Characterization of tomato genes for resistance to *Oidium neolycopersici*

Alireza Seifi

Thesis committee

Thesis supervisor

Prof. dr. R.G.F. Visser

Professor of Plant Breeding

Wageningen University

Thesis co-supervisor

Dr. Yuling Bai

Assistant professor, Laboratory of Plant Breeding

Wageningen University

Other members

Prof. dr. A.H.J. Bisseling, Wageningen University

Prof. dr.ir. J. Bakker, Wageningen University

Prof. dr. B.J. Zwaan, Wageningen University

Dr. A.F.J.M. van den Ackerveken, Utrecht University

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Thesis

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Alireza Seifi

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Contents

Chapter 1

General Introduction

We assign the label ‘plant pathogen’ to all living organisms which threaten our crop production. Plant pathogens include all kinds of fungi, bacteria, oomycets, and viruses. ‘Pest’ is used for insects and small animals. Plants are equipped with different defense mechanisms to cope with their pathogens and pests. The ultimate output of these defense mechanisms is that only those organisms which are able to pass these defense layers are pathogenic.

Plant defense mechanisms against pathogens and pests

Some of the plant features, like architecture, morphology, chemical and biochemical contents, growth habit, etc., can decrease the plant vulnerability to attacking pathogens. Since these features have evolved for other purposes and indirectly enhance plant fitness in response to pathogens, I refer to the resistance due to these characteristics as indirect defense. In addition to indirect defense, a sophisticated innate immune system has evolved in plant cells which is specialized in pathogen recognition and defense responses triggering.

I. Indirect defense

The surface of the plant is the first battlefield between pathogens and plants, therefore, it seems straightforward to have barriers there to prevent pathogen attack. It is believed that hydrophobicity, hardness, chemical components, and topographical properties of the plant surface are important for pre-penetration processes of many fungal pathogens (Mendgen et al., 1996). The cuticle is a good example of the contribution of the plant surface composition to defense. Plant cuticle forms a hydrophobic layer, 0.02–200 micrometer thick, which covers almost all aerial surfaces of land plants and forms the interface between plant and environment (Nawrath, 2006). Cutin is one of the main components of the cuticle (Nawrath, 2006), and therefore, cutin-deficient tomatoes have a thinner layer of cuticle. It has been observed that fruits of cutin-deficient tomato mutants were more susceptible to pathogens like *Botrytis cinera* (Isaacson et al., 2009).

Stomata are microscopic pores on the plant leaf surface for exchanging gas and water. Some pathogens take advantage of these pores to penetrate the plant. Interestingly,

it has been observed that stomata act as a barrier against bacterial infection, partly in tie with the innate immunity system (Melotto et al., 2006).

Plant morphology also contribute to the response to pathogens. It has been shown that there is a high correlation between resistance to yellow mottle virus and plant architecture related traits in rice (Albar et al., 1998). Another example is that some morphological traits were shown to influence the expression of sheath blight resistance genes in rice indirectly (Han et al., 2003).

Secondary metabolites, which are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways, are known for a long time as antimicrobial plant components (Dixon, 2001). For instance, Arabidopsis root exudates contain antimicrobial metabolites and confer resistance to a wide range of bacterial pathogens (Bais et al., 2005). The pre-formed secondary metabolites, referred to as phytoanticipins versus phytoallexins, like pisatin and phaseollin, which are induced upon pathogen infection (VanEtten et al., 1994). For example, α-tomatine in *Solanaceous* plants is a phytoanticipin that has been shown to have antimicrobial function (Morrissey and Osbourn, 1999). The induction of phytoallexins formation upon pathogen infection has been well-documented by visualizing vesicle trafficking during delivery of vesicles which carry flavonoids toward the pathogen infection site (Snyder and Nicholson, 1990). Usually crops contain lower amounts of secondary metabolites compared to their wild relatives. It is believed that during domestication, crops have lost some secondary metabolites and therefore, crops are more vulnerable to pathogens compared to their wild relatives (Wink, 1988).

II. Direct defense: Plant innate immunity

Innate immunity is the defense strategy which relies on a set of specialized receptors so called pathogen- or 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 (Ausubel, 2005).

PAMP- receptors: pathogen-associated molecular patterns (PAMPs) are evolutionary conserved pathogen-derived molecules that distinguish pathogens from their hosts (Ausubel, 2005). Perception of PAMPs by PAMP-receptors in plants results in PAMP-

triggered immunity (PTI) (Jones and Dangl, 2006). For example, FLS2 which is a transmembrane leucine-rich repeat (LRR) receptor kinase, is a well-characterized PAMP-receptor which percepts eubacterial flagellin (Gómez-Gómez and Boller, 2000).

R proteins: these 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 protein family. In Arabidopsis and rice there are 140 and 500 genes, respectively, that are predicted to encode NBS-LRR proteins (Meyers et al., 2003; Zhou et al., 2004). A preliminary analysis suggests that there are around 140 putative genes encoding NBS-LRR proteins in the tomato genome (Fig. 3). R proteins perceive pathogen effectors (directly or indirectly) and thereby, trigger effector-triggered immunity (ETI) (Jones and Dangl, 2006). In the absence of pathogen effector, activation of NBS-LRR proteins is prevented by autoinhibition mediated by the LRR domain. NBS-LRR protein is bound to ADP (adenosine diphosphate) when it is in "Off" state. Effector recognition would lead to intermediated open state where ATP (adenosine triphosphate) replaces ADP and then the protein retains its "On" state, which is the active form. ATP hydrolysis returns the protein to the autoinhibited "Off" state (Takken et al., 2006). Effector recognition by the LRR domain (Takken et al., 2006) or both N- terminus and LRR (Takken and Tameling, 2009) triggers activation of the R protein. If N-terminus and LRR domain are both involved in pathogen recognition, then the NBS domain could be serving as an interaction platform for the downstream signaling components (Takken and Tameling, 2009).

R proteins are specialized in immune response, however there are few exceptions suggesting pleiotropic roles for some R proteins. There are instances of contribution of TIR-NBS-LRR proteins to response to abiotic stresses (Noutoshi et al., 2005) and shade avoidance response (Faigon-Soverna et al., 2006). In the case of CC-NBS-LRR proteins it has been shown that constitutive activation of such an R protein alters morphogenesis through the cytokinin pathway in Arabidopsis (Igari et al., 2008).

❖ Events after pathogen recognition

PTI and ETI trigger similar processes and most probably the main difference is in magnitude of these processes (Tao et al., 2000). Common events in ETI and PTI include

calcium ion influx, oxidative burst, activation of mitogen-activated protein kinases (MAPKs) cascades, reprogramming of gene expression, reinforcing the cell wall at pathogen attempt sites and, often, programmed cell death (PCD) (Dodds and Rathjen, 2010). Hypersensitivity reaction (HR) is a form of apoptosis-like PCD (Hofius et al., 2009) which is the hallmark for ETI (Nimchuk et al., 2003).

Plant hormones, salicylic acid (SA), jasmonic acid (JA), ethylene (ET), auxin, abscisic acid (ABA), and gibberellic acid (GA), cytokinins, and even brassinosteroids contribute to both ETI and PTI significantly (BR) (Bari and Jones, 2009). In general SA is considered to be mostly involved in response to biotrophic pathogens, while JA and ET trigger immunity responses to necrotrophic pathogens (Glazebrook, 2005). The other phytohormones exert their role in immunity response mostly via SA, JA, and ET pathways (Bari and Jones, 2009).

❖ RNA silencing contribution to plant immunity

RNA silencing, or RNA interference (RNAi), was first described as the immune response of animal (Fire et al., 1998) and plant (Hamilton and Baulcombe, 1999) cells to exogenous double-stranded RNAs. Now we know that RNA silencing is a conserved mechanisms in eukaryotes for transcriptional and post-transcriptional regulation of gene expression (Baulcombe, 2004) and for genome defense and stability (Plasterk, 2002; Moazed, 2009). RNA silencing in the model plant, *Arabidopsis thaliana* involves the production of small RNAs (sRNA), 18-24 nucleotide (nt) in size, from a double-stranded RNA (dsRNA) by one of the four dicer-like (DCL) enzymes (Ruiz-Ferrer and Voinnet, 2009). Depending on the precursor, there are two known groups of small RNAs in Arabidopsis, microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Jones-Rhoades et al., 2006). The miRNAs are diced from single strand RNA molecules that include an imperfect stem-loop secondary structure, whereas siRNAs are processed from long, double-stranded RNAs (Ruiz-Ferrer and Voinnet, 2009). Four different types of siRNAs are known in plants: trans-acting siRNAs (ta-siRNAs), natural antisense transcripts-derived siRNAs (nat-siRNAs), heterochromatic siRNAs (hc-siRNAs), and long siRNAs (lsiRNAs) (Katiyar-Agarwal and Jin, 2010). Both miRNAs and siRNAs are incorporated into silencing complexes, wherein they guide repression of target genes

(Jones-Rhoades et al., 2006). Once RNA silencing starts, it can be amplified (Baulcombe, 2007) especially through 22-nt sRNAs (Chen et al., 2010).

There is overwhelming evidence implicating plant RNA silencing pathways in immunity (reviewed in Jin, 2008; Voinnet, 2008; Padmanabhan et al., 2009; Ruiz-Ferrer and Voinnet, 2009; Katiyar-Agarwal and Jin, 2010). There are instances showing that miRNAs (Navarro et al., 2006; Navarro et al., 2008; Li et al., 2010), nat-siRNAs (Katiyar-Agarwal et al., 2006), lsiRNAs (Katiyar-Agarwal et al., 2007), and hc-siRNAs (Pavet et al., 2006; Agorio and Vera, 2007) play a role in Arabidopsis immunity responses. These pathogen-responsive sRNAs induce post-transcriptional gene silencing by guiding mRNA degradation or translational repression, or may guide transcriptional gene silencing by direct DNA methylation or chromatin modification (Ruiz-Ferrer and Voinnet, 2009; Katiyar-Agarwal and Jin, 2010). Surprisingly, it has recently been shown that the plant-derived sRNAs are even able to silence the pathogen's genes inside the pathogen (Nowara et al., 2010).

❖ Plant innate immunity responses fitness trade-off

As mentioned above, a lot of processes and pathways are involved in immunity response. Of course, activation of these processes is costly for the plant cell and it needs to be kept tightly regulated and in balance. A recent discovery in Arabidopsis showed that a single locus, *ACD6*, is one of the genes which increase defense response by compromising growth (Todesco et al., 2010). Hyperactivity of *ACD6* enhances resistance to pathogens but, on the other hand results in decreasing plant biomass (Todesco et al., 2010). In the *RPP5* resistance gene cluster in Arabidopsis, it was shown that sRNAs derived from this gene cluster regulate the transcript level of *SNC1* gene in the same cluster to restrict the fitness cost associated with constitutive expression of these *R* genes (Yi and Richards, 2007). Therefore, fine-tuning of *R* genes expression and activity is also very important, especially in crop breeding for pathogen resistance. It should not be forgotten that a healthy plant needs to keep the immune response in balance. Crop breeding strategies relying on manipulation of the innate immunity system will increase the risk to mess this balance up.

❖ **Crop breeding for resistance to pathogens and pests**

The prevalence of undernourishment in the world in 2010 was estimated as much as 925 millions (Food and Agriculture Organization, 2010). On the other hand, the world population will be around 8.3 billions by 2030 (http://faostat.fao.org). "The challenge is clear. The world must produce 40 percent more food, with limited land and water, using less energy, fertilizer and pesticide by 2030 at the same time as bringing down sharply the level of greenhouse gases emitted globally, and while coping with the impact of climate changes that cannot be avoided. To do so, we must maximize both the use of those technologies already developed and generate and exploit new scientific discoveries. We need a new and greener revolution, a revolution with science and technology at its heart" (Beddington, 2010).

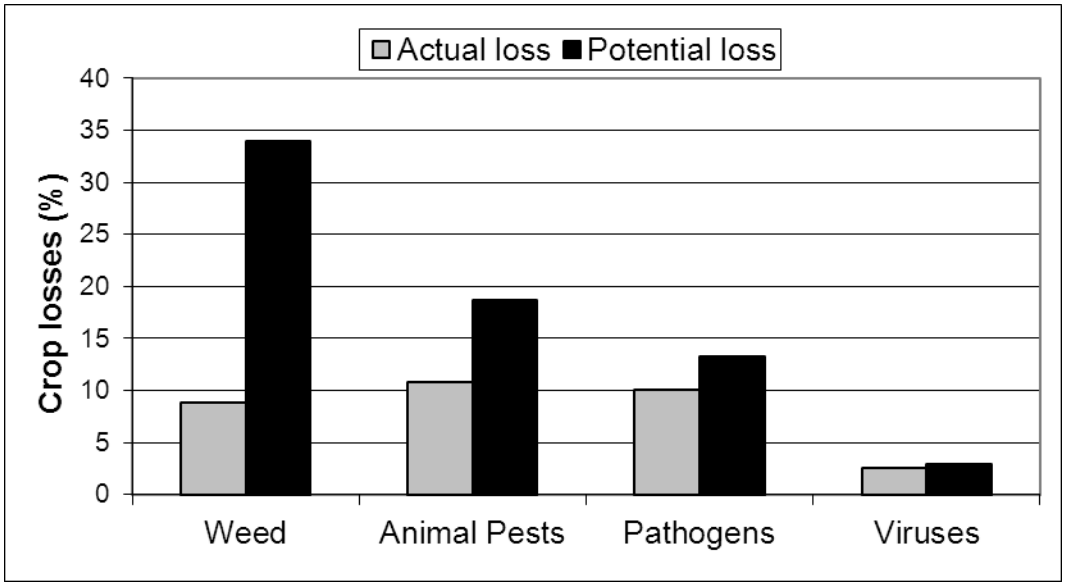

Fig. 1. Worldwide crop production losses due to different pathogen and pest groups in 2001–03. The data represent the average loss in production of 6 major crops (wheat, rice, maize, potatoes, soybean). Animal pests refers to arthropods, nematodes, rodents, birds, slugs and snails. Pathogens refers to fungi and bacteria. Potential loss is the estimated loss if there is no pathogen and pest control applied (adapted from Oerke, 2006).

As demonstrated in Figure 1, pests and pathogens contribute significantly to losses in crop production. Therefore it is clear that efforts towards producing crops with resistances to pests and pathogens are valuable.

As Beddington (2010) suggested, classic crop breeding strategies together with new technologies in genetic engineering and transgenesis are required for a "greener revolution". Classic breeding for resistance to pathogens and pests mostly relies on finding resistance genes in wild relatives of crops and then introgress those genes into crops. It sounds like a straightforward procedure, however, one of the main bottlenecks is that it can take years to deliver the final product, a variety. Introducing molecular biology techniques in the 1980s was the onset of a new and fast-developing era in crop breeding. First, it made it possible to accelerate the selection steps in breeding programs by using molecular markers that are closely-linked to the target trait for selecting the trait. This strategy is called marker-assisted selection. Second, genetic engineering techniques helped to shorten the process of introducing resistance genes to crops. It provided the possibility to clone individual resistance-conferring genes and transform them to crops in order to produce transgenic crops. In this case, genetic linkage drag is not a problem anymore, however, to produce a transgenic line with proper temporal and spatial expression of the resistance gene is the challenge. With transgenic approaches it is possible to introduce genes from other plant species which are not crossable with the target crop or even from other organisms, like bacteria. The classic example is the expression of delta endotoxin proteins derived from *Bacillus thuringiensis* (*Bt*) in the plant which confers resistance to insects (Barton et al., 1987). Since then, *Bt* crops, particularly cotton and corn, have been cultivated widely. In 2008 *Bt* corn and cotton constituted 17% and 18% , respectively, of the cultivated U.S. acreage (Lemaux, 2009).

Tomato -powdery mildew interaction

In this thesis, I will focus on the pathosystem of tomato and tomato powdery mildew to study several tomato resistance genes to this pathogen. Tomato powdery mildew (PM), *Oidium neolycopersici*, is an obligate biotrophic fungus which can parasitize more than 60 species in 13 plant families especially *Solanacea* and *Cucurbitacae* (Jones et al., 2001). In Figure 2, the effect of PM pathogenecity on *S. lycopersicum* cv. Moneymaker (MM) is illustrated.

Tomato resistance to *O. neolycopersici*

Most of the cultivated tomatoes are susceptible to PM, however in wild tomato species several sources of resistance have been identified and introgressed into cultivated tomato (Lindhout et al., 1993). Till now, 9 loci have been identified which confer resistance to PM. *Ol-1* which has been introgressed from *S. habrochites* G1.1560 (van der Beek et al., 1994) and was 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 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* has been introgressed from *S. habrochites* G1.1290 (Huang et al., 2000) and is located on the same chromosomal region as *Ol-1* and there are some evidences suggesting that *Ol-1* and *Ol-3* are probably allelic variants (Bai et al., 2005). *Ol-4* was introgressed from *S. peruvianum* LA2172 and mapped on the short arm of chromosome 6 (Bai et al., 2004). *Ol-5* is introgressed from *S. habrochites* PI247087 and was mapped closely linked to *Ol-1* and *Ol-3* on the long arm of chromosome 6 (Bai et al., 2005). *Ol-6* was found in an advanced breeding line with unknown origin and is mapped in the same position as *Ol-4* (Bai et al., 2005). In addition, three quantitative trait loci (*Ol-qtls*) were identified for PM resistance in *S. neorickii* G1.1601 (Bai et al., 2003). *Ol-qtl1* was mapped on chromosome 6 in a chromosomal region where also *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 conferring resistance to another powdery mildew (*Leveillula taurica*) (Bai et al., 2003). The approximate locations of these *Ol* loci on the tomato chromosomes are shown in Figure 3.

Scope of the thesis

This thesis reports the efforts undertaken to obtain a better understanding of tomato response to *O. neolycopersici*, with the main focus on the dominant *Ol* genes located on tomato chromosome 6. The tomato *Mi-1* gene confers resistance to root-knot nematodes, aphids and whiteflies. This gene is located in a gene cluster (*Mi-1* gene cluster) on the short arm of tomato chromosome 6. Previously we mapped *Ol-4* and *Ol-6* in this cluster.

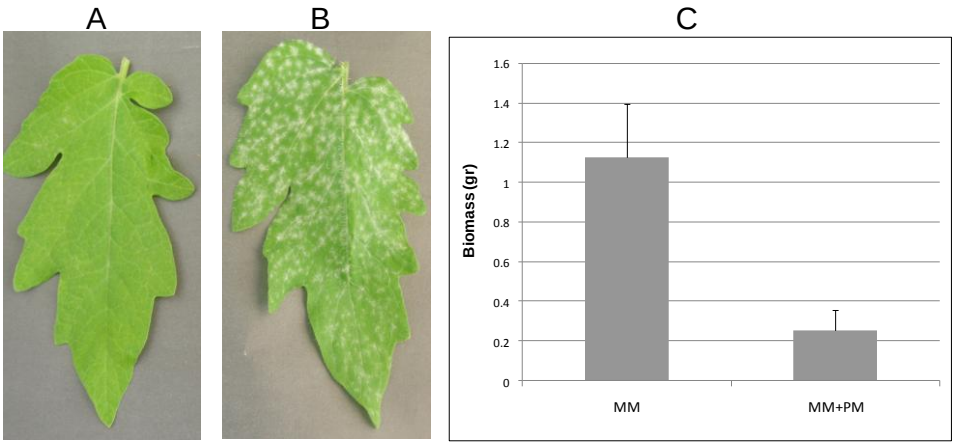

Fig.2. Tomato-powdery mildew interaction. **A**. A leaflet of the resistant tomato near-isogenic line carry the *Ol-4* gene, showing no powdery mildew (PM) sporulation. **B**. Sporulation of PM on a leaflet of the susceptible tomato line, *Solanum lycopersicum* cv. Moneymaker (MM). Pictures were taken two weeks after PM inoculation. **C**. Biomass loss in MM seedlings due to heavy inoculation of PM. The seedlings were heavily inoculated with PM spores 10 days after germination and 10 days later, 20 seedlings were weighted. The average weight of the control (MM) seedlings and inoculated with PM (MM+PM) seedlings is shown here.

In **Chapter 2** we show that near-isogenic lines (NILs) harboring *Ol-4* (NIL-Ol-4) and *Ol-6* (NIL-Ol-6) are also resistant to nematodes and aphids. We also demonstrate that the resistance to both nematodes and tomato powdery mildew in these two NILs is governed by linked (if not the same) *Mi-1* homologues in the *Mi-1* gene cluster.

Chapter 3 is also devoted to the *Mi-1* gene cluster, showing the potential involvement of *transport inhibitor like* (*TIR-like*) genes embedded in this cluster in tomato response to root-knot nematodes. TIR1 is an auxin receptor that plays a pivotal role in auxin signaling. Since auxin is involved in the pathogenicity of tumor-inducing pathogens like pseudomonads and nematodes, the co-localization of *TIR-like* genes with the *Mi-1* gene was intriguing to check if *TIR-like* genes play a role in the resistance conferred by the *Mi-1* gene. We monitored the *TIR-like* transcript abundance (TTA) in both roots and leaves of nematode-resistant and –susceptible tomato lines. TTA was lower in the roots, but not in the leaves, of nematode-resistant plants compared to that in nematode-susceptible plants. In order to check whether the *TIR-like* genes are involved in the *Mi-1* gene resistance pathway, we transiently silenced the *Mi-1* homologues in the nematode-resistant tomato line and measured TTA. Results showed that knocking down

the expression of the *Mi-1* homologues had no effect on *TIR-like* transcription regulation. Our results show that there is an association between TTA and the tomato response to nematodes.

In **Chapter 4** we studied *Ol-1* and *Ol-5,* two closely-linked genes, located on the long arm of chromosome 6. *Ol-1* mediates delayed cell death which is different from HR in the magnitude and in the timing of the response. We fine-mapped the *Ol-1* locus to a 73 Kb interval. Interestingly, our results show that another locus, located in the chromosomal region where the *Ol-5* locus is mapped, is required for *Ol-1*-mediated resistance. Furthermore, delayed cell death associated with *Ol-1*- and *Ol-5*-mediated resistance is regulated by the interaction of both loci. Loss of the *S. habrochaites* allele of the *Ol-5* locus results in abolishing the delayed cell death, while loss of *S. habrochaites Ol-1* allele reduces the effectiveness of delayed cell death probably by disturbing the right timing of this process.

Chapter 5 shows that phytohormone pathways are involved differently in resistance conferred by the *Ol* genes, *Ol-1*, *ol-2*, *Ol-4* and *Ol-qtls*. We investigated the role of SA, ABA, JA, and ET pathways, in the response of these *Ol* gene to PM. We monitored the trend of these phytohormone pathways based on the quantification of the expression of marker genes for these pathways. We also crossed the NILs carring these *Ol* genes with tomato mutants that have altered responses to a subset of these phytohormones. Results suggest that the SA pathway is the main hormone pathway that is recruited HR-based resistance mechanism. Ethylene pathway is associated with delayed cell death. The resistance mechanism relying on callose deposition require ABA pathway. We provide a comparative analysis on the contribution of different phytohormone pathways to different forms of plant defense mechanisms in the same pathosystem.

In **Chapter 6**, I have summarized all our data and discussed our findings in the context of known mechanisms of plant response to pathogens.

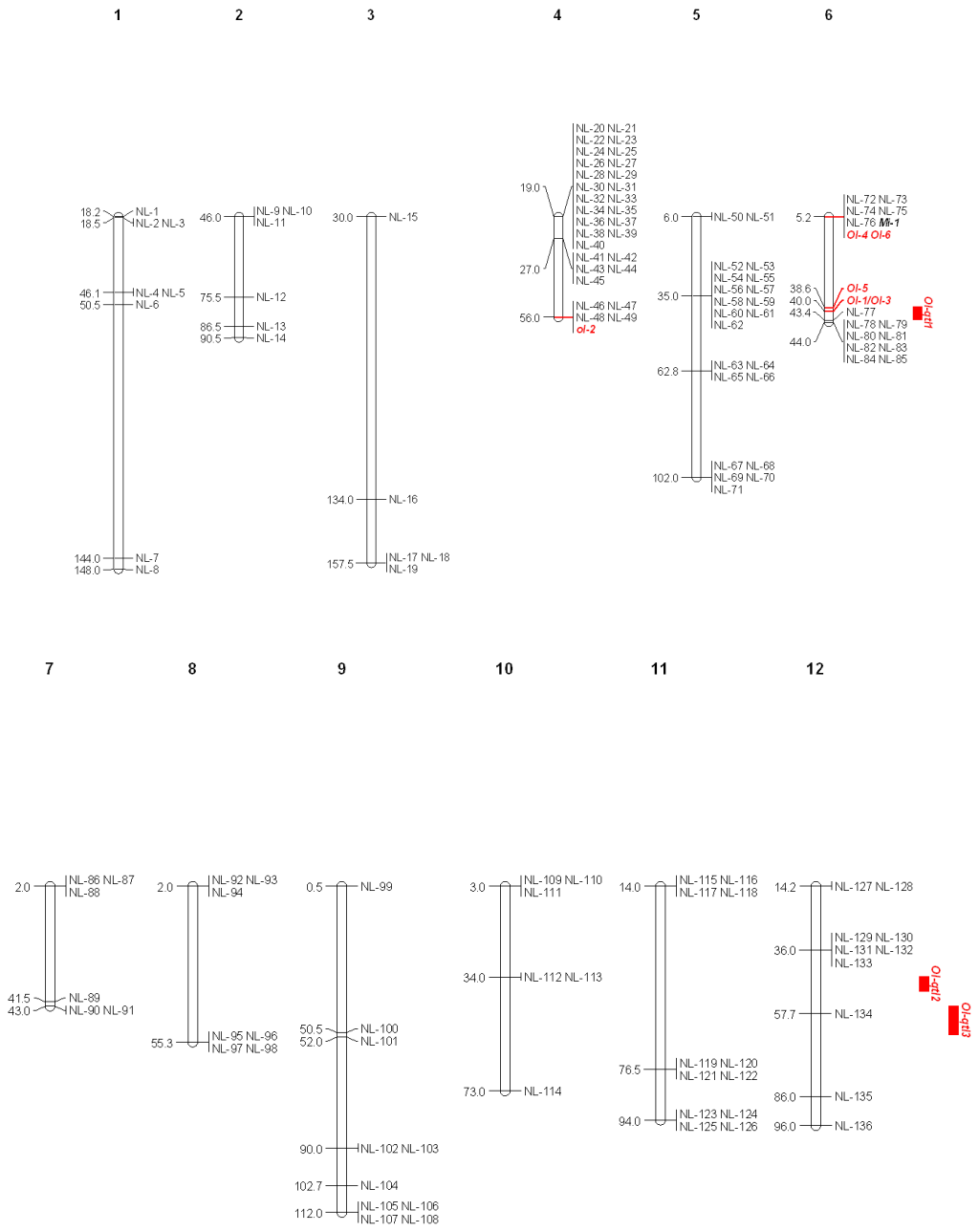

Fig.3. Estimated locations of the putative NBS-LRR coding genes (depicted with NL followed by a number) in the tomato genome. NBS-LRR domain of the *Mi-1.2* gene (AAC67238.1) was used for TBLSTN against tomato genome sequences (WGS version 1.03, http://solgenomics.net). The cut off for E value was set at – 4 (Meyers et al., 2003). Based on this simple analysis there are 142 putative NBS-LRR in the tomato genome of which 6 could not be mapped on any chromosome. The distances are in cM based on Tomato-EXPEN 2000 map (http://solgenomics.net). The approximate locations of the *Ol* loci for resistance to tomato powdery mildew are also included and shown in red color.

Chapter 2

Linked, if not the same, *Mi-1* homologues confer resistance to tomato powdery mildew and root-knot nematodes

Alireza Seifi[1], Isgouhi Kaloshian[2], Jack Vossen[1], Daidi Che[3], Kishor K. Bhattarai[2], Junmei Fan[1], Zabun Naher[1], Aska Goverse[4], W. Freddy Tjallingii[5], Pim Lindhout[1], Richard G. F. Visser[1,6] and Yuling Bai[1,6]

[1]Wageningen UR Plant Breeding, Wageningen University and Research Center, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

[2]Department of Nematology, University of California, Riverside, California 92521, USA

[3] Horticulture College of Northeast Agricultural University, Haerbin 150030, China

[4]Laboratory of Nematology, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

[5]Laboratory of Entomology, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands/EPG systems, Dillenburg 12, 6703CJ Wageningen, The Netherlands

[6]Centre for BioSystems Genomics, The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research, P.O. Box 98, 6700AA Wageningen, the Netherlands

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Linked, if not the same, *Mi-1* homologues confer resistance to tomato powdery mildew and root-knot nematodes

Abstract

On the short arm of tomato chromosome 6, a cluster of disease resistance (*R*) genes has evolved harboring the *Mi-1* and *Cf* genes. The *Mi-1* gene confers resistance to root-knot nematodes, aphids and whiteflies. Previously we mapped two genes, *Ol-4* and *Ol-6* for resistance to tomato powdery mildew in this cluster. The aim of this study was to investigate whether *Ol-4* and *Ol-6* are homologues of the *R* genes located in this cluster. We show that near-isogenic lines (NILs) harboring *Ol-4* (NIL-Ol-4) and *Ol-6* (NIL-Ol-6) are also resistant to nematodes and aphids. Genetically, the resistance to nematodes co-segregates with *Ol-4* and *Ol-6*, which are further fine-mapped to the *Mi-1* cluster. We provide evidence that the composition of *Mi-1* homologues in NIL-Ol-4 and NIL-Ol-6 is different from other nematode-resistant tomato lines, Motelle and VFNT harboring the *Mi-1* gene. Furthermore, we demonstrate that the resistance to both nematodes and tomato powdery mildew in these two NILs is governed by linked (if not the same) *Mi-1* homologues in the *Mi-1* gene cluster. Finally, how *Solanum* crops exploit *Mi-1* homologues to defend themselves against distinct pathogens is discussed.

Introduction

A cluster of disease resistance (*R*) genes has evolved on the short arm of tomato (*Solanum lycopersicum*) chromosome 6 (Fig.1), which harbors *R* genes (*Cf-2*, *Cf-5* and *Mi-1*) that mediate resistance to distinct pathogens. *Cf-2* (originating from *S. pimpinellifolium*) and *Cf-5* (found in *S. lycopersicum var. cerasiforme)* confer resistance to the fungus *Cladosporium fulvum*. *Mi-1* (originating from *S. peruvianum*) mediates resistance to three very different organisms; root-knot nematodes (*Meloidogyne incognita*, *M. arenaria* and *M. javanica*; hereafter refer to as *Meloidogyne spp.),* aphids (*Macrosiphum euphorbiae*) and whiteflies (*Bemisia tabaci*) (Dickinson et al., 1993); (Kaloshian et al., 1998). *Mi-1* belongs to the largest class of *R* genes that encodes a protein containing a nucleotide-binding site plus leucine-rich repeats (NBS-LRR) (Milligan et al., 1998). *Cf-2* and *Cf-5* encode receptor-like proteins with LRR and transmembrane domains (Dixon et al., 1996). Cloning of these *R* genes uncovered the presence of seven *Mi-1* homologues (Seah et al., 2007), three *Cf-2* homologues (Dixon et al., 1996) and four *Cf-5* homologues (Dixon et al., 1998) in this *R* gene cluster. The cluster of *Mi-1* homologues spans about 430 Kb (Fig.1B) and consists of two pseudogenes, one truncated and four intact genes (Seah et al., 2007). Among these seven *Mi-1* homologues only *Mi-1.2* has been shown to be functional and confer resistance to nematodes (Milligan et al., 1998), aphids (Rossi et al., 1998) and whiteflies (Nombela et al., 2003).

