Identification of susceptibility determinants in the tomato- *Clavibacter michiganensis* pathosystem

Eleni Koseoglou

Propositions

1.Resistance to *Clavibacter michiganensis* in tomato can only be achieved through transgenic approaches.

(this thesis)

2. Virulence of *Clavibacter michiganensis* is independent of population size.

(this thesis)

3. It is necessary to study the microbiome of the host as part of a pathosystem.

4. Evolution is a process governed by anarchy.

5. Agriculture acts as a catalyst for the downfall of human civilization.

6. Coffee is served with a side of sexism.

Propositions belonging to the thesis, entitled

"Identification of susceptibility determinants in the tomato-*Clavibacter michiganensis* pathosystem"

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Identification of susceptibility determinants in the tomato- *Clavibacter michiganensis* pathosystem

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Thesis

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CHAPTER 1

General introduction

From domestication to genome editing and back

Plant domestication is inarguably one of the greatest technological and evolutionary innovations of humankind [1, 2]. The transition from foraging to sedentary agriculture, that was brought about by plant domestication, has led to one of the most successful animal-plant symbiotic relationships [1, 3]. Plant domestication and agriculture have provided humans with the means to form complex settlements, eventually giving rise to cities and contemporary human culture [3-5]. Such is the impact of plants on man, that historian Yuval Noah Harari controversially argues that in their relationship plants were the ones domesticating humans [6].

The origins of plant breeding can be traced back to plant domestication and the settlement of the first farmers. During the process of domestication, early farmers selected naturally occurring variants that resulted in favourable phenotypes [4]. The scientific basis of plant breeding and genetics, however, came much later with the discovery of Mendel's laws of heredity in 1866 [7]. After the (re)discovery and validation of Mendel's laws, the field of plant genetics has undergone a rapid evolution. Unlike the domestication of the first crops, plant breeders today have an array of technologies to help them in the improvement of crop varieties [8]. From cross breeding to the discovery of the three-dimensional DNA structure, the engineering of the first recombinant DNA molecules, and from random mutagenesis to targeted genome editing, the field of plant genetics and breeding has met several milestones in the last two centuries (Figure 1) [9, 10].

For millennia, plant domestication has relied on natural genetic variation. The generation of favourable mutations during plant domestication was unpredictable [11]. For decades, traditional plant breeding has relied on the introgression of natural variants from wild species to elite cultivars through extensive crossing and selection [12].To create novel heritable mutations in plant genomes breeders have used various mutagens, such as irradiation and chemical compounds. The random nature of mutations introduced this way leads to several drawbacks, such as a large number of simultaneous mutations. The recent advances in genome editing technologies offer geneticists the opportunity to introduce targeted mutations on genomic sites of interest. CRISPR/Cas9, in particular, has been the frontrunner of genome editing tools in the last decade. CRISPR/Cas systems have allowed scientists to create heritable changes in plants that vary from SNPs [13, 14] to large scale chromosomal inversions [15, 16].

