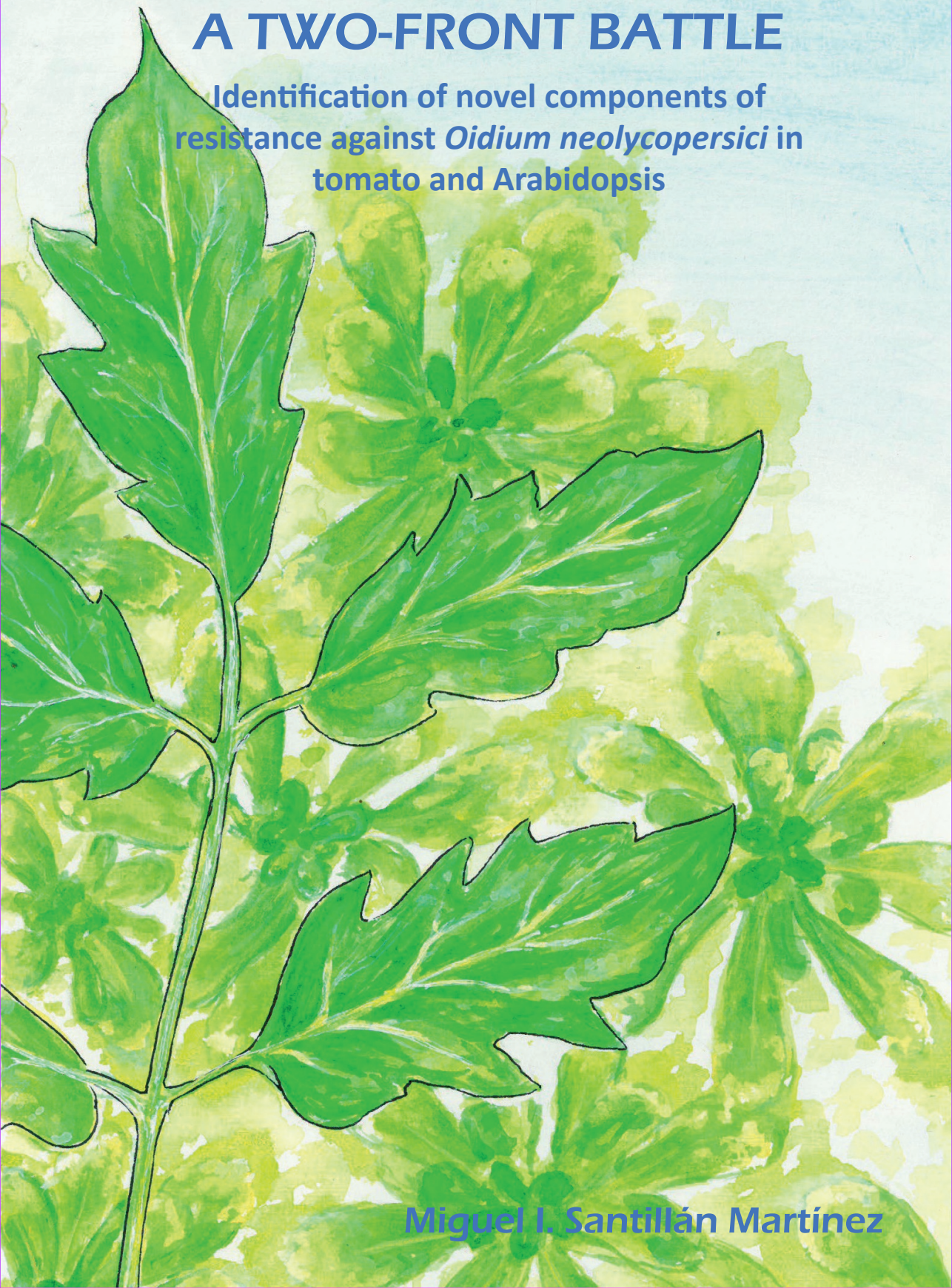
A TWO-FRONT BATTLE

Identification of novel components of resistance against *Oidium neolycopersici* in tomato and Arabidopsis



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Propositions

1. In Arabidopsis, different genetic components are required for resistance against *Oidium neolycopersici* than for resistance against other powdery mildew pathogens. (this thesis)
2. Targeted mutagenesis is essential to characterize incomplete resistance. (this thesis)
3. Loss of biodiversity will become the most severe bottleneck in plant breeding.
4. Not reporting negative results in research causes an unsustainable waste of resources.
5. The most important purpose of a Ph.D. research is to create questions, rather than answering them.
6. More conscious consumers are fostered by involving children in taking care of a garden.
7. Playlists create lazy listeners.

Propositions belonging to the PhD thesis, entitled

A two-front battle: Identification of novel components of resistance against *Oidium neolycopersici* in tomato and Arabidopsis

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A two-front battle:

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A two-front battle:

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Thesis

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Chapter 1

General introduction

The unwanted guests: Powdery mildew disease

Powdery mildew (PM) is the common name given to one of the most widely distributed plant diseases in the world. All pathogens causing PM are fungi belonging to a group of more than 400 ascomycetes of the Erysiphales order, which evolved over 100 million years ago (Takamatsu, 2004; Kiss *et al.*, 2005). This group of pathogens can infect almost 10,000 different plant species (Braun *et al.*, 2002; Glawe, 2008), including many important food and ornamental crops. All monocot PM pathogens belong to the species *Blumeria graminis* and are highly specialized, as most of the *formae speciales* (ff. spp.) are able to colonize only a single plant species (Wyand & Brown, 2003). On the other hand, some dicot PM have a broad host range and are able to infect plants from different families (Takamatsu, 2004; Takamatsu *et al.*, 2013).

Figure 1. Powdery mildew infection caused by *Oidium neolycopersici* in Arabidopsis accession Col-0 (A) and a leaf of tomato cultivar Moneymaker (B).

The PM disease is easily identified by the development of white powdery spots on above-ground parts of the plants that are the result of the abundant sporulation of the pathogen (Figure 1). PM pathogens establish an obligate biotrophic interaction with the host plants, relying on the host's living tissues to feed and complete their life cycle. PM conidiospores spread through air and the disease cycle of these pathogens starts when a viable conidiospore lands on a host surface. Around 6 hours later, a germination tube is formed and develops into a swollen hyphal structure called appressorium (Braun *et al.*, 2002). This structure differentiates into a narrow protuberance known as a penetration peg that is able to breach the host epidermal cell. Once a host cell is breached, the penetration peg extends to form a haustorium, the main interaction organ between the pathogen and the host that is responsible for the uptake of nutrients and the delivery of molecules to maintain a biotrophic relationship with the host (Bushnell, 1972). A fully formed haustorium can be observed at 12-14 hours post inoculation (hpi; Kuhn *et al.*, 2016). However, haustoria are not directly in contact with the host cell cytoplasm as they are surrounded by a

plant-derived membrane, called extrahaustorial membrane. This structure has been shown to share some structural components with the endoplasmic reticulum and to have unconventional secretory pathways (Kwaaitaal *et al.*, 2017). It has been shown that secreted molecules from both the fungus and host are targeted to the space between the haustorium and the extrahaustorial membrane (reviewed by Hückelhoven & Panstruga, 2011). Once a primary haustorium is established, secondary hyphae and secondary haustoria can rapidly develop, which are visible at 24-48 hpi (Kuhn *et al.*, 2016). Finally, conidiophores are formed, which are able to produce new airborne conidia (Figure 1). Although most PM species develop epiphytically, infecting only the epidermal cells of their hosts, some species breach through the stomata, forming haustoria in the mesophyll cells and partially extending their hyphae endophytically. Such is the case for *Leveillula taurica*, a pathogen that affects the production of tomato, pepper and eggplant (Braun *et al.*, 2002; Zheng *et al.*, 2013b).

To establish a biotrophic interaction, plant pathogens need to circumvent plant defence mechanisms and prevent host cell death to allow the uptake of nutrients. To do this, they produce and deliver molecules called effectors into the host. Effectors are generally regarded as molecules that are targeted to the host and contribute to the establishment of the biotrophic relationship (reviewed by Cook *et al.*, 2015). PM pathogens deliver effectors to the host plant cells via the haustorium (Bushnell, 1972). The functional characterization of effectors in PM pathogens is particularly difficult due to their obligate biotrophic characteristics, making it unfeasible to culture them in media, and the lack of effective methods for stable transformation. Nevertheless, transcriptome profiling and proteomic data of *Blumeria graminis* f. sp. *hordei* (*Bgh*) growing on barley (Bindschedler *et al.*, 2009, 2016; Godfrey *et al.*, 2009) have aided the identification of candidate effector-secreted proteins (CSEPs). Further functional characterization of these candidate effectors has been carried out by downregulation via Host-Induced Gene Silencing (HIGS; reviewed by Bourras *et al.*, 2018; Barsoum *et al.*, 2019). Although identification of PM effectors has mostly been focused on monocot PM more research on the dicot PM is also underway. A comparative genome study by Wu *et al.* (2018) between monocot and dicot PM, showed that the number of genes encoding effectors in the genomes of different PM species seems to be related to their host specificity and host-pathogen arms race. By comparing the genome of monocot and dicot PM pathogens it was found that in monocot PM pathogens, CSEPs account for 8% of the total number of genes in *Bgh* (Spanu *et al.*, 2010) and 9% in *B. graminis f.sp*. *tritici* (*Bgt*; Pedersen *et al.*, 2012), while in the five dicot-infecting PM pathogen genomes analysed (*Golovinomyces cichoracearum* UCSC1, *G. cichoracearum* UMSG1, *Oidium neolycopersici* UMSG2, *G. cichoracearum* UMSG3, and *Erysiphe necator* C-strain) only 1.8 - 2.5% of the genome encode candidate effector proteins (Wu *et al.*, 2018). However, it is possible that the limited information on the identity and characteristics of dicot-infecting PM effectors can affect these estimations.

The reluctant hosts: resistance to powdery mildew

First layer of defence

Several plant immune responses have been characterized to be able to suppress colonization by PM pathogens. The plant immune system is described to consist of two layers of defences (Jones & Dangl, 2006). The first layer is activated by the recognition of pathogen-associated molecular patterns (PAMPs) by the plant's pattern recognition receptors (PRRs). PAMPS are usually conserved across pathogens, and therefore their recognition by PRRs can result in basal and broad-spectrum immune responses such as the production of reactive oxygen species, the formation of papillae and callose deposition, or the activation of mitogen-activated protein kinase (MAPK) cascades (Dodds & Rathjen, 2010). PAMP-triggered immunity (PTI) is achieved when these responses are able to abolish the pathogen infection. The formation of papillae is known to be a first defence against adapted and non-adapted PM pathogens in different plant species (Aghnoum & Niks, 2010). The timing of the occurrence of papillae induced upon PM infection, as well as a high concentration of callose, cellulose and arabinoxylan have been associated with effective resistance (Chowdhury *et al.*, 2014).

A number of cell surface receptors involved in basal immunity have been characterized in resistance against PM in *Triticeae* species (reviewed by Saur & Hückelhoven, 2021) such as *Rnr9*/*LEMK1* in barley (Rajaraman *et al.*, 2016), and *RLKV1* (Hu *et al.*, 2018), *LecRK-V* (Wang *et al.*, 2018), and *RLK1/RLK2* in wheat (Chen *et al.*, 2016). Additionally, it has been shown that the Arabidopsis Lorelei-like GPI-Anchored Protein1 (LLG1) protein regulates PRR signalling during infection with the PM pathogen *G. cichoracearum* (Shen *et al.*, 2017).

Second layer of defence

Some pathogens are able to subdue PTI by delivering effectors into the host cells that inhibit defence responses, resulting in effector-triggered susceptibility (ETS) (Jones & Dangl, 2006). Plants, however, have evolved what is considered a second layer of immune response by producing receptors that directly or indirectly recognize such effectors and trigger downstream cascades that result in a stronger set of immune responses such as a hypersensitive response (HR) (Dodds & Rathjen, 2010). When such responses are successful, they lead to effector-triggered immunity (ETI). These plant receptors, also known as R-proteins, are typically nucleotide-binding site leucine-rich repeat (NLR) proteins (Jones & Dangl, 2006).

Interestingly, the number of typical *R* (*NLR*) genes identified in monocots compared to those in dicots is much higher. Wu *et al.* (2018) suggested that this, together with the evidence on the proportion of genes that represent the effectorome of dicot PMs to be much lower than those of monocot PM pathogens, is indicative of the host specificity and higher level of arms race of monocot PMs and their hosts. The barley *Mla* locus in barley was first identified in 1960 (Moseman & Sohaller, 1960) and since then, more than 20 allelic variants have been described to confer resistance against

different isolates of *Bgh* (Seeholzer *et al.*, 2010; Saur & Hückelhoven, 2021). Likewise, 17 allelic variants of the *Pm3* locus in wheat have been found to recognize different isolates of *Bgt* (reviewed by Saur & Hückelhoven, 2021). In dicots, however, only the tomato genes *Ol-4* and *Ol-6*, conferring resistance against PM, have been shown to encode an NLR (Seifi *et al.*, 2011). In Arabidopsis the *RPW8* locus, containing two paralogs (*RPW8.1* and *RPW8.2*), encoding non-typical NLR proteins (possessing a putative N-terminal transmembrane domain and a coiled-coil motif), is known to confer dominantly-inherited resistance against three PM pathogens by triggering defence responses that include HR and induction of pathogenesis-related (*PR-1*) genes (Xiao *et al.*, 2003; Wang *et al.*, 2009, 2013).

Susceptibility factors

For PM pathogens to develop a functional haustorium and establish an obligate biotrophic relationship with their host, they need to overcome the plant's immune responses and reprogram some of their cellular processes (Schulze-Lefert & Panstruga, 2003). Plant genes responsible for these processes and whose absence results in the inability of the pathogens to complete their life cycles, are considered *Susceptibility* (*S*) genes (Vogel & Somerville, 2000; van Schie & Takken, 2014). The most studied example of a susceptibility gene is the *Mildew Resistance Locus O* (*MLO*), discovered in the 1930's in barley and whose impairment confers resistance against PM (Jørgensen, 1992; Kusch & Panstruga, 2017). Natural and induced loss-of-function of *MLO* is known to confer resistance against PMs in several plant species including Arabidopsis, tomato , pea, pepper and cucumber (Consonni *et al.*, 2006; Bai *et al.*, 2008; Humphry *et al.*, 2011; Zheng *et al.*, 2013a; Berg *et al.*, 2015).

In order to screen for other loss-of-function-based resistance to PM in the model plant Arabidopsis, a forward genetic assay with 26,000 mutagenized plants challenged with *G. cichoracearum*, allowed the identification of six *powdery mildew resistant* (*pmr*) mutants (Vogel & Somerville, 2000). Four genes from this study were later identified and cloned. The resistance in the *pmr2* mutant was found to be conferred by the impairment of *MLO2* (Consonni *et al.*, 2006). Resistance in the *pmr4* mutant was due to the impairment of *GLUCAN SYNTHASE-LIKE5* (*GSL5*), also known as *CALLOSE SYNTHASE12* (*CalS12*) (Jacobs *et al.*, 2003; Nishimura, 2013). Remarkably, silencing of the closest ortholog of *PMR4* in tomato was also found to confer resistance against the tomato powdery mildew *Oidium neolycopersici* (Huibers *et al.*, 2013. See Chapter 4 of this thesis). *PMR5* has recently been found to encode a protein involved in acetylation of pectin (Chiniquy *et al.*, 2019). *PMR6* encodes a Pectate-Lyase-Like gene (Vogel *et al.*, 2002).

The enemy in question: *Oidium neolycopersici*

Oidium neolycopersici (*On*) is one of the pathogens causing PM in tomato. Currently this fungal species is also known as *Pseudoidium neolycopersici*. As most other PM pathogens, *On* grows epiphytically mainly on the adaxial side of tomato leaves and can extend to the stem and calyx. As the infection progresses, *On* causes premature

senescence and leaf chlorosis that affects yield and fruit quality (Whipps *et al.*, 1998; Jones *et al.*, 2001). Although an outbreak of PM in tomato was first reported in Europe in the late 1980's (Fletcher *et al.*, 1988), it was only in 2001 that the *On* species was identified as the causal pathogen of this and subsequent outbreaks of PM in cultivated tomatoes outside Australia (Kiss *et al.*, 2001).

Like other dicot PM pathogens, *On* has a wide host range. Whipps *et al.* (1998) reported that plant species from up to 13 families are hosts of *On.* Recently, *On* has been reported to infect Croton (*Codiaeum variegatum* var. *pictum*) (Liu *et al.*, 2015) and to cause severe crop damage in greenhouse Moringa (*Moringa oleifera*) in China (Liu *et al.*, 2019). Furthermore, differences in pathogenicity of *On* isolates in tomato species suggest the existence of different pathotypes (ff. spp.) (Lebeda & Mieslerová, 2002).

The worthy opponent: resistance to *Oidium neolycopersici* in wild relatives of tomato

The urge to look for resistance against *On* in tomato came with the outbreak in the late 1980's, when all tomato cultivars turned out to be susceptible to this pathogen (Fletcher *et al.*, 1988; Huang *et al.*, 1998). Since then, a total of nine loci have been identified in wild relatives of tomato to confer resistance against *On* (Table 1, reviewed by Seifi *et al.*, 2014).

Dominant resistance is known to be conferred by five loci (*Ol-1 to -6*, excl. *ol-2*). *Ol-1* (see **Chapter 5** of this thesis) was identified in *S. habrochaites* G1.1560 and further mapped to the long arm of chromosome 6 (van der Beek *et al.*, 1994; Bai *et al.*, 2005). Later efforts to fine-map *Ol-1* located it to a 71-kb region bordered by markers scaff6 and TAG (Seifi, 2011). *Ol-3* was identified in *S. habrochaites* G1.1290, mapped to the same region as *Ol-1* and suggested to be allelic to the latter (Huang *et al.*, 2000; Bai *et al.*, 2005). *Ol-4* was identified in *S. peruvianum* (currently *S. arcanum*) LA2172 and mapped to the short arm of chromosome 6 to a cluster of *R* genes that harbours the *Mi-1* gene that confers resistance to root-knot nematodes and potato aphids (Bai *et al.*, 2004; Seifi *et al.*, 2011). *Ol-5* was found in *S. habrochaites* PI247087 and was mapped to the long arm of chromosome 6, but not allelic to *Ol-1*/*Ol-3* (Bai *et al.*, 2005). Finally, *Ol-6* was found in an advanced breeding line of unidentified origin, mapped to the same position and probably a homologue of *Ol-4* (Bai *et al.*, 2005; Seifi *et al.*, 2011). At the start of this research, *Ol-1*, *Ol-3* and *Ol-5*, had not been cloned. In **Chapter 5**, we describe the fine-mapping and functional analysis of the *Ol-1* locus. Additionally, recessively-inherited resistance to *On* is conferred by *ol-2*, identified in *S. lycopersicum* var. *cerasiforme* LA1230, mapped to chromosome 4 (Ciccarese *et al.*, 1998), and further identified as a homologue of the *MLO* gene in barley (Bai *et al.*, 2008). Furthermore, three quantitative trait loci are known to confer resistance against *On* (*Ol-qtl*s). These were found in *S. neorickii* G1.1601 (Bai *et al.*, 2003). *Ol-qtl1* has been mapped to an interval that co-localizes with the *Ol-1*

(*Ol-3* and *Ol-5*) region on chromosome 6. *Ol-qtl2* and *Ol-qtl3* were mapped on chromosome 12, near the *Lv* locus, which confers resistance to another PM pathogen, *Leveillula taurica* (Yordanov *et al.*, 1975).

Table 1. Resistance loci to powdery mildew (PM) pathogens in tomato and Arabidopsis. Locus name, origin, PM pathogen, mode of inheritance, and resistance response are listed.

	Locus	Origin	PM Pathogen	Inheritance	Resistance response	Reference
Tomato	*Ol-1*	*S. habrochaites* G1.1560	*O. neolycopersici*	Dominant	Slow (multicellular) HR	(van der Beek *et al.*, 1994)
	ol-2	*S. lycopersicum* var. *cerasiforme*	*O. neolycopersici*	Recessive	Papillae formation	(Bai *et al.*, 2008)
	Ol-3	*S. habrochaites* G1.1290	*O. neolycopersici*	Dominant	Slow (multicellular) HR	(Huang *et al.*, 2000)
	Ol-4	*S. peruvianum* LA1272	*O. neolycopersici*	Dominant	Fast (unicellular) HR	(Seifi *et al.*, 2011)
	Ol-5	*S. habrochaites* PI247087	*O. neolycopersici*	Dominant	Slow (multicellular) HR	(Bai *et al.*, 2005)
	Ol-6	Advanced breeding line	*O. neolycopersici*	Dominant	Fast (unicellular) HR	(Bai *et al.*, 2005)
	Ol-qtl1	*S. neorickii* G1.1601	*O. neolycopersici*	*QTL*		
	Ol-qtl2	*S. neorickii* G1.1601	*O. neolycopersici*	*QTL*		
	Ol-qtl3	*S. neorickii* G1.1601	*O. neolycopersici*	*QTL*		(Bai *et al.*, 2003)
	Ol-qt2, 3	*S. neorickii* G1.1601	*O. neolycopersici*	*QTL*		
	Ol-qtls 1, 2, 3	*S. neorickii* G1.1601	*O. neolycopersici*	*QTL*	Fast + slow HR + Papillae	
Arabidopsis	*RPW1*	Kas-1	*E. cichoracearum*	Semi-dominant	Chlorotic/necrotic flecks	(Adam & Somerville, 1996)
	RPW2	Wa-1	*E. cichoracearum*	Semi-dominant	Chlorotic/necrotic flecks	(Adam & Somerville, 1996)
	RPW4	Stw-0	*E. cichoracearum*	Semi-dominant	Small necrotic flecks	(Adam & Somerville, 1996)
	RPW5	Su-0	*E. cichoracearum*	Semi-dominant	Small necrotic flecks	(Adam & Somerville, 1996)
	RPW6	Ms-0	*E. cruciferarum*		Localized necrosis	(Xiao *et al.*, 1997)
	*RPW7**	Ms-0	*E. cruciferarum*		Localized necrosis	(Xiao *et al.*, 1997)
	RPW8	Ms-0	*E. cichoracearum*, *E. cruciferarum*	Dominant	HR-like response	(Xiao *et al.*, 1997)
	*RPW10**	Kas-1	*E. cichoracearum*	Semi-dominant	Chlorotic/necrotic flecks	(Wilson *et al.*, 2001)
	RPW11	Kas-1	*E. cichoracearum*			(Wilson *et al.*, 2001)
	RPW12	Kas-1	*E. cichoracearum*			(Wilson *et al.*, 2001)
	*RPW13**	Wa-1	*E. cichoracearum*			(Schiff *et al.*, 2001)
	RPW14	Wa-1	*E. cichoracearum*	Minor QTL		(Schiff *et al.*, 2001)

**RPW8 allele*

The enemy of my enemy: the Arabidopsis – *On* interaction

The model plant Arabidopsis offers many advantages for research due to the availability of a large number of scientific resources, tools, experimental protocols and information (Somerville & Koornneef, 2002). However, it was only during the decade of the 1990's, that PM species able to infect this model organism were identified (reviewed by Micali *et al.*, 2008). Since then, four PM species have been used to study their interaction with Arabidopsis, namely *Erisyphe cruciferarum* (Koch & Slusarenko, 1990), *Golovinomyces* (syn. *Erysiphe*) *cichoracearum* (Adam & Somerville, 1996), *Golovinomyces* (syn. *Erysiphe*) *orontii* (Plotnikova *et al.*, 1998) and the tomato-adapted PM pathogen *On* (Xiao *et al.*, 2001). Resistance in Arabidopsis has been found in several accessions and a number of *Resistance to Powdery Mildew* (*RPW*) loci has been identified (Table 1). At the start of this study, the *RPW8* locus, carrying two paralogs (*RPW8-1* and *RPW8-2*) of a non-typical *R* gene, was the only dominantly-inherited locus to confer resistance against PM in Arabidopsis. This resistance was first found in accession Ms-0 (Xiao *et al.*, 2001) and has been found to be functional in many other ecotypes against three PM species (Schiff *et al.*, 2001; Göllner *et al.*, 2008). Notably, accession Shakdara (Sha), carrying both Ms-0-like paralogs of *RPW8* was found to be susceptible to *On* (Göllner *et al.*, 2008). Furthermore, additional *RPW* loci have been found to confer semi-dominant resistance to *E. cichoracearum* in accessions Kas-1, Wa-1, Stw-0 and Su-1 (Adam & Somerville, 1996; Xiao *et al.*, 1997).

The Arabidopsis-PM pathosystem has been studied extensively to elucidate the genetic and molecular components of resistance and susceptibility to these biotrophic pathogens. Particularly, some of the susceptibility components have been found to be conserved in other plant species, as is the case for *mlo* and *pmr4*, and to be broad spectrum. However, most of the studies have focused on the resistance against *E. cruciferarum*, *G. cichoracearum* and *G. orontii*, and limited research has been done on the specific interaction between Arabidopsis and *On*. In order to explore natural resistance in this specific pathosystem a set of 123 Arabidopsis accessions was challenged with the Wageningen isolate of *On.* Through this screening, a natural loss-of-function allele of *EDR1* was found to confer resistance in Arabidopsis accession C24 (Gao *et al.*, 2015).

Scope of this thesis

The work of this thesis focuses on the exploration and identification of new mechanisms of resistance against *On* in two pathosystems. In the first half, we make use of the interaction with the model plant Arabidopsis to identify a novel component of resistance in two accessions. Secondly, we use the crop plant tomato to further characterize a susceptibility factor and to identify the gene responsible for the *Ol-1* resistance.

In **Chapter 2** we mapped the natural, dominantly-inherited resistance found in Arabidopsis accession Bla-6 to a single locus in chromosome 1. Through several rounds of recombinant analyses, we narrowed-down the candidate region and used CRISPR/Cas9 on the candidate genes to elucidate the one responsible for the resistance. We show that targeted mutagenesis of *ZED1-RELATED KINASE 13* (*ZRK13*) in a Bla-6 background results in a susceptible phenotype, thus identifying this gene as a novel component of resistance against *On* in Arabidopsis.

In **Chapter 3** we explored the resistance to *On* observed in Arabidopsis accession Litva. Through a quantitative trait loci (QTL) analysis, we identified two major loci determining the resistance. Through independently executed recombinant analyses, we found one of the QTLs to overlap with the resistance locus found in accession Bla-6. Using CRISPR/Cas9-targeted mutagenesis, we show that the resistance in Litva also requires *ZRK13*. Furthermore, by testing a number of informative recombinants we show that full resistance in this accession also requires a locus on chromosome 3.

In **Chapter 4** we used CRISPR/Cas9-targeted mutagenesis in a susceptible tomato cultivar to elucidate if the full knock-out of the susceptibility gene *PMR4* results in a higher level of resistance than RNAi-silenced transgenic lines. We identified five mutation events in the mutant lines and characterized their resistance. The mutants showed reduced but not complete loss-of-susceptibility to *On*.

In **Chapter 5** we fine-mapped and characterized the candidate genes for *Ol-1*, a dominant resistance found in *S. habrochaites* G1.1560. We show that targeted mutagenesis of Solyc06g060800, a gene encoding a 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein, results in increased susceptibility to PM.

Finally, in the general discussion (**Chapter 6**), I show that the combined research on Arabidopsis and tomato has resulted in a better and more complete understanding of factors that are important in obtaining resistance against *On*.

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Chapter 2

ZED1-RELATED KINASE 13 (*ZRK13*) is required for resistance against *Oidium neolycopersici* in Arabidopsis accession Bla-6

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Abstract

To explore specific components of resistance against the tomato-adapted powdery mildew pathogen *Oidium neolycopersici* (*On*) in the model plant Arabidopsis, we performed a disease assay in 123 accessions. When testing the resistance in the F_1 from crossings between resistant accessions with susceptible Col-0 or Sha, only the progeny of the cross between accession Bla-6 and Col-0 displayed a completely resistant phenotype. The resistance in Bla-6 is known to be specific for *Oidium neolycopersici*. QTL analysis and fine-mapping through several rounds of recombinant screenings allowed us to locate a major resistance QTL in an interval on chromosome 1, where three candidate genes were predicted. We used targeted mutagenesis via CRISPR/Cas9 to knock out these candidates. After infecting the transformants with *On*, we found that *ZED-1 RELATED KINASE 13* (*ZRK13*) is required for the *On* resistance in Bla. Several polymorphisms are observed in the ZRK13 allelic variant of Bla-6 when compared to the Col-0 protein.

Introduction

Powdery mildew (PM) is one of the most widely occurring plant diseases in the world, observed in almost 10,000 different plant species, including several economically important crops (Takamatsu, 2004; Glawe, 2008). Pathogens causing PM are a diverse group of more than 400 species of fungi of the *Erysiphales* order (Takamatsu, 2004) that establish an obligate biotrophic interaction with their hosts, thus depending on living plant cells to complete their life cycles (Glawe, 2008). However, plants have evolved resistance mechanisms to interrupt and overcome this infection. Plant immunity relies on two interconnected layers of defence (Jones & Dangl, 2006; Dodds & Rathjen, 2010). The first layer is activated upon the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), located at the cell surface, and leading to PAMP-triggered immunity (PTI) (Dodds & Rathjen, 2010). The defence responses associated with PTI include oxidative bursts and callose deposition. If PTI is compromised, a second layer may be activated upon recognition of pathogen effectors by the plant's resistance (R) proteins, leading to effector-triggered immunity (ETI). *R* genes typically encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (Jones & Dangl, 2006). A faster and stronger defence response is produced in ETI compared to PTI, such as a hypersensitive response (HR), a fast and localized type of cell death (Dodds & Rathjen, 2010).

The model plant Arabidopsis has been an important resource to study plant-pathogen interactions. Specifically, the Arabidopsis-PM pathosystem has been useful to identify and characterize host resistance mechanisms (Kuhn *et al.*, 2016). Arabidopsis is known to be infected by four PM species, namely *Erisyphe cruciferarum* (Koch & Slusarenko, 1990), *Golovinomyces* (syn. *Erysiphe*) *cichoracearum* (Adam & Somerville, 1996), *Golovinomyces* (syn. *Erysiphe*) *orontii* (Plotnikova *et al.*, 1998), and the tomato PM pathogen *Oidium neolycopersici* (*On*) (Xiao *et al.*, 2001). Resistance to PM in Arabidopsis has been shown to be predominantly polygenic (Göllner *et al.*, 2008) and to date, the only dominantly-inherited *R* locus against powdery mildew in Arabidopsis is *RPW8*. This locus contains two paralogs (*RPW8.1* and *RPW8.2*), it is located on chromosome 3 and the functional *R* paralogs are absent in the reference accession Col-0 (Xiao *et al.*, 2001). Interestingly, the proteins encoded by these genes share only a limited homology to NBS-LRR-like proteins but are able to recruit components used by other NB-LRRs, such as the activation of the salicylic acid-dependent pathways (Xiao *et al.*, 2003, 2005). *RPW8.2* is known to be induced and targeted to the extrahaustorial membrane to activate the defence responses including occurrence of HR, oxidative burst and induction of pathogenesis-related (*PR-1*) genes (Wang *et al.*, 2009). The resistance conferred by *RPW8* is known to be broad-spectrum (Xiao *et al.*, 2001, 2003). However, when testing several accessions of Arabidopsis carrying allelic variants of the *RPW8* paralogs, not all accessions were resistant to the tomato PM pathogen *On*, while being resistant to the other three PM pathogens known to infect

Arabidopsis (Göllner *et al.*, 2008). In particular, accession Sha, which harbors the functional allele of *RPW8* and is fully resistant against *E. cruciferarum*, *G. cichoracearum* and *G. orontii*, is highly susceptible to *O. neolycopersici* (Göllner *et al.*, 2008). Additionally, heterologous expression of *RPW8* genes in tomato failed to confer enhanced resistance against *On* (Xiao *et al.*, 2003). This may imply unique features for the Arabidopsis-*On* pathosystem. This specific interaction has been less extensively characterized compared to that between Arabidopsis and the other three PM species. Therefore, studying this biotrophic relationship may provide new insights to unravel differential genetic components of resistance and susceptibility to *On*.

On the other hand, different PM pathogens seem to be able to exploit common susceptibility factors across plant species. For instance, through screening of an ethyl methanesulfonate- (EMS-) mutagenized population, six powdery mildew resistant *(pmr)* susceptibility factors were identified (Vogel & Somerville, 2000; Vogel et al. 2002, 2004). Specifically, the susceptibility factor *PMR4*, whose impairment resulted in resistance associated with salicylic acid accumulation to both *G. cichoracearum* and *G. orontii*, was further identified as the *Glucan Synthase-Like 5* (*GSL5*) gene (Nishimura, 2013). By silencing the closest *PMR4* ortholog in tomato (*SlPMR4*) Huibers *et al.* (2013) found the resistance observed in Arabidopsis mutants to be conserved in tomato against the adapted PM pathogen *On*.

With the objective of further exploring the resistance in Arabidopsis against *On*, we previously performed a disease assay on 123 Arabidopsis accessions, of which 40 showed to be fully resistant. Further studies of these accessions allowed us to identify a natural mutation of *EDR1* conferring resistance to *On* in accession C24 (Gao *et al.*, 2015). In the present study we describe the mapping and identification of the gene responsible for the resistance against *On* observed in accession Bla-6. Previously, Adam *et al.* (1999) performed disease assays using *G. cichoracearum* and *E. cruciferarum* and reported this accession to be susceptible to these PM pathogens. We report the finding of a novel dominant resistance gene in Arabidopsis accession Bla-6 against *On*. To our knowledge, this is the first Arabidopsis dominant PM resistance to be characterized through its interaction with *On*. We fine-mapped the candidate region through several rounds of recombinant screenings and used CRISPR/Cas9 targeting the candidate genes to elucidate the gene responsible for the resistance.

Results

Resistance in Bla-6 is governed by a dominant locus on chromosome 1

Previously, a set of 123 Arabidopsis accessions (five plants per accession) were inoculated with *On* in order to investigate natural variation in resistance (Gao *et al.*, 2015). The disease symptoms were evaluated using a disease index (DI) score ranging from 0 (resistant) to 3 (susceptible), starting at 12 days post-inoculation (dpi). From

this set of plants, 40 accessions were found to be fully resistant. We selected 19 of these accessions and crossed them with susceptible accessions Col-0 or Sha. Five F_1 plants from each cross were inoculated with *On* to assess the DI. The F_1 plants from 18 of the crosses were found to be susceptible (DI>0). Only the progeny from the Bla-6 x Col-0 cross was found to be completely resistant (DI=0) (Gao *et al.*, 2015; Fig 1A).

The F_2 progeny (92 plants) of the Bla-6 x Col-0 cross was tested in a disease assay with *On*. Of these, 72 plants were resistant and 20 plants were susceptible, fitting a segregation ratio of 3:1 (resistant : susceptible) (Fig. 1A, S1A). Information on the markers used in the fine-mapping are provided in Table S1. A preliminary QTL indicated a major peak at the end of chromosome 1, between markers RH473/474 and RH481/482 (Fig. S1B). We performed a first recombinant screening using 768 F_2 plants, in which we identified 52 recombinants between markers RH473/474 and RH481/482 (Fig. 1Bi). From these, F_3 progenies of nine informative recombinants (17 plants per family) were tested in a disease assay and genotyped using 14 additional markers between RH473/474 and RH481/482 (Fig. S2A). As a result, the QTL region was shown to be bordered on the right side by marker SNP51. However, the left border could not be determined (Fig. S2A). Therefore, a second recombinant screening was performed with markers RH453/454 and RH565/566, using 528 F_3 plants derived from an F_2 plant heterozygous for the markers in the QTL region (plant F-4F, Fig. S2B). Three informative recombinants (#188, 100 and 102) and, as a control, one F3 plant heterozygous for the QTL region (#270) were then selfed for further confirmation and genotyping. For each of these four F_4 families, 40 plants were tested for their response to *On* and further genotyped with four additional markers within the region between RH453/454 and RH565/566 (Fig. S2B). This allowed us to reduce the interval to a 185-kb region between markers RH777/780 and SNP51 (Fig. 1Bii).

Finally, we performed a third recombinant screening with 747 plants of the segregating F_4 family from the F3 plant #270 using markers M6 and RH555/556. A total of 15 recombinants were found (Fig. S2C) and further genotyped with markers M14, M24 and M36. Two recombinants (Rec1 and Rec2), showing contrasting phenotypic response to *On* were both found to carry a recombination event between markers M24 and M36 (66-kb region). To confirm these results, we performed a disease assay in the F_5 progeny (37 plants per genotype) of Rec1 and Rec2, along with informative recombinants 19.9, 8.24 and 13.4 (Fig. S2C). We confirmed a 3:1 (resistant:susceptible) segregation in disease response for Rec1 and a susceptible phenotype for all progeny of Rec2. Furthermore, by sequencing of PCR products (L11, P26, P27 and P33, Table S1) from DNA of homozygous F_5 plants from each line, we pinpointed the recombination events between markers M24 and L11 (5945-bp region) for both families (Fig. 1Biii). Subsequently, homozygous F_5 plants fixed for each allele were selfed to produce F_6 families. We confirmed the expected phenotype of these families in a disease assay using 10 plants per family. By PCR-

based sequencing of F_6 plants, we located the recombination events of these lines at the intergenic region between genes At1g65190 and At1g65200 for Rec1, and 188 bp within the CDS of At1g65180 for Rec2 (Fig. 1Biv).

The region between the two recombination events was subsequently amplified in wild-type (WT) Bla-6 and Col-0, and resistant and susceptible progeny plants from Rec1 and Rec2, using primers L1F (located within At1g65180, Fig. 1Biv) and L11R (located within At1g65200, Fig. 1Biv). This yielded a 5,986-bp PCR fragment for Col-0. However, we obtained larger products for Bla-6 and resistant progeny from Rec1, suggesting the presence of an insertion in these plants. Larger (~4.5 kb) PCR products than the expected 2,502-bp fragment in Col-0 were also obtained when using primers L1F and L33R (Table S2), as shown in Fig. 1C. Using additional in-between primers (Table S2), we sequenced the PCR product of primers L1F and L11R from Bla-6, allowing us to identify a 1967 bp-long insertion (Fig. S5) within the intergenic region between At1g65180 and At1g65190. A prediction of a potential gene with a high similarity to a translation protein SH3-like family protein (AT1G57860.1) was retrieved by analysing the sequence of this insertion using the FGENESH webtool (Solovyev *et al.*, 2006). Thus, three candidate genes are located in the chromosomal region responsible for the resistance to *On* in Bla-6: At1g65180, annotated as a Cysteine/Histidine-rich C1 domain family protein; a putative gene in the intergenic 1967-bp insertion; and At1g65190 (Protein kinase superfamily protein, *ZRK13*).

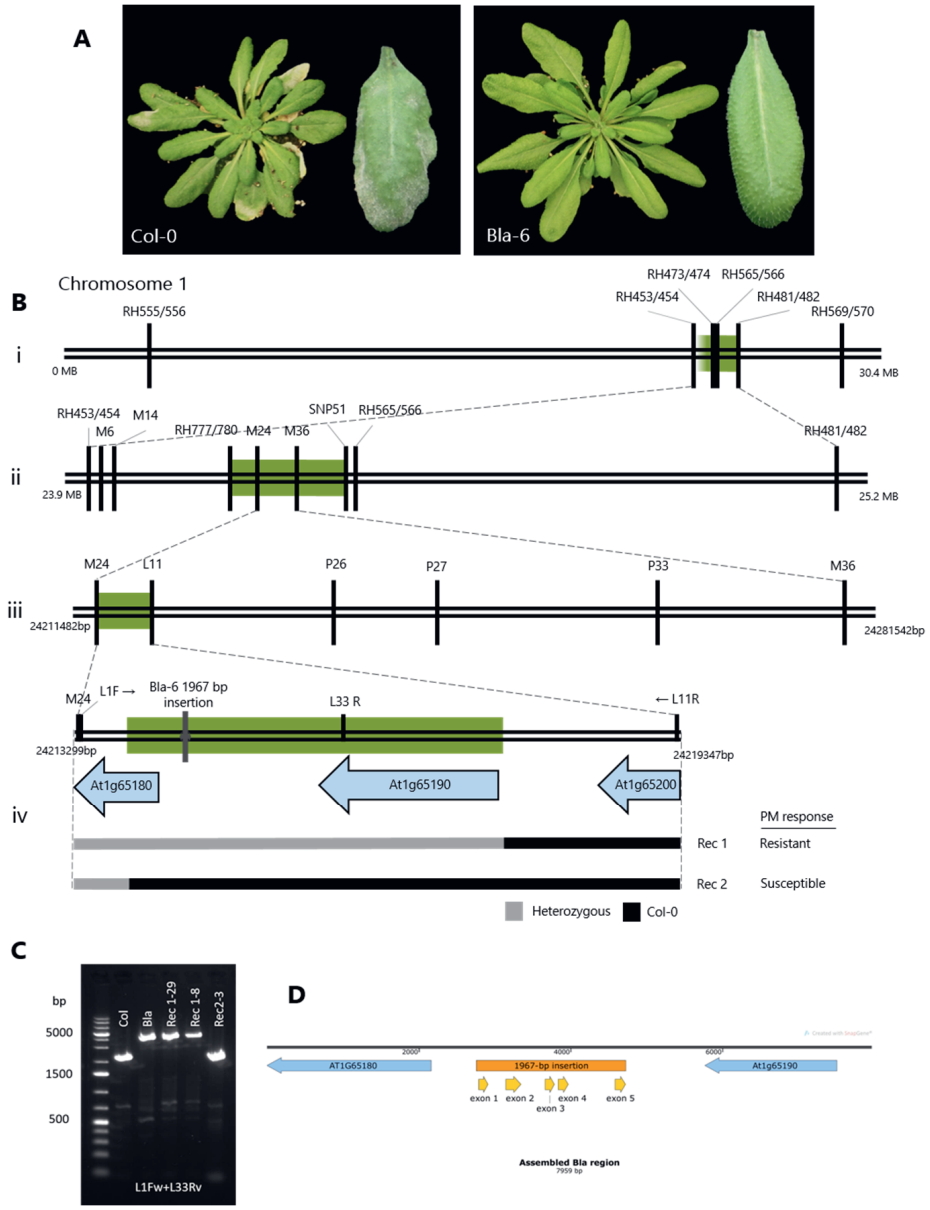


Figure 1. Fine-mapping of the resistance to *Oidium neolycopersici* (*On*) in Arabidopsis accession Bla-6. **A**. Disease response of Col-0 and Bla-6 to *On* at 12 days post-inoculation (12dpi): heavy sporulation over the infected leaves of susceptible accession Col-0 and lack of visible symptoms in accession Bla-6. **B**. Location of markers for fine-mapping of the resistance locus in chromosome 1: green bars indicate the candidate regions for each of the three recombinant screenings (i to iii) and PCR-based sequencing of F_5 informative recombinants Rec1 and Rec 2 with contrasting phenotypic response upon *On* inoculation (iv). **C**. Agarose gel electrophoresis of the PCR product using L1F and L33R for wild-type Col-0 (2502-bp), Bla-6

(4469-bp) and F_6 progeny of Rec1 (4469-bp) and Rec2 (2502-bp). **D.** Assembled candidate region showing the candidate genes At1g65180 and At1g65190 (blue), and the 1967-bp insertion (orange) with its predicted exons (yellow).