In addition to the cloned *Cf* and *Mi-1* genes, *Mi-9* (Ammiraju et al., 2003), *Ty-1* (a locus for resistance to tomato yellow leaf curling virus, TYLCV) and *Cm6.1* (Zhang et al., 2002) have been mapped in the *Mi-1* cluster (Ammiraju et al., 2003; Zhang et al., 2002). Interestingly, *Mi-9* in tomato has been shown to be a *Mi-1* homologue (Jablonska et al., 2007). In addition, *Mi-1* homologues have been identified at syntenic positions in other solanaceous crops. For example, the potato *Rpi-blb2* gene conferring late blight resistance is a *Mi-1* homologue on the short arm of potato chromosome 6 (Vossen et al., 2005). It is intriguing to investigate whether other resistance genes mapped in this cluster are also homologues of *Mi-1* or *Cf* genes.

Previously we mapped two resistance genes, *Ol-4* originating from *Solanum peruvianum* LA2172 and *Ol-6* with unknown origin, on the short arm of tomato

chromosome 6 in the *Mi-1* cluster. These *Ol* genes confer resistance to the fungus *Oidium neolycopersici*, causal agent of tomato powdery mildew by triggering hypersensitive response (HR) (Bai et al., 2005). In this study, we show that near-isogenic lines (NILs) harboring *Ol-4* and *Ol-6* are resistant to *O. neolycopersici*, *M. incognita* and *M. euphorbiae* but not to TYLCV or *C. fulvum*. We provide the evidence that resistance to nematodes co-segregates with *Ol-4* and *Ol-6*. Further, we demonstrate that silencing *Mi-1* homologues in these NILs compromises the resistance to both *O. neolycopersici* and *M. incognita*, suggesting that *Ol-4* and *Ol-6* are *Mi-1* homologues and the resistance to nematodes in these two NILs is also controlled by *Mi-1* homologues.

Results

NIL-Ol-4 and NIL-Ol-6 are resistant to *O. neolycopersici*, nematodes and aphids

Two NILs were generated with an introgression on the short arm of tomato chromosome 6 that contains either *Ol-4* (NIL-Ol-4) or *Ol-6* (NIL-Ol-6) in the genetic background of *S. lycopersicum* cv Moneymaker (MM) (Bai et al., 2005). In these two NILs, the introgression resides only on the short arm including at least the chromosomal region between markers T1198 and cLET-2-H1 (Fig. 1A), which embraces the *Mi-1* gene cluster harboring *Mi-1*, *Cf-2* and *Cf-5*. In addition, *Ty-1* is also mapped in this cluster (Zamir et al. 1994). To test whether NIL-Ol-4 and NIL-Ol-6 have functional alleles of these *R* genes, these two NILs were challenged with the corresponding pathogens; *M. incognita*, *M. euphorbiae*, *C. fulvum* (race 2, 5 and 2.4.5) and one TYLCV strain. In the disease assays with *C. fulvum* and TYLCV, the susceptible control MM as well as NIL-Ol-4 and NIL-Ol-6 were equally susceptible to the three races of *C. fulvum* and TYLCV, except for an intermediate level of resistance to race 5 of *C. fulvum* in NIL-Ol-6 (Table 1 and Fig.2). Since *C. fulvum* race 5 contains *Avr2*, the intermediate level of resistance in NIL-Ol-6 might imply that the allele of *Cf-2* in NIL-Ol-6 is functional to confer a certain level of resistance. As to nematode resistance, NIL-Ol-4 and NIL-Ol-6 were as resistant as Motelle, the resistant control which contains the *Mi-1.2* functional homologue of the *Mi-1* gene originating from *S. peruvianum* PI 128657. No galls or egg masses were observed on the roots of NIL-Ol-4 and NIL-Ol-6 plants in contrast to more than 50 egg masses observed on the roots of MM plants. As to the aphid assay, MM and Motelle were used as

susceptible and resistant controls, respectively. Seven days after confining aphids on the plants with clip cages, the number of adult aphids was similar among the lines. However, the number of nymphs was significantly less on Motelle than that on other lines (Fig.3a).

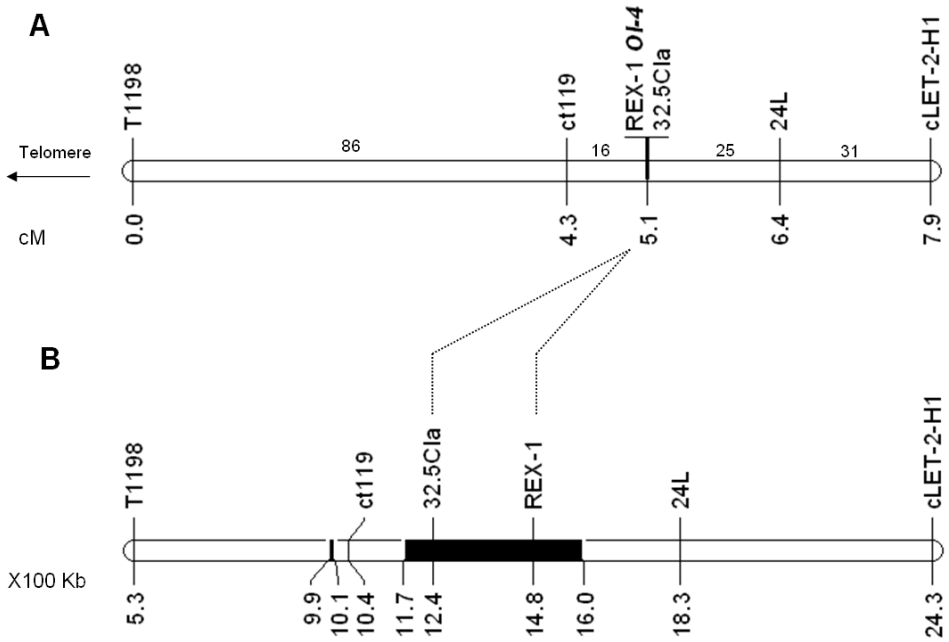

Fig. 1. Genetic and physical maps showing the part of the short arm of tomato chromosome 6 which harbors the *Mi-1* gene cluster. The introgression in NIL-Ol-4 and NIL-Ol-6 is defined on the short arm covering at least the chromosomal region between markers T1198 and cLET-2-H1. A). Genetic map was based on recombinant screening for the *Ol-4* gene by using a BC_3S_1 population derived from interspecific crosses of *Solanum lycopersicum* cv. Moneymaker with *S. peruvianum* LA2172. Genetic distances (cM) are shown in the lower part, while the number of recombinants obtained in each marker interval is mentioned in the interval. B). Physical map of the same region in tomato (*S. lycopersicum*) based on the whole genome shotgun sequencing release 1.05 (www.solgenomics.net). The distances in 100 Kb scale are shown in the lower part. The segment spanning the *Mi-1* gene cluster is shown in solid black (from 1170-1600 Kb) as well as the *Cf* gene cluster which is located above ct119 (from 990 to 1010 Kb).

After the second seven days, comparable numbers of adult aphids were found on Motelle and NIL-Ol-4, which were significantly lower than that on NIL-Ol-6 and MM (Fig. 3b). Though the number of adults and nymphs on NIL-Ol-6 was not as low as that on NIL-Ol-4 and Motelle, it was still significantly lower than that on MM. Similar results were obtained in another aphid assay without using clip cages (Fig. 3c). Furthermore, Electrical Penetration Graph (EPG) (Tjallingii, 1988) was applied to monitor the feeding

behavior of aphids as a different method for aphid assay. The proportion of time (recorded in second (s)) that aphids spent ingesting the phloem sap on MM, NIL-Ol-4, NIL-Ol-6 and Motelle was 10227s, 4737s, 9534s and 4324s, respectively. Comparing to MM, Motelle and NIL-Ol-4 but not NIL-Ol-6 showed significant reduction on the proportion of time. Although NIL-Ol-4 and Motelle showed a comparable level of resistance, substantial differences were observed between these two lines in the performance test (Fig. 3a). Thus, the performance and the feeding behavior of aphids showed that these two NILs were not as resistant as Motelle, suggesting that either the allele of *Mi-1.2* in these two NILs is not as strong as the allele in Motelle or another *Mi-1* homologue (rather than *Mi-1.2*) is involved in response to aphids in these two NILs.

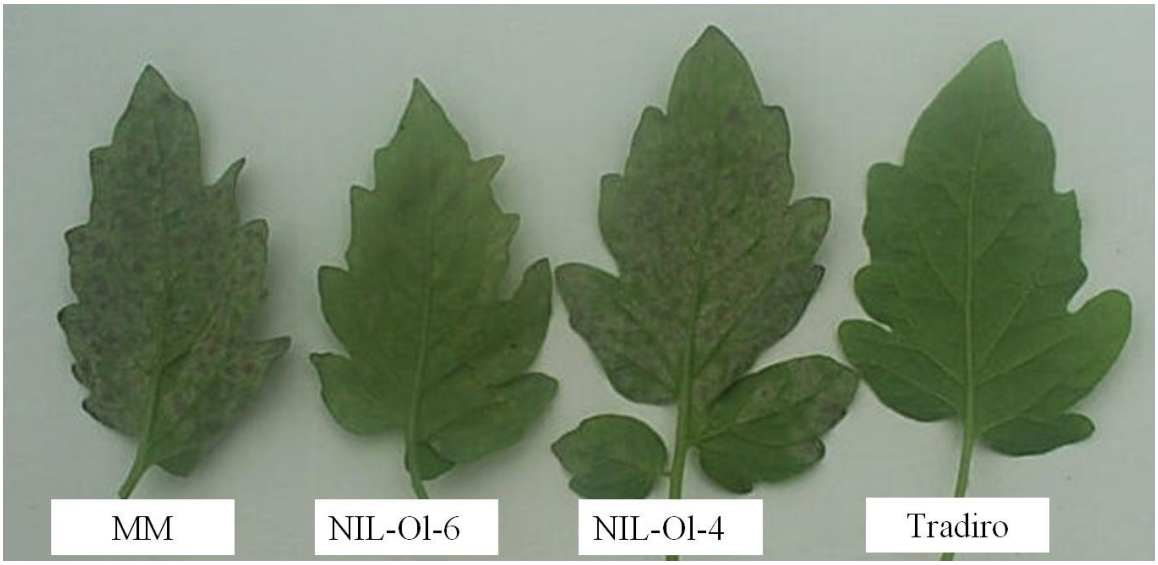

Fig. 2. The abaxial side of leaves of different tomato lines inoculated with *Cladosporum fulvum* race 5. No symptoms of fungal infection are visible on the resistant control Tradiro, while there is heavy sporulation on susceptible control Moneymaker (MM) and NIL-Ol-4. Slight fungal sporulation is visible on NIL-Ol-6, suggesting an intermediate level of resistance in this line.

The composition of *Mi-1* homologues in NIL-Ol-4 and NIL-Ol-6 is different from other nematode-resistant tomato lines

The size and sequence of the first intron of *Mi-1* is variable among *Mi-1* homologues and has been used to differentiate *Mi-1.2* from other *Mi-1* homologues (Seah et al., 2007). To check the presence or absence of *Mi-1.2* in NIL-Ol-4 and NIL-Ol-6, the first intron of *Mi-*

1 homologues was amplified in these two NILs. The size of the three easily discernable amplified introns from NIL-Ol-4 and NIL-Ol-6 appeared to be different from those amplified in MM and Motelle (harboring the functional *Mi-1.2* homologue of the *Mi-1* gene) (Fig. 4A). Furthermore, we sequenced the amplified intron fragments from these

Table 1. Qualitative evaluation of responses of different tomato lines to the tested pathogens and pests.

	Oidium neolycopersici	*Meloidogyne incognita*	TYLCV	*Cladosporium fulvum* race		
				2	5	2.4.5
Moneymaker	S[a]	S	S	S	S	S
NIL-Ol-4	R	R	S	S	S	S
NIL-Ol-6	R	R	S	S	IR	S
Resistant controls	R	R	R	R	R	R
Plants tested per line	20	20	20	10	10	10

[a]R stands for resistant, S for susceptible and IR for intermediate resistant. For disease tests with *O. neolycopersici* and *C. fulvum*, plants with no fungal sporulation were regarded as R, while plants with heavy sporulation as S, plant with weak fungal sporulation as IR. For disease assay with *M. incognita*, plants were scored as resistant if the number of egg masses were less than 10% of the egg masses on the susceptible control Moneymaker. For TYLCV test, plants showing symptoms like curling and yellowing of young leaves (resulting in stop of growth sometimes) were scored as S and plants without any symptom as R. Resistant control for *M. incognita* disease test was Motelle, a cultivar carrying the *Mi-1* gene. In the disease test with TYLCV, we used a breeding line carrying the *Ty-1* gene as resistant control. A commercial hybrid, Tradiro, was the resistant control in *C. fulvum* disease test.

NILs and performed sequence alignment along with available *Mi-1* first intron sequences from VFNT cherry tomato (carrying the *Mi-1.2* homologue and therefore nematode-resistant) (Seah et al., 2004), *S. arcanum* LA2157 (donor of the nematode resistance gene *Mi-9*) and LA392 (nematode susceptible) (Jablonska et al., 2007). The introns of these two NILs were different from the *Mi-1.2* intron and formed two clades which were clearly distinct from other clades derived from VFNT, *S. arcanum* LA2157 and LA392 (Fig. 4B). Thus, we conclude that the *Mi-1.2* allele of the *Mi-1* gene is not present in NIL-Ol-4 and NIL-Ol-6. Furthermore, the intron sequences were not exactly the same in NIL-Ol-4 and NIL-Ol-6, implying that these two NILs have introgressions from different donors.

Resistances to root-knot nematodes and powdery mildew co-segregate

So far, genes conferring resistance to root-knot nematodes in tomato have been mapped on both chromosome 6 and 12. Though we were sure that NIL-Ol-4 and NIL-Ol-6 have introgression on tomato chromosome 6, we could not exclude the possibility that they also contain introgressions on chromosome 12. To verify whether genes for nematode resistance in these two NILs are located on chromosome 6, two BC_3S_1 populations (80 individuals each) segregating for *Ol-4* and *Ol-6* were tested for response to both *O. neolycopersici* and *M. incognita*. By making cuttings, two disease tests were performed on the same individual plant (see M&M). The nematode resistance segregated in both populations following a 3R:1S ratio (58R:22S with $\chi^2 = 0.27$ for the *Ol-4* population, and 64R:16S with $\chi^2 = 1.07$ for the *Ol-6* population). In addition, the nematode resistance in these two populations fully co-segregated with the resistance to *O. neolycopersici* as well as markers linked to *Ol-4* and *Ol-6* loci (Table 2 and Fig. 1A). These results raised the possibility that *Ol-4* and *Ol-6* govern resistance to both nematodes and *O. neolycopersici* or that genes conferring nematode resistance in these NILs are linked to *Ol-4* and *Ol-6*.

***Ol-4* is located in the *Mi-1* gene cluster**

Previously our mapping results suggested that *Ol-4* and *Ol-6* are likely allelic variants of the same gene (Bai et al., 2005). Therefore, we performed fine-mapping only for the *Ol-4* gene. A recombinant screening was carried out in a BC_2S_1 population by using markers located on the short arm of chromosome 6 (Table 2, Fig.1A). From 2000 plants screened, 16 recombinants were found between the *Ol-4* locus and ct119 marker that is tightly linked to the *Cf* gene cluster (Fig. 1A), suggesting that *Ol-4* is not located in this cluster. No recombination events occurred between *Ol-4* and an interval flanked by markers 32.5Cla and REX-1 where the *Mi-1* gene is located (Fig.1A), showing that *Ol-4* is located in the *Mi-1* gene cluster.

Ol-4* and *Ol-6* are homologues of *Mi-1

Besides seven *Mi-1* homologues, several other genes including transport inhibitor response-1 (*TIR-1*), jumonji transcription factors, Na+/H+ antiporter, transposase, as well

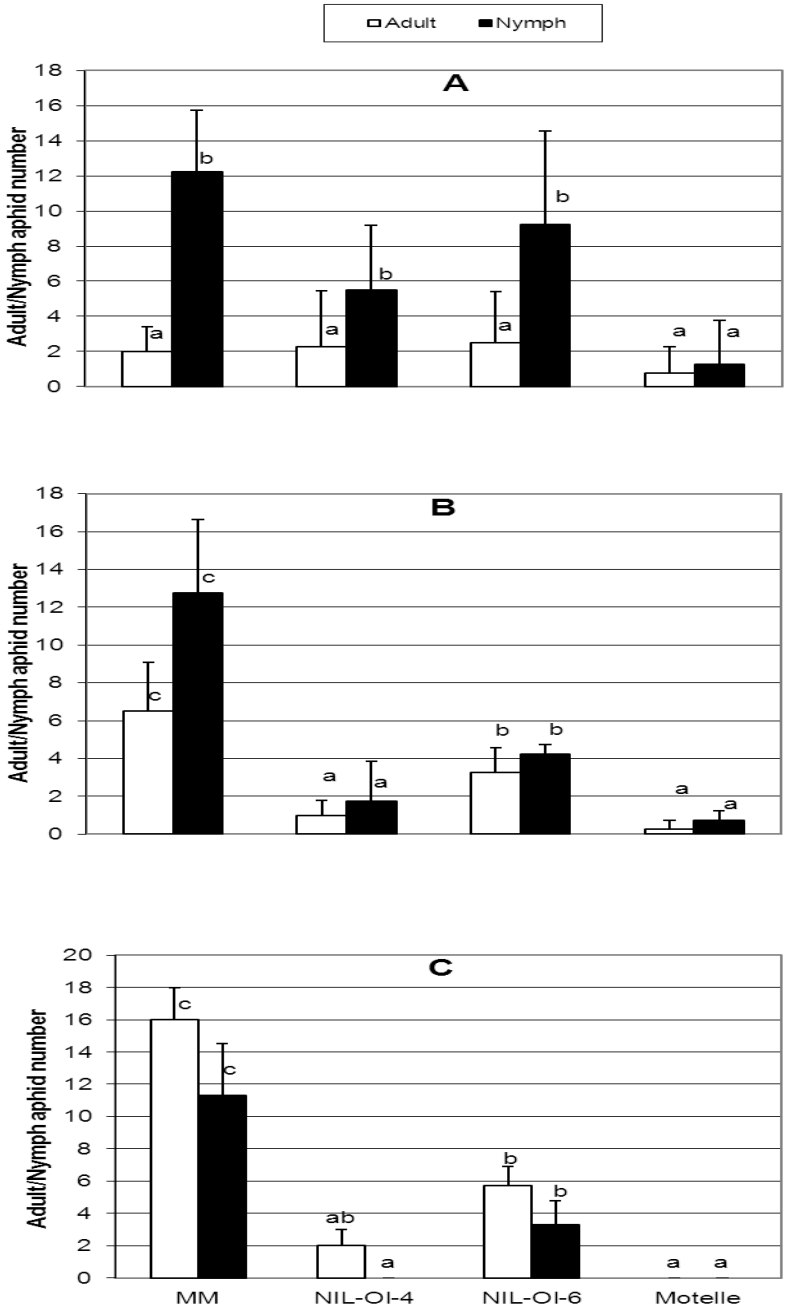

Fig. 3. Aphid tests on NIL-Ol-4 and NIL-Ol-6 compared to Moneymaker (MM; susceptible control) and Motelle (resistant control). Mean value per line is presented and means with a different letter show significance at 5% level ($P<0.05$). A and B, Performance of aphids within clip cages. On each plant (four plant per line in total) 10 aphids were mounted with a clip cage. After 7 days the number of aphids (adults and nymphs) wase counted and then adults were removed. Seven days later (14 days time point) counting was repeated. C, Performance of aphids without clip cage. Data was collected 10 days after placing 10 aphids on the abaxial leaf surface of each plant (three plants per line).

as genes with unknown function are present in the *Mi-1* gene cluster (Seah et al., 2007). Considering *Ol-4* and *Ol-6* are located in the *Mi-1* gene cluster, the first possibility wouldbe that the *Ol-4* and *Ol-6* genes are *Mi-1* homologues. To test this possibility, transient silencing of *Mi-1* homologues was carried out through Virus-Induced Gene Silencing (VIGS). A conserved domain of *Mi-1* homologues was used for VIGS, which had been utilized previously to silence successfully all the *Mi-1* homologues on tomato chromosome 6 (Jablonska et al., 2007; Li et al., 2006). Upon inoculation with *O. neolycopersici,* all MM plants infiltrated with the empty vector showed heavy fungal sporulation suggesting that VIGS did not influence the susceptibility of tomato to *O. neolycopersici* (Fig. 5A). No fungal sporulation was observed on NIL-Ol-4 and NIL-Ol-6 plants infiltrated with the empty vector (Fig. 5A). In contrast, clear fungal sporulation was observed on plants which were infiltrated with the *Mi-1* silencing construct (Fig. 5A). We extracted RNA from the leaves of these infected plants and monitored the *Mi-1* homologues transcript level. In these *Mi*-silenced plants, up to four fold reduction of *Mi-1* transcript levels was detected by qRT-PCR (Fig. 5B). Similar results were obtained in three independent experiments. Thus, silencing *Mi-1* homologues compromised resistance to *O. neolycopersici* in NIL-Ol-4 and NIL-Ol-6, demonstrating that at least one of the *Mi-1* homologues in the *Mi-1* gene cluster is required for resistance to *O. neolycopersici*.

The same *Mi-1* silencing constructs were used to infiltrate another set of MM, NIL-Ol-4 and NIL-Ol-6 plants to test whether silencing *Mi-1* homologues could also compromise resistance to root-knot nematodes in these NILs. More than 200 egg masses appeared on the roots of the susceptible MM plants and almost no egg masses on the roots of the two resistant NILs infiltrated with the empty vector (Fig. 6). However, half of the *Mi*-silenced NIL plants showed egg masses, ranging between 10 to 50 for NIL-Ol-4 and 10 to 100 for NIL-Ol-6 (Fig. 6). Since the nematode resistance in these two NILs is complete, presence of more than 10 egg masses was a clear indication that the resistance was compromised by silencing *Mi-1* homologues. Thus, the resistance to both *O. neolycopersici* and root-knot nematodes in these two NILs is conferred by *Mi-1* homologues.

Table 2. Information of PCR markers.

Marker	Primer Sequence(5'-3')	Annealing temperature °C	Enzyme produced polymorphism	Reference
T1198	F- tagtgggtatggtggctcaatg R- gatggcttccgatggtaggtg	56	*Hha*I	This study
GP79L	F- cactcaatgggggaagcaac R- aatggtaaacgagcgggact	53	*Apo*I	Bai *et al*., 2005
ct119	F- ctattctcacgtaaggggacac R- gtgtacatgtatgaaactctagc	60	*Rsa*I	Dixon *et al*., 1995
REX-1	F- tcggagccttggtctgaatt R- gccagagatgattcgtgaga	55	*Taq*I	Williamson et al., 1994
32.5Cla	F- acacgaaacaaagtgccaag R- caccaccaaacaggagtgtg	56	*Hinf*I	Bai *et al*., 2005
24L	F- tctggggaaggtagtatgtatgc R- aagccggggcgtggttc	64	*HpyCH4*IV	This study
cLET-2-H1	F-cttcttcttcttcaccctaacaca R- ctcgctgctgcactcgtctcttc	56	*HpyF10*VI	This study

Discussion

Mapping of the tomato genes *Ol-4* and *Ol-6* in the *Mi-1* gene cluster and co-segregation of resistance to tomato powdery mildew with resistance to root-knot nematodes put forward the hypothesis that *Ol-4* and *Ol-6* are homologues of the *Mi-1* gene. Transient silencing of *Mi-1* homologues on tomato chromosome 6 indeed compromised both powdery mildew (Fig. 5) and nematode (Fig. 6) resistances in NIL-Ol-4 and NIL-Ol-6 supporting our hypothesis. Analysis of the first intron of *Mi-1* homologues showed that the composition of *Mi-1* gene cluster in NIL-Ol-4 and NIL-Ol-6 is different from the cluster in MM (nematode-susceptible), Motelle and VFTN (nematode-resistant) (Fig. 4). This difference in the *Mi-1* gene cluster was also reflected by the response of NIL-Ol-4 and NIL-Ol-6 to aphids (*M. euphorbiae)*. These two lines were not as resistant as Motelle (Fig. 3). It is worthwhile to mention that the aphid clone (the same clone as used by Kaloshian et al, 1998) was not very well adapted to MM in this study when clip cages were applied (Fig. 3A and 3B). A high number of adult aphids was observed without using clip cages (Fig. 3C). Nevertheless, performance and EPGs showed distinct differences between plant genotypes. Therefore, we conclude that the *Mi-1.2* allele in

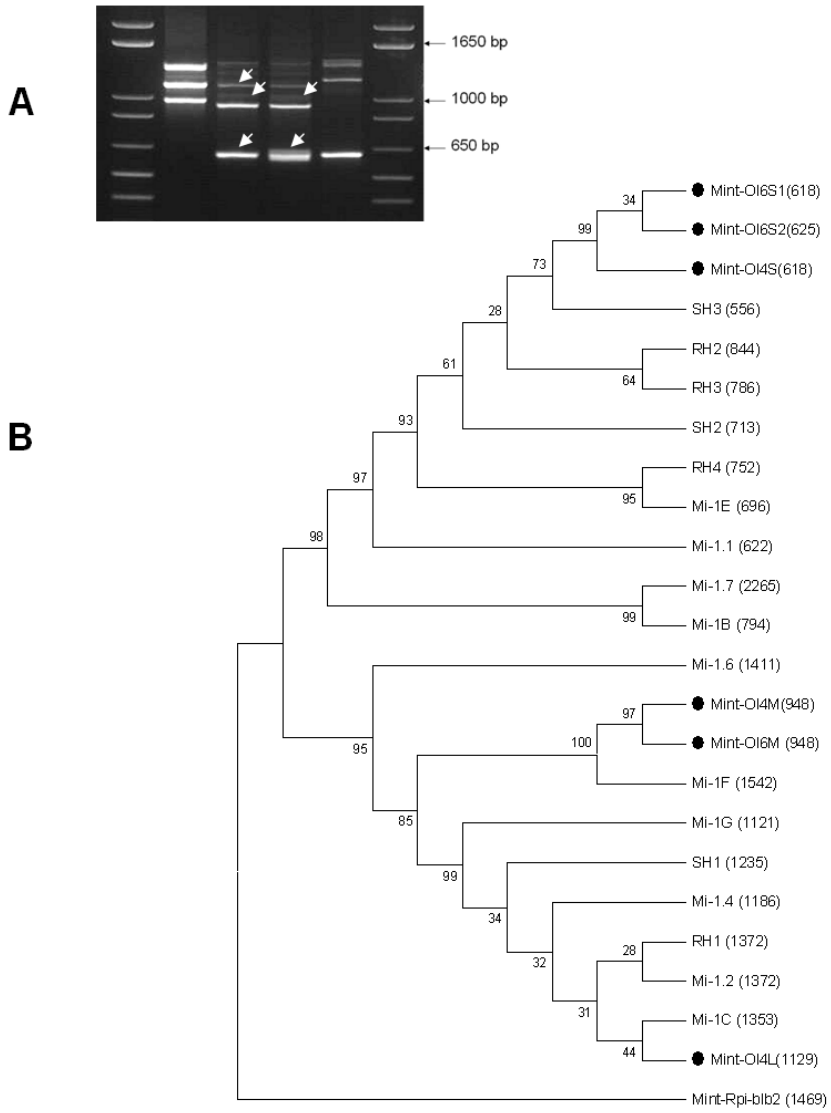

Fig. 4. Analysis of the sequence of first intron of *Mi-1* gene. A) The first intron pattern of the *Mi-1* gene in MM, NIL-Ol-4, NIL-Ol-6, and Motelle (from left to right, respectively). PCR fragments indicated by arrows were sequenced. B) Similarity tree was constructed based on *Mi-1* first intron sequences in susceptible cv. Heinz1706 (assigned as Mi-1B, Mi-1C, Mi-1E, Mi-1F, and Mi-1G), VFNT (Mi-1.1, Mi-1.2, Mi-1.4, Mi-1.6, and Mi-1.7), *S. arcanum* LA2157 (RH1, RH2, RH3, and RH4), *S. arcanum* LA392 (SH1-SH2, SH3, and SH4), NIL-Ol-6 (Mint-Ol6S1, Mint-Ol6S2 and Mint-Ol6M), and NIL-Ol-4 (Mint-Ol4S, Mint-Ol-4M and Mint-Ol4L). The size of each intron is mentioned in the bracket. The introns from NIL-Ol-4 and NIL-Ol-6 are indicated by dots. The bootstrap values are shown. The intron from potato *Rpi-blb2* gene (referring to as Mint-Rpi-blb2) was used as the outgroup sequence. Motelle and VFNT both carrying introgression from *Solanum peruvianum* PI 128657, the donor of *Mi-1* gene. Therefore, the *Mi-1* gene cluster in these two cultivars is the same.

NIL-Ol-4 and NIL-Ol-6 is different from that in Motelle.

It remains to be answered whether, in NIL-Ol-4 or NIL-Ol-6, the same *Mi-1* homologue on the short arm of tomato chromosome 6 governs the resistance to root-knot nematodes, tomato powdery mildew and partial resistance to aphid. It has been suggested that though many homologues of a specific *R* gene are located in a cluster, usually only one of the homologues is functional (Michelmore and Meyers, 1998). For example, in *S. peruvianum* PI 128657, the donor of the *Mi-1* gene, only one (*Mi-1.2*) out of seven homologues has been shown to be functional which confers resistance to three different pathogens including nematodes, aphids and whiteflies (Williamson and Kumar, 2006). Currently, we are in the progress of cloning of *Ol-4* and *Ol-6*, which will reveal the identities of these two genes and also shed light on the genome structure and organization of related homologues in the *Mi-1* gene cluster.

Since the donor of the *Ol-6* gene is unknown, it is unclear whether *Ol-4* and *Ol-6* are alleles of the same *Mi-1* homologue or they are different homologues of *Mi-1*. Though the marker pattern and resistance spectrum to *O. neolycopersici* indicate that *Ol-6* is possibly identical or allelic to *Ol-4*, differences between these two genes have been observed in the level of resistance to tomato powdery mildew (Bai et al. 2005) and aphids (Fig. 3). Furthermore, the *Mi-1* first intron sequences were not exactly the same in NIL-Ol-4 and NIL-Ol-6 (Fig. 4), suggesting that the *Mi-1* gene cluster composition is different in these two lines. So, we conclude that *Ol-4* and *Ol-6* are not identical but are allelic variants, although we could not exclude the possibility that they are different *Mi-1* homologues.

