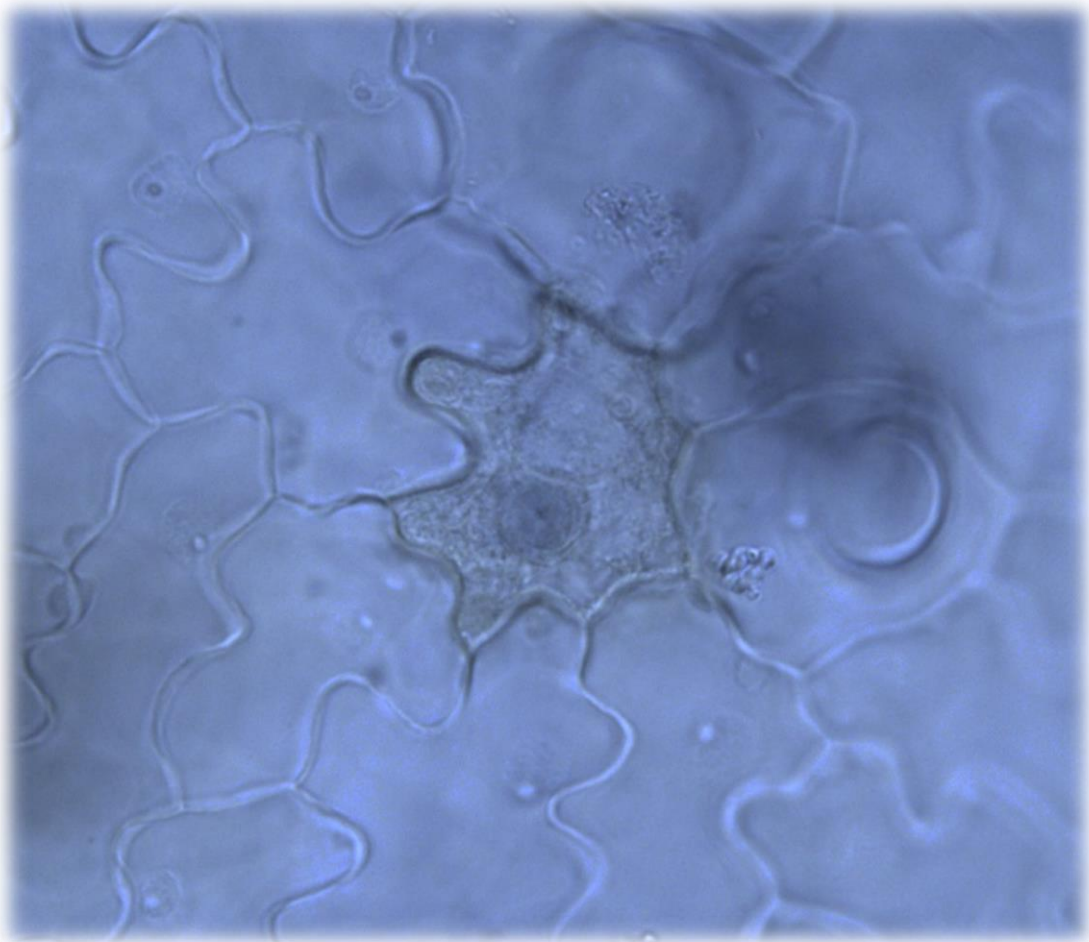




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The study of *SIPMR4* CRISPR/Cas9- mediated  
tomato allelic series for resistance against  
powdery mildew



Eleni Koseoglou  
MSc Minor Thesis Report

Cover photo: Hypersensitive response induced by the formation of a haustorium by the fungus, *Oidium neolycopersici*, in an epidermal cell of a resistant *slpmr4* mutant.

**The study of *SIPMR4* CRISPR/Cas9-mediated tomato allelic series for  
resistance against powdery mildew**

MSc Minor Thesis Report

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by

Eleni Koseoglou

921005469020

PBR-80424

Supervisors:

Dr. Yuling Bai

Miguel Santillán Martínez

Dr. Michela Appiano

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Wageningen University and Research,

Wageningen

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## **Abstract**

Powdery mildews are among the most common phytopathogenic fungi. They are able to infect more than 10000 plant species. Tomato is host to the powdery mildew pathogen *Oidium neolycopersici*, which is considered to cause one of the most devastating diseases of cultivated tomatoes.

*PMR4* is a gene encoding for a callose synthase. Loss-of- function of this gene has been shown to lead to resistance against powdery mildew in Arabidopsis and tomato. The mechanism of resistance in Arabidopsis has been associated with the constitutive activation of the SA pathway and the induction of HR upon pathogen challenge.

In this project, we studied the effect of the CRISPR/Cas9- mediated knockout of the *PMR4* ortholog (*SIPMR4*) in tomato allelic series on resistance against *O. neolycopersici*. To this purpose microscopic analyses of T1 mutant plants, disease assays and gene expression analyses on T2 progenies of the mutant lines were performed.

The outcome of this study contributes to the understanding of the resistance mechanism of *slpmr4* mutants against powdery mildew. Through our experiments we were able to confirm the involvement of SA and the induction of HR in the resistance mechanism of the mutants. Furthermore, our results provide evidence on the nature of the resistance, suggesting that impairment of the *SIPMR4* gene leads to partial resistance against the powdery mildew pathogen *O. neolycopersici*. Lastly, through our results the use of CRISPR/Cas9 as a tool to produce targeted inversions is demonstrated.

**Key words:** *PMR4* gene, tomato, powdery mildew, susceptibility, HR

## **Introduction**

Powdery mildews (PM) are among the most commonly found phytopathogenic fungi (Glawe, 2008; Ale-Agha et al., 2008). They are obligate biotrophic fungi that belong to the *Eryshipales* order and are capable of infecting more than 650 monocot and more than 9000 dicot species (Schulze-Lefert and Vogel, 2000), causing diseases to many economically important plant species (Braun, 2011). Tomato (*Solanum lycopersicum*) is host to three powdery mildew pathogens, namely *Leveillula taurica*, *Oidium lycopersicum* and *Oidium neolycopersici* (Jacob et al., 2008). The disease caused by *O. neolycopersici* is one of the most devastating diseases of cultivated tomatoes (Lebeda et al., 2014), as all tomato cultivars have been found to be susceptible to this pathogen (Seifi et al., 2014).

Traditional resistance breeding is based on the introgression of dominant resistance genes (R genes) from wild species into elite cultivars (Gawehns et al., 2013). R genes mostly encode for receptors of the nucleotide-binding leucine-rich-repeat (NB-LRR), that are able to recognise corresponding products of pathogen avirulence (*Avr*) genes. This recognition leads to a complex signalling cascade resulting in resistance, often associated with hypersensitive response (HR) and cell death (Jones and Dangl, 2006). However, because the resistance that R genes confer is most of the times highly race specific, it can be easily overcome by the pathogen (Pavan et al., 2010).

An alternative to R genes in resistance breeding is the impairment of susceptibility genes (S genes) (Pavan et al., 2010). S genes include all plant genes manipulated by the pathogen for its proliferation and the promotion of disease development (Gawehns et al., 2013; van Schie and Takken, 2014). Loss-of-function of S genes is expected to lead to durable, broad spectrum, recessively inherited resistance (Pavan et al., 2010; Berg et al., 2015). However, because S genes may have conserved functions in plant development, their mutations can also lead to adverse pleiotropic effects. For this reason, it is important that side effects caused by mutation of S genes are carefully assessed (van Schie and Takken, 2014).

Possibly, the most well-known example of an S gene is the *MLO* gene in barley (Büschges et al., 1997). Loss-of-function *mlo* mutants in barley have been used for the production of PM resistant barley cultivars for the last 40 years (Büschges et al., 1997; Jørgensen, 1992). Since its identification, the *MLO* gene has been shown to be conserved across many plant species, including Arabidopsis, tomato, pepper, wheat, apple and grape (Consonni et al., 2006; Zheng et al., 2013; Elliott et al., 2002; Pessina et al., 2014; Feechan et al., 2009).

Besides *MLO*, multiple *S* genes whose impairment leads to resistance against powdery mildew have been identified and characterized in *Arabidopsis* (Huibers et al., 2013). One of these genes is Powdery Mildew Resistance 4 (*PMR4*). *PMR4* (or *GSL5*) was first identified in an *Arabidopsis* mutant screen for loss of susceptibility to the PM pathogen *Erysiphe cichoracearum* (Vogel and Somerville, 2000). *PMR4* belongs to the Glucan Synthase- Like (GSL) gene family, which comprises 12 genes in *Arabidopsis* (Barratt et al., 2011). *PMR4* encodes for a callose synthase responsible for the production of callose in response to biotic and abiotic stresses (Nishimura et al., 2003). Callose deposition in papillae is an induced defense response that can limit the entry of the pathogen. Overexpression of the *PMR4* gene leads to complete resistance against PM in *Arabidopsis*, due to early elevated callose deposition (Ellinger et al., 2013). However, loss- of- function *pmr4* mutants that are unable to deposit callose at the site of the attempted fungal penetration, surprisingly show enhanced resistance to PM (Jacobs et al., 2003; Nishimura et al., 2003). The resistance exhibited by the *pmr4* mutants has been attributed to the constitutive activation of the salicylic acid (SA) pathway. Double- mutant analysis has led to the observation that blocking of the SA pathway is enough to restore full susceptibility in the mutants. Thus, it has been suggested that *PMR4* acts as a negative regulator of the SA pathway (Nishimura et al., 2003). In tomato two *PMR4* homologs annotated as *Solyc02g078230* and *Solyc07g053980* are found (Huibers et al., 2013). Recent studies have shown that silencing through RNAi of the *Solyc07g053980* homolog in tomato leads to enhanced resistance against *O. neolyopersici* (Huibers et al., 2013). Moreover, silencing of the ortholog in potato, namely *Sotub07g019600*, led to partial resistance against the oomycete *Phytophthora infestans* (Sun et al., 2016). In both cases, no significant pleiotropic effects were observed (Huibers et al., 2013; Sun et al., 2016).