Targeted mutagenesis of *ZRK13* in Arabidopsis accession Bla-6 results in susceptible phenotype upon inoculation with *On*

To elucidate the identity of the gene in the chromosomal region responsible for the resistance in Bla-6, we designed three CRISPR/Cas-9 constructs, each containing four sgRNAs targeting one of the candidates (Fig. 2A). The transformation construct included FAST-Red as a marker to optimize the selection of transformed seeds. Transformation was performed by floral dripping. Seeds were harvested and selected under fluorescent light and subsequently stratified to break dormancy before being sown to be tested in a disease assay with *On*. In total, we tested 49 plants from the seeds obtained from the transformation with the construct targeting At1g65180, 53 plants from the seeds obtained from the transformation with the construct targeting the putative gene in the insertion and 75 plants from the seeds obtained from the transformation using the construct targeting *ZRK13*.

We phenotyped the disease response to *On* in the transformants. We expected a small number of the transformants to be bi-allelic or homozygous mutants for the CRISPR-targeted gene as well as chimeric plants with somatic mutations. No disease symptoms were visible in the plants obtained from the transformation with the constructs targeting At1g65180 or the intergenic insertion. However, from the 75 transformed plants obtained using the construct targeting *ZRK13*, five plants showed clear disease symptoms (Fig. S3A). PCR-based sequencing using primers LZRK13F and LZRK13R, amplifying the region containing the target sites of the sgRNAs, revealed the presence of double peaks at the sgRNA ZRK13-1 target site. Furthermore, analysis of the sequencing data using the TIDE webtool (Brinkman *et al.*, 2014) to detect sequence trace decomposition showed aberrant sequences at the expected sites (Fig. S3B). Additionally, we screened at least 15 CRISPR transformants in which At1g65180 or *ZRK13* was targeted to check for mutations at the target sites of sgRNAs. We detected aberrant sequences in two plants that showed no disease symptoms; one At1g65180 CRISPR transformant (2.25) and one *ZRK13* CRISPR transformant (3.36).

In a second disease assay with *On* we tested the selfing T_2 progeny of the five susceptible *ZRK13* CRISPR plants (2.11, 2.7, 2.26, 3.6, and 3.21), along with the selfing progenies of the resistant *ZRK13* CRISPR plant (3.36) and of the At1g65180 CRISPR transformant (2.25) that showed aberrant sequences of the targeted genes. We expected heritable mutations to be found in this generation. We sowed 40 seedlings from each family and infected them with *On.* The total number of plants with clear symptoms for each family are listed in Table 1. To pinpoint mutation events, we used PCR-based sequencing using primers Det_ZRK13F and Det_ZRK13R (Table S2). We identified three homozygous mutation events in three different lines (Fig. 2B, 2C). In

nine susceptible plants of family 2.7, we identified a 1-bp insertion (G) within sgRNA ZRK13-1. Furthermore, PCR-based sequencing of resistant plant 2.7-30 yielded a WT allele. In line 3.21 we identified four susceptible plants carrying a homozygous 25-bp deletion, as well as 1-bp insertion (C) within sgRNA ZRK13-3. Additionally, sequencing of one plant without symptoms (3.21-8), yielded aberrant sequences starting from the site of sgRNA ZRK13-1. Finally, in family 3.6-23 we identified four susceptible plants carrying a 1-bp insertion (G) within sgRNA ZRK13-1. Sequencing of four resistant plants in family 3.6 yielded WT alleles.

In families 2.11, 2.26 and 3.36 we identified the presence of aberrant sequences in susceptible plants but no homozygous mutation events. In family 2.11, we observed only one plant with disease symptoms after inoculation. PCR-based sequencing of this plant showed the presence of aberrant sequences in sgRNA ZRK13-1. In family 2.26, three susceptible plants showed the presence of aberrant sequences from sgRNA ZRK13-1, while three resistant plants yielded a WT allele. Lastly, in family 3.36, we identified aberrant sequences starting at sgRNA ZRK13-1 in five susceptible plants, while sequencing of one resistant plant yielded a WT allele. In none of the progeny of the transformant obtained using the CRISPR construct targeting At1g65180 we observed disease symptoms, and therefore resistance was not compromised. Sequencing of five plants from this family yielded only WT alleles.

Table 1. Phenotype upon inoculation with *Oidium neolycopersici* (*On*) in the T_2 progeny of the CRISPR/Cas9 transformants. Gene target, family number and number of susceptible and resistant plants per family are shown.

Gene target	Family	Susceptible	Resistant
ZRK13	2.11	1	39
	2.7	25	14
	2.26	17	23
	3.6	8	31
	3.21	5	30
	3.36	24	16
At1g65180	2.25	0	34

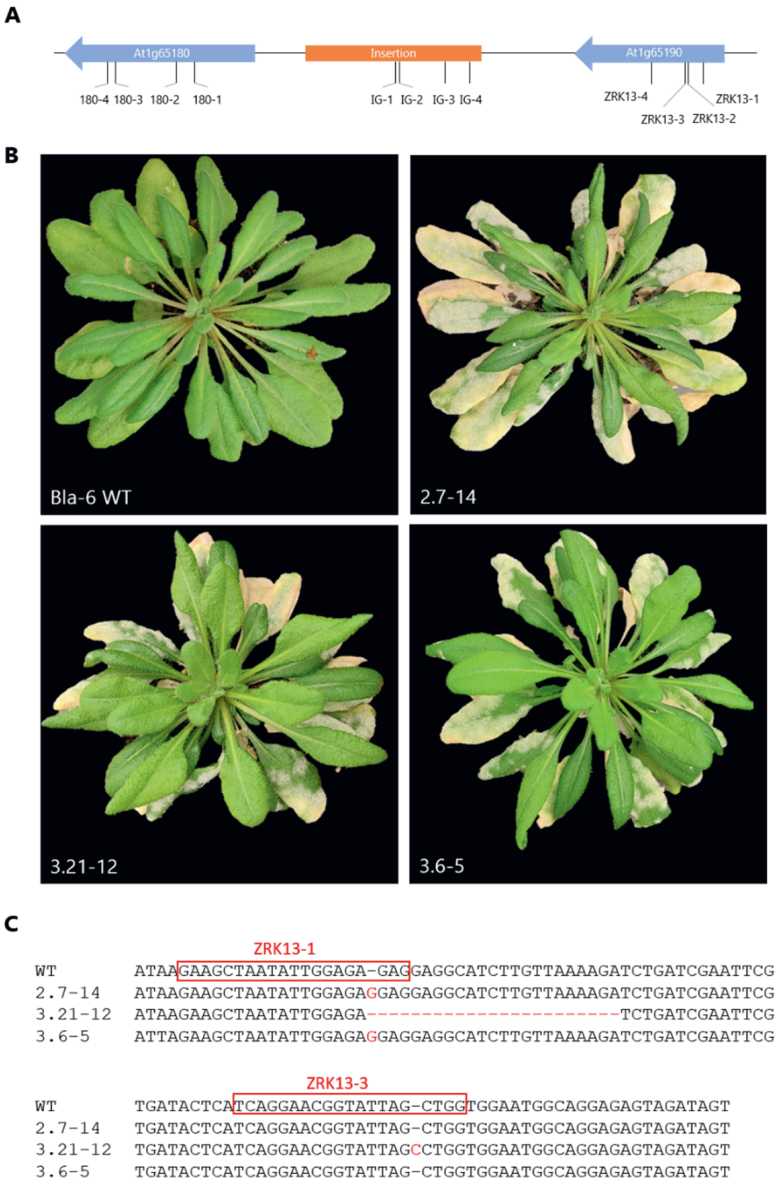


Figure 2. Targeted mutagenesis of *ZRK13* in Bla-6 results in a susceptible phenotype to *Oidium neolycopersici*. A. Location of the single guide RNAs (sgRNAs) in the candidate region of Bla-6. Blue arrows indicate the two genes in the region. Orange square indicates the intergenic region insertion present in Bla-6. Black bars indicate the location of the sgRNAs. **B**. Phenotype at 26 days post inoculation of wild-type (WT) Bla-6 and three T_2 plants from susceptible transformants. **C.** Mutation events identified in the T_2 plants shown in B. Deletions and insertions in CRISPR mutants are indicated with letters in red. sgRNAs ZRK13-1 and ZRK13-3 are indicated inside red boxes.

Polymorphisms in the *ZRK13* allele of Bla-6

Using NCBI's ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/), the predicted ORF of the Bla-6 allele of *ZRK13* was determined to encode a protein of 346 amino acids (aa), contrasting with the 396 amino acids-long Col-0 protein (Fig. 3, Fig. S4). The predicted ZRK13 protein of Bla-6 shows 81.2% similarity with the Col-0 allele and, notably, 86.7% similarity with ZRK14 (At1g65250) of Col-0. The relatively low level of similarity of the ZRK13 protein sequence of Bla-6 compared to the Col-0 protein is caused by many amino acid substitutions and additionally, a 2-aa deletion at position 8, an 8-aa deletion at position 270 and a 4-aa insertion at position 90 (Fig. 3A). Furthermore, a premature translation termination codon at position 357 causes a lack of the 49-aa disordered region reported to be present in the Col-0 protein according to the domain annotation retrieved from Uniprot (https://www.uniprot.org/). Importantly, when compared with the protein sequences of members of the ZRK family which are characterized to recognize type-3 bacterial-secreted effectors to trigger ETI (ZED1, ZRK1 and ZRK3), the ZRK13 allele of Bla-6 clusters in a different clade (Fig. 3B).

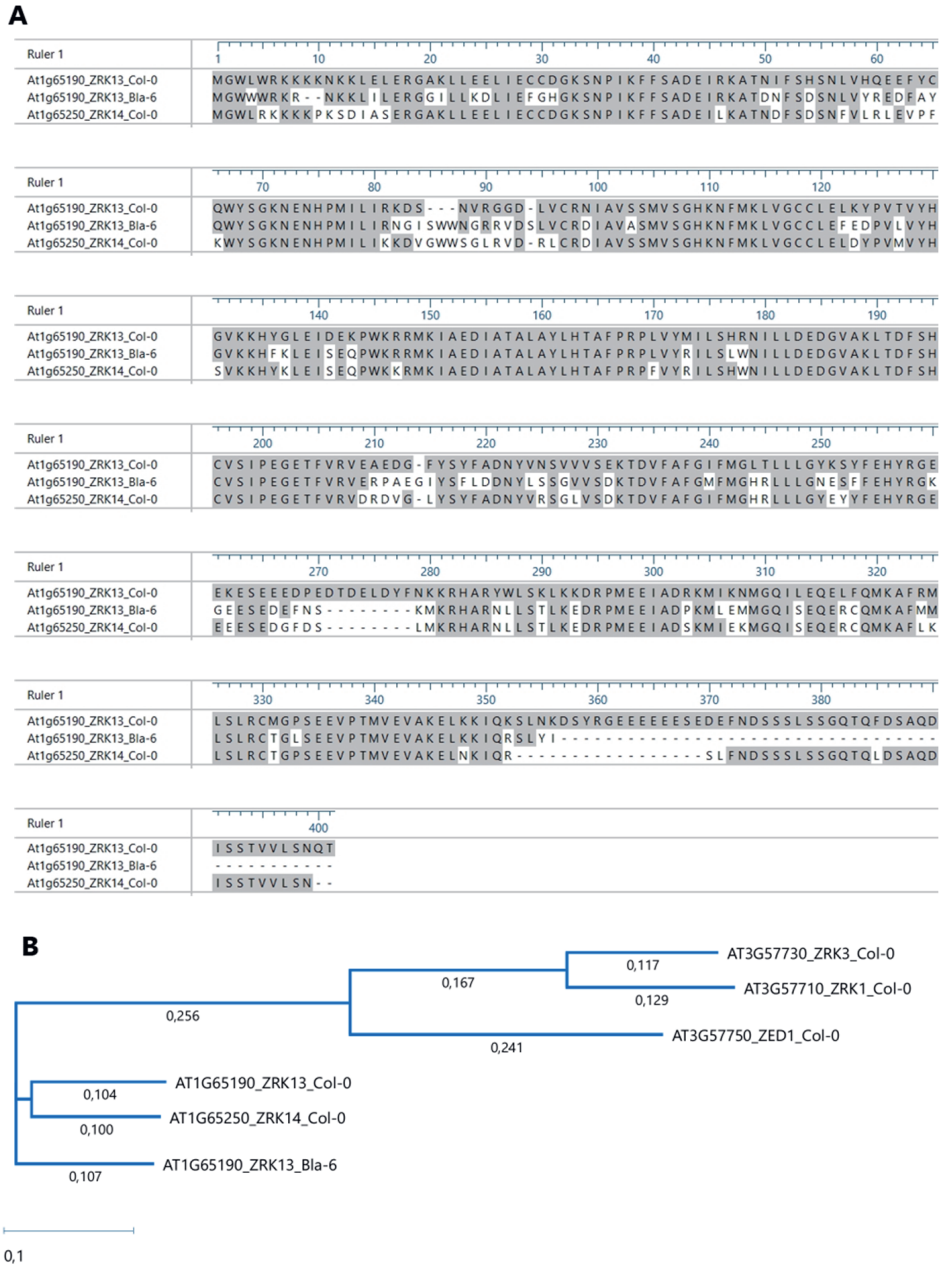


Figure 3. Alignment of the predicted protein sequence of ZRK13 allele of Bla-6 with the ZRK13 and ZRK14 alleles of Col-0. A. Conserved amino acids are shown in grey. **B.** Phylogenetic tree of some of the ZRK genes on chromosome 1 and 3.

Discussion

The model species *Arabidopsis thaliana* has been extensively used for screening and characterization of mechanisms of resistance against pathogens. With the aim of uncovering new mechanisms and sources of resistance against *On*, we screened 123 accessions of Arabidopsis for their response against this disease. From the 19 crosses that we performed between resistant accessions and susceptible Col-0 and Sha, the Bla-6 x Col-0 cross stood out as it was the only F_1 that was completely resistant to *On*. To date, the only reported dominant resistance to PM in Arabidopsis is conferred by the *RPW8* locus on chromosome 3 and first found in accession Ms-0 (Xiao *et al.*, 2001).

In this study we mapped the resistance against *On* in Bla-6 to chromosome 1. Through several rounds of recombinant analyses, we fine-mapped the candidate region and found three predicted candidate genes. We used CRISPR/Cas9 in the candidate genes to identify mutations that would lead to a susceptible phenotype in a Bla-6 background. By testing the T_1 from plants transformed with constructs targeting each of the candidate genes in a disease assay with *On*, we identified susceptible phenotypes (*i.e.* showing compromised resistance) only in the progeny of plants transformed with the construct targeting *ZRK13*. Analysis of the T_2 through PCR-based sequencing allowed us to identify several mutation events. The segregation ratios in disease response in families originating from susceptible plants did not correspond to a mendelian segregation. It has been reported that mutagenesis using CRISPR/Cas9 through flower dipping in Arabidopsis may lead to somatic mutations that are not inherited in the sexual offspring (Feng *et al.*, 2014; Jiang *et al.*, 2014). We speculate that some of the mutations in the T_1 were indeed somatic and therefore were not inherited in the selfing T_2 progeny. For this reason, it has been suggested that screening of heritable mutations should be done in T_2 generations or later (Feng *et al.*, 2014). It is important to mention that we were not able to identify mutation events in the other two candidate genes (At1g65180 and the intergenic insertion). However, we sequenced only a small number of CRISPR T_1 transformants. No symptoms were found in any of the plants obtained from the transformation using the constructs targeting these loci. Therefore, we were able to identify *ZRK13* as essential for the resistance found in Bla-6.

Up until now, four members of the ZED1-RELATED KINASE (ZRK) family in Arabidopsis have been characterized. *ZRK* genes encode receptor-like cytoplasmic kinases (RLCK). Subfamily XII-2 (RLCK XII-2) consists of 13 members (Lewis *et al.*, 2013), eight of which cluster together on chromosome 3 while additional members of the family are located on chromosome 1. All four characterized *ZRK* members (*ZED1*, *ZRK1*, *ZRK2* and *ZRK3*) are closely related and are part of the chromosome 3 cluster (Lewis *et al.*, 2013). With a forward genetics approach, Lewis *et al.* (2013) found that the hopZ-ETI-deficient1 (*zed1*) mutant was specifically impaired in the recognition of HopZ1a, a *Pseudomonas syringae* type-3 secreted effector (T3SE), and therefore

unable to elicit a functional ETI response through the nucleotide-binding leucine-rich repeat (NLR) protein ZAR1 (Lewis *et al.*, 2010). Three additional members of the ZRK family have been shown to be required for the activation of ZAR1-dependent ETI by recognition of T3SEs: RKS1/ZRK1 for recognition of *Xanthomonas campestris* effector AvrAC (Wang *et al.*, 2015), ZRK3 for recognition of *P. syringae* effector HopF2 (Seto *et al.*, 2017), and ZRK2 for recognition of *P. syringae* effector HopBA1 (Martel *et al.*, 2020). Recently the interaction of RKS1/ZRK1 and ZAR1 has been studied via cryo-electron microscopy (Wang *et al.*, 2019), which has allowed the elucidation of the biochemical steps that result in the assembly of the ZAR1 resistosome. This is a pentameric funnel-shaped structure that binds to the plasma membrane eliciting cell death and ultimately resulting in resistance to *X. campestris*. Additionally, it has been reported that ZRKs play a role in the ambient-sensitive immune response in the absence of pathogens. Wang *et al.* (2017) showed that *zed-1D* mutant displayed a severe phenotype when grown at high temperature by triggering an autoimmune ZAR1-dependent response. Interestingly, in the same study, overexpression of *ZRK13* could partially rescue the *zed1-D* phenotype, strongly suggesting that ZRK13 is able to interact with the ZAR1 resistosome. However, it remains to be shown whether the interaction of the ZRK13 protein from Bla-6 with the ZAR1 resistosome upon infection with *On* is responsible for the resistance.

The ZAR1 resistosome has been found to have an ancient origin and to be atypically conserved across plants, being found in more than 80 species including monocots, magnoliids and eudicots (Adachi *et al.*, 2020). Therefore, an evolutionary model has been proposed in which ZAR1 remains as a conserved activator of immune responses while RLCKs evolved into a variety of pathogen sensors (Schultink *et al.*, 2019; Adachi *et al.*, 2020; Martel *et al.*, 2020). It would be interesting to study the allelic variation of *ZRK13* across different accessions of Arabidopsis. We have found that the predicted protein of *ZRK13* in Bla-6 holds many polymorphisms when compared to Col-0. However, confirmation of the allelic variant in Bla-6 at the mRNA level will help to confirm this information.

Although the Arabidopsis-*On* interaction is less well-characterized compared to the other PM pathogens infecting Arabidopsis, our study shows that it represents a robust model that allows analysis of new mechanisms of resistance against biotrophic pathogens. The identification of *ZRK13* as the gene conferring resistance against *On* in Bla-6 opens new opportunities to elucidate the molecular mechanisms of this interaction. Most importantly, the interaction of ZRK13 with ZAR1 can be tested by generating *zar1* mutants in a Bla-6 background and test their response against *On*. Additionally, overexpression of the ZRK13 allele of Bla-6 should be tested in a Col-0 background to confirm its function in resistance.

Materials and methods

Plant material, growth conditions and disease assays

Arabidopsis accessions Bla-6 (accession ID 6621) and Col-0 were obtained from the Max Planck Institute in Köln, Germany. The Arabidopsis plants were grown in soil substrate in a growing chamber with day/night cycles of 16h/8h and 10h/14h in a temperature of 21°C and relative humidity of 70%. For the disease assays, 3 to 4-week old plants were inoculated by spraying a conidiospore suspension (2.5 to 5.8 × 10^5 spores/mL) of the Wageningen isolate of *On*, which was maintained on tomato *cv* Moneymaker plants. The disease symptoms were scored visually at 8-12 days post inoculation with a score from 0 to 3 (0, no sporulation; 1, slight sporulation; 2 moderate sporulation; 3 abundant sporulation) for the QTL mapping and with a qualitative score of R (resistant) or S (susceptible) for the fine mapping and mutant screening.

Mapping and PCR-based sequencing

A preliminary QTL analysis in the F_2 was performed using Indel markers RH555/556, RH473/474, RH565/566, RH481/482 and RH569/570 (Table S1), based on genome sequence differences between accessions Columbia (Col-0) and Landsberg erecta (Ler) (Hou *et al.*, 2010). Additional Indel markers were tested for polymorphisms between accessions Col-0 and Bla-6. SNP markers for mapping were developed based on PCR sequencing results. CAPS markers M24, M36, M6 and M14 (Table S2) were developed based on polymorphisms between Col-0 and Bla-6 retrieved from the Gramene SNP query database (https://archive.gramene.org/db/diversity/snp_query). Primers for PCR-based sequencing for fine mapping and sequencing of the candidate region (Table S2) were designed based on the TAIR reference genome of Col-0 (www.arabidopsis.org).

CRISPR/Cas9 targeted mutagenesis

PCR-based sequencing was used to obtain the sequence of the candidate region using the primers described in Table S2. A set of four single guide RNAs (sgRNAs) was designed to target each of the three candidate loci in the candidate region (Table S3, Fig. 2). All sgRNAs were designed using the CRISPOR (Concordet & Haeussler, 2018) and CC-Top (Stemmer *et al.*, 2015) webtools. G + C content of the sgRNAs was calculated using the ENDMEMO webtool (http://www.endmemo.com/bio/gc.php) and folding of the gRNAs was predicted using the Mfold web server (Zuker, 2003). The sgRNAs were selected following the criteria described by (Liang *et al.*, 2016) and assessing the efficiency (CCTop, Wu-Crispr, Gpp and Doench16) and specificity (MIT specificity score, CRISPRater efficacy score) provided by CRISPOR and CC-Top.

CRISPR/Cas9 constructs for transformation were built in two steps (levels) via the Golden Gate cloning system (Weber *et al.*, 2011; Engler & Marillonnet, 2014). Level 0 constructs contained promoters, 5' untranslated regions, coding sequences, signal peptides and terminators. Level 1 constructs contained complete gene expression

units which were later transferred to the level 2 construct, carrying the multiple gene expression unit (Weber *et al.*, 2011). Each of the sgRNAs were ligated to specific backbones of the CRISPR-Pink system vectors, provided by Mark Youles (TSL Norwich, Synbio, Table S4).

Cloning of level 1 and 2 constructs was done using *E. coli* DH5α chemically competent cells. The final level 2 CRISPR constructs were transformed to *Agrobacterium* strain AGL1 + virG. Arabidopsis Bla-6 plants were transformed using a modified method of flower dipping (Clough & Bent, 1998). Instead of dipping floral buds, drops of the infiltration medium [sucrose (50g/L) and Silwet L-77 (200µl/L)] containing Agrobacterium carrying the level 2 CRISPR construct were deposited onto unopened flower buds in two events within seven days. After harvesting, transformed seeds were selected using a binocular microscope based on the FAST Red fluorescence.

Supplementary information

Table S1. Primers used for QTL analysis and mapping of the candidate region on chromosome 1 of Bla-6. Name, location in the reference Col-0 genome and sequence are provided.

Name	Type	Location (TAIR10)	Forward primer	Reverse primer	Restriction enzyme
RH555/556	Indel	3212189-3212389	GGCTTTCTCGAAATCTGTCC	TTACTTTTTGCCTCTTGTCATTG	
RH473/474	Indel	24329048-24329148	TTACCAAAAGGTTGCGAACA	TGTTTTGTCTTTATCAACTCCACAA	
RH565/566	Indel	24370345-24370540	CTGCCTGAAATTGTCGAAAC	GGCATCACAGTTCTGATTCC	
RH481/482	Indel	25154371-25154490	GCCCAGAGAACTAAGTCAGCA	GAAGCCAGAGAAAAGGCAAG	
RH569/570	Indel	29011992-29012148	GCATCGCTCTTAAACAACCAT	CGTTGCAAAACCGTATCAGAA	
RH777/780	SNP	24171640-24172127	CGACAAAAGTGAAGAAATAAATGTG	CGCAAAAACGAATGATGGTT	
M24	CAPS	24213389-24213762	CCCTTCCCACAAGTAAAGCA	AATCGGTGGGGTAAATGTTG	*MspI*
M36	CAPS	24278646-24279530	GCATTTGCACAAATTAAGAAAAA	GCCACCATGGGAATAGGTT	*HinfI*
SNP51	SNP	24357325-24357444	GTCTAAACCGCACTCGCAAT	CTCGCACTTTGCCACTATCA	
M6	CAPS	23981483-23981846	CCCCTGACACCACAAGAGAT	TGGATGAGTTGTATGATTTGAGAGA	*RsaI*
M14	CAPS	24029663-24030011	CGTTGAGGGTAGGACACAGG	AAATTCTCACTCCCACACACG	AluI/*DdeI*
Bla-6-1	Indel	24347660-24347770	CCAAGAAATTGAACGCAACA	GCCGATTTTTGTTTTCTTAGCA	
Bla-SNP42	SNP	24364379-24364533	TGTCAGCCATTGGAGACAAG	CTTATTGCAGGTCCCGATTG	
Bla-SNP45	SNP	24385803-24385973	AAAAATGCGCCAATTCAAAG	GGAAATAGCAAAAAGCGAAAA	
Bla-SNP17	SNP	24430385-24430507	TATGGACCCTGGAGCAAATC	ATTTCTTGCATGGGGATGAA	
Bla-6-4	Indel	24507047-24507181	GTGAGGGAATAGGGACATGC	GCCAGCTTGCAATTCATTTA	
Bla-SNP30	SNP	24576433-24576627	TTCAACCCCACTTTTGATGA	GCCCGACTCGATGAATAGAA	
Bla-SNP14	SNP	24615974-24616076	GGCTCTTCCCTCCTCTGTCT	ATGAACGATTGCGGTTGTG	
Bla-SNP19	SNP	24666538-24666702	CCTGAACCTGGTGCTGCTAT	CGACCAAGGTCGCTAGATTT	
Bla-SNP12	SNP	24710590-24710736	TCATCCAAATGGTGTTTTCG	AGCTTAGTCACCCGGGATCT	
Bla-SNP21	SNP	24764515-24764661	TCACTGGGCTGTCTTCTGGT	TACCAACCAAGACGGCTTTT	
Bla-SNP9	SNP	24808129-24808249	GGAGCGGTCAAGACCATATT	TTGCATTTGCTTTTTGCATC	
Bla-SNP22	SNP	24854680-24854876	TCCCAACCATGTTTAGGTAGC	TGCCTTCAATTCACTCACTCC	
L11	Sequencing	24218618-24219333	TCAGCTCAAGAAAGACGAAGG	TGCAGATTCAATCAGAGACCTG	
P26	Sequencing	24234230-24235115	TCAGCAGACCCAAATTTCCTA	TGAGACTAGGCCACTCCTAACG	
P27	Sequencing	24243231-24244005	GGTGCTAACATCTTGGCAGTT	CTACCTTGGCGCCTGCTC	
P33	Sequencing	24262525-24262858	CGTCGATTCTCCGATAGCTC	CCACAAAGCTCTGATCAAAGAAG	
L1	Sequencing	24213348-24213897	CCAAATCGCATTCATGGCAT	TCTCCGTCACATGAGCTAAGAG	

Table S2. Primers used for sequencing of the candidate region on chromosome 1 of Bla-6. Name, location in the reference Col-0 genome and sequence are provided.

Name	Location TAIR10	Primer sequence
L21 Fw	24213536	TTCACAGCGCTGACATTCAT
L22Fw	24214060	CAACATCAACGAAGCACGAT
L23 Fw	24214533	CCAAATTATGCAACGGATGA
L24 Rv	24214862	TGTTGACTTGAAGAAATAAAGTTTTGA
L25 Rv	24215376	TCTCCGATCCAAAACCCATA
Det_ZRK13F	24215449	GGTGTTGTGCGTCACCTAGA
LZRK13F	24215788	GGAGGACGTCAATGTTGAAAA
L26 Rv	24215726	TGTGAATCTTGTATTGATGATTGTCT
L27 Rv	24216285	GGGTCAAATCTTAGAACAAGAGC
L28 Rv	24218678	AACCGGTGGATCAGTCTCAG
L31 Rv	24214344	GGGTAACATGATTTAGTTATGAGGGTA
L32 Rv	24215306	TGTCCAAAATAAAGAATGATGTTCA
L33 Rv	24215849	AGTGCTACGTTACTTTTGATTCATT
L34 Rv	24216115	CGACTCTTCTTCTCTTTCTTCAGG
L35 Fw	24214616	TTCTTGGCACTTCCTTAATCG
Det_ZRK13R	24217397	TGAATTTTCGCGGGAGGA
LZRK13Rv	24217402	CCCAAACACACATACACAAACTC
L36 Fw	24217571	TTCAAACACTCGGCTGGAAT
L37 Fw	24218024	CTTTGATTGGAAGTCTCCCTCT

Table S3. Single guide RNAs used for CRISPR constructs. Name, sequence and target gene/locus are indicated.

Name	Sequence	Target
180-1	TTCCCATCAGTATAAGCCGG	At1g65180
180-2	ATGGGCTTCGATCTCCATGG	
180-3	TTTGGTGATGTGTAAAACAA	
180-4	TAGAGCGCATTTGAAATCCA	
IG-1	TCAGGAAGAAGCAGAACGAG	Intergenic Insertion
IG-2	GAGCATGTGCAACAGTCTCA	
IG-3	GCAATTGTGTGAAGCTGCAA	
IG-4	GGAAACGAACAAAGAAACCTG	
ZRK13-1	AAGCTAATATTGGAGAGAGG	At1g65190 (*ZRK13*)
ZRK13-2	CCGTTCCTGATGAGTATCAT	
ZRK13-3	CCGTTCCTGATGAGTATCAT	
ZRK13-4	CCTCTGCTGGTCTCTCAACC	

Table S4. Plasmids used for CRISPR/Cas9 construct. The name of the plasmid, description and sources are indicated.

Plasmid	Description	Source
pICSL12015	Promotor for Cas9	Jonathan D. Jones (Castel *et al.*, 2019)
pICSL60004	Terminator for Cas9	Jonathan D. Jones (Castel *et al.*, 2019)
pICSL70008	FastRed (FastR) Construct	Nicola Patron (Engler & Marillonnet, 2014)
pICSL11024	NPTII	Jonathan D. Jones
pICH47742	Backbone for Cas9	Sylvestre Marillonnet (Weber *et al.*, 2011)
pICH47751	Backbone for FastR	Sylvestre Marillonnet (Weber *et al.*, 2011)
pICH47761	Backbone for sgRNA1	Sylvestre Marillonnet (Weber *et al.*, 2011)
pICH47772	Backbone for sgRNA2	Sylvestre Marillonnet (Weber *et al.*, 2011)
pICH47781	Backbone for sgRNA3	Sylvestre Marillonnet (Weber *et al.*, 2011)
piCH47791	Backbone for sgRNA4	Sylvestre Marillonnet (Weber *et al.*, 2011)

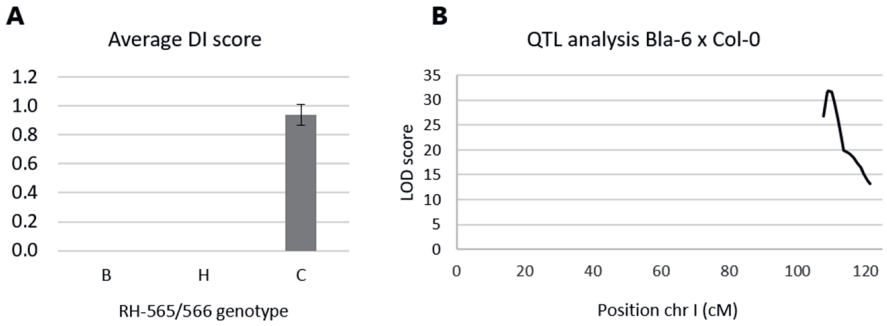


Figure S1. Analysis of Bla-6 x Col-0 F2 progeny. **A**. Average disease index (DI) score on the F2 per genotype class for marker RH565/566 (B: homozygous Bla-6; H: heterozygous; C: homozygous Col-0). **B**. Preliminary QTL analysis of the F_2 of the Bla-6 x Col-0 cross. LOD score and position in centimorgans (cM) are indicated.

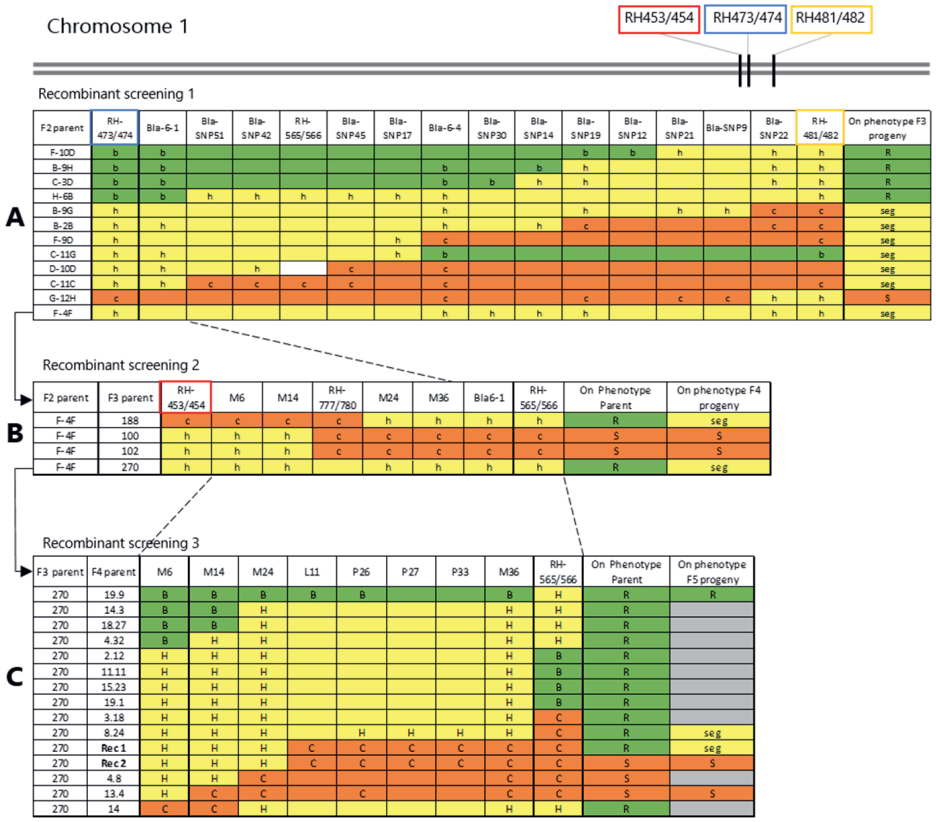


Figure S2. Informative recombinants in each of the three screenings carried out to map the resistance locus in chromosome 1.. A. Recombinant screening 1. **B.** Recombinant screening 2, performed using the progeny of recombinant F-4F. **C.** Recombinant screening 3, performed using the progeny of recombinant F-4F-270. B: homozygous Bla-6 allele, C: homozygous Col-0 allele, H: heterozygous, R: resistant, S: susceptible, seg.: segregating for resistance against *Oidium neolycopersici* (*On*).

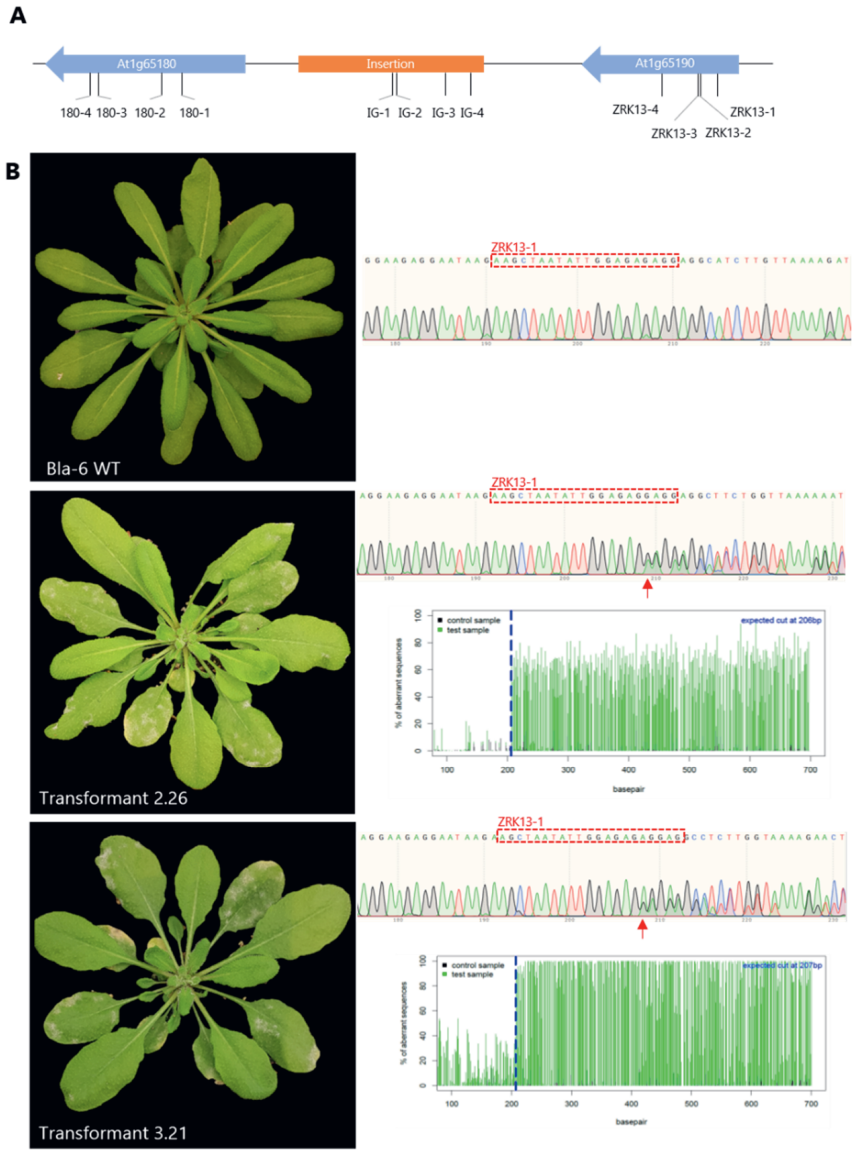
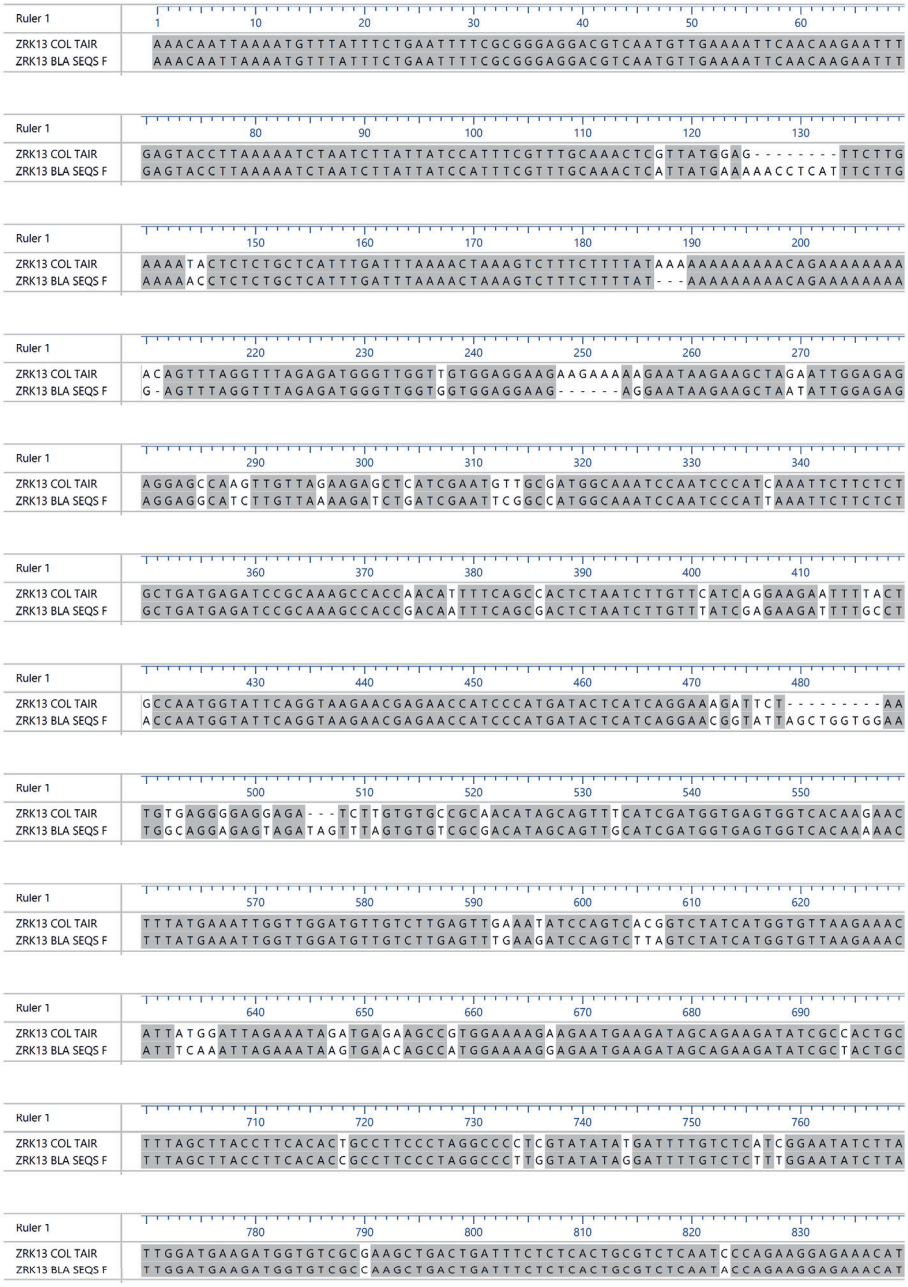