The *Mi-1* gene triggers HR to root-knot nematodes (Dropkin et al., 1969; Roberts and Thomason, 1986), but not to aphids (de Ilarduya et al., 2003). Interestingly, *Ol-4* and *Ol-6* also mediates HR in response to powdery mildew (Bai et al., 2005; Li et al., 2007). HR is a hallmark of *R* gene mediated response (Nimchuk et al., 2003) to biotrophic, but not necrotrophic, pathogens (Glazebrook, 2005). Therefore it is not a surprise to observe HR in the resistance mediated by *Mi-1* gene to powdery mildew and nematodes. No HR has been detected in the *Mi-1*–mediated resistance to potato aphids (de Ilarduya et al.,

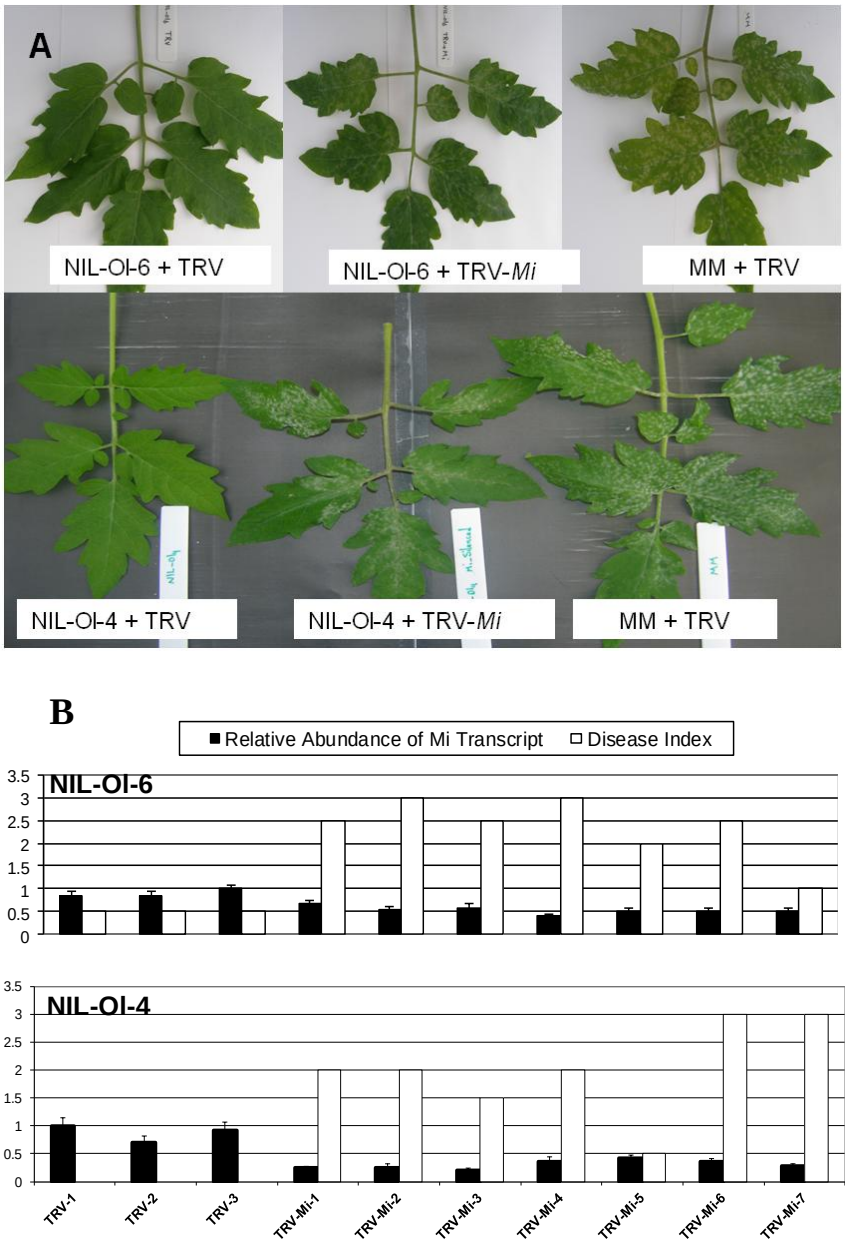

Fig. 5. Silencing of *Mi* homologues compromised resistance to *Oidium neolycopersici* in NIL-Ol-4 and NIL-Ol-6. A) NIL-Ol-4, NIL-Ol-6 and Moneymaker (MM) were infiltrated with empty vector (TRV) or Mi-silencing constructs (TRV-Mi) that silence all seven *Mi-1* homologues on tomato chromosome 6. Pictures were taken of the fourth leaves 14 days after fungal inoculation. B) The association of *Mi-1* transcript levels with *O. neolycopersici* sporulation on the same NIL-Ol-4 and NIL-Ol-6 plants. White bars show the disease index (scale from 0 to 3; 0: no sporulation, 3: heavy sporulation). Black bars show the relative abundance of the *Mi-1* transcripts as measured by the normalized fold expression of the *Mi-1* transcript. TRV plants (3 replicates; TRV1- to TRV-3) were infiltrated with an empty vector and TRV-Mi plants (7 replicates; TRV-Mi-1 to TRV-Mi-7) were infiltrated with a TRV-Mi construct. As is shown on the graph, the disease index for TRV-1 to TRV-3 is zero.

2003). Data from previous EPG study on feeding behavior indicate that aphids ingest only limited amounts of vascular fluids from resistant tomato plants carrying the *Mi-1* gene (Kaloshian et al. 2000). In our study, results showed that the proportion of time that aphids spent ingesting the phloem sap was significantly reduced on NIL-Ol-4 and Motelle. Further experiments are needed to study the nature of the *Mi-1*–mediated resistance mechanism to potato aphids. The *Mi-1* gene fails to confer nematode resistance at higher temperatures (above 28°C), which is a well-documented phenomenon for NBS-LRR proteins (Whitham et al., 1996; Yang and Hua, 2004; Wang et al., 2009). Another *Mi-1* homologue, *Mi-9* that is located in *Mi-1* gene cluster, confers heat-stable resistance to nematodes. It would be interesting to test the heat-stability of nematode resistance in NIL-Ol-4 and NIL-Ol-6.

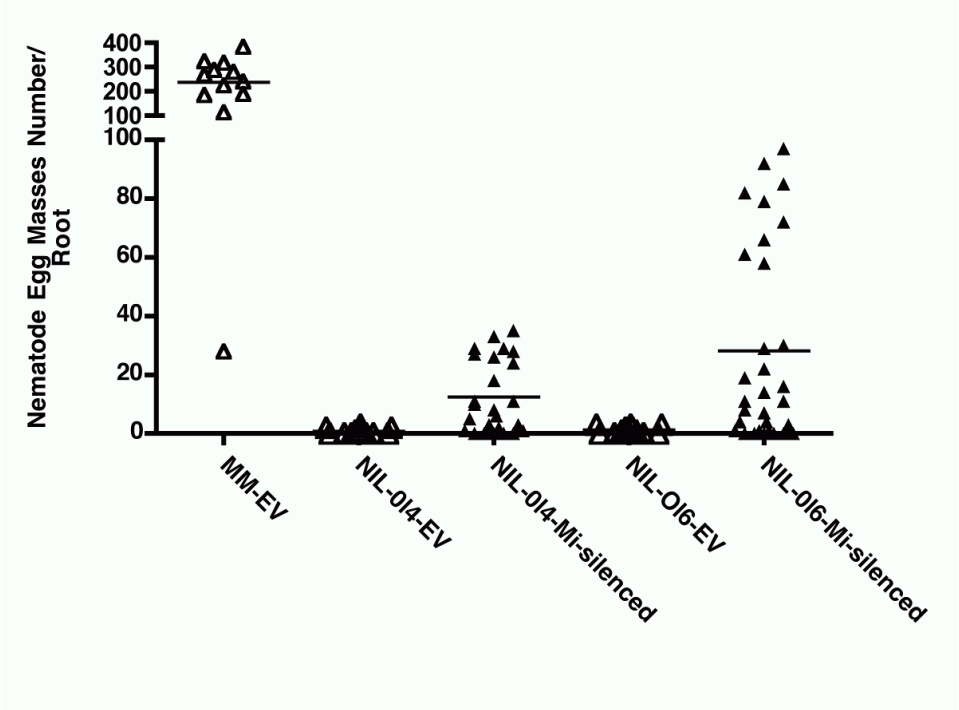

Fig. 6. Silencing *Mi-1* homologues compromised resistance to *Meloidogyne incognita* in NIL-Ol-4 and NIL-Ol-6. Number of egg masses of *M. incognita* on the roots of NIL-Ol-4 and NIL-Ol-6 plants and susceptible control Moneymaker (MM) plants. For MM, NIL-Ol-4-Mi-silenced, NIL-Ol-6-Mi-silenced, NIL-Ol-4-EV and NIL-Ol-6-EV, 12, 25, 30, 12, and 12 plants were tested, respectively. EV plants were infiltrated with the empty vector and Mi-silenced plants were infiltrated with a construct which silences all seven *Mi* homologues on tomato chromosome 6. The horizontal bar indicates the mean.

The most intriguing question is how *Mi-1* homologues can recognize different pathogens. In addition to tomato powdery mildew, *Mi-1* homologues can confer resistance to a wide range of pathogens including root-knot nematodes (*Meloidogyne spp.*), insects (*M. euphorbiae* and *B. tabaci*) and oomycete (*P. infestans*). Dual or multiple specificities of *R* genes can be explained by the guard hypothesis (Dangl, J., and Jones, J. 2001) that *R* gene guards one single virulence target which is modified by multiple effectors. Examples are *Rpm1* in Arabidopsis (Bisgrove et al., 1994) and *Pto* in tomato (Kim et al., 2002) that recognize different effectors of the same bacterial pathogen. Also, the *RRS1* gene in Arabidopsis confers resistance to two different pathogens (bacteria and fungus), by interacting with another *R* gene, *RPS4* (Narusaka et al., 2009). The finding that *Mi-1* homologues in *Solanum* recognize a wide array of pathogen and pest species may suggest that they guard a common or structurally similar virulence target(s). On the other hand, embedding several homologues in an *R* gene cluster could be a mechanism of making a haplotype of *R* genes to give resistance to several different pathogens simultaneously. One example is the *Rx/Gpa2 R* gene cluster in which two homologues confer resistance to two distinct pathogens, virus and nematode (van der Vossen et al., 2000). The distinct functionality of *R* gene homologues could be explained by amplification or reduction in the number of LRR motifs in LRR domains that could modify *R* gene recognition specificity (Staskawicz et al., 1995). For instance, 10 of the 11 alleles that express different rust resistance specificities, showed large variation in the LRR domain (Ellis et al., 1999). Comparing different *Mi-1* homologues in the *Mi-1* gene cluster revealed diversifying nucleotide changes in the LRR domain of these homologues (Seah et al., 2007), which supports the possibility that different homologues in *Mi-1* cluster could be involved in recognition of different effectors. Isolation and functional characterization of pathogen effectors and/or their virulence targets interacting with homologues the *Mi-1* gene family in *Solanum* will deepen our understanding on how *Solanum* crops exploit *Mi-1* homologues to defend themselves against distinct pathogens.

Materials and Methods

Plant materials

Plant material used for mapping and disease tests were described previously (Bai et al., 2005). In short, backcross selfing populations (BC_2S_1 and BC_3S_1) and near-isogenic lines (NILs) harboring *Ol-4* and *Ol-6* (NIL-Ol-4 and NIL-Ol-6) were derived from interspecific crosses of *S. lycopersicum* cultivar (cv.) Moneymaker (MM) with *S. peruvianum* LA2172 and an advanced breeding line, respectively. MM was used as the recurrent parent. Motelle harboring the *Mi-1* gene introgressed from *S. peruvianum* PI128657 (Ho et al., 1992), was kindly provided by the Laboratory of Cell Biology of Wageningen University, the Netherlands.

Disease test

Leaf mold assay: Plants were inoculated with *Cladosporum fulvum* isolate 2 (containing Avr4, 5 and 9), isolate 5 (containing Avr2, 4 and 9), and isolate 2.4.5 (containing Avr9). MM was used as susceptible control and the commercial hybrid Tradiro (resistant to these three races) as resistant control. Leaflets were inoculated as described by Lindhout and associates (1993).

TYLCV test: Agroinoculation was done using an Agrobacterium clone (Agroclone) with 1.8 copy of the TYLCV virus (Israel strain) cloned in Ti plasmid. To prepare the inoculum, Agoclone was first grown for 48 hours at 28°C in LB medium with antibiotics (Kanamycin and Rifampicin, both 100 µg/mL), then the culture was washed three times with water to remove culture media and finally the bacterium was dissolved in water (30 ml culture was dissolved in 3 mL water). The plants were inoculated at 2-3 true leaf stage by infiltrating the inoculum using a syringe with needle. After inoculation plants were grown in 16 hours light at 25˚C and after 14-20 days were evaluated for TYLCV symptoms including curling and yellowing of the young leaves and in the extreme situation stopping of growth. MM was used as susceptible control and a breeding line carrying the *Ty-1* gene originating from *S. chilense* LA1969 (Zamir et al., 1994) as resistant control.

Nematode Assay: Nematode (*M. incognita*, Laboratory of Nematology, Wageningen University) inoculation was performed on two-month-old plants by injecting nematode suspension (300-350 second-stage juveniles per mL, 3 mL per plant) into the sand around the roots. The second inoculation was followed one week later to ensure successful infection. Plants were grown in silver sand and maintained at 25˚C. One day before the inoculation watering of the plants was stopped. Inoculated plants were watered from the tray under the pots for at least one week, in order to prevent washing nematodes away. The phloxine-B staining (10 ng/100 mL) was used to visualize the nematode egg masses on the roots 60 days after inoculation. Plants were scored as resistant if the mean number of egg masses was less than 10% of the susceptible controls (Veremis et al., 1999). For nematode inoculation in VIGS experiments, each plant was inoculated once with 10,000 juveniles.

Powdery mildew assay: Wageningen isolate of *O. neolycopersici* was used (Bai et al., 2005). Fungal spores were washed off from heavily infected tomato leaves and diluted to the concentration of 2.5×10^4 spores per mL. The inoculum was evenly sprayed on the 4-weak-old plants.

Co-segregation test: Two BC2S1 populations (80 individuals each) segregating for *Ol-4* and *Ol-6* were tested with *O. neolycopersici* and *M. incognita*. Plants were first tested with nematodes. Then, a cutting of each individual was made and challenged with *O. neolycopersici*. Tomato cultivars MM, Motelle (carrying the *Mi-1* gene), Poldje (resistant to nematodes) as well as NIL-Ol-4 and NIL-Ol-6 were used as controls.

Aphid assay: Two different methods, performance assay and monitoring aphid feeding behavior by Electrical Penetration Graph (EPG) method, were used to compare response of different tomato lines to aphid (*M. euphorbiae)*. For performance assay, two experiments were carried out on 6-week old plants of MM, Motelle, NIL-Ol-4 and NIL-Ol-6. In the first one, a randomized block design was used with four replications (four plants per line in total). On each plant, a clip cage with 10 aphids was mounted on the fully expended youngest leaf. After 7 days the number of aphids (adults and nymphs) was counted and then adults were removed. After another 7 days aphids were counted again. In the second performance test, three plants per line were placed in one tray containing water to prevent the movement of aphid from one plant to another. Ten adults were

placed on the abaxial leaf surface of the fully expanded young leaf of each plant. Data was taken 10 days after infestation by counting surviving adults and nymphs. For both experiments, the data was transformed to $(X+0.5)^{0.5}$ and then used for ANOVA analysis. Means of all the four lines were compared by using Duncan's multiple range test. As to the EPG experiment, aphids were tested on young, almost fully expanded leaves of 6-week old plants (four plants per line and two aphids per plant) and EPG recording was carried out for 10 hours (h) based on the standard procedure (Tjallingii, 1988). By using non-parametric Mann-Whitney test, the data was analyzed separately for the first 4, 6, 8, and total 10 h of the recordings. The analysis of the first 6 h provided the best distinction between genotypes and is presented here.

Virus-induced gene silencing

The TRV construct and method that have been successfully used to silence *Mi-1* homologues in tomato (Li et al., 2006) and *S. arcanum* LA2157 (Jablonska et al., 2007) was used for silencing *Mi-1* homologues in NIL-Ol-4 and NIL-Ol-6. In brief, the TRV vector carrying a 300 bp fragment of 3'-end of *Mi-1* cDNA, was transformed into *Agrobacterium tumefaciens* GV3101. For VIGS with powdery mildew assay, agroinfiltration was done on cotyledons of 10 day-old seedlings using needle-less syringe and plants were maintained at 21/19°C (day/night). After 21 days, the plants were inoculated with tomato powdery mildew. For VIGS with nematode assay, agroinfiltration was done on 20 day-old seedlings using needle-less syringe and plants were maintained at 21/19°C (day/night). After 27 days, each plant was inoculated with 10,000 juveniles of *M. incognita*. Then, plants were maintained at 26/24°C (day/night) and evaluated for presence/absence of egg masses 12 weeks after nematode inoculation.

Quantitative Real-time PCR

Total RNA was extracted from the leaves of the Mi-silenced and TRV- inoculated plants by using RNeasy™ Plant RNA extraction kit (Invitrogen). Total RNA (1-3 micrograms) was used for cDNA synthesis using Superscritp™ III first strand cDNA sysnthesis kit (Invitrogen). Real-time PCR was done by using iQ SYBR Green Supermix (Bio-Rad). The fold change of the target genes were normalized to Elongation factor 1-alpha (the

internal control gene). Data was analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and reported as normalized fold expression.

Mi-1 gene first intron analysis

NIL-Ol-4 and NIL-Ol-6 genomic DNA was used as the template to amplify the first intron of *Mi-1* using primers, 5'-TTCTCTAGCTAAACTTCAGCC-3' and 5'-TTTTCGTTTTTCCATGATTCTAC-3' (Jablonska et al. 2007), at 50°C as annealing temperature. The amplified fragments were gel-purified and cloned into pGEM-T-Easy vector (Promega). Three clones per each fragment were sequenced. In NIL-Ol-4 we named these fragments as Mint-Ol4S, Mint-Ol-4M, and Mint-Ol-4L, referring to short (S), medium (M), and long (L) size fragments, respectively. In NIL-Ol-6 we only sequenced short and medium size fragments named as Mint-Ol-6S1, Mint-Ol-6S2, and Mint-Ol6M (there are two short fragments in NIL-Ol-6). These sequences were deposited in GenBank under following accession numbers: HQ259295 (Mint-Ol4S), HQ259296 (Mint-Ol4M), HQ259297 (Mint-Ol4L), HQ259298 (Mint-Ol6S1), HQ259299 (Mint-Ol6S2), and HQ259300 (Mint-Ol6M). Intron sequences from VFNT, Heinz 1706 (similar to MM in response to nematode and powdery mildew), *S. arcanum* LA1257 and LA392 were downloaded from GeneBank. By using Mega4 software(Tamura et al., 2007), alignment and construction of similarity tree were performed. The Neighbor-Joining method (Saitou and Nei, 1987) was used to obtain the optimal tree with the sum of branch length=1.99122550. The distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) in the units of the number of base substitutions per site. All positions containing gaps were eliminated from the dataset (Complete deletion option). The intron from potato *Rpi-blb2* gene (a homologue of *Mi*-1, referring to as Mint-Rpi-blb2) was used as the outgroup in this analysis.

Acknowledgements

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Chapter 3

Differential expression of *TIR-like* genes embedded in the *Mi-1* gene cluster in nematode-resistant and –susceptible tomato roots

Alireza Seifi, Richard G. F. Visser and Yuling Bai

Wageningen UR Plant Breeding, Wageningen University and Research Center, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

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Differential expression of *TIR-like* genes embedded in the *Mi-1* gene cluster in nematode-resistant and –susceptible tomato roots

Abstract

Transport inhibitor 1 (TIR1) is an auxin receptor that plays a pivotal role in auxin signaling. It has been reported that *TIR-like* genes are present in a gene cluster carrying the *Mi-1* gene that confers resistance to nematodes, aphids and whiteflies. Since auxin is involved in the pathogenicity of tumor-inducing pathogens like pseudomonads and nematodes, the co- localization of *TIR-like* genes with the *Mi-1* gene may imply a role of *TIR-like* genes in the resistance conferred by the *Mi-1* gene. In this study, we first monitored the *TIR-like* transcript abundance (TTA) in both roots and leaves of nematode-resistant and –susceptible tomato lines. Our results demonstrated that in tomato roots, but not in leaves, the *TIR-like* gene transcript abundance (TTA) was lower in nematode-resistant plants than that in nematode-susceptible plants. Further, we found that the *TIR-like* genes could be cleaved by miR393 in both nematode-resistant and -susceptible plants. These results suggest a possible role for *TIR-like* genes in resistance to root-knot nematodes in tomato. In order to test whether the *TIR-like* genes are involved in *Mi-1* gene resistance pathway, we transiently silenced the *Mi-1* homologues in the nematode-resistant tomato line and measured TTA. Results showed that knocking down the expression of the *Mi-1* homologues had no effect on *TIR-like* transcription regulation. Therefore, we concluded that, involvement of *TIR-like* genes in the response of tomato roots to nematodes, is probably independent of the *Mi-1* homologues. Altogether, our results show that there is an association between TTA and the tomato response to nematodes. We further propose two different scenarios that could explain how TTA are connected to resistance to root-knot nematodes in tomato.

Introduction

Plant nematodes manipulate the morphogenesis and development of root cells of their susceptible host to produce nematode feeding sites (NSF) which appear as cysts or root-knots on the roots (Williamson and Hussey, 1996). There are evidence implicating auxin in the initiation and development of NFSs (Goverse *et al.*, 2000; Viglierchio, 1971). In Arabidopsis, analysis of gene expression in roots infected by *Meloidogyne incognita* (*M. incognita*) revealed significant changes in expression of auxin transporter genes (Hammes *et al.*, 2005). In both tomato and Arabidopsis, formation of NFSs was restricted on roots of auxin-insensitive mutants or on roots treated by auxin transport inhibitors, suggesting that local accumulation of auxin is required for NFS formation (Goverse *et al.*, 2000). Interfering with both expression and polarity of PIN (pin-formed) proteins, particularly PIN1 and PIN7, has been suggested as the mechanism by which cyst nematodes block auxin efflux (Grunewald *et al.*, 2009). Nematode proteins like chorismate mutase 1 (Doyle and Lambert, 2003) could interfere with PIN proteins, through probably manipulating the flavonoid pathways in the plant cell (Wasson *et al.*, 2006).

Transport inhibitor response1 (TIR1) is an auxin receptor which binds to auxin directly and triggers ubiquitination of IAA/AUX proteins, resulting in the activation of auxin responsive factors (ARFs) and therefore triggering expression of auxin-responsive genes (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). TIR1 belongs to the F-box protein family that is negatively regulated by certain plant microRNAs (miRNAs). In Arabidopsis, perception of Flg22, a well-characterized bacterial PAMP (pathogen-associated molecular pattern), triggers resistance to *Pseudomonas syringae* by repression of auxin signaling through manipulating the expression of *TIR1* and *AFB1/2/3* (*TIR1* homologues), as well as the miR393 (Navarro *et al.*, 2006). Interestingly, genomic association of *TIR1-like* genes with nematode resistance gene, *Mi-1*, has been reported in tomato (Seah *et al.*, 2007). *Mi-1* is an NBS-LRR gene conferring resistance to root-knot nematodes (Milligan *et al.*, 1998), aphids (Rossi *et al.*, 1998) and whiteflies (Nombela *et al.*, 2003). On the short arm of tomato chromosome 6, the *Mi-1* gene cluster has been identified consisting of seven *Mi-1* homologues spanning about 400 Kb. In the *Mi-1* gene cluster of the nematode-resistant tomato line VFNT, there is only one *TIR-like* gene while

in the susceptible lines, Heinz 1706 or Moneymaker (MM), at least three homologues of *TIR-like* genes are present (Seah *et al.*, 2007).

Since it has been demonstrated that, in both Arabidopsis and tomato, cyst nematodes manipulate auxin distribution network to facilitate the infection process, it is very likely that auxin plays a similar role in establishing NFS by root-knot nematodes. In addition, miR393 is a highly conserved miRNA and present in different plants including tomato. The presence of *TIR-like* genes in the *Mi-1* gene cluster may suggest a possible role of *TIR-like* genes for resistance to root-knot nematodes in tomato, via repression of auxin signaling. In the present study, our objective was to investigate whether there is any association between *TIR1-like* genes embedded in the *Mi-1* gene cluster and tomato resistance to *M. incognita*. We provide evidence that, in tomato, the expression level of *TIR-like* genes in roots of nematode-resistant plants is lower than that of nematode–susceptible plants. Also we show that the mRNA of *TIR-like* genes in the *Mi-1* gene cluster is cleaved by miR393. Though the transcript abundance of the *TIR-like* and *Mi-1* gene seems to be independent from each other, the possible involvement of *TIR-like* genes in the response of tomato roots to root-knot nematodes is further discussed.

Results

TIR-like genes in the *Mi-1* gene cluster

On the short arm of tomato chromosome 6, six *TIR-like* genes have been identified in the *Mi-1* gene cluster in *S. lycopersicum* (susceptible to nematodes) (Fig. 1). According to the prediction, three of the six *TIR-like* genes encode TIR-like proteins, which we refer to as *TIR-D*, *TIR-E*, and *TIR-G* (Fig. 1, Seah *et al.*, 2007). In contrast, in the *Mi-1* gene cluster introgressed from *S. peruvianum* (donor of the *Mi-1* gene and resistant to root-knot nematodes), four *TIR-like* sequences have been identified and only one of them (referred to as *TIR-1*) encodes TIR-like protein. Figure 1 shows the deduced amino acid sequences of these *TIR-like* genes compared with TIR1 in *S. lycopersicum* cv. Microtom (SlTIR) that is used as the out-group sequence. As is depicted in Fig 1B, these TIR-like protein sequences in the *Mi-1* gene cluster are quite diverged from SlTIR. The F-box domain that is required for the interaction with the ubiquitin ligase complex (Schulman *et al.*, 2000) is missing in *TIR-1* as well as *TIR-D* and *TIR-E* (Fig. 1A), suggesting that even if these two

homologues are expressed they are probably not involved in protein degradation. TIR-G has part of the F-box and probably is able to interact with ubiquitin complex.

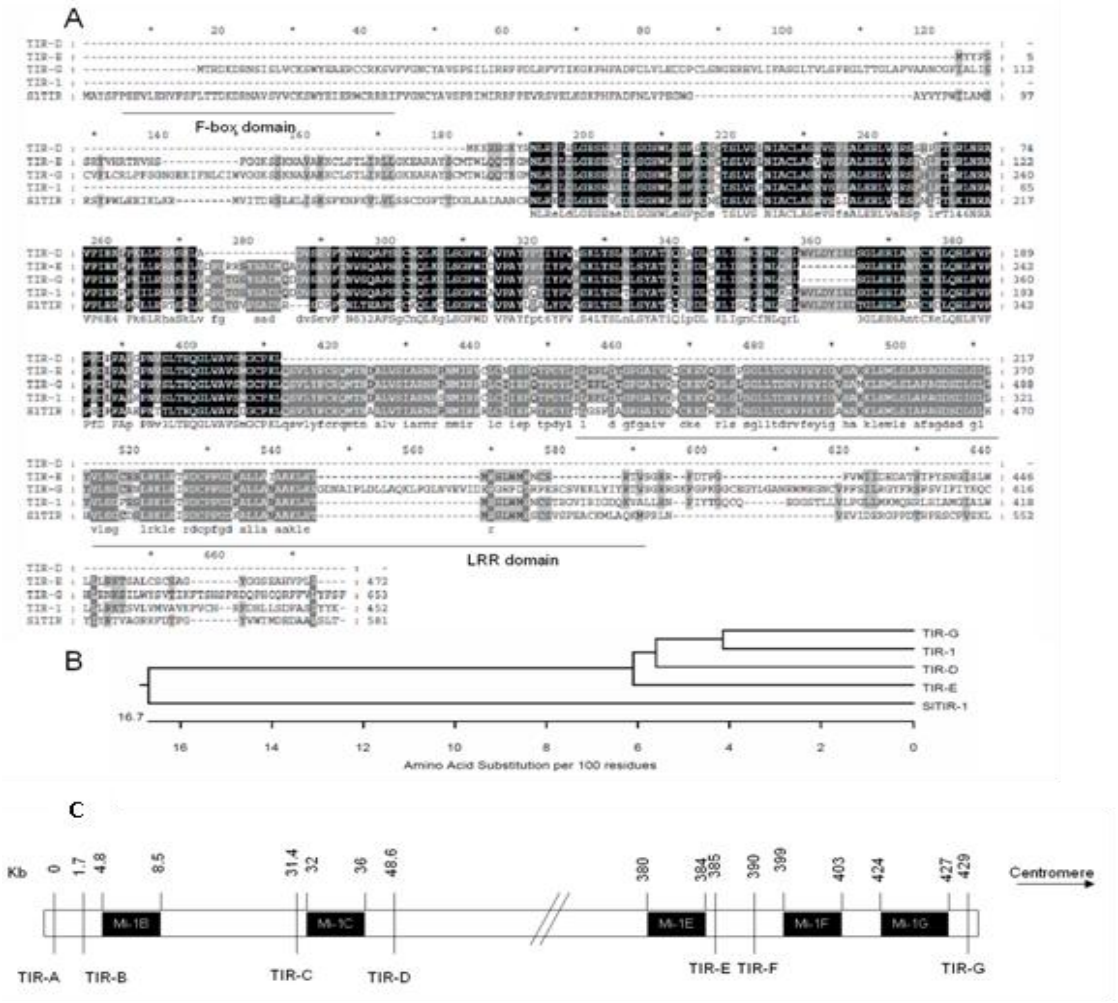

Fig. 1. Comparison of the deduced amino acid sequences of the *TIR-like* genes which are present in the *Mi-1* gene cluster on the short arm of tomato chromosome 6. The tomato *TIR-like* which is located on chromosome 4 (referred as to *SlTIR*) is also included in the analysis. A. Alignment of the deduced amino acid sequences of the *TIR-like* genes. The F-box and LRR domains are shown on the alignment. B. Similarity tree based on the number of amino acid substitutions. C. Relative locations of *TIR-like* genes and the *Mi-1* gene homologues (black boxes) in the *Mi-1* gene cluster of *S. lycopersicum* cv. Heinz 1706. The relative distances are in Kb.

TIR-like transcript abundance is lower in roots of nematode-resistant tomato lines

To test if the genomic differences in the number of *TIR-like* genes influence the transcript level of these genes, we carried out quantitative RT-PCR to compare the TIR-like

transcript abundant (TTA) in both leaves and roots of nematode-resistant and -susceptible tomato lines. The primers were designed to amplify *TIR-1*, *TIR-D*, *TIR-E*, and *TIR-G* in the *Mi-1* gene cluster, but not other *TIR-like* sequences (Table 2). Interestingly, lower TTA was observed in three nematode-resistant lines (Motelle, NIL-Ol-4 and NIL-Ol-6) compared to nematode-susceptible lines (NIL-ol-2 and MM) (Fig. 2). This result was verified on selected F_2 plants (n=12) of a population segregating for resistance to nematodes. Again, TTA was lower in the roots of resistant plants than in susceptible plants (Fig. 3). These results could be due to the difference in the number of *TIR-like* genes between nematode-resistant and –susceptible plants. To check this possibility, we monitored TTA in the leaves of a subset (n=6) of the F_2 plants. Surprisingly, comparable TTA was observed in the leaves of all these plants. It seems that TTA was even higher in the leaves of nematode-resistant plants (Fig. 4). This result implies that probably other factor(s) rather than the number of *TIR-like* genes is/are involved in regulating the transcript level of the *TIR-like* genes.

Table 1. The list and sequences of the primers used for qRT-PCR.