The use of genome editing tools for targeted mutation of genes is a new method that has been used in many organisms (Wang et al., 2015). Genome editing with sequence specific nucleases (SSNs) is a powerful tool for plant biology research (Samanta et al., 2016; Bortesi and Fischer, 2015). It allows the conduction of reverse genetics, genome engineering and targeted transgene integration experiments in a precise and efficient way (Bortesi and Fischer, 2015). Among the SSNs, CRISPR/Cas9 is the most recently characterized and rapidly evolving genome editing tool with applications in a wide range of organisms. CRISPR/Cas9 uses a bacterial-derived DNA endonuclease, namely Cas9 (Samanta et al., 2016). In the CRISPR/Cas9 system, the Cas9 endonuclease is directed by a

20 bp sequence at the 5' end of a single- guide RNA (sgRNA). The sgRNA acts as a guide of the Cas9 on a specific site on the genome. There the endonuclease is able to cleave double-stranded DNA, leading to deletions, insertions or substitutions at the target sites (Wang et al., 2015). Double stranded breaks (DSBs) created by the SNNs, generate modifications through homologous recombination (HR) or non- homologous end- joining (NHEJ) repair mechanisms (Pan et al., 2016; Bortesi and Fischer, 2015).

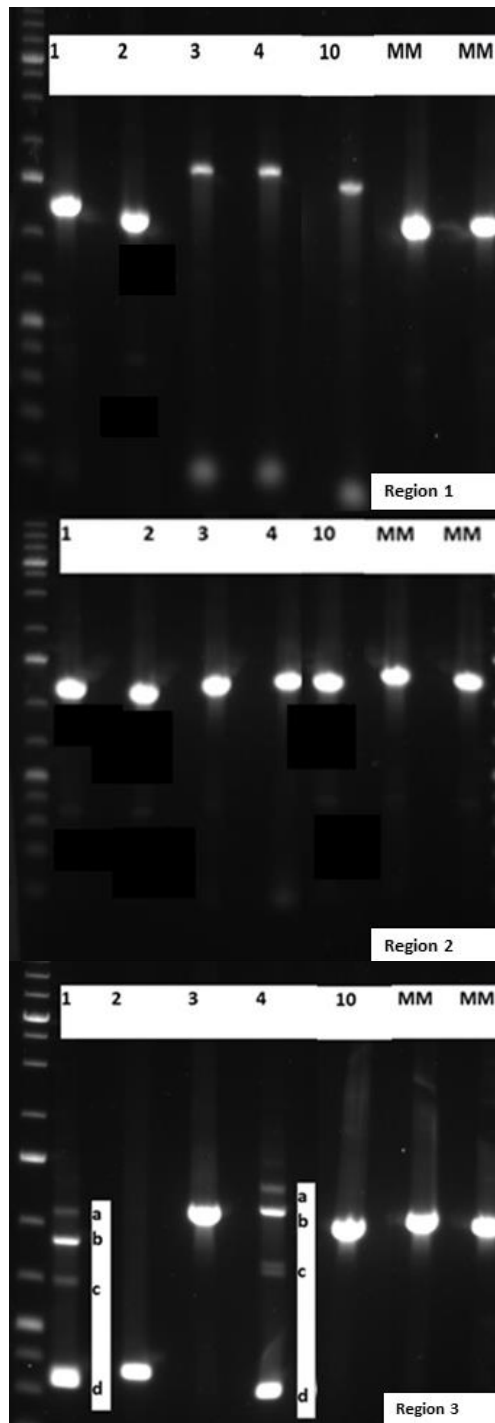
In this study, the CRISPR/Cas9 system was employed to engineer disease resistant tomato allelic series of the tomato *Solyc07g053980* homolog, referred to as *SIPMR4* in this paper. Genotyping, microscopic analyses and gene expression analyses of the generated mutant plants were done, in order to gain insight into the resistance mechanism against powdery mildew in tomato.

## *Results*

### ***Characterization of SIPMR4 mutations in T1 mutants and their segregating progenies***

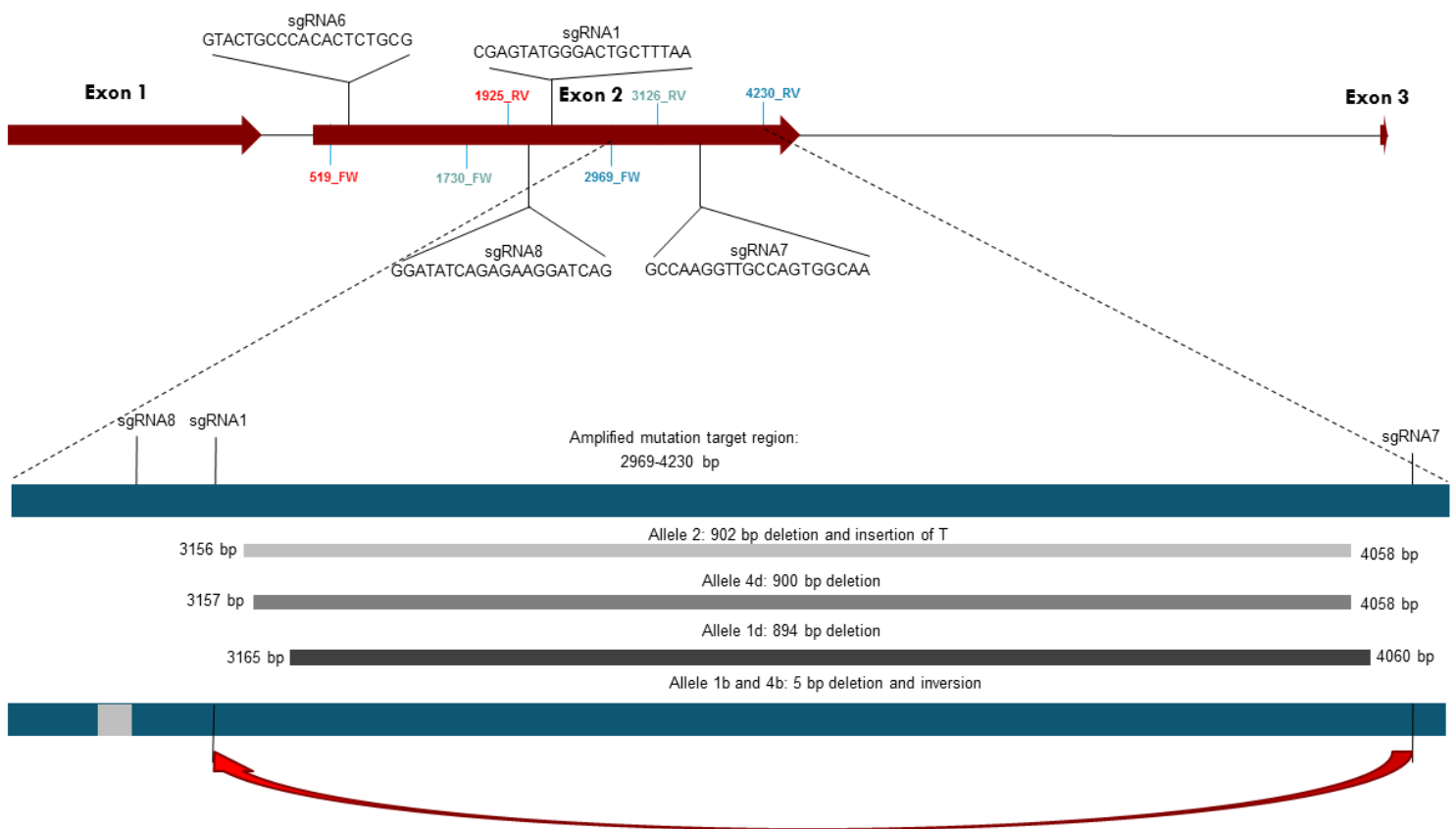
For the genotyping of the mutation target region, three primer pairs giving overlapping products (see Materials and Methods section) were used to amplify the full length of the target region. Twelve mutants were generated. However, for this project only four were studied. The characterization of the mutations was done in the T1 plants of these mutant lines. Among the four T1 lines that were studied, one of them was found to be biallelic homozygous (mutant 2 called TV171010), two biallelic heterozygous (mutant 1 called TV171009 and mutant 4 named TV161212) and the last one did not carry any mutations (mutant 3 called TV161196) (Figure 1). Sequencing of the fragments revealed that different mutations were induced in the *SIPMR4* gene. No mutations were found to be present in mutant TV161196. For the rest of the mutants, deletions and in some cases inversions were induced in the amplified region 3, but no other change was found in the amplified regions 1 and 2 (Figure 1).