Figure S3. Targeted mutagenesis of *ZRK13* in Bla-6 results in a susceptible phenotype in the T_1. A. Location of the single guide RNAs (sgRNAs) in the candidate region of Bla-6. Blue arrows indicate the two genes in the region. Orange square indicates the insertion present in Bla-6. Black bars indicate the location of the sgRNAs. **B**. Left: phenotype of wild-type (WT) Bla-6 and two susceptible transformants at 12 days post-inoculation. Right: Chromatograms showing the sgRNA ZRK13-1 (red box) region with double peaks present within the targeted region. For the two susceptible transformants: output from the TIDE webtool indicating the expected cut site (blue dashed line) and the presence of aberrant sequences (green).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ZRK13 BLA SEQS F AAACAATTAAAATGTTTATTTCTGAATTTTCGCGGGAGGACGTCAATGTTGAAAATTCAACAAGAATTT
Ruler 1
80 90 100 110 120 130
ZRK13 COL TAIR GAGTACCTTAAAAATCTAATCTTATTATCCATTTCGTTTGCAAACTCGTTATGGAG--------TTCTTG
ZRK13 BLA SEQS F GAGTACCTTAAAAATCTAATCTTATTATCCATTTCGTTTGCAAACTCATTATGAAAAACCTCATTCTTG
Ruler 1
150 160 170 180 190 200
ZRK13 COL TAIR AAAATACTCTCTGCTCATTTGATTTAAAACTAAAGTCTTTCTTTTATAAAAAAAAAAAACAGAAAAAAAA
ZRK13 BLA SEQS F AAAAACCTCTCTGCTCATTTGATTTAAAACTAAAGTCTTTCTTTTAT---AAAAAAAAACAGAAAAAAAA
Ruler 1
220 230 240 250 260 270
ZRK13 COL TAIR ACAGTTTAGGTTTAGAGATGGGTTGGTTGTGGAGGAAGAAGAAAAAGAATAAGAAGCTAGAATTGGAGAG
ZRK13 BLA SEQS F G-AGTTTAGGTTTAGAGATGGGTTGGTGGTGGAGGAAG------AGGAATAAGAAGCTAATATTGGAGAG
Ruler 1
290 300 310 320 330 340
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ZRK13 BLA SEQS F AGGAGGCATCTTGTTAAAAGATCTGATCGAATTCGGCCATGGCAAATCCAATCCCATTAAATTCTTCTCT
Ruler 1
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ZRK13 BLA SEQS F GCTGATGAGATCCGCAAAGCCACCGACAATTTCAGCGACTCTAATCTTGTTTATCGAGAAGATTTTGCCT
Ruler 1
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Ruler 1
500 510 520 530 540 550
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ZRK13 BLA SEQS F TGGCAGGAGAGTAGATAGTTTAGTGTGTCGCGACATAGCAGTTGCATCGATGGTGAGTGGTCACAAAAAC
Ruler 1
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Ruler 1
640 650 660 670 680 690
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Ruler 1
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Ruler 1
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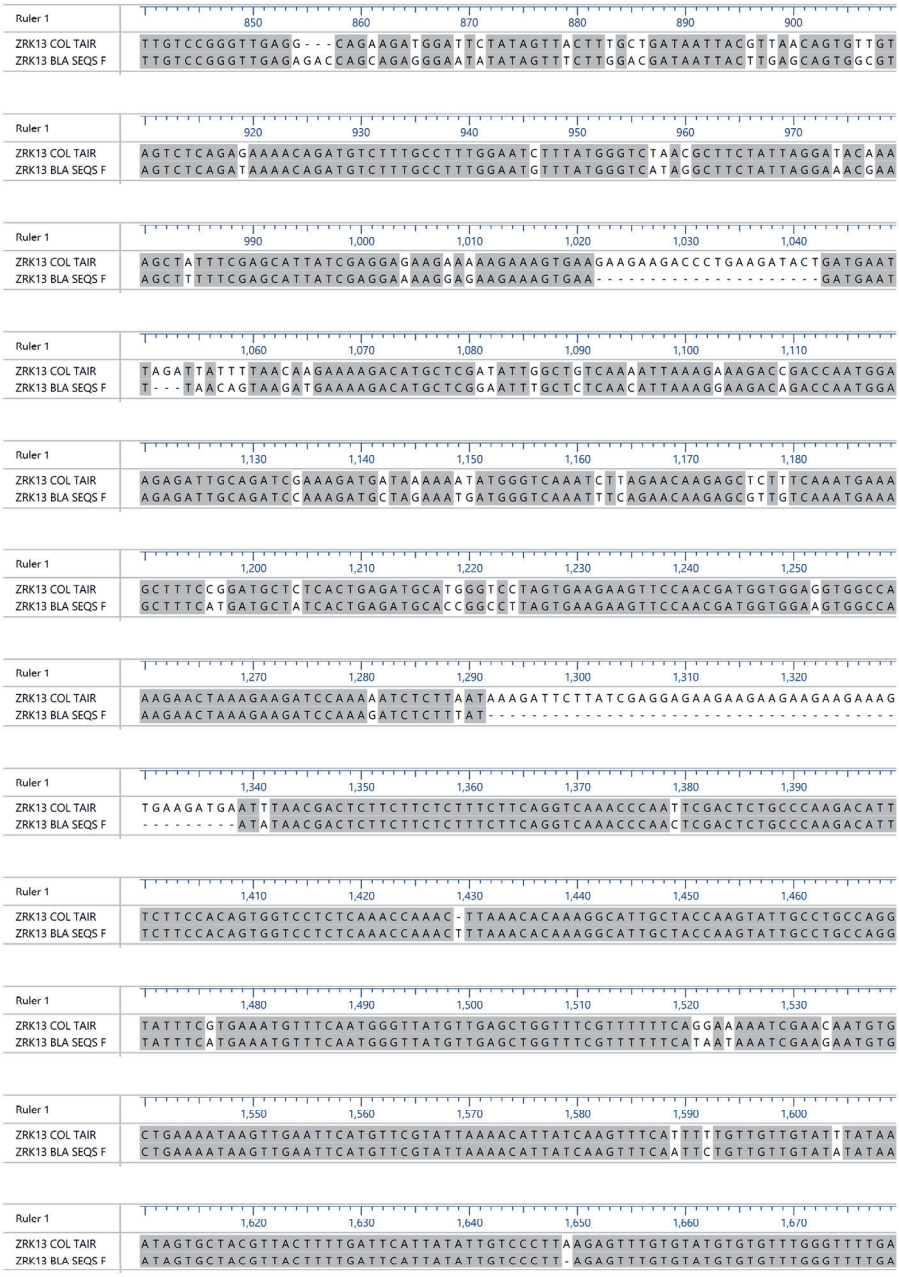
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ZRK13 BLA SEQS F AGAGATTGCAGATCCAAAGATGCTAGAAATGATGGGTCAAATTTCAGAACAAGAGCGTTGTCAAATGAAA
Ruler 1
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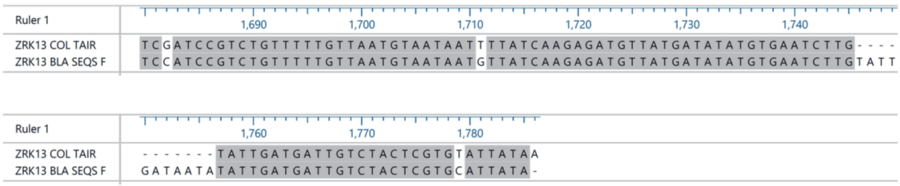
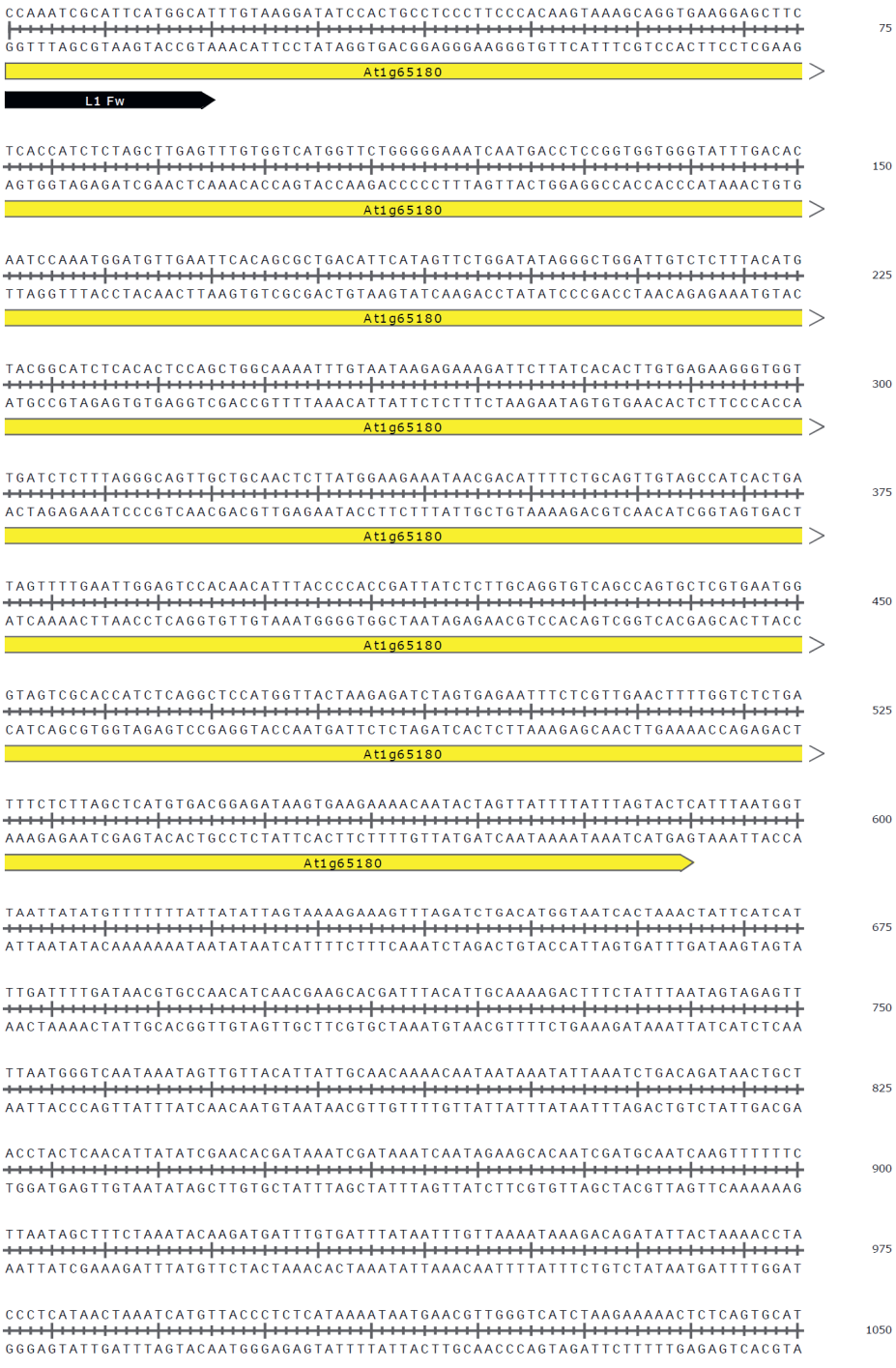


Figure S4. Pairwise alignment of the genomic sequence of ZRK13 of Col-0 and Bla-6.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L1 Fw
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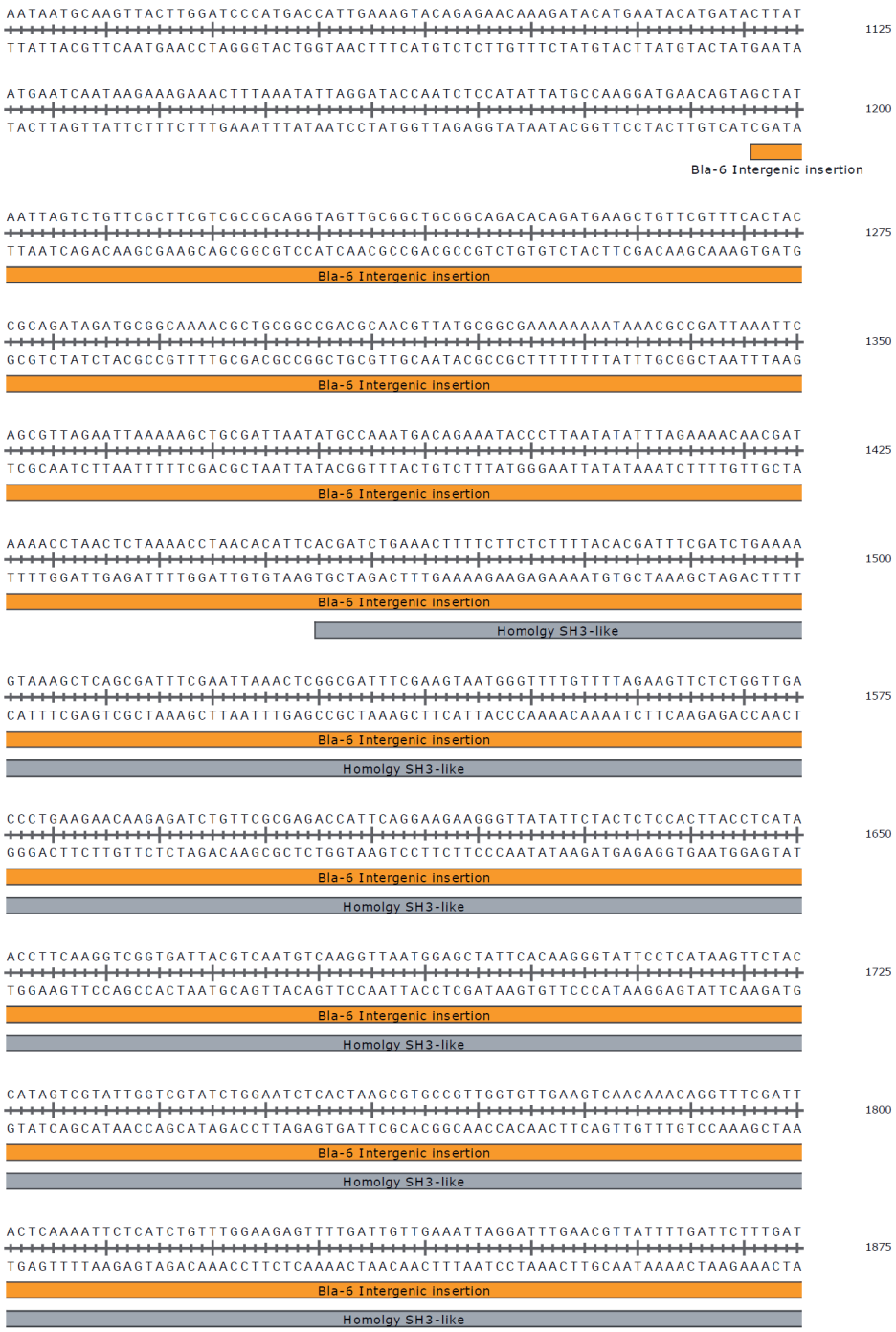
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1725
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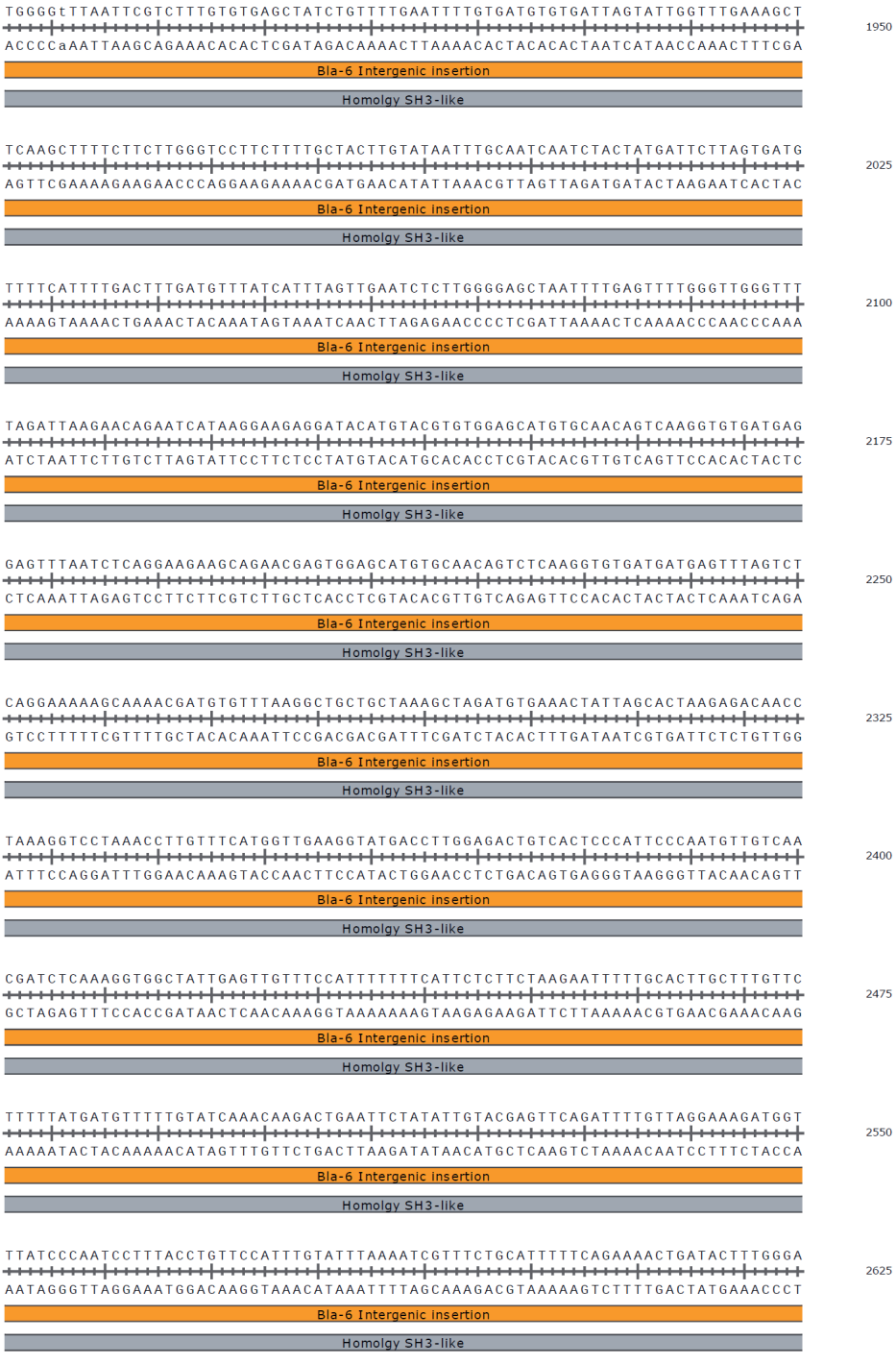
Homolgy SH3-like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2175
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Homolgy SH3-like
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2325
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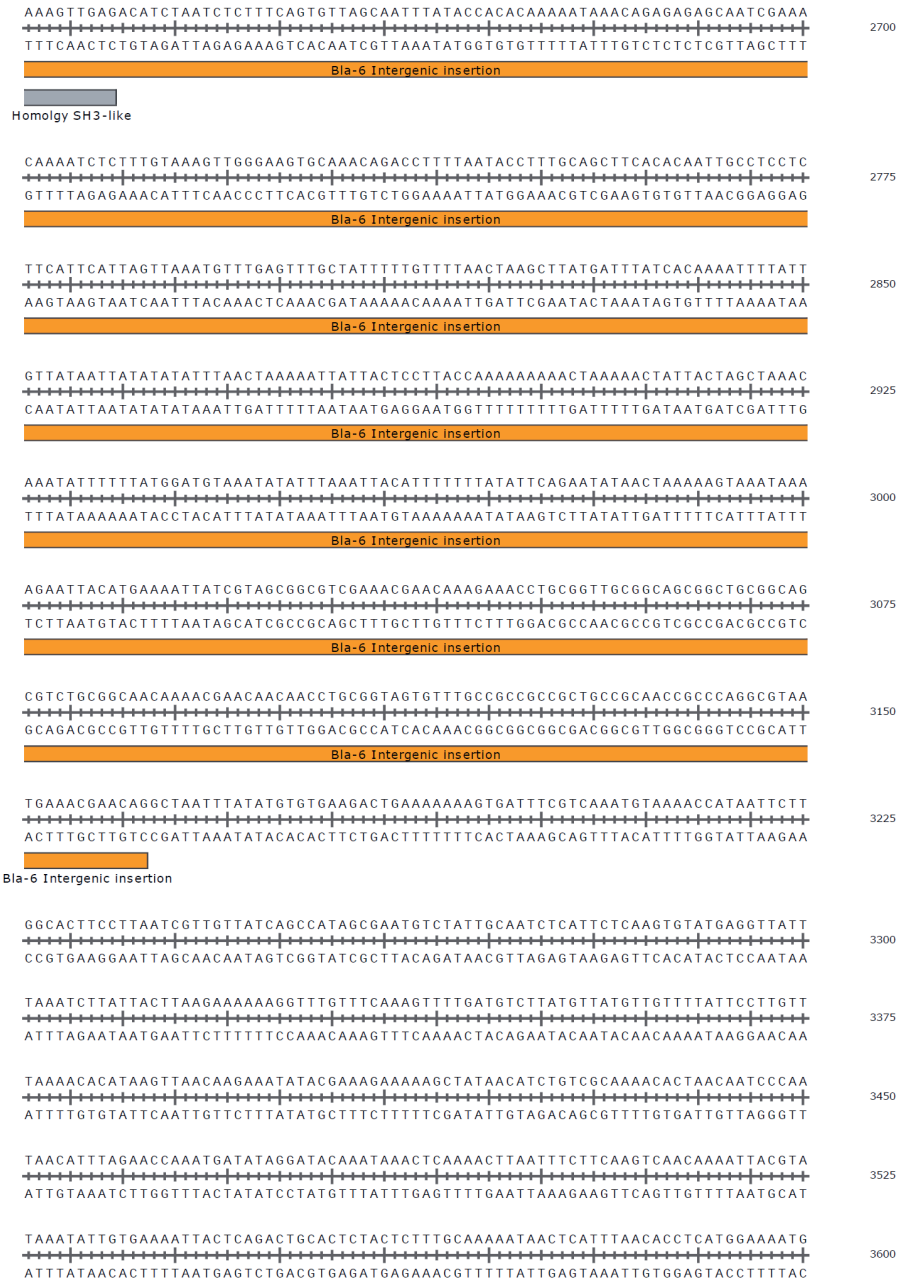
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3600

Figure S5. Sequence flanked by primers L1Fw and L33Rv. Genes At1g65180 and At1g65190, Intergenic insertion and predicted homology with an SH3-like protein are indicated.

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Chapter 3

Resistance to *Oidium neolycopersici* in Arabidopsis accession Litva requires *ZED1-RELATED KINASE 13* (*ZRK13*) and an additional locus on chromosome 3

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Abstract

The tomato-adapted powdery mildew pathogen *Oidium neolycopersici* is able to infect and complete its life cycle in the model plant *Arabidopsis thaliana*. In order to investigate genetic components of host resistance to this economically important pathogen, a disease assay was performed in 123 Arabidopsis accessions. The accession Litva displayed full resistance and was then crossed with susceptible accession Col-0. Quantitative trait locus (QTL) analysis was used on their progeny to determine the loci associated with its resistance. After several recombinant screenings, the QTL with the highest logarithm of the odds (LOD) score (QTL-2) was fine-mapped to a 13.7-kb region on chromosome 1. To identify the gene responsible for the resistance, we used CRISPR/Cas9 on two candidate genes in this region. A disease assay on the transformants revealed that *ZED-1 RELATED KINASE 13* (*ZRK13*) is essential for resistance in Litva. Furthermore, analyses of recombinants revealed that, in addition to *ZRK13*, QTL-4 on chromosome 3 is required for full resistance in Litva.

Introduction

The fungal disease Powdery Mildew (PM) occurs in many economically important crops worldwide including grape, apple, wheat, barley, tomato and cucumber. Symptoms of this disease are characterized by the accumulation of mycelial and reproductive structures that results in the development of white powder-like patches extending along the aerial parts of the plants (Glawe, 2008). More than 400 species of Ascomycetes can cause PM (Panstruga & Schulze-Lefert, 2002; Takamatsu, 2004; Glawe, 2008). These pathogens are obligate biotrophs and thus feed on the living tissue of the host plant by specialized feeding structures called haustoria (O'Connell & Panstruga, 2006). Haustoria are the main interaction sites between the pathogen and the host and are responsible for the uptake of nutrients and the delivery of effectors to maintain a biotrophic relationship (Bushnell, 1972).

For a pathogen to successfully establish this biotrophic interaction, it must overcome several defence responses orchestrated by the two well-described plant immunity layers (Jones & Dangl, 2006). The first layer relies on the identification of pathogen-associated molecular patterns (PAMPs) by the plant's pattern recognition receptors (PRRs) which may result in PAMP-triggered immunity (PTI) by the activation of responses such as the production of reactive oxygen species, the activation of mitogen-activated protein kinase (MAPK) pathways, the formation of papillae and the deposition of callose (Dodds & Rathjen, 2010). The second layer of disease responses can be activated when the plant's resistance (R) proteins, typically nucleotide-binding site leucine-rich repeat (NB-LRR/NLR) proteins, recognize effectors secreted by the pathogen (Jones & Dangl, 2006). This recognition activates fast defence responses such as the hypersensitive response (HR) (Dodds & Rathjen, 2010) and results in effector-triggered immunity (ETI). For instance, the barley NB-LRR protein MLA10 is known to interact with the AVR_{A10} (avirulence A10) effector secreted by the PM pathogen *Blumeria graminis* f. sp. *hordei*, triggering HR (Ridout *et al.*, 2006).

The interaction between PM pathogens and Arabidopsis has been used extensively to characterize different plant defence responses at the genetic and molecular levels (reviewed by Kuhn *et al.*, 2016). Four PM pathogens are known to be able to infect Arabidopsis: *Erisyphe cruciferarum* (Koch & Slusarenko, 1990), *Golovinomyces* (syn. *Erysiphe*) *cichoracearum* (Adam & Somerville, 1996), *Golovinomyces* (syn. *Erysiphe*) *orontii* (Plotnikova *et al.*, 1998) and the tomato-adapted PM pathogen *Oidium neolycopersici* (*On*) (Xiao *et al.*, 2001). Broad-spectrum resistance to PM across Arabidopsis accessions has been found to be mainly polygenic and to occur in an additive manner (Schiff *et al.*, 2001; Wilson *et al.*, 2001; Göllner *et al.*, 2008). Dominant and semi dominantly-inherited resistance against PM caused by *G. cichoracearum* is known to be conferred by

the loci of *RESISTANCE TO POWDERY MILDEW (RPW), including RPW1*, *RPW2*, *RPW4*, *RPW5* and *RPW8* (Adam & Somerville, 1996).

To date, only the *RPW8* locus, carrying two paralogs (*RPW8-1* and *RPW8-2*) of a non-typical resistance gene with a putative N-terminal transmembrane (TM) domain and a coiled-coil (CC) domain, has been studied in detail (Xiao *et al.*, 2001). *RPW8* was first identified in accession Ms-0 (Xiao *et al.*, 2001) and functional *RPW8* alleles are present in many Arabidopsis ecotypes (Schiff *et al.*, 2001; Göllner *et al.*, 2008). The *RPW8* paralogs are known to confer resistance against three of the PM species that can infect Arabidopsis (Xiao *et al.*, 2001, 2003). However, there are conflicting reports on the effectiveness of the *RPW8*-based resistance against *On*. Xiao et al. (2001) reported this locus to be effective against *Oidium lycopersici* (isolate Oxford), later identified as *On* (Kiss *et al.*, 2001; Jones *et al.*, 2001), while Göllner *et al*. (2008) found that accession Shakdara (Sha), carrying both Ms-0-like paralogs of *RPW8*, displayed a susceptible phenotype when inoculated with *On* (Wageningen isolate). Resistance conferred by the *RPW8* locus has been hypothesized to require specific factors that may not be present in all accessions. Furthermore, this resistance may be race- or pathotype-specific in the Arabidopsis-*On* interaction (Göllner *et al.*, 2008), which is less well-characterized than interactions with the other PM species.

To investigate the causal genes and their associated mechanisms for *On* resistance we performed crosses between resistant Arabidopsis accessions with susceptible accessions Col-0 or Sha. Analysis of the F2 progeny of these crosses has previously allowed us to identify a natural loss-of-function mutation of *EDR1* (At1g08720), resulting in resistance in accession C24 (Gao *et al.*, 2015), as well as to functionally characterize *ZRK13* as the gene conferring dominant resistance in accession Bla-6 (Chapter 2 of this thesis). Here, we report the identification of the resistance loci found in accession Litva, as well as the fine-mapping and identification of the gene underlying QTL-2.

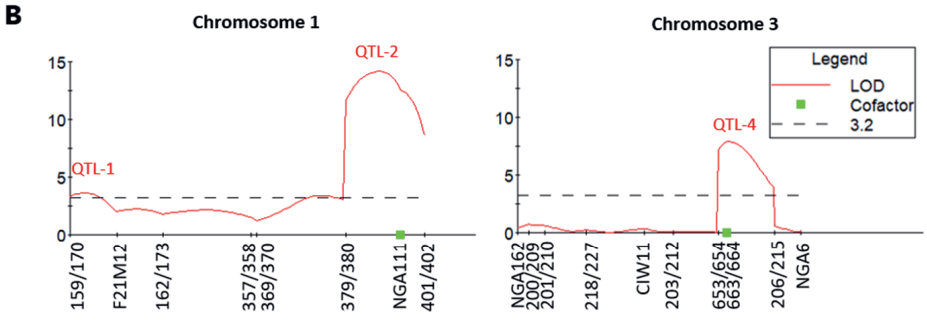
Results

Resistance to *On* in Arabidopsis accession Litva is polygenic

In order to investigate the natural variation of resistance in Arabidopsis, we inoculated a set of 123 accessions (five plants per accessions) with *On*. Disease symptoms were scored using a disease index (DI) from 0 (no symptoms/resistant) to 3 (abundant symptoms/susceptible) starting at 12 days post-inoculation (dpi). Out of the tested accessions, 40 were found to be fully resistant. We crossed 19 of the resistant accessions with susceptible accessions Col-0 or Sha and tested five plants from each cross in a disease assay with *On* (Gao *et al.*, 2015, Chapter 2 of this thesis). The F_1 of 18 of these crosses displayed a susceptible phenotype upon infection. In order to elucidate whether the resistance was mediated by a single gene, we performed a χ^2 test in the F_2 generations. One of the resistant

accessions was Litva (Figure 1A), whose F_2 progeny showed a segregation ratio compatible ($p>0.05$) with a 1:3 (resistant : susceptible) ratio.

To locate the genomic loci responsible for the resistance, we performed a QTL analysis on 96 plants of the F_2 progeny of the Litva x Col-0 cross (Fig. S1), using the genotypic information of 39 indel markers covering all five chromosomes (Table S1). Interval Mapping (IM) analysis revealed four QTLs: QTL-1 and -2 on chromosome 1, QTL-3 on chromosome 2 and QTL-4 on chromosome 3 (Fig. S2). The highest LOD scores were assigned to QTL-2 and QTL-4. When Multiple QTL Mapping (MQM) was performed using marker NGA111 on chromosome 1 and marker RH663/664 on chromosome 3 as co-factors QTL-3 disappeared (Fig. 1B). The QTL-2 region on chromosome 1, flanked by markers RH379/380 and NGA111, showed the highest LOD score peak with 42.7% of explained variance, while the QTL-4 region on chromosome 3, between markers RH653/654 and RH206/215 showed the second highest LOD score peak with 21.2% of explained variance. QTL-1 on chromosome 1 accounted for 8.1% of explained variance. Interestingly, when combining the data on the phenotypic response of the F_2 plants upon *On* inoculation with the genotypic data of markers NGA111 (within QTL-2) and RH663/664 (within QTL-4) we observed a dosage effect for both loci, with the highest level of resistance (lowest DI scores) observed in plants homozygous for the Litva allele for both loci (Figure 1C).



C

Average DI score F2		QTL-4 RH663/664			
		L	H	C	Total
QTL-2 NGA111	L	0.09	0.49	1.06	0.42
	H	0.63	1.35	1.44	1.15
	C	1.27	1.38	1.67	1.39
	Total	0.56	1.13	1.39	0.99

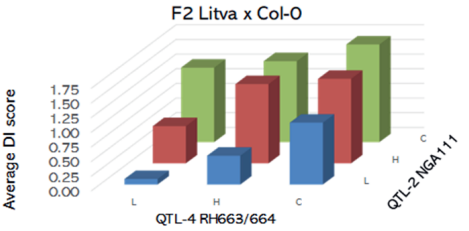


Figure 1. Resistance to *Oidium neolycopersici* (*On*) in Arabidopsis accession Litva. A. Phenotypic response to inoculation with *On* in adult plants of Col-0 and Litva at 12 days post inoculation (dpi). **B**. Multiple QTL mapping (MQM) analysis in the F_2 of a cross between Litva and Col-0. **C**. Table and 3-D graph of the average disease index (DI) score across the genotypes for markers NGA111 (QTL-2) and RH663/664 (QTL-4) in the F_2 progeny of the Litva x Col-0 cross. L, homozygous for the Litva allele; H, heterozygous; C, homozygous for the Col-0 allele.

Fine-mapping of QTL-2

We decided to fine-map the QTL with the highest LOD score (QTL-2 on chromosome 1) with 43% explained variance. For this, we selected an F_2 plant (F_2#21, Fig. 2A) that was heterozygous in the QTL-2 region and homozygous for the Litva alleles in the other QTL regions, to produce F_3 seeds. Previous results

from the F_2 showed that the DI of heterozygous plants carrying both a Litva allele and a Col-0 allele in QTL-2 was on average half of the DI value scored for plants homozygous for the Col-0 allele (Fig. 1C). This suggested that QTL-2 inherited co-dominantly when other QTLs are fixed with the Litva alleles. Therefore, we expected that, when the F3 plants were infected with *On*, only plants homozygous for the Litva allele of QTL-2 were almost symptomless.

In order to fine-map QTL-2, we first performed a recombinant screening using 255 F_3 plants, progeny of the plant F_2#21 (Fig. 2A). Using markers RH379/380 and NGA111 (Fig. 2B), we identified seven informative recombinants (Fig. S3A). Further genotyping of these recombinants using seven additional markers allowed us to narrow-down the region to 230 kb between markers RH461/462 and RH473/474 (Fig 2C). In a second recombinant screening using an additional ~200 F_3 plants from F_2#21 and genotypic information of markers RH461/462 and RH473/474, we identified three new informative recombinants. The F_4 progeny of all informative F_3 recombinants was tested in a disease assay and further genotyped using nine additional in-between markers (Fig. S3B). With this information, the borders of the region were established at markers RH950/951 and RH876/877, spanning 97.3 kb.

We then performed a third recombinant screening using the F_4 progeny of the F_3 recombinant M24 (Figure S3B). We screened 2400 plants and initially genotyped them using markers RH461/462 and RH473/474. We identified 13 informative recombinants, whose progeny was tested in a disease assay, and genotyped with nine markers within the candidate region (Fig. S3C). The candidate interval was then reduced to 73 kb between markers RH866/867 and RH872/873 (Fig. 2D). Additionally, the F_5 progeny of three informative F_4 recombinants, 3-6F, 20-10F and 1-5E, were further genotyped using 10 additional markers within the region (Fig. S3D) and phenotyped in a disease assay. This allowed us to establish the candidate locus between markers RH1056/1057 and RH1070/1071, spanning 13.7 kb (Fig. 2E).

The 13.7-kb candidate locus contains four genes in the reference Col-0 genome: At1g65170 to At1g651200. Notably, this region overlaps with the chromosomal interval harbouring the PM resistance locus identified in accession Bla-6 (Chapter 2 of this thesis). More specifically, the fine-mapped regions of Bla-6 and Litva both contain genes At1g65180 and At1g65190. Therefore, we hypothesized that the gene responsible for the resistance within QTL-2 of Litva was an allelic variant of the gene mapped in accession Bla-6. To test this hypothesis, we decided to generate CRISPR mutants of the two candidate genes At1g65180 and At1g65190 in Litva, to assess their role in powdery mildew resistance.

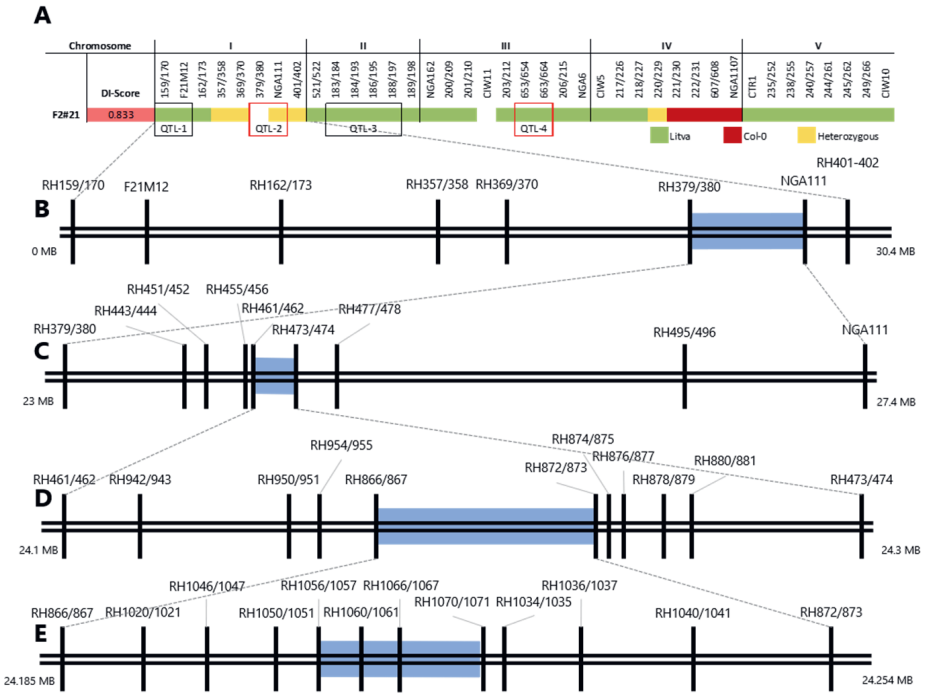


Figure 2. Fine-mapping of QTL-2 in Litva. A. Fine-mapping was carried out using the selfing progeny of recombinant F_2#21, heterozygous for QTL-2 while homozygous for Litva alleles for the remaining QTLs. B-E. Fine mapping of the candidate region after recombinant analyses.

Targeted mutagenesis of *ZRK13* in Litva results in a susceptible phenotype

Before designing CRISPR constructs targeting the candidate genes At1g65180 and At1g65190 (*ZRK13*) in Litva, we performed PCRs to amplify the genes. In addition, we amplified the intergenic region between At1g65180 and At1g65190. This resulted in a PCR product with a similar size as expected for Col-0. Thus, we did not obtain a larger product as detected in accession Bla-6 (Chapter 2 of this thesis), suggesting no (large) insertion was present in Litva compared to Col-0. Sequences of the PCR products were used to select sgRNAs for generating CRISPR/Cas9 constructs to target each of the two genes (Fig. 3A).

Transgenic seeds were selected with the FAST-red marker in the CRISPR constructs (see Materials and Methods) and stratified before being sown in soil. Three-week-old plants were infected with *On* and the DI was evaluated at 8 and 12 dpi. None of the 120 tested plants obtained from the transformation with the construct targeting At1g65180 displayed visible disease symptoms upon

inoculation. However, by screening ~20 plants by PCR sequencing of the target gene (At1g65180) and using the TIDE webtool (Brinkman *et al.*, 2014), we identified two plants that showed aberrant sequences (2.14 and 2.24) at the expected sites. In contrast, three out of the 79 plants from the transformation with the construct targeting *ZRK13* showed clear disease symptoms (1.3, 1.21 and 1.23) when compared to the wide-type (WT) Litva control.

We obtained selfing T_2 progenies from the two putative transformants of At1g65180 (2.14 and 2.24) and the transformants of ZRK13 that displayed a susceptible phenotype (1.3, 1.21 and 1.23). We sowed 40 seedlings from each family and infected with *On*. We screened the plants for symptoms after inoculation at 12 and 25 dpi. The response to *On* of the tested plants are listed in Table 1. To pinpoint mutation events, we used PCR-based sequencing using primers Det_ZRK13F and Det_ZRK13R (Table S1). We expected to find heritable mutations in this generation. We identified one homozygous mutant in family 2.24 from the transformant of At1g65180, carrying a 230-bp deletion within sgRNA 180-1 and three additional transformants with aberrant sequences starting from the site sgRNA 180-1 (Fig. S4). None of these plants showed visible increased susceptibility. For the *ZRK13* transformants we could not identify homozygous mutations. However, PCR-based sequencing of susceptible plants 1.23.22 and 1.3.9 (Fig. 3B) revealed the presence of aberrant sequences at the site of sgRNA ZRK13-2 and ZRK13-1, respectively (Fig. 3C). Sequencing of two plants from families 1.23 and 1.3 with no visible increase in susceptibility yielded WT alleles.

Thus, targeted mutagenesis of At1g65180 did not result in increased susceptibility against *On*, whereas the resistance in CRISPR transformants carrying aberrant sequences in *ZRK13* was compromised, indicating a role for *ZRK13* in resistance conferred by QTL-2.

Table 1. Phenotype upon inoculation with *Oidium neolycopersici* (*On*) in the T_2 progeny of the CRISPR/Cas9 transformants. Gene target, family number and number of susceptible, intermediate and resistant plants per family are shown.

Gene target	Family	Susceptible	Intermediate	Resistant
ZRK13	1.3	5	11	8
	1.21	12	14	14
	1.23	8	18	10
At1g65180	2.14	0	0	40
	2.24	0	0	39

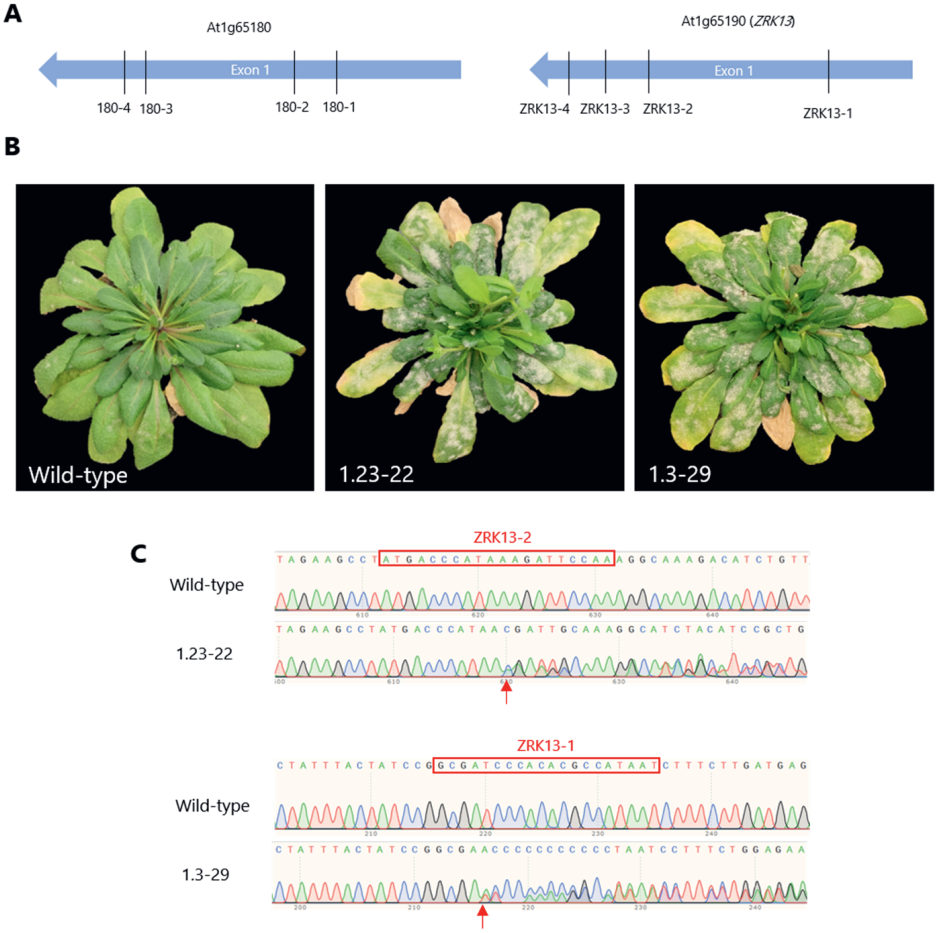


Figure 3. Targeted mutagenesis of ZRK13 in Litva results in a susceptible phenotype to *Oidium neolycopersici*. A. Graphical representation of the position of the four single guide RNAs (sgRNAs) targeting each candidate gene. **B**. Phenotypic response upon inoculation with *Oidium neolycopersici* of wild-type (WT) Litva and T_2 transformants 1.23-22 and 1.3-29 at 25 days post-inoculation. **C**. PCR-based sequencing in the sgRNAs ZRK13-1 and ZRK13-2 of WT and transformants 1.23-22 and 1.3-29. Red box indicates the sgRNAs positions and red arrows indicate the start of the aberrant sequences.