Gene	Sequence(5' to 3')
TIR-like	F- CTTTGCAGCATTAGCCAACA
	R- ATCGAGCCTCAAACTCCTGA
Mi	F- AACTGTTGATTTCCTATTCG
	R AACCAATGTTGCTCTGTTG
Elongation Factor 1-Alpha	F- GAGGGTATTCAGCAAAGGTCTC
	R- ACAGGCGTTCAGGTAAGGAA
SAUR-1	F- GAGCGTTACTGGGTGGATGT
	R- TTTTGTTTTTCGCCATCTCC
SAUR-2	F- GTTCGTCTCCGTCAAATGCT
	R- CCGACGTTTACAGCTACGTG
GH3	F- GCCAACAACAGAGGAAGAGC
	R- TACATTCCTTTGCCCGTCTC
AJ937282	F- GCCACCCATTCGATCTTTTA
	R- TCAAATAGGGAGCACCATCC

Transcripts of the *TIR-like* genes are cleaved by miR393

The recognition site for miR393-mediated cleavage is present in the sequence of the *TIR-like* genes (Fig. 5A). To test whether the transcript of the *TIR-like* genes is cleaved by

tomato miR393, a rapid amplification of cDNA ends (RACE) assay was performed. PCR-amplified cleavage products from the *TIR-like* genes were detected in the roots of both susceptible and resistant tomato lines (Fig. 5B). Sequencing of the cleaved fragments confirmed that these fragments are the result of miR393 cleavage.

Difference in TTA is not mirrored in the expression level of auxin-responsive genes

Since the F-box protein TIR1 is an auxin receptor and mediates auxin signaling (Kepinski and Leyser, 2005) (Dharmasiri *et al.*, 2005), in the next step we tested the possibility that higher expression of TIR-like sequences in nematode-susceptible tomatoes, might trigger higher level of auxin signaling. Three immediate auxin-responsive genes were selected (Navarro *et al.*, 2006) and their expression was compared in the roots of resistant (Motelle) and susceptible (MM) lines (Fig. 6). Although the expression level of *SAUR* and *GH3* was different between resistant and susceptible lines, these differences were not as significant as one might expect for downstream responsive genes. Thus, we concluded that this set of auxin-responsive genes were not affected by differences in TTA under the tested conditions.

Table 2. *TIR-like* sequences in the tomato genome. The first column shows the scaffold numbers on which *TIR-like* genes are identified.

Scaffold number	E value	chromosomal location	homologues number
SL1.03sc00192	1.00E-148	4	1
SL1.03sc01319	1.00E-116	6 (*Mi-1* gene cluster)	6
SL1.03sc02256	1.00E-106	4	1
SL1.03sc00215	1.00E-76	2	1
SL1.03sc00446	6.00E-64	6 (long arm)	1
SL1.03sc00002	1.00E-30	5	1
SL1.03sc00208	5.00E-27	5	1

***TIR-like* transcript abundance is not affected by the expression of the *Mi-1* homologues**

The *Mi-1* gene is required for nematode resistance in Motelle. In NIL-Ol-4 and NIL-Ol-6, we previously showed that the resistance to nematodes is also controlled by *Mi-1* homologues in the *Mi-1* cluster on the short arm of tomato chromosome 6 (Chapter 2). In

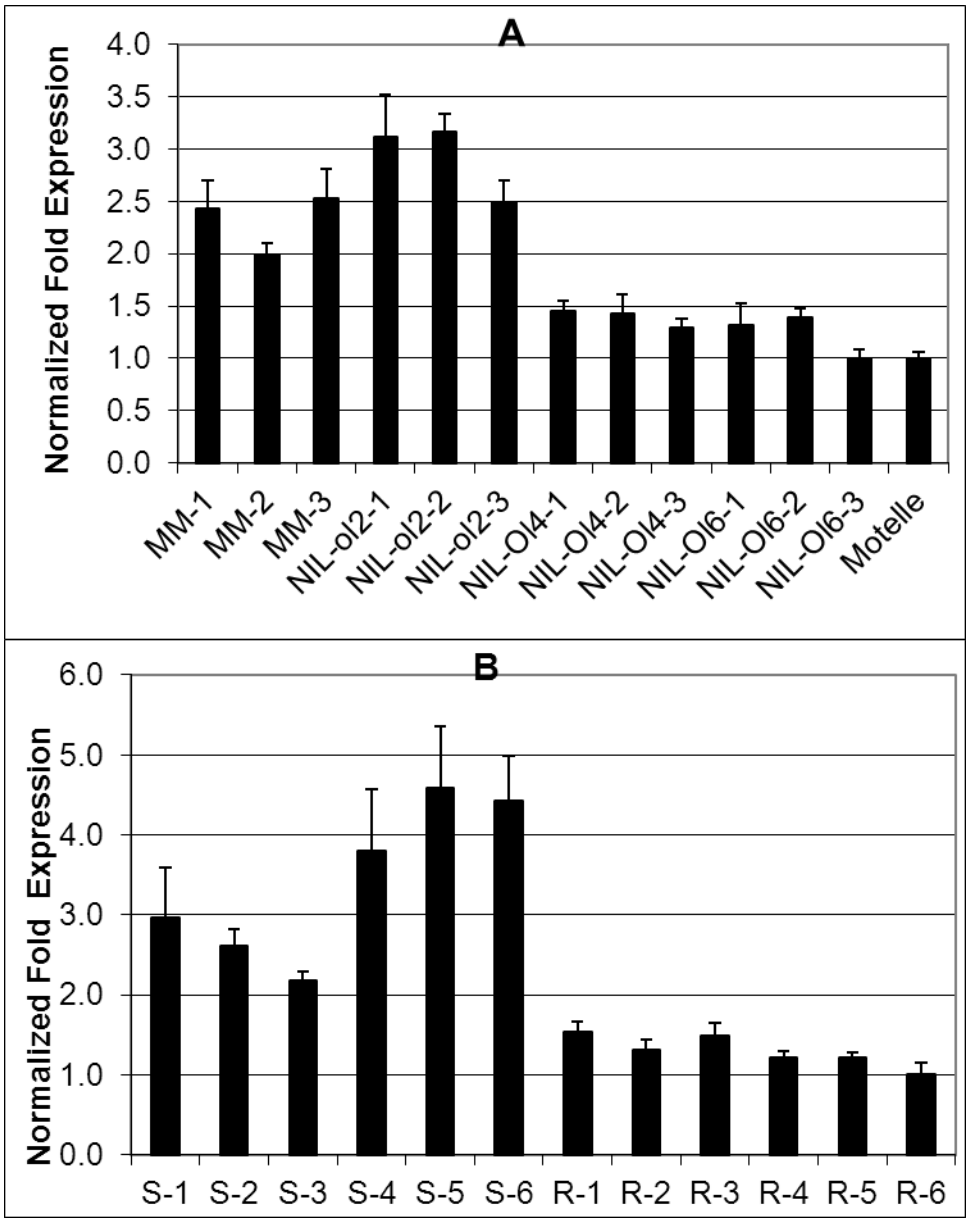

Fig. 2. A. Comparison of *TIR-like* transcript abundance (TTA) in the roots of nematode-resistant and -susceptible tomato lines. MM and NIL-ol-2 are the susceptible lines, while Motelle, NIL-Ol-4 and NIL-Ol-6 are resistant lines. Error bars show the standard deviation. B. Comparison of *TIR-like* transcript abundance (TTA) in the roots of nematode- resistant and -susceptible tomato plants of an F_2 population that segregates for resistance to nematodes. Resistant (R) and susceptible (S) plants were selected by a marker that is fully linked with the *Mi-1* homologue. Error bars show the standard deviation.

order to explain an association between TTA and nematode resistance, we considered the possibility that *TIR-like* genes act in the resistance pathway triggered by *Mi-1* homologues. We knocked down the expression of the *Mi-1* homologues transiently in the

roots of NIL-Ol-4 plants and then measured the TTA. Virus-induced gene silencing (VIGS) was performed to silence all the *Mi* homologues in the *Mi* gene cluster on the short arm of the tomato chromosome 6 (Chapter 2). Results showed that silencing the *Mi-1* gene homologues up to 75% didn't change TTA significantly (Fig. 7). We have shown that this level of silencing of *Mi-1* homologues is enough to abolish *Mi*-mediated resistance to root-knot nematodes and powdery mildew in tomato (Chapter 2). Therefore we concluded that the transcript level of the *Mi-1*gene homologues has no effect on the *TIR-like* genes transcript abundance.

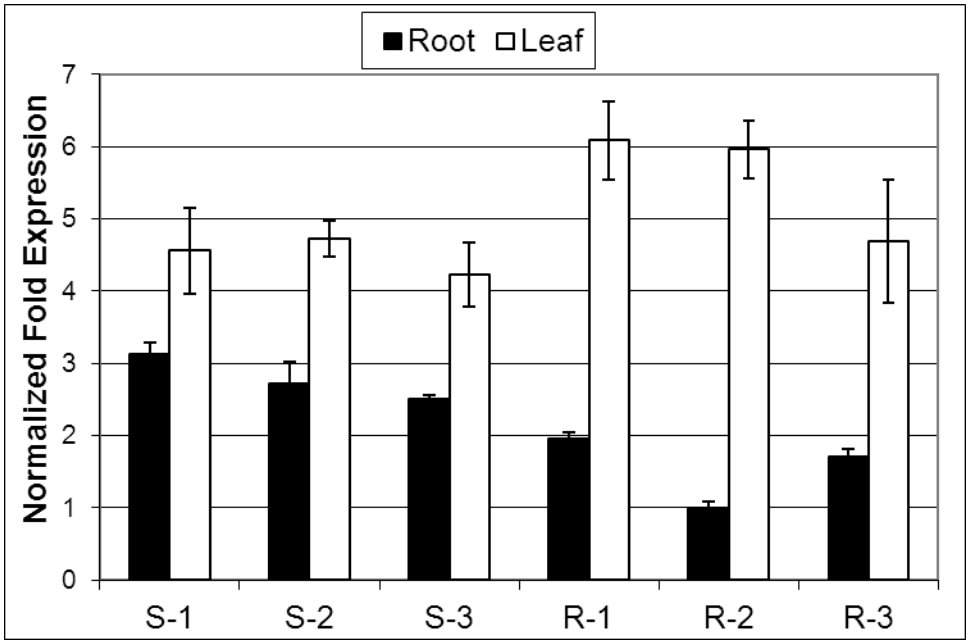

Fig. 4. Comparison of *TIR-like* transcript abundance (TTA) in roots and in leaves of nematode-resistant and –susceptible tomato plants. From an F_2 population segregating for resistance to nematodes, resistant (R) and susceptible (S) plants were selected based on a marker which is fully linked to the *Mi-1* gene homologue. Error bars show the standard deviation.

Discussion

The tumor-inducing pathogen, *Pseudomonas syringae*, elevates the auxin receptor protein TIR1 in *Arabidopis thaliana* cells, resulting in higher activation of auxin signalling and eventually tumour induction (Navarro *et al.*, 2006). The root-knot nematode, *M. incognita*, also produces galls on roots of their host plants, which needs manipulation of the plant hormones especially auxin (Viglierchio, 1971). The gene for resistance to *M.*

incognita, *Mi-1*, is located in the *Mi-1* gene cluster on the short arm of tomato chromosome 6, wherein *TIR-like* genes are also embedded (Seah *et al.*, 2007). Interestingly, the number of *TIR-like* genes differ between nematode-resistant and -susceptible tomato lines (Seah *et al.*, 2007). Moreover, instability of the resistance to

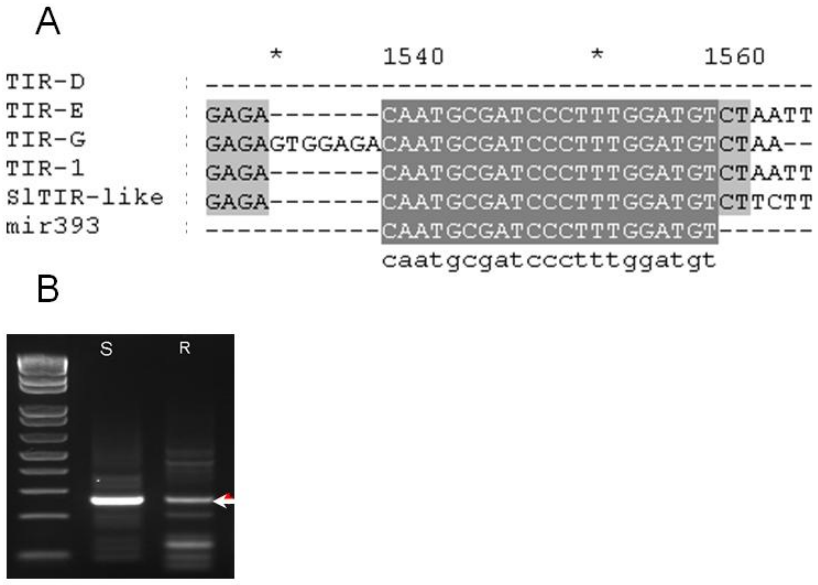

Fig. 5. Vulnerability of *TIR-like* genes to miR393-mediated cleavage. A. Presence of miR393 recognition site on *TIR-E*, *TIR-G*, and *TIR-1* as well as *SlTIR*. B. Detection of cleaved fragments of the *TIR-like* genes in both nematode-resistant (R) and -susceptible (S) lines. The arrow shows the expected cleaved fragment (about 260 bp).

nematodes has been reported in the tomato lines which have been transformed by *Mi-1* gene (Goggin *et al.*, 2004), suggesting that the *Mi-1* gene is probably not the only factor needed for resistance to nematodes. These evidences intrigued us to hypothesize that *TIR-like* genes might have a role in the response to nematodes. Our results showed the association between *TIR-like* transcript abundance and nematode-resistance in the roots of tomato plants (Figs. 2 & 3). Interestingly, this association was not observed in the leaves (Fig. 4), suggesting that the number of *TIR-like* homologues is probably not the only factor influencing the TTA. Further, we demonstrated that these *TIR-like* genes have the recognition site for miR393 and that cleavage of these *TIR-like* genes by miR393 occurs. Whether the efficiency of the cleavage is the same in both nematode-resistant and -susceptible tomato lines needs to be further investigated. Next, we tested the possibility

that *TIR-like* genes play a role in conferring resistance to root-knot nematodes downstream of the *Mi-1* gene. A reduction in the transcript level of the *Mi-1* gene in the nematode-resistant tomato line did not influence the TTA (Fig. 7). Our conclusion is that, if *TIR-like* genes are involved in the response of tomato roots to nematodes, it seems to be independent of the *Mi-1* gene. Also, we compared the expression level of four auxin-responsive genes in nematode-resistant and –susceptible roots. As is depicted in Fig. 6, results showed that differences in TTA did not influence the expression of these auxin-responsive genes.

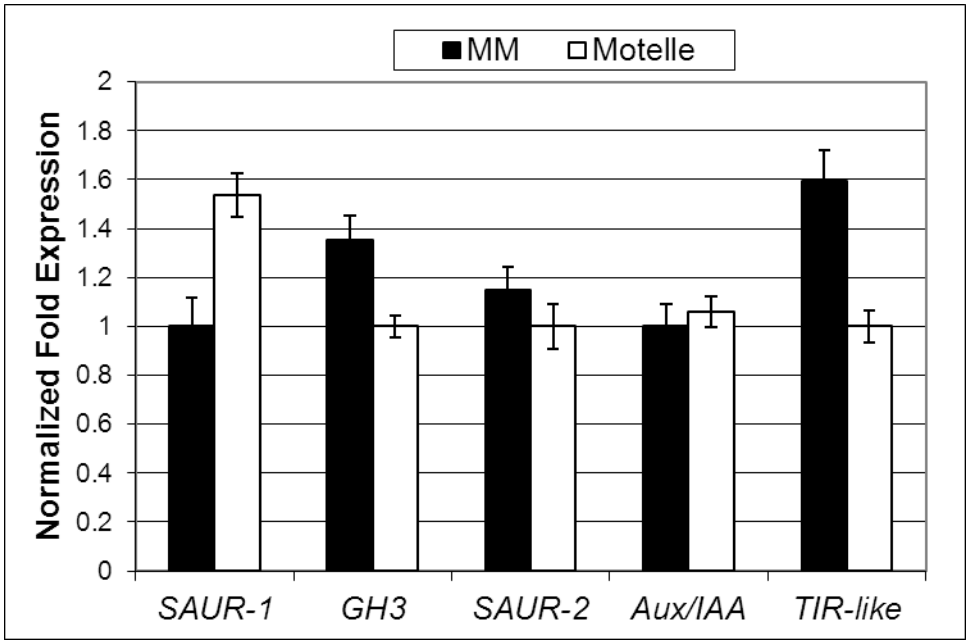

Fig. 6. Relative expression of four auxin-responsive genes and *TIR-like* genes in nematode-resistant (Motelle) and -susceptible (MM) lines. Error bars show the standard deviation.

To explain our observations we propose a model for the possible involvement of these *TIR-like* genes in tomato response to root-knot nematodes. Nematodes attack tomato root cells (resistant or susceptible) and trigger local accumulation of auxin by blocking auxin efflux (Goverse *et al.*, 2000), through manipulation of PIN proteins

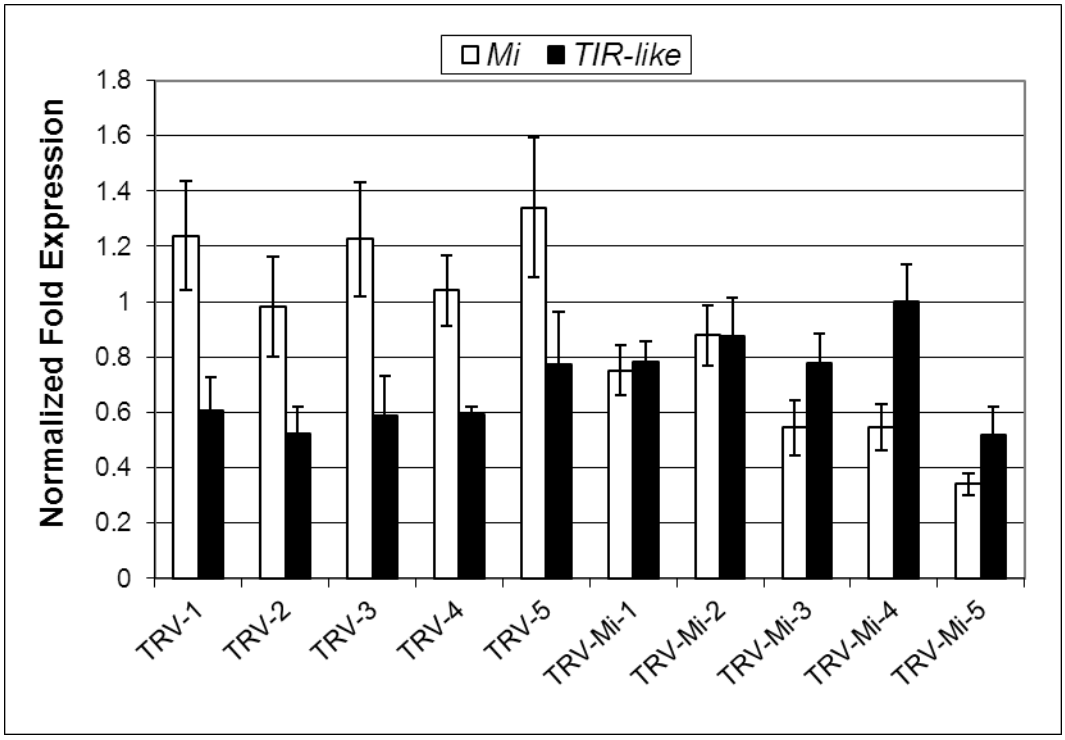

Fig. 7. Effect of silencing the *Mi-1* homologues on *TIR-like* transcript abundance (TTA). NIL-Ol-4 plants were infiltrated with empty vector (TRV) or Mi-silencing constructs (TRV-Mi) that silence all seven *Mi-1* homologues on the short arm of tomato chromosome 6. White bars show the relative expression of the *Mi-1* homologues and black bars show TTA. Error bars show the standard deviation. TRV plants (5 replicates; TRV-1 to TRV-5) were infiltrated with an empty vector and TRV-Mi plants (5 replicates; TRV-Mi-1 to TRV-Mi-5) were infiltrated with a TRV-Mi construct.

(Grunewald *et al.*, 2009). With a higher amount of TIR protein present in the roots of nematode-susceptible tomato plants, accumulation of auxin would lead to an activation of auxin-responsive genes and eventually gall production on the roots. In contrast, a lower amount of TIR protein present in the roots of nematode-resistant plants may prevent further changes in auxin signalling even though nematode could probably accumulate auxin locally. So we speculate that the level of TIR protein in the root cells influences the auxin signalling leading ultimately to the success or failure of the formation of NFS.

The other possibility of the involvement of the *TIR-like* genes in response to nematodes might be a regulatory role of these *TIR-like* genes on the expression of the *Mi-1* homologues. The *Mi-1* gene cluster consists of seven *Mi-1* homologues, of which five (including a pseudogene) are expressed (Seah *et al.*, 2007). In the *RPP5* gene cluster in Arabidopsis, RNA silencing could regulate the transcript level of resistance (*R)* genes to

restrict the fitness cost associated with constitutive expression of the genes (Yi and Richards, 2007). The *TIR-like* genes which are located in the *Mi-1* gene cluster have some interesting features. Firstly, they are all located on the antisense DNA strand in relation to their corresponding *Mi-1* gene. Secondly, the F-box domain is missing at least in TIR-D, TIR-E and probably TIR-1 (Fig. 1), suggesting that these proteins are probably not involved in protein degradation in the auxin signaling pathway. Thirdly, *TIR-like* genes are cleaved by miR393 (Fig. 5). We speculate that these *TIR-like* genes might act as a source for producing small RNAs to regulate the transcript level of the *Mi-1* homologues. For instance, the miRNA-cleaved transcripts could be a source of production of the trans-acting small RNAs (tasiRNAs) (Vaucheret *et al,* 2005). Besides *TIR-like* genes, there are also jumonji transcription factors in the *Mi-1* gene cluster (Seah *et al.,* 2007). Recently, it has been shown that a member of the jumonji gene family is involved in RNA silencing (Searle *et al.*, 2010). Decreasing of susceptibility to cyst nematodes in Arabidopsis RNAi mutants, *dcl*1 and *rdr,* also suggests a role for small RNAs in plant-nematode interaction (Hewezi *et al.*, 2008).

Based on our preliminary results we propose that *TIR-like* genes embedded in the *Mi-1* gene cluster probably have a role in tomato response to root-knot nematodes. We propose two different scenarios which could explain this role. These speculations demand further investigations. For instance, monitoring TTA in *Mi*-transgenic lines which showed decreased levels of resistance to nematodes may reveal the importance of *TIR-like* genes in resistance to nematodes. It is also tempting to monitor the mechanisms of transcriptional and translational regulation of *Mi-1* homologues, whether there is a footprint of *TIR-like* genes there. Future studies will shed light on the complexity of *Mi-1* gene cluster and the genes embedded there including *TIR-like* genes.

Material and Methods

Plant Materials

S. lycopersicum cv. MoneyMaker (MM) and NIL-ol-2 were used as nematode-susceptible tomato lines. NIL-ol2 is a near-isogenic line in which the *ol-2* gene is introgressed in MM background (Bai *et al*. 2005). Motelle, NIL-Ol-4 and NIL-Ol-6 which have

introgression from *S. peruvianum*, were used as nematode resistant lines (Chapter 2). One F_2 population segregating for the nematode-resistance derived from a cross between MM and *S. peruvianum*. Nematode-resistant (R) and susceptible (S) plants were selected by using a marker fully co-segregating with resistance conferred by the *Mi*-1 gene homologue (Chapter 2).

TIR-like sequences analysis

Arabidopsis TIR1 protein sequence was used for TBLASTN against tomato genome sequences(http://solgenomics.net) to find the TIR1 orthologs in tomato. Protein sequence alignment and similarity tree construction was done by DNASTAR™ software. The level of similarity is reported based on number of amino acid substitutions.

Quantitative Real-time PCR

Total RNA was extracted from the tomato roots or leaves by using RNeasy™ Plant RNA extraction kit (Invitrogen). 1-3 micrograms of total RNA was used for cDNA synthesis using Superscritp™ III first strand cDNA sysnthesis kit (Invitrogen). Real-time PCR was done by using iQ SYBR Green Supermix (Bio-Rad). The fold change of the target genes were normalized to Elongation factor 1-alpha (the internal control gene). Data was analyzed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and reported as normalized fold expression.

Auxin-responsive genes in tomato

Arabidopsis proteins GH3 (AT1G48690) and SAUR (AT1G75590) were used for TBLASTN to find orthologs in the tomato Unigene database (http://solgenomics.net). SGN-U326221 and SGN-U330915 were the best hits for SAUR which we called SAUR1 and SAUR2, respectively. SGN-U325385 was the best hit for GH3. Tomato AUX/IAA was already available in GenBank under accession number AJ937282.

Oligonucleotides

Primers for qRT-PCR were designed by using OligoPerfect™ Designer (http://tools.invitrogen.com/content.cfm?pageid=9716). The primers (Table 1) have the

Tm at about 60 °C and amplify a fragment between 100-150 bp in size. The primers for *TIR-like* genes amplify only *TIR-E, TIR-D, TIR-G* and *TIR-1* but not *SlTIR* or any other TIR-like genes from other chromosomes.

Virus-Induced Silencing of the *Mi-1* gene homologues

The method and construct which has been used successfully by Jablonska *et al*. (2007) was applied for silencing of the *Mi-1* homologues. In brief, the TRV vector carrying a 300 bp fragment of 3'-end of *Mi-1* cDNA, was transformed into *Agrobacterium tumefaciens* GV3101 and used for agroinfiltration of four-week-old tomato seedlings using needleless syringe. Three weeks later, roots were sampled for RNA extraction.

RACE

To detect cleaved fragments of *TIR-like* genes, RACE was carried out by using GeneRacer™ kit (Invitrogen).

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We would like to thank Prof. Valerie Williamson (Department of Nematology, University of California, Davis), Dr. Isgouhi Kaloshian (Department of Nematology, University of California, Riverside) and Dr. Aska Goverse (Laboratory of Nematology, Wageningen University, The Netherlands) for valuable discussions about this work.

Chapter 4

Two interacting loci on the long arm of tomato chromosome 6 confer resistance to tomato powdery mildew by coordinating delayed cell death

Alireza Seifi[1], Teruo Nonomura[2], Dongli Gao[1], Wosene G. Abtew[1], Hideyoshi Toyoda[2], Richard G. F. Visser[1] and Yuling Bai[1]

[1]Wageningen UR Plant Breeding, Wageningen University and Research Center, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

[2]Laboratory of Plant Pathology and Biotechnology, Faculty of Agriculture, Kinki University, Nara 631-8505, Japan

Two interacting loci on the long arm of tomato chromosome 6 confer resistance to tomato powdery mildew by coordinating delayed cell death

Abstract

On the long arm of tomato chromosome 6, two closely linked loci, *Ol-1* and *Ol-5*, have been mapped conferring resistance to *Oidium neolycopersici*. Both *Ol-1* and *Ol-5* are identified from the wild tomato species *Solanum habrochaites.* We performed fine-mapping coupled with physical mapping for the *Ol-1* gene and narrowed down this locus to a 73 Kb interval. This interval contains at least 10 putative genes, of which none belongs to resistance (*R*) gene family members cloned so far. Microscopically, the response mediated by *Ol-1* is delayed cell death that allows limited fungal colonization.. Interestingly, our results show that another locus, located in the chromosomal region where the *Ol-5* locus is mapped, is required for *Ol-1*-mediated resistance, suggesting that the interaction between *Ol-1* and *Ol-5* is needed to confer resistance to *O. neolycopersici*. In fine-mapping of the *Ol-5* locus, we observed that resistance mediated by *Ol-5* requires an additional locus that is located in the *Ol-1* interval. Furthermore, our data show that the interaction between *Ol-1* and *Ol-5* regulates delayed cell death. Loss of the *S. habrochaites* allele of the *Ol-5* locus results in abolishing the cell death, while loss of *S. habrochaites Ol-1* allele reduces the effectiveness of cell death probably by disturbing the right timing of this process. Altogether our results suggest that *Ol-1*, by interacting with *Ol-5*, confers resistance to *O. neolycopersici* by regulating delayed cell death upon pathogen attack. Preliminary functional analysis suggests that *Ol-1* encodes a homeodomain-leucine zipper transcription factor, which silencing comprised resistance mediated by both *Ol-1* and *Ol-5*.

Introduction

Plant cell death is one of mechanisms of resistance against biotrophic pathogens by delimiting pathogen progress on plant tissues. The hypersensitivity response (HR) is a form of cell death which is typically triggered upon recognition of pathogen avirulence (Avr) proteins by plant resistance (R) proteins (Nimchuk et al., 2003). It has been reported that HR is the main resistance mechanism of tomato (*Solanum lycopersicum*) to *Oidium neolycopersici*, the causal agent of tomato powdery mildew (PM) (Huang et al., 1998). Particularly, two different forms of HR have been observed in tomato response to PM. 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*. The *Ol-4* gene is derived from *S. peruvianum* LA2172 and homologous to *Mi-1* encoding an NBS-LRR R protein (Chapter 2). 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 the *Ol-1* or *Ol-5* gene. The *Ol-1* and *Ol-5* genes are identified from the wild tomato species *S. habrochaites* G1.1560 and PI247087, respectively (Huang et al., 2000; Bai et al., 2005) and mapped as a cluster on the long arm of chromosome 6 of tomato (Bai et al., 2005). Resistance mediated by *Ol-1* or *Ol-5* is incomplete and race-non-specific, since near-isogenic lines carrying these genes (NIL-Ol-1 and NIL-Ol-5) showed variable levels of resistance to all different PM isolates which were tested (Bai et al., 2005).

Map-based cloning of genes in some plant species have been accelerated by availability of the genome sequence of these plant species. Tomato genome sequence is available now (http://solgenomics.net) and makes it easy to narrow down the interval of the gene of interest. However, the remained challenge to clone the gene is functional confirmation, which demands genetic complementation of the candidate genes by performing stable transformation. Alternatively, virus-induced gene silencing (VIGS) has been widely used as a fast and relatively simple method for functional analysis of genes in plants (Liu et al., 2002; Lu et al., 2003; Brigneti et al., 2004; Chapter 2). This technique involves cloning of a short fragment of the target gene into a viral-based vector that can be infiltrated into the target plant (Liu et al., 2002). Virus propoagte and spread systemically in the plant and produce double-stranded RNA of the fragment of the target

gene, which will be processed by RNA silencing machinary of the plant cell to knock down the transcript level of the targeted gene (Liu et al., 2002).

Here we report that *Ol-1* triggers delayed cell death, which is microscopically discernible from HR mediated by *Ol-4*. Then, based on genetic and physical mapping data, we show that *Ol-1* is not an *R* gene and also it requires another locus, *Ol-5,* for conferring resistance to *O. neolycopersici*. Data from VIGS experiments suggest the possibility of involvement of a homeodomain-leucine zipper (HD-Zip) transcription factor in *Ol-1*-mediated resistance.

Results

Ol-1 confers broad-spectrum resistance to PM by triggering delayed cell death

Previously, we reported that a near-isogenic line carrying *Ol-1* (NIL-Ol-1) showed variable levels of resistance to all the tested isolates of *O. neolycopersici*, compared to its background, Moneymaker (MM) that was fully susceptible (Bai et al., 2005). However, MM and NIL-Ol-1 show similar fungal growth patterns as defined by the number of primary haustoria, hyphae, and secondary appressoria and haustoria per infection unit (Li et al., 2007). In this study, we microscopically compared the type of cell death that occurs in the wild species, *S. habrochaites* G1. 1560 and *S. peruvianum* LA2172 that are donors of *Ol-1* and *Ol-4*, respectively. The result revealed a clear distinction in cell death between these two species (Fig.1). In *S. peruvianum*, HR occurred in invaded epidermal cells at the stage when primary appressoria and haustoria were formed. At 48 hour post inoculation (hpi), all epidermal cells attacked by PM in *S. peruvianum* showed HR, which was effective to prevent secondary hyphal production. However, in *S. habrochaites*, delayed cell death happened in epidermal cells invaded by PM haustoria. In this case, epidermal cell death occurred after the conidia produced functional primary haustoria and elongated secondary hyphae. At the primary penetration sites, the percentage of cell death in *S. habrochaites* was 70% at 48 hpi and 80% at 72 hpi (Table 1), compared to 100% HR in *S. peruvianum* at 48 hpi. Obviously, delayed cell death in *S. habrochaites* was less severe and did not engulf the entire cell (Fig. 1B, D), which enabled the fungus to produce secondary hyphae to attack other cells. Thus, cell death triggered by *Ol-1* is

delayed and allows limited fungal colonization, leading to moderate but not complete resistance of *S. habrochaites* to *O. neolycopersici*.