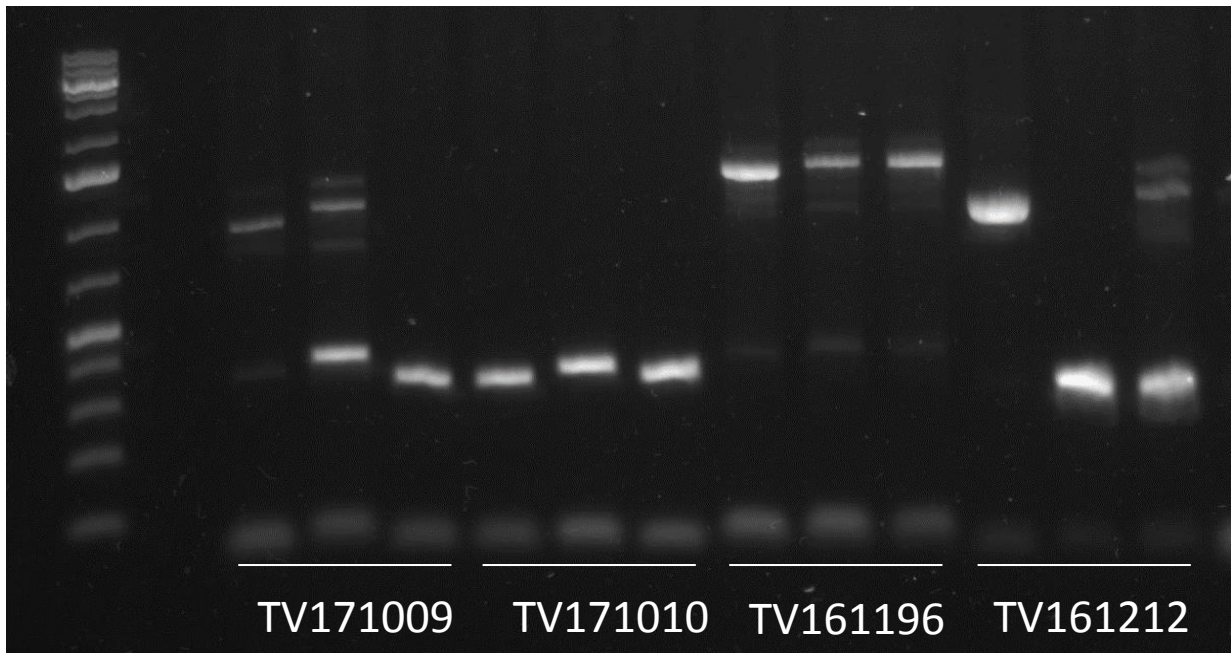
**Fig. 1 Amplification of the second exon targeted by the sgRNAs of the mutation target region.** The mutation target region was divided into three parts by overlapping primer pairs (Region 1-3). Each PCR product was visualized on 1% agarose gel. Numbers and capital letters represent different genotypes: 1-TV171009; 2-TV171010; 3-TV161196; 4-TV161212; 10-TV161209; MM-Moneymaker. Lowercase letters represent the different alleles in heterozygous biallelic mutants. From this point on we will refer to alleles using the number given to their respective mutants and the lowercase letter indicating the allele, in the case of biallelic heterozygous mutants. PCR bands a and c were found to be unspecific after a second amplification of the gDNA of mutants.

Deletions in mutant alleles 1b, 1d and 2 were found to be out-of-frame, leading to an early stop codon and truncation of the produced protein. In alleles 1d and 2 an 894 bp and a 902 bp deletions were found in positions 3165–4060 bp and 3156–4058 bp, respectively. In addition, in allele 2 the insertion of a T was found at the point of the deletion. In allele 4d, a 900 bp in-frame deletion in position 3157–4058 bp, leading to the removal of 300 amino acids in the produced protein was found. Interestingly, multiple mutations were found in allele 4b and 1b. A 5 bp deletion (AAGGA) was found in sgRNA8 and an inversion between sgRNA1 and sgRNA7 starting in position 2873 bp were found, in both cases (Figure 2).



**Fig. 2 Mutations produced by the CRISPR/Cas9 system on the second exon of the *SIPMR4*.** A schematic representation of the *SIPMR4* gene and the mutations induced by the CRISPR/Cas9 system. Red arrows represent the three exons of the gene. The blue line represents the amplified region 3 of the exon where the mutations were found. The four sgRNAs including their sequences and the primers used for the amplification of the exon are given on the scheme. Primers sharing the same colour on the scheme represent primer pairs. The grey lines represent the deletions induced by the CRISPR/Cas9 system. The red arrow indicated the position of inversions between sgRNAs 1 and sgRNAs in alleles 1b and 1d. For each mutation the positions and the size of the deletions are given.

Seeds of the *slpmr4* mutants were obtained after self-fertilization. Since all the mutations were found in region 3, primer pair SIPMR4\_Fw\_2969 (5'-GCG AAT GCG TAG AGA AGG AA-3')/ SIPMR4\_Rv\_4230 (5'-CCC CAC TAA GTG CCA GGT AA-3') was used to genotype the segregating progenies of mutants 1 and 4, named TV171009 and TV161212, respectively. Genotyping confirmed the segregation of the different alleles in the T2 mutants (Figure 3).



**Fig. 3 Segregating alleles in the T2 progenies.** The primer pair amplifying region 3 that was used for genotyping of the T1 mutants was used for the genotyping of the T2 progenies. As expected, the progenies of biallelic heterozygous mutants (TV171009 and TV161212) segregated for the two alleles. Progenies of biallelic homozygous mutants (TV171010 and TV161196) did not segregate.

### ***Microscopic analyses of T1 mutants***

The effect of knocking-out *SIPMR4* was evaluated by means of microscopy in the T1 mutant lines. Compared to MM, fungal growth was significantly reduced in the mutant lines, due to the presence of HR beneath the appressorium. The rate of primary HR was 22%, 81.8%, 91.6% and 84.7% in genotypes cv. MM, TV171009, TV171010 and TV161212, respectively (Table 1). However, in some cases the pathogen was able to penetrate the epidermal cells. The rate of primary haustorium formation was significantly different between the genotypes, being equal to 11.3%, 47.9% and 36.9% in TV171009, TV171010 and TV161212, respectively (Table 1). Intriguingly, the development of spores was remarkably different

among the genotypes. On TV171009 the fungal growth was predominantly stopped after the formation of one hypha (90% of total infection units) (Table 1). On genotypes TV171010 and TV161212 individual fungal spores were able to form two or more secondary hyphae (44% and 32% of total infection units, respectively). Interestingly, attachment of spores seemed to be hampered on genotype TV161196. Only a few non germinated spores were found on the microscopic slides (Table 1).

**Table 1 *Oidium neolycopersici* growth.** Development of *Oidium neolycopersici* on the susceptible genotype Moneymaker and the four knockout lines carrying different mutations on the SIPMR4 gene.

Genotype	Per AP		Per Primary HS			Hyphae per IU					
	Primary AP per IU <sup>y</sup>	Primay HR <sup>y</sup>	Primary HS <sup>y</sup>	Secondary Hyphae	Secondary HR	Secondary HS	1	2	3	4	5
MM	100	22	80	100	10	85	20	8	30	42	0
TV171009	88	81.8	11.3*	80	20	60*	90	10	0	0	0
TV171010	96	91.6*	47.9	95.6	52.1	47.8	56	16	26	2	0
TV161196	No attachment of spores										
TV161212	92	84.7*	36.9	94.1	29.4	47.05	68	16	12	4	0

