. (2013) was used. Primary transformants (T1) were selfed to generate T2 progeny. For each segregating T2 family, a PCR using NPTII primers Fw-NPTII-TTCCCCTCGGTATCCAATTA and Rv-NPTII-GATTGTCTGTTGTGCCCAGT was performed to select transgenic progeny.

Quantitative RT-PCR and data analysis

In each experiment, three biological replicates per genotype were used. Samples were prepared from Arabidopsis rosette leaves or pooled 3rd and 4th leaves per tomato plant. For quantification of fungal biomass, DNA or RNA was used. For quantification of transcript levels, RNA was used. DNA was isolated with the DNeasy plant mini kit (Qiagen). Total RNA was extracted using the RNeasy kit (Qiagen). After removal of DNA with DNase I (Invitrogen), 1ug total RNA was used for cDNA synthesis using superscript II reverse transcriptase kit (Invitrogen). Quantitative real-time PCR was conducted using the iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). The PCR amplification consisted of an initial denaturation step of 3 min at 95℃, followed by denaturation for 15 sec at 95 ℃, annealing and extension for 1 min at 60℃ for 39 cycles, then a final melt step from 65°C to 95°C ramp with 0.5°C increments per cycle to monitor specificity. Primers used for fungal quantification were Fw-On-CGCCAAAGACCTAACCAAAA and Rv-On-AGCCAAGAGATCCGTTGTTG. Primers for detection of relative transcript levels were Fw-TGAAGGAGCCAGAAAATCCA and Rv-

TCTTCCCATGGAATCTCACA for *Solyc01g097980*; Fw-TTCATGGGAGCTGTTACTCG and Rv-ACTGATTGTTGGGTCGATGG for *Solyc06g068980*; Fw-*EF*-GGAACTTGAGAAGGAGCCTAAG and Rv-*EF*-CAACACCAACAGCAACAGTCT for tomato reference gene Elongation Factor 1α (Løvdal et al. 2009); Fw-GAACACGTGCAATGGAGTTT and Rv-GGTTCCACCATTGTTACACCT for Arabidopsis *PR1* gene At2G14610; Fw-CCCGTAGCATACTCCGATTT and Rv-AAGGAGCTTAGCCTCACCAC for Arabidopsis *PR2* gene At3G57260; Fw-AATCACAGCACTTGCACCA and Rv-GAGGGAAGCAAGAATGGAAC for Arabidopsis reference gene actin (*act2*) At3G18780.

For analysis of the relative expression level and fungal biomass the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001) was used. Data were statistically examined using independent-samples t-test and one-way analysis of variance (ANOVA) based on Post-hoc comparisons using Tukey's HSD test (P <0.05). All analyses were performed using SPSS Statistics 20 following the instructions of SPSS Survival Manual 4th edition (Pallant 2010).

Acknowledgements

We thank Prof. Maarten Koornneef of the Max Planck Institute in Köln, Germany for providing seeds of *Arabidopsis thaliana* accessions. We thank Prof. Roger Innes (Indiana University, Bloomington, USA) for providing *edr1* mutant seeds. Thanks also to Hanzi He (Wageningen University) and Dr. Guido van den Ackerveken (Utrecht University) for providing C24 seeds.

Supplemental table 1 Disease Index (DI) scores of Arabidopsis accessions inoculated with *On*

No.	Accession-ID	Accession	DI
1	291	Zal-1	0
2	903	Kas-0	2
3	905+	Ms-0	2
4	**906+**	**C24**	**0**
5	913	RLD1	2-3
6	925	Litva	0
7	926	Pet-0	0
8	929	Shah / Sha	3
9	931	Sorbo	2
10	1005	Bsch-2	1-2
11	1014	Bu-5	2
12	1065	Can-0	1
13	1072	Chi-0	0-1
14	1074	Chi-1	1
15	1094	CT-1	1
16	1116	Dra 0	2
17	1138	En-2	2
18	1184	Gd-1	2
19	1187	Ge-0	0
20	1211	Gre-0	1
21	1212	GU-0	0
22	1214	GU-1	1
23	1249	Ji-1	2
24	1260	Jm-1	2
25	1264	Kas- 2	0
26	1622	Yo-0	1
27	1629	Zu-1	0
28	1635	Cnt	2
29	1636	Nd-1	2
30	1637	Ema-1	2
31	1639	Wei-1	2
32	1640	Tsu-1	2
33	1656	Alc-0	2
34	2360	WS-2	3
35	6042	Car 1	3
36	6044	FLO 1	1-2
37	6045	KL-PW-1	1
38	6047	Mst- 1	2
39	6048	Ken-1	2
40	6182	Wei-0	2
41	6600	Aa-0	0
42	6608	Bay-0	0-1
43	6613	Be-0	3
44	6621	Bla-6	0
45	6626	Br-0	2
46	6627	Bs-1	2
47	6643	Bur-0	0
48	6645	Blh-1	1-2
49	6659	Cal-0	0
50	6669	Co-1	0
51	6672	Co-4	1-2
52	6689	Ei-2	0
53	6693	Eil-0	2
54	6699	Es-0	0
55	6700	Est-0	0
56	6705	Fi-1	1
57	6714	Ga-0	0
58	6732	Gy-0	0
59	6752	Ka-0	1
60	6754	Kil-0	2
61	6755	Kin-0	1-2
62	6799	Mt-0	0-1

No.	Accession-ID	Accession	DI
63	6800	Mz-0	0
64	6810	Nok-3	0
65	6818	Ob-2	2
66	6832	Pi-0	0
67	6848	Rsch-0	2
68	6849	Ri-0	2-3
69	6865	Stw-0	1-2
70	6868	Ts-2	0
71	6884	Van-0	1-2
72	6885	Wa-1	1
73	6918	Te-0	0
74	8068	Berk	2
75	8070	Lim	2
76	8144	Lin	1
77	8580	CVI	2
78	10038	Driel-1	2
79	10169	Fei-0	0
80	10172	Vil-0	2
81	10175	FK	1-2
82	10182	Aa-0	0
83	10183	Ag-0	2
84	10184	Bl-1	0
85	10185	Bla-10	2-3
86	10187	Fl-1	2
87	10189	Pog-0	0
88	10210	Hi-0	2
89	10212	Pak-1	2
90	10214	Pak-3	0
91	10215	Yam-1	0
92	10217	Izumo	1-2
93	10219	JW113	2
94	10221	TY	1-2
95	10222	TKS	0
96	10223	IK	2
97	10224	AK	2
98	10225	OY	1
99	10226	NG	2
100	10227	ES	1-2
101	10229	Sendai-1	1
102	10230	Eniwa	0
103	10232	RIB-1	2
104	10237	Ost-0 (Navot)	0
105	10255	Kam (Navot)	0
106	10256	Strand	2
107	10257	Byn	0
108	10258	Orn	0
109	10265	Pont L'Eveqeu	0
110	10270	Son Stefano	2
111	10281	Sij-1	1-2
112	10282	Sij-2	2
113	10296	Daejeon	0
114	10297	Suwon	0
115	22351	HS-1	2
116	22363	Ith-1	0
117	22401	Kno-1	1
118	22419	Csh-1	2
119	22436	KZ-2	1-2
120	22445	KZ-13	2
121	22446	Puz-2	0
122	22456	Sapporo	2
123	22491	Konchezero	1-2

Supplemental table 2 Segregation of resistance to *On* in Arabidopsis accessions. Chi-square tests were performed in all respective F_2 generations. The P value is only shown when higher than 0.05, which means that the segregation ratio fits the indicated pattern.

Cross	F_1 phenotype[1]	F_2 (R:S) [1]	Segregation 3 : 1	Segregation 1 : 3	F_2 (R:I:S) [2]	Segregation 1 : 2 : 1
			P (χ^2 test)	*P* (χ^2 test)		*P* (χ^2 test)
Bla-6 (♀) x Col-0 (♂)	R	(72:20)	*P*=0.470		(72:7:13)	
Bay-0 (♀) x Sha (♂)	S	(22:72)		*P*=0.721	(22:55:17)	*P*=0.196
Kas-2 (♀) x Col-0 (♂)	S	(17:66)		*P*=0.342	(17:33:33)	
Litva (♀) x Col-0 (♂)	S	(17:79)		*P*=0.099	(17:51:28)	*P*=0.235
Es-0 (♀) x Ws-2 (♂)	S	(14:78)			(14:32:46)	
Zal-1 (♀) x Col-0 (♂)	S	(10:62)			(10:33:29)	
Pak-3 (♀) x Sha (♂)	S	(38:56)			(38:50:6)	
C24 (♀) x Sha (♂)	**S**	**(53:43)**			**(53:30:13)**	
GU-0 (♀) x Sha (♂)	S	(52:38)			(52:37:1)	
Cal-0 (♀) x Col-0 (♂)	S	(49:43)			(49:37:6)	
Ga-0 (♀) x Sha (♂)	S	(53:34)			(53:34:0)	
Nok-3 (♀) x Col-0 (♂)	S	(6:83)			(6:78:5)	
Pi-0 (♂) x Col-0 (♀)	S	(87:5)			(87:4:1)	
Te-0 (♀) x Sha (♂)	S	(38:58)			(38:53:5)	
Fei-0 (♂) x Col-0 (♀)	S	(12:83)			(12:73:10)	
Bl-1 (♀) x Col-0 (♂)	S	(81:13)			(81:13:0)	
TKS (♀) x Col-0 (♂)	S	(44:51)			(44:42:9)	
Eniwa (♀) x Col-0 (♂)	S	(95:5)			(95:4:1)	
10265 (♀) x Col-0 (♂)	S	(38:52)			(38:45:7)	

Supplemental table 3 Indel marker primers for preliminary QTL analysis

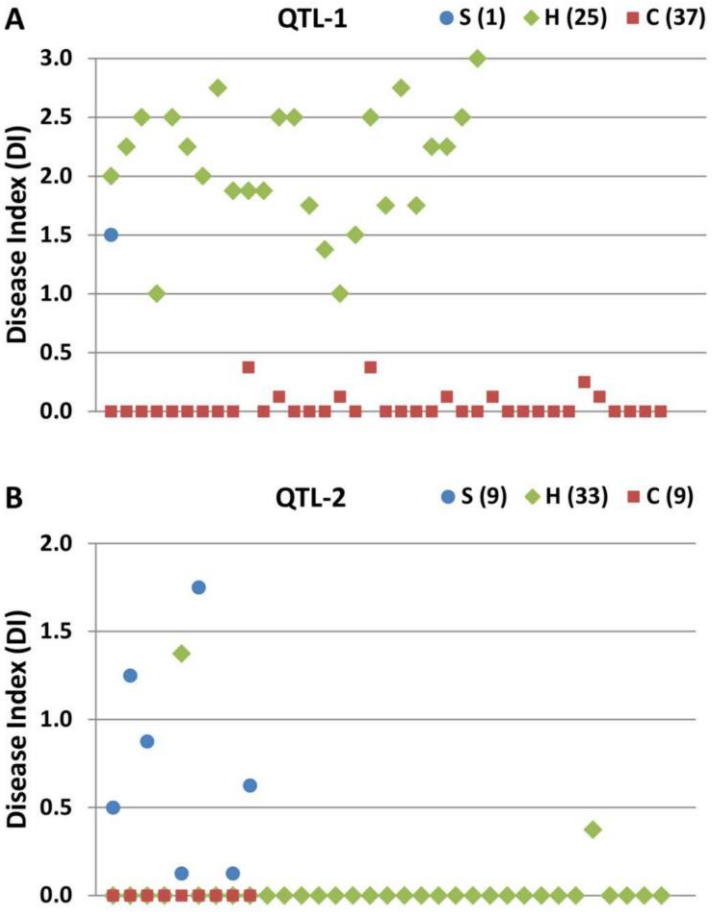
Indel	Chromosome	Forward	Reverse
159	1	GATGAATTCTTCCTTTTCACGTT	TGTTGTACTTAAATGTAACCAGTCAG
F21M12	1	GGCTTTCTCGAAATCTGTCC	TTACTTTTTGCCTCTTGTCATTG
162	1	CATACATACAATTCACTAACCAAAA	TGGATCTCCTTAATAGTTTAAAAGG
561	1	GGACAACGTCTCAAACGGTT	GGAGGCTATACGAATCTTGACA
CIW1	1	ACATTTTCTCAATCCTTACTC	GAGAGCTTCTTTATTTGTGAT
567	1	CAACCACCAGGCTC	GTCAAACCAGTTCAATCA
169	1	CAGAATTTCTATCTGAAGAATCGAG	GTGAAGGTTTAGAGAGAATCAAAGG
515	2	ATCTTCCTCCGACGACATTG	TGATGATATGTTTCCCCTCGT
187	2	GAGAAATATCGACGGGAAAAA	ATGCTCATCTTACAACAACACTAAA
188	2	AAAGAGTCAAGGAAAAGTATGTGTG	TTAAGATAGAAACCAAAACCAAGC
189	2	GTTTGCGTTTAATAGTCAAGATATG	CAAATGTTTAAGGTTTGTGGTTG
585	3	AGCTGCTTCCTTATAGCGTCC	CATCCGAATGCCATTGTTC
200	3	AAATAAGATTTGTAATGTAAGACGAA	TTCCACCTAACCTAATAATAACAAG
591	3	GCACTTGCAGCTTAACTT	CGTGACTGTCAAACCG
203	3	GAACAATAAAGAGGAAGAAGAAAGC	GCATTACAACGTATAACGTAATGAAA
661	3	ATGGATGCATTTGGAAGAAA	TTGTGTAATTGATTTTACGTCATTTT
NGA6	3	TGGATTTCTTCCTCTCTTCAC	ATGGAGAAGCTTACACTGATC
218	4	GACATAACTTCGAATTGTTGGATAG	AATTTCGCCGGAATAAACAG
605	4	TTTCTTGTCTTTCCCCTGAA	GACGAAGAAGGAGACGAAAA
611	4	CGTTTCATCAAGTTCCGA	TAGGAGGTTATCATGCGTG
245	5	GCAATATCAGGGTCTTGTAAAGATA	CCATTGGATATAATTAAGAAGAAGAA

Supplemental table 4 Marker Primers located on chromosome 1 for genotyping recombinants to fine-map QTL-1