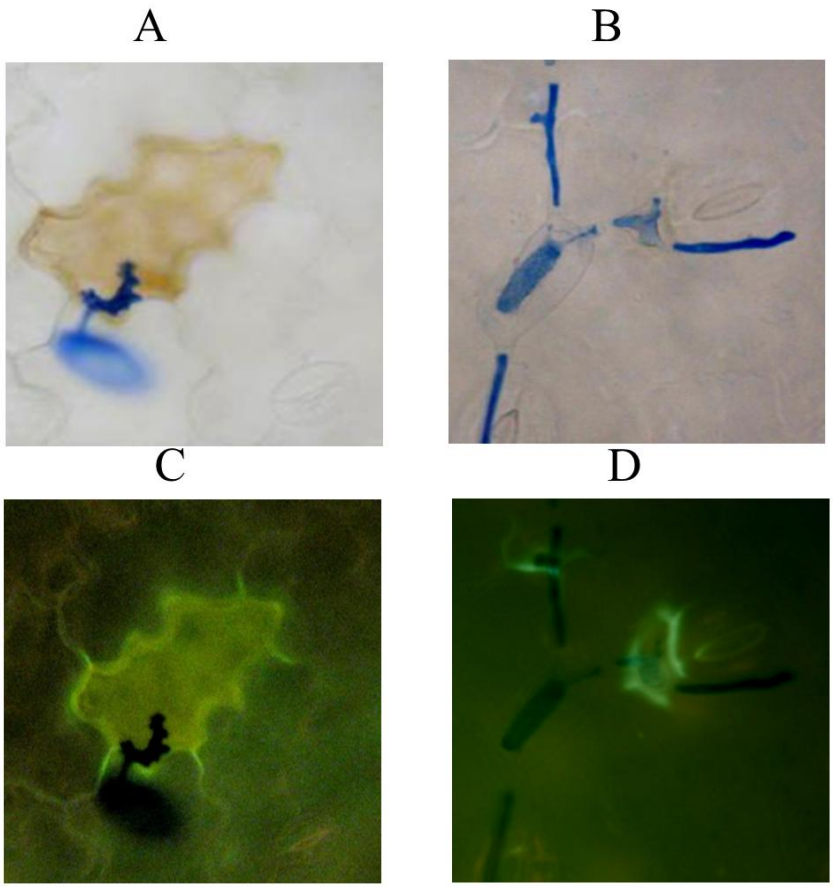


Fig.1. Death of cells attacked by *Oidium neolycopersici*. A. A tomato epidermal cell attacked by infection pegs from an appressorium of *O. neolycopersici* (stained in blue) and committed hypersensitive response (HR). B. Tomato epidermal cells which respond to *O. neolycopersici* attack by triggering slow cell death allowing limited fungal growth. C and D are the same as A and B, respectively, but under the florescent microscope. The samples for microscopy were taken 10 days after inoculation with *O. neolycopersici*.

Fine-mapping of the *Ol-1* gene

The *Ol-1* gene was previously mapped on the long arm of tomato chromosome 6 between markers P13M49 and H9A11 (Bai et al., 2005) which spans about 210 Kb on the tomato genome (Fig. 2C). In this study, screening of about 1350 BC_2S_3 plants for recombinants in this marker interval resulted in identification of several recombinants carrying four different crossing-over events (Fig. 2C). Recombinant inbred lines (RILs) were generated

by selfing these recombinants. Based on the sequences in the P13M49-H9A11 interval, new molecular markers were developed (Table 2 and Fig. 2B and C) and used to narrow

Table 1. Percentage of epidermal cells in *S. habrochites* G1. 1560 showing cell death upon fungal attack at primary penetration sites.

	48 hours post inoculation					72 hours post inoculation				
leaflet	Plant number					Plant number				
	1	2	3	4	5	1	2	3	4	5
1	70	70	80	60	70	80	70	70	80	80
2	60	90	70	60	70	80	70	80	70	90
3	70	70	70	70	70	70	80	60	80	90
4	70	80	70	60	80	100	90	70	90	80
5	80	70	60	70	70	80	70	90	90	90
Mean	70.4 ± 4.3					80.0 ± 4.9				

down the *Ol-1* locus by genotyping these four RILs. By applying disease tests, resistance or susceptibility of the RILs was determined (Fig. 2). As shown in Figure 2C, RIL1 was resistant, showing that the resistant allele of the *Ol-1* gene is present. In this RIL, markers in the chromosomal interval between markers scaff4 and P13M49 have resistant genotype, indicating that the *Ol-1* gene is located in this marker interval. In contrast, the other three RILs were all susceptible, showing the resistant allele of the *Ol-1* gene is not present. All these three RILs have susceptible genotype for markers in a chromosomal region below marker scaff6 towards H9A11, indicating that the *Ol-1* gene is below marker scaff6. Altogether, results of these four RILs showed that *Ol-1* is located between markers scaff6 and TAG, a chromosomal interval of about 73 Kb (Fig. 2D). Gene prediction programs suggested that there are at least ten putative genes in the 73 Kb *Ol-1* interval, based on the genomic sequence of Heinz 1706 cultivar which is susceptible to PM (Fig. 2D). The best hints for these genes in protein database and also in the Unigene database are presented in Table 3. One of these genes (gene number 2) has no match in the Unigene database and no match in experimentally-evidenced proteins in NCBI, suggesting that it is probably a wrongly predicted gene. The other one is a polyprotein. Interestingly, there is no *R* gene in this genomic interval.

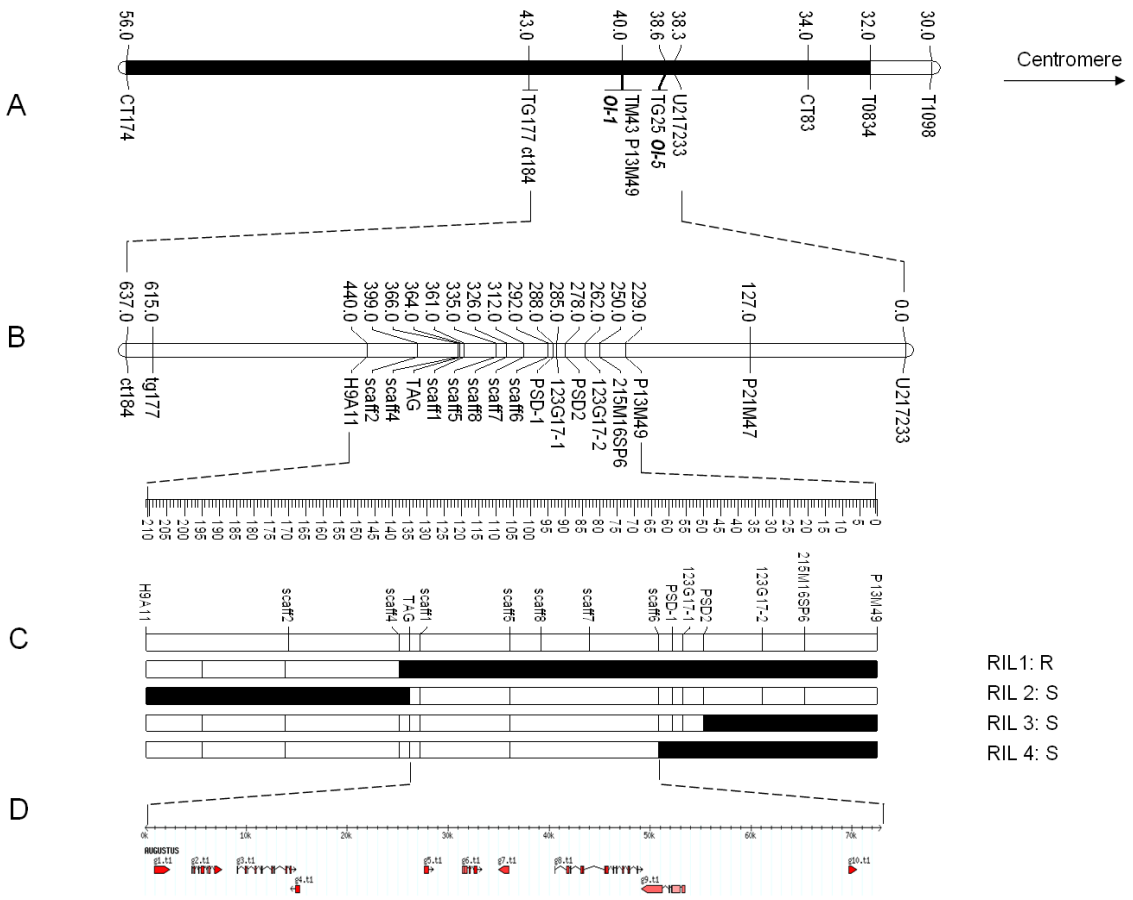
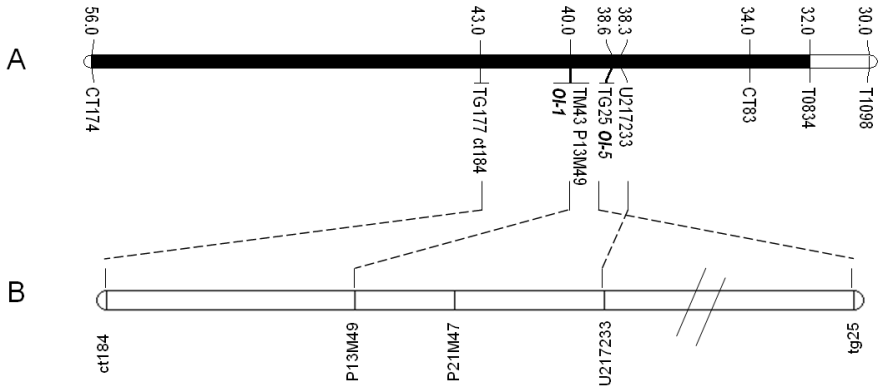


Fig. 2. Genetic and physical map of *Ol-1* locus on the long arm of tomato chromosome 6. **A**. Part of the long arm of chromosome 6 which harbors *Ol-1* and *Ol-5* loci. The introgression region in NIL-Ol-1 is shown as filled bar. **B**. Physical map of the part of the chromosome which harbors *Ol-1* locus. The relative distances in Kb are shown. **C**. Physical map of the previous *Ol-1* interval, P13M49-H9A11. The four recombinant inbred lines (RIL 1-4) which resulted in new interval for *Ol-1* (TAG- scaff6) are also shown. The phenotype of each RIL is indicated as R (resistant) or S (susceptible). The black color indicates markers are homozygous for the resistant parental genotype, white homozygous for the susceptible parental genotype. **D**. Illustrates the predicted genes in the *Ol-1* interval based on the AGUSTUS software (http://augustus.gobics.de/).

An additional locus interacts with the *Ol-1* locus

In the recombinant screening for *Ol-1* fine-mapping we encountered a controversial phenomenon. One recombinant inbred line, Line 6 in Fig. 4, homozygous for the resistant parental allele for the markers in the *Ol-1* interval was fully susceptible to PM. The genotype and susceptibility were confirmed in the selfed progeny of this line. We

compared the size of the introgression from the donor species, *S. habrochaites* in NIL-Ol-1 and in Line 6, by genotyping them with markers on the long arm of chromosome 6 (Tables 2 and 4). In NIL-Ol-1, the *S. habrochaites* introgression is between markers



lines	ct184	P13M49	P21M47	U217233	tg25	Response to PM	PCD level
1	h	h	h	h	h	IR	IM
2	a	a	a	a	h	S	none
3	a	a	a	h	h	S	IM
4	h	h	h	h	a	S	nd
5	a	a	a	h	h	S	nd
6	b	b	b	b	a	S	none
7	b	b	b	b	b	R	strong

Fig. 3. Informative recombination events in *Ol-5* region. A. The genetic map showing part of the long arm of tomato chromosome 6 harboring *Ol-1* and *Ol-5* loci. B. Genotypes and phenotypes of the recombinants identified in the region between ct184 and tg25. The relative location of the markers on the physical map is shown. The phenotype of each recombinant is indicated as IR (intermediate resistant), S (susceptible), or R (resistant). The level of cell death is shown as strong, intermediate (IM), no necrosis (none), or not determined (nd). The black color indicates markers are homozygous for the resistant parental genotype ('b' genotype), white homozygous for the susceptible parental genotype ('a' genotype), and grey as heterozygous genotype ('h' genotype). As is shown, tg25 is mapped distal to U217233, while on the physical map it is located proximal to U217233. The physical map in the region between markers tg25 and U217233 is still gapped.

ct174 and T0834 (Fig.1A), while in Line 6 the introgression starts from a region below marker ct174 and stops between markers U217233 and tg25 (Fig. 4). Previously we have shown that *Ol-5* locus is linked to *Ol-1* and mapped between tg25 and P21M47 (Bai et al., 2005). Thus, this result is striking as the introgression in NIL-Ol-1, but not that in Line 6 covers a chromosomal region where the *Ol-5* locus is located (Bai et al., 2005)

(Fig. 2A). This raised the possibility that *Ol-1* requires another locus located in the *Ol-5* interval for conferring resistance.

Table 2. Markers which were developed based on *Solanum lycopersicon* cv. MM and *Solanaum habrochites* G. 1560

Marker Name	Primer Sequence	Annealing Temp. (°C)	Polymorphisym
2C17	F- ATGCTGCAACTCCACTGATG R- TGACAGAAGCAGCAAAAGGA	55	*HypF3*I
123E21	F- ATGCCCTTTTGGTGTTCTTG R- AAGTTTGGCCTTGACACCAG	55	*Rsa*I
135H21	F- GAGCGTTGCTGAAAACATCA R- ATGCACGCTGTGTTCAGTTC	55	*HypF3*I
123G17	F-TCAAACATTTTTCCCCGTGT R-CACGAGAGTGCCAAAGAGTG	55	*HypF3*I
177K13	F-TTGGCACTGGGACATTCATA R-GCGTACACCACATGTTCAGG	55	dominant SCAR
215M16	F- TCTACGGAGGGTAGATCGTG R- GAGACTACGCCTGTAGGATC	62	dominant SCAR
PSD-1	F-ACGAGCAGCAGGTGTGATAGAC R- GTGGTTAATGAGATGGGTGGAC	62	SCAR
PSD-2	F-AAGAAATAATATCCCCATCGTG R- AACAAAAACATCCGCAGTGAGA	62	dominant SCAR
123G17-2	F-AGAATCCGCGCTACAACTACA R-ACAGGACCTGATGGAAGTGTG	55	*Spe*I
P13M49	F-TGCTAAGAATCAGAAACCACACCT R-ACAACAAGCTGATCCACCTAAAGA	56	*Xcm*I
ct184	F-TTTCCGTGTATTGCCAACAA R-ACCAAAGAGTCAATGGATGG	56	*Dde*I
tg25	F- TAATTTGGCACTGCCGT R-TTGTYATRTTGTGYTTATCG	52	SCAR
161G17	F- CATAGGTGGGCAGTGTTGAGT R- ATGGGCTTAGGAAATGAGGTG	55	*Xap*I
H9A11	F- TGCTCTAACAAAATCACCAAAATC R- AAATGGTCAAACAAAGTCTATTGAG	52	SCAR
scaff1	F- GGGACAGAAAGTCAGCTTCG R- CACTGTCGGTTCCTTCTGGT	60	*FspB*I
scaff2	F- AAATTTGGAAGGCCTCTGCT R- ATATTCCCCTGCCCAATTTC	55	SCAR
scaff5	F- ATCCTGTTCACGTCGATTCC R- CCTTGCAAACACTCTTGCTC	60	dominant SCAR
scaff4	F- CGTTTTACACCTCCGACCAT R- ACTTGGCCGTGATTAGATGC	55	*Xap*I
TAG	F- ATGCAGATCGGAACAGCACTTC R- TCATGATGATGGACTAGGCA	60	*CfrI13*I

The *Ol-5* locus needs likely *Ol-1* for resistance to PM

To verify whether *Ol-5* also requires *Ol-1* for resistance to PM we performed a recombinant screening for fine-mapping of *Ol-5*. By screening about 700 plants from a BC_2S_2 population we identified six informative recombinants representing four different

crossing-over events between markers tg25 and P21M47 (Line 2, 3, 4 and 5 in Fig. 3). We scored the disease resistance level and macroscopically the severity of cell death in

Table 3. The best hints of predicted genes in the *Ol-1* interval in protein and Unigene databases (http://solgenomics.net/).

No.	Arbitrary name	BLASTP Hints	E value	Unigene	E value
1	TGA	Triacylglycerol lipase (*Nicotiana attenuata*)	0	U603232	0
2	-	conserved hypothetical protein (*Ricinus communis*)	4.00E-27	none	none
3	UCH	UCH3 (Ubiquitin C-terminal hydroxylase3)(*Arabidopsis thaliana*)	1.00E-30	U570457	1.00E-138
4	Ole	Oleosine (*Coffea canephora*)	3.00E-45	U577353	0
5	ZT	Zinc transporter, putative (*Asparagus officinalis*)	4.00E-16	U564556	1.00E-154
6	HD-Zip	homeodomain-leucine zipper protein HD2 (*Gossypium hirsutum*)	4.00E-90	U573653	0
7	HPRG	hydroxyproline-rich glycoprotein family protein (*Arabidopsis lyrata*)	3.00E-12	U296457	3.00E-80
8	FS	Flavonol synthase/flavanone 3-hydroxylase, putative (*Ricinus communis*)	1.00E-75	U574730	0
9	-	putative polyprotein (*Solanum lycopersicum*)	0	U107559	0
10	ID	putative 3-isopropylmalate dehydrogenase small subunit (*Capsicum annuum*)	2.00E-115	U430720	2.00E-26

these lines. Results are presented in Fig. 3. Lines 2, 3 and 5 were susceptible to PM, pointing *Ol-5* to the chromosomal region below marker P13M49 towards marker ct184, where markers have the susceptible genotype. Since *Ol-1* is located in between markers P13M49 and ct184, it is possible that *Ol-5* is allelic to *Ol-1*. However, Line 4 that shows the resistant genotype for markers in the *Ol-1* interval was fully susceptible. The result of Line 4 implicated that *Ol-5* is located between markers U217233 and tg25, which is in agreement with previously obtained results that *Ol-5* was between P21M27 and tg25. Interestingly, we observed resistance in Line 1 and 7 (Fig. 3), in which the *S. habrochaites* introgression covers the chromosomal region between markers ct184 and tg25, flanking both *Ol-1* and *Ol-5* intervals. Thus, we concluded that the *Ol-5* locus

requires likely *Ol-1* for conferring resistance to PM. This conclusion is supported by the recombinant event identified in fine-mapping of the *Ol-1* gene (Line 6, Fig. 3).

Table 4. Markers which were developed based on *Solanum lycopersicon* cv. MM and *Solanaum habrochites* PI247087.

Marker Name	Primer Sequence	Annealing Temp. (°C)	Polymorphisym
23B17	F- AAGGTGCATCGAGAATGTCC R- CACACCCACACCATATCCAA	55	*Rsa*I
40F08	F- TATGCTTGCTTGGACTGTCG R- CTTGATCGGACACAACATGG	55	*Rsa*I
76N05	F-GGACATAGGTTGSGGGGCT R-GTCACAGTTCCGCTCCAGAT	55	SCAR
116O16	F- GAAAGTGAGCCATTCCCGTA R- GGCAAGAACAGAAGCAATCA	55	*HypF3*I
5H10	F-AAATCACCTTCCACAGTGCAG R- CTGGCCATAAAGTCTGGACAA	55	*Rsa*I
167M06	F- TTGGGAGAAGGGAAGAGAGAG R- AAGGAACCCACCAGTGAAATC	55	*Xap*I
U217233	F-AGGCATAGCAATTCTATGGATGGG R-TTGGAACGTGCAGCAGATTGTC	55	*Rsa*I
P13M49	F-TGCTAAGAATCAGAAACCACACCT R-ACAACAAGCTGATCCACCTAAAGA	56	*Xcm*I
ct184	F-TTTCCGTGTATTGCCAACAA R-ACCAAAGAGTCAATGGATGG	56	*Dde*I
tg25	F- TAATTTGGCACTGCCGT R-TTGTYATRTTGTGYTTATCG	52	SCAR
216A18	F-CACCACAAGGCTACCATCCT R-GCATTACATGGGTTGGGTTG	58	*Sdu*I

Further, the cell death level, measured as necrotic spots surrounding fungal colonies, varied between these lines. Plants of Line 7 carrying *S. habrochaites* alleles of both loci in homozygous status were completely resistant to PM and showed a very severe cell death (Line 7 in Fig. 3B). Plants of Line 1 harboring heterozygous *S. habrochaites* alleles of both loci showed intermediate levels of resistance accompanied with also an intermediate level of cell death (Line 1 in Fig. 3). However when the *S. habrochaites* introgression does not cover the *Ol-5* interval, the cell death was abolished (Line 6, Fig. 3). On the other hand, when the *S. habrochaites* introgression does not cover the *Ol-1* locus (Line 3, Fig. 3), there was a level of cell death, but no PM resistance.

Functional analysis of the putative genes in the *Ol-1* interval

To identify which of the putative genes is the *Ol-1* gene, we performed virus-induced gene silencing (VIGS) experiment to silence transiently all the genes in the *Ol-1* interval except for the putative zinc transporter, because we only identified this gene in the later genome sequence release. For each gene, one construct was made (Table 5) and applied

Table 5. Primers used for silencing constructs in virus-induced gene silencing experiments.

Gene	Primers Sequence	Amplicon Size (bp)
ID	F- TGTTTCCAGTTCCATCACCA R- GATGATCCGCATCCAAAGTT	438
DDS	F- TGGGCTTAGGAAATGAGGTG R- CAACCAAAGTCCCATGATCC	411
HPRG	F- GCCAGTGGTTGTTGTTGCTA R- CTCTTGCAGCCCCTATGAAC	413
HLZ	F- AAAGAAGATCTGGGGTTGAGC R- AATAGTGCTGTTCGGAGACGA	255
Ole	F- ATGAGCAGCACCTTTCTCGT R- TAATCTGCTGCTGTGCTTGG	378
UCH	F- GCGTATGCAGTTCTTGGTGT R- TCACGACAGCAGCCAATAAA	260
PLA	F- GATTCAACGAATGGGAAGGA R- AATCATCTGCGACCAAAAGG	314
HLZutr	F- GGGGTCTAGAAAATCTCTTGC R- CTCTTCTCCACTCCCCACAA	146
FS	F- AATCGACCTTACTTCCCCTGA R- AATCCTCCATGGTTGATCTCC	494

on seven plants of MM and NIL-Ol-1 for silencing. The number of PM colonies was counted for NIL-Ol-1, but not for MM due to the spreading of colonies. All MM plants were heavily infected by PM, showing that silencing these nine genes did not influenced the susceptibility of MM. To compare the effect of silencing *Ol-1* candidate genes on PM sporulation in NIL-Ol-1, colony numbers were compared between plants in which a specific candidate gene was silenced and control plants infiltrated with an empty vector (TRV). Transient silencing is not homogenous and the rate of silencing is variable

between different plants. Assuming that silencing a given gene results in increased PM sporulation, we expect to observe more sporulation on those plants in which the given gene is silenced better. For each gene we selected 3 plants with the highest number of PM colonies. Then the average numbers of colonies on these 3 plants were compared to the average colony number of the 3 empty vector plants which showed highest sporulation rate. Results showed that silencing all of these genes compromised resistance in NIL-Ol-1 at different levels (Fig. 4A). The highest number of PM colonies was observed on plants in which the homeodomain-leucine zipper (HD-Zip) transcription factor gene was silenced.

Since our fine-mapping results suggested that *Ol-5* requires likely *Ol-1* for conferring resistance to PM, we expect that silencing the potential candidate of *Ol-1*, HD-Zip would also compromise *Ol-5*-mediated resistance. We further preformed a VIGS experiment to silence the HD-Zip gene in NIL-Ol-5 that carries a *S. habrochaites* introgression covering both *Ol-1* and *Ol-5* intervals. Results demonstrated that silencing HD-Zip also compromised the PM resistance in NIL-Ol-5 (Fig. 4B), supporting the conclusion drawn from the fine-mapping results that *Ol-1* and *Ol-5* jointly confers PM resistance.

Discussion

By fine-mapping of *Ol-1* and *Ol-5*, we demonstrated that *Ol-1* mediates delayed cell death, by interacting with the *Ol-5* locus. The fact, that *Ol-1* confers resistance to a broad range of PM isolates and that no *R* genes are identified as the candidate for *Ol-1*, suggests that *Ol-1* is an enhancer of the basal defense. Transient gene silencing results suggested that a homeodomain-leucine zipper transcription factor is probably involved in resistance mediated by both *Ol-1* and *Ol-5*.

During the recombinant screening for *Ol-1* and *Ol-5,* we found that an *S. habrochaites* introgression covering both *Ol-1* and *Ol-5* intervals is required for PM resistance showing that these two genes require each other for their function. In those plants where the *S. habrochaites* introgression covers only the *Ol-1* interval, plants were completely susceptible and showed no necrotic spots (Line 6 in Fig. 3), suggesting that *Ol-5* is the gene which potentiates cell death. On the other hand, when only the *Ol-5*

interval is introgressed from *S. habrochaites*, an intermediate level of necrotic spots were observed which could not prevent PM growing (Line 3 in Fig. 3), indicating that *Ol-1* regulates the timing of cell death occurrence. Presence of *S. habrochaites* alleles of both loci resulted in strong cell death associated with complete resistance (Line 7 in Fig. 3). Thus, we concluded that *Ol-1* and *Ol-5* coordinate jointly the effective cell death upon PM infection and therefore confer resistance.

In the *Ol-1* interval of 73 Kb, 10 genes are predicted (Table 3). This prediction is based on the sequence of a tomato cultivar susceptible to PM, so we cannot exclude the possibility of the presence of additional gene(s) in this interval in the resistant line. VIGS experiments showed that silencing these genes in NIL-Ol-1 compromised resistance to PM with different levels (Fig. 4). Nevertheless, the highest PM colony number was observed on the HD-Zip-silenced plants of NIL-Ol-1. Moreover, silencing this HD-Zip gene compromised resistance in NIL-Ol-5 (Fig. 4), showing that this gene is also required for *Ol-5*-mediated resistance. This supports the results obtained in fine-mapping that *Ol-1* and *Ol-5* jointly confer PM resistance. This HD-zip belong to the class II of HD-Zip transcription factors featured by their CPSCE motif, located downstream of the leucine zipper motif and is involved in sensing the changes in the cellular redox status (Chan et al., 1998; Tron et al., 2002; Ariel et al., 2007). Such a transcription factor, NbHB1, was shown to be a positive regulator of pathogen-induced cell death in *Nicotiana* via JA signaling pathway (Yoon et al., 2009). Another HD-Zip transcription factor located on the tomato chromosome 4, is involved in limiting the cell death in response to pathogen and its silencing resulted in cell death due to oxidative burst (Mayda et al., 1999). Based on these evidence, we speculate that the HD-Zip transcription factor in the *Ol-1* interval plays a role in regulating the cell death and thereby, conferring resistance to PM.

Two major kinds of cell death have been described in plant cells, apoptosis-like PCD (AL-PCD) and autophagic PCD, and timing is the factor determines which kind of cell death should be undergone (Love et al., 2008). HR is a form of AL-PCD cell death (van Doorn and Woltering, 2005; Hofius et al., 2009) which is typically triggered upon recognition of pathogen avirulence (Avr) proteins by plant resistance (R) proteins (Nimchuk et al., 2003). Previously, the cell death in *S. habrochaites* (Huang et al., 2000)

and NIL-Ol-1 (Bai et al., 2005; Li et al., 2007) have been defined as slow HR. In this study, we would like to suggest that the cell death associated with *Ol-1*-mediated resistance resemables autophagic PCD, and is not HR. Our suggestion is based on the fact that the cell death mediated by *Ol-1* is slow and not complete, which is discernable from the HR mediated by *Ol-4*, an *R* gene. Furthermore, transient gene silencing results suggested that a homeodomain-leucine zipper transcription factor is probably involved in resistance mediated by both *Ol-1*. Autophagic PCD is believed to be an exaggerated form of autophagy and happens during senescence or those responses that do not demand a fast reaction (Love et al., 2008). Autophagy is an evolutionary conserved process in eukaryotes in which unwanted cellular components (including proteins, lipids, entire organelles and invading pathogens) are engulfed by double-membraned vesicles called autophagosomes and delivered to lysosomes where they are destroyed (Levine and Ranganathan, 2010). It has been shown that autophagy negatively regulates cell death in plant cells and limit it to the pathogen-infected cells (Diaz et al., 2005; Yoshimoto et al., 2009). Further investigations are needed to characterize the cell death triggered by *Ol-1* and *Ol-5* in more details, in order to get a better idea of the involvement of autophagy in the pathogen resistance mediated by these genes.

Based on gene expression profiling, our previous results suggested that the molecular events underlying *Ol-1*-mediated response to PM is more similar to the response which occurs in MM than that triggered by *Ol-4* (Li et al., 2007). Moreover, MM and NIL-Ol-1 showed similar fungal growth patterns as defined by the number of primary haustoria, hyphae, and secondary appressoria and haustoria per infection unit, with the exception that cell death happens in NIL-Ol-1 preventing further pathogen progress (Li et al., 2007). All these evidence suggest the delayed cell death associated with *Ol-1*-mediated resistance is not dependent on R-Avr protein cognition. The fact that no *R* genes are identified as the candidate for *Ol-1*, suggests that *Ol-1* is an enhancer of the basal defense.

Apart from the HD-Zip gene, silencing the other seven genes in *Ol-1* interval also compromised resistance to PM with lower levels (Fig. 4). One explanation for this observation may be the contribution of these genes to basal defense. For example, Hydroxylproline-rich glycoprotein (HRGP) are one of the main protein groups in plant

cell walls, which cross-linking strengthens the cell wall and therefore provides added resistance to pathogen-derived cell wall depredating enzymes (Bradley et al., 1992).