<sup>y</sup> represent percentages

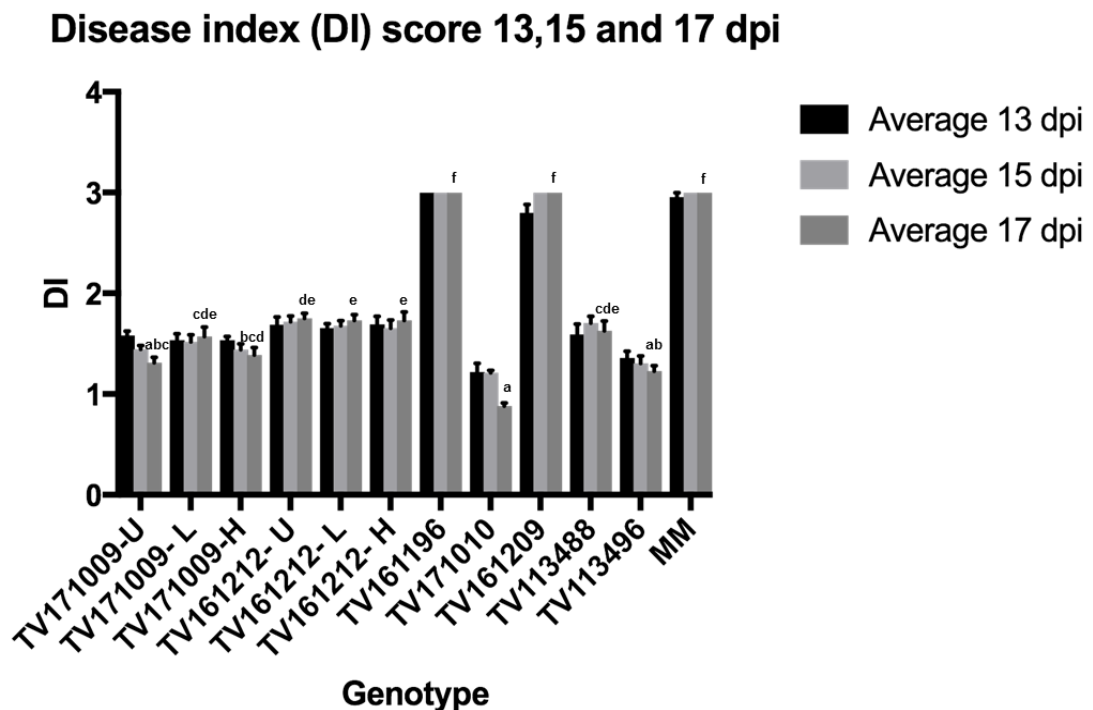
Asterisks represent statistically significant differences between the genotypes and cv. MM as it was calculated by a t- test. \*: p<0.05

IU= infection units, AP= appressorium, HS= haustorium

### Phenotypic evaluation of T<sub>2</sub> CRISPR/Cas progenies

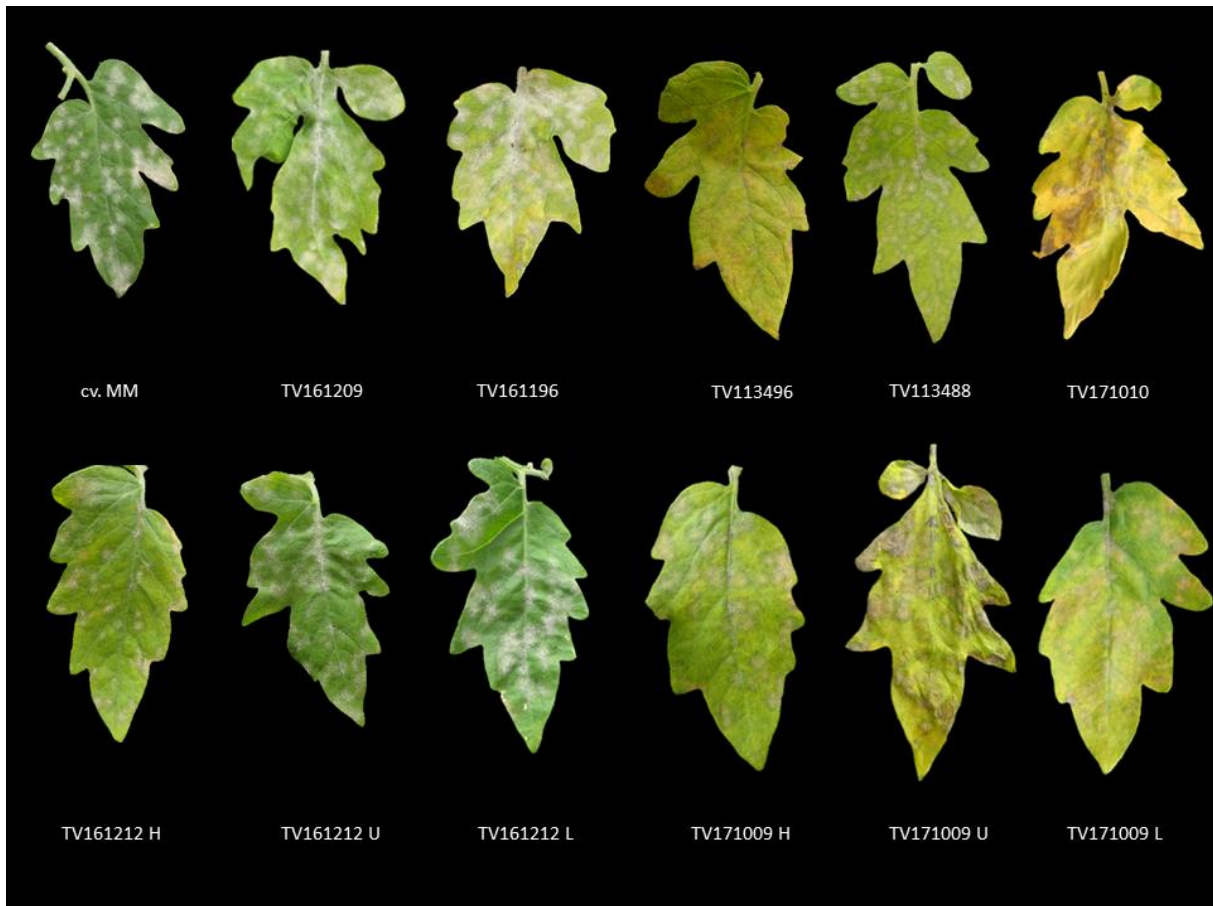
By the time the experiments began seeds of four of the T<sub>1</sub> mutant lines were available, namely TV171009, TV171010, TV161196 and TV161212. The progenies of these mutants were used in the following experiments (S. Table 1).

In the disease assay, T<sub>2</sub> mutant plants and RNAi silenced lines were used to assess the effect of impairing the *SIPMR4* gene on resistance against PM. The Wageningen *O. neolyopersici* strain was used in the assay. Overall, the *slpmr4* mutant plants and the RNAi lines were more resistant to the pathogen compared to the susceptible cv. Moneymaker. A general decline in the disease index (DI) score of the mutant plants was recorded between 13,15 and 17 dpi (Figure 4). Phenotypically, the biallelic homozygous mutant line TV171010, carrying a 902 bp out-of-frame mutation, was found to be the most resistant line. However, statistically the resistance level of TV171010 was comparable to line TV171009 and the RNAi line TV113496. Line TV161196, that was found not to carry any mutations, was as susceptible as the control cv. MM plants. A clear association between the mutated alleles and the resistance level of the plants was not found (Figure 4).



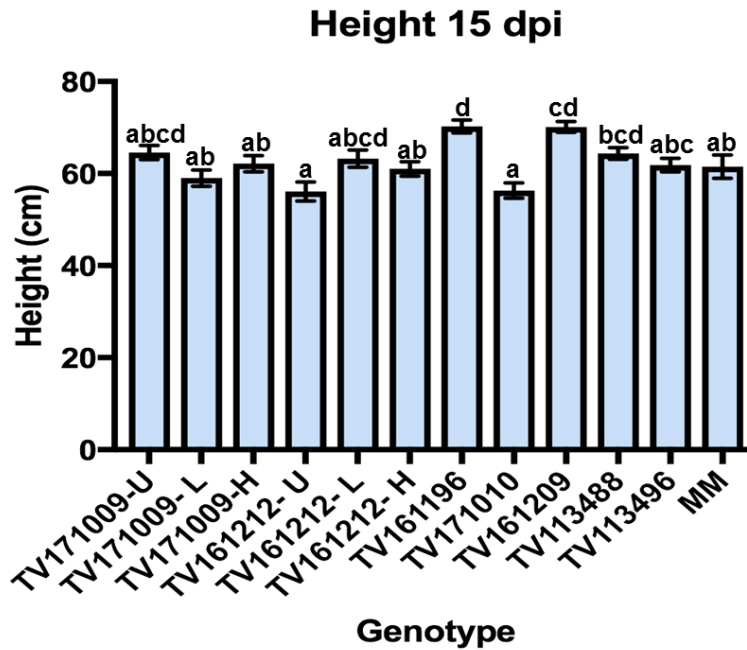
**Fig. 4 Average disease index score 13, 15 and 17 dpi.** The average DI for each genotype 13, 15 and 17 days after pathogen inoculation is given in the graph. At least 14 biological replicates for each genotype were used in the disease assay. Bar represent standard errors. Letters refer to significant differences at 17 dpi as inferred by a Tukey's post-hoc test ( $p < 0.05$ ).

Less sporulation of the pathogen was observed on the *slpmr4* mutants and the RNAi lines compared to MM plants. Moreover, we generally observed in CRISPR/Cas9 mutants chlorosis and wilting of the infected leaves and the formation of a necrotic ring around the fungal colony (Figure 5). In contrast, no necrotic rings were observed on the RNAi lines (Figure 5).



**Fig. 5. Powdery mildew evaluation on plants of segregating T2 lines obtained after targeted knockout of the *SIPMR4* gene to test the involvement of the gene in resistance against *O. neolyopersici*.** The figure shows the phenotypic evaluation at 14 dpi of the pathogen growth on the leaves of different T2 individuals. The genotype of each individual is given beneath its photo.