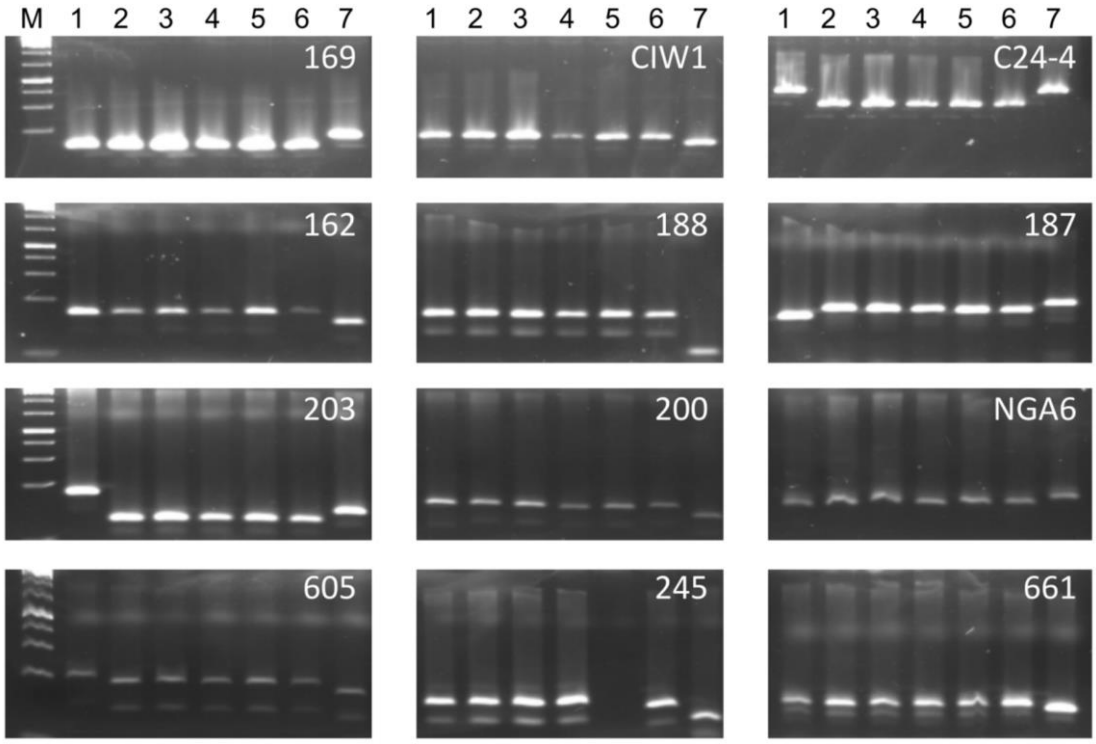
Marker	Type	Chromosome location	Forward	Reverse
159	Indel	440401	GATGAATTCTTCCTTTTCACGTT	TGTTGTACTTAAATGTAACCAGTCAG
30	Indel	2579701	CTCTTGGTGGTGTCCCAAGT	TCGACGCAGTTTTTCATCAG
SNP1	SNP	2754401	GCCGTGGATCAAACCCTTAT	TGCATTCATGAAAGGGGAAT
SNP23	SNP	2801501	TGTTGTCGATTGGCTGAGAA	AATGGTAGCCGCAGCAATAG
SNP50	SNP	2811983	ATAAACGTGCCTGCGATTTC	GTGGTTCCAATGGCATCTTT
SNP54	SNP	2825828	TCTGGTGATTGAAGAGAACCTC	TTCTTGCAGGGCCTCTATTG
SNP46	SNP	2829144	CATTGGTCTCCAGGGCTAAA	GCTTTGAGCCACACTAAGCTC
73	Indel	2890801	TTGTGGATTATGAAGGAAAAACA	CGGCACAAAAGTGTTAACGAG
F21M12	Indel	3212189	GGCTTTCTCGAAATCTGTCC	TTACTTTTTGCCTCTTGTCATTG
38	Indel	3319641	TTGCCAATTATAGGTTGACACG	TTCAATTGTTGCCACGCATA
162	Indel	8103701	CATACATACAATTCACTAACCAAAA	TGGATCTCCTTAATAGTTTAAAAGG

Supplemental table 5 Indel markers primers for genotyping different sources of C24

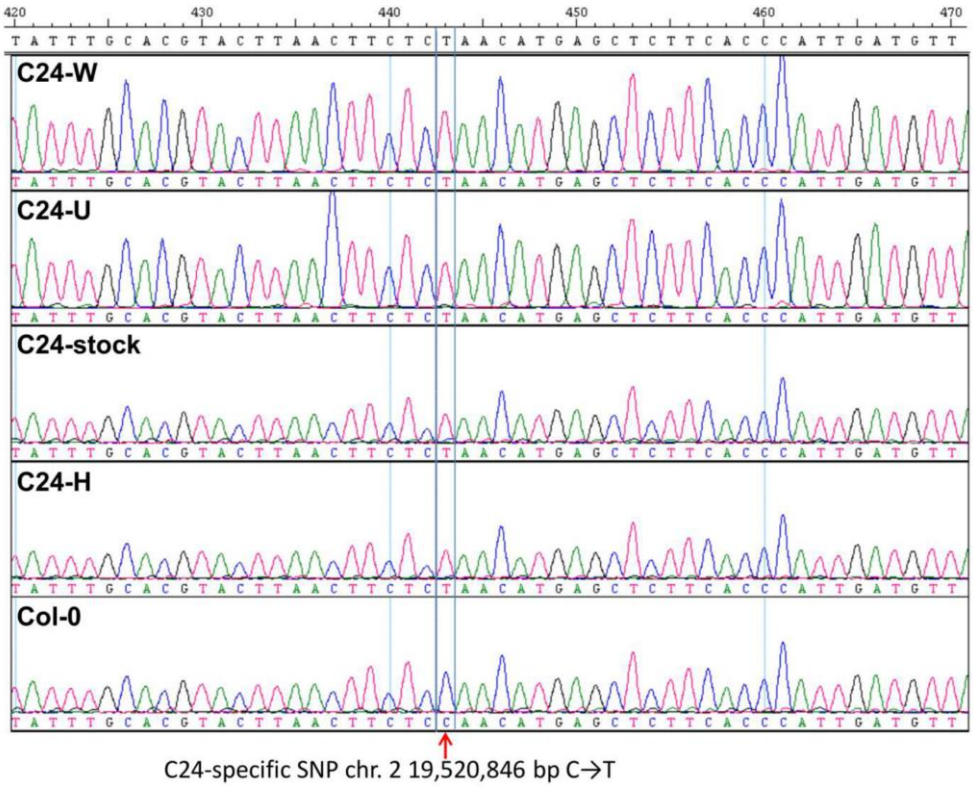
Indel	Chromosome	Forward	Reverse
169	1	CAGAATTTCTATCTGAAGAATCGAG	GTGAAGGTTTAGAGAGAATCAAAGG
CIW1	1	ACATTTTCTCAATCCTTACTC	GAGAGCTTCTTTATTTGTGAT
C24-4	1	AAGCCAAGTACCTCCAAGCA	TTTCCCTCAAGGGTTCTTCA
162	1	CATACATACAATTCACTAACCAAAA	TGGATCTCCTTAATAGTTTAAAAGG
188	2	AAAGAGTCAAGGAAAAGTATGTGTG	TTAAGATAGAAACCAAAACCAAGC
187	2	GAGAAATATCGACGGGAAAAA	ATGCTCATCTTACAACAACACTAAA
203	3	GAACAATAAAGAGGAAGAAGAAAGC	GCATTACAACGTATAACGTAATGAAA
200	3	AAATAAGATTTGTAATGTAAGACGAA	TTCCACCTAACCTAATAATAACAAG
NGA6	3	TGGATTTCTTCCTCTCTTCAC	ATGGAGAAGCTTACACTGATC
605	4	TTTCTTGTCTTTCCCCTGAA	GACGAAGAAGGAGACGAAAA
661	4	ATGGATGCATTTGGAAGAAA	TTGTGTAATTGATTTTACGTCATTTT
245	5	GCAATATCAGGGTCTTGTAAAGATA	CCATTGGATATAATTAAGAAGAAGAA



Supplemental figure 1 Relation between genotype of F_3 progeny for QTL-1 and QTL-2 and resistance to *On*. Disease Index (DI) was scored for plants showing C24 (C), heterozygous (H) or Sha (S) genotypes. A, the QTL-1 region (both markers 159 and 162). B, the QTL-2 region (both markers 515 and 187). Each data point represents the average value from two time points of scoring per F_3 plant. The total number of plants with the designated genotype is shown between brackets.



Supplemental figure 2 Application of Indel markers to verify the identity of C24-W. For each marker (see Table S5), seven plants were tested. Lane 1, Col-0; 2, C24-W; 3, C24-stock; 4, C24-U; 5, C24-H; 6, C24; 7, Sha. M = marker. DNA from lanes 6 and 7 was used for developing all the markers used for mapping in this population. Genotyping was repeated twice, and data from one replicate are presented here.



Supplemental figure 3 Sequence of PCR products containing MIR164A SNP in C24 sources and Col-0.

Chapter 5

Activation tagging of *ATHB13* in *Arabidopsis thaliana* confers broad-spectrum disease resistance

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Submitted

Activation tagging of *ATHB13* in *Arabidopsis thaliana* confers broad-spectrum disease resistance

Abstract
Powdery mildew species *Oidium neolycopersici* (*On*) can cause serious yield losses in tomato production worldwide. Besides on tomato, *On* is able to grow and reproduce on Arabidopsis. In this study we screened a collection of activation-tagged Arabidopsis mutants and identified one mutant line, 3221, which in addition to reduced stature and serrated leaves displayed resistance to *On*. Additional disease tests demonstrated that 3221 mutant exhibited resistance to downy mildew (*Hyaloperonospora arabidopsidis)* and green peach aphid (*Myzus persicae)* but retained susceptibility to bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000. The resistance trait and morphological alteration were mutually linked in 3221. Identification of the activation tag insertion site and microarray analysis revealed that *ATHB13*, a homeodomain-leucine zipper (HD-Zip) transcription factor, was constitutively overexpressed in 3221. Silencing of *ATHB13* in 3221 resulted in the loss of both the morphological alteration and resistance, whereas overexpression of the cloned *ATHB13* in Col-0 and Col-*eds1-2* backgrounds resulted in morphological alteration and resistance. Microarray analysis further revealed that overexpression of *ATHB13* influenced the expression of a large number of genes. Previously, it was reported that *ATHB13*-overexpressing lines conferred tolerance to abiotic stress. Together with our results, it appears that *ATHB13* is involved in the crosstalk of abiotic and biotic stress resistance pathways.

Keywords *Arabidopsis thalian,* Overexpression, HD-Zip transcription factor, *ATHB13*, broad-spectrum disease resistance

Introduction

Powdery mildews pose a threat to the production of a wide variety of plant species. As a model host *Arabidopsis thaliana* is extensively employed to explore powdery mildew resistance and the underlying mechanism. Four powdery mildew species can infect *A. thaliana*: *Golovinomyces* (formerly *Erysiphe*) *cruciferarum* (Koch and Slusarenko 1990), *G. cichoracearum* (Adam and Somerville 1996), *G. orontii* (Plotnikova et al. 1998), and *Oidium neolycopersici* (*On*) (Xiao et al. 2001). Arabidopsis genes conferring powdery mildew resistance have been identified by screening natural resistant accessions e.g. *RPW8* (Xiao et al. 2001) and by means of mutagenesis mainly causing impaired function of genes, e.g. *enhanced disease resistance* (*edr1-3,* Frye and Innes 1998; Tang et al. 2005; Tang et al. 2006) and *powdery mildew resistance (pmr1-6,* Vogel and Somerville 2000; Vogel et al. 2002; Vogel et al. 2004).

Compared to the three other powdery mildew species, *On*-Arabidopsis pathosystem is not well characterized. *On* is a worldwide disease on tomato, and a limited number of tomato loci (*Ol-1*, *-3*, *-4*, *-5*, *-6*, *ol-2* and three QTL) conferring resistance to this pathogen have been identified by screening wild and cultivated tomato germplasm (Bai et al. 2003; Bai et al. 2005). The recessive *ol-2* gene has been revealed to be a loss-of-function allele of the tomato *Mlo* ortholog (Bai et al. 2008). In addition, we demonstrated that silencing of *PMR4* ortholog in tomato results in resistance against *On* (Huibers et al. 2013). These results indicate that different powdery mildew species may make use of the same plant susceptibility genes to cause disease in plants (Pavan et al. 2010). However, Arabidopsis *RPW8* is not effective to *On* although it confers resistance to three other powdery mildew species infecting Arabidopsis (Xiao et al. 2003; Göllner et al. 2008). Thus, the genetic factors for *On* resistance in Arabidopsis could be also different from the ones involved in resistance to other powdery mildews. The possibility of uncovering novel resistance traits in Arabidopsis and transferring knowledge to tomato prompted us to exploit the large Arabidopsis mutant population.

There are several mutagenesis systems to create mutants including ethyl methanesulfonate (EMS), fast neutron, gamma, X-ray and insertion of foreign DNA like transposon and T-DNA. Two types of mutation can arise, *i.e.* loss-of-function or gain-of-function mutation. Loss-of-function mutagenesis has been shown to be a powerful approach to identify novel resistance genes as mentioned above and dissect resistance signalling pathways (Glazebrook 2001). Regarding to gain-of-function mutation, activation-tagged mutants have been successfully employed to identify Arabidopsis genes conferring resistance, such as *AHL19* to the soil borne fungal pathogen *Verticillium* (Yadeta et al. 2011), *ADR1* and *ADR2* to biotrophic but not necrotrophic pathogens (Grant et al. 2003; Aboul-Soud et al. 2009), *CDR1* to a bacterial pathogen (Xia et al. 2004), and *IRM1* to aphids (Chen et al. 2013). Activation tagging involves the random integration of regulatory sequences distributed by T-DNA or transposons in a plant genome (Weigel et al. 2000). Gain-of-function mutants are obtained as a result of transcriptional activation of genes in the vicinity of the integrations.

In this study we screened an En-1 transposon-based activation tag population (Marsch-Martinez et al. 2002) in the background of Wassilewskija (Ws) to identify genes affecting growth and reproduction of *On*. The mutant line 3221, which was of reduced stature and increased leaf serratedness, showed enhanced resistance to *On*. This mutant line also displayed elevated resistance to downy mildew pathogen *Hyaloperonospora*

arabidopsidis (*Hpa*) and the green peach aphid *Myzus persicae*. In 3221, constitutive expression of the homeodomain and leucine zipper (HD-Zip) transcription factor *ATHB13* was found to cause resistance.

Results

The mutant line 3221 shows altered leaf morphology and resistance to pathogens and aphids

To identify genes conferring resistance to tomato powdery mildew *On*, around 8000 En-I transposon-based activation tag mutants in the Arabidopsis accession Ws (Marsch-Martinez et al. 2002) were screened. This resulted in the identification of one mutant, 3221, showing reduced growth of *On* and morphological alteration (reduced stature and serrated leaves). To investigate the association between morphological and *On* resistance traits, the 3221 mutant was crossed with the parental line Ws. Examination of the F2 population of 135 plants three times showed that the morphological trait was segregating in a 1:2:1 ratio (Wild-type : intermediate : 3221-like; Figure 1A, B and C respectively). F2 plants with a 3221-like leaf morphology showed significantly less fungal biomass compared with F2 plants with wild-type appearance (Figure 1D). While F2 plants with an intermediate leaf phenotype showed approximately intermediate fungal biomass accumulation, suggesting a gene dose-dependent control of the morphological and resistance traits by a single locus.

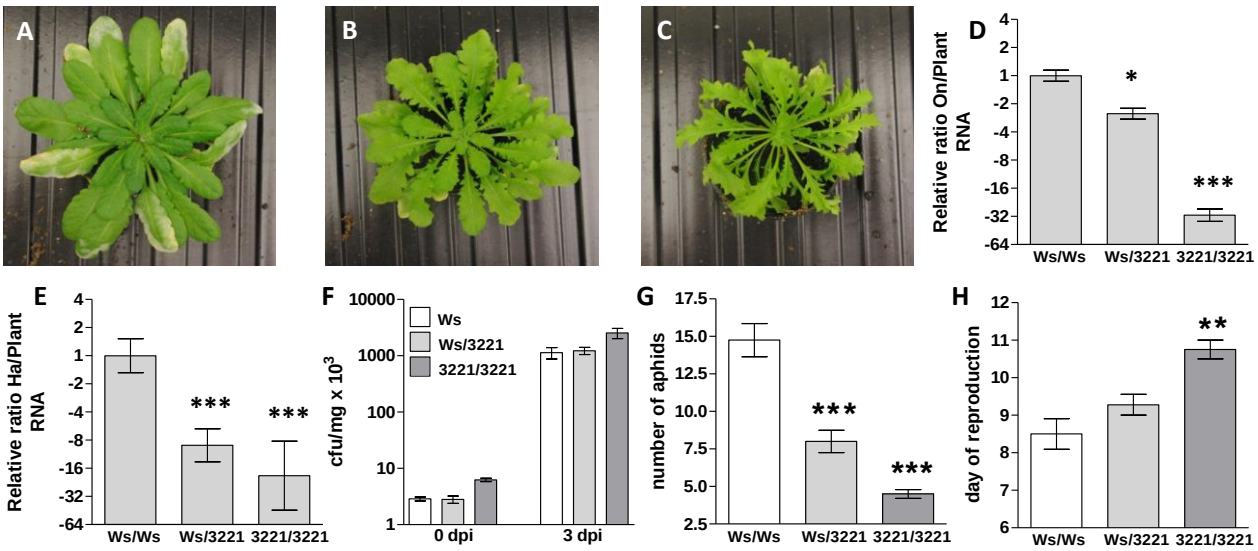

Figure 1 The activation tag mutant 3221 showed altered leaf morphology and broad-spectrum disease resistance. (A-C) Photographs were taken for F2 plants derived from the cross of 3221 and its background Ws-3 showing (A) wild-type, (B) intermediate or (C) mutant phenotype. (D-H) Fungal biomass was quantified for (D) *On*, (E) *Hpa*, (F) *p.syringae* pv *tomato* DC3000. For green peach aphid, (G) degrees of reproduction and (H) development were indicated. Data indicate the mean of 3 or more biological replicates with error bars representing the standard error. Asterisk indicates significant difference between wild-type plants and plants showing intermediate or mutant phenotype according to one way analysis of variance. Number of asterisks indicate the degree of significance (*p<0.05; **p<0.01; ***p<0.001). dpi, days post infection. Ws/Ws: F2 plants with the wild-type leaf morphology as Ws; 3221/3221: F2 plants with the mutant leaf morphology as 3221; Ws/3221: F2 plants with an intermediate leaf morphology.