ZRK13 allele of Litva

PCR-based sequencing of genomic DNA of Litva allowed us to identify several polymorphisms compared to other *ZRK13* alleles. The predicted ZRK13 protein of Litva spans 349 amino acids and holds an 80.4% similarity with the Col-0 allele and a 90% similarity with the allele of On-resistant accession Bla-6 (Chapter 2 of this thesis). Interestingly, it holds an 85.6% similarity with the Col-0 allele of ZRK14 (At1g65250). Furthermore, a BLASTP analysis showed a high similarity with

the hypothetical protein AXX17_AT1G58850 of accession Landsberg *erecta* (Ler; GenBank: OAP14971.1) and an unnamed protein product of accession An-1 (GenBank: VYS50054.1) (Fig. 4A).

When compared with the other members of the ZRK family, a phylogenetic tree shows that the Litva allele of ZRK13 clusters together with ZRK12 (At1g67470), ZRK13 and ZRK14 (At1g65250) of Col-0, along with the hypothetical protein from Ler and the unnamed protein product from accession An-1, in a clade apart from other members of the ZRK family characterized as pseudokinases that recognize type 3 bacterial-secreted effectors to trigger ETI (ZED1, ZRK1 and ZRK3; Fig. S5). Within the ZRK12-14 clade the ZRK13 allele of Litva clusters together with the ZRK13 allele of Bla-6 and the unnamed protein of accession An-1 (Fig. 4B).

By predicting the topology of the putative protein using the Protter webtool (Omasits *et al.*, 2014), the Litva ZRK13 allelic variant does not appear to harbour the two transmembrane domains present in the Col-0 variant, therefore predicting it to be an intracellular protein (Fig 4A). As in the case of the Bla-6 allele of ZRK13 (Chapter 2 of this thesis), the Col-0 allele of ZRK14, and the unnamed protein of An-1 a premature translation termination codon in the Litva allele of ZRK13 causes the lack of a 49 amino acid-long disorder region reported to be present in the Col-0 protein according to the domain annotation retrieved from Uniprot (https://www.uniprot.org/). Furthermore, similarly to Bla-6, the ZRK13 predicted protein of Litva appears to lack critical domains for kinase catalytic activity including the GxGxxG, VAIK, HRD, and DFG, suggesting an atypical kinase identity (Huard-Chauveau *et al.*, 2013).

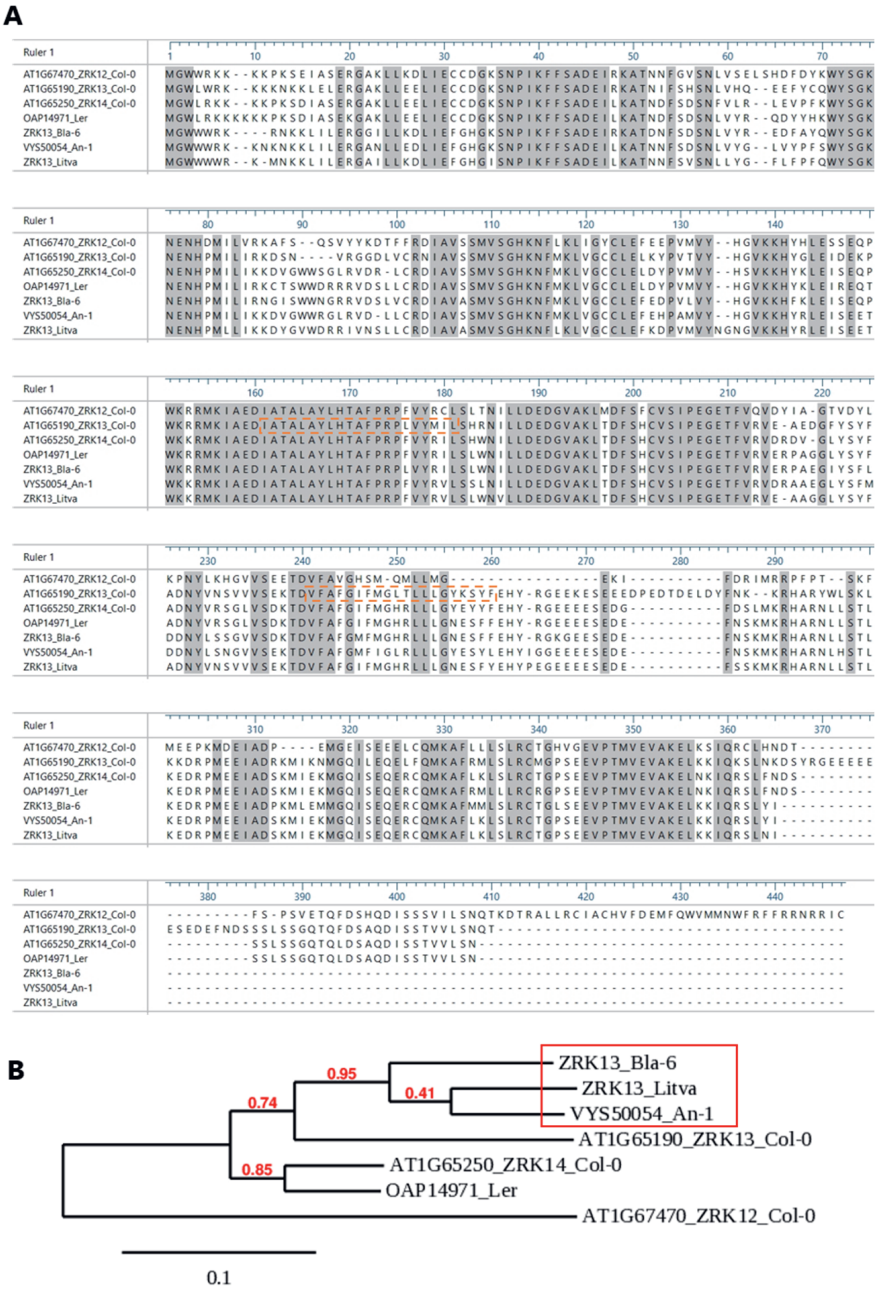


Figure 4. Allelic variant of ZRK13 in Litva. **A.** Alignment of the protein sequences of ZRK12, ZRK13 and ZRK13 from reference accession Col-0; OAP14971 from accession Ler; predicted protein from the genomic sequence of *ZRK13* from resistant accession Bla-6; VYS50054 from accession An-1 and predicted protein from the genomic sequence of

ZRK13 from accession Litva. Predicted transmembrane domains using Protter (Omasits *et al.*, 2014) in the Col-0 ZRK13 protein are shown in orange boxes. Conserved residues are shown in gray. **B**. Phylogenetic tree of the proteins listed in A showing the ZRK13 alleles of Bla-6, Litva and VYS50054 from An-1 clustering together.

Resistance to *On* in Litva requires *ZRK13* and QTL-4

While performing confirmation assays in the recombinant analysis for fine-mapping of QTL-2, we tested two F_4 families derived from recombinant F_2#77, which was genotyped to be heterozygous for both QTL-2 (*ZRK13*) and QTL-4 (Fig. 5A, Fig. S1). When the progeny of this plant was genotyped using marker LZRK13 (in-gene marker for *ZRK13*), not all plants homozygous for the Litva allele of *ZRK13* showed resistance. We speculated that this segregation in resistance was due to the effect of QTL-4, which prompted us to verify these results. To separate the effects of both QTLs, we selected a set of families derived from selfings of two informative recombinants: F_2#21, previously used to fine-map QTL-2, and F_2#77 to compare the disease response of plants carrying Litva alleles for either or both *ZRK13* and QTL-4 in homozygous and heterozygous state (Table 2, Fig. 5). For this, we obtained the genotypic information from marker LZRK13, and two markers in the QTL-4 region on chromosome 3: ID760 (located close to marker RH653/654) and RH663/664.

In total, five F_6 families (Table 5) derived from F_2#21 (1-5E-8-28, 1-5E-8-29, 20-10F-8-4, 20-10F-8-6 and 20-10F-8-7) and four F_5 families derived from F_2#77 (A5-10-5, A5-10-6, A5-30-2 and A5-30-8) were tested. The genotypes of the parents of each of these families for markers LZRK13 (QTL-2) and ID760 (QTL-4) are presented in Table 2, along with the phenotypic response of the progeny obtained in disease assays with *On* in two independent experiments. We tested at least 10 plants from each family and scored them as susceptible, intermediate or resistant (S, I, R, respectively). Interestingly, upon inoculation with *On,* only families carrying the Litva allele of both *ZRK13* and QTL-4 in homozygous state displayed resistance comparable to WT Litva (Table 2, Fig. 5B). Families carrying the Col-0 allele of either *ZRK13* or QTL-4 in homozygous state exhibited a susceptible phenotype (Table 2, Fig. 5B). Furthermore, for families derived from parents heterozygous for either of the aforementioned loci, the response corresponded with that of the fixed lines: only plants homozygous for the Litva allele in both loci displayed resistance comparable to WT Litva, while plants carrying Col-0 alleles in either of these loci were susceptible (Fig. S6). Additionally, as previously seen in the initial F_2 disease assay (Figure 1C), heterozygous plants for one of the two tested loci but homozygous Litva for the other locus displayed an intermediate phenotype, indicating a dosage effect for both *ZRK13* and QTL-4 (Fig. S6). Together these results indicated that resistance in Litva requires both *ZRK13* and QTL-4.

As mentioned previously, the QTL-4 region is flanked by markers RH653/654 and RH206/255. This is a 3.1-Mbp region on chromosome 3 between 17.2 and 20.3 Mbp, from gene At3g46870 to At3g54900 (~800 genes in Col-0). These include homologs of *RPW8* (At3g50450 to At3g50480), and the *HOPZ-ACTIVATED RESISTANCE 1* (*ZAR1*) gene (At3g50950).

Table 2. Arabidopsis F_5 and F_6 families used to dissect the effect in resistance to *Oidium neolycopersici* (*On)* of QTL-2 and QTL-4 individually and together in Litva. Family code, F_2 parent, generation, genotypic information of *ZRK13* and ID760 (L, homozygous Litva; H, Heterozygous; C, homozygous Col-0) in the parental plant, and phenotypic response of the progeny upon inoculation with *On* are listed.

F_2 Parent	Plant	Generation	Genotype		*On* response in selfing progeny
			ZRK13 (Chr.1)	ID760 (Chr. 3 - QTL-4)	
F_2#21	1-5E-8-28	F_5	L	L	Resistant
	20-10F-8-4		L	L	Resistant
	20-10F-8-6		H	L	Segregating
	1-5E-8-29		C	L	Susceptible
	20-10F-8-7		C	L	Susceptible
F_2#77	A5-30-2	F_4	L	L	Resistant
	A5-30-8		H	L	Segregating
	A5-10-6		L	H	Segregating
	A5-10-5		L	C	Susceptible

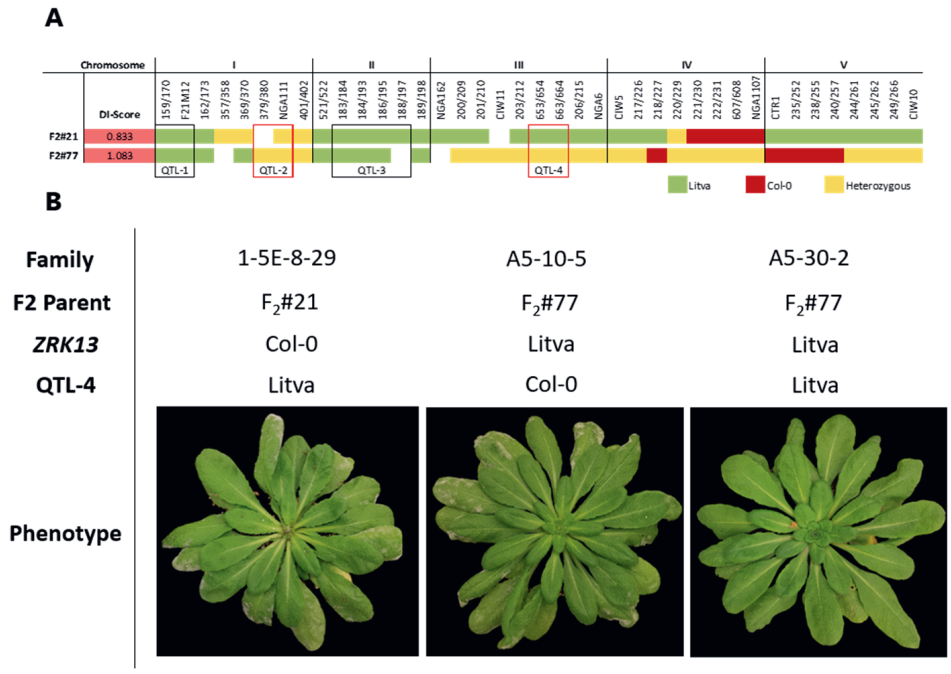


Figure 5. Phenotypic response of fixed lines carrying *ZRK13* and QTL-4 individually and together. A. Genotypic information across all chromosomes of informative recombinants F_2#21 and F_2#77 (L, Litva; H, Heterozygous; C, Col-0). Disease index (DI) score is the average of three observations starting at 8 days post inoculation (dpi). **B**. Genotypic information and disease response of fixed families carrying ZRK13 and QTL-4.

Discussion

Although the model plant Arabidopsis has been extensively used to study the genetic and molecular basis of resistance to adapted PM pathogens, the interaction with the tomato-adapted PM pathogen *On* has received less attention in the past. The analyses of progeny of crosses between Arabidopsis accessions resistant and susceptible to *On* have allowed us to identify novel components of resistance (Gao *et al.*, 2015, Chapter 2 of this thesis). As previously suggested, the resistance to PM in Arabidopsis is mostly polygenic (Göllner *et al.*, 2008). In the present study, QTL analysis of the progeny of a cross between resistant Arabidopsis accession Litva and susceptible accession Col-0 allowed us to identify two major loci determining resistance against *On*. By selecting an F_2 recombinant carrying the QTL with the highest LOD score (QTL-2) in heterozygous state while carrying the other resistance loci in homozygous state for the Litva allele (F_2#21), and through subsequent recombinant analyses of its progeny, we were able to fine-map the gene behind this resistance. Targeted mutagenesis using CRISPR/Cas-9 of At1g65180 in a WT Litva background had no effect in resistance

to *On*, while targeted mutagenesis of *ZRK13* resulted in a susceptible phenotype, indicating that this gene is essential for the resistance response in this accession. Segregation ratios of the T_2 families originating from susceptible plants did not follow a mendelian segregation. We speculate that the susceptibility is the result of somatic mutations may not be inherited to the offspring. Chimerism and somatic mutagenesis have been reported to occur in Arabidopsis when transformed via flower dipping (Feng *et al.*, 2014; Jiang *et al.*, 2014). Therefore, the screening for heritable mutations has been suggested to be done in generations later than T_2 (Feng *et al.*, 2014). Although we did not find homozygous deletions in the progeny of *ZRK13* transformants, by detecting aberrant sequences in susceptible plants at the sites of two different sgRNAs, we were able to identify *ZRK13* as essential for the resistance found in Arabidopsis accession Litva. Analysis of the progeny of these transformants will determine if the mutations found correspond to somatic events or heterozygous mutants.

ZRK13 belongs to the receptor-like cytoplasmic kinases (RLCKs) XII-2 subfamily of the *ZED1-RELATED KINASE* (*ZRK*) family (Lewis *et al.*, 2013). Until now, four other members of this subfamily have been characterized to be necessary for the recognition of different type 3 bacterial-secreted effectors (T3SE) to trigger an ETI response dependent on the interaction with the ZAR1 resistosome: ZED1 required for the recognition of HopZ1 and HopX1 of *Pseudomonas syringae* (Lewis *et al.*, 2013; Bastedo *et al.*, 2019; Martel *et al.*, 2020), RKS1 (ZRK1) for the recognition of AvrAC of *Xanthomonas campestris* (Wang *et al.*, 2015, 2019), ZRK3 for the recognition of HopF1 and HopO1 of *P. syringae* (Seto *et al.*, 2017; Martel *et al.*, 2020), and ZRK2 for the recognition of HopBA1 of *P. syringae* (Martel *et al.*, 2020). Furthermore, ZRK6 and ZRK15 have also been shown to interact with ZAR1 (Lewis *et al.*, 2013; Wang *et al.*, 2015). Interestingly, an autoimmune ZAR1-dependent response in the *zed-1D* mutant, causing a severe phenotype when grown at high temperature, was shown to be partially rescued by the overexpression of *ZRK13* (Wang *et al.*, 2017), suggesting that ZRK13 is also able to interact with ZAR1. However, whether the resistance to *On* found in Litva relies on the interaction between ZRK13 and the ZAR1 resistosome remains to be studied. Co-immunoprecipitation assays and challenging silenced or knocked-out plants for *ZAR1* with *On* in a Litva background will help to answer this question at molecular and phenotypic levels.

In the previous chapter of this thesis we describe the fine-mapping and identification of *ZRK13* as the gene conferring resistance against *On* in Arabidopsis accession Bla-6. In the present chapter we report the identification, via an independently-executed fine-mapping study, of an allelic variant of the same gene conferring resistance to *On* in accession Litva. However, important differences between both cases should be taken into consideration. Notably, the resistance in Bla-6 was found to be inherited in a dominant manner, unlike the situation in Litva, where the F_3 displayed a recessive or co-dominant inheritance.

We hypothesised that the 1:3 (resistant: susceptible) segregation of the tested progenies may had been the result of either the recessive inheritance of the trait or a dominant resistance supressed by another required genetic locus. We have shown that at least one additional gene on chromosome 3 is required for full resistance in Litva. Moreover, analysis of the individual effects of the two major QTLs in Litva by testing recombinant lines segregating for each of these loci in disease assays with *On* suggested a recessive inheritance. Two possibilities could account for this phenomenon: firstly, that the full resistance in Litva is the result of an additive effect of *ZRK13* and the locus in QTL-4, or secondly, that this resistance is the result of an interaction between ZRK13 and a gene underlying QTL-4. With respect to the first scenario, other PM resistance genes have been identified on chromosome 3, including *RPW2*, *RPW3* (Adam & Somerville, 1996) and *RPW8* (Xiao *et al.*, 1997). However, the semi-dominant gene *RPW2* has been mapped between markers NGA152 and GL1, located at 4.6 and 10.4 Mbp, respectively; while the recessive gene *RPW3* is located between markers NGA172 and CHIB, at 0.8 and 3.9 Mbp, respectively. Therefore, both of these genes are located outside the candidate QTL-4 region, which is bordered by markers RH653/654 and RH206/215, at 17.3 and 20.3Mbp, respectively. Yet, the *RPW8* locus, although not present in the reference accession Col-0, is located close to At3g50470, at 18.7 Mbp and thus within the borders of QTL-4. Although *RPW8* has been shown to be ineffective against the Wageningen isolate of *On* in accession Sha, there is a possibility that this interaction may require specific factors (Göllner *et al.*, 2008) that could be present in Litva. Therefore, the presence of functional *RPW8* allele(s) in Litva should be tested by specific amplification of the locus.

For the second scenario, a candidate for QTL-4 is *ZAR1*, located on chromosome 3 at position 18.9Mbp (within the borders of QTL-4). If ZRK13 requires interaction with a specific allelic variant of ZAR1 in Litva to trigger an immune response, it would explain why the tested recombinants carrying the Litva allele in only one of the two loci display a susceptible phenotype upon inoculation with *On* (Figure 5). Although the crucial domains for the interaction between ZAR1 and RLCKs are still being elucidated (Baudin *et al.*, 2020b; Martel *et al.*, 2020), a recent study on the natural diversity in the ZAR1/ZED1 immunocomplex (Baudin *et al.*, 2020a) showed that natural and induced polymorphisms in ZAR1 can affect the affinity of the interaction with ZED1 and may result in a weaker recognition of the pathogen effector. Further studies on the polymorphisms between the *ZRK13* alleles of Litva and Col-0 and their effect in resistance to *On* are required. Nevertheless, for either of the two scenarios, further identification of the locus responsible for the resistance in QTL-4 can be pursued using the selfing progeny of recombinant F_2#77.

On the other hand, we mapped QTL-1 at the top end of chromosome 1, flanked by markers RH159/170 and F21M12 (0.4 – 3.2 Mbp). Previously, a QTL with minor

effect in resistance to *E. cichoracearum* (*RPW14*) has been mapped to the top of chromosome 1 in accession Warschau-1 (Schiff *et al.*, 2001), the same region established for minor QTL-1 in Litva (Figure 1B). Additionally, *EDR1* (At1g08720), whose impairment is known to confer resistance to *E. cichoracearum*, is located at position 2.8Mbp in chromosome 1. It will be interesting to study if *EDR1* contributes to the resistance conferred by QTL-1.

It is intriguing to investigate if and how the polymorphisms between the Litva and the Bla-6 alleles determine the different levels of resistance against *On* displayed in both accessions. Previously, polymorphisms among the natural alleles of *RKS1* (*ZRK1*) have been found to be associated with different levels of resistance to *X. campestris* (Huard-Chauveau *et al.*, 2013). Interestingly, the resistant *RKS1* haplotype was found to be strongly associated with high expression levels. We have found that in the case of *ZRK13* from Litva, a dosage effect is evident in plants derived from parents heterozygous at this locus. It will be interesting to study polymorphisms in the promoter of the Litva allele, and whether these affect the expression level compared to the Bla-6 allele of *ZRK13*. A lower expression level of the Litva allele might explain the dosage effect on the level of resistance to *On*. Alternatively, specific amino acid changes may influence the functionality of the two different *ZRK13* alleles.

Although the predicted ZRK13 protein of Litva holds many polymorphisms when compared to that of the reference Col-0 genome, it shows a high similarity with the predicted protein of *On*-resistant accession Bla-6. Furthermore, a BlastP query revealed a high similarity of the ZRK13 alleles of both *On*-resistant accessions with the hypothetical protein AXX17_AT1G58850 of accession Ler (GenBank: OAP14971.1). A de novo assembly of the complete genome of Arabidopsis accession Ler has shown hundreds of rearranged regions compared to the reference Col-0 genome, including 564 transpositions, 47 inversions, as well as 105 unique genes for the genome of Ler (Zapata *et al.*, 2016). Further sequencing of the genomic region of ZRK13 in Litva and other Arabidopsis accessions, as well as cDNA sequencing will confirm these polymorphisms. Moreover, we found high similarity between the Litva allele with an unnamed protein product in accession An-1 (GenBank: VYS50054.1). It will be interesting to investigate whether accession An-1 shows resistance to *On*. This might aid in the elucidation of crucial polymorphisms influencing the resistance level conferred by different *ZRK13* alleles. Accession An-1, as well as Bla-6, is reported to be susceptible to PM pathogens *E. cruciferarum* and *E. cichoracearum* (Adam *et al.*, 1999). Additionally, the ZRK13 protein from Col-0 is predicted to be localized in the plasma membrane, as two transmembrane domains are present. Interestingly, the Litva and Bla-6 alleles carry polymorphisms that result in the lack of both of these domains and are therefore predicted to be an intracellular protein. This is intriguing, as all RCLKs characterized for their interaction with ZAR1 are known to be localized intracellularly. Furthermore, although the kinase

activity of ZRK13 in Litva remains to be studied, the predicted protein lacks complete kinase domains, suggesting its pseudokinase identity. The identification of *ZRK13* as an essential gene for the resistance in Litva, opens the possibility of further exploration of mechanisms and identity of other genetic components involved in the resistance in this accession.

Materials and methods

Plant material, growth conditions and disease assays

Arabidopsis accession Litva and Col-0 were obtained from the Max Planck Institute in Köln, Germany. Plants were grown in pots or trays with soil substrate in a growth chamber at Unifarm. Wageningen University & Research, The Netherlands, at a relative humidity of 70% at 16 h/8 h day/night cycle at 21°C/18 °C day/ night temperature for the mapping studies, and at 10 h/14h day/night cycle at 21°C temperature for the analysis of mutants and fine-mapping. Disease assays with PM were done using the Wageningen *On* isolate, maintained on susceptible tomato plants (cultivar Moneymaker). Spore solutions for inoculation were prepared by washing spores from tomato leaves and diluting them to concentrations between 2.5 and 4.5 x 10^5 spores/mL. Arabidopsis 25- to 30-day-old plants were inoculated by spraying the spore solution on the leaves. DI was assessed starting at 8 days after inoculation. For the QTL and further fine-mapping DI was scored in a range from 0 to 3 depending on the level of sporulation observed on the leaves: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; 3 abundant symptoms (Gao *et al.*, 2015). For the confirmation assays and disease assays of CRISPR transformants, a simplified score of susceptible (S), intermediate (I), and resistant (R) phenotype was recorded.

Mapping and PCR-based sequencing and phylogenetic analysis

For QTL analysis, Joinmap 4 and 5 (Van Ooijen, 2006) and MapQTL6 (Van Ooijen, 2009) were used. Molecular markers for mapping were obtained from Bell and Ecker (Bell & Ecker, 1994) and Lukowitz (Lukowitz *et al.*, 2000). Additional SNP and CAPS markers for fine mapping were designed using the data available from the 1001 genome database (http://10001genomes.org). Amplification of the candidate loci was made using the 65180F/R and LZRK13F/R primers described in Table S1 and sequencing was obtained by Sanger. For the phylogenetic analysis, protein sequences were obtained from TAIR for the members of the ZRK family (ZRK1, ZRK2, ZK3, ZRK4, ZED1, ZRK6, ZRK7, ZRK10, ZRK11, ZRK12, ZRK13, ZRK14 and ZRK15), and NCBI for the hypothetical protein AXX17_AT1G58850 of accession Landsberg *erecta* (Ler; GenBank: OAP14971.1) and unnamed protein product of accession An-1 (GenBank: VYS50054.1). Alignments were made using Megalign (Clewley & Arnold, 1997). Phylogenetic trees were retrieved using the Phylogeny.fr webtool (Dereeper *et al.*, 2008)

CRISPR/Cas-9-targeted mutagenesis

A set of four single guide RNAs (sgRNAs) was designed to target each of the two candidate loci in the candidate region (Table 2). Design of the CRISPR/Cas-9 constructs and transformation of Arabidopsis accession Litva plants were carried out using the methodology described in Chapter 2 of this thesis.

Table 2. Sequences of sgRNAs targeting At1g65180 and At1g15190 (*ZRK13*) in Litva.

Name	Sequence	Target
180-1	TTCCCATCAGTATAAGCCGG	At1g65180
180-2	ATGGGCTTCGATCTCCATGG	
180-3	TTTGGTGATGTGTAAAACAA	
180-4	TAGAGCGCATTTGAAATCCA	
ZRK13-1	ATTATGGCGTGTGGGATCGC	At1g65190 (*ZRK13*)
ZRK13-2	ATGACCCATAAAGATTCCAA	
ZRK13-3	CTGCAATCTCCTCCCATGGT	
ZRK13-4	AAGCTATCACTGAGATGCAC	

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Supplementary information

Table S1. Molecular markers used in this study. INDEL, SNP and CAPS markers across all chromosomes in Litva for QTL, fine-mapping and confirmation studies.

Name	Type	Location TAIR10	Forward primer	Reverse primer
RH159/170	INDEL	chr1:440418-440555	GATGAATTCTTCCTTTTCACGTT	TGTTGTACTTAAATGTAACCAGTCAG
F21M12	INDEL	chr1:3212189-3212389	GGCTTTCTCGAAATCTGTCC	TTACTTTTTGCCTCTTGTCATTG
RH162/173	INDEL	chr1:8103703-8103817	CATACATACAATTCACTAACCAAAA	TGGATCTCCTTAATAGTTTAAAAGG
RH357/358	INDEL	chr1:13828045-13828127	CAATATCACTTTTTCTCAATTGCTT	CCAATTGAATGGACTTGATACAC
RH369/370	INDEL	chr1:16370974-16371098	TATAGCAAAGCTTTCGATCCAT	GTATTCTATGATAGGCACAAGACCA
RH379/380	INDEL	chr1:23102164-23102277	GCAACCTCATAACTAAACTAAACAGA	CCATTATATAACCCGATCATGG
RH443/444	INDEL	chr1:23738343-23738427	CAGCTGCGGAAAAGAAGAAG	ACAAACCGGAGTGTCATGGT
RH451/452	INDEL	chr1:23847296-23847405	ATATTCACGGGCTTTCTTCC	CAGGTCAGGTCACTGATGAAG
RH455/456	INDEL	chr1:24060258-24060404	TATGGCAAATGGGAAAAAGG	TTTTCTGTTGATTGGTCGTCA
RH461/462	INDEL	chr1:24098863-24098977	GAGAAAAGTTGGAATCTTTCATTTG	GACGAGCTGAAATCCCTGAA
RH942/943	SNP	chr1:24120931-24121101	GATATGGTTACTTATCTTGGGCTCT	ACATAGTTGTTGAAACCACCTAAAC
RH950/951	SNP	chr1:24163798-24163965	TAAAGCCCAACTATTTCTTTGTCTA	CTACAGGACATATTAGAAGGGTCAA
RH954/955	SNP	chr1:24172590-24172763	ATACCTACGTTTGCTTCATTGTAAA	AACTAATTGAACACAAAAACAGAGG
RH866/867	SNP	chr1:24189465-24189614	TGAAATGTGGTGTTTTGTTTATGA	CAACCACAAAAAGATGTGTCAAA
RH1020/1021	SNP	chr1:24195979-24196200	TGATAATAACAACAGAGATGAAGCA	CGAAATTAAATAGAAGCTGTTGTGT
RH1046/1047	SNP	chr1:24201323-24201498	GATATCACGAATCGATATAATCAGG	ATAGGGTTAGGAGTTAGTTGGTTTG
RH1050/1051	SNP	chr1:24207069-24207205	TATGATAACCAAACTTCCATCTGTT	AGTGCTAAACAATACGAGAGAAAAA
RH1056/1057	SNP	chr1:24210603-24210730	TAACTAGTTCGTTTCAGTTCCATTT	CTATTGTCTTCATAAGCTACGCAGT
RH1060/1061	SNP	chr1:24214058-24214237	GATTTACATTGCAAAAGACTTTCTA	GCATCGATTGTGTTTTTATTGAT
RH1066/1067	SNP	chr1:24217355-24217469	TTTTAAGGTACTCAAATTCTTGTTGA	TGTGATAAATCCTTCCAACTCTAAA
Det_ZRK13F/R	Seq.	Chr1:24215449-24217397	GGTGTTGTGCGTCACCTAGA	TGAATTTTCGCGGGAGGA
LZRK13F/R	CAPS	chr1:24215788-24217402	GGAGGACGTCAATGTTGAAAA	CCCAAACACACATACACAAACTC
65180F/R	Seq.	chr1:24213874-24211765	TCCGTCACATGAGCTAAGAGAA	CAAACACACTTACAAATCTAAATCACA
RH1070/1071	SNP	chr1:24224224-24224343	TACCAAATTGATCCAATACAAAAAT	TTTTGGTCTTTGCTTTATCAGTAGT
RH1034/1035	SNP	chr1:24225913-24226156	ATTTCTATATAACCATCAACCATGC	GAAATCTGAGAACTCAAAAATACGA
RH1036/1037	SNP	chr1:24232310-24232543	TAGTTGTGAAAGGACAGAGGTATTT	ACAAGTTACTGGCGATCTTAAAACT
RH1040/1041	SNP	chr1:24241544-24241765	GTTATCTTTAATGTTTCGTTGTGCT	AGAGTTTTGCAAGTTTTTGAGTCTA
RH872/873	SNP	chr1:24252969-24253165	GGGACATCATAAAGTTGTCTTGG	CAATCACCAATAGACTTGGTGTACTAT
RH874/875	SNP	chr1:24256729-24256870	CCTTCCTATGATGTCAAATACTGAAA	GAAATTCAATTGGTATAAAAACATGG
RH876/877	SNP	chr1:24261066-24261194	TGTTTCATCATCTCCAAAGCA	TGCAGTGAATTTGATGTGTCTG
RH878/879	SNP	chr1:24272364-24272496	CATTAAGAGCCCAACCGAAA	TTGTACGATTGAGCCTCGTG
RH880/881	SNP	chr1:24280624-24280753	TTGGATTCTTTGTAACATCCAAAA	ACTGTGATCGCCGACTCTT
RH473/474	INDEL	chr1:24329048-24329148	TTACCAAAAGGTTGCGAACA	TGTTTTGTCTTTATCAACTCCACAA
RH477/478	INDEL	chr1:24549376-24549522	TCTGCGAAGTGGAAGAGAAAA	CCTGTGTTGCTGTGGAGTGT
RH495/496	INDEL	chr1:26392741-26392915	GGCGTCTTGTCGATGTTACC	GTGCCTTGTTCATCGTCTCA
NGA111	INDEL	chr1:27353212-27353339	CTCCAGTTGGAAGCTAAAGGG	TGTTTTTTAGGACAAATGGCG
RH401/402	INDEL	chr1:28922583-28922712	TAATGATCCGTGTGGAACTTAACTA	TTACTTCTCTAACTTCACTCCAAGG
RH-521/522	INDEL	chr2:358201-358317	AATTGCCAAACGATCAAAGG	CACTTGCCCAGTGTCAAAGTT
RH-183/184	INDEL	chr2:4751701-4751835	AACAATAGTAAATTTGCGAATACCA	ATCTACTTCTCTCTCTCTCGCTGAT

Name	Type	Location TAIR10	Forward primer	Reverse primer
RH-184/193	INDEL	chr2:7283601-7283737	ATCTACTTCTCTCTCTCGCTGAT	GAAACAGTAAAACGGCAAAGC
RH-186/195	INDEL	chr2:9965601-9965761	GTAACGGTTTATCTATCTTTTTCCA	AGAAGCACCAGACACAAGAAAC
RH-188/197	INDEL	chr2:13929901-13930039	AAAGAGTCAAGGAAAAGTATGTGTG	TTAAGATAGAAACCAAAACCAAGC
RH-189/198	INDEL	chr2:18328201-18328380	GTTTGCGTTTAATAGTCAAGATATG	CAAATGTTTAAGGTTTGTGGTTG
NGA162	INDEL	chr3:4608201-4608308	CATGCAATTTGCATCTGAGG	CTCTGTCACTCTTTTCCTCTGG
RH-200/209	INDEL	chr3:5707001-5707139	AAATAAGATTTGTAATGTAAGACGAA	TTCCACCTAACCTAATAATAACAAG
RH-201/210	INDEL	chr3:7441901-7442039	AAGCCGTACAGAAACATCAGTTAG	ACCTACAAAGTTGAGGTAAGGTCAT
CIW11	INDEL	chr3:9774301-9774480	CCCCGAGTTGAGGTATT	GAAGAAATTCCTAAAGCATTC
RH-203/212	INDEL	chr3:12557701-12557858	GAACAATAAAGAGGAAGAAGAAAGC	GCATTACAACGTATAACGTAATGAAA
ID760	CAPS	chr3:17222162-17222850	GCAACCAAAGGGTTCAAAGA	CCTTCCAATCTAGCGTCTCG
RH-653/654	INDEL	chr3:17262301-17262419	CCTCAACTACCCATCCATGA	GAGCCTTTTGGGAAGGTATC
RH-663/664	INDEL	chr3:17801201-17801303	TGGGGCAAATTTGTGAAAGT	GGAATGGGTTCAGGGTTTTT
RH-206/215	INDEL	chr3:20342501-20342624	TGGGTTTAAAAATAAAAATTAGAGGA	TAAACCGAAATACTAATACGATCCA
NGA6	INDEL	chr3:23031101-23031244	TGGATTTCTTCCTCTCTTCAC	ATGGAGAAGCTTACACTGATC
CIW5	INDEL	chr4:738001-738165	GGTTAAAAATTAGGGTTACGA	AGATTTACGTGGAAGCAAT
RH-217/226	INDEL	chr4:1589201-1589322	CTGACGGAGAGATAGTTATCTGGTT	GAAGGGGAACTACTGAGATTTGTA
RH-218/227	INDEL	chr4:2697501-2697623	GACATAACTTCGAATTGTTGGATAG	AATTTCGCCGGAATAAACAG
RH-220/229	INDEL	chr4:6179701-6179865	CCTCCACAAGAGATTTGAGTTTAT	CTTTTAGTGTTTGGTTTTTGGTTTA
RH-221/230	INDEL	chr4:9309501-9309650	TCATGTTTATTTCACTGTTTCAA	ACTCCCATTAGAGCAGTTCAAAGT
RH-222/231	INDEL	chr4:10871901-10872033	CTGCGTTTACACAAGACAACAC	ACTCGCATTGTTCTCCAAACT
RH-607/608	INDEL	chr4:11966293-11966451	TGTAAATATCGGCTTCTAAG	CTGAAACAAATCGCATTA
NGA1107	INDEL	chr4:18096201-18096351	GCGAAAAAACAAAAAAATCCA	CGACGAATCGACAGAATTAGG
CTR1	INDEL	chr5:979701-979860	CCACTTGTTTCTCTCTCTAG	TATCAACAGAAACGCACCGAG
RH-235/252	INDEL	chr5:1035701-1035851	TTTAGAAACTCTCTCTCACCAAATC	TGGTTAAGTGTTTGAAGTGTAATGA
RH-238/255	INDEL	chr5:5464101-5464256	TCGTATTAAAAGTCATTCAGTAAAGG	CTCTATATGGTTTCAAGCCTCTGAT
RH-240/257	INDEL	chr5:9445201-9445358	ATGAGTTCAGAACCTAAAAATTGTG	TCTCACTAGAAAAAGCAGAACTAGG
RH-244/261	INDEL	chr5:16765001-16765186	CTGCTTATTGTAAGCTTGCTTGT	TCATTTGTTATCATCACTTATTCACTT
RH-245/262	INDEL	chr5:17869901-17870033	GCAATATCAGGGTCTTGTAAAGATA	CCATTGGATATAATTAAGAAGAAGAA
RH-249/266	INDEL	chr5:22398901-22399019	GCTACAGGTAAAGGAATGCATAAAT	GTAACGACTAACGAGGTAAAAGGTC
CIW10	INDEL	chr5:24530801-24530941	CCACATTTTCCTTCTTTCATA	CAACATTTAGCAAATCAACTT

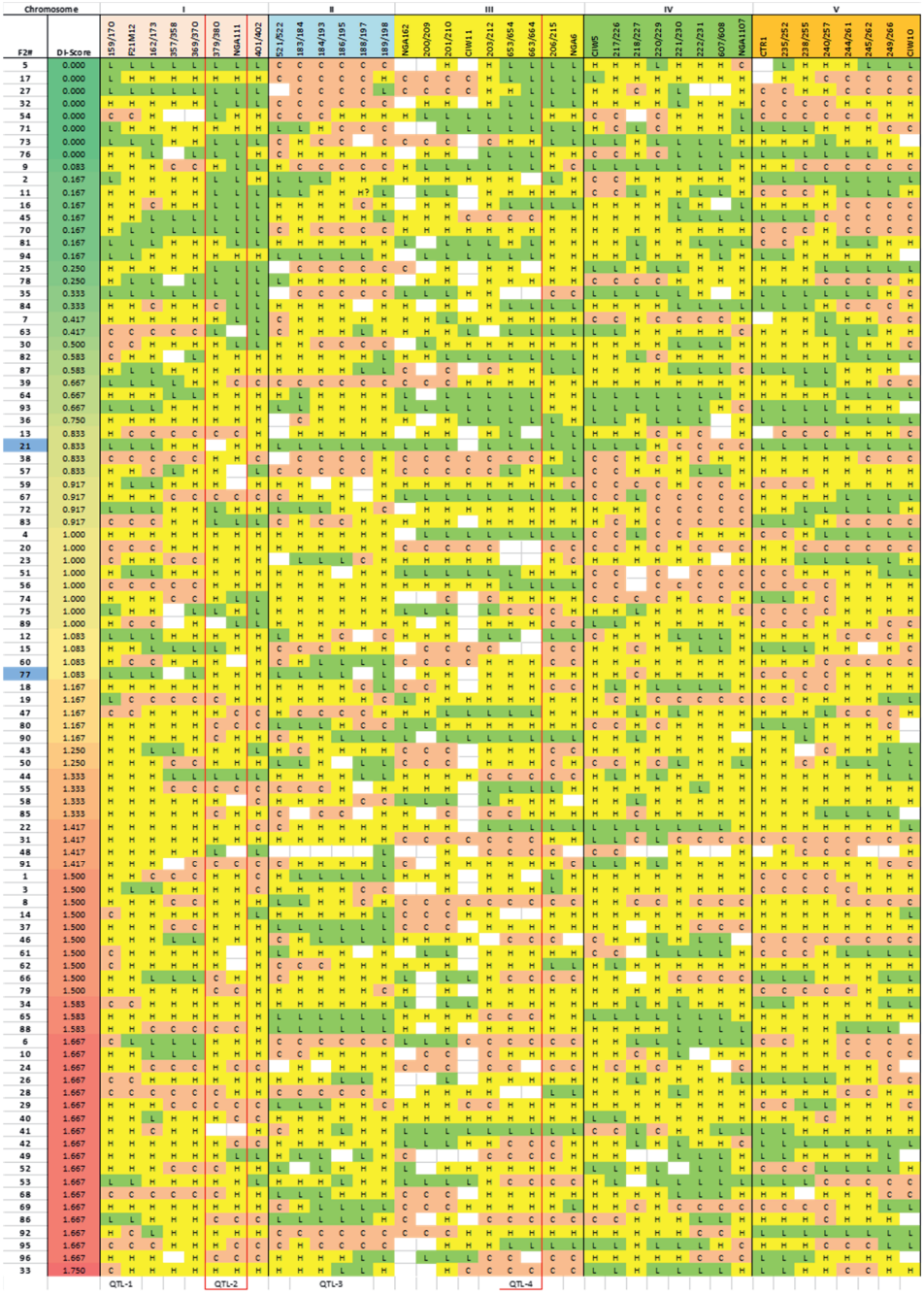


Figure S1. Genotypic and phenotypic information of the F_2 progeny of Litva x Col-0. Genotyptic information (L, Litva; H, Heterozygous; C, Col-0) of markers across all five chromosomes. Disease index (DI) score is the average of three observations starting at 8 days post inoculation (dpi). Identified QTLs are indicated in the bottom row. In blue:

recombinants 21 and 77 used for fine mapping and recombinant analyses of QTL-2 and QTL-4.