To better determine possible pleiotropic effects of silencing *SIPMR4* on plant growth, the height of the mutant and wild type plants was measured. Overall, a decrease in the height of the mutant plants was observed. However, between the average height of mutants and of MM there was no statistically significant difference. Lines TV161196 and TV161009 that were as susceptible as MM, were found to be significantly higher than MM (Figure 6).



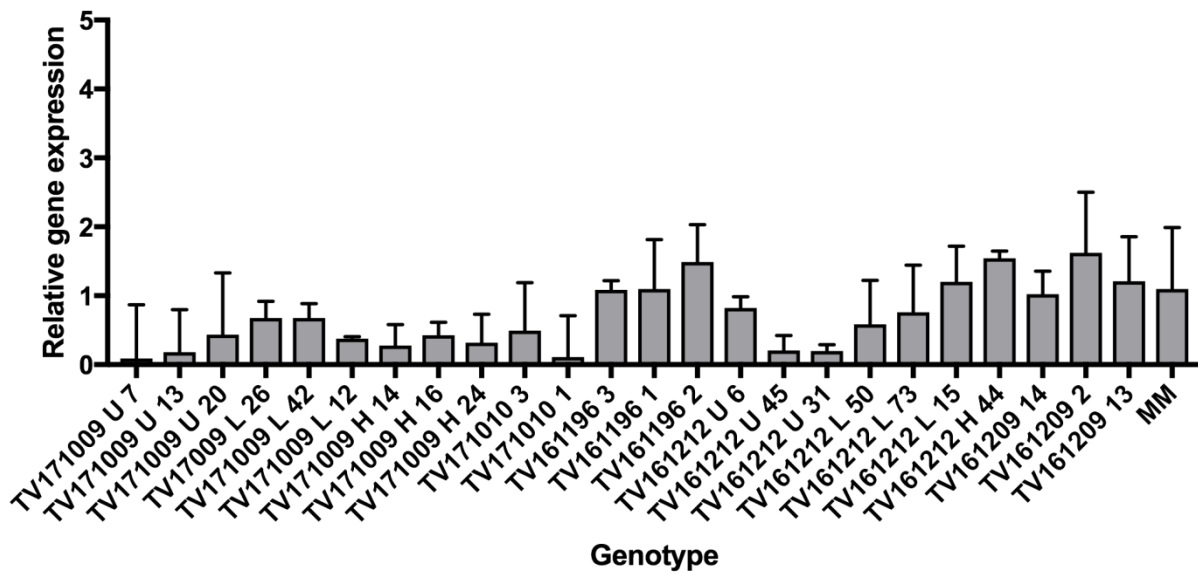
**Fig. 6 Average plant height 15 dpi.** The average height of each genotype 15 days post inoculation is given in the graph. At least 14 biological replicates for each genotype were used. Bars represent standard errors. Letter refer to significant differences, as they were calculated after a Tukey's post-hoc test ( $p < 0.05$ ).

### ***Gene expression of PMR4 and PR1 in T<sub>2</sub> CRISPR/Cas9 progenies***

The possible residual expression of the *SIPMR4* gene was assessed by RT-qPCR in samples of the CRISPR/Cas9 mutants collected before the PM inoculation. Overall, a noticeable variation of gene expression was found (Figure 7). However, the residual expression detected in the more resistant plants of lines TV171009 and TV171010 was significantly lower than the cv. MM and the susceptible lines TV161196 and TV171009. The expression level of the gene in these two susceptible lines was comparable to the expression in MM plants (Figure 7). Surprisingly, the mutants in line TV161212 had a higher expression of the *SIPMR4* gene compared to the other resistant genotypes. In most of the TV161212 plants, the expression of the gene was comparable to MM plants.

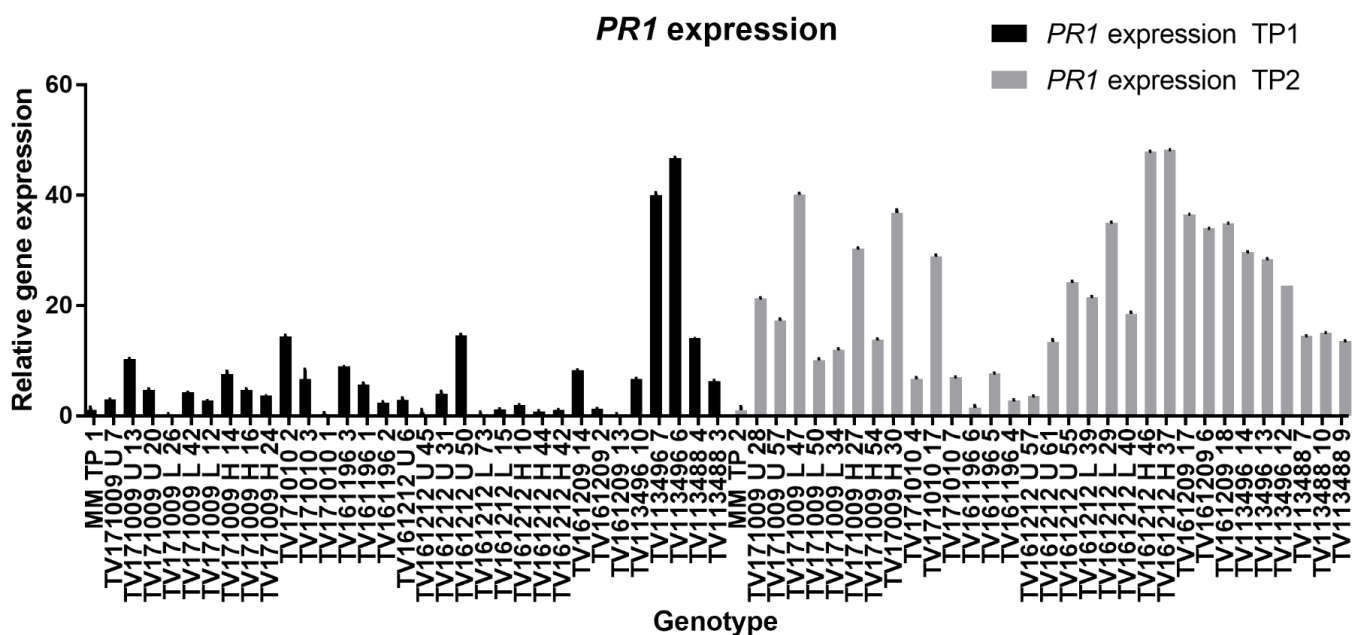


### SIPMR4 expression



**Fig. 7 Relative *SIPMR4* gene expression.** Three individual biological replicates per genotype were used for the quantification of the relative gene expression. Each column represents the average relative gene expression of two technical replicates. Bars represent standard errors.

In order to confirm the constitutive activation of the SA pathway (Nishimura et al., 2003), the expression of the *PR1* marker gene was analysed before (TP1) and after the inoculation (44 hpi indicated as TP2). Our data confirmed that the *PR1* gene is significantly higher expressed in the CRISPR/Cas9 mutants and the RNAi lines compared to the susceptible plants in both time points (Figure 8). Nevertheless, great variation in the expression of the *PR1* gene was found among the *SIPMR4* silenced plants. The *PR1* expression of in the TV113496 RNAi line was found to be significantly higher than in the other silenced genotypes. Overall, the *PR1* expression increased significantly at 44 hpi compared to prior inoculation (MM TP1), except for TV161196. The average  $2^{-\Delta\Delta C_t}$  values of the relative gene expression in the TV161196 progenies were 5.69 and 4.00, in TP1 and TP2, respectively. A t-test between the values of the two time points gave a p-value equal to 0.56. In MM, the expression of *PR1* did not change after the inoculation. The average  $2^{-\Delta\Delta C_t}$  values of the relative gene expression in MM were 1.10 and 1.0 in TP1 and TP2, respectively.



**Fig. 8 Relative PR1 gene expression at TP1 and TP2.** Three individual biological replicates per genotype were used for the relative gene quantification. Each column represents the average relative gene expression of two technical replicates. Bars represent Error bars. Black columns represent the relative gene expression at the first time point (before inoculation). Grey columns represent relative gene expression at the second time point. Labels indicate the genotype of each plant.

## Discussion

In this study, we describe the obtainment of tomato allelic series of *SIPMR4* through CRISPR/Cas9 and their involvement in resistance against powdery mildew. Our results were consistent with previous studies reporting that silencing of the *PMR4* gene leads to enhanced resistance against powdery mildew pathogens (Vogel and Somerville, 2000; Nishimura et al., 2003; Huibers et al., 2013). Moreover, through the results of the *PR1* gene expression analyses and microscopic observations, we were able to further confirm that the main defence mechanism of *pmr4* mutants is based on constitutive activation of the SA pathway and the induction of HR upon pathogen challenge.

Intriguingly, it was observed that different mutations on the *SIPMR4* gene lead to different plant-pathogen interactions. Our microscopic data of the T1 mutants suggest that plants carrying different mutations allow different growth of the pathogen (Table 1). This indicates that in each T1 mutant a faster or slower HR reaction was involved in the defence mechanism. A fast HR leads to arrest of fungal growth at the primary haustorium stage, whereas a slow HR response allows further growth of the pathogen and the formation of both primary and secondary haustoria (Li et al., 2007). As defined by Li et al. (2007), a fast HR reaction was

observed in mutant TV171009, whereas a slow HR reaction was observed in mutant lines TV171010 and TV161212. Furthermore, the attachment of spores on line TV161196 was completely hampered.