To assess whether enhanced disease resistance of 3221 mutant is specific to *On* or broad spectrum, the mutant was challenged with downy mildew *Hpa*, bacterial pathogen *P. syringae* pv *tomato* DC3000 and green peach aphid *M. persicae*. Both reproduction (Figure 1G) and development (Figure 1H) of aphids were significantly reduced on 3221-like plants compared to wild-type plants. Regarding the resistance to *Hpa*, F2 plants with the mutant/intermediate phenotype showed significantly reduced biomass of *Hpa* compared to F2 plants with wild-type phenotype (Figure 1E). While proliferation of *P. syringae* bacteria did not differ between wild-type and 3221-like F2 plants (Figure 1F), indicating that the 3221 mutant does not provide effective protection against this pathogen.

Constitutive overexpression of *ATHB13* causes both altered leaf morphology and resistance in 3221

To determine the transposon insertion location, the flanking DNA was isolated by inverse PCR, sequenced and analysed by BLASTN against the Arabidopsis genome sequence. Results revealed that the transposon was inserted 906bp upstream of the predicted translational start codon of At1g69780 encoding a HD-Zip transcription factor *ATHB13* (Figure 2A). By microarray analysis *ATHB13* was also identified as the gene most highly up-regulated in the mutant (34-fold) relative to Ws, whereas the neighbouring genes At1g69770 and At1g69790 showed only around 2-fold increase in expression (Supplemental table 1, 2). The constitutive overexpression of *ATHB13* was confirmed by qPCR using RNA isolated from non-inoculated Ws and 3221 plants (Figure 2B), suggesting that hyperactivation of *ATHB13* was causative to the observed mutant phenotypes.

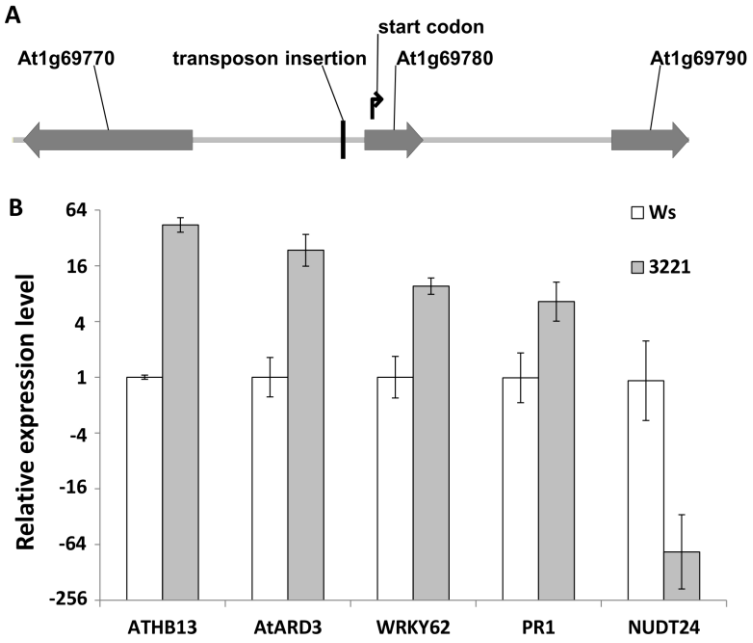

Figure 2 Transposon insertion in Arabidopsis genome causing changes in gene expression. (A) Schematic representation of the transposon insertion at 906 bp upstream of At1g69780 encoding a HD-Zip transcription factor *ATHB13*. (B) qPCR verification of the expression of five genes showing up- or down-regulation in the microarray analysis. Error bars represent standard error of 4 biological replicates.

To verify that the increased *ATHB13* expression in 3221 resulted in morphological alteration and pathogen resistance, 3221 plants were transformed with a silencing construct specifically targeting *ATHB13*. All obtained T1 transformants showed wild-type leaf morphology and size (Figure 3A, B). T2 progenies from two independent T1 plants were analysed for the presence or absence of the silencing construct, *ATHB13* expression and *On* resistance. T2 plants of both families harbouring a silencing construct showed wild-type leaf morphology and susceptibility to *On* (Figure 3G, H). Further, these T2 plants showed significantly higher level of *On* susceptibility and lower level of *ATHB13* expression as compared to T2 plants not inheriting a silencing construct (-) (Figure 3G, H). In addition, we transformed plants of Arabidopsis accession Col-0 and Col-*eds1-2* (carrying a null allele of immune regulator *EDS1*) with an *ATHB13* overexpression construct (Figure 3D, F). Quantification of the *ATHB13* expression and fungal biomass in one T2 family showed that plants harbouring the overexpression construct showed significantly higher *ATHB13* expression and lower fungal biomass in both genetic backgrounds (Figure 3I, J). Together, these data clearly demonstrated that high level of *ATHB13* transcript caused pathogen resistance, reduced stature and leaf serratedness as observed in the 3221 mutant. In addition, *ATHB13*-overexpression induced *On* resistance was independent of the immune regulator *EDS1* since T2 plants harbouring the overexpression construct in the background of Col-*eds1-2* acquired significantly higher level of *On* resistance (Figure 3I, J).

Substantial changes in gene expression in the mutant line 3221

As *ATHB13* encodes a transcription factor we expected up- or down-regulation of other genes under direct or indirect control of *ATHB13* in the mutant line 3221. In an attempt to explore this, microarray analysis was conducted using non-inoculated 3221 and Ws plants. In total, 495 genes were up- or down-regulated by 2.5-fold or greater (Supplemental table 1). To assess the validity of microarray data, expression of *ATHB13* and four additional genes was determined by qPCR and obtained results were in agreement with the microarray analysis (Figure 2B).

The genes with altered expression were functionally categorized using MAPMAN software (Thimm et al. 2004). It was shown that these genes were involved in diverse biological processes, and notably genes belonging to "regulation of transcription" constituted the second largest group next to the unclassified group (Figure 4). In addition to transcription factors, many genes involved in hormone pathways and responses to (a)biotic stresses were induced. For example, expression of *EDS5*, which is required for salicylic acid (SA) synthesis (Nawrath et al. 2002), and *PR1*, a maker gene for SA-responsive defense pathway, were elevated in 3221 compared to Ws (Supplemental table1, 2). Interestingly, dramatic transcriptional changes were observed for a group of genes reported to be involved in resistance to insects, such as *AtARD3*, At2g26390 encoding a serine protease inhibitor, as well as *AtVSP1* and *AtVSP2* (Supplemental Table 1, 2).

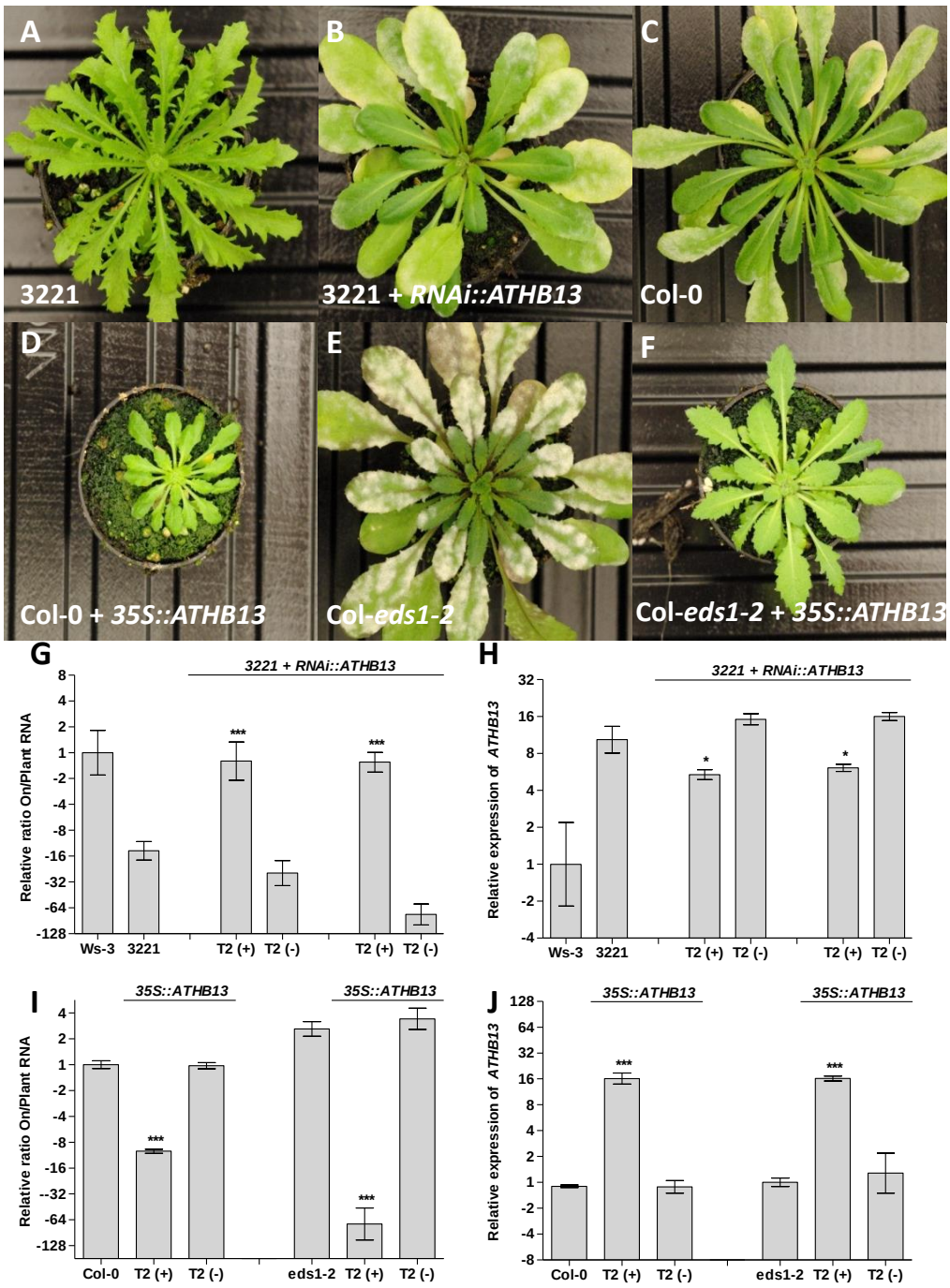

Figure 3 High expression of *ATHB13* resulted in reduced stature, leaf serratedness and powdery mildew resistance in Arabidopsis. (A-F) Phenotypes of 5-week-old (A) 3221, (B) 3221 + *RNAi::ATHB13*, (C) Col-0, (D) Col-0 + *35S::ATHB13*, (E) Col-*eds1-2* and (F) Col-*eds1-2* + *35S::ATHB13* plants 14 days post *On* inoculation. (G-H) Quantification of (G) fungal biomass and (H) *ATHB13* expression in Ws-3, 3221 plants and T2 plants carrying a *RNAi::ATHB13* silencing construct (+) or not (-) in the 3221 genetic background. (I-J), Quantification of (I) fungal biomass and (J) *ATHB13* expression in Col-0 and Col-*eds1-2* plants and T2 plants carrying a *35S::ATHB13* construct (+) or not (-) in respectively the Col-0 or Col-*eds1-2* genetic backgrounds. Data indicate the mean of 3 or more biological replicates with error bars representing the standard error. Asterisk indicates significant difference between T2(+) and T2(-) and the respective untransformed parents according to one way analysis of variance. Number of asterisks indicate the degree of significance (*p<0.05; **p<0.01; ***p<0.001).

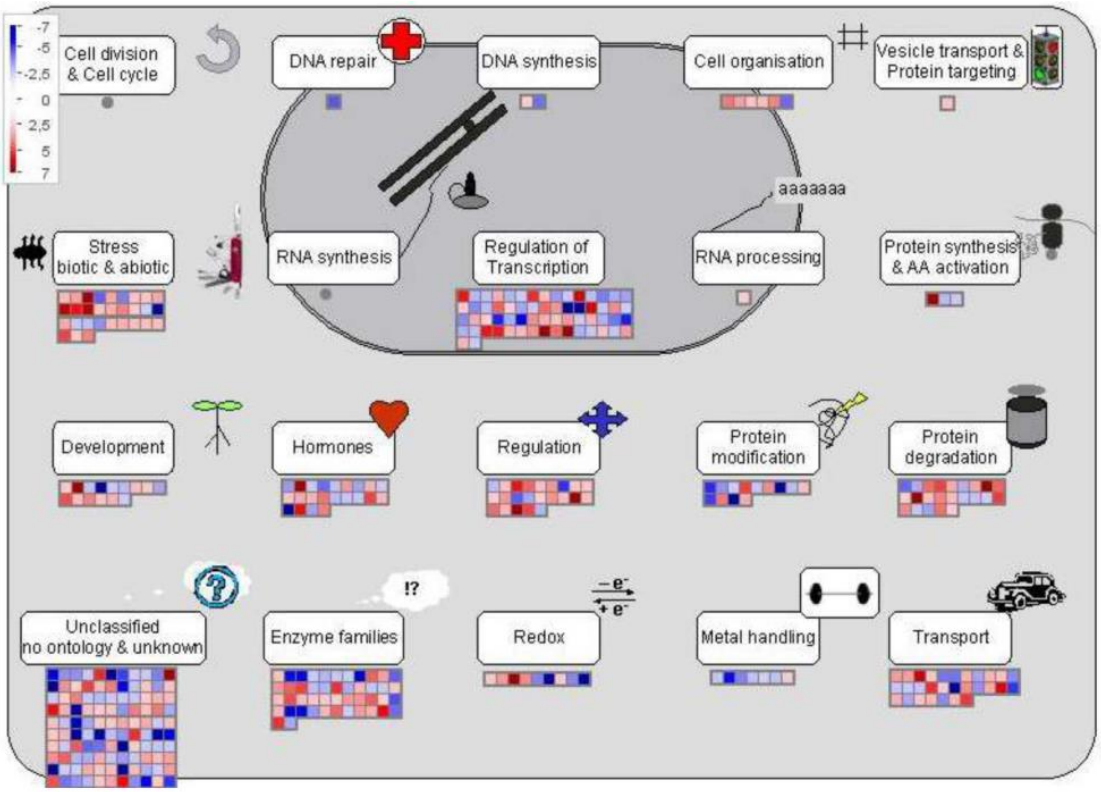

Figure 4 Functional categorization of differentially expressed genes in the mutant 3221 relative to Ws using MAPMAN. Each box represents a gene; blue box indicates repression and red indicates induction of gene expression in 3221 plants as compared with Ws under non-inoculated conditions.

Discussion

HD-Zip proteins are unique to higher plants. The Arabidopsis genome contains 47 HD-Zip genes (Henriksson et al. 2005), which have been grouped into four classes I – IV (Sessa et al. 1994). *ATHB13* belongs to HD-Zip class I (Ariel et al. 2007), which form dimers that recognize the pseudopalindromic sequence CAATNATTG (Palena et al. 2001). In this study we showed that overexpression of *ATHB13* in Arabidopsis activation tag mutant 3221 resulted in resistance to pathogens and insects, including tomato powdery mildew *On*, downy mildew *Hpa* and the green peach aphid *Myzus persicae*. Many characterized HD-Zip genes regulate developmental processes in response to environmental conditions (Henriksson et al. 2005), while tomato HD-Zip I gene *H52* is the only one found to mediate disease resistance when its expression was suppressed (Mayda et al. 1999). Our results demonstrated that *ATHB13* overexpression in the mutant line 3221 led to both broad-spectrum disease resistance and altered leaf morphology (Figure 1).