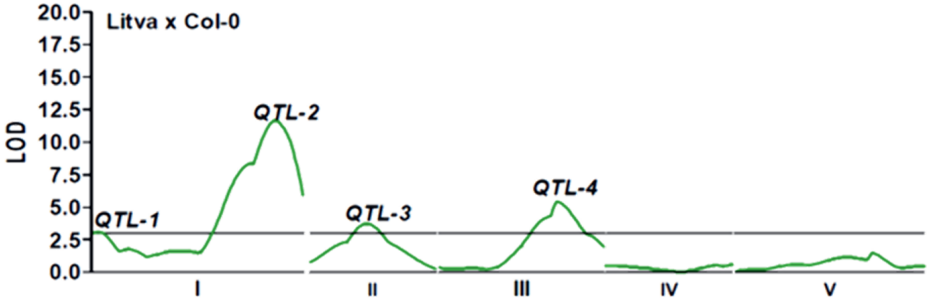
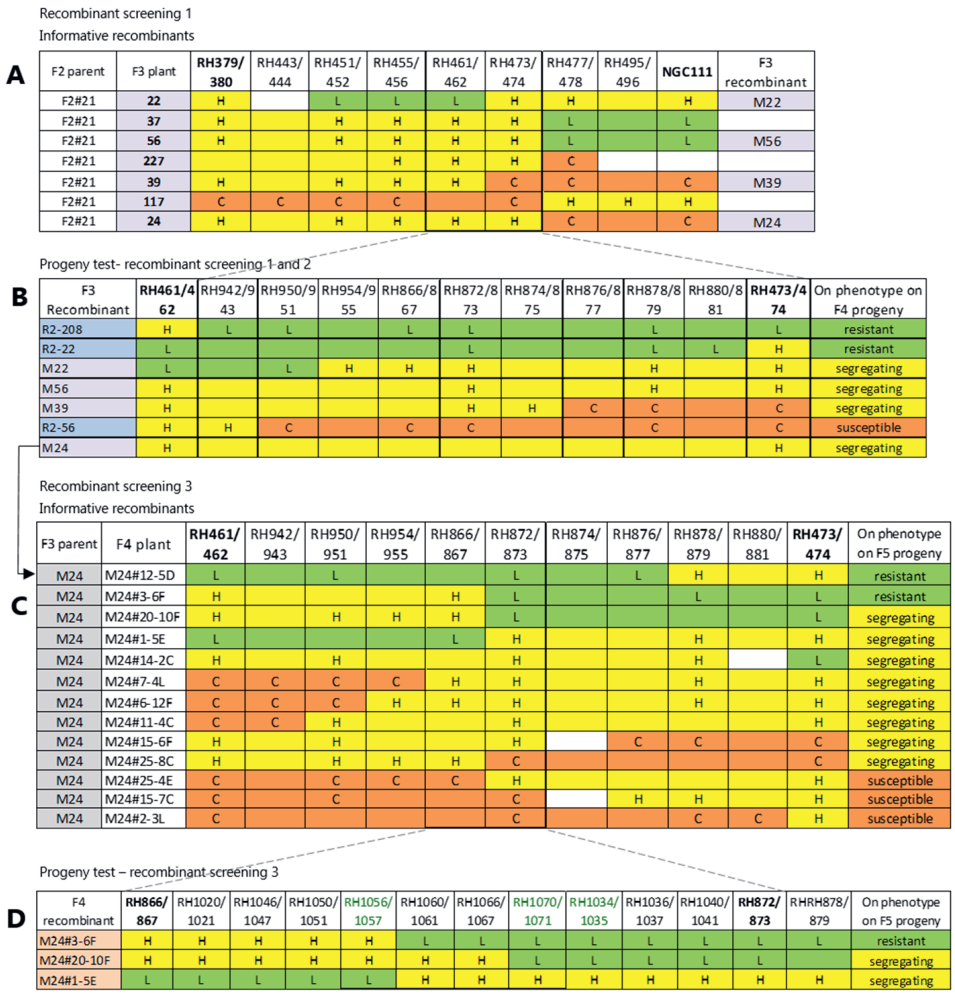


Figure S2. QTL analysis of the F_2 of the Litva x Col-0 cross showing the LOD scores for the four loci responsible for the resistance. Result of Interval Mapping (IM) is shown.



Recombinant screening 1
Informative recombinants

A

F2 parent	F3 plant	**RH379/ 380**	RH443/ 444	RH451/ 452	RH455/ 456	RH461/ 462	RH473/ 474	RH477/ 478	RH495/ 496	**NGC111**	F3 recombinant
F2#21	**22**	H		L	L	L	H	H		H	M22
F2#21	**37**	H		H	H	H	H	L		L	
F2#21	**56**	H		H	H	H	H	L		L	M56
F2#21	**227**				H	H	H	C			
F2#21	**39**	H		H	H	H	C	C		C	M39
F2#21	**117**	C	C	C	C		C	H	H	H	
F2#21	**24**	H		H	H	H	H	C		C	M24

Progeny test- recombinant screening 1 and 2

B

F3 Recombinant	**RH461/4 62**	RH942/9 43	RH950/9 51	RH954/9 55	RH866/8 67	RH872/8 73	RH874/8 75	RH876/8 77	RH878/8 79	RH880/8 81	**RH473/4 74**	On phenotype on F4 progeny
R2-208	H	L	L		L	L			L		L	resistant
R2-22	L					L			L	L	H	resistant
M22	L		L	H	H	H			H		H	segregating
M56	H					H			H		H	segregating
M39	H					H	H	C	C		C	segregating
R2-56	H	H	C		C	C			C		C	susceptible
M24	H										H	segregating

Recombinant screening 3
Informative recombinants

C

F3 parent	F4 plant	**RH461/ 462**	RH942/ 943	RH950/ 951	RH954/ 955	RH866/ 867	RH872/ 873	RH874/ 875	RH876/ 877	RH878/ 879	RH880/ 881	**RH473/ 474**	On phenotype on F5 progeny
M24	M24#12-5D	L		L			L		L	H		H	resistant
M24	M24#3-6F	H				H	L			L		L	resistant
M24	M24#20-10F	H		H	H	H	L					L	segregating
M24	M24#1-5E	L				L	H			H		H	segregating
M24	M24#14-2C	H		H			H			H		L	segregating
M24	M24#7-4L	C	C	C	C	H	H			H		H	segregating
M24	M24#6-12F	C	C	C	H	H	H			H		H	segregating
M24	M24#11-4C	C	C	H			H					H	segregating
M24	M24#15-6F	H		H			H		C	C		C	segregating
M24	M24#25-8C	H		H	H	H	C					C	segregating
M24	M24#25-4E	C		C	C	C	H					H	susceptible
M24	M24#15-7C	C		C			C		H	H		H	susceptible
M24	M24#2-3L	C					C			C	C	H	susceptible

Progeny test – recombinant screening 3

D

F4 recombinant	**RH866/ 867**	RH1020/ 1021	RH1046/ 1047	RH1050/ 1051	RH1056/ 1057	RH1060/ 1061	RH1066/ 1067	RH1070/ 1071	RH1034/ 1035	RH1036/ 1037	RH1040/ 1041	**RH872/ 873**	RHRH878/ 879	On phenotype on F5 progeny
M24#3-6F	H	H	H	H	H	L	L	L	L	L	L	L	L	resistant
M24#20-10F	H	H	H	H	H	H	H	L	L	L	L	L		segregating
M24#1-5E	L	L	L	L	L	H	H	H	H	H	H	H	H	segregating

Figure S3. Recombinant analyses for fine mapping of QTL-2 in Litva. Genotypic information (L, Litva; H, Heterozygous; C, Col-0) of markers in the candidate region of informative recombinants. Parental lines of each informative family are indicated in the first column. A. Informative recombinants of screening 1. B. Progeny test of recombinant screening 1(M) and 2 (R2). C. Informative recombinants from screening 3. D. Progeny test of recombinant screening 3.

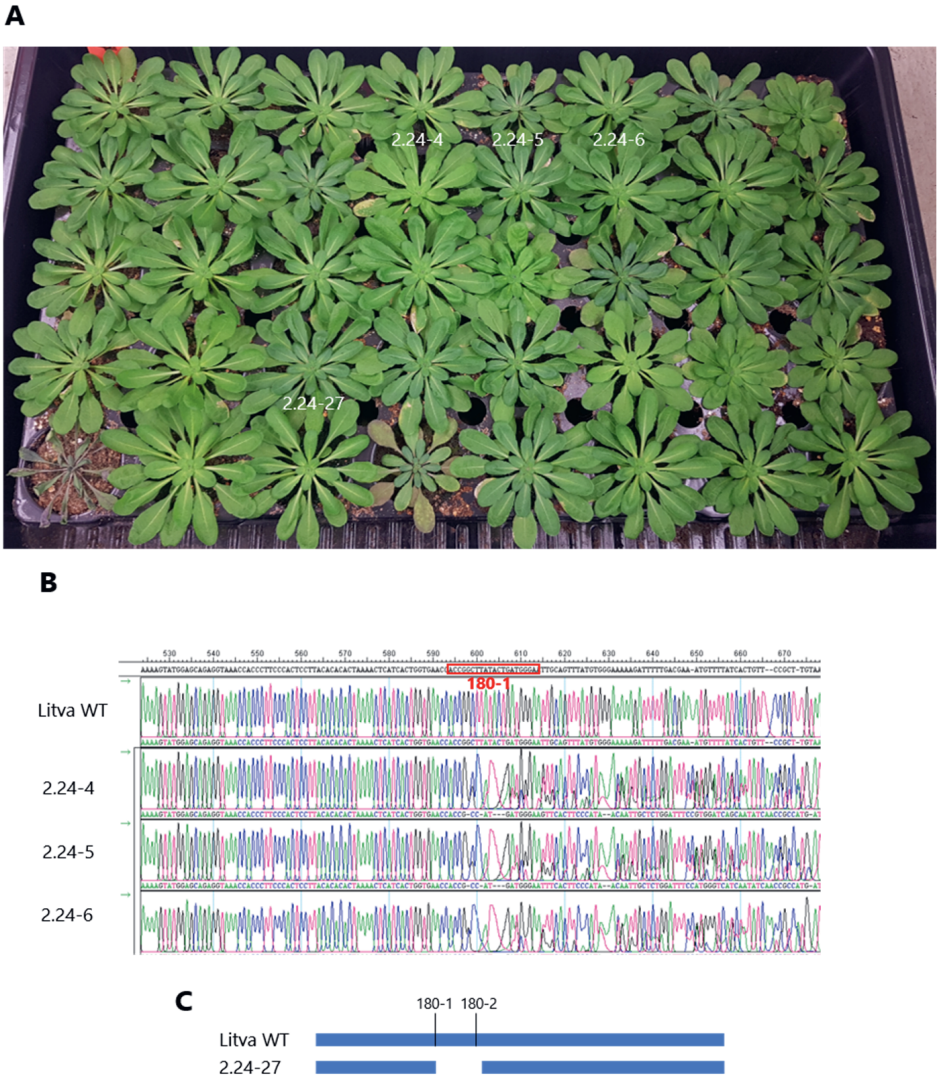


Figure S4. Targeted mutagenesis of At1g65180 in Litva does not affect the resistance against *Oidium neolycopersici* (On). **A**. Phenotypic response of T_2 family 2.24 to *On* at 25 days post inoculation. Sequenced plants 2.24-4, 2.24-5, 2.24-6 and 2.24-27 are indicated with white letters. **B**. PCR-based sequencing of plants 2.24-4, 2.24-5 and 2.24-6 revealed the presence of aberrant sequences starting at the site of sgRNA180-1 (indicated in red). **C**. Schematic representation of the homozygous deletion found in plant 2.24-27.

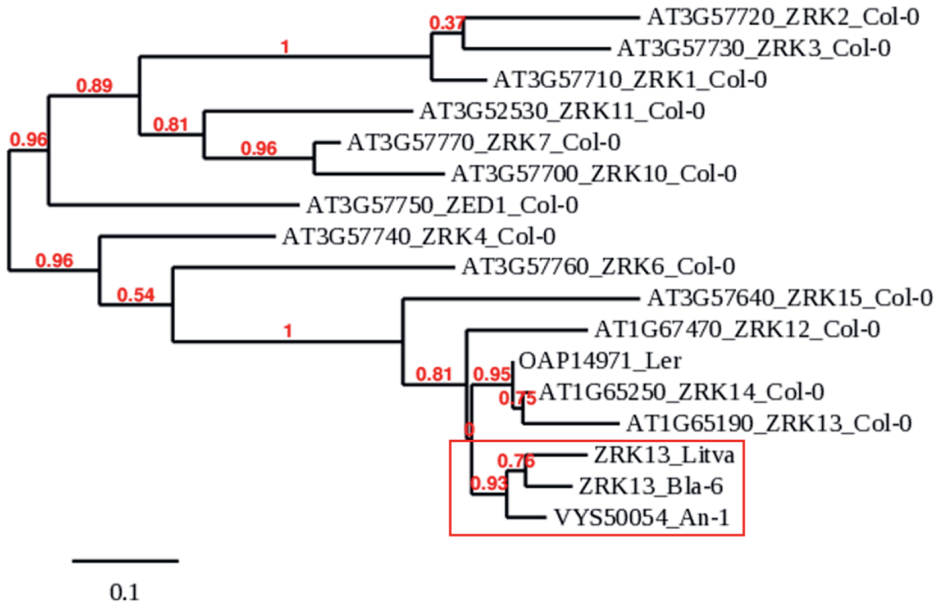
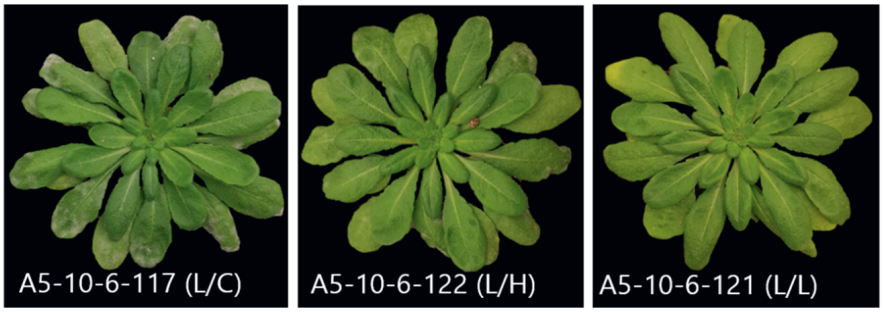


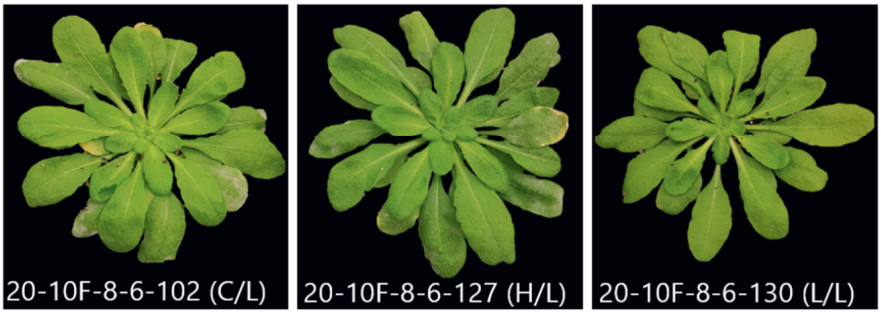
Figure S5. Phylogenetic tree of the members of the ZRK family and the hypothetical protein AXX17_AT1G58850 of accession Landsberg erecta (Ler; GenBank: OAP14971.1) and unnamed protein product of accession An-1 (GenBank: VYS50054.1). Red box indicates the ZRK13 alleles of *On*-resistant accessions Bla-6 and Litva clustering together with VYS50054 of accession An-1.

A



Phenotype	A5-10-6 QTL-4 genotype C	H	L
S	12	5	0
I	0	6	0
R	0	2	4

B



Phenotype	20-10F-8 ZRK13 genotype C	H	L
S	5	3	0
I	0	11	0
R	0	1	10

Figure S6. Recombinant analysis of a family segregating for QTL-4 while homozygous for the Litva allele of *ZRK13* (A) and a family segregating for *ZRK13* while homozygous for the Litva allele of QTL-4 (B). For each plant the genotype of the two QTLs is shown (ZRK13/QTL-4). (L, Litva; H, Heterozygous; C, Col-0). Tables below each family indicate the number of plants for each genotype and phenotype (S: susceptible, I: intermediate, R: resistant). A skewed segregation 12C:13H:4L was seen in family A5-10-5-6 (A) and a 1:2:1 segregation was observed in family 20-10F-8-6 (B).

Chapter 4

CRISPR/Cas9-targeted mutagenesis of the tomato susceptibility gene *PMR4* for resistance against powdery mildew

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Abstract

Background

The development of CRISPR/Cas9 technology has facilitated targeted mutagenesis in an efficient and precise way. Previously, RNAi silencing of the susceptibility (*S*) gene *Powdery Mildew Resistance 4* (*PMR4*) in tomato has been shown to enhance resistance against the powdery mildew pathogen *Oidium neolycopersici* (*On*).

Results

To study whether full knock-out of the tomato *PMR4* gene would result in a higher level of resistance than in the RNAi-silenced transgenic plants we generated tomato *PMR4* CRISPR mutants. We used a CRISPR/Cas9 construct containing four single-guide RNAs (sgRNAs) targeting the tomato *PMR4* gene to increase the possibility of large deletions in the mutants. After PCR-based selection and sequencing of transformants, we identified five different mutation events, including deletions from 4 to 900-bp, a 1-bp insertion and a 892-bp inversion. These mutants all showed reduced susceptibility to *On* based on visual scoring of disease symptoms and quantification of relative fungal biomass. Histological observations revealed a significantly higher occurrence of hypersensitive response-like cell death at sites of fungal infection in the *pmr4* mutants compared to wild-type plants. Both haustorial formation and hyphal growth were diminished but not completely inhibited in the mutants.

Conclusion

CRISPR/Cas-9 targeted mutagenesis of the tomato *PMR4* gene resulted in mutants with reduced but not complete loss of susceptibility to the PM pathogen *On.* Our study demonstrates the efficiency and versatility of the CRISPR/Cas9 system as a powerful tool to study and characterize *S*-genes by generating different types of mutations.

Key words: CRISPR/Cas9, targeted mutagenesis, *PMR4*, Powdery Mildew, Susceptibility gene.

Background

Powdery mildew (PM) in tomato, caused by the obligate biotrophic fungus *Oidium neolycopersici* (*On*), is a world-wide disease that threatens the production of greenhouse- and field-grown tomatoes [1, 2]. Over the last few decades, research focused on breeding for resistance against PM in tomato has resulted in the identification of five dominant resistance (*R*) genes (*Ol*-genes) from wild tomato species [3]. These genes were introgressed into the susceptible tomato cultivar Moneymaker and near-isogenic lines (NILs) were made [4]. Histological studies of these NILs after powdery mildew infection has allowed the identification of two different types of host responses associated with resistance: unicellular hypersensitive response (HR) in *Ol-4* and *Ol-6*, leading to complete resistance against PM; and slow multicellular HR in *Ol-1*, *Ol-3* and *Ol-5*, leading to incomplete resistance [4–6].

In addition to these dominantly-inherited resistance genes a recessive gene named *ol-2* was identified [7]. This is a loss-of-function mutant allele of the powdery mildew susceptibility (*S*) gene, *MILDEW RESISTANCE LOCUS O* (*MLO)* [7]. In homozygous state this *ol-2* allele confers broad-spectrum resistance to different PM species by inducing papilla formation and callose deposition, which block fungal development at penetration stage [6]. The loss-of-function allele of *MLO* is one of the best studied examples of recessively-inherited resistance against PMs [8]. In recent years, many other examples of resistance conferred by the impairment of *S* genes have been described in different pathosystems [9]. The use of such *S* genes in plant breeding, due to their broad-spectrum and potentially durable resistance characteristics, represent a promising alternative to the introgression of *R* genes that has been driving traditional resistance breeding [9–11].

In Arabidopsis, several mutants showing resistance against the adapted PM pathogen *Golovinomyces cichoracearum* have been identified after screening an EMS population [12]. These mutants were named *powdery mildew resistant 1* (*pmr1*) to *pmr4*. The *pmr4* mutant showed resistance not only against *G. cichoracearum*, but also against *G. orontii* and downy mildew *Hyaloperonospora arabidopsidis*. *PMR4* was shown to be the callose synthase gene At4g03550, also known as *GLUCAN SYNTHASE-LIKE 5* (*GSL5)* or *CALLOSE SYNTHASE 12* (*CalS12*) [13, 14]. The *GSL*/*CalS* gene family comprises 12 genes in Arabidopsis [15-17]. The callose synthase encoded by *PMR4* is responsible for the production of callose in response to biotic and abiotic stresses [14]. Both the *pmr4* and a *gsl5* mutant (homozygous for a T-DNA insertion in the *PMR4* gene) showed almost complete lack of callose in infected leaves [12, 13]. Histological analyses proved that these mutants form papillae after PM infection, even in the absence of callose [13, 14].

It would seem strange that mutant plants lacking callose in the papillae formed at attempted penetration sites show PM resistance, especially because Arabidopsis plants overexpressing *PMR4* show complete penetration resistance against *G. cichoracearum* [18, 19]. This latter resistance is based on increased callose

deposition at infection sites, which acts as a physical barrier against PM-secreted cell wall hydrolases [19].
However, the *pmr4* mutant develops lesions reminiscent of hypersensitive cell death after PM infection [14]. This cell death likely results from activation of the salicylic acid (SA) signal transduction pathway as genes in this pathway were upregulated in the *pmr4* mutant compared to the wild type control, and blocking the SA pathway in the *pmr4* mutant (using double mutants) was enough to restore full susceptibility to PM [14]. Probably, PMR4 is not only involved in callose synthesis at specific sites after attempted fungal penetration, but also negatively controls the SA-associated defense pathway [20]. In plants overexpressing *PMR4* the SA pathway is not induced [18] . Thus, different mechanisms are involved in resistance by overexpressing or knocking-out the *PMR4* gene.
To investigate whether the PMR4 function is conserved in other plants species than Arabidopsis, the closest tomato ortholog of *PMR4* (Solyc07g053980; *SlPMR4*) was silenced by RNAi [21]. Transgenic plants in which the gene was well-silenced showed enhanced resistance against the tomato PM pathogen (*On*) and a slight reduction in plant size when compared to the non-silenced controls [21]. These RNAi plants still showed a low level of expression of the *SlPMR4* gene. Knock-down-based methods for characterization of *S* genes, such as RNAi or virus-induced gene silencing (VIGS), usually result in residual expression of the targeted genes, which typically causes partial phenotypes complicating functional analysis of the genes [22]. Mutations resulting in full knock-out of gene function sometimes produce a stronger phenotype than knock-down transgenic plants obtained by RNAi silencing [23, 24] . We set out to produce tomato *SlPMR4* mutants to investigate whether complete resistance against *On* could be obtained in this way, compared with the substantial but not complete resistance in the RNAi transgenic plants [21] . For this, targeted mutagenesis is the preferred method. The generation of precise, stable and heritable knock-out alleles of *S* genes is now possible with the development of the clustered regularly interspaced short palindromic repeats (CRISPR) technology. This technology has already been used to generate PM-resistant *slmlo1* tomato mutants [25], and PM-resistant wheat by the simultaneous modification of the three *EDR1* homologues [26]. In addition, host resistance in other pathosystems has been achieved using CRISPR-induced targeted mutation [27, 28]. In this study, we report the successful generation and characterization of five different mutation events via CRISPR/Cas9 in the tomato ortholog of the *PMR4* gene. Further, we show that PM resistance in these *pmr4* mutants is associated with cell death upon PM infection.

Results

CRISPR/Cas-9-targeted mutagenesis of *SlPMR4*

To produce mutants of the tomato *PMR4* ortholog (Solyc07g053980) [21] a single CRISPR/Cas-9 construct containing four sgRNAs (sgRNA6, sgRNA8, sgRNA1 and sgRNA7; Supplementary Table 1) was made. We used four sgRNAs in order to

increase the chances of obtaining mutants with large deletions [29, 30] . The position of the four sgRNAs in the *SlPMR4* genomic sequence and in the predicted SlPMR4 protein can be seen in Fig. 1. *PMR4* contains two known protein domains according to NCBI Conserved Domains (https://www.ncbi.nlm.nih.gov/cdd/): FKS1dom1 [31] and the Glucan-synthase domain [32]. One of the four sgRNAs (sgRNA6) targets the FKS1dom1 domain in the N-terminal region. The three remaining sgRNAs target the intracellular part of the Glucan-synthase domain. The *SlPMR4* CRISPR/Cas9 construct was used to transform the susceptible tomato cultivar Moneymaker (MM).
In total, 37 primary transformants (T1) were obtained and analyzed. Individual T1 plants containing mutations within the *SlPMR4* gene were selected via PCR amplification. Two different primer combinations were used: primers Fw519+Rv1925 flanking sgRNA6 and primers Fw2969+Rv4230 flanking sgRNA8, sgRNA1 and sgRNA7 (Fig. 1). When a large deletion has occurred smaller PCR products than the expected ones from wild-type (WT) controls will be visible on an agarose gel. Additionally, the sgRNA6 target site plus PAM contains the recognition sequence for restriction enzyme *Xcm*I (CCANNNNN↓NNNNTGG). When a small indel has occurred within this target site the PCR product will not be digested by *Xcm*I.

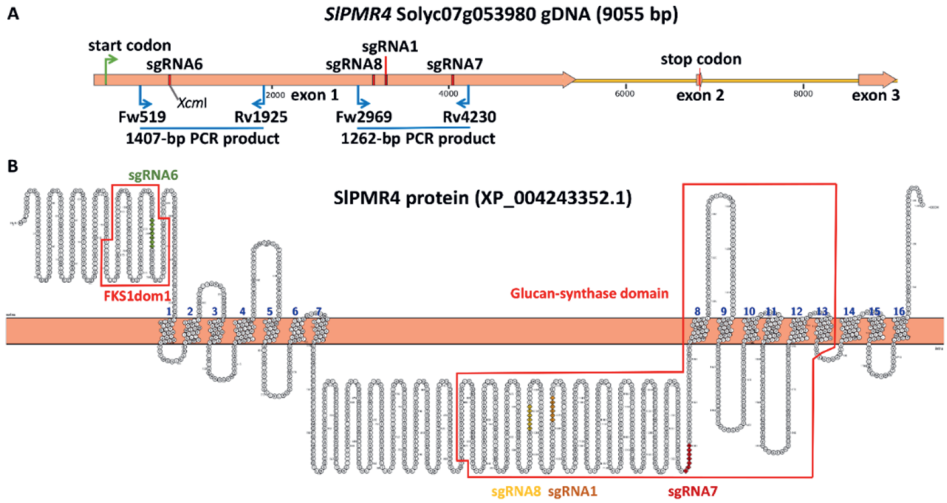

Figure 1. Position of target sites of the sgRNAs in *SlPMR4*. A. Representation of genomic sequence of *SlPMR4* showing the position of four single guide RNAs (sgRNAs) that were designed to produce knock-out mutants of *SlPMR4*. PCR primers Fw519+Rv1925 and Fw2969+Rv4230 were used to identify mutants. **B.** PROTTER [53] representation of the SlPMR4 protein. sgRNA6 (green) is located in the N-terminal FKS1dom1 domain of the protein; sgRNA8 (yellow), sgRNA1 (orange) and sgRNA7 (red) target sites in the intracellular part of the Glucan-synthase domain.

The PCR with primers Fw519+Rv1925 flanking sgRNA6 (Fig. 1) did not yield PCR products obviously smaller than the 1,407-bp WT PCR product in any of the primary transformants, indicating no large deletions had occurred in this region. In contrast,

PCR with primers Fw2969+Rv4230 flanking the three other sgRNAs resulted in the selection of 17 T1 transformants with large deletions compared to the 1,262-bp PCR product of the WT allele. These were subsequently selfed to produce T2 progeny. T2 progenies were analyzed by PCR and individual T2 plants homozygous for the mutation were selected to produce T3 progenies to facilitate further characterization of the mutations (results shown in the next section).
To analyze whether any of the selected T2 or T3 mutants contained a small indel at the sgRNA6 target site PCR products obtained with primers Fw519+Rv1925 were digested with *Xcm*I. For all tested plants digestion was complete, suggesting that no mutation had occurred at the sgRNA6 target site. To be sure none of the mutants contained a mutation in the sgRNA6 region the PCR products were sequenced. No differences with the WT allele were observed. These results indicate that sgRNA6 in the FKS1dom1 domain was not effective in producing mutations, whereas one or more of the three sgRNAs in the Glucan-synthase domain were successful.

Characterization of CRISPR/Cas-9-mediated mutants in *SlPMR4*

We characterized the mutation events in T2 and T3 progenies derived from the original transgenic T1 plants (Table 1). From segregating T2 families plants homozygous for potential mutant alleles were selected that showed size differences in the PCR amplified products (using primers Fw2969+Rv4230) when compared to the WT allele. In addition, homozygous T3 lines were obtained and the presence of mutant alleles was verified by repeating the PCR amplification (Fig.2).

Table 1. Overview of the mutation events in the tomato *slpmr4* CRISPR mutants.

Event	Mutation	T2 plants	T3 lines
1	900-bp deletion	TV171009-L	TV171365
		TV171030-L	TV171366
			TV171355
2	5-bp deletion and 277-bp deletion	TV161212-U	TV171358
		TV171033-U	
3	895-bp deletion	TV161212-L	TV171356
		TV171033-L	
4	902-bp deletion and T insertion	TV171010	TV171370
			TV171371
5	4-bp deletion and 892-bp inversion	TV171009-U	TV171367
		TV171030-U	TV171368
			TV171359

For each mutation event the mutation is described and the homozygous plants (T2) and homozygous lines (T3) are listed.
L, lower band of PCR products in heterozygous plants; U, upper band of PCR products in heterozygous plants.

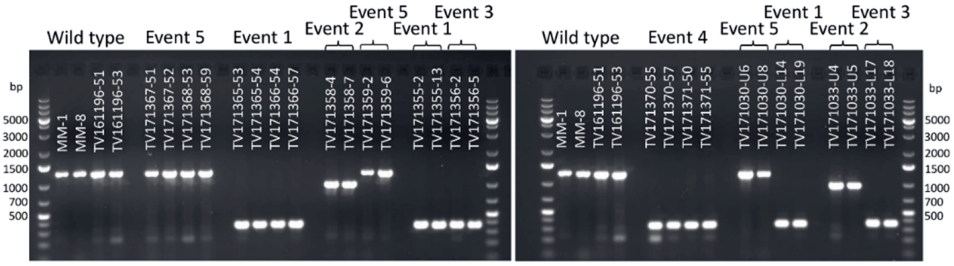

Figure 2. PCR amplification of the CRISPR tomato mutants. Selection of the mutants was done by amplifying the region containing sgRNAs 8, 1 and 7 using flanking primers Fw2969 and Rv4230 (Fig.1). A 1262-bp wild-type allele was amplified in Moneymaker (MM) and transformant TV161196 (non-mutant). Smaller fragments than the wild-type allele indicate deletions between sgRNAs. Different mutation events are indicated and sequence details of events 1 to 5 are given in Figure 3.

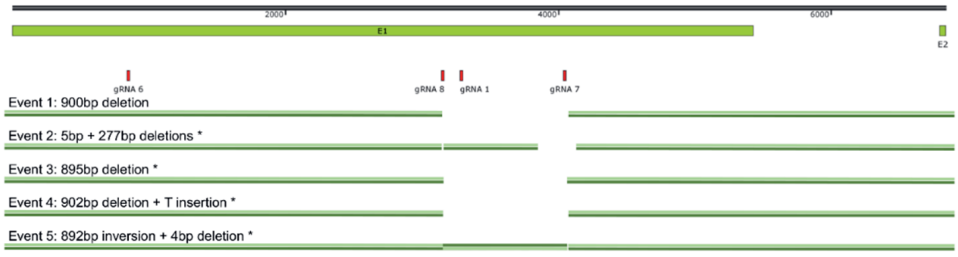

Figure 3. Schematic representation of the mutation events in *SlPMR4* mutants. Two exons (E1 and E2) of the *SlPMR4* gene are shown. The positions of the four sgRNAs are shown in red. The mutation for each event is represented at the genomic level. Deletions ranging from 4-bp to 902-bp were observed. Additionally, a 892-bp inversion is present in the event 5 mutants. * indicates the presence of premature translation termination codons (PTTCs) in the predicted protein.

By sequencing of PCR fragments we identified five different mutation events ("events") in the mutant lines (Fig. 3, Table 1 and Supplementary Document 1). Event 1 contains a 900-bp in-frame deletion between sgRNA8 and sgRNA7. Event 2 shows deletions of 5 bp at sgRNA8 and 277 bp at sgRNA7. Event 3 carries a 895-bp deletion between sgRNA8 and sgRNA7. Event 4 has a 902-bp deletion between sgRNA8 and sgRNA7 and the insertion of a T at the site of the mutation. Event 5 is a special case that has a 4-bp deletion and a 892-bp inversion next to the deletion between sgRNA8 and sgRNA7. With the exception of event 1, all the mutation events are predicted to

generate premature translation termination codons (PTTCs) in the transcript (Supplementary figure 1). The predicted protein of event 1 is lacking 300 amino acids in the Glucan-synthase domain.
Phenotypically, the CRISPR/Cas9 *pmr4* mutants were similar to non-transformed MM. However, they displayed a slight reduction in size when compared to MM and WT allele-carrying transformants. An exception to this is line TV171370 that showed segregation of plants with a dwarf phenotype (three out of eight T3 progeny). To investigate the cause of this dwarf phenotype a PCR was performed to check for the presence of the Cas9 sequence and possible association with T-DNA integration site. All eight plants carried the Cas9 gene; therefore no association was found with T-DNA integration. Next, the sgRNA6 target site was investigated by sequencing of the Fw519+Rv1925 PCR product. No additional mutation(s) were observed in the dwarf plants.
One of the criteria for selection of the sgRNAs was a minimal chance of off-targets (unintended mutations elsewhere in the genome instead of *SlPMR4*). When using the Cas-OFFinder program [33] with a threshold of 3 or less mismatches between sgRNA and (off-) target sequence no putative off-targets were predicted for sgRNA 6, one for sgRNA8 (in an intergenic region), one for sgRNA1 (in an intron of gene Solyc09g009800) and three for sgRNA7 (Supplementary table 2). These off-targets all contain 3 mismatches, most of which are present in the seed of the sgRNA. From the three off-targets of sgRNA7 two are in intergenic regions and one in the coding sequence of gene Solyc02g078230. This gene encodes a callose synthase-like gene designated *SlPMR4*-h2 [21]. Because of this we investigated whether any mutation had occurred in the *SlPMR4*-h2 gene in our selected *SlPMR4*(-h1) mutants. For this, a PCR was performed using primers PMR4_h2_Fw1 and PMR4_h2_Rv1 yielding a 765-bp PCR product containing the putative off-target site of sgRNA7. In total, PCR products of 36 individual T2 and T3 mutant plants and 6 control plants (MM and WT-allele carrying transgenic plants) were sequenced. No sequence differences were found between control and mutant (including the three dwarf) plants, indicating no off-target mutation had occurred in the *SlPMR4*-h2 gene.

Resistance to powdery mildew in slpmr4 mutants

A previous study with RNAi lines showed that the knock-down of *SlPMR4* enhances resistance against powdery mildew [21]. To evaluate whether our *slpmr4* mutant lines showed increased or full resistance against PM, we inoculated them with *On* to assess the disease index (Fig.4A and B). Additionally, we quantified the disease severity by measuring the relative *On* biomass in the mutants to confirm the phenotypic observations (Fig. 4C). Two unsuccessful transgenic lines (TV161196 and TV161209) carrying the WT allele were used as controls. No significant differences in the disease index or the relative fungal biomass were observed among the mutants. However, all the mutants displayed reduced susceptibility compared to the controls as indicated by a lower disease index and significantly lower fungal biomass (Fig. 4, Supplementary figure 2).

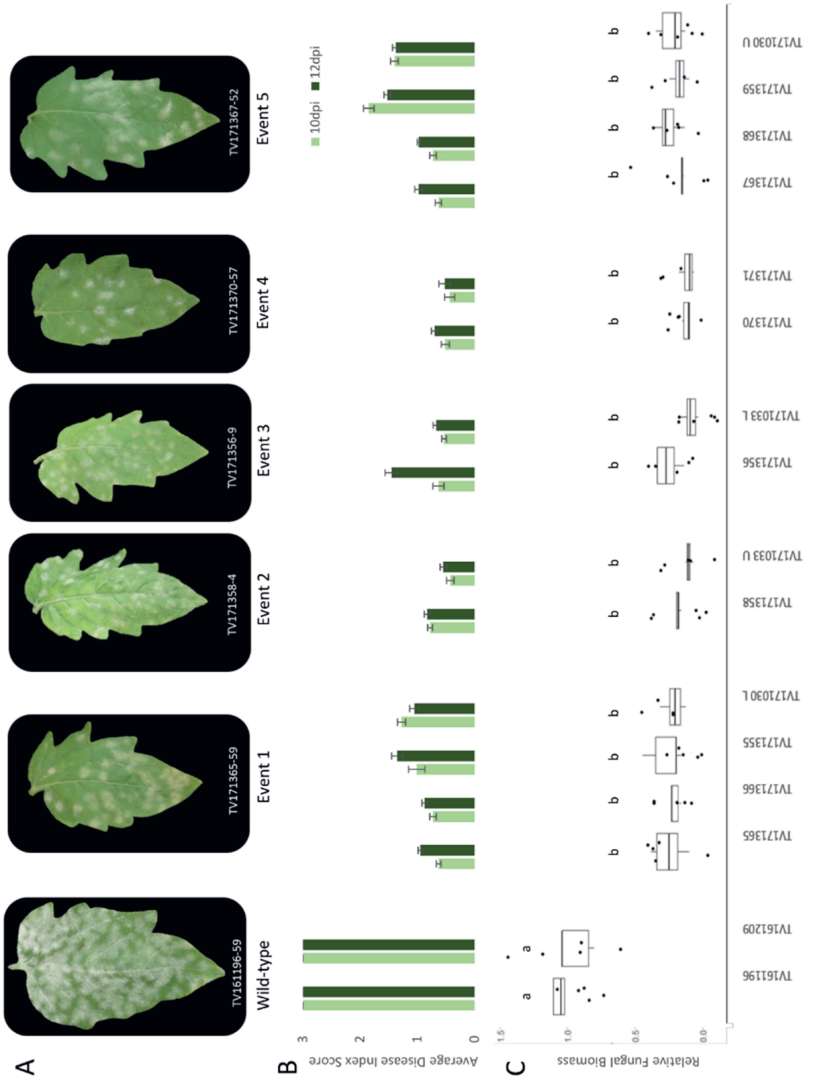

Figure 4. Phenotypic response of the *pmr4* mutants to infection with *Oidium neolycopersici*. **A**. Powdery mildew symptoms observed on the leaves of wild-type plants and mutants (one genotype is given from each of the five mutation events). Photos were taken at 21 days post inoculation (dpi). **B**. Average disease index score of the mutant lines at 10 and 12 dpi. **C**. Relative fungal biomass quantification on at least three individual plants of the mutant lines. This is calculated as the ratio of fungal ITS gene amplification in comparison with tomato EF1a and normalized with the values of the wild-type genotype TV161196. Significant differences were observed between the different mutation events and the control lines (Tukey HSD test, $\alpha = 0.05$).

Histological analysis of the infection sites of Arabidopsis *pmr4* mutants revealed the presence of hypersensitive response (HR)-like cell death [14]. To investigate whether this phenomenon could also be observed in our *slpmr4* mutants, we performed histological studies using heavily infected leaf samples of 21 plants representing all five mutation events in addition to wild-type MM plants. The samples were taken at 44 hours post infection (hpi) and stained with trypan blue. Fungal structures and plant cell death were quantified in both mutant and wild-type plants (Table 2). No papillae were observed in any of the samples. HR-like cell death was visible at a much higher percentage of the infection sites in all *pmr4* mutants compared to the wild-type allele-carrying MM (Table 2 and Fig. 5). Simultaneously, the percentage of primary haustoria and the number of hyphae per infection unit was decreased in the mutants compared to MM. These results corroborate the finding that tomato *pmr4* mutants show reduced susceptibility to *On*, but not complete resistance.

Table 2. Histological study of *Oidium neolycopersici* growth.

Genotype	*SlPMR4* mutation	Mutation event	Primary AP per IU (%)	Per AP		# Hyphae per IU (%)			
				Primary HS (%)	Primary HR (%)	1	2	3	4
MM	wild type	none	100	80	22	20	8	30	42
TV171009	bi-allelic	1+5	88	11.3*	81.8	90	10	0	0
TV171010	homozygous	4	96	47.9	91.6*	56	16	26	2
TV161212	bi-allelic	2+3	92	36.9	84.7*	68	16	12	4

Development of *Oidium neolycopersici* on the susceptible genotype Moneymaker (MM) and three *slpmr4* CRISPR mutant lines carrying different mutation events.
Asterisks represent statistically significant differences between the mutant genotypes and cultivar MM as calculated by a t-test. *: $p<0.05$
IU, infection unit; AP, appressorium; HS, haustorium; HR, hypersensitive response-like cell death

In Arabidopsis it was shown that PM resistance in the *pmr4* mutant is associated with an activation of the SA signal transduction pathway [14]. To analyze whether the SA pathway is also activated in our *slpmr4* mutants expression level of the tomato *PR1* gene 44 hours after PM infection was determined by qPCR. Infected leaf samples were taken from individual homozygous mutant T2 plants representing all five mutation events and control plants (MM and unsuccessful transgenic lines TV161196 and TV161209). Disease index scores of this experiment are shown in Fig. 6A and relative *PR1* gene expression in Fig. 6B. Plants from all five mutation events show reduced susceptibility (but not complete resistance) to PM compared to the control lines (Fig. 6A). All mutant plants showed a significant increase in *PR1* gene expression at 44 hours post infection (hpi) compared with the non-mutant controls (Fig. 6B).

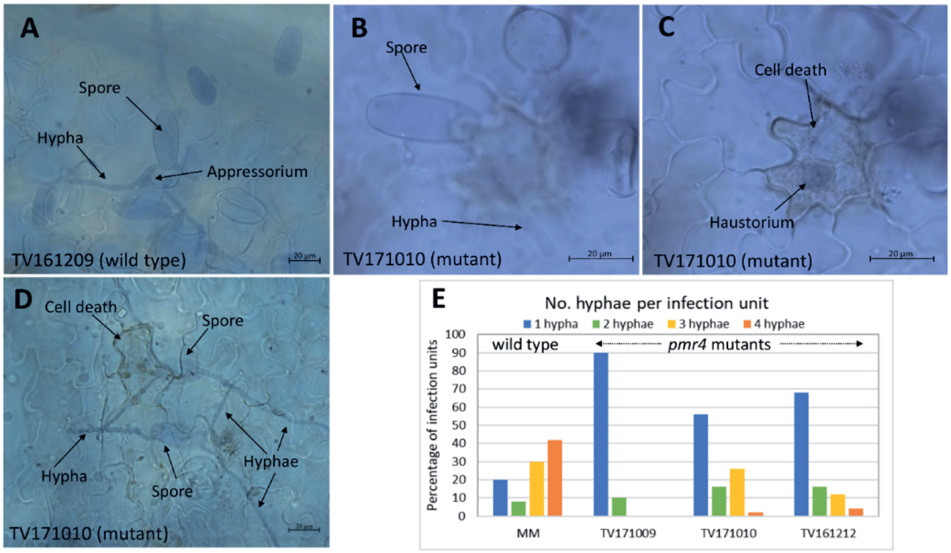

Figure 5. Microscopic observations on powdery mildew infection at 44 hours post inoculation (hpi). A. In the wild-type allele-carrying plant (TV161209) a normal development of the spores occurs; appressorium and hyphae are developed. **B,C.** In the mutant plant (TV171010; event 4) cell death is observed in epidermal cells invaded by the fungus. **C** shows a deeper focal plane involving the same infection unit as in **B;** a haustorium is present in the cell showing cell death. **D.** Both cell death and hyphal growth in mutant plant TV171010. **E.** Comparison of number of hyphae per infection unit between wild type and *pmr4* mutant plants.