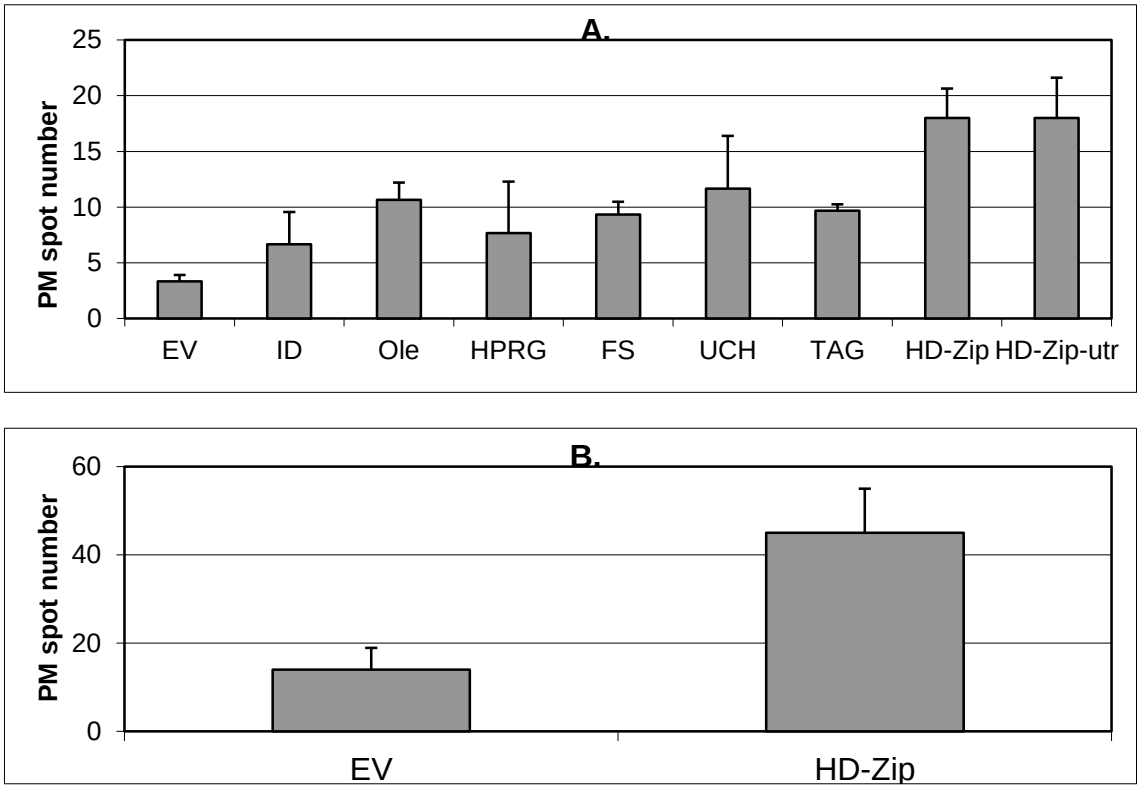


Fig. 4. Silencing the predicted genes in the *Ol-1* interval compromised resistance in NIL-Ol-1 with different levels (A). Silencing the HD-Zip gene reduced the level of resistance in NIL-Ol-5 (B). Seven plants per construct were infiltrated with TRV empty vector (EV, as the control) or silencing constructs harboring a small fragment of the target gene. Two weeks after sowing, agroinfiltration was done on the cotyledonary leaves and 2 weeks later, PM spores were sprayed on the plants. Fungal colonies were counted two weeks after fungal inoculation. For each construct, the average number of colonies on three plants bearing the highest colony number was calculated. The result is shown on the graph. Error bars show standard deviation.

Ubiquitin carboxyl-terminal hydrolase (UCH) enzyme is involved in protein deubiquitination (Wilkinson, 1997). The role of ubiquitination in plant immunity has attracted more attentions recently (Trujillo and Shirasu, 2010). It would be also expected that reverse reaction, deubiquitination, play a role in plant immunity, for instance, by regulating phytohormones signaling pathways. *FS* encodes a putative 2OG-Fe(II) oxygenase. Such a gene, *DMR6*, has been reported to enhance Arabidopsis susceptibility

to *Hyaloperonospora parasitica* (Van Damme et al., 2008). However, in our pathosystem, *FS* seems to be positively involved in PM resistance in tomato since silencing *FS* resulted in partial loss of resistance conferred jointly by *Ol-1* and *Ol-5*. We expect that oxygenase activity of FS is involved in regulating the ROS levels in the cell and thereby, influence the PCD associated with resistance mediated by *Ol-1* and *Ol-5*. *ID* encodes the small subunit of 3-isopropylmalate/(R)-2-methylmalate dehydratase. This enzyme is involved in amino acids valine, leucine, and isoleucine biosynthesis pathway. This gene also has an aconitase domain which has been shown to be involved in the activation of both resistance-associated HR and disease-associated cell death (Moeder et al., 2007). *Ole* encodes a protein with similarity to Oleosine proteins which are structural proteins in oilbodies (Frandsen et al., 2001), containing triacylglycerol (TAG). TAG is the substrate for glycerolipid pathway in chloroplast which is necessary for induction of systemic acquired resistance (Nandi et al., 2004) and is a source of production of molecules like phosphatidic acid, a well-known signaling molecule in plant defense (Distéfano et al., 2010). In *Nicotiana attenuate*, it has been shown that TAG is essential for JA biosynthesis (Kallenbach et al., 2010). Based on above listed evidence we conclude that silencing these genes would impair the basal defense to certain level and thus reduce the level of *Ol-1*-mediated resistance.

In sum, our data suggest that *Ol-1* mediates autophagic PCD, but not HR, against tomato PM. The *Ol-1* gene is affected by *Ol-5*, another locus conferring resistance to PM in tomato. Future work focused on the fine-mapping of the *Ol-5* gene in populations where the *Ol-1* gene is present will help to identify candidates for the interacting genes. Further, monitoring the expression of the genes regulating autophagy in NIL-Ol-1 and NIL-Ol-5 and in the RILs carrying both *Ol-1* and *Ol-5* genes, will shed light on the regulation of autophagy by these genes which results in delayed cell detah and thereby, resistance to the pathogen. Moreover, experiments on functional analysis need to be performed to verify whether *Ol-1* encodes a homeodomain-leucine zipper transcription factor.

Materials and Methods

Plant materials

We used a BC_2S_3 population developed from crossing *Solanum lycopersicum* cv. Moneymaker (MM) and *S. habrochaites* G.1560 for recombinant screening in the *Ol-1* fine-mapping experiments. For fine-mapping of the *Ol-5* gene, a BC_2S_2 population derived from the crossing of MM with *S. habrochaites* PI247087 was used. In both cases, MM was used as the recurrent parent. Near-isogenic lines (NILs) harboring the *Ol-1* and *Ol-5* genes (Bai et al. 2005) in the genetic background of MM were used.

Powdery mildew assay

For disease tests, Wageningen isolate of *O. neolycopersici* was used (Bai et al., 2005). Fungal spores were washed off from heavily infected tomato leaves and diluted to the concentration of 2.5×10^4 spores per mL. The inoculum was evenly sprayed on the 4-weak-old plants. We scored the plants based on the macroscopic observation of PM sporulation. Plants showing no sporulation were scored as resistant. Plants with a high level of sporulation, comparable to that on the susceptible control MM plants, were considered as susceptible. Plants showing sporulation with necrosis surrounding the colonies, were considered as intermediate resistance.

Microscopic observation

To monitor cell death, Japanese isolate KTP-01 was used for inoculation (Kashimoto et al., 2003). Inoculation was conducted by collecting conidia on conidiophores with an electric spore collector and suspending them in water containing Mixpower (10^{-5} diluted) to produce inoculum (Nonomura et al., 2009). Tested leaves (3^{rd} and 4^{th} true leaves) of one-month-old plants were spray-inoculated. Two and three days post inoculation (dpi), five leaflets of the 3^{rd} and 4^{th} true leaves were harvested from five individual plants, decolored in a boiling alcoholic lactophenol solution for 1–2 min, and stained with aniline blue, according to a previous method (Sameshima et al., 2004). The leaves were observed under an Olympus light and fluorescence microscope (B excitation, B absorption filter and O-515 barrier filter). Ten conidia per leaflet were observed.

Marker development

Based on the bacterial artificial chromosome (BAC) sequences which were available in the region of interest, primers were designed by using Primer 3 software (Rozen and Skaletsky, 2000). If amplified fragments derived from MM and NIL-Ol-1 or NIL-Ol-5 were not polymorphic, PCR products were digested with different restriction enzymes to reveal polymorphism (Tables 2 and 4).

Gene prediction and annotation

The gene prediction for the *Ol-1* interval (73 Kb) was done by using AGUSTUS (http://augustus.gobics.de/) and Fgenesh (http://linux1.softberry.com/berry.phtml) web-based gene prediction softwares. Then the predicted amino acid sequence were used for BLASTP against protein database in NCBI. To check if these predicted genes are expressed, the predicted cDNA was used for BLASTN against tomato Unigene database (http://solgenomics.net/) (Table 3).

Virus-induced gene silencing (VIGS)

The TRV vector that has been developed for transient gene silencing in tomato (Liu et al., 2002b) was used. In brief, the TRV vector carrying a fragment between 150-500 bp of the cDNA of each of the 9 candidate genes (primer used for preparing the construct are listed in Table 5) in the *Ol-1* interval, was transformed into *Agrobacterium tumefaciens* strain GV3101. Agroinfiltration was done on cotyledons of 10 day-old seedlings using needle-less syringe and plants were maintained at 19-21°C. After 21 days, the plants were inoculated with tomato powdery mildew.

Chapter 5

Tomato recruits phytohormone pathways in different ways to withstand *Oidium neolycopersici*

Alireza Seifi, Zhe Yan, Zheng Zheng, Luigi Faino, Stefano Pavan, Fien Meijer-Dekens, Richard G.F. Visser and Yuling Bai

Laboratory of Plant Breeding, Wageningen University, PO box 386, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands

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Tomato recruits phytohormone pathways in different ways to withstand *Oidium neolycopersici*

Abstract

Phytohormones are involved in different aspects of plant life including response to biotic stresses. We investigated the role of different phytohormones, salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), and ethylene (ET) in tomato in response to *Oidium neolycopersici* infection. A set of tomato (*Solanum lycopersicum*) near-isogeneic lines (NILs) which carry different resistance genes were used. These NILs show different levels of resistance that are based on hypersensitive response (HR), delayed cell death (DCD), callose deposition and also a combination of DCD and callose deposition. First we monitored the trend of the phytohormone pathways by quantification of the expression of marker genes for these pathways. Second, we crossed the NILs with tomato mutants that have altered responses to a subset of these phytohormones. Our results suggest that the SA pathway is the main hormone pathway that is recruited in HR-based resistance. For the resistance associated with DCD, the ethylene pathway is essential. The ABA pathway is crucial for the resistance mechanism relying on callose deposition. Our data provide a comparative analysis on the contribution of different phytohormone pathways to different forms of plant defense mechanisms in the same pathosystem, tomato and *O. neolycopersici*.

Introduction

Plant-pathogen studies based on gene-specific interactions have distracted attentions from the role of phytohormones in plant responses to attacking pathogens. Recent findings show that to establish a successful defense response, plants need to attenuate any pathogen-induced hormone perturbations (Grant and Jones, 2009). Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathways are considered as the backbone of phytohormone networks in plant immune system, with which auxin, abscisic acid (ABA), and gibberellic acid pathways interact (Pieterse et al., 2009). In general, SA and JA are believed to be signaling molecules in defense against biotrophic and necrotrophic pathogens, respectively (Glazebrook, 2005). SA pathway is well-documented as an essential component in effector-triggered immunity (ETI), PAMP-triggered immunity (PTI) and systemic acquired resistance (SAR) (Vlot et al., 2009). JA in the presence of low levels of ET is only able to trigger response to herbivores and wounding, while in combination with high ET levels, it triggers responses to necrotrophes 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 signaling pathways (Anderson et al., 2004).

Tomato response to tomato powdery mildew (PM), *Oidium neolycopersici*, is conditioned by several resistance genes including *Ol-1*, *ol-2*, *Ol-4*, and *Ol-qtls* which trigger different resistance mechanisms (Bai et al., 2003; Li et al., 2006). *Ol-1* enhances basal defense by inducing delayed cell death (DCD) in the late stages of pathogen infection (Li et al., 2007; Chapter 4). A recessive gene, *ol-2*, which encodes a membrane protein homologous to barley *mlo*, mediates resistance to PM by inducing callose deposition and cell wall fortification to stop PM at penetration stage (Bai et al., 2008). We also have shown that an *R* gene, *Ol-4*, which is an NBS-LRR gene homologue to *Mi-1* (Chapter 2) triggers hypersensitivity reaction (HR) and thereby prevents the PM colonization after formation of primary haustoria. Interestingly, a combination of callose deposition and cell death is triggered by three combined quantitative trait loci, *Ol-qtls*, conferring resistance to PM (Bai et al., 2003; Li et al., 2006). We had previously developed near-isogenic lines (NILs) harboring *Ol-1*, *ol-2*, *Ol-4* and *Ol-qtls* (NIL-Ol-1,

NIL-ol-2, NIL-Ol-4 and NIL-Ol-qtls, respectively) in the genetic background of *S. lycopersicum* cv. Moneymaker (MM) which is susceptible to PM (Bai et al., 2003).

In this study, we took advantage of the above mentioned tomato-PM interactions to study the involvement of phytohormone pathways in four different kinds of plant defense mechanisms. Two complementary strategies were adopted in this work. First we monitored the expression of marker genes for different phytohormone pathways by using NILs that carry each of the different *Ol* genes (including *Ol-1*, *ol-2*, *Ol-4* and *Ol-qtls*). Then we evaluated whether PM resistance in these NILs would be compromised if JA, ET and ABA pathways are impaired. We show in this chapter that SA, JA, ET and ABA pathways are involved differently in the different tomato-PM interactions.

Results

To monitor changes in JA, SA, ET, and ABA pathways in different tomato-PM interactions, we measured the expression level of marker genes (Table 1) for these pathways in different tomato NILs and in MM, in a time-course from 1 to 9 days post inoculation (dpi) with PM. For NIL-Ol-qtls, the time-points were 1, 5 and 7 dpi. The level of marker genes expression was quantified by quantitative RT-PCR. The results showed differences in the trend and in the induction magnitude of some of these pathways among NILs and MM (Fig.1 and Fig. 2).

Early and late induction in SA pathway

SA induces expression of a group of pathogenesis-related genes (*PR* genes) in Arabidopsis, among them *PR-2* is often used as a marker gene for SA pathway (Uknes et al., 1992). Tomato *PR2* (*TomQ'b*) gene (Domingo et al., 1994) was shown to be induced in response to BTH, an analog of SA (Beyer et al., 2001). Therefore, we used this *PR2* as a marker gene for SA pathway in this study. The trend of SA pathway fluctuations, based on the changes in the expression of this *PR-2*, is depicted in Figure 1A and Figure 2A. At 1 dpi, there was an induction in the *PR-2* expression in NIL-Ol-4 and NIL-ol-2. In NIL-Ol-1 and MM (Fig. 1A) this induction was very low, and in NIL-Ol-qtls it was absent (Fig. 2A). At the latest time-point (9 dpi), *PR-2* expression increased in MM, NIL-Ol-1 and NIL-Ol-4, with the highest level in NIL-Ol-1 (Fig. 1A). As to NIL-Ol-qtls,

considerable induction in *PR-2* expression was observed at 7dpi, which was earlier than that in the other lines (Fig. 2A).

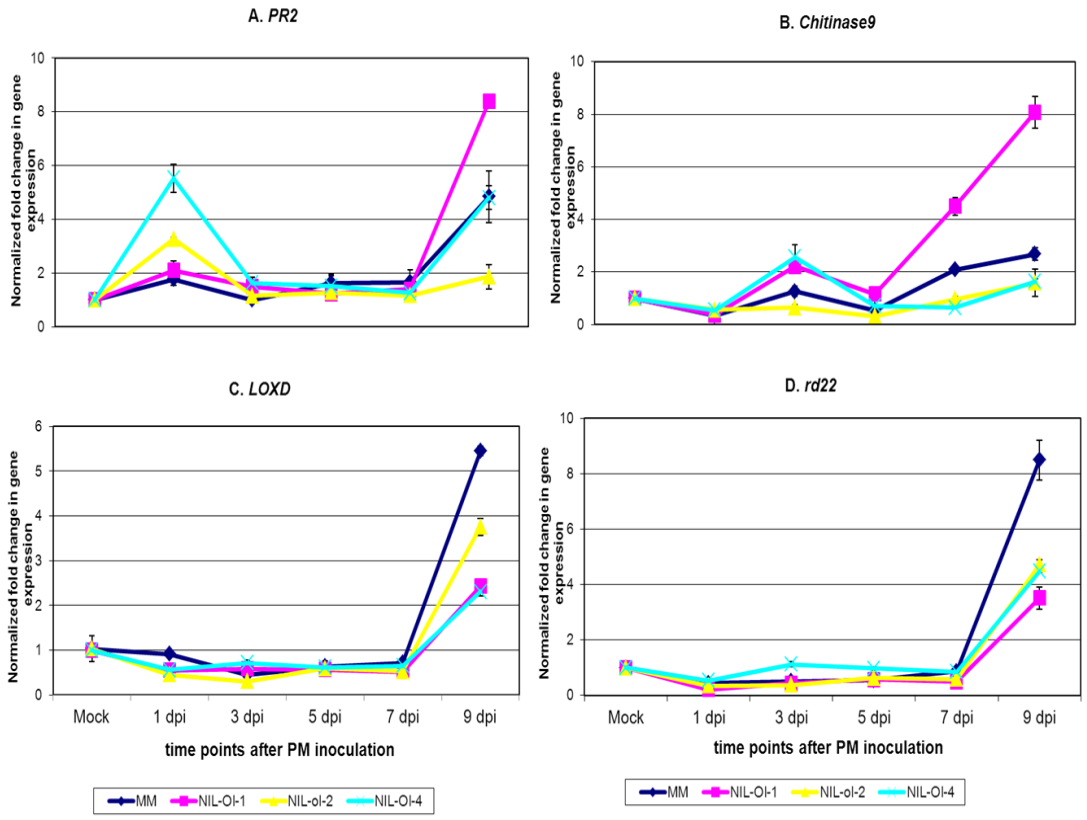

Fig.1. Expression of *PR-2* (A), *Chitinase9* (B), *LOXD* (C), and *rd22* (D), in MM, NIL-Ol-1, NIL-ol-2, and NIL-Ol-4 in a time-course after inoculation with PM. These genes are the markers for SA, ET, JA, and ABA pathways, respectively. Second and third leaves were sampled at 1, 3, 5, 7, and 9 days post inoculation (dpi) from powdery mildew-inoculated and -non-inoculated (Mock) plants. RNA extracted from these leaf samples were used for monitoring the expression of the marker gene. Error bars show standard deviation.

Late induction in ET pathway in NIL-Ol-1 and NIL-Ol-qtls

The ET pathway was monitored based on the expression of the *Chitinase9* (*Chi9*), which has been used as a marker gene for ET pathway in toamto (Barry et al., 2001). As shown in Figure 1B, the expression level of *Chi9* was rather constant during the time-course in all lines except in NIL-Ol-1 and NIL-Ol-qtls. After 5 dpi, the expression level of *Chi9* increased steadily in NIL-Ol-1 and NIL-Ol-qtls (Fig. 2B).

Induction of ABA and JA pathways at the late time-points

The *lipoxygenase D* (LOXD) has been shown to be induced by jasmonic acid in tomato (Heitz et al., 1997), thus we used this gene as a marker for JA pathway. In Arabidopsis, *rd22* is an ABA-responsive gene (Shinozaki et al., 2003). We found the ortholog of *rd22* in tomato (see M&M) and used it as a marker gene for ABA pathways in our study. Results showed that the trends in ABA and JA pathways were similar in all the lines (Fig.1C and D). These trends included a steady-state level of expression of *rd22* and *LOXD* till 7 dpi, followed by an exponential induction at 9 dpi. The highest level of this induction occurred in MM for both *rd22* and *LOXD*. We monitored the marker genes expression only till 7 dpi in NIL-Ol-qtls (Fig. 2C, D), thus presence or absence of this late exponential induction is not known in NIL-Ol-qtls.

Effect of hormone mutants on the resistance mediated by different *Ol* genes

In order to evaluate the effect of phytohormones on different interactions of tomato with PM, we tested the effect of impairing the pathways of these hormones on resistance mediated by different *Ol* genes, as well as on basal resistance (represented by the level of resistance in MM, the susceptible line). Therefore, we crossed different NILs that carry different *Ol* genes (including *Ol-1*, *ol-2*, *Ol-4* and *Ol-qtls*) and MM, with available tomato hormone mutants; *def1* (JA-deficient) (Howe et al., 1996), *not* (ABA-deficient) (Burbidge et al., 1999), *epi* (ET overproducer) (Fujino et al., 1988), and *Nr* (ET-insensitive) (Wilkinson et al., 1995). Except *Nr*, all the other mutants are recessive, and therefore, we evaluated the F_2 generations of these crosses for response to PM. We selected F_2 plants which were homozygous for each *Ol* gene *(Ol/Ol)* based on the markers that are closely linked to these genes (see M&M). These selected F_2 plants were expected to be all resistant to PM if resistance in these NILs was independent of JA, ET and ABA pathways. As we expected that the mutant locus is randomly segregating among these selected F_2 plants, if the response to PM was also segregating among the selected F_2 plants, we could conclude that the mutant has influenced the response to PM. From each population we selected 16 plants homozygous for the corresponding *Ol* gene and tested them for the response to PM. In the case of *epi* mutant, it was possible to select also for the homozygous mutant (*epi/epi*) based on the plant morphology; dark-grown

epi/epi seedling were shorter and thicker ((Fujino et al., 1988) and Fig. S1). Therefore, from the F_2 populations derived from crosses with *epi* mutants, we selected at least three plants homozygous for both *Ol* and *epi* (*Ol/Ol/epi/epi*) and tested them for response to PM.

These mutants and their background lines were all susceptible to PM. We did not observe discernable effect of these mutants on the level of susceptibility of MM, while the resistance mediated by some of the *Ol* genes was compromised in crosses with some of these mutants, as described below.

Table 1. Marker genes used for monitoring different signaling pathways in this study.

Marker gene	Pathway	Primer sequences (5'- 3')
PR-2	SA	GCTACATACTCGGCCCTTGA
		TGTTGTAAGTCCTCGCGTTG
LOXD	JA	TTGTGCCTGAAAAAGCAGTG
		GTTCTAGCGCGACATTCCTC
rd22	ABA	ACGTGGCGTTATTTTTCCTG
		ATCTCCGGCATCTTCTCTGA
Chitniase9	ET	GAAATTGCTGCTTTCCTTGC
		AGTAATCGCCAGGGCTACCT
GAST1	GA	AGCAGCAGCAACAACAGAGA
		TCTTTGAACACCGGTACGTG
GH3	Auxin	GCCAACAACAGAGGAAGAGC
		TACATTCCTTTGCCCGTCTC

JA-deficiency compromises *ol-2*-mediated resistance

Segregation of response to PM was observed among 16 selected F_2 plants derived from the cross between NIL-ol-2 and *def1* mutant line. As mentioned in Table 2, the ratio of susceptible (S) to resistance (R) was 1: 3 which agrees with a ratio expected for a segregating recessive gene. Thus, we concluded that *def1* mutation, and thereby JA-deficiency, compromised resistance mediated by the *ol-2* gene. None of the selected F_2 plants from the cross between NIL-Ol-4 and *def1* mutant line was susceptible, suggesting that JA-deficiency had no obvious effect on the *Ol-4*-mediated resistance. The F_2 plants carrying *Ol-1* gene and segregating for *def1* locus, were all as resistant as NIL-Ol-1, suggesting that there is no influence of *def1* mutation on *Ol-1*-mediated resistance.

ABA-deficiency compromises *ol-2*- and *Ol-qtls*-mediated resistance

Susceptible plants were found in the selected F_2 plants from the crossing of *not* mutant with NIL-ol-2 and NIL-Ol-qtls (Table 2). The ratio of S to R plants agrees with a 1: 3 ratio (χ^2= 0.33, *P* >0.05), suggesting that recessive *not* mutation compromised resistance mediated by *Ol-qtls* and *ol-2*. No susceptible plants were observed among the F_2 plants selected from crossing between *not* mutant with NIL-Ol-1 and NIL-Ol-4.

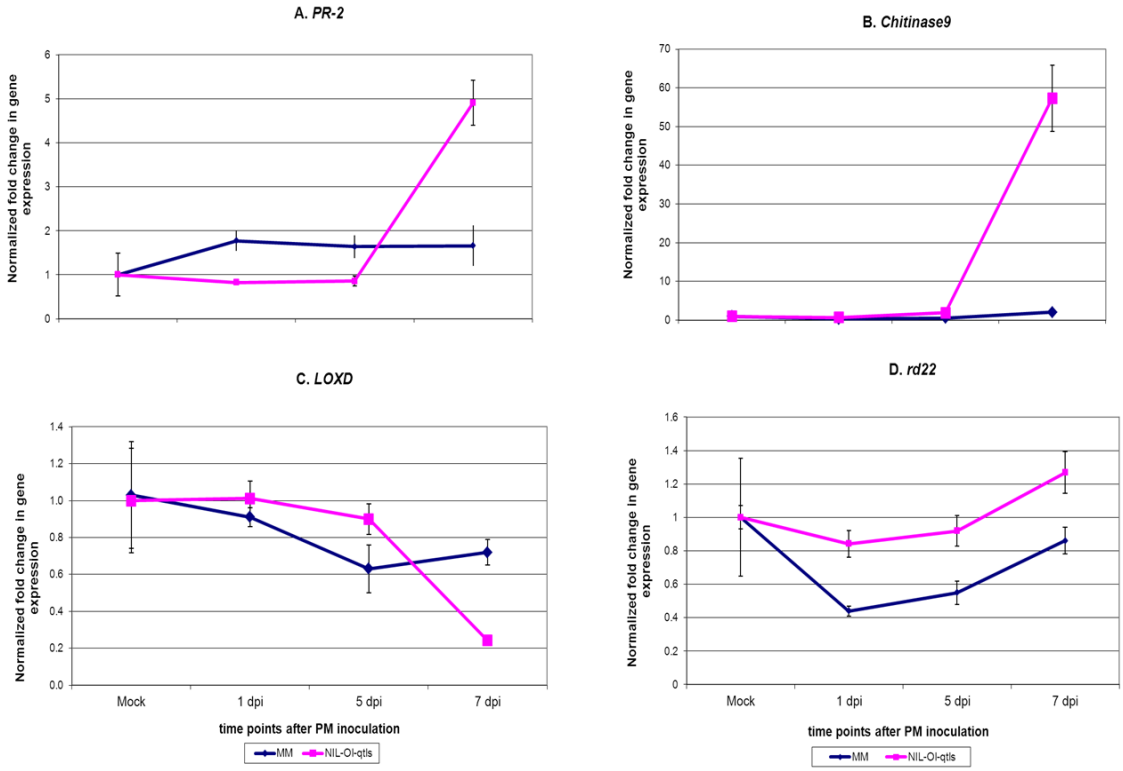

Fig.2. Expression of *PR-2* (A), *Chitinase9* (B), *LOXD* (C), and *rd22* (D) in MM, and NIL-Ol-qtls in a time-course after inoculation with PM. These genes are the markers for SA, ET, JA, and ABA pathways, respectively. Second and third leaves were sampled at 1, 3, 5, 7, and 9 days post inoculation (dpi) from powdery mildew-inoculated and -non-inoculated (Mock) plants. RNA extracted from these leaf samples were used for monitoring the expression of the marker gene. Error bars show standard deviation.

ET mutants influence PM resistance conferred by *Ol-1* and *Ol-qtls*

For the ET pathway we had two mutants, one was *Nr* that decreases tomato cell sensitivity to ET (Lanahan et al., 1994) and the other one was *epi*, which has been

described as ET-overproducing mutant (Fujino et al., 1988). We found susceptible plants among the 16 plants selected from the F_2 populations derived from crosses of *Nr* mutant with NIL-Ol-1 and NIL-Ol-qtls. Since *Nr* is a dominant mutation (Lanahan et al., 1994), we expected to observe 75% of the selected F2 plants to be susceptible if ET mutant had a negative effect on the response to PM. However as shown in Table 2, this was not the case since the number of susceptible plants was less than that was expected. From crosses of the *Nr* mutant and NIL-o*l-2* or NIL- *Ol-4*, no susceptible plants were observed among the selected plants.

In crosses with *epi* mutant, susceptible plants were also found for NIL-Ol-1 and NIL-*Ol-qtls*, but not for NIL-ol-2 or for NIL-Ol-4 (Table 2). Though all the selected F_2 plants showed *epi* phenotype, they were not all susceptible. As mentioned in Table 2, out of seven plants which were homozygous for the *Ol-1* gene and showed *epi* phenotype, two were fully susceptible and the other five showed the same level of resistance as NIL-Ol-1. Also, from three plants homozygous for *Ol-qtls* and showing *epi* phenotype, two were fully susceptible and one was as resistant as NIL-Ol-qtls.

Table 2. Summary of the disease evaluation on the selected F_2 plants derived from crosses of hormone mutants with different tomato lines. 'S' stands for susceptible, 'R' stands for resistant.

	MM	NIL-Ol-1	NIL-ol-2	NIL-Ol4	NIL-Ol-qtls
def1	16S	16R	4S:12R	16R	nd[a]
not	16S	16R	3S:13R	nd[b]	4S:12R
Nr	16S	1S:15R	16R	16R	6S:10R
epi[c]	5S	2S: 5R	1S: 5R	5R	2S:1R

[a]not determined because the F_2 population for this cross was not available.
[b]lethality was observed in the F2 population derived from this cross. As the ratio suggested (1/4), probably homozygous genotype for *not* is not tolerated in this population.
[c]in all crosses, 16 plants homozygous for individual *Ol* gene were selected. In the case of *epi* mutant, it was further possible to select for *epi* mutant, therefore we selected at least 3 plants which were homozygous for *epi* and also the *Ol* genes.

The response of plants to pathogens is a continuum of possible responses from extreme resistance to extreme susceptibility (Glazebrook, 2005). The tomato–PM pathosystem provides an opportunity to investigate such a wide range of different responses. We performed a comparative analysis of phytohormone pathways in different kinds of response of tomato to PM, from susceptibility in MM to intermediate levels of resistance in NIL-Ol-1 and NIL-Ol-qtls, to a high level of resistance in NIL-ol-2 and

complete resistance in NIL-Ol-4. On one hand, we compared trends of several phytohormone pathways in different NILs based on the appropriate marker genes expression (Table 1) during their interaction with PM. On the other hand, we crossed these NILs with available tomato hormone mutants and evaluated the effect of the mutants on the response to PM.

A level of resistance which is present in a susceptible plant is called basal resistance (Glazebrook, 2005; Jones and Dangl, 2006). MM is the background of the NILs were used in this experiment and is susceptible to PM, therefore, the level of resistance in MM represents basal resistance in our study. We did not observe any discernable effect of the tomato mutants on the basal resistance. However, others reported that for example, ABA-deficiency (Achuo et al., 2006) or ET-insensitivity (Lund et al., 1998) enhances basal resistance in tomato against biotrophes. The susceptibility of MM to PM was not altered in our experiments, which is probably due to the low resolution of our disease scoring system to reveal minor changes in the susceptibility.

One of the first physiological responses upon perception of pathogens by the plant cell is the occurrence of oxidative burst (OB) (Lamb and Dixon, 1997). A weak and transient OB occurs in both resistant and susceptible cells, followed by a stronger phase of OB only in resistant cells, which in turn, results in accumulation of SA and reactive oxygen species (ROS) (Lamb and Dixon, 1997). We detected an early induction in SA pathway in NIL-Ol-4 and NIL-ol-2 (Fig.1A and Fig. 2A). This induction is probably the result of massive accumulation of SA upon occurrence of the second OB. Accordingly, our previous data showed that at 1 dpi in NIL-Ol-4 and NIL-ol-2, HR and callose deposition occur, respectively (Li et al., 2007). Fig. 1A suggests that also in MM and NIL-Ol-1, such an induction happens but much weaker.