Previously, it was shown that constitutive expression of *ATHB13* causes a decrease in the width/length ratio of cotyledons and true leaves with increasing sucrose concentration in the growth medium (Hanson et al. 2001). These phenotypes were only observed in 35S::*ATHB13* seedlings that were sugar-treated, suggesting a sugar-dependent control mechanism for *ATHB13* activation. Altered leaf morphology in mutant 3221 (Figure 1) and in 35S::*ATHB13* Col-0 and Col-*eds1-2* plants generated in this study (Figure 2), was independent of glucose supplementation. However, altered leaf

morphology (and pathogen resistance) was caused by extremely high *ATHB13* expression levels (being 34-fold, Supplemental table 1, 2) which are much higher than that reported for the *35S::ATHB13* plants generated by Hanson et al. (2001, being 3-6-fold). This suggests that sugar-dependent *ATHB13* activation can be overcome by extremely high constitutive expression. *ATHB13* is normally expressed in the vasculature in the basal parts of cotyledons, rosette and cauline leaves and flower organs, but not in developing leaves (Hanson et al. 2002). Although many genes involved in hormone metabolism and development showed altered expression according to the MAPMAN categorization, three characterized genes *PIN1*, *MIR164A* and *CUC2* (Bilsborough et al. 2011; Nikovics et al. 2006), controlling serrated leaf formation were similar in their transcript levels between 3221 and Ws (Supplemental table 2). Thus, it is likely that alterations in leaf morphology are a consequence of neomorphic alleles (Zhang 2003).

ATHB13 overexpression influences the expression of a large array of genes including transcription factors and stress-inducible genes (Figure 4 and Supplemental table 1). Thus, the alteration in resistance and morphological traits in 3221 is likely the result of a transcriptional reprogramming in many genes (in)directly regulated by *ATHB13*. For example, WRKY transcription factors have long been associated with regulation of defense gene induction (Rushton et al. 2010). In 3221, seven WRKY transcription factors (*WRKY62*, *38*, *18*, *58* and *46*, *59* and *75*, Supplementary table 1, 2) showed altered expression and they all were up-regulated. The first five have been shown to be induced by treatment with SA or SA analogue benzothidiazole S-methylester, supporting their roles in defense response (Kim et al. 2008; Wang et al. 2006; Hu et al. 2011). In addition to transcription factors, a few genes responsive to SA or jasmonate (JA) were reprogrammed. The level of *PR1* transcript was enhanced in 3221 (Figure 2), while genes having defined roles in SA biosynthesis, such as *AtICS1* and *AtPAL1-4* (Dempsey et al. 2011), showed a similar expression level in 3221 and Ws (Supplemental table 2). The JA-responsive *VSP* genes, both *AtVSP1* and *AtVSP2,* were up-regulated in 3221. However, *AtLOX2* (JA-synthesis related gene) and *PDF1.2* (a marker gene for JA-regulated defense pathway) were slightly down-regulated according to microarray analysis (Supplemental table 2). The inconsistence may reflect complex regulation of gene transcripts and may also indicate that SA/JA pathways are not constitutively activated in 3221. The lack of pronounced up-regulation of marker genes sets 3221 apart from the mutants in which SA or JA/ethylene-dependent signalling pathway is constitutively activated, such as activation tag mutants containing overexpressed *ADR1*, *ADR2* and *CDR1* (Grant et al. 2003; Aboul-Soud et al. 2009; Xia et al. 2004). In 3221 up-regulation of *PR1* may be associated with the enhanced expression of *EDS5*. It was demonstrated that overexpression of *EDS5* leads to accumulation of SA, which can induce the expression of *PR1* (Ishihara et al. 2007). Also, elevated expression of *PR1* may be modulated by WRKY or other transcription factors. It was shown that overexpression of *WRKY18* does not alter endogenous levels of free SA, but results in constitutive expression of PR genes under non-induced conditions (Chen and Chen 2002).

In 3221, both *AtVSP1* and *AtVSP2* transcripts were elevated (Supplemental table 1, 2), which may contribute to resistance against aphids. Arabidopsis *VSP* transcripts are lowly expressed in leaves (Utsugi et al. 1998). They are rapidly induced by wounding, JA, or insect feeding (Utsugi et al. 1998; Berger et al. 1995; Berger et al. 2002), and anti-insect functionality of AtVSP2 was demonstrated (Liu et al. 2005). Furthermore, a

remarkably enhanced expression was observed for *AtARD3* in 3221 (Supplemental table 1, 2). *AtARD3* is involved in methionine recycling during ethylene synthesis but is not regulated by ethylene (Bürstenbinder et al. 2007). It was shown that *AtARD3* is induced by aphid treatment but not by *P. syringae* infection in Col-0 (Barah et al. 2013). The expression of AT2g26390, encoding a serine protease inhibitor, was increased by 10-fold in 3221 (Supplemental table 1, 2). Serine protease inhibitors presumably function in defense against insects by affecting the degradation of relevant proteins (Koiwa et al. 1997).

NUDT24 showed very strong down-regulation (-64-fold in Supplemental table 1, 2), which was confirmed by qPCR (Figure 2B). *NUDT24* belongs to Nudix family which contains 29 putative members in Arabidopsis (Kraszewska 2008). By far the most prominent and best studied member is *NUDT7*. Its loss-of-function mutation results in enhanced resistance to several pathogens, which is attributable to constitutive activation of defense-related genes (Jambunathan and Mahalingam 2006) and a higher level of SA (Bartsch et al. 2006), or associated with redox homeostasis (Ge et al. 2007). *NUDT24* was shown to be in a different clade than *NUDT7*, and likely involved in thiamine metabolism (Goyer et al. 2013). Interestingly, in the *nudt7* mutant growing in suboptimal conditions, genes associated with systemic acquired resistance and cell death pathways were induced, whereas JA-responsive genes were down-regulated, and additional Nudix hydrolases were repressed, such as *NUDT24* (Jambunathan et al. 2010). The strongly reduced expression of *NUDT24* in 3221 hinted that *NUDT24* might be a potential susceptibility gene (Pavan et al. 2010) and that its loss-of-function would lead to resistance. Unfortunately, the Arabidopsis *NUDT24* knockout mutant (KO-24, T-DNA mutant CS856946 with insertion occurring at the eighth exon of *NUDT24*) did not show a decrease in fungal sporulation compared with the background Col-0 upon *On* challenge (Supplemental figure 1).

Besides conveying broad-spectrum disease resistance, ectopic expression of *ATHB13* also confers tolerance to cold, drought and salt stresses (Cabello et al. 2012; Cabello and Chan 2012). And expression of *ATHB13* ortholog in sunflower was proven to be effective in abiotic stresses (Cabello and Chan 2012; Cabello et al. 2012). Further understanding of how *ATHB13* modulates the crosstalk of multiple stress conditions will not only provide insights into the molecular mechanisms, but also the opportunity of utilizing it in crop breeding for engineering increased resistance to abiotic and biotic stresses.

Materials and Methods

Arabidopsis lines and growth conditions

The Arabidopsis En-I transposon-based activation tag population was originally developed at Wageningen University and described previously (Marsch-Martinez et al., 2002). Only Ws activation tag lines were screened for powdery mildew resistance, due to the high level of susceptibility of Ws to *On*. Arabidopsis plants were grown in soil in a growth chamber at 21ºC and 19ºC during the 8h day and 16h night periods respectively, a relative humidity of 70% and a light intensity of 100 W/m^2.

Pathogen and Aphid bioassays

On isolate *On*-Ne (Bai et al. 2005) and *Hpa* isolate Waco9 were maintained on tomato cv Moneymaker and Arabidopsis Col-0 plants respectively. Spore suspensions were obtained by washing heavily infected leaves in water. For disease assays, plants were sprayed with an inoculum of 2.5 x 10^5 spores per mL. Fungal and oomycete quantifications were performed 8-14 days post inoculation on 4-6 weeks old plants. *Pseudomonas syringae* pv *tomato* DC3000 assays were performed as described previously (Tornero and Dangl 2001) using 4-6 weeks old plants and bacterial suspensions with an OD_{600} of 0.04. For bioassays with the aphid *Myzus persicae*, synchronized one-day-old nymphs were used to infest three-week-old Arabidopsis plants with one nymph per plant. For the pre-reproductive period, the aphids were monitored twice a day at 9 in the morning and at 3 in the afternoon from 6 till 12 days post infestation (dpi). The time point when a nymph began to reproduce was recorded. For the population development, the total number of aphids was counted at 14 dpi.

Isolation of transposon-flanking DNA by inverse PCR

Genomic DNA of plant leaves was extracted using the DNeasy Plant Mini kit (Qiagen) and then digested with restriction enzyme EcoRI (Thermo, product # ER0275) or BamHI (Thermo, product # ER0051). Digested gDNA was recovered and self-ligated with T4 DNA ligase (Fermentas, product # EL0011). Five µl of the ligation products was used as templates in inverse PCR (iPCR) reactions of 50 µl total volume using proof-reading Phusion™ DNA polymerase (Finnzymes, Product codes: F-530S, 100U). The iPCR conditions consisted of 30 seconds at 98°C followed by 35 cycles of 98°C for 10 sec, 64°C for 10 sec, and 72°C for 3 min with a final extension at 72°C for 10 min. Primers used for transposon flanking sequence isolation were designed based on the sequences of the BAR gene located on the transposon (Marsch-Martinez et al. 2002) being: F051-CTGGCAGCTGGACTTCAGCCTG and F049-GCGTCGTTCTGGGCTCATGGT. The PCR product was sequenced and the sequence was analysed by BLASTN against the Arabidopsis genome at http://www.arabidopsis.org/.

Microarray analysis

Three biological replicates were used for 4-week-old Ws and 3221 plants, each including 5-10 pooled plants. Total RNA was isolated from leaves and prepared for hybridization using a 12x135k Arabidopsis microarray containing 39,042 genes with 4 probes per target gene. The whole process of microarray including sample preparation, labelling, hybridization and scanning was performed at Roche Nimblegen (http://www.nimblegen.com/). Microarray data analysis including background subtraction, normalization and elimination of false positives was processed at the Micro Array Division (MAD) of University of Amsterdam. Log_2-transformed data were produced, and to identify genes showing significant change in expression RankProdIt (Laing and Smith 2010) was used. Genes that meet two criteria: pfp (percentage of false prediction) <0.05, and differentially expressed by 2.5-fold or greater, were imported to MAPMAN for pathway reconstruction (Thimm et al. 2004).

Generation of transgenic Arabidopsis plants

To generate an *ATHB13* (At1g69780) RNAi silencing construct, a 384-bp fragment was amplified from Ws-derived cDNA with primers: Fw-*ATHB13*-caccTTTGCTTCGTTTCTAGGTAAGAGA and Rv-*ATHB13*-TCAGCTTGGAGTTTCTGATTATGA. To generate *ATHB13* overexpressing construct, full length coding sequences were amplified from Ws cDNA with primers: Fw-*ATHB1*3- cacctgttgcaaaacagaagaagatg and Rv-*ATH*B13- tctgatcaaaattccaaatacaaaa. PCR products were cloned into pENTR/D-TOPO vector (Invitrogen) and the derived plasmids were sequenced. Subsequently, the inserts were recombined into pHellsgate8 (silencing vector) or pK7WG2 (overexpressing vector) via LR reactions (Invitrogen). Agrobacterium strain GV3101 was used for Arabidopsis transformation using the floral-dip method (Logemann et al. 2006). Kanamycin-resistant T1 plants were selected and selfed to produce T2 families (Harrison et al. 2006). For each segregating T2 family, NPTII gene cassette primers Fw-NPTII-TTCCCCTCGGTATCCAATTA and Rv-NPTII-GATTGTCTGTTGTGCCCAGT were used in a PCR analysis to select transgenic plants.

Selection of homozygous plants of the T-DNA line

Knock-out T-DNA line CS856946 for *NUDT24* was ordered from Arabidopsis biological resource center (ABRC). To select homozygous plants, primers based on the gene and vector sequences were used: Fw-*NUDT24*-ATGTAACGCTTAACATGATGCTTG, Rv-*NUDT2*4-TCATGAAGGCACATGAAGACT and Fw-vector-AACGTCCGCAATGTGTTATTAAGTTGTC. A plant was considered homozygous when a PCR product was obtained with vector primer and *NUDT24* reverse primer, but not with *NUDT24* forward and reverse primers. The homozygous plants were referred as KO-24.

Quantitative RT-PCR and data analysis

For quantification of fungal biomass, DNA or RNA extracted from Arabidopsis plants was used. For quantification of transcript levels, RNA was used. DNA was isolated with the DNeasy Plant Mini kit (Qiagen). Total RNA was extracted from leaflets using the RNeasy kit (Qiagen). After removal of DNA with DNase I (Invitrogen), 1 ug total RNA was used for cDNA synthesis using Superscript II reverse transcriptase kit (Invitrogen). Relative biomass or transcript levels were determined using the iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). Arabidopsis *Act2* (At3g18780) transcript level was used for normalisation with primers Fw-*Act2*-AATCACAGCACTTGCACCA and Rv-*Act2*-GAGGGAAGCAAGAATGGAAC. For *On* quantification, primers were used amplifying part of the internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA being Fw-*On*-CGCCAAAGACCTAACCAAAA and Rv-*On*-AGCCAAGAGATCCGTTGTTG (Kiss et al. 2005). For *Hpa* quantification, primers were used amplifying part of the *HpACT* gene being: Fw-*HpAct*-GTGTCGCACACTGTACCCATTTAT and Rv-*HpAct*-ATCTTCATCATGTAGTCGGTCAAGT (Huibers et al.2009). Primers used for determining relative transcript levels were: Fw-*ATHB13*-TGAAGGATCTTGCAGTAACAGA, Rv-*ATHB13*-GGCCACCGGTTAATGTACTG; Fw-*WRKY62*-GTCCATGGAAAGGGAGGATT, Rv-*WRKY62*-GGATTGATCGTCTTGGTGGT; Fw-*AtARD3*-CAAACTGAAGAAGTGATTCAAGC and Rv-*At*ARD3-TTGTCCAAAGATACAAATTCCTTA; Fw-*PR1*- GAACACGTGCAATGGAGTTT and Rv-*PR1*-GGTTCCACCATTGTTACACCT; Fw-*NUDT24*-ATGTAACGCTTAACATGATGCTTG, Rv1-*NUDT24*-TCCCAGGGATGATTCCTTT. For analysis of the relative expression level and

fungal biomass the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (2001) was used. Values were normalized relative to *Act2,* and calibrated to levels in the control plants, which were set as 1. Data were statistically examined using independent-samples t-test and one-way analysis of variance (ANOVA) based on Post-hoc comparisons using Tukey's HSD test. All analyses were performed using SPSS Statistics 20 following the instructions of SPSS Survival Manual 4th edition (Pallant 2010).

Acknowledgements
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Supplemental table 1 A list of differentially expressed genes between 3221 and Ws under non-inoculated conditions.