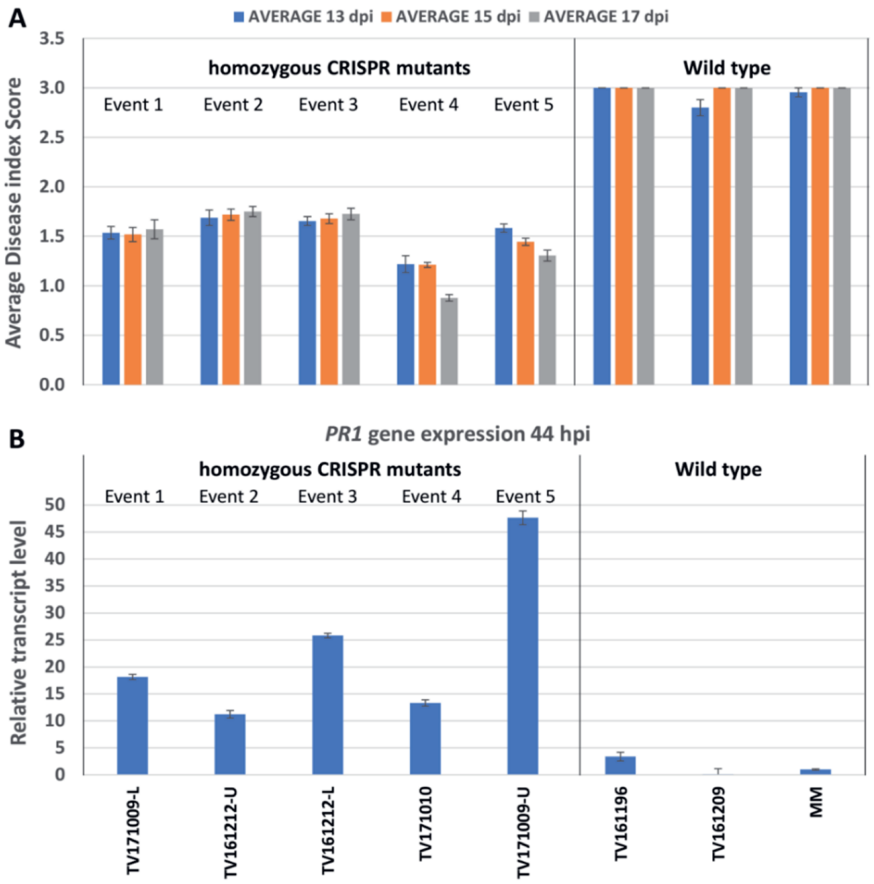

Figure 6. PM-resistance level and *PR1* gene expression of *slpmr4* mutant lines. A. Average disease index score of mutant and control lines at 13, 15 and 17 days post inoculation (dpi). **B.** Relative *PR1* gene expression in leaf samples of mutant and control lines 44 hours post inoculation (hpi).

Discussion

Effectiveness of multiple guide RNAs to obtain knock-out mutants.

The use of loss-of-function alleles of *S*-genes in plant breeding is a promising alternative to the traditional *R*-gene-based introgression breeding because of its durable and broad-spectrum characteristics [10]. In this study, we successfully produced CRISPR/Cas-9-mediated knock-outs of the susceptibility gene *PMR4* in tomato against the PM pathogen *On*. Our results showed that the use of four sgRNAs for CRISPR-induced mutation and selection using PCR amplification to screen for visible (large) deletions was efficient to obtain the five described mutation events. Analysis of the different target sites indicated a difference in effectiveness of the four

sgRNAs although all four sgRNAs were selected based on the same stringent criteria such as GC content, secondary structure and base pairing scores (Supplementary table 1). No mutations were found close to the sgRNA6 target site, thus it was not efficient in guiding the Cas9 protein to induce double-stranded breaks. However, all five described mutation events seem to be the result of double-stranded breaks at target sites of both sgRNA8 and sgRNA7, as deletions were found at or between these positions. Therefore, sgRNA8 and sgRNA7 seem to be highly effective. We cannot judge whether sgRNA1 was effective, as the target site of this sgRNA is positioned between those of sgRNA8 and sgRNA7, and was deleted in three of the five mutation events (Fig. 3). As we focused on the selection of mutants with large deletions in the *SlPMR4* gene we have not checked whether any additional mutants with small indels (insertion or deletions of a few nucleotides) at the targets sites of sgRNA1, 7 or 8 were present among the original primary transformants. Such an analysis would allow a better comparison of the effectiveness of sgRNAs1, 7 and 8.

One of the five mutation events we characterized contained a 892-bp inversion, showing that chromosome re-arrangement can occur by using CRISPR/Cas9 technology. In agreement with our results, genome editing via the CRISPR/Cas9 system has recently been reported to produce inversions between sgRNAs [34, 35]. Induction of inversions are of particular interest in plant breeding due to its potential to allow the fixing or breaking of linkages [35].

Putative pleiotropic effects of *SlPMR4* mutation

Similar to the observation on transgenic plants in which *SlPMR4* (Solyc07g053980) was silenced by RNAi [21], a slight reduction in plant size was observed in the CRISPR/Cas9 *slpmr4* mutants. This may be due to an elevated SA level in the mutants. Constitutively high levels of SA can result in growth impairment in Arabidopsis [36, 37]. Although we did not measure SA levels in our mutant lines, expression of the *PR1* gene (as an indicator of SA levels) was increased in PM-infected leaves of the *slpmr4* mutants compared to controls containing WT alleles of *SlPMR4*.

Three out of eight individuals of T2 family TV171370 showed a dwarf phenotype. We tried to identify the cause for this dwarf phenotype. None of the plants showed additional mutations at the sgRNA6 target site of *SlPMR4* or at the possible off-target site in Solyc02g078230 (*SlPMR4*-h2). Furthermore, all eight plants were positive for the Cas9 gene. Therefore, no obvious cause for the occurrence of dwarf plants was identified. However, it is still possible that the site of T-DNA integration plays a role. When the T-DNA of the CRISPR/Cas9 construct has integrated within a gene influencing plant growth, dwarf plants are obtained only when the T-DNA insertion is present in homozygous state. Another possibility is that multiple integrations of the T-DNA had occurred in the parental T1 plant of TV171370, one of which would be in a crucial gene for plant development.

As dwarf plants were only found in one T2 family and not in any of the others this phenotype does not seem to be caused by mutation of the *SlPMR4* gene. The possible pleiotropic effects in loss-of-susceptibility lines have been discussed as a

setback to the deployment of *S*-genes in plant breeding [9]. Clearly, further phenotypic analyses in standard greenhouse conditions and yield estimation should determine whether the gain in resistance of *pmr4* tomato plants also carries a fitness cost in the plants.

Resistance to PM in knock-out *SlPMR4* plants

We aimed to investigate whether the full knock-out of *SlPMR4* would result in a higher level of resistance than RNAi-silenced transgenic plants [21] . In this study we have shown that our *slpmr4* mutants in the susceptible cultivar Moneymaker background displayed enhanced resistance to tomato PM compared to control plants containing WT alleles of *SlPMR4*, but not complete resistance. We evaluated the plants for disease index at 10-17 dpi (Figs. 4 and 6) and determined fungal biomass at 21 dpi. It could be argued that susceptibility in the mutants is not reduced but delayed. However, we kept the inoculated *pmr4* mutants till 30-35 dpi and still the plants showed less infection than the WT control plants. Therefore, the *pmr4* mutants show reduced susceptibility to PM.

In agreement with previous observations in Arabidopsis *pmr4* mutants [14] we observed a higher occurrence of HR-like cell death at infection sites in tomato CRISPR *pmr4* mutants compared to wild type plants. This increased HR likely resulted from activation of the SA signaling pathway, as *PR1* gene expression was significantly increased in the *pmr4* mutants compared to the controls. Still, this cell death did not completely block fungal growth in the tomato *pmr4* mutants. In barley, RNAi was used to downregulate gene *HvGsl6*, which is the closest ortholog of *AtGSL5* (*PMR4*) [38]. As expected, this resulted in a lower accumulation of papillary and wound callose. However, contrary to what has been found in Arabidopsis and tomato, silencing of *HvGsl6* led to a higher susceptibility of barley to PM *Blumeria graminis* f. sp. *hordei* compared with control lines. It was found that silencing of *HvGsl6* does not lead to activation of the SA-dependent defense pathway. Whether reduced susceptibility to powdery mildew species in *pmr4*/*gsl5* mutants is a plant species-specific phenomenon remains to be investigated. We have not been able to check for presence of callose at the cell wall near the sites of fungal penetration in the CRISPR mutants, although we would expect absence or a lower level of callose compared to wild-type plants, depending on redundancy of callose synthase genes. In order to verify whether the PM resistance observed in the tomato *pmr4* mutants is callose-independent, quantification of callose deposition should be included in future experiments with these mutants after PM attack.

In tomato an additional possible ortholog of *PMR4*, designated *SlPMR4*-h2 (Solyc02g078230), has been found [21]. This gene is the closest tomato ortholog to Arabidopsis gene At4g04970 [21], also known as *GSL1*/*CalS11*. Both Solyc07g053980 (*GSL5*/*CalS12*-like) and Solyc02g078230 (*GSL1*/*CalS11*-like) are reported to function in callose formation during pathogen infection [39]. It would be interesting to investigate whether these genes show functional redundancy in relation to PM resistance/susceptibility, and thus whether knocking out both genes simultaneously would result in higher resistance to PM. However, GSL1 and GSL5 also play

redundant roles in plant development [15] . In Arabidopsis, a *gsl5* mutant with one mutant allele of *GSL1* is severely stunted and shows highly reduced fertility, and double mutants could not be obtained [15].

Perspectives for slpmr4 mutants in breeding for resistance against PM

We have transformed susceptible tomato cultivar Moneymaker with the *SlPMR4* CRISPR construct and observed reduced susceptibility in the obtained mutants associated with the activation of the SA signaling pathway. It would be interesting to know what effect the *slpmr4* mutation would have in different genetic backgrounds, and especially in resistant tomato backgrounds, with the aim to pyramid different resistance genes. As resistance conferred by the *pmr4* mutation is associated with an elevated SA pathway defense response we expect that PM resistance in tomato lines carrying *Ol-4* or *Ol-6* would not be influenced by mutation of the *SlPMR4* gene. *Ol-4* and *Ol-6* encode NB-LRR type resistance genes [40] and lines containing these genes show a fast HR after PM infection. This strong resistance is not expected to become even stronger because of further elevation of SA levels caused by the *pmr4* mutation. Resistance genes *Ol-1*, *Ol-3* and *Ol-5* are not cloned yet, but lines containing these genes show a slow HR response. Possibly, combining one of these genes with the *pmr4* mutation might result in a stronger or faster HR response. PM resistance conferred by the recessive gene *ol-2*, containing a mutation in the *SlMlo1* gene, is associated with formation of papillae and increased callose deposition. It would be interesting to analyze the PM resistance level in double mutants *slmlo1 slpmr4*. The resistance conferred by *ol-2* might be compromised when the callose synthase gene *SlPMR4* is mutated and there is no redundancy with another callose synthase gene (e.g. *SlPMR4*-h2). However, in Arabidopsis the *atmlo2 atpmr4* double mutant displayed the same level of resistance to PM *G. orontii* as the single *atmlo2* mutant [41]. Therefore, the *atmlo2* resistance was independent of PMR4-mediated callose deposition [41]. Whether the same holds true in tomato and the *slmlo1 slpmr4* double mutant would still show the same level of resistance as the single *slmlo1* (*ol-2*) mutant remains to be tested.

Conclusions

The use of *S*-genes in plant breeding stands as a promising alternative due to its durable and broad-spectrum characteristics. In this study, we used CRISPR/Cas-9 targeted mutagenesis to knock-out the *S*-gene *PMR4* in tomato. We characterized five different mutation events and confirmed the reduced susceptibility of the mutant lines against *On.* Our study demonstrates the efficiency and versatility of the CRISPR/Cas9 system as a powerful tool to study and characterize *S*-genes.

Methods

Design of sgRNAs and transformation

The full-length CDS of the tomato *PMR4* homolog (Solyc07g053980) was retrieved from the Sol Genomics Network database [42]. Four single guide RNAs (sgRNAs) targeting the gene were selected using the guidelines described by Liang *et al.* [34] (Supplementary Table 1). A first list of gRNAs was generated using the CC-Top CRISPR/Cas9 Target Prediction Tool [43]. The G + C content of the sgRNAs was calculated using the ENDMEMO webtool (http://www.endmemo.com/bio/gc.php). The folding of the gRNAs was predicted using the Mfold web server [44]. Additionally, the activity of the gRNAs was predicted using the sgRNA scorer [45]. Four sgRNAs were selected (sgRNA1:TTAAAGCAGTCCCATACTCG, sgRNA6: GTACTGCCCCACACTCTGCG, sgRNA7: GCCAAGGTTGCCAGTGGCAA, and sgRNA8: GGATATCAGAGAAGGATCAG) for transformation. The analysis of the location of the sgRNAs and topology of the predicted protein was made using a set of twelve plasmids obtained from Addgene was used to build the construct used for transformation: pICH86966 (as template for amplification); pICSL01009 (as level 0 plasmid); pICH47751, pICH47761, pICH47772, pICH47781 and pICH47732 (as level 1 plasmids); pICH41766, pICH41780 and pICH41822 (as linkers); and pAGM4723 (as level 2 binary vector) (Supplementary figure 3). The plasmids were cloned using *E. coli* DH5α and transformed to Agrobacterium strain AGL1. Susceptible tomato cultivar Moneymaker (from WUR-Plant Breeding seed collection) was used for transformation according to the method described in [46] according to Dutch legislation under GMO licence 01-135.

PCR-based selection of slpmr4 mutants and characterization of mutation events

Selection of plants carrying deletions in *SlPMR4* was done by analyses of PCR products flanking the sgRNA target regions. PCR using primers Fw519 (5'-TGGTGCTCTTTTCTCGGTCT-3') and Rv1925 (5'-CAACTGCTCTTCTGGCATCA-3') yields a 1,407-bp product flanking the sgRNA6 target for the WT allele, and PCR with primers Fw2969 (5'-GCGAATGCGTAGAGAAGGAA-3') and Rv4230 (5'-CCCCACTAAGTGCCAGGTAA-3') yields a 1,262-bp PCR product flanking sgRNAs8, 1 and 7 for the WT allele. Smaller sizes of the amplified fragments in transgenic plants compared to the WT allele indicated deletions in the targeted region. The sgRNA6 target site together with the PAM site contains the recognition sequence for restriction enzyme *Xcm*I. Fw519+Rv1925 PCR products were digested with this enzyme (New England Biolabs) yielding 1069-bp and 338-bp fragments for the WT *SlPMR4* allele. The PCR products were sequenced to further characterize the mutation events. Primary transformants (T1) carrying mutant alleles were selected using these methods and selfed to produce T2 progeny. Homozygous plants from two T2 bi-allelic lines (TV171030 and TV171033) were selected for use in the disease assay. Homozygous mutant T2 plants derived from other mono-or bi-allelic T1

transformants were selected and selfed to obtain T3 progeny. These included TV161212-U, TV161212-L, TV171009-U, TV171009-L and TV171010 (where U stands for upper band of PCR products in the agarose gel, and L for lower band). Plants from the individual T2 plants and T3 mutant lines (TV171367, TV171368, TV171359, TV171365, TV171366, TV171355, TV171358, TV171356, TV171370 and TV171371) derived from the selfing of previously selected T2 plants, were also tested in the disease assay.

Off-target analysis

The program Cas-OFFinder [33] at http://www.rgenome.net/cas-offinder/ was used to check for possible off-targets of the four sgRNAs of *SlPMR4*. Mismatch number was set at 3 or less. To analyze possible off-target mutations in gene Solyc02g078230 (*SlPMR4*-h2) a PCR was performed using primers PMR4_h2_Fw1 (5'-AACGTGTTCTTGCCGATCCTC-3') and PMR4_h2_Rv1 (5'-CAAAGTGGCTGCGAGCATACA-3'), yielding a 765-bp PCR product for the WT *SlPMR4*-h2 allele. PCR products of *slpmr4* mutants were sequenced and compared with WT control sequences.

Disease assay and quantification of relative fungal biomass

Ten plants homozygous for each of the alleles in the T2 lines and ten plants from the homozygous T3 lines were inoculated with the Wageningen University isolate of *On* by spraying four weeks-old plants with a suspension of conidiospores obtained from leaves of infected tomato Moneymaker plants and adjusted to a concentration of $3.5*10^4$ spores per ml. Two transgenic lines (TV161196 and TV161209) obtained from the same CRISPR transformation experiment, but carrying the wild-type allele were used as controls. Inoculated plants were grown at 20 ± 2˚C with 70 ± 15 % relative humidity and day length of 16 h in a greenhouse of Unifarm of Wageningen University & Research, The Netherlands. Disease index scoring was carried out 10 and 12 days after inoculation. Powdery mildew symptoms were scored visually using a scale from 0 to 3 as described by Bai *et al*. [7]. For the quantification of relative fungal biomass, plant and fungal genomic DNA was isolated from infected leaves collected at 21 dpi, using an adapted CTAB protocol [47]. 10 ng of DNA were used as a template for amplification. Relative fungal biomass was quantified by real-time PCR using the primer pairs On-Fw/On-Rev amplifying the internal transcribed spacer sequence (ITS) of *Oidium neolycopersici* [48] and Ef-Fw (5'-GGAACTTGAGAAGGAGCCTAAG-3')/Ef-Rev (5'-CAACACCAACAGCAACAGTCT-3') amplifying tomato reference gene *Elongation Factor 1α* (*Ef1α*) [49]. The $2^{-\Delta\Delta Ct}$ method [50,51] was used to calculate the fold-change of the ratio between fungal and tomato gDNA.

Histological analysis

At least two plants of each line were grown together with the plants in the disease assay described above but were infected using a heavier inoculation of $3*10^5$ spores per ml. Infected leaf samples of 4 cm^2 were collected 44 hours post inoculation, bleached in a 1:3 (v/v) acetic acid/ethanol solution, and stained 48 h later by boiling

in 0.005% trypan blue in lactophenol : ethanol (1:2 v/v) solution for 3-5 min and cleared in a nearly saturated aqueous solution of chloral hydrate (5:2 w/v) as described by [52]. The slides were mounted on glass slides with a 1:1 (v/v) glycerol-water solution. Analysis of the slides was done using a Zeiss Axiophot bright field microscope. For quantification of fungal structures and host cell death 50 infection units (*O. neolycopersici* conidia) were analyzed per genotype, from two slides obtained from two different plants per genotype.

Analysis of PR1 expression

Expression level of the tomato *PR1* gene was measured 44 hpi after infection with PM by qPCR. Infected leaf samples were taken from individual homozygous mutant T2 plants representing all five mutation events and control plants (MM and unsuccessful transgenic lines TV161196 and TV161209). Leaf samples were frozen in liquid nitrogen and stored at -80°C before being grinded into a fine powder using a pestle and mortar. Total RNA isolation was done using the MagMax™ 96 Total RNA Isolation Kit (Qiagen, Germany). cDNA synthesis was done using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, U.S.A.). 10 ng of cDNA were used as template for the reaction. Expression levels of *PR1* were measured using primers SlPR1a_Fw (5'-GTGTCCGAGAGGCCAGACTA-3') and SlPR1a_Rev (5'-CATTGTTGCAACGAGCCC GA-3'), and compared to the expression of tomato *Ef1α* reference gene using primers Ef_Fw (5'-ATTGGAAACGGATATGCCCCT-3') and Ef_Rev (5'-TCCTTACCTGAACGCCTGTCA-3') [21].

Availability of data and materials

The sequences obtained by Sanger sequencing in this study are available in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under reference accession numbers MT521499 to MT521504. The data produced for this article are included within the manuscript and additional files, and the raw data are available from the corresponding author on reasonable request.

Author's contributions

MISM was involved in designing experiments, characterizing, selecting and testing the mutants, interpreting the results and drafting the manuscript. VB designed the transformation constructs, generated, tested and did the initial characterization of the mutants. EK and MA were involved in selecting and characterizing the mutation events, expression analysis, histological analysis and carrying out the disease assays. EJ, RGFV and YB conceived the idea for the experiments. AMAW was involved in interpreting the results. AMAW and RGFV critically reviewed the paper. AMAW and YB edited the manuscript. All authors read and approved the final manuscript.

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Additional files

Additional files described in this chapter can be accessed through:
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Supplementary Document 1. Alignment of sequences of PCR products of the tomato *PMR4* CRISPR mutant alleles. A. Sequence alignment of PCR products obtained by using primers Fw2969 and Rv4230 for wild type Moneymaker (MM) and five *PMR4* CRISPR mutation events. Primers and sgRNA1, 7 and 8 are indicated. In red, nucleotides differing form the MM allele are shown. Deletions are indicated by dashes. Event 5 contains a large inversion, indicated in red. **B.** Plot showing the inversion (blue line) of the sequence between sgRNA8 and sgRNA7 in mutation event 5 compared to MM.

Supplementary Figure 1. Alignment of predicted proteins of the tomato *PMR4* CRISPR mutant alleles. Protein sequences are based on DNA sequencing data from the fragments amplified by the region flanked by primers Fw2969 and Rv4230.

Supplementary Figure 2. Panel of phenotypes upon infection with *Oidium neolycopersici.* Leaves from wild-type allele-carrying controls and individual plants of the different *slpmr4* mutation classes are shown. Heavy fungal sporulation is present on the leaves of the wild-type plants, while less infection is seen on the leaves of the mutant plants.

Supplementary Figure 3. Map of the level 2 vector for CRISPR/Cas9 transformation. The NPTII, Cas9, the four sgRNAs and AtU6 promoters are highlighted.

Supplementary Table 1. Characteristics of four selected sgRNAs for *SlPMR4*. sgRNAs were selected using CCTop program. PAM, protospacer adjacent motif.

Supplementary Table 2. *SlPMR4* sgRNAs off-targets. Off-targets with a maximum of three mismatches were found for sgRNA8 and sgRNA7 with Cas-OFFinder [33]. crRNA, sgRNA sequences. DNA, off-target sequences. Mismatches are indicated in red.

Chapter 5

Identification and characterization of candidate genes for *Ol-1*, a dominant gene conferring resistance to *Oidium neolycopersici* in tomato

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Abstract

The dominantly-inherited gene *Ol-1* confers resistance in tomato against the powdery mildew (PM) pathogen *Oidium neolycopersici*. *Ol-1* was identified in *S. habrochaites* G1.1560 and mapped to the long arm of chromosome 6. In the present study we fine-mapped the resistance locus and identified candidate genes. Analysis of the expression profile and virus-induced gene silencing of the candidate genes did not provide indications of their involvement in resistance to PM. However, we performed a second fine-mapping study and used CRISPR/Cas-9 to characterize the candidate genes. We show that targeted mutagenesis of Solyc06g060800, encoding a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, results in increased susceptibility to PM, strongly suggesting that this gene is responsible for the *Ol-1* resistance.

Introduction

In tomato (*Solanum lycopersicum*), powdery mildew (PM) can caused by three different species namely, *Oidium neolycopersici* (*On*), *Oidium lycopersici* and *Leveillula taurica* (Seifi *et al.*, 2014). *On* has been found to be present across the entire world, while *O. lycopersici* has been reported to occur only in Australia (Kiss *et al.*, 2001). *L. taurica* is an endophytic PM pathogen that is also able to infect other Solanaceaous species (Braun *et al.*, 2002; Zheng *et al.*, 2013). In the late 1980's an outbreak of *On* occurred in Europe, which later spread through America and Africa (Braun *et al.*, 2002; Lebeda *et al.*, 2015, 2017). The urgent need to control this disease in tomato prompted the research on the identification of resistance and susceptibility genes, making it the third well-characterized plant system to study the obligate biotrophic interaction between plants and PM after barley and Arabidopsis (Seifi *et al.*, 2014).

Resistance to *On* has been found in several wild accessions of tomato (Lebeda *et al.*, 2014) and, so far, five dominant resistance (*R*) genes (*Ol*-genes) have been identified (Bai *et al.*, 2005; reviewed by Seifi *et al.*, 2014). *Ol-1* was found in *S. habrochaites* G1.1560 (van der Beek *et al.*, 1994) and mapped to the long arm of chromosome 6 (Bai *et al.*, 2005). Further fine-mapping efforts to identify *Ol-1* located it between markers scaff6 and TAG, a region of 71-kb (tomato Heinz SL4.0 genome) lacking any typical *R* genes in the reference genome (Seifi, 2011). *Ol-3* was found in *S. habrochaites* G1.1290, it was mapped to the same chromosomal region as *Ol-1*, and it has been speculated to be an allelic variant of this gene (Huang *et al.*, 2000). *Ol-4* was identified in *S. arcanum* (previously classified as *S. peruvianum*) LA2172 and mapped to the short arm of chromosome 6 (Bai *et al.*, 2004). *Ol-4* belongs to a cluster of *R* genes that includes the NBS-LRR protein-encoding *Mi-1* gene (Seifi *et al.*, 2011), which is known to confer resistance against root-knot nematodes (*Meloidogyne* spp.) (Milligan *et al.*, 1998), potato aphids (*Macrosiphum euphorbiae*) (Rossi *et al.*, 1998), and whiteflies (*Bemisia tabaci*) (Nombela *et al.*, 2003). *Ol-5* was found in *S. habrochaites* PI247087 and mapped to the long arm of chromosome 6, close to the chromosomal region containing *Ol-1* and *Ol-3* (Bai *et al.*, 2005). *Ol-6* was identified in an advanced breeding line of unknown origin, mapped to the short arm of chromosome 6 (Bai *et al.*, 2005) and further identified as a likely allelic variant of *Ol-4* (Seifi *et al.*, 2011). Additionally, three quantitative trait loci (*Ol-qtl*s) have been mapped in *S. neorickii* G1.1601 (Bai *et al.*, 2003). *Ol-qtl1* mapped to the region of chromosome 6 co-localizing with *Ol-1*, *Ol-3* and *Ol-5*. *Ol-qtl2* and *Ol-qtl3* mapped to chromosome 12, close to the *Lv* gene, a dominant gene conferring resistance to *L. taurica*. Furthermore, the recessively-inherited *ol-2* gene, identified in *S. lycopersicum* var. *cerasiforme* LA1230, was mapped to chromosome 4 (Ciccarese *et al.*, 1998) and later cloned and identified as a homologue of the barley *MLO* susceptibility gene (Bai *et al.*, 2008).

Histological characterization of the cellular events underlying the resistance against *On* has allowed the identification of two main types of immune response (Huang *et al.*, 1998; Bai *et al.*, 2005; Li *et al.*, 2007). Firstly, a fast, single-cell hypersensitive response (HR) that occurs in all epidermal cells where a primary fungal appressorium is developed, which results in complete inhibition of fungal development (Bai *et al.*, 2005). This response is observed in plants carrying *Ol-4* and *Ol-6* (Li *et al.*, 2007; Seifi *et al.*, 2012). Secondly, a slow, multicellular HR that leads to an incomplete resistance has been observed in tomato plants carrying *Ol-1*, *Ol-3* and *Ol-5*. This multicellular response is described to occur later in time after spore germination, allowing an initial establishment of fungal haustorium but partially preventing the further development of the pathogen in later stages (Li *et al.*, 2007).The characteristics of this late response have led to the suggestion that *Ol-1* encodes an enhancer of basal defence (Li *et al.*, 2006; Seifi *et al.*, 2014).

Although the *Ol-1* gene has not yet been cloned, a number of studies has been carried out to dissect its associated resistance mechanism. By measuring the expression of marker genes for hormonal pathways in resistant *Ol-1* plants upon *On* inoculation, a slight induction of the salicylic acid (SA) reporter gene *PR-2* was observed at 1 day post-inoculation (dpi) and notably, a late and strong up-regulation of the ethylene pathway marker gene *Chi9* was observed after 5 dpi (Kissoudis *et al.*, 2017). In the same study, the resistance conferred by *Ol-1* was shown to be compromised under salt stress conditions. Furthermore, the *Ol-1* resistance was shown to be severely compromised in plants carrying the *epinastic* (*epi*) recessive mutation. Additionally, a number of genes has been identified to play a role in the *Ol-1*-mediated resistance. Transient silencing, using virus-induced gene silencing (VIGS), of two acetolactate synthase (*ALS*) genes (Gao *et al.*, 2014), or a glutathione S-transferase (*GST*) gene (Pei *et al.*, 2011), in Near-Isogenic Line-*Ol-1* (NIL-*Ol-1*) plants resulted in an increased susceptibility to *On*. Moreover, *Solanum habrochaites Oidium Resistance Required-1* (*ShORR-1*), an ortholog of Solyc06g059860 identified via a cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis (Li *et al.*, 2006, 2007), has been shown to be required for the PM resistance conferred by *Ol-1*, as transient silencing of the *ShORR-1* gene in *S. habrochaites* G1.1560 resulted in increased *On* susceptibility (Zhang *et al.*, 2019). These studies suggest unique molecular components of the *Ol-1*-conferred resistance. In this study, we fine-mapped the candidate *Ol-1* region and made use of VIGS and CRISPR/Cas9 targeted mutagenesis to identify the gene underlying this resistance.

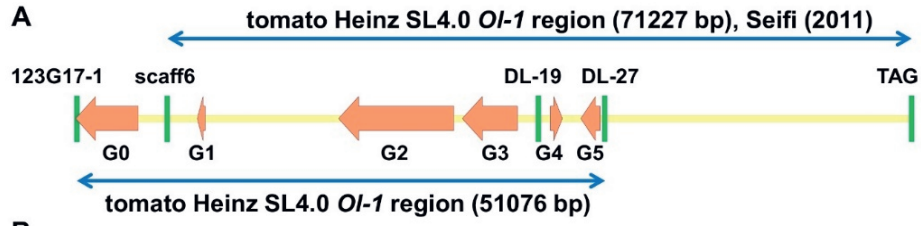
Results

First fine-mapping of the *Ol-1* region

The *Ol-1* region was previously fine-mapped to chromosome 6, in a region spanning 71-kb (tomato Heinz SL4.0 genome) between markers scaff6 and TAG (Seifi, 2011; Fig. 1A, Table S1). In order to further narrow down this region we screened

approximately 4000 plants derived from two segregating F_3 families of Moneymaker (MM) x *S. habrochaites* G1.1560 with markers 123G17-1 and TAG (Table S1) to identify recombinants. New markers were developed in the region between 123G17-1 and TAG (DL-19 and DL-27, Fig. 1A, Table S1). To verify linkage between markers and *On* resistance, progenies from recombinants were preferred. However, these recombinants were unable to produce seeds by selfing. Therefore, they were crossed with MM. Backcross (BC_1) families derived from three resistant recombinants (F_3 plants 152, 212 and 223) were genotyped and phenotyped. All three families showed segregation for *On* resistance (Fig. 1B). Based on the marker scores *Ol-1* was fine-mapped to a 51-kb region (SL4.0ch06: 36472191..36523267) between markers 123G17-1 and DL-27 (Fig. 1A). This region contains six annotated genes (Table 1) in the cultivated tomato Heinz genome, from Solyc06g060790 (G0) till Solyc06g060830 (G5). None of these is a typical NB-LRR type of resistance gene.

A



B

F3 family	Plant #	Phenotype On test	Marker					BC1 progeny	Phenotype On test
			123G17-1	scaff6	DL-19	DL-27	TAG		
PV94084	152	R	h	h	h	b	b	PV123059	segregating
PV94083	212	R	b	h	h	h	h	PV123056	segregating
PV94083	223	R	h	h	h	a	a	PV123057	segregating

Ol-1 region

Figure 1. Fine-mapping of *Ol-1*. **A**. Graphical representation of *Ol-1* region. *Solanum lycopersicum* Heinz region between markers 123G17-1 and TAG is indicated, with markers shown as green vertical bars, and annotated genes as orange arrows. The 71-kb fine-mapped region by Seifi (2011) between markers scaff6 and TAG is shown above. The further fine-mapped 51-kb region between markers 123G17-1 and DL-27 is shown below. **B**. Genotyping results of *On*-resistant recombinant F_3 plants, and phenotyping results of their BC_1 progeny (a: homozygous Moneymaker allele; h: heterozygous; b: homozygous *S. habrochaites* allele).

Table 1. Genes present in the candidate *Ol-1* region.

Gene	Code	Annotation ITAG4.1
Solyc06g060780	G0	Phosphatidylserine decarboxylase
Solyc06g060790	G1	3-isopropylmalate dehydratase small subunit 3
Solyc06g060800	G2	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
Solyc06g060810	G3	Dehydrodolichyl diphosphate synthase complex subunit NUS1
Solyc06g060820	G4	Hydroxyproline-rich glycoprotein family protein
Solyc06g060830	G5	Homeobox-leucine zipper protein HAT2

In order to study the sequence of the fine-mapped region from the *Ol-1* donor *S. habrochaites*, we first performed long-range PCRs spanning the predicted 51-kb region. Sequences were obtained from the region including exon 10 of G2 until G5. This region from *S. habrochaites* was highly syntenic to the cultivated tomato Heinz region and contained orthologs of genes G2, G3, G4 and G5. However, a region between G1 and G2 could not be amplified. Therefore, we developed a Bacterial Artificial Chromosome (BAC) library containing *Hin*dIII genomic fragments from the NIL-Ol-1 BC_3S_5 line PV103045. The library was screened with marker 45 (Table S1) yielding a 447-bp product, located within Solyc06g060790 (G1). A positive BAC clone (BAC23) was identified and sequenced. The 69-kb insert contained the *S. habrochaites* orthologs of genes Solyc06g060750 till Solyc06g060790 (G1), and the last exon (exon 11) plus part of intron 10 of Solyc06g060800 (G2). It included markers 123G17-2, PSD-2, 123G17-1, PSD-1 and scaff6 (Seifi, 2011), besides marker 45. We identified an insertion of 4755 bp in intron 10 of G2 of the NIL-Ol-1 line compared with the sequence of tomato cultivar Heinz. This insertion was found to consist of a retrotransposon. The presence of such a transposon within a gene may influence gene expression level.

Expression profile of the candidate genes

To examine whether the six annotated genes (Table 1) showed a differential response upon PM infection, their relative expression levels were monitored in NIL-Ol-1 and MM during a time course of 0 (before inoculation), 1, 3 and 5 days post inoculation (dpi). Results are shown in Figure 2. Analysis using two-way between groups ANOVA indicated that there was no significant difference in gene expression levels for NIL-Ol-1 and MM for any time point.

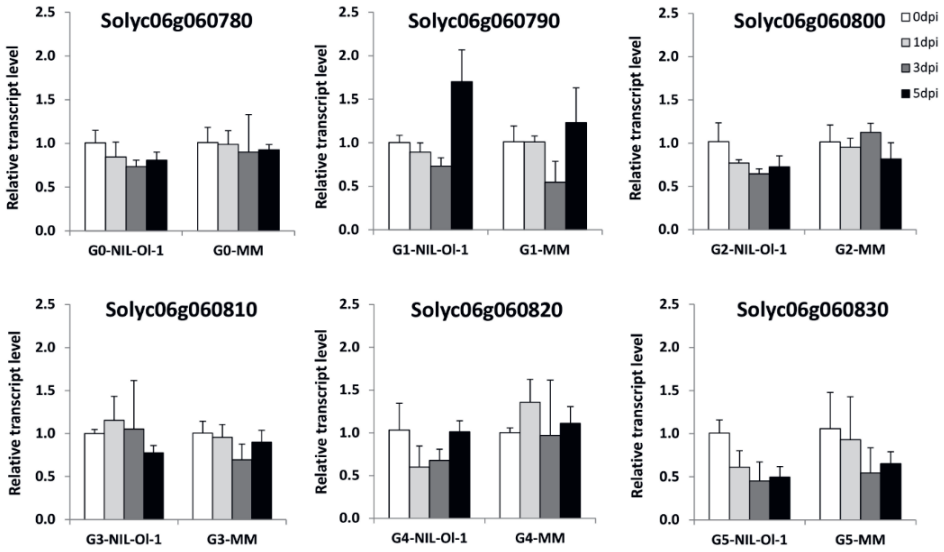


Figure 2. Relative expression levels of six candidate genes (see Table 1) in NIL-*Ol-1* and MM at different time points after *On* infection: 0, 1, 3 and 5 days post inoculation (dpi). Values were normalized relative to the reference gene EF1α, and per genotype expression levels of each gene were calibrated relative to the level at 0 dpi. Error bars indicate standard deviation of three samples. For each sample, third and fourth leaves were pooled from three plants.

Virus-induced gene silencing (VIGS) of candidate genes

To study which of the candidate genes might be involved in resistance against powdery mildew *On*, we used virus-induced gene silencing (VIGS) to transiently silence each of these genes. We designed constructs targeting the candidate genes and used constructs with β-glucuronidase (GUS) and phytoene desaturase (PDS) as controls. After infiltration with Agrobacterium carrying the VIGS constructs, we noticed pleiotropic phenotypes in plants treated with the constructs targeting Solyc06g060790 (Gene 1) and Solyc06g060810 (Gene 3). For Solyc06g060790, yellowing (chlorotic-like) tissue was observed extending from the main nerve to the young leaves that, at later stages, resulted in leaves severely reduced in size (Fig. S1B). For Solyc06g060810, necrotic areas developed and expanded on the leaves and later resulted in stunted plants that lost the younger leaves (Fig. S1B). After infection with *On*, we were unable to accurately score the plants agroinfiltrated with these two constructs, particularly with the construct targeting Solyc06g060810, in which case inoculated leaves had died by the time of the assessment. PM symptoms were scored at 12 and 15 days post-inoculation (dpi) (Fig. S1A). Although significant differences were found for four genes compared to the GUS control in PV033373 (Fig. S1A), these were not corroborated in PV0333375. Therefore, we were unable to identify the *Ol-1* gene using the designed VIGS constructs.

Second fine-mapping of the *Ol-1* region

In order to confirm and further narrow down the candidate region we tested a set of seven recombinant lines in two separate disease assays with *On* (Table 3). BC_1S_1 lines PV133256, PV133277, PV133264, PV133266 originated from the backcross and subsequent selfing used for the first fine-mapping effort (see First fine-mapping of the Ol-1 region), while BC_3S_2 line PV033357, and BC_1S_3 lines PV033369 and PV033371 originated from an earlier MM x *S. habrochaites* G1.1560 cross (Seifi, 2011). We genotyped them using newly developed molecular markers (Table S1) and through PCR-based sequencing (Fig. 3). For the lines in Fig. 3B the marker scores of the parental genotype are shown. Line PV133256 was found to be homozygous for the MM allele (a) at marker 123G17-2, while segregating (h) for Cfr421_rs_0 and markers to the right. This line showed co-segregation of resistance with markers to the right of Cfr421_rs_0. Lines PV133264 and PV133266 were found to be segregating for scaff6 and markers to the left, while being homozygous for the MM allele for marker DL-27. PCR-based sequencing of Solyc06g060830 (G5) (Fig. 3A) in one plant from each of these lines allowed us to pinpoint the recombination event within this gene. The resistance in both lines (PV133264 and PV133266) was found to co-segregate with the markers in the heterozygous region. Lines PV033369 and PV033371 were homozygous for the *S. habrochaites* allele (b) for markers 123G17-2 and KsapAI_rs_f and homozygous for the MM allele for 123G17-1 and markers to the left. All plants from these lines were found to be susceptible. Moreover, the parent of line PV133277 was found to be homozygous for the MM allele for marker scaff6 and heterozygous for marker DL-27. Further PCR-based sequencing in a plant of this line of gene Solyc06g060820 (G4) showed a complete MM-like sequence, while sequencing of Solyc06g060830 (G5) resulted in a complete *S. habrochaites*-like sequence (Fig. 3B). As all plants from this line displayed a susceptible phenotype upon inoculation with *On* (Fig. 3C), we determined the right border of the candidate region at Solyc06g060830 (G5). Additionally, line PV033357 was genotyped to be homozygous for the *S. habrochaites* allele for all markers between and including 123G17-2 and 45, the latter located within gene Solyc06g06790 (G1). PCR-based sequencing in five plants of a fragment at the intergenic region (IG_1_2) between Solyc06g06790 (G1) and Solyc06g06800 (G2) showed allelic segregation at this position. The resistance in this line was found to co-segregate with marker DL-19 (Fig. 3C). Therefore, we were able to determine the left border of the candidate region at marker 45 in Solyc06g06790. Altogether, these data allowed us to establish the candidate region (18.3-kb in tomato Heinz SL4.0 genome) to include the genes Solyc06g06800 (G2), Solyc06g06810 (G3) and Solyc06g06820 (G4).

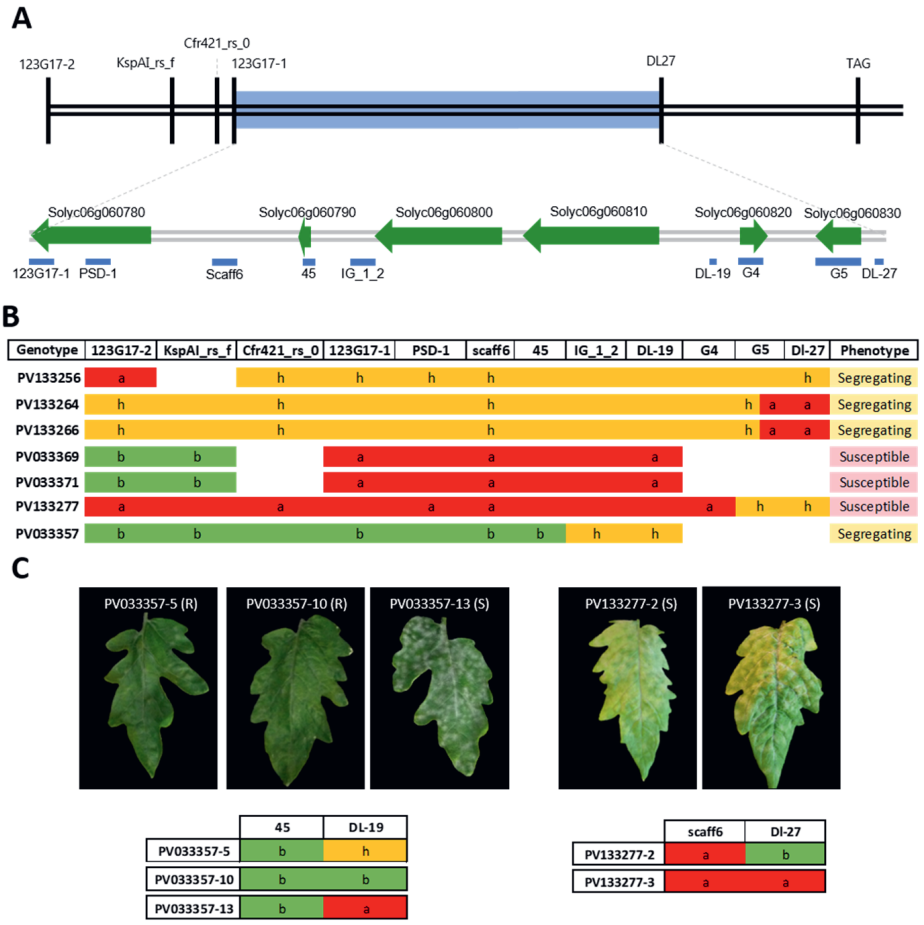


Figure 3. Confirmation of the fine-mapped region. A. Schematic representation of the candidate region . Above: region bordered by markers 123G17-2 and TAG . Below: region bordered by markers 123G17-1 and DL-27 (33.7-kb) containing six genes (green arrows) in the reference genome. Markers used for the confirmation are shown in blue. **B.** Informative recombinant lines. Parental genotype is shown for the markers described in **A**; a: homozygous Moneymaker allele; h: heterozygous; b: homozygous *S. habrochaites* allele), together with phenotypic response of the progeny upon inoculation with *Oidium neolycopersici* (*On*). **C.** Infection on the leaves and genotype of informative plants from lines PV033357 and PV13277. Letter in brackets indicates the resistant (R) and susceptible (S) phenotype upon *On* inoculation.

CRISPR/Cas-9-targeted mutagenesis

Having determined the boundaries of the candidate *Ol-1* region, and knowing that transient silencing yielded no conclusive results regarding compromised *On* resistance, we set out to generate knockout mutants of the candidate genes using

CRISPR/Cas9. Due to the severe phenotypic effect of transient silencing of Solyc06g060810 (G3) (Fig. S1), we decided to exclude this gene from the mutation experiment. Therefore, we designed CRISPR/Cas-9 constructs containing four sgRNAs per gene targeting Solyc06g060800 (G2) and Solyc06g060820 (G4) (Fig. 4). We used the NIL-*Ol-1* BC_1S_3 line PV033374 for transformation.