However, when disease assays were performed on T2 mutants containing the same mutations of their corresponding T1 mutants, the phenotypic observations did not fully mirror the progression of PM growth observed on their respective T1. The results of our disease assay on the T2 mutants indicate partial resistance of the mutants and a general decline of the DI score between 13, 15 and 17 dpi (Figure 4). Moreover, the resistant phenotype exhibited by the mutants was associated with chlorosis of leaves and the formation of necrotic lesions at the sites where fungal colonies were present. By extrapolating the data from the microscopic analyses of the T1 mutants, one would expect that progenies of TV161196, followed by the progenies of TV171009, would be the ones with the highest resistance level. However, T2 progenies of TV161196 were found to be fully susceptible to the pathogen and progenies of the remaining mutant lines were partially resistant to the pathogen. Nevertheless, the results of the disease assay, indicating that the T2 mutants are partially resistant, are in accordance with the results of Vogel and Sommerville (2000) and Nishimura et al. (2003) in *Arabidopsis*. Pathogen growth on *Arabidopsis pmr4* mutants was found to be slowly inhibited. Conidia were able to germinate and begin growing in a regular way. On the mutants less colonies than in the wild-type plants were observed. Moreover, the colonies grew slower on the mutants compared to wild-type plants. Eventually, the colonies in the mutants appeared to be dead or dying. Nevertheless, a subset of colonies on all mutants was able to grow and produce conidiophores, indicating that the mutants were partially resistant and they do not completely block fungal development (Vogel and Sommerville, 2000). Resistance exhibited by the *pmr4* *Arabidopsis* mutants was also associated with the development of lesions reminiscent of hypersensitive cell death. Necrotic lesions were developed under fungal penetration sites and conidia, without spreading of the necrotic spots beyond the infection site (Nishimura et al., 2003). Taking together all these evidences, we suggest that the partial resistant T2 phenotype and the reduced fungal growth could be associated with a slow HR reaction (Li et al., 2007). Hence, the fully resistant genotype of the T1 mutants could be attributed to alterations caused by the *in vitro* conditions, as *in vitro* propagation can result in plantlets with altered morphology, anatomy and physiology (Chandra et al., 2010). To accurately assess the disease resistant phenotype of the T2 mutant plants fungal biomass quantification and microscopic analyses are recommended. Since the resistance phenotype was partial and the scoring of the

plants was based on qualitative estimates, comparison of fungal biomass between phenotypically resistant and susceptible plants will provide more subjective results (Ayliffe et al., 2014). The microscopic observations will confirm the type of HR reaction involved in resistance of the T2 mutants.

Importantly, scoring of the T2 mutants segregating for the different alleles suggested that progenies of line TV171010 were phenotypically the most resistant. However, no statistically significant difference was found to exist between the segregating alleles and the level of resistance they confer (Figure 4).

Proteins are gene products consisting of different domains. Domains are independent functional units of proteins (Praksh and Bateman, 2015). These domains can either act independently or in cooperation with other protein domains and are very important in determining the function of a protein through their interactions (Vogel et al., 2004). Since all mutations were induced on the second exon of the gene, this would suggest that the position of the mutation on the second exon leading to truncation of the protein is not important in the case of *SIPMR4*. However, it would be possible that a mutation on a different exon of the gene, potentially encoding for a different domain of the protein, could be involved in the plant-pathogen interaction, leading to a different resistant phenotype. To assess that, targeted mutations on different parts of the gene could be generated through the CRISPR/Cas9 system. The resistance phenotype of the new mutant plants can then be studied through disease assays.

An alternative explanation for the partially resistant phenotype of the mutant plants could be that the susceptibility factor is encoded by multiple genes (Appiano, 2016). For example, 15 *MLO* genes are present in Arabidopsis. Mutation of the *AtMLO2* gene leads to partial resistance against adapted powdery mildew pathogens. However, the triple mutant *Atmlo2 Atmlo6 Atmlo12* is completely resistant to these pathogens (Acevedo- Garcia et al., 2014). In tomato, two homologs of the *PMR4* gene have been identified (Huibers et al., 2013). In our study, only the homolog on chromosome 7 was targeted and knocked-out. Therefore, the partially resistant phenotype exhibited by the T2 mutants could be attributed to the second homolog being functional and still sustaining the plant-pathogen interaction. To better characterize the role of *PMR4* in resistance, the effect of silencing each homolog on the phenotype and eventually the effect of silencing both homologs, in order to obtain a complete loss-of-function mutant should be evaluated (Appiano, 2016). This can easily be done using the CRISPR/Cas9 system, as it allows the genome engineering in multiple loci, by using multiple sgRNAs bearing different sequences (Samanta et al., 2016).

### ***PMR4 residual expression in mutants***

In literature the residual expression of knockout genes in CRISPR/Cas9 mutants is not addressed. However, since the gRNAs leading to mutations were located on the second exon, we measured the eventual *SIPMR4* expression through RT-qPCR. Residual expression was found to exist in all mutant genotypes. Overall, it was higher in progenies of mutants TV161196 and TV161209. This coincides with the phenotypic results indicating that the progenies of these lines were highly susceptible to the pathogen. Moreover, in mutant TV161196 no mutations were found to exist on the *SIPMR4* gene, suggesting that the gene was fully functional. However, the level of transcripts between resistant mutants carrying the same mutation was variable.

Most of the mutations produced by the CRISPR/Cas9 system in our lines led to a premature stop codon and predicted truncated proteins. In eukaryotes a highly conserved regulatory pathway for the degradation of aberrant transcripts carrying a premature stop codon exists (Shaul, 2015; Nguyen et al., 2014). The nonsense-mediated mRNA decay (NMD) pathway is one of the most studied RNA surveillance pathways, leading to the rapid degradation of mRNA that would translate truncated proteins with potentially harmful consequences for the organism (Hug et al., 2016). Activation of the pathway in plants by recognition of the aberrant mRNA results in low accumulation of mutant mRNA. If the mutation leading to the mutant mRNA is not recognised by the pathway, then the accumulation of the mutant mRNA is expected to be the same as the standard mRNA (Hori and Watanabe, 2007). Thus, results of our *SIPMR4* expression level suggest the activation of the pathway and degradation of the aberrant mRNA. However, RT-qPCR is only used for the quantification of the level of mRNA and no information about the protein degradation is provided. Ideally, western blotting should be performed to quantify the levels of protein still present in the mutants (Holmes et al., 2010).

### ***PR1 gene expression***

Resistance exhibited by the *Arabidopsis pmr4* mutants has been linked to increased expression of the defence related gene *PR1* and the activation of the SA pathway, as *PMR4* has been suggested to be a negative regulator of this pathway (Vogel and Somerville, 2000; Nishimura et al., 2003). To confirm the increased expression of the *PR1* gene in our mutant lines, RT-qPCR was performed using samples collected at two different time points (TP) (before inoculation and at 44 hpi). Overall, expression of the gene in resistant mutants was found to be higher than in the susceptible cv. MM. The expression of the gene in progenies of mutant line TV161212 was generally lower than in other mutant lines. This could be

attributed to the fact that mutant TV161212 was the only line containing an in-frame mutation, leading to the removal of 300 amino acids from the produced protein and not its truncation. Thus, the produced protein could still be partially functional. Moreover, the expression of the *PR1* gene was significantly higher in the RNAi line TV113496. According to our initial hypothesis, RNAi knockdown plants should have lower expression of the *PR1* gene when compared to the knock-out plants, as in many cases the gene expression is not completely inhibited through RNAi (Holmes et al., 2010). The RNAi lines used in this study were previously reported to have a >5-fold less expression of the *SIPMR4* gene and no occurrence of cross silencing of the second homolog (*SIPMR4\_h2*) (Huibers et al., 2013). However, when repeating the RT- qPCR using the same pair of primers as in Huibers et al. (2013), it was found that the silencing effect was visible, although not in all tested plants. When looking in more details in the RT- qPCR output, it was observed that the melt curves of the primers gave multiple peaks (S. Figure 1). Hence, this suggested that the primers were not specific enough, which would make the results unreliable. Therefore a careful validation of the RNAi lines used in this study has to be performed, also considering the possibility that *SIPMR4\_h2* could be silenced. If, absurdly, that is the case, one could hypothesize that *SIPMR4\_h2* leads to higher expression of *PR1*, meaning that silencing of the second homolog has also an effect on the resistance level of the plants. To confirm this, new primer pairs specific for the second homolog should be designed, in order to quantify its expression level in these lines.

### ***Pleiotropic effects***

A major drawback in the application of impaired S genes in breeding is that S genes might have evolutionary conserved functions in plant development (Pavan et al., 2010). To better assess the possible pleiotropic effects of knocking-out *SIPMR4* on plant growth, the height of the mutant plants was measured 15 dpi. Overall, the mutant plants were slightly shorter than the cv. MM control plants. However, this difference in height was not statistically significant (Figure 6), suggesting that the use of mutated *SIPMR4* alleles in breeding could be feasible. Nevertheless, the effects of inactivating the *SIPMR4* gene on agronomically important traits and the development of the mutants under conditions resembling those of agricultural environments should be better studied.