Locus	Fold 3221/Ws
AT1G69780	33.61
AT2G26400	15.32
AT5G01900	13.91
AT2G26390	10.21
AT5G22570	9.80
AT2G03130	9.06
AT4G15990	8.60
AT4G19230	8.51
AT2G14610	7.86
AT3G50770	7.68
AT1G03850	7.57
AT5G24780	7.46
AT3G28510	7.18
AT3G59220	7.03
AT1G19230	6.72
AT4G33730	6.60
AT4G31330	6.47
AT1G08090	6.35
AT1G62975	6.25
AT5G06800	6.16
AT2G25810	5.97
AT3G22600	5.77
AT3G61198	5.62
AT3G16460	5.57
AT5G44574	5.56
AT1G08590	5.50
AT4G39030	5.50
AT5G03680	5.48
AT2G36080	5.45
AT2G21900	5.39
AT2G24850	5.37
AT5G44585	5.33
AT1G52750	5.23
AT3G54990	5.19
AT1G67910	5.18
AT2G42990	5.15
AT4G23700	5.14
AT2G29470	5.13
AT5G25420	5.06

Locus	Fold 3221/Ws
AT3G61390	4.99
AT2G45760	4.94
AT4G11320	4.94
AT3G14370	4.92
AT4G37150	4.91
AT1G70890	4.90
AT1G21310	4.87
AT4G18430	4.87
AT5G09290	4.83
AT2G30770	4.79
AT3G49780	4.77
AT3G50140	4.73
AT3G13610	4.63
AT2G14560	4.58
AT5G47950	4.56
AT5G19880	4.53
AT2G31110	4.49
AT2G44480	4.48
AT1G26390	4.48
AT5G51780	4.47
AT3G16420	4.46
AT4G18250	4.45
AT3G12220	4.44
AT3G48350	4.36
AT5G44460	4.35
AT5G26930	4.34
AT5G13080	4.29
AT5G07610	4.27
AT1G76090	4.23
AT5G22355	4.14
AT5G26010	4.10
AT1G10340	4.10
AT2G43820	4.07
AT3G20380	4.07
AT4G10500	4.04
AT2G44380	4.03
AT4G28040	4.02
AT2G34700	4.00
AT1G01680	3.96

Locus	Fold 3221/Ws
AT1G58390	3.96
AT4G37010	3.95
AT1G66960	3.91
AT1G28480	3.89
AT5G05280	3.87
AT4G21840	3.85
AT2G41690	3.82
AT1G15520	3.82
AT5G07380	3.81
AT1G32350	3.78
AT2G36890	3.77
AT2G30930	3.75
AT2G39410	3.74
AT4G31800	3.73
AT1G14430	3.72
AT1G73805	3.71
AT3G46080	3.68
AT4G21230	3.65
AT1G48660	3.64
AT5G19970	3.63
AT2G37130	3.61
AT3G09940	3.61
AT5G61290	3.60
AT5G26660	3.60
AT1G51850	3.60
AT5G24910	3.58
AT5G03350	3.57
AT1G08230	3.56
AT3G48630	3.54
AT4G33740	3.54
AT2G44383	3.53
AT1G19250	3.52
AT2G41178	3.51
AT5G22545	3.51
AT4G35180	3.51
AT2G38750	3.50
AT2G02100	3.47
AT5G57550	3.45
AT3G18250	3.44
AT3G15536	3.40
AT4G25780	3.38
AT2G43570	3.38
AT2G22890	3.36
AT3G51330	3.35

Locus	Fold 3221/Ws
AT1G44608	3.34
AT5G04330	3.33
AT1G28660	3.33
AT5G59310	3.32
AT3G60966	3.31
AT5G38970	3.29
AT1G44160	3.28
AT3G21520	3.28
AT5G03995	3.28
AT3G62270	3.27
AT1G47760	3.26
AT5G43060	3.25
AT5G60280	3.24
AT2G35585	3.24
AT1G43160	3.22
AT5G36910	3.22
AT1G47400	3.20
AT3G48920	3.19
AT1G76930	3.19
AT4G25434	3.18
AT5G49620	3.16
AT5G52720	3.16
AT1G16370	3.16
AT3G24300	3.16
AT1G70850	3.15
AT5G13320	3.15
AT5G49850	3.14
AT5G23660	3.10
AT4G29270	3.09
AT5G38900	3.09
AT5G10760	3.06
AT3G46500	3.05
AT3G43800	3.05
AT1G77660	3.04
AT1G22150	3.04
AT5G17700	3.04
AT1G17060	3.04
AT3G16400	3.02
AT2G46400	3.02
AT1G56270	3.01
AT4G35160	3.01
AT5G04010	3.01
AT3G03230	3.00
AT3G23560	2.99

Locus	Fold 3221/Ws
AT3G26200	2.99
AT3G61190	2.98
AT3G28320	2.96
AT1G70830	2.96
AT1G60470	2.94
AT3G16670	2.94
AT1G34170	2.93
AT1G05880	2.92
AT3G01830	2.91
AT1G55780	2.91
AT1G70885	2.90
AT5G07460	2.90
AT3G01080	2.89
AT3G22240	2.89
AT3G56000	2.89
AT5G55170	2.89
AT2G36307	2.89
AT1G28670	2.88
AT1G70860	2.88
AT5G39970	2.87
AT5G45000	2.86
AT2G30750	2.85
AT1G61120	2.85
AT1G54095	2.85
AT1G62660	2.85
AT4G39670	2.85
AT4G11500	2.84
AT1G70880	2.84
AT4G34380	2.84
AT5G42380	2.84
AT2G32530	2.83
AT4G02700	2.82
AT2G44390	2.81
AT5G14750	2.81
AT2G45220	2.81
AT5G07780	2.80
AT3G50470	2.80
AT1G02450	2.80
AT5G20960	2.80
AT4G39795	2.78
AT1G18140	2.78
AT5G50760	2.78
AT2G04495	2.76
AT1G60390	2.76

Locus	Fold 3221/Ws
AT3G22231	2.76
AT1G56120	2.76
AT2G34940	2.74
AT5G24530	2.74
AT1G15790	2.74
AT4G31100	2.74
AT5G24080	2.73
AT2G04100	2.73
AT3G46490	2.73
AT2G22330	2.71
AT2G38760	2.70
AT3G22235	2.70
AT4G13280	2.70
AT4G12170	2.69
AT1G08100	2.69
AT1G56060	2.67
AT3G60470	2.67
AT3G28310	2.66
AT2G01530	2.65
AT5G24770	2.65
AT2G21550	2.65
AT2G43000	2.64
AT4G14610	2.64
AT3G45650	2.64
AT5G22540	2.63
AT1G33840	2.62
AT3G44970	2.62
AT3G26320	2.61
AT4G15680	2.61
AT4G39700	2.60
AT1G27020	2.60
AT4G03820	2.60
AT1G71390	2.59
AT5G10570	2.59
AT4G37430	2.58
AT2G25780	2.58
AT4G00700	2.57
AT5G42860	2.55
AT5G05600	2.55
AT4G09770	2.55
AT1G65810	2.55
AT5G53592	2.55
AT3G19350	2.55
AT1G47395	2.53

Locus	Fold 3221/Ws
AT3G63470	2.52
AT4G31110	2.52
AT1G18570	2.51
AT5G51790	2.51
AT5G24290	2.51
AT5G60890	2.50
AT2G39420	2.50
AT1G05310	2.50
AT1G13750	2.50
AT2G34930	-2.50
AT4G10120	-2.50
AT2G18196	-2.50
AT3G48510	-2.51
AT3G10910	-2.51
AT3G20340	-2.51
AT5G05890	-2.52
AT3G24520	-2.52
AT3G20362	-2.52
AT4G19850	-2.53
AT1G78070	-2.54
AT2G14247	-2.54
AT2G25820	-2.54
AT3G46670	-2.54
AT5G18030	-2.54
AT2G30432	-2.55
AT5G50800	-2.56
AT2G43700	-2.56
AT4G01390	-2.56
AT4G33905	-2.57
AT1G78460	-2.57
AT2G27402	-2.57
AT1G69500	-2.58
AT1G49640	-2.58
AT1G76820	-2.58
AT5G37540	-2.58
AT4G36700	-2.59
AT5G49330	-2.60
AT1G51090	-2.61
AT3G02480	-2.61
AT1G28230	-2.61
AT5G54060	-2.62
AT1G52880	-2.62
AT4G13840	-2.63
AT5G54470	-2.64

Locus	Fold 3221/Ws
AT1G66000	-2.64
AT5G17220	-2.64
AT2G29950	-2.65
AT5G54585	-2.65
AT1G71000	-2.65
AT3G09450	-2.65
AT5G35480	-2.66
AT4G15550	-2.66
AT4G37220	-2.66
AT1G18400	-2.67
AT5G59050	-2.67
AT2G45600	-2.68
AT2G46790	-2.68
AT2G06002	-2.68
AT1G79520	-2.68
AT5G47220	-2.69
AT5G47610	-2.69
AT3G47340	-2.69
AT2G22810	-2.70
AT5G52900	-2.70
AT4G17550	-2.70
AT1G21000	-2.70
AT2G18550	-2.70
AT3G16800	-2.71
AT1G01580	-2.72
AT5G43150	-2.72
AT3G28270	-2.75
AT1G30860	-2.75
AT2G37950	-2.75
AT5G55250	-2.77
AT1G09240	-2.78
AT1G68870	-2.78
AT1G18265	-2.78
AT4G08570	-2.79
AT5G06760	-2.79
AT1G22810	-2.81
AT4G15210	-2.81
AT5G47590	-2.83
AT1G67860	-2.84
AT1G11850	-2.85
AT5G45840	-2.85
AT5G05420	-2.86
AT5G10300	-2.87
AT1G73830	-2.87

Locus	Fold 3221/Ws
AT5G39860	-2.91
AT1G69490	-2.92
AT5G03640	-2.92
AT2G36750	-2.92
AT4G25830	-2.94
AT3G02040	-2.94
AT3G55760	-2.95
AT1G76720	-2.95
AT1G01250	-2.98
AT5G12050	-2.98
AT4G35720	-2.99
AT4G14090	-3.00
AT1G02340	-3.01
AT1G46768	-3.01
AT1G32900	-3.01
AT2G34655	-3.01
AT5G03130	-3.05
AT5G58310	-3.07
AT1G78970	-3.10
AT5G43860	-3.11
AT1G62290	-3.11
AT1G75250	-3.15
AT4G32280	-3.16
AT1G67260	-3.16
AT5G20790	-3.16
AT3G47965	-3.17
AT2G29490	-3.17
AT4G22880	-3.17
AT1G07180	-3.18
AT2G35070	-3.19
AT1G66380	-3.20
AT1G31173	-3.20
AT1G62710	-3.23
AT2G36120	-3.23
AT1G20190	-3.24
AT3G56970	-3.25
AT3G14210	-3.28
AT1G26945	-3.29
AT1G73700	-3.30
AT3G28007	-3.31
AT4G28700	-3.32
AT5G01740	-3.34
AT1G56650	-3.35
AT1G73870	-3.36

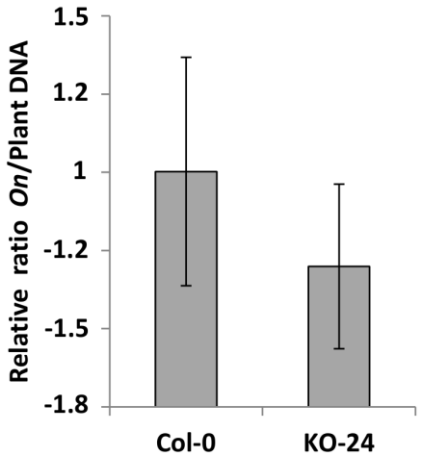
Locus	Fold 3221/Ws
AT3G44120	-3.39
AT1G66690	-3.39
AT4G24450	-3.40
AT3G53250	-3.40
AT2G40610	-3.40
AT4G25000	-3.43
AT3G56980	-3.43
AT4G22870	-3.49
AT1G24735	-3.50
AT3G01960	-3.52
AT2G40670	-3.54
AT4G19170	-3.56
AT3G29639	-3.59
AT1G73040	-3.60
AT1G67865	-3.64
AT3G60160	-3.65
AT1G15410	-3.65
AT3G55646	-3.65
AT3G17520	-3.66
AT1G66700	-3.69
AT5G20830	-3.72
AT1G29395	-3.72
AT1G31258	-3.73
AT1G23130	-3.73
AT4G01080	-3.75
AT1G24260	-3.80
AT5G15160	-3.82
AT1G61800	-3.82
AT4G13800	-3.84
AT5G16570	-3.89
AT5G18600	-3.89
AT2G47880	-3.93
AT4G39800	-3.95
AT5G49740	-3.97
AT2G04170	-4.00
AT3G03480	-4.04
AT3G21330	-4.05
AT5G13930	-4.05
AT4G23600	-4.07
AT5G57760	-4.14
AT1G12940	-4.14
AT1G31490	-4.15
AT5G09730	-4.17
AT2G21650	-4.17

Locus	Fold 3221/Ws
AT1G64780	-4.19
AT1G14520	-4.19
AT3G55240	-4.20
AT2G18050	-4.23
AT4G33790	-4.26
AT2G16990	-4.26
AT3G53980	-4.30
AT1G05560	-4.32
AT5G15500	-4.34
AT5G59130	-4.38
AT5G15190	-4.42
AT2G15020	-4.44
AT2G15220	-4.44
AT5G03545	-4.44
AT1G31690	-4.46
AT4G18422	-4.49
AT3G46370	-4.51
AT4G34060	-4.59
AT3G10150	-4.71
AT1G69530	-4.84
AT5G16030	-4.88
AT1G77960	-4.93
AT5G25110	-4.94
AT1G04570	-5.07
AT4G04293	-5.10
AT4G23290	-5.10
AT1G07430	-5.17
AT1G18710	-5.29
AT4G39250	-5.33
AT1G53100	-5.54
AT5G49730	-5.58
AT1G11785	-5.71
AT2G36970	-5.72

Locus	Fold 3221/Ws
AT1G66390	-5.75
AT4G01985	-5.79
AT3G28220	-5.91
AT2G04460	-5.93
AT1G52000	-5.99
AT1G52040	-6.09
AT1G02850	-6.31
AT1G23110	-6.49
AT3G21460	-7.11
AT5G44260	-7.23
AT1G09350	-7.30
AT3G19550	-7.32
AT2G32870	-7.49
AT1G64360	-7.85
AT3G58070	-7.86
AT2G16367	-7.93
AT5G58770	-8.44
AT2G25625	-8.57
AT1G74670	-8.62
AT4G25100	-8.81
AT5G02760	-8.91
AT5G01180	-9.40
AT1G80130	-9.76
AT1G66060	-9.92
AT1G34060	-10.72
AT1G53160	-11.28
AT4G19430	-11.36
AT5G28080	-11.51
AT3G27250	-12.12
AT1G56600	-14.88
AT1G70640	-20.22
AT5G19470	-64.39

Supplemental table 2 A list of genes that are present in the microarray analysis and specifically mentioned in the text

Locus	Fold 3221/Ws	Encoding gene
At1g69780	33.61	ATHB13
At1g69770	2.33	CMT3
At1g69790	2.34	putative protein kinase
At4g39030	5.50	EDS5
At2g14610	7.86	PR1
At2g26400	15.32	ATARD3
At2g26390	10.21	serine protease inhibitor
At5g24780	7.46	AtVSP1
At5g24770	2.65	AtVSP2
At1g73590	-1.03	PIN1
At2g47585	-1.16	MIR164A
At5g53950	1.25	CUC2
At5g01900	13.91	WRKY62
At5g22570	9.8	WRKY38
At4g31800	3.73	WRKY18
At3g01080	2.89	WRKY58
At2g46400	3.02	WRKY46
At2g21900	5.39	WRKY59
At5g13080	4.29	WRKY75
At1g74710	1.54	AtICS1
At2g37040	-1.03	AtPAL1
At3g53260	-1.18	AtPAL2
At5g04230	1.93	AtPAL3
At3g10340	1.11	AtPAL4
At3g45140	-2	AtLOX2
At5g44420	-2.39	PDF1.2
At5g19470	-64.39	NUDT24



Supplemental figure 1 Quantification of *On* biomass in Col-0 and homozygous KO-24 plants. Data indicate mean of 3 biological replicates with error bars representing the standard error.