We first screened for mutations in regenerated T_1 plants based on size differences in PCR fragments from the targeted genes. Primer combination G2D (Table S1) was used to amplify a 2570-bp region flanking the first seven exons of Solyc06g060800 and the sites targeted by the four sgRNAs (Fig. 4A). Primer combination G4D (Table S1) was used to amplify a 1126-bp region in the monoexonic gene Solyc06g060820 containing the sites targeted by the four sgRNAs (Fig. 4B). In this way we identified four transgenic plants for Solyc06g060800 and five plants for Solyc06g06820 harbouring a mutant allele (Fig. S2, Table 2). These plants were subsequently selfed to obtain T_2 seeds. However, for one of the plants originating from the transformation using the construct targeting Solyc06g060800 (TV191201) and one using the construct targeting Solyc06g060820 (TV191208) we obtained fruits with no seeds. For another plant obtained from the transformation using the construct targeting Solyc06g060800 (TV191202) we obtained only six seeds. For the screening, we sowed all six available seeds from TV191202 and 80 seeds from each of the other available lines in order to select homozygous mutants by screening for difference in sizes of the amplified targeted sequence for each line.

Table 2. Mutant plants obtained by CRISPR/Cas-9 of Solyc6g060800 and Solyc6g060820.
Targeted gene, code of the T1 plant and number of T2 seeds obtained are described.

Targeted gene	T1 plant	T2 seeds obtained
Solyc06g060800 (G2)	TV191199	More than 200
	TV191200	More than 200
	TV191201	0
	TV191202	6
	TV191203	More than 100
Solyc06g060820 (G4)	TV191204	More than 200
	TV191205	More than 100
	TV191206	More than 200
	TV191207	More than 200
	TV191208	0

For the CRISPR::Solyc06g060800 lines, when amplifying the targeted region using DNA from T_2 plants belonging to line TV191199, only three heterozygous plants were identified. We kept these three plants, along with 19 plants of this family that

showed only a wild-type size band. For lines TV191199 and TV191200, amplification of the targeted region in all samples yielded only WT-size bands. For the six T_2 plants from TV191202 line, amplification of the targeted region in all samples only yielded a smaller fragment of 419-bp, suggesting they were homozygous mutant. For line TV191203, only plants with the wild-type size band were detected, from which we kept 17 plants to be tested in the disease assay. For the CRISPR::Solyc06g060820 (TV191204, TV191205, TV191206) lines, we selected 10 T_2 plants from each line that carried only the lower (L) mutant bands when amplifying the targeted sequence (Fig. S2). All plants from line TV191207 showed only a smaller fragment when compared to the WT allele; thus we concluded they were all mutant.

For the selected plants, the mutations in lines TV191202, TV191204, TV191205, TV191206 and TV191207 were characterized by PCR-based sequencing. Plants from line TV191202 (G2) were found to carry a homozygous 2151-bp deletion (Fig. 4A). Sequencing of the original TV191201 transformant showed that it carried the same homozygous deletion as TV191202. For the lines originating from the transformation using the construct targeting Solyc06g060820, four mutation events were characterized with deletions ranging from 301 bp to 727-bp (Fig. 4B).

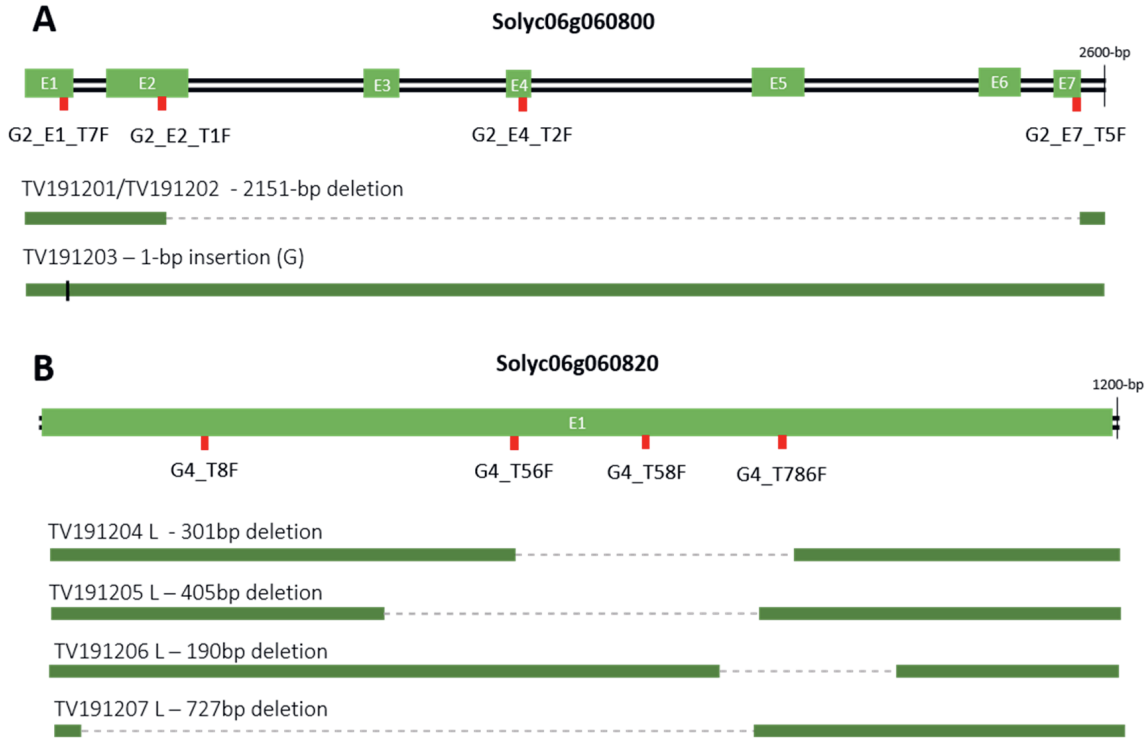


Figure 4. Mutation events in genes Solyc06g060800 (A) and Solyc06g060820 (B) in CRISPR transformants. The first seven exons (E) of Solyc06g060800 and the only exon of Solyc06g060820 are shown. Position of sgRNAs are indicated in red. L: allele representing lower PCR fragment in agarose gel as observed in Fig. S2.

We used the afore mentioned selected T_2 plants in a disease assay with *On*. NIL-*Ol-1* BC_1S_4 line PV195142 (selfing progeny from PV033374) was used as a control. The infection was scored at 13 days post inoculation (dpi) (Fig. 5). The T_2 plants from TV191199 (CRISPR::G2) that carried alleles similar in size as the WT allele displayed a disease index score similar to control plants of PV195142. Notably, the three heterozygous plants of line TV191199, and all plants of TV191202 and TV191203 showed increased susceptibility compared to the control. We were not able to sequence the individual alleles of the heterozygous TV191199 plants. The results from line TV191203 prompted us to check for mutation events in this line, as we had not found differences in the size of the amplified targeted region during the first screening based on gel electrophoresis results. Through PCR-based sequencing, we identified an insertion of 1bp (G nucleotide) within the sgRNA1 target sequence, located in the first exon (Fig. 4A). In contrast to the CRISPR::G2 lines the CRISPR::Solyc06g060820 (G4) lines (TV191204, TV191205, TV191206 and TV191207) displayed no increase in susceptibility compared to the control (Fig. 5).

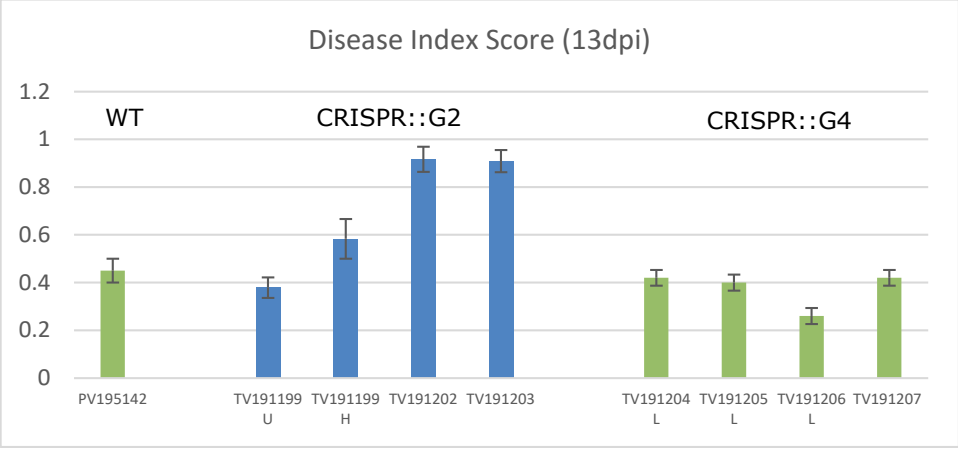


Figure 5. Disease index score at 13 days post inoculation (dpi) of CRISPR mutants. NIL-*Ol-1* PV195142 (WT) was used as a control. CRISPR::Solyc06g060800 (G2) transformant lines TV191199, TV191202 and TV1912903; and CRISPR::Solyc06g060820 (G4) transformant lines TV191204, TV191205, TV191206 and TV191207 were tested. Plants carrying the upper (U), lower (L) allele or heterozygous (H) were selected by PCR-based selection by amplifying the region targeted by the sgRNAs.

We kept some of the plants to assess progress of the secondary infection (Fig. 6A) and scored the disease symptoms after 22 days (Fig. 6B). We observed a similar response to that of the primary infection, with the heterozygous plants of TV191199, and all plants from lines TV191202 and TV191203 showing an increased susceptibility when compared to those of the wild-type control PV195142. Additionally, relative fungal biomass from the secondary infection was quantified. The results showed an

increased fungal biomass in CRISPR::G2 lines TV191202 and TV191203 compared to wild type control PV195142 and CRISPR::G4 line TV191206 (Fig. 6C).

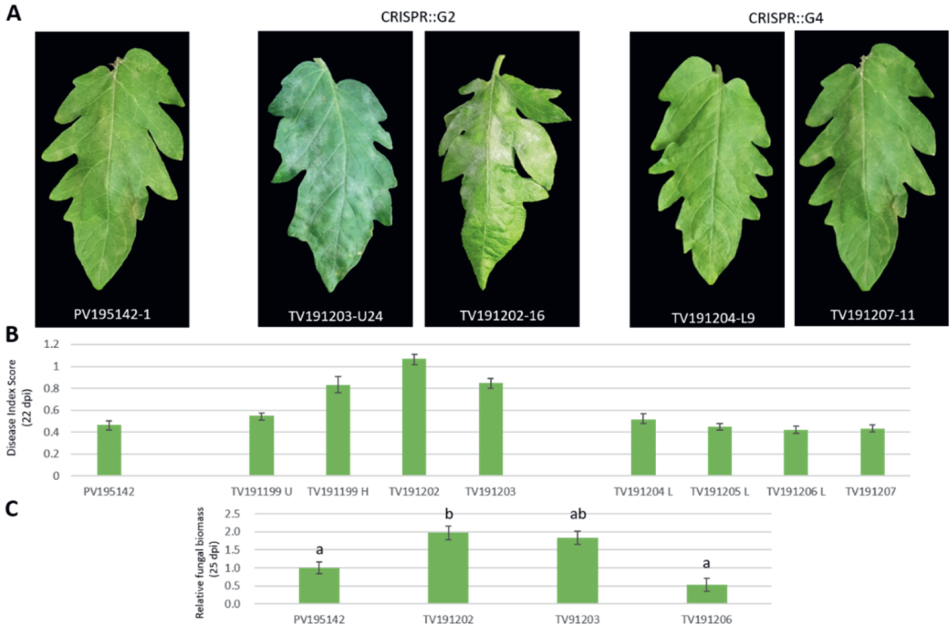


Figure 6. Phenotypic response of the CRISPR::Solyc06g060800 and CRISPR Solyc06g060820 lines. A. Powdery mildew symptoms observed on a leaf of wild-type control PV195142, and CRISPR lines at 25 days post inoculation (dpi). **B.** Average disease index score at 22 dpi. **C.** Relative fungal biomass quantification on at least three individual plants of the mutant lines, calculated as the ratio of fungal *ITS* gene amplification compared with the tomato *EF1α* gene and normalized with the values of the wild-type genotype PV195142. Significant differences were observed across the different mutant lines (Tukey HSD test, α=0.05).

A second disease assay was performed with T_2 plants from line TV191203 (20 plants). Due to the lack of seeds for the lines TV191201 and TV191202, we decided to use cuttings of the two original mutant in vitro T_1 plants TV191201 and TV191202. In total, five cuttings from each of the mother plants were used. These cuttings were identified as TV191260 and TV191261, respectively. Additionally, we used 20 plants grown from seeds from line TV191207, which was previously found to carry a homozygous deletion in Solyc06g060820. In this second disease assay we evaluated the disease response from the primary inoculation at 22 dpi. Fungal biomass quantification at this timepoint confirmed a higher fungal biomass accumulation in lines TV191203, TV191260 and TV191261 compared to WT PV195142 and TV191207 (Figure 7).

Together, the results for the mutant lines in both disease assays gave a clear indication that the impairment of Solyc06g060800 (G2) in the NIL-Ol-1 line leads to more severe disease symptoms after *On* infection.

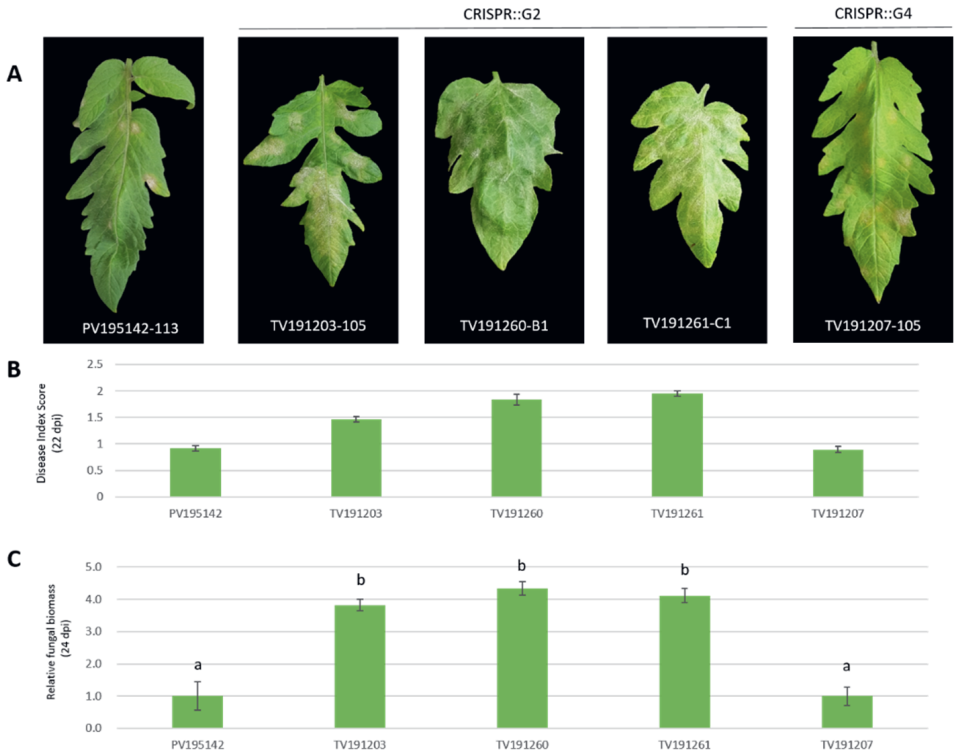


Figure 7. Phenotypic response of the CRISPR::Solyc06g060800 and CRISPR Solyc06g060820 lines in the second disease assay. A. Powdery mildew symptoms observed on a leaf of wild-type control PV195142, and CRISPR lines at 22 days post inoculation (dpi). **B.** Average disease index score at 22 dpi. **C.** Relative fungal biomass quantification on at least three individual plants of the mutant lines, calculated as the ratio of fungal *ITS* gene amplification compared with the tomato *EF1a* gene and normalized with the values of the wild-type genotype PV195142. Significant differences were observed across the different mutant lines (Tukey HSD test, α=0.05).

Solyc06g060800 allele in *Ol-1*

Solyc06g060800 is annotated as a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (ITAG4.1; Table 1). To identify sequence differences between Solyc06g060800 cDNA from cultivated tomato and its ortholog in the *Ol-1* line we isolated RNA from NIL-*Ol-1* PV103045 and synthetized cDNA. When comparing the *Ol-1* sequence to the tomato Heinz cDNA sequence, we found 11 SNPs (Fig. S3). After *in silico* translation, we identified 4 amino acid substitutions (G22R, T85A, S166N and K213E) (Fig. 8). Two of these substitutions (S166N and K213E) were

found within the predicted Fe2OG dioxygenase domain. However, when comparing the predicted protein with orthologs of several solanaceous species (Fig. 8) we found only T85A to be unique to *Ol-1*. For all four aminoacid changes found the effect in protein function was predicted to be neutral by the Protein Variation Effect Analyzer (PROVEAN; Choi & Chan, 2015).The putative ortholog of this gene in the model plant Arabidopsis is identified as AT4G16765 (Fig. S3).

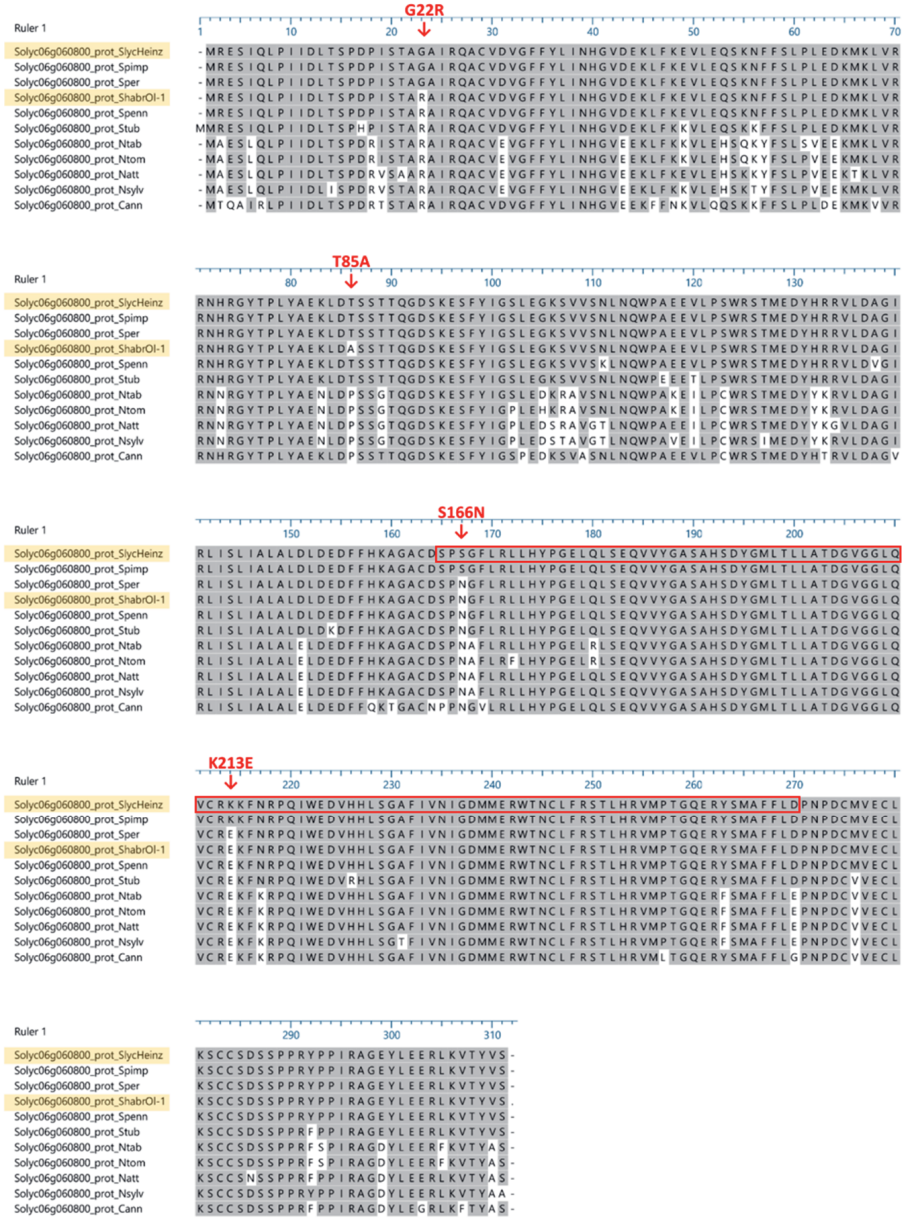


Figure 8. Alignment of the predicted protein sequence of Solyc06g060800 (G2) in Ol-1 and orthologs of several Solanaceous species. SlycHeinz, *Solanum lycopersicum* Heinz, Spimp; *S. pimpinellifolium*; Sper, *S. peruvianum*; ShabrOl-1, *S. habrochaites* G1.1601 *Ol-1*; Spenn, *S. pennellii*; Stub, *S. tuberosum*; Ntab, *Nicotiana tabacum*; Ntom, *N. tomentosiformis*; Natt, *N. attenuata*; Nsylv, *N. sylvestris*; Cann, *Capsicum annuum*. Amino acid changes in S. habrochaites Ol-1 compared with the S. lycopersicum Heinz reference sequence are indicated in red letters. Fe2OG domain is indicated inside the red box.

Discussion

The dominant resistance gene *Ol-1* was first identified in *S. habrochaites* G1.1560 and later mapped to the long arm of chromosome 6 (van der Beek *et al.*, 1994; Bai *et al.*, 2005). Since then, several efforts have been made to fine-map and identify this gene. In this study we fine-mapped the candidate region and phenotyped plants after transient silencing and stable mutation via CRISPR/Cas9 of the candidate genes to identify the *Ol-1* gene. Our approach using virus-induced gene silencing (VIGS) did not yield a clear indication of the identity of the gene. Two factors may have influenced these results. Firstly, the residual expression of the targeted genes (Langner *et al.*, 2018) may have prevented an effect upon infection with *On*. Secondly, the incomplete nature of the resistance conferred by *Ol-1* may have made the effect of the transient and patchy gene silencing even less clear.

However, after confirmation of the fine-mapped region, we were able to narrow-down the candidate region to include three candidate genes. Upon infection with *On*, mutants of Solyc06g060800 generated via CRISPR Cas/9 targeted mutagenesis in a NIL-*Ol-1* background displayed an enhanced susceptibility when compared to the wild type NIL-*Ol-1*. Relative *Oidium* biomass quantification confirmed the higher susceptibility in plants with an impaired *S. habrochaites* Solyc06g060800 allele. Although further analyses are needed, the results of this study provide a strong indication that this gene is responsible for the *Ol-1* resistance.

By measuring the levels of expression of the candidate genes, we were unable to detect significant differences between susceptible MM and resistant NIL-Ol-1 line at five time points after inoculation with *On*. However, previous measurement of the relative expression of reporter genes for plant hormones after *On* inoculation (Kissoudis *et al.*, 2017) revealed a significant upregulation of Chitinase9 (*Chi9*) in NIL-*Ol-1* after 5dpi. *Chi9* has been used in tomato as a marker gene for the ethylene pathway (Barry *et al.*, 2001). However, it is unclear if the upregulation of the ethylene pathway is responsible for the resistance against *On* in *Ol-1*-carrying plants. In any case, it will be interesting to know whether the expression of Solyc06g060800 remains stable at time points later than 5dpi.

After sequencing of Solyc06g060800 in a NIL-*Ol-1* line, we were able to identify 11 polymorphisms. Four of them resulted in amino acid substitutions in the predicted protein when compared to the refence sequence in Heinz. However, when comparing the predicted protein with orthologs of several solanaceous species, we found only one substitution (T85A) to be unique to *Ol-1*. Additionally, a neutral effect was predicted for all four amino acid substitutions. Solyc06g060800 is annotated as a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (ITAG4.1). Members of this family are known to catalyze a diverse range of oxidative reactions (reviewed by Islam *et al.*, 2018). Remarkably, the putative ortholog of Solyc06g060800 in Arabidopsis (AT4G16765) has recently been reported as a putative 1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO) (Ahmadizadeh *et*

al., 2020). ACO genes are known to catalyse the last step in the production of ethylene in plants and have been shown to be the rate-limiting step in ethylene production (Houben & Van de Poel, 2019). This holds relevance due to the observed upregulation of the ethylene pathway reporter gene *Chi9* upon inoculation with *On* in NIL-*Ol-1* (Kissoudis *et al.*, 2017). It is intriguing to investigate whether Solyc06g060800 may act as an ACO in tomato and whether there is a difference in functionality between the *S. habrochaites Ol-1* allele and the cultivated tomato allele. The involvement of the plant hormone ethylene in defence response is better understood in the interaction with necrotrophic pathogens (Thomma *et al.*, 1998, 2001; Glazebrook, 2005). However, recently, Zheng *et al*. (2020) demonstrated that *Triticum urartu ACO3* (*TuACO3*) regulates ethylene biosynthesis and enhances the defence response against the PM adapted pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*). In this study, the authors show that the upregulation of *TuACO3* or the transcription factor *TuMYB46L*, as well as the exogenous application of ethylene results in reduced microcolony development of *Bgt* in the host cells. It will be interesting to test if the exogenous application of ethylene in our CRISPR::Solyc06g060800 mutants can restore the resistant phenotype.

Additionally, we have identified a transposon present in the last intron of Solyc06g060800 in our NIL-*Ol-1*. Transposable elements are known to be able to reprogram host genes and may lead to changes in the exonic regions, as well as alter the epigenetic regulation of the genes (reviewed by Negi *et al.*, 2016). Further studies on the activity and effect of this transposable element on Solyc06g060800 are needed.

The resistance response observed in *Ol-1* is described as incomplete (Li et al. 2007), allowing the pathogen to establish a haustorium but preventing a normal fungal development observed in susceptible plants. It will be very interesting to know how this response is altered at the histological level in Solyc06g060800 CRISPR mutant plants. Additionally, to further confirm this gene as the one responsible for the *Ol-1* resistance, it is essential to carry out complementation studies by overexpressing this gene in a susceptible background and analyze whether the transformants show resistance against *On*. Due to the mechanisms and specificities in resistance observed in *Ol-1*- carrying plants, this gene has been suggested to be an enhancer of basal resistance (Li *et al.*, 2006; Seifi *et al.*, 2014). Furthermore, downregulation of *ALS*, *ShGST* and silencing of *ShORR-1-G* have been shown to compromise the *Ol-1*-based resistance (Pei *et al.*, 2011; Gao *et al.*, 2014; Zhang *et al.*, 2019). These studies, together with our results, suggest that the resistance conferred by *Ol-1* involve unique mechanisms that are not common to other described resistance genes against PMs.

Materials and Methods

Plant materials and fine-mapping

Tomato lines used in the present studies are listed in Table 3. For the second fine-mapping, plants from seven lines used in previous studies (Seifi, 2011) were re-tested to confirm their genotype and phenotypic response upon PM inoculation. BC_1S_1 lines PV133256, PV133277, PV133264 and PV133266 were obtained from the backcross and subsequent selfing used for the first fine-mapping effort, while BC_3S_2 line PV033357, and BC_1S_3 lines PV033369 and PV033371, originated from a Moneymaker (MM) x *S. habrochaites* G1.1560 cross (Seifi, 2011). Available seeds from each of these lines were sown and grown at 20 ± 2 °C with 70 ± 15% relative humidity and day length of 16 h in a greenhouse of Unifarm of Wageningen University & Research, The Netherlands. In total 35 plants of line PV133264, 31 plants of line PV133266, 10 plants of line PV133277, 5 plants of line PV133256, 10 plants of line PV033369, 10 plants of line PV033371, and 34 plants of line PV033357 were tested. NIL-*Ol-1* PV033374 and susceptible cultivar MM were used as controls. Three weeks-old plants were inoculated by spraying with a suspension of *O. neolycopersici* conidiospores obtained from leaves of infected tomato MM plants. The suspension concentration was adjusted to of $3.5*10^4$ conidiospores per ml. Plants were genotyped with molecular markers listed in Table S1. The disease symptoms were evaluated at least twice starting at 8 dpi and were visually scored using a scale from 0 to 3 described by Bai *et al.* (2007).

Table 3. Lines used for fine-mapping, confirmation and characterization studies. Line code, origin and generation are listed.

Line	Origin	Generation
PV123059	*S. habrochaites* (G1.1560)	BC_1
PV123056	*S. habrochaites* (G1.1560)	BC_1
PV123057	*S. habrochaites* (G1.1560)	BC_1
PV133256	*S. habrochaites* (G1.1560)	BC_1S_1
PV133264	*S. habrochaites* (G1.1560)	BC_1S_1
PV133266	*S. habrochaites* (G1.1560)	BC_1S_1
PV033369	S&G Seeds	BC_1S_3
PV033371	S&G Seeds	BC_1S_3
PV133277	*S. habrochaites* (G1.1560)	BC_1S_1
PV033357	S&G Seeds	BC_3S_2
PV103045	S&G Seeds	BC_3S_5
PV033373	S&G Seeds	BC_1S_3
PV033374	S&G Seeds	BC_1S_3
PV033375	S&G Seeds	BC_1S_3
PV195142	S&G Seeds	BC_1S_4
Moneymaker		

Construction and screening of Bacterial Artificial Chromosome (BAC) library and sequencing of selected BAC clone

A pooled bacterial artificial chromosome (BAC) library was developed by Bio S&T Inc. (St-Laurent, Quebec, Canada). DNA fragments of NIL-Ol-1 BC3S5 line PV103045 were cloned in pIndigoBAC (*Hin*dIII) vector (5x genome coverage) and transformed to *Escherichia coli* DH10β cells. The average insert size was 140-kb. Colonies were distributed into six 96-deep well plates (pooled BAC library). Each well contained about 400 independent primary clones. This BAC library was screened at Bio S&T Inc. by PCR using primer combination 45 (Table S1), yielding a 447-bp PCR product from gene Solyc06g060790, to identify individual BACs containing sequences from the *Ol-1* introgression region. A single positive BAC clone (BAC23) was isolated.

This BAC clone was subsequently sequenced at INRA-CNRGV (Toulouse, France) on a PacBio RS sequencer using PacBio RS libraries with 8–12-kb insert size. A coverage of approximately 60x read depth per BAC was obtained. Sequences were aligned to *S. lycopersicum* Heinz genome SL2.50 by BLASTN analyses at National Center for Biotechnology Information (NCBI) and SGN (Sol Genomics Network) webpages and DNASTAR Lasergene (https://www.dnastar.com/software/lasergene/) programs. Subsequently, gene prediction was performed using the FGENESH module from SoftBerry (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind) and Augustus (http://bioinf.uni-greifswald.de/augustus/).

Expression analysis

For determination of transcript level for each predicted gene, total RNA was isolated from leaflets using the RNeasy kit (Qiagen). After removal of DNA with DNase I (Invitrogen), 1µg total RNA was used for cDNA synthesis using superscript II reverse transcriptase kit (Invitrogen). Quantitative real-time PCR was conducted using the iQ SYBR Green supermix (Bio-Rad) and the CFX96 Real-Time system (Bio-Rad). Primers for expression analysis of the candidate genes are listed in Table S2. The PCR amplification consisted of an initial denaturation step of 10 min at 95℃, followed by denaturation for 15s at 95 ℃, annealing for 30s at 60℃, and extension for 30s at 72℃ for 30 cycles. Sizes of the amplicons ranged from 99-105bp. The generated threshold cycle (Ct) was used to calculate the transcript abundance relative to tomato EF1α gene, using primers F-GGAACTTGAGAAGGAGCCTAAG and R-CAACACCAACAGCAACAGTCT (Livak & Schmittgen, 2001; Løvdal & Lillo, 2009).

Virus-induced gene silencing

Virus-induced gene silencing (VIGS) was performed using the TRV-based vector system (Liu *et al.*, 2002). For building the constructs the sequence of the six predicted genes was retrieved from Sol Genomics Network. Primers were designed to amplify a fragment size of 300-400 bp (Liu & Page, 2008; Table S3). The sequence of the primers for amplification of the targeted sequence were BLASTed on the SGN

"Tomato Genome CDS (ITAG release 2.40)" database (Fernandez-Pozo *et al.*, 2015) with an e-value of 1 on the default (BLOSUM62) substitution matrix. This was done to note the specificity of the primers and assure that there were no off-targets Primers used to build the VIGS constructs can be found in Table S3. Additionally, the SGN VIGS Tool (http://vigs.solgenomics.net/) was used to check the ideal primer positions. To enable directional cloning into the pENTR-TOPO vector a four nucleotide sequence consisting of "CACC" was added at the 5' end of the forward primer (Thermofisher). VIGS fragments were cloned using pENTR D-TOPO vectors (Thermofisher) according to the manufacturer's protocol using One Shot TOP10 Chemically Competent *E. coli* cells. Positive colonies were selected by plating in LB containing kanamycin (1 µl/ml [50 mg/ml]) and verified by sequencing. The constructs for agroinfiltration were made by transforming *Agrobacterium tumefaciens* electrocompetent cells (strain GV3101). In the VIGS experiment, eight different constructs were used to test five candidate genes, including two controls pTRV2::GUS and pTRV2::PDS, and pTRV1 (Table S4). Two homozygous *Ol-1* BC1S3 lines PV033373 and PV033375 were used. VIGS experiments were performed as described by Gao et al. (2014).

Targeted mutagenesis and analysis of mutants

The full-length coding sequence of Solyc06g060800 was cloned from a plant from line PV103045, homozygous for the *Ol-1* candidate region (Figure S4). Additionally, the full-length genomic sequence of Solyc06g060820 was amplified from a plant of the same line (Figure S5). In order to design the single guide RNAs (sgRNAs) targeting the candidate genes we followed the guidelines described by Liang *et al.*, (2016). The CC-Top CRISPR/Cas9 web tool (Stemmer *et al.*, 2015) was used to retrieve a list of gRNAs. G+C content of selected sgRNAs was calculated using the ENDMEMO webtool (http://www.endmemo.com/bio/gc.php). To further select the sgRNAs, the sgRNA scorer (Chari *et al.*, 2017) was used. Prediction of the folding of the sgRNAs was analyzed using the Mfold web server (Zuker, 2003). The sgRNAs used to target G2 and G4 are listed in Table S5. CRISPR constructs were made using Golden Gate cloning (Engler *et al.*, 2008). Twelve plasmids obtained from Addgene were used to build the construct for transformation: pICH86966 (as template for amplification); pICSL01009 (as level 0 plasmid); pICH47751, pICH47761, pICH47772, pICH47781 and pICH47732 (as level 1 plasmids); pICH41766, pICH41780 and pICH41822 (as linkers); and pAGM4723 (as level 2 binary vector). *E. coli* DH5α were used to clone the plasmids, which were then transferred to *Agrobacterium tumefaciens* strain AGL1 for transformation. NIL-*Ol-1* BC_1S_3 line PV033374 was used for transformation based on the methodology described by McCormick *et al.* (1986).

For selection of T_1 transformants, we screened for difference in sizes of PCR products using primers flanking the regions targeted by the sgRNAs: G2D for Solyc06g060800 and G4D for Solyc06g060820 (Table S1). In the WT sequence, primer combination G2D yields a 2570-bp product and primer combination G4D yields a 1126-bp product.

After amplification, smaller sizes compared to the WT alleles indicated deletions in the targeted regions. The selected transformants were then selfed to produce T_2 seeds. For selection of T_2 transformants, 80 seeds from each of the available lines, along with the six seeds available from line TV191202 were screened for difference in sizes of PCR products using the aforementioned primer combinations to test these plants in a disease assay with *On*.

In the first disease assay, 10 plants from NIL-*Ol-1* BC_1S_4 line PV195142 (selfing progeny from the line used as background for transformation, PV033374) were used as controls. For the CRISPR::Solyc06g060800 lines, we used three identified heterozygous mutants from line TV191199, along with 19 plants from this family that carried a WT size band; six plants from line TV191202 that carried smaller 419-bp mutated alleles; and 17 plants from line TV191203 that carried only a WT size band. For the CRISPR::Solyc06g060820 lines TV191204, TV191205, and TV191206, 10 plants homozygous for the lower (L) allele from each family were used. Finally, all plants from line TV191207 showed a smaller fragment when compared to the WT allele, from which 10 were used in the disease assay.

In the second disease assay, we used 10 plants from line PV195142 as controls. For the CRISPR::Solyc06g060800 we used 20 plants from family TV191203; and five cuttings from each of the original TV191202 and TV191203 mother plants, which were identified as TV191260 and TV191261, respectively.

Relative fungal biomass quantification

For fungal biomass quantification, DNA was isolated with an adapted protocol using the CTAB method (Doyle & Doyle, 1987) from infected leaves collected at 25 dpi for the first disease assay and 24 dpi for the second disease assay. 10ng of DNA were used as template for amplification. Relative fungal biomass was quantified using the method described by Santillán Martínez *et al.* (2020; Chapter 4 of this thesis).

Supplementary material

Table S1. Molecular markers and primers used for fine-mapping and sequencing of the *Ol-1* region. Name, type of primer/marker, forward and reverse sequence and digestion enzyme (for the case of CAPS markers) are listed.

Name	Type	Forward	Reverse	Digestion
123G17-2	CAPS	AGAATCCGCGCTACAACTACA	ACAGGACCTGATGGAAGTGTG	*Spe1*
KspAI_rs_f	CAPS	CTTTCAAGTAACCGAATCACATGA	GAAATGAGCTGTATTTTTCTTCA	*KspAI*
Cfr421_rs_0	CAPS	GACTTAAGGATCATACCTCATCT	TGAGTACATTGCCATGGCTA	*Cfr421/SacII*
123G17-1	CAPS	AAGTACTTAACCCGGCTGGAA	TGGGTGATGCCTCAAACTATC	*Bg1II*
PSD-1	SCAR	ACGAGCAGCAGGTGTGATAGAC	GTGGTTAATGAGATGGGTGGAC	
scaff6	CAPS	TGCCTCTGAGATCGACAATG	TTCTCAATCCGGCGTAATTC	*Hin1II*
45	CAPS	ATCCATTAATCTCCCATTCCGTCT	GCGGATAAACTTCACCAGTCGAAA	*EcoRI*
IG_1_2	Seq.	CCCAGCTATGGTTGAGCTTC	TTCCGAATACATGCGAATCA	
G2D	Seq.	TTGGAGTGACGAAGAAAAGC	TCGCCTTCTACTCCGATCAT	
DL-19	CAPS	ATGCCTCTTTCTTGCACTGA	CACGAATGAACGCATGTGTTT	*DdeI*
G4	Seq.	ATGGATCAAGATTCAGCTCCTT	TTACTGGATTAACATTTTAGCAGG	
G4D	Seq.	TGGATCAAGATTCAGCTCCTT	GCCGATGTACGAATGGACTT	
G5	Seq.	AAAAATCCTTGTTTGAGGAAGA	CTACTTTTGTCTTGCAAGTGAAGG	
DL-27	CAPS	GCAGCACTATACGAGGTTGAGA	TTGATTCTTACGCGATTGGA	*XapI*
TAG	CAPS	ATGCAGATCGGAACAGCACTTC	TCATGATGATGGACTAGGCA	*Cfr13I*

Table S2. Primers used for expression analysis.

Gene	Code	Primer sequence
Solyc06g060780	G0	F-ACCAACCCTGCGAACAAATA
		R-AGCAAAACACCAGTGCCTCT
Solyc06g060790	G1	F-CCAAATCATCCCAGCAGAAT
		R-GGTACGATGAGGGAAGTCCA
Solyc06g060800	G2	F-GGCATGTGATTCACCAAGTG
		R-CTGAATGAGCAGAAGCACCA
Solyc06g060810	G3	F-TGTAGGTTATGGGGGACCAG
		R-CGATCTCAGTGTACCGGATTC
Solyc06g060820	G4	F-GGCTGCAAGAGCCAGATTAT
		R-GCTGGCTCTAACACTTGCATC
Solyc06g060830	G5	F-GCCCCACAAATACACCTGTC
		R-GTGGAGGATGAGTCCCACAT

Table S3. Primers used for amplification of sequences for virus-induced gene silencing (VIGS) constructs.

Gene	Name		Primer sequence	Fragment size
Solyc06g060780	G0	Fw	caccCTCAGCGCAAAATTCAGAAATTT	300 bp
		Rv	TTCTGATCATCATAGAGACGTCGA	
Solyc06g060790	G1	Fw	caccCCAAATCATCCCAGCAGAAT	440 bp
		Rv	CTTCAATGACAGGACCAGCA	
Solyc06g060800	G2	Fw	caccTGGAGTGACGAAGAAAAGCA	552 bp
		Rv	TAATCGGGACCAACTCAACC	
Solyc06g060810	G3	Fw	caccGCTCACTTTTGGTTTTGGACTT	525 bp
		Rv	AAAACTAGCCAGGAACTTGACA	
Solyc06g060820	G4	Fw	caccCGAACAATTAGGCCGTTCAT	349 bp
		Rv	CAGGCTGTGTAGCCGATGTA	
Solyc06g060830	G5	Fw	caccAAAAATCCTTGTTTGAGGAAGA	415 bp
		Rv	AATAGTGCTGTTCGGAGACGA	

Table S4. List of virus-induced gene silencing constructs used for transient silencing of candidate genes.

Construct	Target
pTRV2:780-3	Solyc06g060780
pTRV2:790	Solyc06g060790
pTRV2:800	Solyc06g060800
pTRV2:810	Solyc06g060810
pTRV2:820	Solyc06g060820
pTRV2::GUS	β-glucuronidase, used as a negative control
pTRV2::PDS	Phytoene desaturase, control for agroinfiltration, causes photobleaching in silenced tissue
pTRV1	RNA1 of TRV, encodes replicase and movement proteins.

Table S5. Single guide RNAs (sgRNAs) used for targeted mutagenesis of the candidate genes.