### ***Characterization of mutations by CRISPR/Cas9***

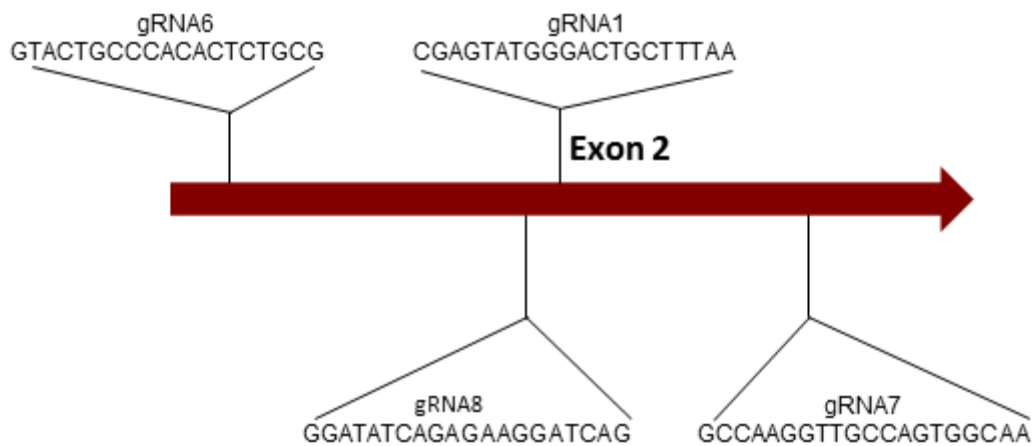
The use of the CRISPR/Cas9 system to generate targeted mutations has rapidly been adopted by scientists in a wide variety of species. CRISPR/Cas9 is able to target a specific DNA sequence leading to a double stranded break (DSB). The resulting breaks are then primarily

repaired by the error prone pathway of non-homologous-end-joining (NHEJ) (Samanta et al., 2016). Genotyping of our mutant plants further illustrates the ability of the system to precisely induce targeted mutations. However, due to the error prone repair mechanism of NHEJ, during the repair of the DSB small sequence changes can be generated (Rodgers and McVey, 2016). This is well demonstrated by the insertion of a T at the point of the deletion in mutant TV171010. More interestingly though, the ability of the CRISPR/Cas9 system to create targeted inversions in plants was first reported by Zhang et al. (2016). In order to achieve inversions Zhang et al. (2016) designed a dual sgRNA targeting two different locations on the gene to create two DSBs. These two DSBs are then capable of inducing a variety of mutations including deletions and inversions. However, inversions are a rare phenomenon and account for only one tenth of the deletions rate (Zhang et al., 2016). Genotyping of our plants led to the discovery of two mutant alleles carrying such inversions. The 1b and 4d alleles both contained an inversion between sgRNA1 and sgRNA7, leading to a premature stop codon and truncation of the produced protein. This rare case of inversions can be attributed to the fact that four sgRNAs were delivered to the plants to create deletions on the second exon. A dual sgRNA as described by Zhang et al. (2016) could therefore, have been unintentionally developed between the sgRNAs leading to the inversions. Nevertheless, the ability of inducing targeted inversions through CRISPR/Cas9 is a new, important tool of studying the function of such type of mutations in plants (Zhang et al., 2016). To our knowledge this is the second report of such inversions created by the CRISPR/Cas9 system in plants.

## ***Materials and methods***

### ***Plant material***

Starting plant material was provided by dr. Valentina Bracuto. Four *SIPMR4* CRISPR/Cas9 mediated knockout plants (TV171009, TV171010, TV161196 and TV161212), carrying different mutations in MoneyMaker (MM) background, their segregating T2 mutant progenies and two *SIPMR4* RNAi silenced lines (TV113496 and TV113488) in MM background (Huibers et al., 2013) were used in this study. For the generation of the CRISPR/Cas9 mutants four guide RNAs (sgRNA) were designed to generate mutations in the coding sequence (exon 2) of the *SIPMR4* gene (Figure 9). The susceptible *S. lycopersicum* cv. MM, carrying the fully functional *SIPMR4* homolog, and a susceptible transformant were used as controls. The plants were grown in a standard greenhouse compartment.



**Fig. 9 sgRNAs on the second exon of SIPMR4.** The approximate location and sequence of each of the four sgRNAs on the second exon of the SIPMR4 gene are depicted in the scheme.

### ***Histological analysis***

Leaf samples cut 44 hours post inoculation from heavily inoculated leaves of the T1 mutants were provided by dr. Valentina Bracuto. The concentration of the inoculum was equal to  $3 \times 10^5$  conidia/ml.

In the follow up experiment, three leaves from three plants of the RNAi lines and each of the segregating alleles of the T2 mutants were collected at the same time point.

In all cases, a 4 cm<sup>2</sup> segment was cut from the inoculated leaves. Bleaching of the leaves was done by using a 1:3 (v/v) acetic acid- ethanol solution. Samples were stained by heating them at 90-92° C in a 1:2 (v/v) lactophenol/ ethanol solution, containing 0.03% trypan blue, for five to ten minutes. For the clarification of the samples, a 5kg: 2L (w/v) saturated chloral hydrate was used. The samples were left in this solution for at least 48 h. Afterwards, each leaf sample was mounted on a glass slide with a 1:1 (v/v) glycerol-water solution. For each genotype a total of 50 infection units (IU), defined as a germinated spore that produced at least a germ tube, were counted. Histological analysis of the samples was done using a Zeiss Axiophot bright field microscope and photographs were taken with an Axiocam ERc5s. For each IU the presence of an appressorium, the number of hyphae, the absence/presence of primary and secondary haustoria and the presence/absence of primary and secondary HRs were recorded.



### ***Genomic DNA (gDNA) isolation***

gDNA of the original T1 mutants was provided by dr. Valentina Bracuto. gDNA of the T2 mutant plants and the RNAi lines was isolated using a standard CTAB protocol. Half a cotyledon from each plant was collected in a microcentrifuge tube, in which two steel balls were placed. The harvest of the samples was done on ice. 500 µl of CTAB extraction buffer containing 1 µl/1 ml RNase was added in each tube. The samples were then mixed in a shaker for about 5 minutes and were incubated in a water bath at 65° C for 60 min. Subsequently, they were cooled down in ice water for 30 min. 250 µl of chloroform isoamyl alcohol (24:1) were added in the samples and the tubes were mixed by inversion. Centrifugation at 4000 RPM for 15 minutes was then done to separate the sample phases. 400 µl of the water phase were pipetted into new tubes. 200 µl of isopropanol were then added to the suspension and the tubes were mixed by inversion. Following, the tubes were centrifuged for 15 min at 4000 RPM and the supernatant was removed from the tubes. The remaining pellets were washed by adding 300 µl of 70% ethanol and the samples were centrifuged for 15 min at 4000 RPM. The pellets were dried until no ethanol was present in the tubes. Finally, the pelleted DNA was dissolved in 100 µl of MQ water by briefly vortexing.

### ***Genotyping of mutant plants***

The presence of mutations in the T1 mutants and their segregating T2 progeny was assessed by PCR. Furthermore, the RNAi plants were genotyped for the presence of the silencing construct. The CRISPR/Cas9 mutation target region was divided into three fragments. Three primer pairs overlapping at their ends were designed by dr. Valentina Bracuto to amplify the full length of the mutation target region [SIPMR4\_Fw\_519 (5'-TGG TGC TCT TTT CTC GGT CT-3')/ SIPMR4\_Rv\_1925 (5'-CAA CTG CTC TTC TGG CAT CA-3'), SIPMR4\_Fw\_1730 (5'-ATT GCT TTG GCT TCC TGT TG-3')/SIPMR4\_RV\_3126 (5'-GAA AGG CCA GCA TTT TGA GA-3') and SIPMR4\_Fw\_2969 (5'-GCG AAT GCG TAG AGA AGG AA-3')/ SIPMR4\_Rv\_4230 (5'-CCC CAC TAA GTG CCA GGT AA-3')]. Amplification of each fragment was done by using the reagents and PCR conditions described in tables 2 and 3, respectively. Reactions of 20 or 50 µl total volume were used. PCR products were visualised on 1% agarose gel. DNA of homozygous biallelic mutants was sent for sequencing after amplification. Different alleles of heterozygous biallelic mutants were eluted from the agarose gel using a MinElute PCR Purification Kit (QIAGEN) following the manufacturer's instructions and then sequenced. DNA concentrations of  $\geq 20$  ng/µl were used for sequencing. To identify the mutations produced by the CRISPR/Cas9 system, an *in silico*

analysis was performed by aligning the obtained fragment sequences against the predicted full length of the *SIPMR4* gene.