Chapter 6

General discussion

Disease caused by pathogens and pests is a constraint for improving yield and quality of crops. To minimize the loss due to disease, resistance genes have been deployed in the cultivars of many crops species. Frequently *R* genes can be identified from wild species, and isolation of *R*-genes and signal transduction pathway genes provides a basis for understanding the resistance mechanism. Alternatively, model species can be studied to facilitate the identification of genes which upon induction or inactivation confer resistance, and subsequently searching for orthologs or transgenic approaches can be undertaken to achieve resistance in crops. In this PhD research, a combination of these approaches was undertaken by studying resistance to tomato powdery mildew *On* in tomato and *Arabidopsis thaliana.* In tomato *Ol-1*-mediated resistance was dissected. In Arabidopsis, genes conferring resistance when silenced or overexpressed were searched. Here we place our findings concerning the genes that influence or underly the resistance against *On* in a broader context.

Role of amino acid metabolic pathways in disease resistance

Amino acid metabolism influences pathogen nutrition availability and hormone signaling

The primary goal for pathogens and pests is to gain access to nutrients from the hosts for growth and propagation. Chen et al. (2010) identified a class of sugar transporters from several plant species, named *SWEETs*, and showed that fungal and bacterial pathogens induce the expression of different *SWEET* genes. Interestingly, *OsSWEET11* underlies the dominant allele (*Xa13*) of the recessive resistance gene *xa13*, which provides protection against bacterial blight (Yang et al. 2006; Yuan et al. 2009). Pathuri et al. (2011) showed that susceptibility to powdery mildew *Bgh* in barely is altered by manipulating the expression of *ADH* (*alcohol dehydrogenase*), which appears to play an important role in energy metabolism. Amino acid uptake is vital for a successful compatible interaction (Buell et al. 2003), so the altered production of certain amino acids presumably influences the outcome of plant-pathogen interactions (Figure 1). Knock-out mutations in five amino acid permeases (AAP) negatively affected nematode infestation levels in Arabidopsis, and reduced availability of leucine in the *aap6* mutant was likely contributing to the resistance (Marella et al. 2013; Elashry et al. 2013). Arabidopsis plants carrying mutations in *AK2* (*ASPARTATE KINASE 2*) and *DHDPS2* (*DIHYDRODIPICOLINATE SYNTHASE 2*) (Figure 1) were resistant to downy mildew. These mutants have higher levels of threonine, which may make the host inappropriate for the pathogen colonisation (Stuttmann et al. 2011). Resistance to downy mildew was also acquired in Arabidopsis defective in *DMR1* (*DOWNY MILDEW RESISTANT1*) (Figure 1). The *dmr1* mutant has a higher concentration of homoserine, and exogenous treatment of homoserine in wild type plants rendered resistant phenotypes (van Damme et al. 2009). The resistance mechanism of the *dmr1* mutant is unkown. The conserved role of *dmr1* was demonstrated in tomato; suppression of a *DMR1* ortholog in tomato resulted in resistance to *On* (Huibers et al. 2013). Soybean *Rhg4* (*Resistance to Heterodera Glycines 4*) encodes a serine hydroxymethyltransferase (SHMT) (Liu et al. 2013) (Figure 1), and amino acid changes in *Rhg4* likely affect its catalytic function in glycine and folate metabolism, leading to nutritional deficiency for nematode growth. Both arginase (ARG) and threonine deaminase (TD) (Figure 1) from the tomato act in the midgut of the

herbivore *M. sexta*, and their defensive effects are correlated with the depletion of arginine and threonine respectively, thus reducing larval growth (Chen et al. 2005; Gonzales-Vigil et al. 2011).

On the other hand, amino acid metabolism has a profound impact on SA-dependent signalling, which plays an important role in resistance against biotrophic pathogens. Examples include pepper *CaAS1* (*Asparagine Synthase 1*) (Figure 1), Arabidopsis *LHT1* (*Lysine Histidine Transporter 1*), *AGD2* (*Aberrant Growth and Death 2*) encoding an aminotransferase and *AtCAT1* (*Aationic Amino acid Transporter 1*) (Hwang et al. 2011; Liu et al. 2010; Song et al. 2004a,b; Yang et al. 2013). Disruption of *LHT1* and *AGD2*, and overexpression of *AtCAT1* resulted in SA accumulation and resistance. Only in the case of *LHT1*, the physiological substrate was established, which is Glutamine (Gln). For the remaining genes, amino acid profiling did not reveal significant changes in knockout or overexpression plants. Hence the link whereby the SA pathway is activated is unknown for these genes. In pepper plants with reduced *CaAS1* expression, asparagine biosynthesis was impaired upon *X. campestris* pv. *vesicatoria* (*Xcv*) infection, and the production of H_2O_2, defense-related genes, and SA were affected. In addition, lysine catabolite pipecolic acid is a critical regulator of inducible plant immunity, and it regulates SA biosynthesis in the presence of pathogen-derived and other stimuli (Návarová et al. 2012).

ALS is a key enzyme in the biosynthesis of the branched-chain amino acids (BCAAs) valine, leucine and isoleucine (Figure 1), and it is also a target of commercial herbicides. Tomato genomes contain three *ALS* genes; *ALS1* and *ALS2* show high similarity while *ALS3* is quite different from *ALS1* and *ALS2*. By means of gene silencing and herbicidal application we demonstrated that targeting ALS negatively compromised *Ol-1*-mediated resistance (chapter 3). In contrast, herbicidal application did not result in more fungal growth in susceptible Moneymaker and in resistant NIL-*Ol-4* which carries a NB-LRR type resistance gene (*Ol-4*). The content of BCAAs markedly increased in Arabidopsis leaves following *P. syringae and Xcv* infection (Ward et al. 2010; Návarová et al. 2012), and exogenous application of isoleucic acid (which is closely related to isoleucine) triggered the expression of defense marker gene *PR1* and provided *P. syringae* resistance (von Saint Paul et al. 2011). In chapter 3, exogenous treatment of BCAAs in MM and NIL-*Ol-1* did not affect their responses to powdery mildew infection, although application of homoserine elevated the resistance level in both genotypes. Fine-mapping of *Ol-1* delimited the region to an interval of 73 kb (Seifi 2011). Coincidently, one of the candidate genes (*Solyc06g060790)* for *Ol-1* encodes 3-isopropylmalate dehydratase (IPMD) (Figure 1), which is involved in the biosynthesis of leucine. However, silencing of this gene via VIGS did not compromise the resistance of NIL-*Ol-1*. In the future, cloning of *Ol-1* and measurement of amino acid contents are crucial to clarify the role of ALS in *Ol-1*-mediated resistance.

Application of amino acid metabolism related genes for disease resistance

The elucidation of amino acid metabolic pathways involved in resistance promises new sources for engineering disease resistant crops. Most of the identified Arabidopsis mutants display varying fitness costs, and *On* resistance resulting from suppression of the *Dmr1* ortholog in tomato is associated with reduced leaf size and yellowish colour (Huibers et al. 2013). This trend does not hold true for all the genes. Overexpression of

ARG2 in tomato resulted in less weight of *M. sexta* larvae and less foliage consumption, and the resistance was not associated with obvious morphological or reproductive phenotypes (Chen et al. 2005). Furthermore, the level of arginine was not altered in tomato leaves. Soybean *Rhg4* represents a major source of resistance to cyst nematodes in cultivars (Liu et al. 2013), and is expected to be unburdened with undesirable fitness costs.

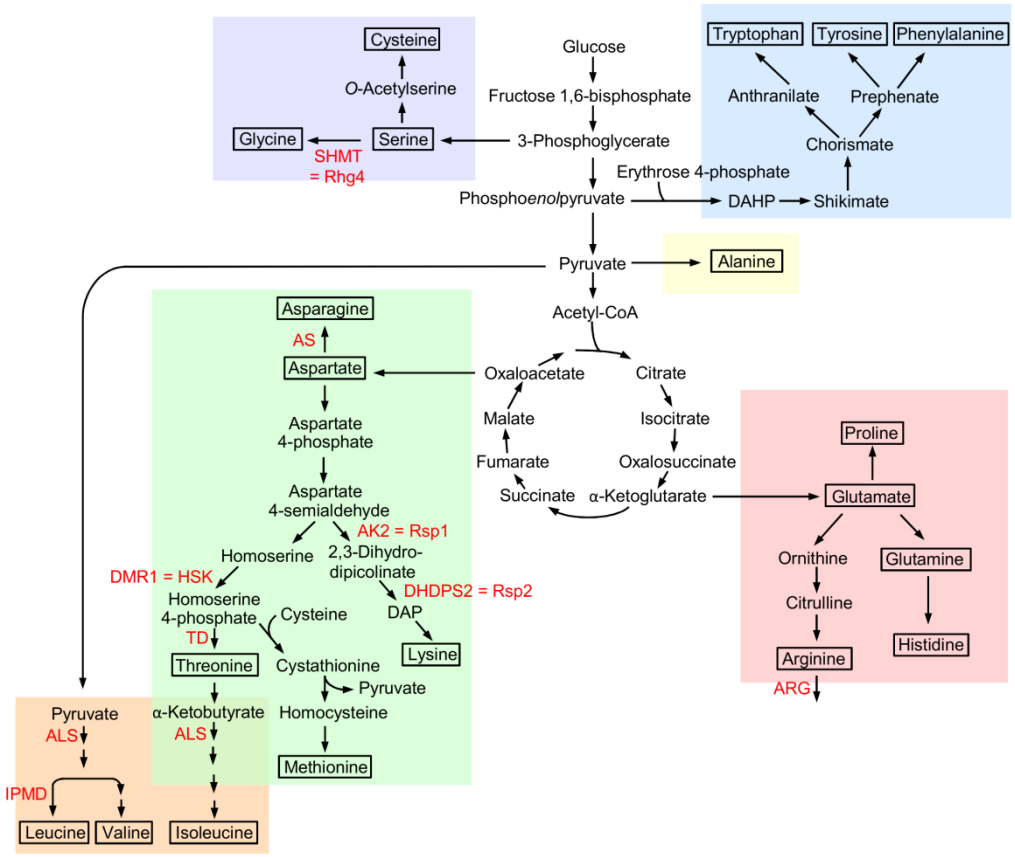


Figure 1 Pathway of amino acid metabolism with overview of the genes (highlighted in red) that (likely) play a role in resistance to different pests and pathogens. The figure is adapted from Buchanan et al. (2000).

One factor to be considered is that plant amino acid metabolism is affected by environmental factors, such as supply of inorganic nitrogen. Gupta et al. (2013) demonstrated that the form of available nitrogen impacts defense responses and resistance to bacterial infection in tobacco. Besides genetic approaches to manipulate relevant genes, the identified substrate metabolites can serve as plant protective agents.

Besides the practical use, the characterized genes offer a perspective of the evolution of plant defenses. One excellent example is TD (Gonzales-Vigil et al. 2011). Many plant species have a single TD gene, while tomato and closely related solanaceous plants contain two TD genes. TD1 is essential for isoleucine synthesis, and defects in this gene impairs plant growth and development. TD2 is 51% identical to TD1. Silencing of TD2 on the other hand did not display any obvious morphological or developmental abnormalities in tomato. Rather the duplicated TD2 adopts a defensive role against insect herbivores related to threonine catabolism. Compared with TD1, TD2 is more resistant to

proteolysis and high temperature, and highly expressed in reproductive tissues. This shows that gene duplication of TD is important in the evolution of plant defenses.

Powdery mildew resistance in natural Arabidopsis accessions

Natural variation in powdery mildew resistance phenotypes has been explored in hundreds of Arabidopsis accessions collected worldwide (Adam and Somerville 1996; Adam et al. 1999). The efforts culminated in the isolation of a phenomenal *R*-gene *RESISTANCE TO POWDERY MILDEW 8* (*RPW8*) from the accession Ms-0, which is structurally atypical and confers broad-spectrum resistance. Later in depth analysis of the genetic basis underlying natural resistance revealed that resistance is either based on *RPW8* (found in nine accessions to date), or of polygenic origin (Willson et al. 2001; Schiff et al. 2001; Göllner et al. 2008). In chapter 4, we observed that four Arabidopsis accessions carry a single resistance locus, and the remaining 15 accessions have polygenic resistance to powdery mildew *On*. Polygenic resistance can be the result of multiple genes all required for resistance, or by a combination of single genes each sufficient to confer resistance. In C24-W, two QTLs were detected, and the recessive one encodes a mutated EDR1 (enhanced disease resistance1) protein. The lack of typical NB-LRR *R*-genes in powdery mildew resistance in Arabidopsis is in contrast to the situation in resistance to bacterial pathogen *Pseudomonas syringae* or downy mildew *Hyaloperonospora parasitica* (Stahl et al. 1999; Mauricio et al. 2003; Rose et al. 2004). Three explanations have been proposed (Schulze-Lefert and Vogel 2000; Micali et al. 2008; Göllner et al. 2008). First, the interaction of Arabidopsis-powdery mildew is young, so the classical *Avr*/*R* gene pairs have not had enough time to evolve. Second, Arabidopsis is not the primary host for powdery mildew. To infect Arabidopsis plants, the inoculum dosage for Arabidopsis was almost 10-fold higher than that for tomato in our disease assay. Third, *RPW8* conveys resistance to several powdery mildew isolates, likely its presence eliminates the evolutionary driving force for acquisition of isolate-specific resistance.

Loss of function *edr1* allele confers broad-spectrum resistance

Accession C24 is exceptional owing to its excellent performance under biotic and abiotic conditions. This accession conveys resistance to four powdery mildew species that colonize Arabidopsis (Göllner et al. 2008; chapter 4), NBS-LRR-type based resistance to cucumber mosaic virus (Takahashi et al. 2002), and isolate-non-specific resistance to downy mildew and dominant resistance against bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (Lapin et al. 2012). C24 is also tolerant to drought and submergence (Bechtold et al. 2010; Vashisht et al. 2011). In chapter 4, we aimed to isolate a novel recessively inherited resistance locus, because *S*-genes are more likely to confer broad-spectrum resistance compared with NB-LRR *R*-proteins. Eventually a natural mutation in *EDR1* was uncovered from C24-W by map-based cloning.

The *edr1* mutant was originally identified in a screen searching for resistance to bacterial pathogen *Pseudomonas syringae,* and this mutant also exhibited resistance to powdery mildew *G. cichoracearum* (Frye and Innes 1998). Premature stop codons in both *edr1* mutant (Frye and Innes 1998) and C24 carrying *edr1* mutation (chapter 4) occurred at the N-terminal region, causing the elimination of C-terminal kinase domain.

EDR1 encodes a protein kinase with similarity to CTR1 (Constitutive Triple Response), a negative regulator of ethylene pathway (Frye et al. 2001). Amino acid sequence analysis of the protein kinase catalytic domain shows that Arabidopsis MAPKKKs (Mitogen-Associated Protein Kinase Kinase Kinases) fall into two large families: MAPKKK-like kinases and Raf-like kinases. CTR1 and EDR1 belong to the Raf-like group. Different from members in MAPKKK-like group, CTR1 and EDR1 are devoid of biochemical and genetic evidence to support that they function upstream of MAPKKs in plant MAPK cascades. Recent studies dismiss CTR1 as a MAPKKK as it directly phosphorylates EIN2, an endoplasmic reticulum (ER) membrance-localized Nramp (Natural Resistance-Associated Macrophage Protein) homolog that positively regulates ethylene responses (Ju et al. 2012). Tang and Innes (2002) also suggested that it was inappropriate to assume that EDR1 functions as a MAPKK kinase, although the kinase activity has been substantiated for EDR1.

Efforts have been undertaken to understand the function of EDR1 and its role in pathogen resistance. In 2008, Wawrzynska et al. identified a mis-sense mutation in the *KEEP ON GOING* (*KEG*) gene that suppresses *edr1*-mediated resistance. *KEG* encodes a RING finger E3-ubiquitin ligase, which was shown to negatively regulate protein levels of the transcription factor ABSCISIC ACID-INSENSITIVE (ABI5) (Stone et al. 2006). This established a link between ABA signaling and *edr1*-mediated resistance. In 2011, Gu and Innes further showed that KEG may interact directly with EDR1 and recruit EDR1 to the trans-Golgi network/early endosome vesicles, suggesting that EDR1 may be involved in vesicle trafficking. In 2012, Pan et al. identified a mutation in *HPR1* that suppresses *edr1*-mediated resistance. Arabidopsis *HPR1* is a homolog of human HPR1, which is a component of the THO/TREX complex. This complex functions in mRNA processing and export, thus it likely mediates the nuclear/cytoplasmic trafficking of the mRNA of genes that play general roles in defense responses. In 2013, Wawrzynska et al. described an enhancer of *edr1* mutant, which encodes *POWDERY MILDEW RESISTANT 4* (*PMR4*). Loss of both *EDR1* and *PMR4* functions has a synergistic effect on both SA and JA signaling pathways, supporting the role of EDR1 in negatively regulating SA and JA production or signalling. It is clear that complex signalling pathways underpin *edr1* phenotypes. The identification of a natural mutation in *EDR1* in a different genetic background than the previously induced *edr1* mutant may provide an additional dimension for unravelling the signalling network for *edr1*-mediated broad-spectrum disease resistance.