Recognition of pathogen effectors by plant R proteins enhances OB (Lamb and Dixon, 1997; Nimchuk et al., 2003). As we reported before, *Ol-4* encodes an R protein (Chapter 2), so the strong OB, reflected in the strong induction of SA pathway in NIL-Ol-4 is probably the result of the function of this R protein. The other components required for OB are heterotrimeric G proteins and calmodulin proteins (Nimchuk et al., 2003). ol-2 is a truncated protein homologous to the barley MLO protein, which has been shown to be binding to calmodulin proteins (Stein and Somerville, 2002) and also interacting with

heterotrimeric G proteins (Lorek et al., 2010). It would be interesting to investigate wheather loss of function of ol-2 influences its binding activity to calmodulin and heterotrimeric G proteins, and thereby influence the quality of OB.

Apart from the induction in SA pathway at 1 dpi, the remaining changes in hormone pathways happened late in the time-course. ET pathway induction culminated from 7dpi and reached a maximum level at 9 dpi in NIL-Ol-1 (Fig. 1B). A similar trend was observed in NIL-Ol-qtls (Fig. 2B), while the other NILs showed the same pattern as that was observed in MM (Fig. 1B). Late induction in SA pathway was also observed in NIL-Ol-1 and NIL-Ol-qtls (Fig.1A, and Fig. 2A), which is distinguishable from that induction in the other lines. We have suggested that *Ol-1* is an enhancer of basal defense that mediates delayed cell death resembeling autophagic-PCD (Li et al., 2007; Chapter 4) in the later stages of PM infection. *Ol-qtls* also mediates cell death similar to autophagic PCD (Li et al., 2007). ET is considered as the hormone which is involved in autophagic PCD, probably through synergistic interaction with SA (O'Donnell et al., 2001; Love et al., 2008; Trobacher, 2009). Our data suggest that DCD in NIL-Ol-1 and NIL-Ol-qtls is probably the output of the late induction in the ET and SA pathways. In agreement with the significant induction in ET pathway in NIL-Ol-1 and NIL-Ol-qtls, we showed that ET-insensitivity compromises resistance in these two NILs. However, the ratio of the identified susceptible plants were not in agreement with what was expected (Table 2), which might be due to the fact that *Nr* mutant is not completely ET-insensitive (Clark et al., 1999). If ET plays a positive role in resistance in NIL-Ol-1 and NIL-Ol-qtls, we expect that ET overproduction increases the resistance level in these two NILs. However, we got contradictory results since *epi* mutant compromised resistance in these two NILs (Table 2). One of the effects of *epi* mutation is that the epidermal cells of this mutant line are different from the wild type by having a more round shape and being swollen (Barry et al., 2001). Keeping in mind that epidermal cells are the target of PM, there is a possibility that changes in the morphology of the epidermal cells resulted in susceptiblity in plants carrying the *epi* mutation.

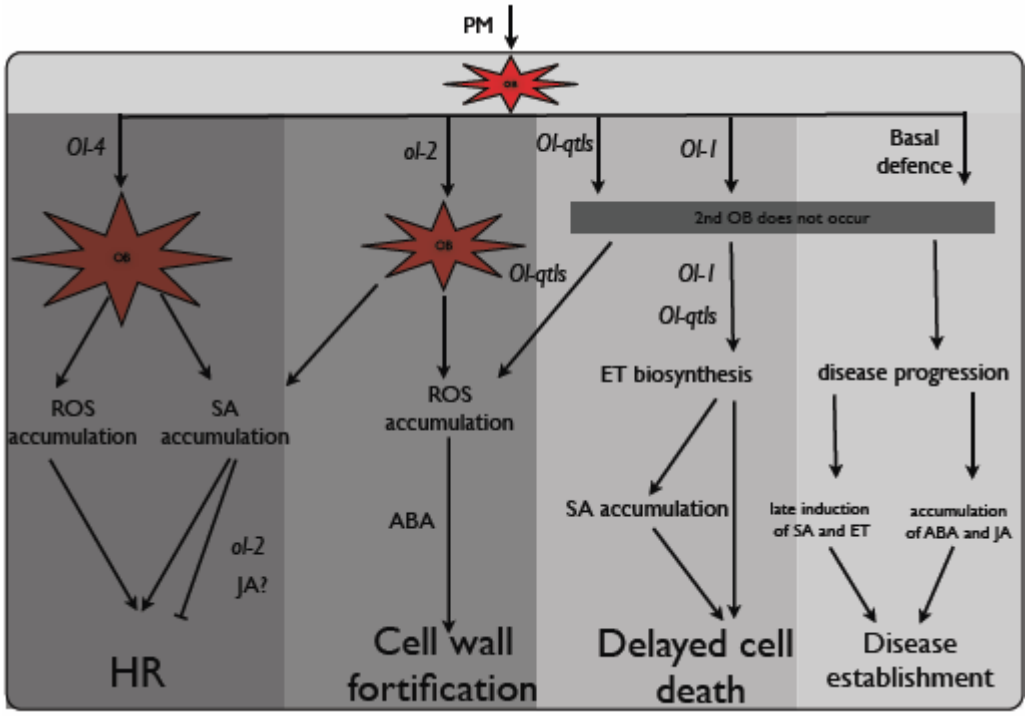

Fig. 3. A proposed model for different tomato responses to PM. Upon infection of a tomato epidermal cell by PM, an oxidative burst (OB) occurs in this cell, regardless of the identity of the cell (resistant or susceptible). In the presence of *Ol-4* or *ol-2*, this initial OB is exaggerated and results 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 exaggerate 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 gray color represents the level of resistance, which is highest in the presence of *Ol-4* and gradually decreases to basal resistance.

ABA and JA pathways (Fig.3 and Fig.4) showed a very similar pattern; a constant level in the period of infection followed by an induction in the later stage of infection with highest rate in MM. Late accumulation of ABA and JA in susceptible tomato 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 might be the result of disease establishment and stress rather than a defense response. Surprisingly, ABA-deficiency compromised both *ol-2*- and *Ol-qtls*-mediated resistance. 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 conclude that a basal level of ABA pathway is required for the process of callose deposition that contributes to the resistance mediated by *ol-2*- and *Ol-qtls*.

JA, ABA, and ET pathways in NIL-Ol-4 showed the same trend as in the basal defense, with the difference in the amplitude of induction (Fig. 1B-D). Accordingly, mutants for ET, JA, and ABA had no effect on *Ol-4*-mediated resistance (Table 2). However, there was a high induction in SA pathway in NIL-Ol-4 (Fig. 1A). We expect to have the *Ol-4*-mediated resistance compromised in crosses with SA-deficient lines, which needs further experimental confirmation.

The model presented in Figure 3 illustrates the summary of our results in the context of known molecular events happening in plant response to pathogens. This model suggests that the same initial events happen in different interactions, compatible or incompatible, and the key factor to determine the fate of the interaction is the ability of the cell to enhance and amplify this initial event and recruit it in order to prevent pathogen penetration and/or development. Our model suggests that the SA pathway is the main phytohormone pathway for HR-associated resistance mediated by *Ol-4* gene. For the resistance associated with DCD, mediated by *Ol-1* and *Ol-qtls*, ethylene is essential. ABA and JA are crucial for the resistance mechanism relying on callose deposition, such as *ol-2*-mediated resistance. In summary, the interaction between tomato and *O. neolycopersici* provides an interesting pathosystem in which the contribution of different phytohormone pathways to different forms of plant defense mechanisms is compared.

Materials and Methods

Plant materials

Never ripe (*Nr*), *epinastic* (*epi*), and *notabilis* (*not*) tomato mutants and their backgrounds AC (Ailsa Craig), and VNF8, were received from Tomato Genetic Resource Center

(TGRC), University of California, Davis, California. The tomato *defenseless1* (*def1*) mutant was obtained from Dr. C.A Ryan, Washington State University. Near-isogneic lines (NILs), NIL-Ol-1 (BC_3S_3), NIL-ol-2 (F_4), NIL-Ol-4 (BC_3S_4), and NIL-Ol-qtls (BC_2S_3) (Bai et al., 2005), and their background cultivar, Moneymaker (MM), were crossed to the mutant lines *Nr*, *def1*, *not*, *epi*, as well as the mutant backgrounds AC and VNF8. The NILs were used as maternal parent. By selfing the F_1 progeny, F_2 populations was produced and used in this study.

Selection for *epi* mutant and *Ol* genes

For crosses with *def1*, *not*, and *Nr* mutants that show no obvious phenotype, we randomly selected 16 plants (per F_2 population) homozygous for *Ol* genes and tested them for response to PM. Selection of homozygous *Ol* genes were done based on the markers which were reported previously (Bai et al., 2003; Bai et al., 2005; Bai et al., 2008). The homozygous *epi* mutant shows a distinct phenotype which is already distinguishable in five days dark-grown seedlings (Fujino et al., 1989). This phenotype was the criteria for screening homozygous *epi* mutants in the F_2 populations. Thus, for *epi* mutant F_2 plants homozygous for both *epi* and *Ol* genes were selected.

Disease assay

Oidium neolycopersici spores were washed off of the infected tomato leaves and diluted to a concentration of 2.5×10^4 spores per mL. The inoculum was evenly sprayed on one-month-old plants. After 10, 12, 15, and 20 days, the progress of the pathogen development was recorded. If it was possible, the number of the pathogen colonies on the leaves was counted; otherwise the severity of pathogen sporulation was scored based on 0 to 3 scale, 0 was complete resistant and 3 was complete susceptible.

Marker genes for hormone signaling pathways

Chitinase 9 (Barry et al., 2001) and *LOXD* (Heitz et al., 1997) and *PR-2* (Beyer et al., 2001) have been reported as marker genes for ET, JA, and SA pathways in tomato, respectively. We used the sequence of these genes for primer designing for qRT-PCR analysis. The Arabidopsis genes, *rd22* is a responsive gene for ABA (Shinozaki et al.,

2003). By performing TBLASTN in NCBI an orthologue of this gene in tomato (EU679376.1) was retrieved and used for primer designing. The primer sequences for these marker genes are listed in Table 1.

qRT-PCR

The same time-series of cDNAs which had been used previously for cDNA-AFLP profiling (Li et al., 2007) were used in this experiment. In brief, this time-series included cDNAs from MM, NIL-Ol-1, NIL-ol-2, NIL-Ol-4, and NIL-Ol-qtls derived from non-inoculated (Mock) and PM-inoculated leaves (2^{nd} and 3^{rd}) at 1, 3, 5, 7 and 9 dpi (for NIL-Ol-qtls the time points were 1, 5 and 7 dpi). For each line, the cDNAs from Mock samples from different time-points were mixed and used as calibrator for qPCR analysis. Quantitative Real-time PCR was done by using iQ SYBR Green Supermix (Bio-Rad). The expression level of the marker genes was normalized to the expression level of Elongation factor 1-alpha (the internal control gene). The expression level in inoculated samples were calculated relative to the expression level in the calibrator (mock sample) based on $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The final results are presented as normalized fold change in gene expression.

Fig. S1. Phenotypic selection of *epi* mutant. The F_2 populations derived from crossing the *epi* mutant line with different NILs as well as MM, were sown and kept in the dark. After 5 days, plants bearing *epi* mutation (right) were shorter and ticker comparing to the wild type (left).

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Chapter 6

General Discussion

Introduction

An interdisciplinary field of research is the playground for innovations and exciting discoveries. As such, researches on plant-microbe interaction have been contributing greatly to our understanding of plant biology as well as to practical resistance breeding in minimizing the loss of crop products due to pathogens.

In this thesis we used the tomato-powdery mildew pathosystem as a model to investigate some aspects of the plant response to biotrophic pathogens. *Oidium neolycopersici* is the causal agent of powdery mildew (PM) on tomato. The beauty of this pathosystem is that it includes different levels and various forms of resistance to PM. Tomato (*Solanum lycopersicum*) has been a model plant for biotic stress studies, its genome is sequenced, and enriched tomato transcriptome and metabolome databases are publicly available. More importantly, there are invaluable collections of plant materials in tomato such as wild species and different kinds of populations like isogenic lines. The bottleneck in this pathosystem is the pathogen, which is an obligate pathogen and therefore, it is not easy to perform genetic studies on it. Sequencing of the genome of this pathogen may overcome this drawback to some extent.

In the previous chapters of this thesis, I described different tomato resistance mechanisms triggered by different resistance genes in the tomato-PM pathosystem. Here I discuss our findings in the context of the consensus knowledge in the field of plant-microbe interaction which is relevant to this thesis.

Plant resistance based on innate immunity

Innate immunity responses start with the recognition of pathogens by pattern or pathogen recognition receptors (PRRs), which are localized in the plasma membrane or in the cytosol (Ausubel, 2005). One group of PRRs, which are called PAMP-receptors, perceives PAMPs (pathogen-associated molecular patterns) that are common molecules of microbes. The immunity responses triggered upon ligand/receptor recognition is called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). The second group of PRRs, which are called resistance proteins (R proteins), recognizes pathogen effectors that, in contrast to PAMPs, are more specialized molecules. This kind of recognition triggers immunity responses which is called effector-triggered immunity (ETI) (Jones and Dangl,

2006). Regardless the kind of PRRs, similar events occur after the perception/recognition of the pathogen, and the main difference is the magnitude of these events, which depends on the type of PRR (Dodds and Rathjen, 2010).

Ol-4 and *Ol-6* trigger ETI in tomato in response to PM

One of the interesting characteristics of *R* genes is that they appear in the plant genome as gene clusters (Hulbert et al., 2001). The clustered arrangement of these genes may be a mechanism to increase the chance of recombination or gene conversion in order to generate new recognition specificities (Hulbert et al., 2001). The *Mi-1* gene cluster spans about 400 Kb of the short arm of tomato chromosome 6, and consists of seven homologues encoding NBS-LRR proteins (Seah et al., 2007). It has been shown that the *Mi-1* gene confers resistance to nematodes, aphids and whiteflies (Kaloshian et al., 1998). In **Chapter 2** of this thesis we showed that tomato *Ol-4* and *Ol-6* resistance genes are *Mi-1* homologues. The *Mi-1* gene cluster in NIL-Ol-4 and NIL-Ol-6 confers resistance to not only PM, but also to nematodes and to some extent, to aphids. *Ol-4* and *Ol-6* confer race-specific resistance to PM by triggering hypersensitive response (HR) (Bai et al., 2005). Race-specificity and occurrence of HR are two hallmarks of ETI (Jones and Dangl, 2006).

There is a possibility that the same *Mi-1* homologue confers resistance to PM, nematodes, and aphids. If it is true, then an interesting question is: how is the *Mi-1* gene able to recognize different pathogens? One possibility could be explained based on the guard hypothesis (Dangl and Jones, 2001); *Mi-1* protein guards the same host target of different pathogens' effectors. It has been shown that the *RRS1* gene in Arabidopsis confers resistance to two different pathgoens (Narusaka et al., 2009). Also the *Pto* gene in tomato (Kim et al., 2002) and the *Rpm1* gene in Arabidopsis (Bisgrove et al., 1994) can recognize multiple effectors from the same pathogen.

Alternatively, it is possible that different *Mi-1* hmologues in the *Mi-1* gene cluster confer resistance to different pathogens. It would imply that *R* gene clusters function as haplotype of resistance genes against diferent pahtogens. There are instances supporting this possibility. For example, in the *Rx/Gpa2* gene cluster in potato, two homologues confer resistance to two distinct pathogens, a virus and a nematode (van der Vossen et al.,

2000). Changes in the LRR domain of *R* genes has been proposed to modify their recognition specificity (Staskawicz et al., 1995); accordingly the main difference in the coding sequence of *Mi-1* gene homologues is in the LRR domain (Seah et al., 2007).

Cloning of *Ol-4* genes will clarify which one of these two scenarios is true. We have made a library of the expressed *Mi-1* homologues in NIL-Ol-4 and sequenced a part of the LRR domain of these homologues. Result suggested that there are about 16 different *Mi-1* homologues expressed in NIL-Ol-4 with different abundance (Fig. 1). To pinpoint which one is the *Ol-4* gene, complementation experiments are required. It would be interesting to have individual *Mi-1* homologues transformed into *Solanum lycopersicum* cv. Moneymaker (MM) which is susceptible to PM, nematodes and aphids, in order to identify the *Mi -1* homologue(s) in NIL-Ol-4 conferring resistance to different pathogens.

Molecular events after recognition of the pathogen

As mentioned above, PTI and ETI trigger similar molecular events including calcium ion influx, oxidative burst, activation of mitogen-activated protein kinase cascades, reprogramming of gene expression, reinforcing cell wall at pathogen attempt sites, and programmed cell death (PCD) (Dodds and Rathjen, 2010). Ion influx and oxidative burst are very early responses which occur in seconds to minutes after pathogen perception and lead to intermediate responses (minutes to hours, including MAPK/CDPK activation, ethylene production, stomatal closure, and transcriptional reprogramming), followed by late responses (hours to days; e.g., salicylic acid accumulation, callose deposition) (Zipfel and Robatzek, 2010). Here, I discuss several of these molecular events in relation to our results presented in other chapters.

- **Oxidative burst**

One of the first events occurs upon pathogen attack is the accumulation of reactive oxygen species (ROS) which is 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). NADPH oxidases are regulated by calcium ion, and in a positive feedback loop, NADPH oxidases increase ROS in outer membrane and thereby induce opening of plasma

membrane channels for calcium influx (Hückelhoven, 2007). In the compatible interactions there is a weak induction of OB, however, in incompatible interactions a second OB with higher magnitude occur (Lamb and Dixon, 1997). What is the function of the OB? The ROS produced in OB are antimicrobial agents, and also H_2O_2 contributes to cell wall fortification, induces HR, and acts as a diffusible signal for induction of systemic defense response (Lamb and Dixon, 1997; Torres et al., 2006). In **Chapter 5** we suggested that the magnitude of OB is a determinant factor in tomato response to PM.

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 (papillae formation) (Hückelhoven, 2007). A very good example of cell wall apposition in our pathosystem has been observed in *ol-2*-mediated resistance (Bai et al., 2008). This gene encode a trasmembrane protein, homologous to barley MLO protein, which has been shown to be interacting with calmadulin proteins (Stein and Somerville, 2002) as well as heterotrimeric G proteins (Lorek et al., 2010). In **Chapter 5**, we suggested that these interactions enable ol-2 protein to trigger the second OB, resulting in higher concentration of H_2O_2 which could be used for cell wall apposition in an ABA-dependent manner. We proposed that the role of MLO in limiting PCD in epidermal cells is also retained in *ol-2*, and therefore, this protein can negatively regulate the PCD upon OB.

- **Plant hormones in plant immunity**

An important part of downstream pathways in immunity response are plant hormones signaling pathways. Recent evidence suggest that SA, JA, ET, ABA, GA, cytokinins, auxin and brassinosteroids signaling pathways play a role in plant immunity (Bari and Jones, 2009; Grant and Jones, 2009).

The importance of plant hormones in plant immunity encouraged us to investigate the involvement of the pathway of a subset of these hormones in tomato response to PM. Plant hormones involvement in tomato response to pathogens has been studied mostly in the basal defense in the absence of resistance genes (Diaz et al., 2002) (Thaler et al., 2004; Achuo et al., 2006). In our study (**Chapter 5**), we compared the hormonal

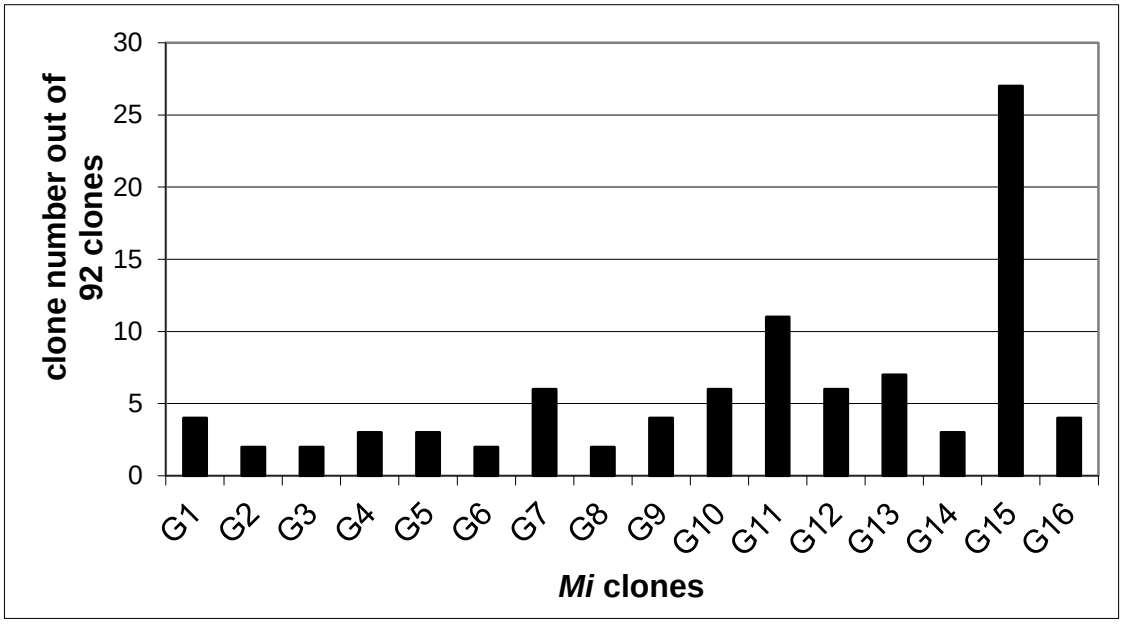

Fig.1. Different *Mi-1* homologues expressed in near-isogenic line carrying *Ol-4* (NIL-Ol-4). The full-length cDNA of the *Mi-1* homologues were amplified and cloned. A fragment (500 bp) of the LRR domain of 92 clones was sequenced. Based on the sequences, the clones were grouped into 16 groups (G). The height of the bars represents the number of clones out of 92 clones; reflecting the transcript abundance of each group of *Mi-1* homologues.

pathways in different kinds of tomato-PM interactions governed by different resistance genes, including HR, delayed cell death (DCD), cell wall apposition. Results showed that SA pathway is induced in the presence of *Ol-4*, which mediates HR-associated response. ET promotes DCD in the presence of *Ol-1* and *Ol-qtls*. ABA is required for the resistance conferred by *ol-2* and *Ol-qtls*, probably in association with cell wall apposition. JA is also required for resistance mediated by *ol-2*, probably because of its role in regulating PCD. However in Arabidopsis it has been sown that JA does not play a role in *mlo*-based resistance (Consonni et al., 2006), which indicates that the molecular mechanism underlying the resistance mediated by MLO homologues in tomato and Arabidopsis are not completely the same.

Auxin is considered as a promoting factor for disease establishment (Grant and Jones, 2009). In **Chapter 3** we discussed the possibility of the involvement of auxin in tomato response to nematodes. In the *Mi-1* gene cluster there are genes similar to *transport inhibitor 1* (*TIR1*), which is an auxin receptor and plays pivotal role in triggering auxin

signaling pathways (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). It has been shown that this gene is important for pathogenecity of gall-producer pathogen *Pseudomonas syringae* in Arabidopsis (Navarro et al., 2006). We showed that in roots of the nematode-resistant tomatoes, *TIR-like* transcript abundance is lower than that in nematode-susceptible ones. We proposed that low level of TIR-like proteins probably prevent nematodes to produce gall (upon local accumulation of auxin), and therefore, plants will be resistant to nematodes. Further experiments, like testing the response of *tir1* Arabidopsis mutant to nematodes, are required to test this possibility.

- **Programmed cell death**

PCD is one the common immunity responses, both in PTI and ETI. Two main kinds of PCD have been described in plant cells, apoptosis-like PCD (AL-PCD) and autophagic PCD (Love et al., 2008). ETI triggers such huge changes in the cell that normally lead to HR, a form of apoptosis-like PCD. In **Chapter 4** we compared these two forms of PCD in our pathosystem. The *Ol-4* gene which encodes an NBS-LRR protein, mediates HR in response to PM infection, contrasting *Ol-1* which triggers a slow-developing PCD resembling autophagic PCD. Fine-mapping of the *Ol-1* gene narrowed down the candidate genes for *Ol-1* to about 10 genes, of which, none is an *R* gene (**Chapter 4**). Among these genes, a homeodomain-lecine zipper (HD-Zip) may play a role in regulating the autophagic PCD that is associated with the resistance conferred by the *Ol-1* gene. This HD-zip belongs to the class II of HD-Zip transcription factors which have the CPSCE motif, located downstream of the leucine zipper motif and is involved in the perception of cellular redox status (Chan et al., 1998; Tron et al., 2002; Ariel et al., 2007). Notably, NPR1 protein, which is the pivotal protein in SA signaling pathway, is also activated upon changes in the redox status in the cell (Mou et al., 2003) and thereafter move to the nucleus to bind to the TGA transcription factors and turn on the expression of pathogenesis-related genes (Després et al., 2003). Interestingly, a class II HD-Zip transcription factor, NbHB1, was shown to be a positive regulator of pathogen-induced cell death in *Nicotiana* via JA signaling pathway (Yoon et al., 2009). Another HD-Zip transcription factor, which is located on the tomato chromosome 4, is involved in limiting the PCD in response to pathogen in tomato and its silencing resulted in oxidative burst-

mediated cell death (Mayda et al., 1999). We speculate that the HD-Zip transcription factor in the *Ol-1* interval plays a role in regulating the autophagic PCD and thereby, conferring resistance to PM.

RNA silencing in plant immunity response

One of the milestones in the field of plant-microbe interaction was set in 2006, when Jones' lab demonstrated that a mircoRNA (miRNA) plays an important role in response of Arabidopsis to *Pseudomonas syringae* (Navarro et al., 2006). Since then, more evidence has accumulated showing that different groups of small RNAs (sRNAs) are involved in Arabidopsis immunity responses (Katiyar-Agarwal et al., 2006; Navarro et al., 2006; Pavet et al., 2006; Agorio and Vera, 2007; Katiyar-Agarwal et al., 2007; Li et al., 2010)**.**

In our pathosystem we also found footprints of RNA silencing in response to PM. From a microarray dataset (unpublished data), we found an interesting gene which has been highly upregulated in the compatible interaction compared to incompatible interactions. This gene encodes a putative suppressor of gene silencing. The orthologue of this gene in tobaco has been shown to be induced in response to tobacco mosaic virus and supppresses the gene silencing machinery of the plant, the resistance mechanism against viruses (Anandalakshmi et al., 2000). We verified the expression of this gene in NILs carrying different *Ol* genes as well as in MM. Results clearly showed that this gene is induced drastically in MM (compatible interaction) in the early time-points (Fig. 2). This suggests that probably PM interrupts the tomato RNA silencing machinery in MM in order to establish a compatible interaction. Recent report on the ability of barley to silence barley PM genes, likely inside the pathogen (Nowara et al., 2010), strongly supports this speculation. It would be interesting to monitor small RNAs populations in the epidermal cells of tomato infected with PM.

It has been shown that sRNAs originated from the *RPM1* gene cluster in Arabidopsis, play a role in regulating the transcript level of *R* genes in this cluster (Yi and Richards, 2007). In **Chapter 3** also we proposed similar situation for the *Mi-1* gene cluster. We suggested that *TIR-like* genes in *Mi-1* cluster might have role a in regulating

the transcription and/or translation of *Mi-1* homologues. A good starting point to test this possibility is to search for cleaved mRNA of the *Mi-1* homologues.

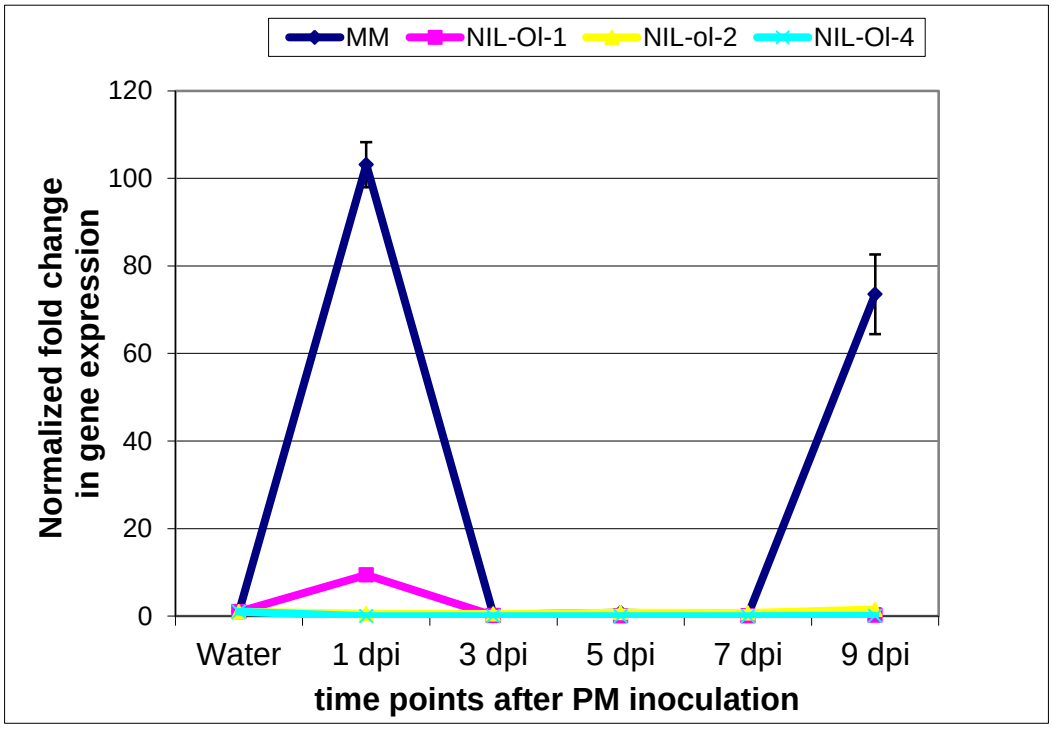

Fig. 2. The expression pattern of a putative gene silencing suppressor in different tomato interactions with powdery mildew (PM). Second and third leaves were sampled at 1, 3, 5, 7, and 9 days post inoculation (dpi) from PM-inoculated and PM-non-inoculated (Mock sample) plants of MM, NIL-Ol-1, NIL-ol-2, and NIL-Ol-4. RNA extracted from these leaf samples were used for monitoring the gene expression. Error bars show standard deviation.

Systemic acquired resistance

After the immune response is triggered, it will be extended to other parts of the plant. About half a century ago, it was observed that pathogen infected plants are less susceptible to subsequent attacks by a broad spectra of pathogens (Ross, 1961). This form of induced resistance is called systemic acquired resistance (SAR). SAR can be activated in many plant species by those pathogens that cause necrosis, either as part of HR or as a symptom of disease (Durrant and Dong, 2004). The resistance conferred upon SAR response is effective against a broad-spectrum of pathogens including viruses, bacteria, fungi, and oomycetes (Durrant and Dong, 2004). Plants expressing SAR are 'primed' to

respond to subsequent infections (Hammerschmidt, 2009) resembling a kind of immunological memory (Parker, 2009) which may last for the lifetime of the plant (Durrant and Dong, 2004).

Bridging the lab to the farm: crop breeding for resistance to pathogens

Translation of the scientific achievements into useful technology for daily life is state of the art. Producing crops with less vulnerability to pathogens is the aim of translating the findings in plant-microbe interaction research.

Different strategies for crop breeding have been summarized in comprehensive reviews (Gurr and Rushton, 2005; Stuthman et al., 2007; Gust et al., 2010). In this chapter I divide these strategies into two groups:

- strategies relying on enhancing indirect defense mechanisms
- strategies based on enhancing direct defense mechanisms; the plant innate immune responses

Breeding strategies based on indirect defense

Phenological traits of the crop could help it to avoid or escape the pathogen. Early maturation results in completing the life cycle before the epidemic of the pathogen. A good practical example for this kind of resistance was the decline in the wheat stem rust damage by using earlier maturing winter wheat cultivars in the 1930s and 1940s (Peterson, 2001).