**Table 2 PCR reagents used for the amplification of each mutation target region.**

PCR reagents	Volume
10x DreamTaq Buffer	2 $\mu$ l
dNTP	1 $\mu$ l
Forward Primer	1 $\mu$ l
Reverse Primer	1 $\mu$ l
DreamTaq	0.1 $\mu$ l
H2O	12.9 $\mu$ l
DNA	2 $\mu$ l
<b>Total</b>	20 $\mu$ l

**Table 3 PCR conditions used for the amplification of each mutation target region.**

PCR conditions		
Temperature	Time	Stage
95° C	5 min	1
95° C	30 sec	} <b>x40</b>
61° C	30 sec	
72° C	1 min, 30 sec	
72° C	10 min	3
10° C	$\infty$	4

### ***Total RNA isolation and cDNA synthesis***

Three fully expanded leaflets from the third and fourth true leaves, before inoculation and 44 hpi were sampled for RNA isolation. The leaves were flash 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) in combination with a KingFisher processor, according to the manufacturer's instructions. RNA

concentration was measured using an Isogen Nanadrop Spectrophotometer ND-100. cDNA synthesis was done using an iScript™ cDNA synthesis Kit (Bio-Rad) following the manufacturer's instructions. The PCR conditions used for the cDNA synthesis are given in table 4. A 1:10 cDNA dilution was used in the following quantitative Real- Time PCR (qRT-PCR) reactions.

**Table 4 PCR conditions used for the cDNA synthesis**

Temperature	Time	Stage
25° C	5 min	1
46° C	20 min	2
95° C	1 min	3
10° C	∞	4

### ***Gene expression analysis***

The expression of the *SIPMR4* and *PR-1* genes was analysed through qRT-PCR. To measure the residual expression of the *SIPMR4* gene a specific pair of primers was designed on the first exon of the gene [*SIPMR4\_qPCR\_Fw* (5'-CGG CTT TAC GCT CTG TAG GT-3')/*SIPMR4\_qPCR\_Rev* (5'-AAA AGA GCA CCA CGA CGA GT-3')]. A pair of primers for the *PR-1* gene was also designed [*SIPR1a\_Fw* (5'-GTG TCC GAG AGG CCA GAC TA-3')/*SIPR1a\_Rev* (5'-CAT TGT TGC AAC GAG CCC GA-3')]. A specific pair of primers for the *Ef* tomato reference gene was used [*Ef\_Fw* (5'-ATT GGA AAC GGA TAT GCC CCT-3')/*Ef\_Rev* (5'-TCC TTA CCT GAA CGC CTG TCA-3')]. Three plants segregating for each of the different alleles were used per genotype. qRT-PCR was performed using a CFX96 Real- Time PCR machine (Bio-Rad Laboratories, USA). A 10 µl reaction was used for the qRT- PCR. The reagents and the reaction conditions are described in table 5 and table 6, respectively. Two technical replicates for each sample were tested. Relative expression of each gene was determined by the  $\Delta\Delta C_t$  method (Pfaffl, 2001).

**Table 5 Reagents used for the RT- qPCR**

Reagents	Volume (ul/ sample)
SYBR Green	5
Forward Primer (uM)	0.3
Reverse Primer (uM)	0.3
Water	3.4
C (1:10 dilution)	1

**Table 6 Conditions used for the RT- qPCR**

<b>Temperature</b>	<b>Time</b>		<b>Stage</b>
95° C	3 min		1
95° C	10 sec	} <b>x39</b>	2
60° C	30 sec		3
95° C	10 sec		4
65 ° C	5 sec		5
95 ° C	5 sec		6

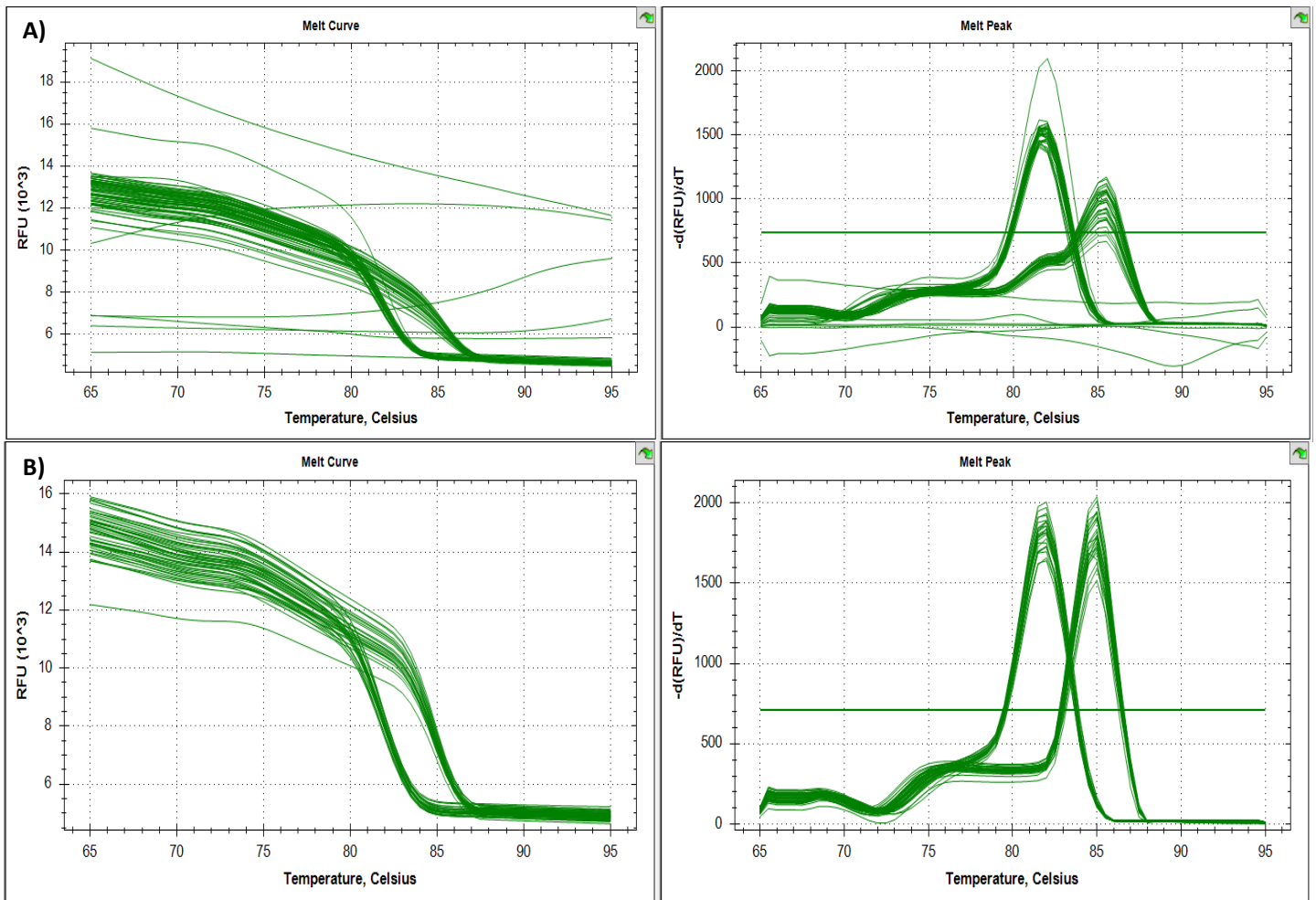
***Plant inoculation for PM disease test and symptom scoring***

Artificial inoculation in the greenhouse was used for the powdery mildew disease assay. The Wageningen *O. neolycopersici* isolate was used. A  $3 \times 10^4$  (conidiospores/ml) conidial suspension of the *O. neolycopersici* was prepared by rinsing freshly sporulating leaves in water. The suspension was sprayed on one month old plants. For the assay, at least 14 plants per genotype were used. Symptom scoring of the plants was done 13,15 and 17 days post inoculation (dpi) by inspecting the third and fourth true leaves of each plant. A 0-3 scale was used for the scoring- 0 indicating that there was no sporulation on the leaves; 1 indicating few fungal colonies; 2 indicating up to 30% leaf coverage by fungal colonies; 3 indicating more than 30% leaf coverage by fungal colonies. For the gene expression study and the microscopic analysis of the T2 mutants and the RNAi lines, a second inoculation was set up. Mutant plants segregating for each allele, *SIPMR4* RNAi silenced plants and cv. MM plants were sprayed with a  $3 \times 10^5$  conidial solution. Three biological replicates per genotype were used for each of the time points (before inoculation and 44 hpi).

## Supplementary data

**S.Table 1. Experimental set up.** Experimental set up of the disease assay, gene expression and histological analyses. The genotype of the plants used, the number of plants per allele and the expected segregation of alleles are given in the table.

TV171010, TV161212				TV161196,TV171010, TV161209	TV133488, TV113496	MM
	Hetero	Allele I	Allele II	Allele I	Allele I	Allele I
Disease assay	8	8	8	10	5	5
Gene expression	6	6	6	6	6	6
Histological	3	3	3	3	3	3
Total	17	17	17	19	14	14
Exp. Segregation	0.5	0.25	0.25	1		



**S. Fig. 1 Melt curves of qPCR primers.** In panel A) the melt curves of the qPCR primers for the SIPMR4 gene designed by Huibers et al. (2013) are given. In panel B) the melt curves of the new primers designed for the quantification of the residual expression of SIPMR4 are given.

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