Disabling of *S*-genes to achieve disease resistance

As suggested in chapter 2, disabling of *S*-genes is a strategy to achieve non-host like resistance. We have shown that suppression of *Dmr1* and *Pmr4* orthologs in tomato results in resistance to *On* (Huibers et al. 2013). The conserved role of *mlo* has been demonstrated in barley, Arabidopsis, tomato, pepper and pea (Zheng et al. 2012). *EDR1* can be considered as a *S*-gene because its loss-of function mutation gives rise to resistance. In chapter 4, we explored whether EDR1 was a good target for powdery mildew resistance in tomato. The results showed that silencing of two putative *EDR1* homologs individually did not result in less fungal growth compared to the susceptible Moneymaker. Although explicitly excluding EDR1 as a good target requires more evidence, it appears that EDR1 function is less conserved in tomato. Another strategy to manipulate EDR1 for disease resistance is to overexpress its kinase-deficient form of the

gene. Tang and Innes (2002) demonstrated that overexpression of a kinase-deficient full-length *EDR1* gene in wild-type Arabidopsis plants promotes resistance to powdery mildew. It would be of considerable interest to test whether this strategy can result in powdery mildew resistance in tomato and other crops. However, the condition for this strategy is that other crops species have functional *EDR1* orthologs.

A major hurdle for application of *S*-genes in breeding is that loss of *S*-genes is often accompanied by pleiotropic effects. For example, the *dmr1* Arabidopsis mutant is smaller in size, and disruption of *SlDMR1* in tomato resulted in resistance at the expense of plant growth (van Damme et al. 2009; Huibers et al. 2013). This can be tempered by use of weak alleles of *S*-genes. Techniques, such as TILLING (targeting induced local lesions in genomes) (McCallum et al. 2000), ZFN (Zinc Figure Nuclease) (Urnov et al. 2010), TALENs (transcription activator-like effetor nucleases) (Wood et al 2011) and Crispr-Cas9 (Jiang et al. 2013) may allow for finding such alleles of *S*-genes that show partial loss of function and cause mild pleiotropy. Similarly, natural diversity of *S*-genes can be exploited (Hückelhoven et al 2013). At present, naturally occurring variation of susceptibility is demonstrated for *mlo* and *ACD6* genes (Piffanelli et al. 2004; Todesco et al. 2010). Therefore it is important to address the natural diversity of *S*-genes by candidate gene sequencing, together with phenotyping data, targeted breeding and stacking of weak *S*-alleles can be realized.

Induced powdery mildew resistance in Arabidopsis mutants

The wide use of mutants has aided in elucidating many important aspects of resistance to powdery mildew. Non-host resistance mechanism is largely unravelled using the *PEN* knock-out mutants as well as mutants defective in defense components. The importance of susceptibility factors in powdery mildew pathogenesis is registered using *pmr*, *edr* and *mlo* knock-out mutants. Complementary to knock-out mutants, activation tag lines can also serve as useful sources to identify genetic components involved in resistance. Compared with knock-out mutants, this collection has several advantages. First, use of four 35S enhancers in tandem considerably affects genes which are temporarily expressed or show tissue-specific expression patterns. An example is *ATHB13* (chapter 5), which was not expressed in developing leaves (Hanson et al. 2002). It was expected that this gene was not responsive to pathogen invasion in wild-type plants, and microarray analysis revealed that expression of *ATHB13* remained unchanged at three days post inoculation. Therefore its function concerning resistance would not be uncovered in the loss-of-function mutant. On the contrary, the tagging construct contains four 35S enhancers and drives *ATHB13* expression ubiquitously (chapter 5). Without screening of an activation tag population, this gene would not have been found to confer broad-spectrum resistance. Second, gain-of-function mutants behave dominantly, so both heterozygous and homozygous state of insertion can cause phenotypes. While in loss-of-function mutants, heterozygous genotypes do not result in the phenotype of interest, reducing the chance of identification of relevant genes (O'Malley and Ecker 2010). Other merits of activation tag mutants include easy isolation of candidate genes technically, and interrogation of the function of redundant genes and genes whose disruption is lethal to the plant.

Overexpression of a HD-Zip transcription factor *ATHB13* results in resistance

Depending on the invaders imposed on the plants, genes assuming divergent roles have been identified using activation tag populations, ranging from a DNA-binding protein, an aspartic protease, a NB-LRR gene, a TIR-NB-LRR gene to an unknown gene (Yadeta et al. 2011; Xia et al. 2004; Grant et al. 2011; Aboul-Soud et al. 2009; Chen et al. 2013). In chapter 5, we screened an activation tag collection and identified one mutant, 3221, which not only exhibits resistance to powdery mildew *On* but also to downy mildew *H. arabidopsidis* and aphid *M. persicae*. In 3221, constitutive expression of *ATHB13* driven by four 35S enhancers causes the resistant phenotype. *ATHB13* encodes a HD-Zip transcriptional factor characterized by a DNA-binding homeodomain and an adjacent leucine zipper motif. Modulating the expression of a transcription factor can alter disease resistance by activating downstream target genes. The downstream target genes should meet the following criteria: (1) altered level of transcripts in the transgenic plants under control conditions; (2) existence of the core motif that the transcription factor can bind to; (3) responsive to external stimuli. For *ATHB13*, relevant information is lacking such as whether it functions as an activator or a repressor and what its binding preference is. This prevents a proper analysis of the transcript data obtained using the microarray (chapter 5). Despite that the downstream target genes are hard to be identified, we could still infer that overexpressed *ATHB13* did not activate response pathways that are dependent on signaling molecules such as SA, JA and ET, because marker genes for these pathways were not induced in 3221 except the moderately increaed expression of *PR1* (chapter 5). The constitutive activation of defense-related hormone pathways is commonly found for loss-of-function and gain-of-function mutants. In this way, a suite of native stress-inducible genes are up-regulated and contributing to the plant's ability to overcome disease, such as *PR1* and *PR2*, which were induced approximately 200-fold in one activation tag mutant (Xia et al. 2004). These mutants are usually associated with growth penalties. 3221 mutant also displayed altered morphology, and we suggested this was due to the high expression of *ATHB13* in leaves where it is not normally expressed. In 3221, overexpression of *ATHB13* caused altered expression for a large array of transcription factors. Cross-coupling of transcription factors was suggested to be involved in regulation of gene expression during the plant defense response (Rushton and Somssich 1998). Possibly *ATHB13* regulates gene expression synergistically with transcription factors that bind to the elements of defense-related genes. Determination of the binding site of *ATHB13* is crucial to examine this scenario and elucidate the molecular mechanism underlying *ATHB13*-overexpresser induced resistance.

Overexpression of transcription factors for resistance to biotic and abiotic stresses

Transcription factors are ideal targets for engineering enhanced disease resistance, because many of them have been found to play a role in the conserved pathogen response pathways in multiple plant species. Particularly, members of the ERF (Ethylene-Response Factor) subfamily of the AP2/EREBP family, when overexpressed, provided resistance to multiple unrelated pathogens (reviewed in Century et al. 2008). For example, *Pti4*, *Pti5* and *Pti6* were identified as interactors with the tomato disease resistance protein Pto in yeast 2-hybrid assays. Overexpression of *Pti5* in tomato promotes disease resistance to bacterial pathogen *P. syringae* pv. *tomato* (He et al. 2001). When these three genes were expressed in Arabidopsis, a wide array of defensive

marker genes were up-regulated, and plants displayed increased resistance to bacterial pathogen *P. syringae* pv. tomato and powdery mildew *G. orontii* (Gu et al. 2002). In addition, other transcription factor families, including WRKYs, bZIPs, and MYBs, are strongly implicated in resistance (Singh et al 2000; Gurr and Rushton 2005; Rushton and Somssich 1998). They serve as potential sources for broad-spectrum disease resistance.

Some transcription factors have been shown to confer both disease resistance and osmotic stress tolerance when overexpressed. Examples are *OPBP1*, *CaPF1*, *CaERFLP1*, *HvRAF*, *TaERF1*, and *ATHB13* (reviewed in Century et al. 2008; Cabello et. 2012; Cabello and Chan 2012). Manipulation of these genes involved in cross-talk may help develop plants with enhanced resistance and tolerance to biotic and abiotic stresses. The ortholog of *ATHB13* in sunflower was shown to confer tolerance to cold, drought and salinity stresses (Cabello et., 2012; Cabello and Chan 2012). In tomato, Solyc05g007180 showed 71% similarity to ATHB13 at the amino acid level. This gene is known as *LeJa1* (*Jasmonic acid 1*) and was shown to activate threonine deaminase in tomato (Eliezer Lifschitz and Limor Broday, pers. comm.). It is worthwhile investigating whether overexpression of *Solyc05g007180* can cause resistance to powdery mildew and tolerance to abiotic stresses in tomato. An alternative approach is to express *ATHB*13 in tomato either under 35S promoter or inducible promoter as shown for *ADR1*. *ADR1* was identified in an activation tag mutant population for resistance to downy mildew and powdery mildew pathogens, and the authors demonstrated that transient *ADR1* gene expression induced by chemical cues can also establish disease resistance in the absence of seed yield decrease in Arabidopsis (Grant et al. 2011).

Conclusion

This study investigated the factors that influence or determine the outcome of interaction between powdery mildew *On* and two hosts, tomato and Arabidopsis. In tomato, we focused on *Ol-1*, which confers race-non-specific resistance. We showed that *Ol-1*-mediated resistance requires ALS activity specifically through an unkown mechanism. In Arabidopsis, we first screened natural accessions and isolated a recessively inherited gene from accession C24-W This gene was shown to be a natural mutant allele of the *EDR1* gene. Then we screened the activation tag mutant collection, and identified one mutant in which a HD-Zip transcription factor *ATHB13* was constitutively expressed. The next step is to transfer the knowledge obtained in Arabidopsis to tomato and other crops for enhanced resistance to powdery mildew and other pathogens. At present, in the list of genes conferring resistance to powdery mildew *O. neolycopersici* (Figure 2), there are *S*-genes (*SlDMR1, Ol-2*, *SlPMR4, EDR1), Ol-1* which likely incorporates amino acid metabolic genes in its resistance pathway, NB-LRR genes (*Ol-4* and *Ol-6*), a transcription factor *ATHB13*, and two QTL and two other dominant *Ol* genes with unknown identities. Utilization of these genes individually or in combination can minimize the loss caused by *O. neolycopersici*, furthermore in the future cloning and identfication of downstream signalling components of these genes allows for a better understanding of the plant and pathogen interactions.

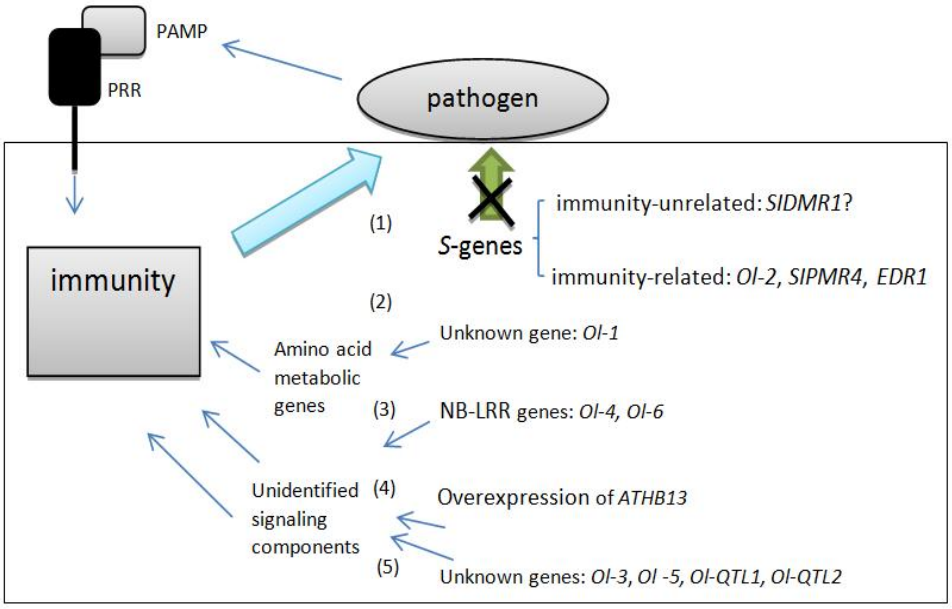


Figure 2 Overview of powdery mildew resistance system with reference to the identified genes conferring resistance to *On*. The perception of PAMP (pathogen-associated molecular patterns) by PRR (pattern-recognition receptors) triggers basal resistance. Resistance level is reinforced through several mechanisms: (1) *S*-genes are supposed to foster susceptibilty (green arrow), and impairment of their functions can cause resistance. When *S*-genes serve to the demands of the pathogen, they are considered immunity-unrelated. When *S*-genes exert negative control of defense pathways, they are considered immunity-related. (2) *Ol*-1-mediated resistance is somehow related to the amino acid metabolic pathway, as indicated by the compromised resistance of NIL-*Ol-1* when both *ALS1* and *ALS2* gnes are silenced. (3) *Ol-4* and *Ol-6* were shown to be homologues of *Mi-1*, a NB-LRR gene. (4) Overexpression of a HD-Zip transcription factor *ATHB13* causes resistance. For genes in groups 3 and 4, their downstream signalling components have not been elucidated. (5) For genes in this group, their identities and downstream signalling components have not been dissected.

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pathway. Plant Cell 13(2):337-350.

Summary

Many plant species are hosts of powdery mildew fungi, including Arabidopsis and economically important crops such as wheat, barley and tomato. Resistance has been explored using induced mutagenesis and natural variation in the plant species. The isolated genes encompass loss-of-function susceptibility genes and dominantly inherited genes encoding NB-LRR proteins, receptor-like kinases or proteins that do not have typical resistance protein domains. Cultivated tomato is susceptible to powdery mildew species *Oidium neolycopersici*, and exploiting the resistance genes present in wild tomato species is a favourable strategy to control the disease. In **chapter 2**, we give an overview of all the identified resistance genes in wild tomato species and their resistance mechanisms inferred from cytological and molecular data. Furthermore, resistance genes and their mechanisms are compared between tomato and other plant species, such as dicot Arabidopsis and monocots barley and wheat. This comparison illustrates that both common and species-specific mechanisms are involved with respect to resistance to powdery mildews in different plant species.

Resistance gene *Ol-1* originates from wild tomato species *S. habrochaites*. It confers race-non-specific resistance to tomato powdery mildew. To elucidate the resistance signalling pathway, we adopted a virus induced gene silencing (VIGS) approach to suppress genes which are differentially expressed when comparing genotypes with and without the *Ol-1* introgression. In **chapter 3**, we showed that ALS (acetolactate synthase) activity is important for *Ol-1*-mediated resistance, as simultaneous silencing of two *ALS* genes attenuated the resistance level of NIL-*Ol-1*. ALS is a key enzyme in the biosynthesis of branched-chain amino acids, and a target of commercial herbicides. Reducing ALS activity via herbicidal treatment did not result in altered responses to powdery mildew infection in susceptible cultivar Moneymaker and resistant line NIL-*Ol-4*, indicating that ALS is not involved in basal defense nor in NB-LRR gene-mediated resistance. Whether the role of ALS in *Ol-1*-mediated resistance is associated with amino acid homeostasis is unknown and needs further investigation.