Some morphological characteristics, like hair or waxes, on the leaf surface change the hydrophobicity of the leaf surface, and therefore repel water which is required for pathogen to germinate and grow (Stuthman et al., 2007). It has been demonstrated that interference with leaf topography results in the reductions in pathogen infection (Walters, 2006). Also evidence implicated both chemical and topographical signals in appressorium induction by wheat stem rust (Collins et al., 2001). Another interesting example is the resistance of rice to *Magnaporthe grisea*, the rice blast pathogen. A high level of silicon in the soil leads to increasing the silicon uptake by plant and deposition in the epidermal cells, which makes it more difficult for the pathogen to penetrate (Winslow et al., 1997).

It would be of interest to develop rice cultivars with higher silicon uptake efficiency to increase the resistance level to blast (Stuthman et al., 2007).

As mentioned in **Chapter 1**, plants produce a diverse range of secondary metabolites with antimicrobial activity (Dixon, 2001). Phytoanticipins are those secondary metabolites that are produced independently of the presence of potential pathogens (VanEtten et al., 1994). It is expected to have higher level of broad spectrum pathogen resistance by selecting for higher amount of secondary metabolites.

Quantitative disease resistance, which is referred to as partial resistance sometime, is controlled by quantitative trait loci (QTLs). It has been hypothesized that the biological function of the genes underlying QTLs for disease resistance could be in regulating morphology and developmental traits, or in production of components of chemical warfare, or in enhancing the basal defense, or encoding weak forms of *R* genes (Poland et al., 2009). There are QTLs for disease resistance which have been mapped on the same position on the linkage maps as *R* genes, leading to the hypothesis that weak form of *R* genes are controlling this QTLs, but up to now none of the cloned QTLs showed similarity to any *R* gene (St Clair, 2010). Therefore, most probably QTLs for disease resistance exerting their effect on enhancing resistance to pathogens by controlling the traits indirectly influence disease resistance. Accumulation of these QTLs is a marvelous strategy for breeding for disease resistance. Breeders, then need to select for other favorite traits, such as for morphology and quality, among the plants carrying these QTLs.

Breeding strategies based on plant innate immunity

Plant innate immunity is a process of pathogen recognition and then triggering signaling pathways that lead to defense responses. Accordingly, strategies to manipulate both recognition and downstream signaling have been suggested for breeding crops for resistance to pathogen (Gust et al., 2010).

Enhancing the pathogen recognition process: PAMP-receptors are the molecules to recognize microbes non-specifically, thus it sounds straightforward to think of enhancing of the ability of these receptors in recognition toward broad spectrum resistance to pathogens. One strategy following this line is to design antibodies against

PAMPs and express it in crops (Gust et al., 2010). The effectiveness of such antibodies has been demonstrated in Arabidopsis and wheat against *Fusarium* (Peschen et al., 2004; Li et al., 2008). Also Overexpression of OsBAK1, a PTI-associated protein, enhanced immunity in rice (Li et al., 2009). Alternatively it has been shown that engineering of the PAMP-receptors confer broad spectrum resistance in crops (Lacombe et al., 2010). EFR is a PAMP-receptor in the *Brassicacae* plant family which is not present in solanaceous plants, and heterologous expression of this gene in solanaceaous plants, tomato and tobacco, enhanced resistance to diverse pathogens (Lacombe et al., 2010). Field trials are needed to check if there is any fitness cost for the manipulation of the PAMP-receptors. One of the drawbacks of this strategy is that the engineered plants may become host to those pathogens that they were nonhost before. For example, there are pathogens for Arabidopsis which are able to suppress PTI response by manipulating EFR mediated pathways in this plant. Presence of EFR in tomato, for instance, may enable those pathogens to interfere with immune system of tomato via manipulation of ERF. There might be a risk that tomato expressing ERF from Arabidopsis becomes a host for some of pathogens from Arabidopsis, which normally tomato is nonhost for. Lacombe and associates (2010) showed that engineered plants are resistant to several pathogens, but they the range of susceptibility of those plants also needs to be checked.

The ability of plant to recognize the pathogen's effectors also could be enhanced. One might envisage the production of antibodies against pathogen effectors in order to block this molecules once they enter the cell. Or even further, one might design catalytic antibodies for the effectors to recognize and degrade the effectors.

More naturally strategy is to use *R* genes for this aim. Adoption of *R* gene to increase resistance to pathogen was one of the first efforts in crop breeding for disease resistance. The problem with this strategy is that *R* genes are normally race-specific and thus, the resistance mediated by these genes is not durable. Pyramiding *R* genes has been proposed as a method for increase durability of *R* gene mediated resistance; however in practice the success rate of this method was low (Pink, 2002). One of the main reason for this failure is the fitness cost which is associated with *R* genes function (Tian et al., 2003). It has been shown that defense responses compromise the plant growth (Todesco et al., 2010).

Therefore, it is expected that high level of defense response associated with activity of several *R* genes, influence the other aspects of plant life.

An alternative strategy is to use multiline cultivars; cultivars which are composed of individual lines that are phenotypically similar but are different in genes for race-specific resistance to an important pathogen (Stuthman et al., 2007).

Enhancing the post-recognition processes: more efforts have been devoted to enhance the events that occurs after recognition of the pathogens. Many different genes have been tried. For example for resistance to fungal pathogens genes encoding for toxin detoxifiers, PR proteins, chitinase, oxalate oxidase, cell death regulators, antimicrobial peptides and metabolites have been used (Collinge et al., 2010). *Ol-1* gene (**Chapter 4**) is a good target for this kind of strategies. This gene, as suggested by the function of the putative candidate genes for *Ol-1*, acts in the post-recognition pathway and confer race-non-specific resistance. *Ol-1* enhances basal defense by triggering dell death, therefore, it is expected that introgression of this gene into elite lines will enhance the level of field resistance. The same holds true for *Ol-qtls* which molecularly showed similar response to *Ol-1* (**Chapter 5**).

SAR is a natural mechanism for conferring broad spectrum and long–lasting resistance. Efforts to enhance SAR in the field by using SA analog chemicals have not been successful (Beckers and Conrath, 2007; Gust et al., 2010). However, recent discoveries in the mechanisms of SAR induction may provide new opportunities for genetic engineering of the crops for enhanced level of SAR (Gust et al., 2010). Alternatively, it would be valuable to assess the genetic diversity for SAR response, if there are genetic resources could be used in classic crop breeding programs.

In summary, two general strategies are used for crop breeding for disease resistance, breeding for traits which indirectly enhance resistance or breeding for enhanced innate immune responses. Breeding strategies based on the accumulating partial resistance are probably the best practical strategy from the first group. Methods based on manipulation of innate immune system are tricky because of the complexity of this system and fitness costs associated with activation of this system. Using of multilines is one of the safest strategies from this category which have been used successfully. Enhancing the immune response pathways is also promising. What is required here is to find suitable components

of these pathways, to enhance their function only at the time and tissue which is needed. Enhancing of the plant capacity to recognize pathogens needs deeper understanding of the plant immune system which future researches will provide us.

Both conventional plant breeding and genetic engineering methods are used nowadays. In most cases genetic engineering has the advantage that it is targeted, and can be accomplished in a shorter time. But for instance, to introduce QTLs for disease resistance, conventional methods based on crossing, is the better strategy.

The sad story in our world is that while people like Norman Borlauge, who devoted his life to fight against food shortage in the world, were shouting out the necessity of adoption of genetic engineering in food production, decision makers are some young well-dressed law-school graduated people, who never knew what is the meaning of food shortage.

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Summary

Tomato, *Solanum lycopersicum*, is a host for *Oidium neolycopersici*, the cause of powdery mildew (PM). Though cultivated tomatoes are susceptible to PM, resistance is reported in wild *Solanum* species. By screening wild tomato species, nine loci conferring resistance to PM have been identified, namely *Ol-1*, *ol-2*, *Ol-3*, *Ol-4*, *Ol-5*, *Ol-6*, *Ol-qtl1*, *Ol-qtl2*, and *Ol-qtl3*. These genes are located on different chromosomes and mediate different levels of resistance by different mechanisms. In this thesis we mainly focused on the *Ol* genes located on chromosome 6 (*Ol-1*, *Ol-4*, *Ol-5* and *Ol-6*) with the aim to fine-map and eventually clone these genes. In addition, we studied the contribution of different phytohormone pathways to the resistance mediated by *Ol-1*, *ol-2*, *Ol-4* and *Ol-qtls*.

We first focused on the *Ol* genes on the short arm of tomato chromosome 6, *Ol-4* originating from *S. peruvianum* LA2172 and *Ol-6* with unknown origin (Chapter 2). We showed that *Ol-4* is a homologue of the *Mi-1* gene. Interestingly, *Mi-1* homologues, which are present in the *Mi-1* gene cluster in the near-isogenic line carrying *Ol-4* (NIL-Ol-4), confer resistance to tomato PM (*O. neolycopersici*), nematodes (*Meloidogyne spp.*) and aphids (*Macrosiphum euphorbiae*). It is intriguing to investigate whether the resistance to different pathogens is conferred by the same *Mi-1* homologue or by different *Mi-1* homologues. Also, we showed that *Ol-6* is a homologue of the *Mi-1* gene. The resistance response to PM, nematodes and aphids is relatively weaker in NIL-Ol-6 compared to that in NIL-Ol-4, suggesting that *Ol-4* and *Ol-6* are different homologues of *Mi-1* genes or different alleles of the same *Mi-1* homologue.

On the short arm of tomato chromosome 6, the *Mi-1* gene cluster is about 400 Kb in size and consists of several other genes besides the *Mi-1* homologues. There are *transport inhibitor responses-like* (*TIR-like*) genes embedded in this cluster. Interestingly, the copy number of these *TIR-like* genes in the nematode-resistant tomatoes is less than that in nematode-susceptible ones (Chapter 3). Furthermore, lower expression of these *TIR-like* genes was observed in roots, but not in leaves, of nematode-resistant plants compared to nematode-susceptible plants. These observations prompted us to suggest and to discuss two different scenarios explaining how *TIR-like* genes could play a role in the

plant response to root-knot nematodes. TIR1 is the pivotal element in auxin signaling and it has been shown that nematodes can manipulate auxin efflux in plant root cells to accumulate auxin locally. Thus, we speculated that auxin accumulation, in the presence of high amounts of *TIR-like* proteins, triggers auxin signaling pathways and allows the nematodes to create feeding sites on the roots of nematode-susceptible tomato plants. The other possibility that we discussed is the potential role of *TIR-like* genes in regulating the expression (and translation) of the *Mi-1* gene homologues. We proposed that *TIR-like* genes might be a source of small RNAs for regulating the transcript level of *Mi-1* homologues.

Then, we studied the *Ol-1* and *Ol-5*, which are located on the long arm of chromosome 6, and originated from different *S. habrochaites* accessions. *Ol-1* is closely linked to *Ol-5*. With fine-mapping, we narrowed down this locus to a 73 Kb interval which contains at least 10 putative genes. Interestingly we observed an interaction between chromosome regions harboring *Ol-1* and *Ol-5*, indicating that the interaction between *Ol-1* and *Ol-5* is needed to confer PM resistance. Both *Ol-1* and *Ol-5* trigger delayed cell death that is distinguishable from hypersensitive response (HR), the hallmark of *R* gene response to biotrophic pathogens. The delayed cell death associated with *Ol-1* and *Ol-5* resembles the autophagic PCD. We observed that *Ol-1* and *Ol-5* were both required for on-time and effective cell death to stop PM. If one of these two genes was not present, cell deathcould not happen or not be effective enough to stop pathogen growth.

Finally, we investigated the involvement of phytohormone pathways in PM resistance conferred by the *Ol* genes, including *Ol-1*, *ol-2*, *Ol-4* and *Ol-qtls* (Chapter 5). There is overwhelming evidence implicating plant hormones in plant responses to pathogens. In this experiment we, in addition to *Ol-1* and *Ol-4*, included other resistance loci for PM resistance in tomato. The first one is *ol-2*, a homologue of the barley *mlo* gene and derived from *S. lycopersicum var cerasiforme* LA1230. This gene confers resistance to PM by triggering callose deposition and, thereby, cell wall fortification. The other is *Ol-qtls*, a combination of three QTLs for PM resistance associated with both delayed cell deathand callose deposition. NILs carrying *Ol-1*, *ol-2*, *Ol-4* and *Ol-qtls*, plus the background of these NILs (*S. lycopersicum* cv Moneymaker, MM), provided us the

possibility to compare the involvement of hormonal pathways in different kinds of tomato responses. These responses include basal defense, cell wall fortification, delayed cell death, and HR. We quantified the expression of marker genes for the pathways of salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET) over a time-course after inoculation with PM. As a complementary approach, we crossed our NILs with tomato mutants for JA, ET and ABA. Our results suggested that *Ol-4*-mediated resistance probably relies on the SA pathway. *Ol-1* and *Ol-qtls* require ET to promote the delayed cell death for PM resistance. JA deficiency can compromise resistance mediated by *ol-2*. Our results also suggested that ABA is required for those interactions demanding callose deposition, resistance associated with *ol-2* and *Ol-qtls*. These results present a nice example of the involvement of different phytohormones in different phases of resistance against PM in tomato. Altogether, this thesis describes different tomato resistance mechanisms triggered by different resistance genes in the same pathosystem, underscoring the plant ability to adopt diverse molecular mechanisms to defense itself against intruders.

Samenvatting

Tomaat, *Solanum lycopersicum*, is een gastheer voor de schimmel *Oidium neolycopersici*, de veroorzaker van de ziekte 'echte meeldauw'. Alhoewel al de gecultiveerde tomaten gevoelig zijn voor deze ziekte zijn er wilde soorten die resistent zijn. Screening van verschillende wilde soorten heeft geleidt tot de identificatie van negen loci die resistentie tegen meeldauw geven. Dit zijn zowel hoofdgenen als zogenaamde quantitatieve genen (QTL), namelijk *Ol-1, ol-2, Ol-3, Ol-4, Ol-5, Ol-6, Ol-qtl1, Ol-qtl2* en *Ol-qtl3*. Deze genen liggen op verschillende chromosomen en geven verschillende niveaus van resistentie. In dit proefschrift heeft het onderzoek zich vooral gericht op de genen gelegen op chromosoom 6 (*Ol-1*, *Ol-4*, *Ol-5* en *Ol-6*) met als doel om hun werking te ontrafelen, maar ook om ze op termijn te isoleren. Daarnaast zijn er experimenten uitgevoerd om de bijdrage van de verschillende planten hormoon routes bij de resistentie veroorzaakt door *Ol-1, ol-2, Ol-4* en *Ol-qtls* te bepalen. In hoofdstuk 2 is gekeken naar resistentie *Ol-4* afkomstig van *S. peruvianum* LA2172 en *Ol-6* met onbekende herkomst. Uit dit onderzoek bleek dat *Ol-4* een homoloog is van het *Mi-1* gen. Dit gen bevindt zich in een cluster van *Mi-1* homologen in de tomatenlijn NIL-Ol-4 die resistentie vertoont tegen meerdere pathogenen waaronder meeldauw (*O. neolycopersici*), nematoden (*Meloidogyne spp.*) en luizen (*Macrosiphum euphorbiae*). Doel was om aan te tonen of de resistentie tegen de verschillende pathogenen wordt veroorzaakt door hetzelfde gen of verschillende genen uit het *Mi-1* cluster. Ook *Ol-6* bleek een homoloog van het *Mi-1* gen te zijn alhoewel het niveau van resistentie minder was in tomatenlijn NIL-Ol-6 vergeleken met dat van NIL-Ol-4, hetgeen suggereert dat *Ol-4* en *Ol-6* verschillende homologen zijn van het *Mi-1* gen of verschillende allelen van hetzelfde *Mi-1* homoloog.

Het *Mi-1* gen cluster, gelegen op de korte arm van chromosoom 6, is ongeveer 400 Kb groot en bevat naast de *Mi-1* homologen verschillende andere genen. Er bevinden zich zogenaamde *transport inhibitor responses-like* (*TIR-like*) genen in dit cluster, waarvan het interessant is om te zien dat in de nematoden resistente tomatenlijnen het aantal kopieën van deze *TIR-like* genen lager is dan in de nematoden vatbare lijnen (Hoofdstuk 3). Daarnaast bleek dat de expressie van deze genen in wortels (het weefsel waar nematoden op aangrijpen) lager was dan in bladeren. Deze observaties kunnen duiden op een mogelijke rol van deze genen in de resistentie tegen nematoden

via auxine signaal transductie. Aangezien het aangetoond is dat nematoden de auxine efflux in wortelcellen van planten kunnen beïnvloeden om een hogere lokale auxine concentratie te bewerkstelligen zou een auxine verhoging in aanwezigheid van grote hoeveelheden *TIR-like* eiwitten ertoe kunnen leiden dat dit de auxine signaal transductie route activeert waardoor de nematoden in staat zijn om zogenaamde 'feeding sites' te creëren op de wortels van nematoden vatbare tomatenlijnen. Het zou ook kunnen zijn dat de *TIR-like* genen een rol spelen in de regulatie van de expressie (en de vertaling in eiwit) van *Mi-1* gen homologen via siRNAs. In hoofdstuk 4 zijn de uitkomsten van experimenten beschreven die tot doel hadden om de resistentie genen *Ol-1* en *Ol-5,* gelegen op de lange arm van chromosoom 6, en afkomstig van verschillende *S. habrochaites* herkomsten nader te karakteriseren. Door middel van fijnkartering kon het interval waarin dit locus zich bevindt verkleind worden tot een gebied van 73 Kb met zeker 10 mogelijke genen. Er bleek een interactie te zijn tussen de regio's die de *Ol-1* en *Ol-5* loci bevatten die nodig bleek om een goed niveau van resistentie tegen echte meeldauw te geven. Zowel *Ol-1* als *Ol-5* veroorzaken vertraagde celdood hetgeen duidelijk onderscheidbaar is van de 'hypersensitive response (HR)', de snelle dood van één of een beperkt aantal cellen als reactie van de resistentiegenen van de plant in antwoord op de aanval door (biotrofe) pathogenen. De vertraagde celdood die geassocieerd is met *Ol-1* en *Ol-5* lijkt heel sterk op de zogenaamde 'autophagic Programmed Cell Death'. Het bleek dat *Ol-1* en *Ol-5* beiden nodig zijn voor een tijdige en effectieve celdood om de echte meeldauw infectie te voorkomen. Indien één van beide genen niet aanwezig was trad er geen of onvoldoende celdood op om de groei van de pathogeen effectief te stoppen.

Tenslotte is in hoofdstuk 5 de betrokkenheid van verschillende plantenhormoon routes bij de resistenties gegeven door *Ol-1*, *ol-2*, *Ol-4* en *Ol-qtls* bestudeerd. Er zijn zeer veel aanwijzingen dat plantenhormonen een rol spelen bij resistentie tegen pathogenen. *ol-2,* een homoloog van het gerst *mlo* gen en afkomstig uit *S. lycopersicum var cerasiforme* LA1230 geeft resistentie tegen echte meeldauw door callose vorming hetgeen de celwand extra versterkt waardoor de pathogeen moeilijker kan binnendringen in de cel. De *Ol-qtls* resistentie wordt veroorzaakt door een combinatie van drie QTLs voor meeldauw die aanleiding geven tot celdood en callose vorming. Tomatenlijnen met *Ol-1, ol-2, Ol-4* en *Ol-qtls*, plus de controle lijn voor deze lijnen (*S. lycopersicum* cv Moneymaker, MM), gaven de mogelijkheid om de betrokkenheid van de verschillende hormoon syntheseroutes in de verschillende

resistenties met elkaar te vergelijken. De verschillende responses van de tomatenlijnen op infectie met de pathogeen vielen uiteen in basale verdediging, celwand versterking, vertraagde celdood en HR. De expressie van merker genen voor de verschillende hormoon synthese routes werden bepaald [salicyl zuur (SA), jasmonzuur (JA), abscisinezuur (ABA), en ethyleen (ET)] in een tijdspad van verschilende uren na infectie met echte meeldauw. Daarnaast werden de verschillende tomaten resistentielijnen gekruist met mutanten voor de JA, ET en ABA routes. De resultaten laten zien dat de op *Ol-4*-gebaseerde resistentie leunt op de SA signalerings route. *Ol-1* en *Ol-qtls* hebben ET nodig om de vertraagde celdood te bevorderen om meeldauw resistentie te verkrijgen. Gebrek aan JA kan de resistentie veroorzaakt door *ol-2* compromitteren. De resultaten laten ook zien dat ABA nodig is bij die resistentie die werken middles callose vorming, zoals bij *ol-2* en *Ol-qtls*. Al deze resultaten tonen de grote flexibiliteit die een plantensoort kan hebben om zich tegen een en dezelfde pathogeen (echte meeldauw) op veel verschillende en vooral effectieve manieren te wapenen.

Acknowledgments

PhD is the highest academic degree which I am about to get it. To reach to this point, many people helped me and many conditions came together. Its time to pay tribute to those people and to express my appreciation for those favorable situations.

I am very glad to be born and grown up in Persian culture, which among the others, has one prominent feature. Persians respect seekers of knowledge very much and its a great value there, which inspired many to venture into an academic career. My parents were those who first guided me in that direction and have always supported me.

To do agricultural sciences in Iran demands lots of motivations and persistence; not everybody is eager to do that. It was the same for me untill I came across a new concept, Plant Biotechnology, and started my MSc in this field. I was lucky enough to be working in the Agricultural Biotechnology Research Institute of Iran (ABRII), a newly-established institute which was open almost every day and every hour to young researchers. Dr. Behzad Gharayaziee was the charismatic character there, who was the source of inspiration for a lot of young researchers. It was a big change in my life to meet him, work under his supervision and learn how great things could be even in a developing country. I learnt that " the value of a man is based on what he wishes for".

Meeting and collaborating with foreign scientists was a bonus for researchers in ABRII. There, I once met a scientist from Wageningen and had a chance to talk to him and ask about the possibilities to go to Wageningen for doing a PhD. Eventually he provided me such a golden opportunity. That Dutch scientist was Dr. Rients Niks, a nice man interested in Persian culture and quite knowledgeable about that. Dear Rients, you helped me to open a new chapter in my life, I can't thank you enough for that!

Five years ago, in Spring of 2006, I came to Wageningen for an interview. Prof. Richard Visser agreed that I could start my PhD under his supervision. It was a wonderful day! Thank you Richard for providing me with such an opportunity and for all supports and constructive comments during my PhD, especially during the writing period.

Though I don't really believe in luck, I am a lucky person most of the time. My co-promoter and daily supervisor in PhD thesis was Dr. Yuling Bai. She was kind, helpful, supportive and always available for discussions. The word "hardworking

person" equals Yuling for me. It happened quite frequent during my PhD that I and Yuling were among the last persons leaving the building late in the evening and even in the weekends. But this was not all. I learned great lessons of humanity from Yuling. Dear Yuling, I am grateful for all the things I learned from you and for all your supports and kindness!

Dr Sjaak van Heusden was my co-supervisor at the beginning and was ready to help always in the PhD thesis or even by providing a bike or househodl stuff. Dear Sjaak, I had lot of fun during scientific and non-scientific discussions with you. More important, I tried to learn from you how to be a source of funs and joys while you are working hard and serious. Thank you for everything!

Colleagues in Plant Breeding were friendly and helpful. There was joyful times with them at work and also in social occassions in Plant Breeding. In the lab, I could easily ask for experiences and there would always be someone to help you. I would like to thank all technicians especially Danny, Doret, Gert, and Johan who made our lab (I. 120) a friendly place to work. Without helps and cares of Irma, Yolanda, Marian, Fien, Koen, Martijn, Gerda, Brian, Hannake, Patrick, and other technicians, it was not possible to work in the labs. Especially, I am thankful to Annelies for her help in tomato transformation which was pretty crucial for me. I also appreciate the helps received from staff in Unifarm, especially Bert, which facilitated my greenhouse experiments significantly.

The secretariat of Plant Breeding is one of the most efficient places, I believe. Most problems could be solved with their help. I would like to thank all staff in the secretary especially Annie, Mariame and Letty.

The circle of good friends is one of the most important requirements for everyone. I had very good friends in Wageningen, particularly in Plant Breeding there were nice friends always around. My officemates were Stefano, Zheng, Luigi, and Maarten plus Estelle (who was almost an officemate). Thank you guys for all the joyful times we had in and out of office. I learned quite a lot from you, too. I would like to thank also Antoine, Pierre, Efstathios, Bjorn D., Bjorn K., Nico, Annoma, Anitha, Pingping, Arwa, Yusuf, Paula, Nasim, Naser, Reza, Hossein, Farhad, Freddy, Xingfeng, Ningwen, Sabaz, Collete, Mathieu, Thierry, Benoite, and many others, for your friendship.

It was a blessing to be able to do a PhD in Europe, where I got to know more of Western culture. There were, and still are, quite a lot of good things there for me to

learn and to try to integrate into my culture. In the first six months after arriving in Wageningen, I was living with a Dutch family, kind Vera and Titus de Meester. I had an enjoyable time in their beautiful house, nice cultural, historical and philosophical discussions over dinner, and much more. Thanks for this experience.

Wageningen is called “the city of life sciences”. For me it was the city for life and for science; nice place to work and also nice environment to live. In Wageningen I met many different people with different cultures and with different good things to learn. I will never forget my life in Wageningen. Another thing I liked particularly in Wageningen, was ‘de Bongerd’, the sport center of Wageningen University. It is a nice place to relax after work and I had a lot of fun there. Thanks to the director, instructors and other staff of ‘de Bongerd’.

Thank you all again for creating such a happy and funny four and half years of my life in Wageningen. I am very much looking forward to being in touch and collaborating with you in the future.

Alireza
Feb. 23, 2011
Wageningen
The Netherlands

About the author

Alireza Seifi was born on January 24, 1978 in Mashhad, Iran. After finishing high school in natural sciences, he started his BSc in Agronomy and Plant Breeding in 1997 in Ferdowsi University of Mashhad, Iran. In 2000 he passed the MSc entrance national exam in Plant Biotechnology by rank number one among the contestants in that branch. For MSc he moved to Karaj, where faculty of Agriculture of Tehran University is located. That translocation was a turning point in his carrier as he got to know outstanding scientists in Plant Breeding and Biotechnology. Alireza performed his MSc thesis in the Agricultural Biotechnology Research Institute of Iran (ABRII), and obtained his master degree on January 1, 2003. He continued to work in ABRII as a research assistant till September 2006. Working in ABRII was a great opportunity for him to develop his skills in tissue culture, gene cloning and transformation, and also to get to meet international scientists who often visited and collaborated with ABRII. Dr. Rients Niks was one of those visitors who eventually helped Alireza to start his PhD in Laboratory of Plant Breeding in Wageningen University in September 2006.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Alireza Seifi
Date: 18 March 2011
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	date
▶ **First presentation of your project**	
Cloning and characterization of *Ol-4* resistance gene in tomato	Dec 19, 2006
▶ **Writing a review or book chapter**	
▶ **MSc courses**	
▶ **Laboratory use of isotopes**	
Safe-handling with radiolable material	Feb 04, 2008
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	date
▶ **EPS PhD student days**	
EPS PhD student day, Wageningen University	Sep 13, 2007
1st Joint Retreat of PhD Students in Experimental Plant Sciences, Wageningen, The Netherlands	Oct 02-03, 2008
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
▶ **EPS theme symposia**	
EPS theme 2 "Interactions between Plants and Biotic agents', Amsterdam university	Feb 02, 2007
EPS Theme 2 "Interactions between Plants and Biotic agents', Utrecht University	Jan 22, 2009
▶ **NWO Lunteren days and other National Platforms**	
NWO-ALW Experimental Plant Sciences, Lunteren (two days meeting)	Apr 02-03 2007
NWO-ALW Experimental Plant Sciences, Lunteren (two days meeting)	Apr 07-08, 2009
NWO-ALW Experimental Plant Sciences, Lunteren (two days meeting)	Apr 19-20, 2010
▶ **Seminars (series), workshops and symposia**	
Diversification of Small RNA Pathways in Plants, Dr. Jim Carrington	Mar 26, 2007
The small RNA revolution, Hubrect symposium, Utrecht	Sep 08, 2007
Mechanisms and function of active DNA demethylation in arabisopsis, Prof. Jian-Kang Zhu	Nov 03, 2008
Science from an editor's view, Pamela J. Hines	Nov 06, 2008
Receptor kinase signalling in plant innate immunity, Dr. Cyril Zipfel	Feb 05, 2009
Seeds, microRNA and Darwin, Dr. Hiro Nongaki	Sep 17, 2009
Mobile RNA silencing in plants, Prof. Sir David Baulcombe	Sep 27, 2010
Seminar Series Plant Sciences 5x	2010
Seminar Isgouhi Kaloshian, Tomato innate immunity to root-knot nematodes and aphids	May 14, 2009
Seminar J.D.H. Keatinge, AVRDC and WUR need to fight the battle against poverty & malnutrition together	Jun 18, 2009
Seminar Valerie Williamson, Connecting genetics and genomics of pathogenicity and behavior in root-knot nematodes	Oct 23, 2009
EPS Seminar Paul Birch, Trying to understand susceptibility and exploit resistance in potato-Phytophthora infestans interactions	May 20, 2010
Plant Research Day (Plant breeding)	Sep 27, 2007
▶ **Seminar Plus**	
Mobile RNA silencing in plants, Prof. Sir David Baulcombe	Sep 27, 2010
▶ **International symposia and congresses**	
Molecular plant-microbe interaction, Quebec , Canada	Jul 19-23, 2009
SOL meeting, Dundee, Scotland	Sep 05-09, 2010
▶ **Presentations**	
poster presentation in MPMI congress, Quebec, Canada	Jul 19-23, 2009
poster presentation in SOL meeting, Dundee, Scotland	Sep 05-09, 2010
▶ **IAB interview**	Dec 05, 2008
▶ **Excursions**	
visiting seed production companies	Jun 11, 2008
Subtotal Scientific Exposure	*11.9 credits**

3) In-Depth Studies	date
▶ **EPS courses or other PhD courses**	
Bioinformatics: A User's Approach	Mar 13-16, 2007
EPS summer school " Environmental signaling"	Aug 24-26,2009
Spring school "RNAi & the world of small RNA molecules"	Apr 14-16, 2010
▶ **Journal club**	
Member of a literature discussion group at Plant Breeding	2007-2009
▶ **Individual research training**	
Advanced protein domain analyses, Lyon University, France	May 18-20, 2008
Subtotal In-Depth Studies	*6.9 credits**

4) Personal development		*date*
▶	**Skill training courses**	
	Techniques for writing and presenting scientific paper	Oct 16-19 , 2007
	PhD competence assesment	Jan 30, 2007
	writing grant proposal	Oct 09 & Nov 13, 2009
	Scientific publishing	Nov 19, 2009
	Search the digital library efficiently	Jan 25, 2011
▶	**Organisation of PhD students day, course or conference**	
	1st Joint Retreat of PhD Students in Experimental Plant Sciences, Wageningen, The Netherlands	Oct 02-03, 2008
	Organizing weekly work discussion meeting in Biotic stress group in Plant Breeding laboratory	2007-2008
	Organizing committee for the Plant Breeding group lab trip	2007
▶	**Membership of Board, Committee or PhD council**	
	Membership PhD Council	Mar 2008 - Dec 2010
	Subtotal Personal Development	*8.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	30.3

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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The photo on the cover illustrates occurrence of HR in a resistant tomato epidermal cell in response to powdery mildew. Photo courtesy Prof. Hideyoshi Toyoda, Kinki University, Japan.

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