Primer Label	Sequence	Target
G2_E1_T7F	CAGCGGTGGAAATAGGATCA	Solyc06g060800
G2_E2_T1F	CTGGTACGAAGGAACCACAG	
G2_E4_T2F	TCTTCTATGGTAATCCTCCA	
G2_E7_T5F	GAAGATGTGCATCACCTCAG	
G4_T8F	CATCACCCAAACAGGATATT	Solyc06g060820
G4_T56F	TTAACAATCGATGATGGCGA	
G4_T58F	TTGTGATAGGGTCAAGAACA	
G4T_78F	AACAATTAGGCCGTTCATAG	

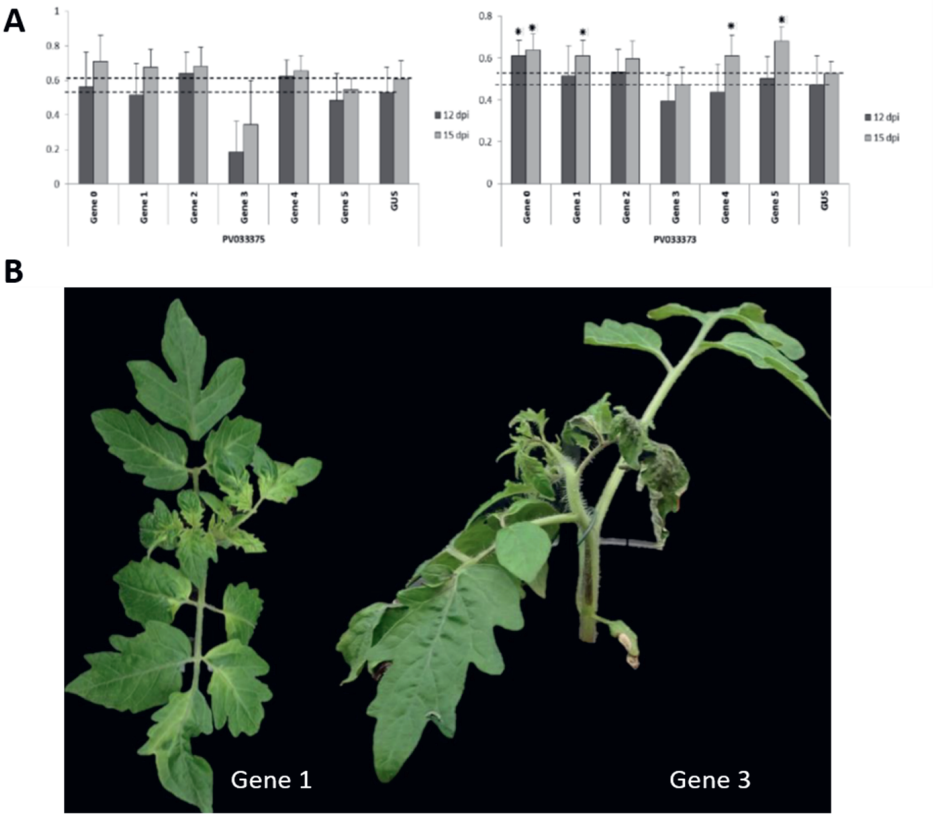


Figure S1. Response upon *Oidium neolycopersici* (*On*) infection of plants in which candidate *Ol-1* genes were transiently silenced via Virus-induced gene silencing (VIGS). A. Average disease index (DI) score at 12 and 15 days post inoculation (dpi) of nine plants per line for each construct targeting the candidate genes Soly06g060780 (Gene 0), Solyc06g060790 (Gene 1), Solyc06g060800 (Gene 2), Solyc06g060810 (Gene 3), Solyc06g060820 (Gene 4), and Solyc06g060830 (Gene 5). Two homozygous *Ol-1* BC1S3 lines PV033373 and PV033375 were used. Bars indicated with an asterisk differ significantly from the GUS control. **B**. Phenotypic responses after silencing using VIGS constructs targeting Solyc06g060790 (Gene 1) and Solyc060810 (Gene 3).

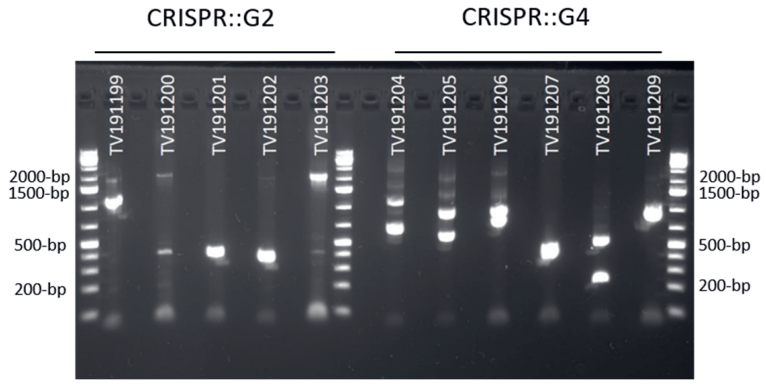


Figure S2. PCR amplification of the targeted sequence in the original mutants for Solyc06g0800 (CRISPR::G2) and Solyc06g0820 (CRISPR::G4).

Figure S3. Alignment of the predicted protein sequence of Solyc06g060800 in NIL-*Ol-1*, reference Heinz sequence and Arabidopsis thaliana putative ortholog AT4G16765. Amino acid substitutions in the NIL-*Ol-1* and AT4G16765 proteins compared to the reference sequence from tomato cultivar Heinz are shown in grey.

```
ATGAGAGAATCAATTCAGCTTCCCATAATCGACCTTACTTCCCCTGATCCTATTTCCACCGCTCGAGCAATTCGTCAGGCATGTGTAGAT     90
GTTGGGTTCTTTTACCTAATAAATCATGGAGTAGACGAAAAGTTGTTTAAGGAAGTGCTTGAACAGAGCAAAAACTTCTTCTCACTGCCC    180
CTTGAAGACAAGATGAAACTGGTACGAAGGAACCACAGAGGTTATACTCCCCTTTATGCTGAGAAACTTGATGCCTCTTCTACTACACAA    270
GGTGACTCAAAAGAAAGCTTCTACATTGGTTCTCTGGAAGGAAAGAGTGTTGTTAGTAACTTGAATCAGTGGCCTGCAGAAGAAGTTCTG    360
CCAAGTTGGAGATCAACCATGGAGGATTACCATAGAAGAGTCTTGGATGCCGGTATAAGACTAATTTCACTGATTGCTTTGGCATTGGAT    450
TTGGACGAGGACTTCTTTCATAAAGCAGGGGCATGTGATTCACCAAATGGATTCCTTCGTCTATTACATTATCCAGGCGAGTTGCAACTA    540
TCTGAACAAGTGGTGTATGGTGCTTCTGCTCATTCAGACTATGGGATGTTAACTCTTCTAGCTACAGATGGTGTTGGTGGACTTCAGGTT    630
TGCCGGGAGAAATTCAACCGACCTCAGATATGGGAAGATGTGCATCACCTCAGCGGGGCTTTCATAGTTAACATTGGAGATATGATGGAG    720
AGGTGGACAAACTGCTTATTTCGGTCTACATTGCATCGAGTTATGCCAACAGGGCAAGAGCGTTATTCGATGGCTTTCTTTTTGGATCCA    810
AATCCAGATTGTATGGTGGAATGCTTGAAGAGCTGCTGTAGTGATTCATCTCCTCCCAGATATCCTCCAATTCGCGCCGGAGAATATTTG    900
GAAGAGCGCTTAAAAGTTACATATGTATCATAG       933
```

Figure S4 cDNA sequence of Solyc06g060800 from NIL-Ol-1


```
ATGGATCAAGATTCAGCTCCTTACATCTCCAAAAAACAAAAAACAAACCATGCAAACTACCCTTGTAAGTTTTACAATAT      80
TTTTCCATTTAAAACTATGCTACTTATCATATTTCTAGTTGCACTTCCACTTTTTCCTTTACAACCTCCTGAAATCATCA     160
CCCAAACAGGATATTGGGAACTTGTTCAGCTTATTTTAGTTGGTATAGCTGTTTCTTATGGTTTATTCAGTAGAAAAACC     240
GAAGATGACGATAACGATGATGATGTTGATGATACAGAAAATGAATATTTATTTAGTTCAAAGATTGATAATGTGCAATC     320
TAGATTACTTGAGGTTTCATCTTTCTTTGAAAACCATCAAGCGTCTGATGAAAACATAGTGAACAATTATCAGTATTGTA     400
GTGGTAAGCCAGTAGTTGTTGTTGCTAAGGAAAATGATGCTATTATTAGTACAAGTTTTGTTGAAAAGCCTTTGCTTTTG     480
CCAATTAGAAGTTTAAAATCCCCTGTTCTTGACCCTATCACAACTACTAATTCACCAAAATCTATGGTTTCATCTCCAAG     560
AAAGATTGATCAGTTTGATCACAAAATTGTGAGGAAACAGAGTTTGCACAAATCCTCTGTTTCACCACCACCTCCTCTGC     640
CATCGCCATCATCGATTGTTAAAAAATCTACTTTGTTGAGATCAAGCTCTATAGTAATGGATGATCATAAAGGTTCTTTT     720
GGTAAGGAATTGAGGAGAAGTACTAGGAGTGTTCCATTTGAATTGAGCTCGTATAAAGGGAAGTCTGTTCGAACAATTAG     800
GCCGTTCATAGGGGCTGCAAGAGCCAGATTATATGCTAAAGATTTCGTAAATGGGATTACAGAGGATGAAAGAAACAAAG     880
AAGTTGGTACACAGATGCAAGTGTTAGAGCCAGCATTGATGGAGTTCTCAGAGGATGAGAAGAAAACATCGAAAAGTGAT     960
GAAGATTCAGACGATTGTATTGAAGAAAGCTCGGAAAATGTAGACGAAGAAAGGAAATGTGGTGTTGATGGTAGTTCAGA    1040
TAATGTAGACAAGAAGGCTGATGAGTTTATAGCTAAATTTAGAGAGCAAATTAGGCTTCAGAGAATTAAGTCCATTCGTA    1120
CATCGGCTAAACAGCCTGCTAAAATGTTAATCCAGTAA         1158
```

Figure S5 G4 gDNA sequence of Solyc06g060820 from NIL-Ol-1

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Chapter 6

General discussion

Plant diseases are a constant threat to agricultural production since they reduce yields and affect crop quality. The dynamic spread of plant pathogens causing these diseases across the world is likely to increase due to human activities in addition to the influence of climate changes (Chakraborty & Newton, 2011; Bebber *et al.*, 2014). At the same time, the reduction in the use of pesticides has become a key target towards the goal of developing sustainable food production systems (Schebesta & Candel, 2020). In this context, the development of vegetable varieties with resistance to pests and diseases stands out as a crucial tool to tackle the challenge of meeting the agricultural production demands in a sustainable manner.

Race-specific resistance to powdery mildew: differences between monocots and dicots

Powdery mildew (PM) is one of the most common plant diseases in the world. Plants affected by this disease are easily recognizable by the characteristic symptoms of white powder extending across the aerial parts of the plants. PM-causing fungi are obligate biotrophs, that thus require the living tissue of their hosts to obtain nutrients and complete their life cycles. PM can occur in more than 10,000 plant species (Braun *et al.*, 2002; Glawe, 2008). The most extensively-studied PM pathogens are the monocot-infecting ones, all belonging to the species *Blumeria graminis*, which are highly specialized and thus have a narrow host range. Among these, the barley- and wheat-adapted pathogens *B. graminis* f.sp. *hordei* (*Bgh*) and *B. graminis* f.sp. *tritici* (*Bgt*), respectively, are perhaps the best understood. Conversely, a large number of *Resistance* (*R*) genes of the typical nucleotide-binding leucine-rich repeat receptors (NLR) family has been characterized to be effective against PM in monocots. These NLRs recognize race-specific pathogen-secreted effectors following a gene-for-gene interaction and trigger a fast hypersensitive response (HR) that results in effector-triggered immunity (ETI). Since the identification, more than 60 years ago, of the NLR-encoding *Mla* resistance locus in barley (Moseman & Sohaller, 1960), over 20 different allelic variants have been identified to confer resistance against *Bgh* (reviewed by Saur & Hückelhoven, 2021). In the case of wheat, 17 functional variants of the NLR-encoding *Pm3* have been identified (Bhullar *et al.*, 2010; Kang *et al.*, 2020). From the pathogen side, advances in next-generation sequencing and ~omics technologies are quickening the elucidation of the genetic basis of hosts specialization of these fungi (Hacquard *et al.*, 2013; Menardo *et al.*, 2016, 2017; Frantzeskakis *et al.*, 2018) and the identification of avirulence (*Avr*) genes recognized by resistant hosts (Bourras *et al.*, 2016). It has been shown that in *Bgh* and *Bgt*, 8% and 9%, respectively, of the total number of genes encode candidate effector secreted proteins (CSEPs) (Spanu *et al.*, 2010; Pedersen *et al.*, 2012; Wu *et al.*, 2018). Saur *et al.* (2019) recently reported the isolation of four *AVR* genes in *Bgh*: $AVR_{\alpha 7}$, $AVR_{\alpha 9}$, $AVR_{\alpha 10}$, and $AVR_{\alpha 22}$, which are recognized by four different barley MLA allelic proteins. Together, studies in both these pathosystems demonstrate an arms race between these PM pathogens and their hosts after domestication (Yahiaoui *et al.*, 2006; Seeholzer *et al.*, 2010).

On the other hand, the degree of specialization differs in the case of dicot-infecting PMs. Some pathogens of this group have a wide host range and are able to infect many different plant species (Křístková *et al.*, 2009; Takamatsu *et al.*, 2013; Wu *et al.*, 2018). Such is the case of *Oidium neolycopersici* (*On*), which can infect plants from up to 13 different families including Solanaceae and Cucurbitaceae (Jones *et al.*, 2001) and first reports of its pathogenesis in other plant species (i. e., *Moringa oleifera* and *Codiaeum variegatum* var. *pictum*) have been published recently (Liu *et al.*, 2015, 2019). But compared to monocot-PM pathosystems, research on dicot-infecting PMs is relatively more recent. It was only in the decade of the 1990's when PM species that are able to cause disease in the model plant Arabidopsis were identified (reviewed by Micali *et al.*, 2008). Since then, four PM pathogens have been used to study their interaction with Arabidopsis, namely *Erisyphe cruciferarum* (Koch & Slusarenko, 1990), *Golovinomyces* (syn. *Erysiphe*) *cichoracearum* (Adam & Somerville, 1996), *Golovinomyces* (syn. *Erysiphe*) *orontii* (Plotnikova *et al.*, 1998) and the tomato-adapted PM pathogen *On* (Xiao *et al.*, 2001). Additionally, in the case of *On* and its host tomato, research was urged by the outbreak of this disease in the late 1980's (Fletcher *et al.*, 1988; Reviewed by Seifi *et al.*, 2014). Two recent comparative studies revealed distinct differences between monocot- and dicot-infecting PMs. Wu *et al*. (2018) found the size of the effectorome of four dicot-infecting PMs to be much smaller compared to that of *Bgh* and *Bgt*. Liang *et al.,* (2018), also analyzed the differences in selection patterns of CSEP families in both PM lineages, observing that the CSEP families in dicot-infecting PM pathogens appear to be shrinking, in contrast with the rapidly-expanding families of monocot-infecting PMs. Accordingly, from the plant side, race-specific resistance appears to be rare in dicot-PM pathosystems and, to date, only the tomato genes *Ol-4* and *Ol-6*, conferring resistance to *On*, have been mapped to a cluster of NLR-encoding genes (Seifi *et al.*, 2011). Additionally, in several accessions of Arabidopsis, resistance to PM has been found to be conferred by the atypical *R* genes *RESISTANCE TO POWDERY MILDEW 8.1* (*RPW8.1*) and *RPW8.2* (Göllner *et al.*, 2008), clustering together in chromosome 3 and identified in accession Ms-0 (Xiao *et al.*, 2001). *RPW8.1* and *RPW8.2* encode a distinct type of R proteins with a characteristic subclass of coiled-coil (CC) domain, the RPW8-like CC (CC_R) (Collier *et al.*, 2011). *RPW8.1* can enhance basal defence and trigger an HR-like cell death (Ma *et al.*, 2014) and has recently been shown to be part of an autoregulatory circuit with the ethylene-signalling pathway by interacting and stabilizing 1-aminocyclopropane-1-carboxilase oxidase 4 (ACO4), an enzyme that catalyses the final step in the biosynthesis of ethylene (Zhao *et al.*, 2020). RPW8.2 is targeted specifically to the extra-haustorial membrane and interacts with several intramolecular trafficking signals to activate cell death (Huang *et al.*, 2019). Although the *RPW8*-based resistance is effective against three of the PM species infecting Arabidopsis, accession Shakdara (Sha), carrying both Ms-0-like *RPW8* alleles, was found to be susceptible to *On* (Göllner *et al.*, 2008). Furthermore, heterologous expression of the *RPW8* genes in tomato failed to confer resistance against *On* (Xiao *et al.*, 2003). This

suggests specific genetic and molecular components are present in the interaction between Arabidopsis and *On*.

With the purpose of exploring specificities of the Arabidopsis-*On* pathosystem, we performed a disease assay in 123 accession of Arabidopsis. From these, 40 accessions were fully resistant, which were then crossed with susceptible accessions Col-0 or Sha. Analysis of the F_2 progeny of these crosses allowed the identification of a natural loss-of-function mutation of *EDR1*, explaining the resistance found in accession C24 (Gao *et al.*, 2015). In the work reported in **this thesis**, I further explored this pathosystem in order to determine specific genetic components of resistance.

The Arabidopsis-*On* interaction reveals a novel component of resistance

Among the Arabidopsis accessions that were found to be resistant to *On* in our screening, accession Bla-6 was previously reported to be susceptible to *E. cichoracearum* and *E. cruciferarum* (Adam *et al.*, 1999). This indicated that so far undescribed genes were responsible for the resistance to *On*. In **Chapter 2** of this thesis I report the identification of *ZED-1 RELATED KINASE 13* (*ZRK13*) as a novel component of resistance against *On*. Furthermore in **Chapter 3**, I report, via an independently executed fine-mapping study, that *ZRK13* is also required for resistance in accession Litva. Four other members of the *ZRK* family, belonging to the Receptor-Like Cytoplasmic Kinase (RLCK) XII subfamily (Lewis *et al.*, 2013), have been characterized to be required for the recognition of type III secreted effectors from *Pseudomonas syringae* and *Xanthomonas campestris* and trigger an ETI response by the activation of the HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) resistosome (Lewis *et al.*, 2013; Wang *et al.*, 2015; Seto *et al.*, 2017; Bastedo *et al.*, 2019; Martel *et al.*, 2020). There is evidence suggesting that ZRK13 is able to interact with ZAR1, as its overexpression was reported to partially rescue a severe ZAR-1-dependant autoimmune phenotype in the *zed-1D* mutant grown at high temperature (Wang *et al.*, 2017).

There are important differences between the resistance in Bla-6 and Litva. In accession Bla-6 the resistance was inherited in a dominant manner, while in Litva, the F_3 was found to display a recessive or co-dominant inheritance. I hypothesized that the 1:3 (resistant: susceptible) segregation in the latter may be the result of a recessive inheritance of the trait or a dominant resistance supressed by another required locus. Furthermore, for full resistance in Litva, we showed that an additional locus in chromosome 3 is required (QTL-4). For this reason, I speculate that full resistance may be the result of either an additive effect between *ZRK13* and QTL-4 or an interaction between both loci. Although QTL-4 has not yet been fine-mapped, this region includes the *ZAR1* gene (At3g50950). If the ZRK13 allele of Litva requires interaction with a specific allelic variant of ZAR1 to trigger an immune response, it would explain why the tested recombinants carrying the Litva allele in only one of

the two loci display a susceptible phenotype. To test whether the resistance to *On* in accessions Bla-6 and Litva is dependent on the interaction of ZRK13 with ZAR1, disease assays in *zar1* mutants in both Bla-6 and Litva backgrounds have to be performed.

It is intriguing to speculate if the ZAR1-dependent resistance could be extrapolated to other plant species. Indeed, ZAR1 is known to be highly conserved among plant species, with orthologs found in monocot, magnoliid and eudicot plants (Adachi *et al.*, 2020). Notably, by forward genetic screening in *Nicotiana benthamiana* the *NbZAR1* was identified to be required for recognition of the XOPJ4 effector of *Xanthomonas perforans* (Schultink *et al.*, 2019). This study also identified a member of the RLCK XII family, named XOPJ4 IMMUNITY 2 (JIM2), to be required for perception. However, the authors further show evidence of a partial functional divergence between NbZAR1 and the Arabidopsis ortholog AtZAR1, by showing that transient expression of AtZAR1, JIM2 and XOPJ4 in a *N. benthamiana zar1-1* mutant did not trigger a visible immune response. Furthermore, the putative ZAR1 ortholog in tomato, *SlZAR1* (Solyc02g084890) also failed to trigger an immune response when transiently co-expressed with JIM2 and XOPJ4. By comparing ZAR1 proteins from several plant species, the authors showed the presence of several missense mutations in SlZAR1, leading them to speculate that it could be nonfunctional. However, more analyses are required to confirm this. When analysing the putative protein-protein interactions of SlZAR1 in the STRING database (Szklarczyk *et al.*, 2019), it was predicted to interact with the ortholog of the Arabidopsis RLK Suppressor Of BIR1 (SOBIR1), a receptor-like kinase (RLK) that, together with a close homologue SOBIR1-like, is known to interact with receptor-like proteins to trigger a hypersensitive response in tomato upon recognition of fungal effectors (Liebrand *et al.*, 2013). It is intriguing to know if the ZAR1 resistosome is indeed present in tomato and which components it may recruit. A BLASTP analysis of ZRK13 and ZRK14 of Arabidopsis against tomato, revealed high homology with two proteins Solyc09g014730 and Solyc06g060680. Both are annotated as Receptor-like protein kinases. Solyc09g014730 is further mentioned to be a Wall-Associated Kinase (Wak) and clusters together with three additional *Wak* genes, one of which (*SlWak1*) was recently characterized to encode a positive regulator in PAMP-triggered immunity against *Pseudomonas syringae* pv. tomato (Zhang *et al.*, 2020). Interestingly, Solyc06g060680 was also predicted to interact with SlZAR1 by the STRING analysis. Solyc06g060680 is located very close to the *Ol-1* gene identified in this thesis, Solyc06g060800 (**Chapter 5** of this thesis). This region also overlaps with the fine-mapped interval of *Ol-qtl1* (Faino *et al.*, 2012). In this sense, it will be interesting to investigate if the gene underlying this *Ol-qtl1* is an RLK-like gene that might be interacting with *SlZAR1,* or an allelic variant of *Ol-1*.

Susceptibility factors to PM

Resistance to PM can be the result of the activation of different components of the plant immune system. Furthermore, monocot- and dicot- PM pathogens seem to be able to exploit common susceptibility factors in their hosts. These host genes whose absence results in the inability of the pathogens to complete their life cycles, are considered *Susceptibility* (*S*) genes. The use of *S* genes is proposed as a suitable alternative to obtain more durable, broad-spectrum resistance and particularly important in the absence of *R* genes in certain crops (Pavan *et al.*, 2010; Gawehns *et al.*, 2013; van Schie & Takken, 2014). Van Schie and Takken (2014) listed more than 30 genes that contribute to susceptibility to PM. However, for *S genes* to be used in breeding, the possible pleiotropic effects that their loss-of-function may have must be taken into account. The classical example of a susceptibility (*S*) gene used in agriculture for durable resistance is precisely found in the context of resistance to the PM pathogen, *Bgh*. For decades, *mlo* mutated alleles have been used in breeding of several barley varieties (Jørgensen, 1992; Lyngkjær *et al.*, 2000; Reinstädler *et al.*, 2010; Reviewed by Kusch & Panstruga, 2017).

Forward genetics screening of mutants has been employed to identify susceptibility factors to PM, as is the case of the six *powdery mildew resistant* (*pmr*) Arabidopsis mutants (Vogel & Somerville, 2000). This screening led to the identification of *pmr4*, the *GLUCAN SYNTHASE-LIKE5* (*GSL5*), also known as *CALLOSE SYNTHASE12* (*CalS12*), whose impairment results in resistance to PM associated with the accumulation of salicylic acid (Jacobs *et al.*, 2003; Nishimura, 2013). PM resistance conferred by impairment of *pmr4* was further shown to be conserved in tomato (Huibers *et al.*, 2013). In **Chapter 4** of this thesis, I describe the generation of tomato *pmr4* mutants via CRISPR/Cas9 that displayed reduced but not complete loss-of-susceptibility to *On*. The resistance observed in our mutants was indeed associated with the activation of the salicylic acid pathway. Host immune system responses activated by this pathway are known to involve a plant growth trade-off (van Butselaar & Van den Ackerveken, 2020). However, further phenotypic analyses and yield estimation of the *pmr4* mutants should be carried out to evaluate this fitness cost.

With the generation of our *pmr4* mutants, we set the basis for the CRISPR/Cas9-based protocol for gene identification that was later used in the other chapters of this thesis. Gene knockout can provide a better resolution than posttranscriptional silencing for functional studies. However, in practice, partial silencing of an *S* gene could sometimes reduce the severity of pleiotropic effects compared to full knockouts. Nevertheless, advances in the CRISPR/Cas9 technology open up additional possibilities for disease resistance engineering, such as single base editing of pathogen effector targets and modification of *S* genes promoter regions (Schenke & Cai, 2020).

Lessons learnt from atypical resistance: the *Ol-1* gene in tomato

Non-NLR-based resistance in the tomato-*On* pathosystem is seemingly the norm. Out of the 9 loci known to confer resistance, the *Ol*-4 gene, originating from *Solanum peruvianum* LA1272, and the likely allelic variant *Ol-6*, are the only identified *typical R* genes (Seifi *et al.*, 2011). A fast HR at the fungal penetration sites completely halts the pathogen development in *Ol-4*- and *Ol-6*-carrying plants upon PM infection (Bai *et al.*, 2005). On the other hand, a slow, multicellular HR is associated with the resistance conferred by *Ol-1*, *Ol-3* and *Ol-5* (Bai *et al.*, 2005; Li *et al.*, 2007). The identification of the *Ol-1* gene has been an ongoing effort since its mapping more than 25 years ago (van der Beek *et al.*, 1994). The characterization of this gene has been particularly challenging due to its incomplete nature. Transient posttranscriptional downregulation using virus-induced gene silencing (VIGS) of the candidate genes was insufficient to provide a clear resolution on the differences in resistance responses. The development of CRISPR/Cas9-targeted mutagenesis offered new opportunities to characterize the candidate genes with a better resolution by analysing the knockout phenotypes upon PM inoculation.

In **Chapter 5** of this thesis I describe the fine-mapping and characterization using CRISPR/Cas9 of the candidate genes for *Ol-1* and the identification of Solyc06g060800, encoding a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, as the putative gene responsible for the *Ol-1* resistance. Genes from this family are known to catalyse a diverse range of oxidative reactions (reviewed by Islam *et al.*, 2018). The closest ortholog of Solyc06g060800 in Arabidopsis is AT4G16765, which has recently been reported as a putative *1-Aminocyclopropane-1-Carboxylic Acid Oxidase* (*ACO*, Ahmadizadeh *et al.*, 2020). ACOs are known to catalyse the last step in the production of the phytohormone ethylene and have been shown to be the rate-limiting step in its production (Houben & Van de Poel, 2019). In a previous study, measurement of the relative expression of reporter genes for plant hormones after *On* inoculation (Kissoudis *et al.*, 2017) revealed an upregulation of *Chitinase9* (*Chi9*), a marker gene for the ethylene pathway (Barry *et al.*, 2001), in NIL-*Ol-1* at 5 days post-inoculation (dpi). Together, this suggests the involvement of the ethylene hormonal pathway in the resistance to PM displayed in *Ol-1*-carrying plants.

The plant hormone salicylic acid is known to be important for the signalling and regulation of immune responses and cell death in resistance against PM and other biotrophic pathogens, (reviewed by Kuhn *et al.*, 2016). Conversely, the antagonistic ethylene/jasmonic acid pathway is known to be involved in the resistance to necrotrophic pathogens (Robert-Seilaniantz *et al.*, 2011). However, evidence suggests that ethylene signalling also contributes to the immune responses to PM. In Arabidopsis, it was shown that constitutive activation of the ethylene pathway in the *cev1* mutant resulted in increased resistance against *E. cichoracearum*, *E. orontii*, and *On* isolate Oxford (Ellis & Turner, 2001). Furthermore, in the diploid einkorn

wheat *Triticum urartu*, it was recently found that *TuACO3* expression is induced upon *Bgt* infection, resulting in ethylene accumulation-associated host defence (Zheng *et al.*, 2020).

Previous studies on the identification and characterization of *Ol-1* suggested that *Ol-1* interacts with *Ol-5*, a close by locus in chromosome 6, to mediate delayed cell death leading to resistance to *On* (Seifi, 2011). Confirmation of such interaction should be carried out. Furthermore, due to the characteristics of the responses associated with *Ol-1*, it has been hypothesized to be an enhancer of an ethylene-involved basal defence. The identification of Solyc06g060800 as the putative gene responsible for the *Ol-1* resistance will help to confirm this interaction. Further studies, most importantly overexpression of this gene in a susceptible background, will aid to confirm these results and the involvement of the ethylene pathway in *Ol-1* resistance. The identity and characteristics of this gene, however, are further evidence that the resistance to *On* in tomato is typically non-reliant on NLR recognition.

Additional to the work of this thesis, we screened a set of 10 commercial tomato cultivars reported to be (incompletely) resistant to *On*. Among these, we identified two cultivars displaying a high level of resistance and two with an intermediate level of resistance against *On* at the young plant stage when compared to the susceptible cultivar Moneymaker. Genotyping with markers linked to the known resistance loci suggested the presence of a resistant *Ol-1* allele in the highly resistant and one of the intermediate resistant cultivars. As this study was performed before the identification of the Solyc06g060800 as the putative *Ol-1* gene, it will be interesting to sequence the alleles of this gene in these two cultivars and compare them with the *Ol-1* allele described in **Chapter 5**. Although *Ol-1* is a non-typical resistance gene, confirmation of the presence of this allele in the tested cultivars would show *Ol-1* to be a suitable gene for commercial breeding in tomato and would further support the notion that non-NLR components of the plant immune system can be used for resistance breeding.

Oidium neolycopersici and the two-front battle

In **this thesis** I studied the resistance responses to *On* from two perspectives: tomato and Arabidopsis. In tomato, *Ol-4* and *Ol-6*, mapped to a cluster of genes encoding NLRs are known to confer resistance against *On* by triggering a fast HR (Seifi *et al.*, 2011). However, the interaction of these proteins with an *On* effector remains to be elucidated (Fig. 1). In the first part of this thesis, we showed that *ZRK13* is required for resistance against *On* in Arabidopsis accessions Bla-6 and Litva. I speculate that this resistance is dependent on the interaction with ZAR1 and an *On* effector (Fig. 1). Additionally, impairment of susceptibility factors such as *MLO* and *PMR4* are known to confer broad-spectrum resistance to PM pathogens. Resistance conferred by the loss-of-function of *PMR4* is associated with the accumulation of salicylic acid (Fig. 1).

Lastly, the dominantly inherited gene *Ol-1* in tomato has been proposed to be an enhancer of basal defence. Our results suggest this response is related to the accumulation of ethylene (Fig. 1).

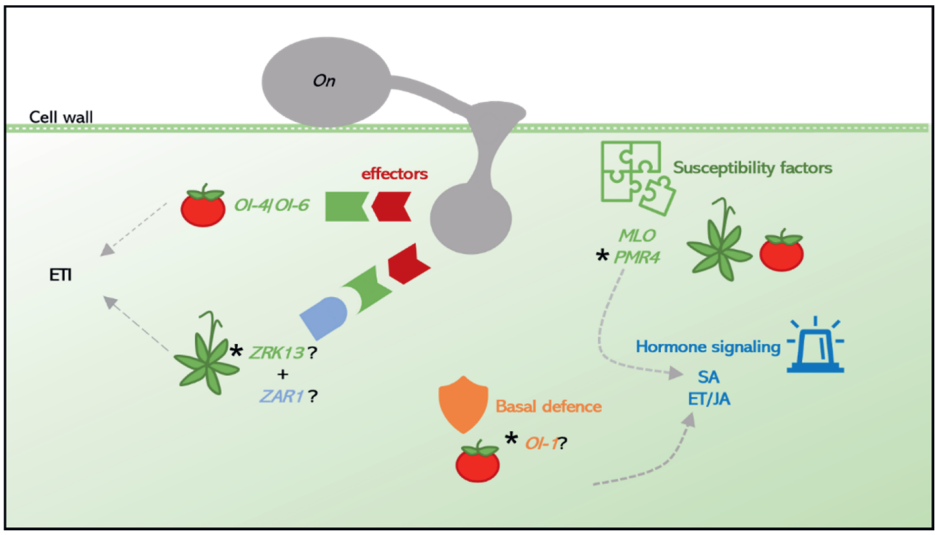

Figure 2. Resistance to *Oidium neolycopersici* (*On*) in tomato and Arabidopsis. Graphical representation of the components of resistance studied in **this thesis**. Components of resistance described in the present work are indicated with a (*). Effector-triggered immunity (ETI) is suggested to be achieved by the perception of *On* effectors by Ol-4 and Ol-6 in tomato. I speculate that ZRK13 interacts with ZAR1 to trigger ETI in Arabidopsis. *Ol-1* is hypothesised to be an enhancer of basal resistance by triggering the ethylene- (ET-) dependent responses. Susceptibility factors are known to be conserved in tomato and Arabidopsis. Resistance in *pmr4* mutants is associated with the accumulation of salicylic acid (SA).

The importance of knowing the enemy

As previously mentioned, dicot-infecting PMs are, in comparison to monocot-infecting ones, less extensively studied and are mainly driven by the research on the model plant Arabidopsis. The research described in the present thesis, by focusing on the Arabidopsis- and tomato-*On* pathosystems, addresses previous questions regarding the host genetic components and responses that result in resistance to this pathogen, but also outlines new questions on the nature of these interactions. Addressing these new questions could be largely aided by more in-depth studies on the pathogen side. Genome sequencing and ~omics studies can help elucidate the effector arsenal that *On* uses to infect its many hosts. The identification and characterization of CSEPs of *On* could improve the understanding on its pathogenesis and aid on the further development of new and sustainable means for control. Once characterized, effectors could be used to screen for interacting or targeted host proteins, which could lead to the identification of *S* gene candidates (Gawehns *et al.*,

2013), or the generation of resistant plants by expressing host-induced gene silencing (HIGS) constructs targeting such effectors (Schaefer *et al.*, 2020).

Conclusion

Resistance to *On* in tomato or Arabidopsis can be the consequence of the activation of a diverse set of immune responses in the host. The work reported in **this thesis** is reflective of such diversity. We have explored the natural resistance in Arabidopsis and identified *ZRK13* as a novel component of resistance in accession Bla-6. Furthermore, through an independently-executed mapping effort, we have found *ZRK13* to contribute to resistance response in accession Litva, together with an additional locus in chromosome 3. Additionally, we made use of the CRISPR/Cas9 technology to further understand the resistance conferred by the loss-of-function of the susceptibility factor *PMR4*. And finally, we made use of this technology to aid in the characterization of the candidate genes for the non-NLR-encoding *R* gene *Ol-1*, identifying Solyc06g060800 as the putative gene responsible for this resistance. Together, these results contribute to the understanding of the diversity of genetic components of resistance against *On* with the aim of aiding in the development of breeding strategies that result in a sustainable management of this disease.

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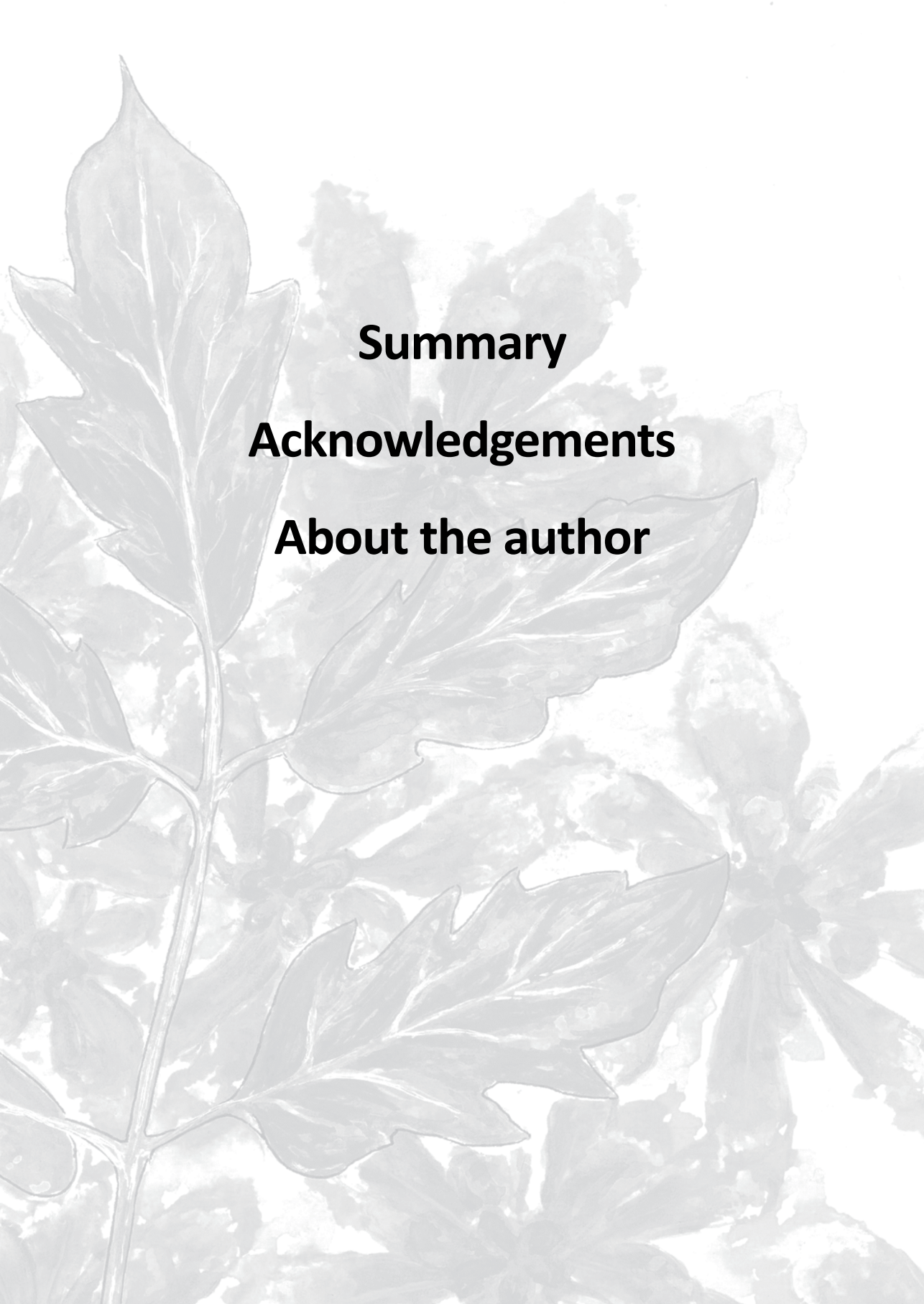

Summary

Powdery mildew (PM) is one of the most common plant diseases in the world, occurring in many economically important food and ornamental crops. The characteristic symptoms of this disease are easily recognizable as a white powder that extends across the aerial parts of the plants. The causal pathogens of PM are obligate biotrophic fungi from the Erysiphales order that obtain nutrients from the living tissue of their hosts. To this day, chemical control of PM remains a common practice in many crops. However, the use of fungicides has a negative impact on the environment. For this reason, breeding for resistant varieties stands out as an essential tool to achieve a sustainable management of this disease.

In tomato, PM is caused by *Oidium neolycopersici* (*On*), a polyphagous fungal species that threatens the production of this crop worldwide. The current understanding of this disease and the mechanisms through which plants are able to withstand it comes from the research done in two pathosystems. On the one hand, in the late 1980's, an outbreak of *On* in cultivated tomato in Europe urged researchers to find natural sources of resistance to this disease. This drove the research on the identification of *Resistance* (*R*) loci in wild relatives of tomato. On the other hand, *On* can also infect the model plant *Arabidopsis thaliana* (Arabidopsis). This model organism offers many advantages for research due to the availability of a large number of scientific resources and information. Apart from *On*, Arabidopsis is a host for three more PM species. Hence, the Arabidopsis-PM pathosystems have been widely used to identify genetic components of resistance and susceptibility. These latter components are particularly interesting, as they seem to be conserved for several species of PMs and can potentially be extrapolated to other plant species. The plant genes whose absence results in the inability of the pathogen to complete their life cycles, are named *Susceptibility* (*S*) genes. The use of *S* genes is proposed as a suitable alternative to obtain more durable, broad-spectrum resistance and is particularly important in the absence of *R* genes in certain crops. The work of this thesis focuses on the exploration and identification of new genetic components of resistance and susceptibility to *On* in both the model plant Arabidopsis and the crop plant tomato.

In the first half of this thesis, I make use of Arabidopsis to identify genetic components of the natural resistance against *On*. In **Chapter 2**, we report the finding of a dominantly-inherited resistance in accession Bla-6. Through a series of recombinant analyses, we fine mapped the locus responsible for the resistance and used CRISPR/Cas9 to knockout the candidate genes. We show that targeted mutagenesis of the *ZED1-RELATED KINASE 13* (*ZRK13*) in Bla-6 results in a susceptible phenotype, thus identifying this gene as a novel component of resistance against *On*. Furthermore, in **Chapter 3**, we found two major QTLs explaining resistance to *On* in accession Litva. Through an independently-executed fine-mapping effort we identified *ZRK13* to be also required for resistance in this accession. However, an additional locus in chromosome 3 is needed for full resistance in Litva. In these two chapters I discuss the identity of this gene and suggest further steps to investigate the molecular mechanisms of this previously uncharacterized resistance component.

The second half of this thesis focuses on the tomato-*On* interaction to study susceptibility and resistance components. In **Chapter 4**, we report the generation, through CRISPR/Cas9-targeted mutagenesis, of tomato knockouts of the susceptibility gene *PMR4*. This gene was first found in a forward genetic screening in Arabidopsis and resistance through its impairment was later found to be conserved in the tomato-*On* pathosystem. We describe the generation and characterization of five different mutation events in the target gene and report a reduced but not complete loss of susceptibility upon inoculation with *On*. With this study, we set the basis for the CRISPR/Cas9-based protocol for gene identification that was later used in the other chapters of this thesis.

In **Chapter 5** we describe the fine-mapping and characterization of the candidate genes for *Ol-1*, a dominant resistance found in *S. habrochaites* accession G1.1560. We show that targeted mutagenesis of Solyc06g060800, a gene encoding a 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily protein, results in increased susceptibility to PM in near-isogenic *Ol-1* background. In this chapter we further discuss the implications of this finding.

In **Chapter 6** I give an overview of the results presented in this thesis and discuss their implications in the plant immunity model. Altogether, the work presented in this thesis contributes to the understanding of the diversity of genetic components of resistance against *On* in both Arabidopsis and tomato, with the aim of aiding in the development of breeding strategies that result in a sustainable management of this disease.

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About the author

Miguel Isauro Santillán Martínez was born in Pachuca de Soto, Mexico on July 24th, 1987, in a family with two older brothers. He had a happy childhood with caring parents that nurtured his curiosity for plants and science from a young age. This curiosity grew into an academic path that led him to obtain the BSc. in Agrobiotechnology Engineering from the Instituto Tecnológico de Monterrey (ITESM), Mexico, in 2011. By the time he finished his Bachelor, he already knew he wanted to pursue a MSc degree. However, shortly before this, he worked as an intern and research assistant in the International Maize and Wheat Improvement Center (CIMMYT). His stay at CIMMYT helped him to find his passion and interest in plant breeding.

In 2013, he started his MSc in Plant Biotechnology with a specialization in Molecular Plant Breeding and Pathology at Wageningen University. He did both of his MSc theses in the Department of Plant Breeding. For his major thesis, he joined the Breeding for Resistance group, where he worked with resistance and non-host interactions to powdery mildew in tomato. For his minor thesis, he joined the Biobased Economy group and worked in the characterization of miscanthus genotypes for biofuel production. During his stay in Wageningen, Miguel made many friends that have been supporting him ever since.

The work on his MSc major thesis encouraged him to continue working in the same group and topic. In 2016, Miguel started his PhD under the supervision of Prof. Yuling Bai, Prof. Richard Visser and Dr Anne-marie Wolters. During this time, he worked on the identification and characterization of resistance and susceptibility genes to powdery mildew in tomato and Arabidopsis. The results of this work are presented in this thesis.

Apart from his own city of birth, Pachuca, Wageningen has been the place where Miguel has lived for the longest period of time in his life. The town, Wageningen University & Research, and the people he has met there have changed his life and have helped him to transform his childhood curiosity for plants and science into a scientific and professional career. He is very excited to see where this path will take him.

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