Besides tomato, Arabidopsis is a host of powdery mildew *O. neolycopersici*. The large collection of Arabidopsis accessions and several mutant collections are valuable resources to identify novel resistance genes. In **chapter 4**, we first screened 123 Arabidopsis accessions for *O. neolycopersici* resistance and then studied the genetic basis of the resistance by segregation analysis in 19 F_2 populations. The results showed that polygenic resistance is the main form of resistance. Accession C24 displays complete resistance with polygenic nature, as shown by QTL analysis of the F2 population derived from the cross between C24 and susceptible accession Sha. The recessively inherited locus on chromosome 1 was fine-mapped by recombinant screening, and analysis of candidate genes resulted in the isolation of the gene conferring resistance. It proved to be a mutant allele of *EDR1*, harbouring a deletion upstream of the kinase domain resulting in a truncated protein. Previously, an induced *edr1* mutation in Col-0 background was obtained. However, the *edr1* mutation in our C24 source (referred to as C24-W) occurred in a different position. The resistance conferred by *edr1* in C24-W was not associated with constitutively expressed pathogenesis-related genes. Remarkably, we observed that although C24-W carried the *edr1* mutation this mutation was absent in other C24 sources. In addition, C24-W was smaller in size than C24 from other sources.

Since the *edr1* mutation confers resistance to tomato powdery mildew in Arabidopsis, we investigated whether this resistance system is conserved in tomato. The results showed that individual silencing of two tomato *EDR1* candidate genes in susceptible cultivar Moneymaker did not result in decreased sporulation of tomato powdery mildew.

In **chapter 5**, we screened an activation tag Arabidopsis mutant collection. In these mutants, tagged genes are overexpressed by the strong 35S enhancers resulting in a dominant gain-of-function phenotype. One mutant line, 3221, was identified due to its resistance to powdery mildew *O. neolycopersici*. Additional disease tests showed that 3221 displayed resistance to the downy mildew *Hyaloperonospora arabidopsidis* and the aphid *Myzus persicae*, but susceptibility to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000. The mutant line 3221 also showed reduced size and serrated leaves, and the altered morphology was associated with resistance. Inverse PCR and expression analysis revealed that the gene underlying the resistance was *ATHB13*, a HD-Zip transcription factor. Suppression of *ATHB13* in 3221 by RNAi transformation resulted in the loss of resistance and altered morphology, while overexpression of *ATHB13* in wild-type plants induced resistance and altered morphology. Microarray analysis of 3221 and the parental line Ws resulted in the identification of a large number of genes showing differential expression. Analysis of these results did not give a clear indication that the resistance phenotype in 3221 is due to the activation of classical hormone pathway genes involved in resistance. The possibility of utilizing *ATHB13* for engineering pathogen resistance in tomato needs to be investigated in the future.

Finally, in **chapter 6** the results from the previous chapters are discussed in a broader context.

Samenvatting

Echte meeldauw is een pathogene schimmel die kan groeien op vele plantensoorten, waaronder Arabidopsis en economisch belangrijke gewassen zoals tarwe, gerst en tomaat. Resistentie tegen echte meeldauw kan verkregen worden door mutanten te induceren of door gebruik te maken van natuurlijke variatie in de plantensoorten. De geïsoleerde genen kunnen gegroepeerd worden in twee klassen: gemuteerde susceptibiliteitsgenen die hun functie hebben verloren, en dominant verervende resistentiegenen, coderend voor onder meer NB-LRR eiwitten, receptor-achtige kinases of eiwitten zonder karakteristieke resistentiegen-domeinen. De gecultiveerde tomaat (*Solanum lycopersicum*) is vatbaar voor de echte meeldauwsoort *Oidium neolycopersici*, en een favoriete strategie om de ziekte in te dammen is gebruik te maken van resistentiegenen aanwezig in wilde tomaatsoorten. **Hoofdstuk 2** bevat een overzicht van alle geïdentificeerde resistentiegenen tegen echte meeldauw in wilde tomaatsoorten, en de bijhorende resistentiemechanismen afgeleid uit cytologische en moleculaire data. Verder worden de resistentiegenen van tomaat en hun mechanismen vergeleken met die van andere plantensoorten, zoals de dicotiele soort Arabidopsis en de monocotiele soorten gerst en tarwe. Deze vergelijking laat zien dat zowel gemeenschappelijke als soort-specifieke mechanismen een rol spelen in resistentie tegen echte meeldauwsoorten in verschillende plantensoorten.

Resistentiegen *Ol-1* is afkomstig van de wilde tomaatsoort *Solanum habrochaites*. Dit gen geeft niet-isolaat-specifieke resistentie tegen *O. neolycopersici*. Als eerste stap om het resistentiemechanisme van *Ol-1* te ontrafelen is eerder onderzocht welke genen differentieel tot expressie komen wanneer genotypen met en zonder de *Ol-1* introgressie met elkaar vergeleken worden. Vervolgens hebben we een "virus-geïnduceerde gen uitschakeling" (VIGS) methode toegepast om de activiteit van enkele geselecteerde genen uit te schakelen of te verminderen. In **hoofdstuk 3** laten we zien dat ALS (acetolactaatsynthase) activiteit nodig is voor resistentie door het *Ol-1* gen, omdat gelijktijdige vermindering van expressie van twee *ALS* genen het resistentieniveau van een *Ol-1* bevattende NIL (bijna-isogene lijn) verlaagt. ALS is een sleutelenzym in de biosynthese van vertakte aminozuren, en een doelwit van commerciële herbiciden. Verlaging van ALS activiteit door behandeling met herbicide resulteerde niet in een veranderde respons op echte meeldauwinfectie in de vatbare cultivar Moneymaker, noch in de resistente lijn NIL-*Ol-4* die een NB-LRR type resistentiegen tegen echte meeldauw bevat. Dit duidt erop dat ALS niet betrokken is bij basale afweer, noch bij resistentie afkomstig van een NB-LRR gen. Het is nog niet bekend of de rol van ALS in *Ol-1* afhankelijke resistentie geassocieerd is met een veranderde balans van aminozuurgehaltes. Dit vereist verder onderzoek.

Naast tomaat kan ook Arabidopsis geïnfecteerd worden door *O. neolycopersici*. De grote collectie van Arabidopsis accessies en verschillende mutantencollecties zijn waardevolle bronnen om nieuwe resistentiegenen te identificeren. In **hoofdstuk 4** hebben we 123 Arabidopsis accessies getest op resistentie tegen *O. neolycopersici*. Daarna hebben we de genetische basis van resistentie van 19 accessies bestudeerd door analyse van de uitsplitsing in F_2 populaties. Hieruit bleek dat de resistentie in de meeste gevallen polygeen was. Accessie C24 vertoonde complete resistentie. Deze was polygeen, zoals bleek uit QTL (kwantitatieve eigenschap locus) analyse van een F2 populatie

verkregen na kruising van C24 met vatbare accessie Sha. Er werden twee QTLs gevonden, waarvan QTL1 op chromosoom 1 een recessieve overerving liet zien. QTL1 werd fijngekarteerd door analyse van recombinanten. De kandidaatgenen in de verkregen regio werden geanalyseerd, en uiteindelijk werd het gen verantwoordelijk voor resistentie geïsoleerd. Dit bleek een mutant allel van het *EDR1* (*Enhanced Disease Resistance 1*) gen te zijn, omdat het een deletie van twee nucleotiden stroomopwaarts van het kinasedomein bevat, resulterend in een voortijdig stopcodon, en een verkort eiwit. Er is eerder al een *edr1* mutant in een Col-0 achtergrond beschreven. De *edr1* mutatie in onze C24 bron (C24-W genoemd) bevindt zich echter op een andere positie. De resistentie verleend door *edr1* in C24-W was niet geassocieerd met constitutief tot expressie komende pathogenese-gerelateerde genen (*PR* genen). Een opmerkelijke waarneming was dat de *edr1* mutatie waargenomen in C24-W niet aanwezig was in andere C24 bronnen. Bovendien waren C24-W planten kleiner dan C24 planten van andere bronnen. Omdat de *edr1* mutatie in Arabidopsis resulteerde in resistentie tegen echte meeldauw van tomaat (*O. neolycopersici*) hebben we onderzocht of dit resistentiemechanisme ook in tomaat aanwezig was. Op basis van homologie bleken er twee kandidaatgenen voor EDR1 aanwezig te zijn in tomaat. Verminderde genexpressie van elk van deze genen afzonderlijk in het vatbare tomatenras Moneymaker leidde echter niet tot verminderde infectie door *O. neolycopersici*.

In **hoofdstuk 5** hebben we een Arabidopsis mutantencollectie met inserties van een activeringsconstruct geanalyseerd. In deze mutanten wordt de expressie van genen in de buurt van een insertie geactiveerd door 35S versterkers. Deze overexpressie kan resulteren in nieuwe fenotypen die dominant overerven. Mutant 3221 bleek resistent te zijn tegen echte meeldauw *O. neolycopersici*. Uit additionele ziektetoetsen bleek dat deze mutant ook resistent was tegen valse meeldauw *Hyaloperonospora arabidopsidis* en tegen de luis *Myzus persicae*, maar niet tegen de bacteriële ziekteverwekker *Pseudomonas syringae* pv *tomato* DC3000. Mutant 3221 planten waren kleiner dan niet-gemuteerde planten met dezelfde genetische achtergrond (wild type), en de bladeren waren meer ingesneden dan die van het wild type. Deze veranderde morfologie was geassocieerd met resistentie. Inverse PCR en expressie-analyse toonden aan dat het gen verantwoordelijk voor de resistentie *ATHB13* was, een HD-ZIP transcriptiefactor. Vermindering van *ATHB13* expressie in mutant 3221 door RNAi transformatie resulteerde in verlies van zowel resistentie als veranderde morfologie, terwijl overexpressie van *ATHB13* in wild-type planten leidde tot resistentie en veranderde morfologie. Een microarray analyse van mutant 3221 en de wild-type accessie Ws resulteerde in de identificatie van een groot aantal genen die differentieel tot expressie komen. Bestudering van deze lijst van genen gaf geen duidelijke aanwijzing dat de resistentie in mutant 3221 afhankelijk is van de activering van klassieke hormoon-geïnduceerde resistentiemechanismen. De mogelijke toepassing van *ATHB13* overexpressie om resistentie tegen verschillende pathogenen in tomaat te verkrijgen moet in de toekomst onderzocht worden.

Tenslotte worden in **hoofdstuk 6** de resultaten van de eerdere hoofdstukken bediscussieerd in een ruimere context.

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About the author

Dongli Gao was born on 6th December, 1983 in Shanxi province, China. She started her BSc study in the field of Agronomy at Northwest A&F University. She then obtained a master degree in plant cultivation and faming system at Northwest A&F University. Starting from September 1st, 2009, she received PhD training in the Laboratory of Plant Breeding at Wageningen University.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Dongli Gao**
Date: **18 June 2014**
Group: **Plant Breeding, Wageningen University&Research Center**

1) Start-up phase	*date*
► **First presentation of your project**	
Genes required for resistance and susceptibility to tomato powdery mildew	Jan 26, 2010
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Genetics and molecular mechanisms of resistance to powdery mildews in tomato (Solanum lycopersicum) and its wild relatives, Eur. J. Plant Pathol. 138 (2014), pp 641-665	Nov 2013
► **MSc courses**	
Plant-Microbe Interactions (PHP - 30306)	Mar 2010
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Amsterdam	Nov 03, 2012
EPS PhD student day, Leiden	Nov 29, 2013
► **EPS theme symposia**	
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Utrecht	Jan 23, 2009
EPS Theme 4 symposium 'Genome Plasticity", Wageningen	Dec 10, 2010
EPS Theme 2 meeting 'Interactions between Plants and Biotic Agents, Wageningen	Feb 10, 2011
► **NWO Lunteren days and other National Platforms**	
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: breeding for quality	Mar 08, 2011
Invited seminar Scott Nichols: 'origin and ancestry of cell adhesion and communication in animals'	Jan 19, 2012
Invited seminar Graham Seymour: 'the tomato genome: from genes to QTL and networks'	Jan 24, 2012
Invited seminar Paul Fraser: 'Metabolic engineering of high-value industrial and nutritional isoprenoids in plants'	Feb 16, 2012
PBR research Day: next generation sequencing-what is in it for me	Feb 28, 2012
Invited seminar Cornlia Spetea Wiklund: 'lessons from photosynthetic analyses in three widely used Arabidopsis ecotypes'	Feb 21, 2012
Invited seminar Inez Hortenze Slamet-Loedin: 'genetic modification for iron biofortification and drought tolerance in rice'	Jun 29, 2012
Plant breeding 100 year symposia	Nov 12-14, 2012
Invited seminar Hans de Kroon: 'Mechanisms and consequences of belowground interactions between grassland species'	Dec 11, 2012

	Invited seminar Niels Anten:'Tragedies and cooperation in plant communities: the aboveground perspective'	Dec 11, 2012
	Invited semianr Howard S. Judelson: 'Molecular Insights into Spore Biology and Metabolism of Phytophthora infestans, the Potato Blight Pathogen'	May 07, 2013
	Invited seminar Rays H.Y. Jiang: 'Integrative genomics of destructive pathogens from oomycetes to malaria parasites'	May 07, 2013
	Invited seminar Brian Staskawicz: 'Effector-Targeted Breeding for Durable Disease Control of Xanthomonas diseases in Tomato and Cassava'	May 21, 2013
	Invited seminar Martin Weih: 'Biomass for bioenergy - The Swedish experience with Salix grown for biomass on agricultural land'	Nov 23, 2012
	Symposium: 'Intraspecific pathogen variation-implications and opportunities'	Jan 22, 2012
	Invited seminar Alain tissier: 'Glandular trichomes of tomato: from terpene biosynthesis to trichome differentiation'	May 03, 2013
	Invited seminars Guusje Bonnema and Arnaud Bovy: 'The role of plant breeding in improving quality of crop plants'	Jun 11, 2013
	Invited seminar Hanhui Kuang: 'Using the Nicotiana-TMV system to study resistance gene evolution and plant genome stability'	Sep 11, 2013
	Invited seminar Stephane Blanc: 'New insights into the relationship between plant viruses and insect vectors'	Sep 18, 2013
	Invited semianr Augusto Franco Garcia: 'Genetic mapping in autopolyploids, with emphasis in sugarcane'	Oct 04, 2013
	Mini-symoposium: how to write a world-class paper	Oct 17, 2013
	Breeding industry and food security in the Netherlands	Nov 09, 2013
	Invited seminar Jiayang Li: 'Understanding the molecular mechanisms underlying rice tillering'	Nov 15, 2013
►	**Seminar plus**	
►	**International symposia and congresses**	
	8th Solanaceae and 2nd Cucurbitaceae Joint Conference, Japan	Nov 28-Dec 02, 2010
►	**Presentations**	
	Oral: to breeding companies involved in the project	2012
	Poster: Unravelling the involvemnt of WRKY family in the basal defence against powdery mildew in tomato	Nov 01-03, 2010
	Poster: Loss of susceptibility factors as a novel breeding strategy for non-host like resistance	Nov 28, 2010
►	**IAB interview**	Nov 15, 2012
►	**Excursions**	
	YOUNG PSG excursion	Jul 15, 2011
	Subtotal Scientific Exposure	*13.3 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS Autum School - Host-Microbe Interactomics	Nov 01-03, 2010
► **Journal club**	
Participation in literature dicussion group at Plant Breeding, Wageningen UR	2009-2013
► **Individual research training**	
Subtotal In-Depth Studies	*3.9 credits**

4) Personal development	*date*
► **Skill training courses**	
WGS course: PhD Competence Assessments	May 11& Jun 24, 2010
WGS course: Project and Time Management	Jul 13-16, 2010
WGS course: Techniques for Writing and Presenting a Scientific Paper	Sep 04-07, 2012
ExPectionS Day (EPS Career Day)	Feb 01, 2013

► **Organisation of PhD students day, course or conference** ► **Membership of Board, Committee or PhD council**	

Subtotal Personal Development *3.3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.0**
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.*	

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