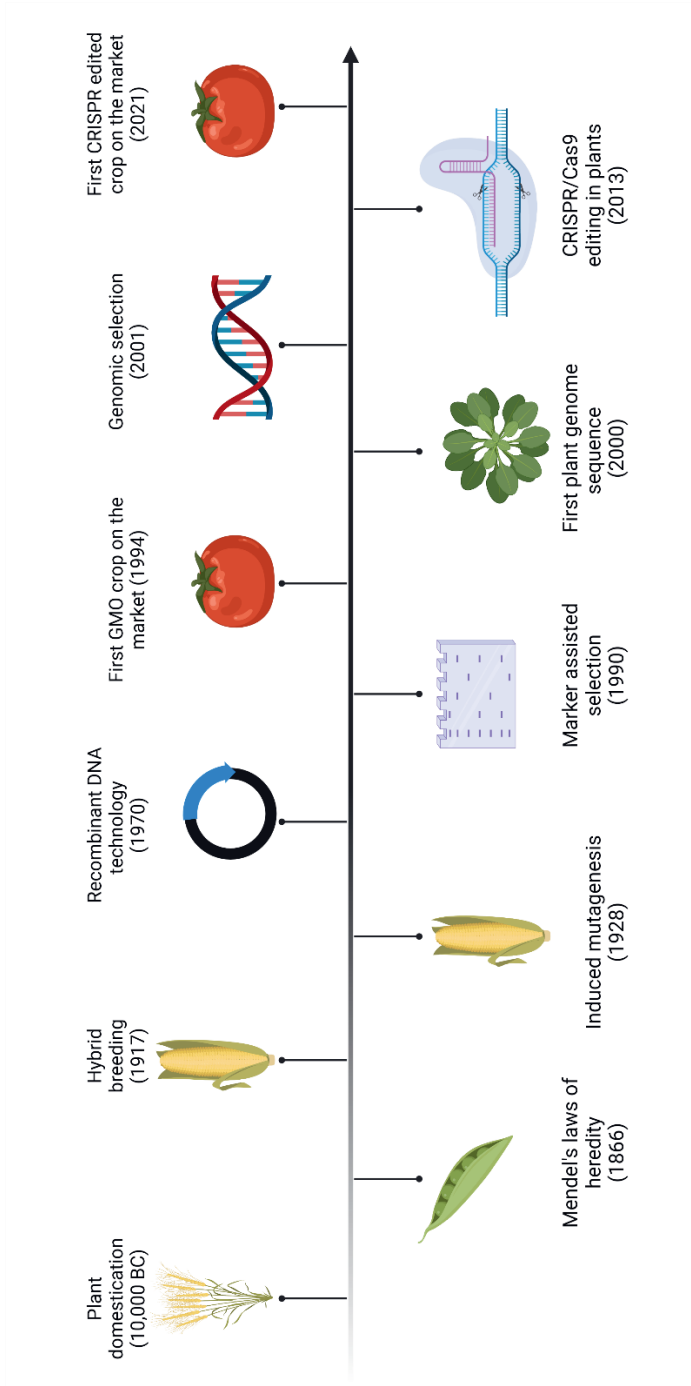

Figure 1| Milestones in plant breeding. Since the domestication of the first plants, plant breeding and genetics has undergone a rapid evolution due to technological advances. Some of the most important milestones in the last two centuries are presented in this figure. Figure made with BioRender.

The applications of CRISPR/Cas systems can, however, reach further than creating desirable mutations in elite cultivars. Wild relatives of crops harbour valuable genetic diversity, such as resistance to pathogens, stress tolerance and nutritional features, that was left unutilized during domestication. The inheritance of many genes involved in domestication traits follows Mendelian patterns, that involve gain- or loss-of-function mutations [17]. Recently, the concept of *de novo* domestication, which aims at the introduction of desirable traits in wild genotypes through genome editing, has been proposed as a novel breeding strategy [18, 19]. Groups working on the *de novo* domestication of wild tomatoes and wild rice, were able to simultaneously alter orthologs of genes known to be important for agronomical traits using CRISPR/Cas9 [17, 18] [20]. In contrast to the past, by combining our current knowledge of functional genomics of domesticated crops, the fast sequencing of plant genomes and the rapid generation of mutations using genome editing, the *de novo* domestication of wild crop relatives can be achieved in a few generations [18]. Genome editing can therefore be used to introduce domestication traits in wild relatives of crops, without losing desirable traits of the wild plants.

The plant innate immune system

To escape invasion of the diverse organisms that constantly challenge them, plants have developed a two-layered innate immune system that is activated on a detection-and-response manner [21, 22]. The first layer of immunity is activated when plasma membrane bound pattern-recognition receptors (PRRs) recognise conserved pathogen associated molecular patterns or host derived damage associated molecular patterns (PAMPs/ DAMPs), resulting in P/DAMP-triggered immunity (PTI). To evade or suppress PTI, adapted pathogens secrete effector proteins that lead to effector-triggered susceptibility (ETS). In response to ETS, plants have evolved a second intracellular immunity level that is based on the direct or indirect recognition of pathogen effectors by resistance (R) genes that results in effector-triggered immunity (ETI) [22, 23]. To counteract and evade ETI, pathogens can rapidly evolve, diversify or lose effectors, in order to avoid detection by R genes [21, 22]. For pathogens to become pathogenic, a high degree of adaptation is required. To achieve a compatible interaction with their host, pathogens manipulate host factors encoded by plant susceptibility (S) genes. S genes belong to diverse gene families and when manipulated they can aid pathogens to exploit host processes to their advantage [24]. Although S genes can lead to the promotion of disease, their loss-of-function can lead to recessively inherited and possibly broad-spectrum resistance [25].

A brief history of tomato breeding

Cultivated tomato, *Solanum lycopersicum* L., belongs to the species-rich family of the nightshades (*Solanaceae*). The genus *Solanum* is one of the ten biggest genera of angiosperms. It contains several crops of economic importance such as potato (*S. tuberosum L.*) and eggplant (*S. melongena L.*) [26]. Wild tomato species are native to the Andean region, which now includes parts of Chile, Bolivia, Ecuador, Colombia and Peru [27, 28]. The globalization of tomato started with its introduction in Europe in the 16th century [26]. Despite the use of tomato as a crop for centuries, its centre of domestication is still under dispute [26-28]. Tomato domestication probably began in the Andean region of Peru and Ecuador, and it was completed in Mesoamerica. Eventually, human selection and breeding led to the extensive phenotypic changes of tomato [29]. As a consequence of the extensive selection tomato has gone through, its genetic variation is considered to be extremely limited compared to its wild relatives. It is estimated that due to severe domestication syndrome, cultivated tomato contains less than 5% of the variation found in wild tomato species. From the 1970s on, however, the need for resistant varieties and consumer favourable traits has drastically increased the diversity across the tomato genome [27, 30-32].

Through the decades, the breeding goals for tomato have shifted. These goals can generally be divided into four phases: breeding for yield in the 1970s, breeding for shelf-life in the 1980s, breeding for taste in the 1990s and currently breeding for its nutritional quality [27, 28, 33]. Tomato is host to more than 200 diseases and pests that can lead to significant economic losses. Therefore, the generation of tomato cultivars carrying resistance has been a major trait for breeding through the decades [27]. Many resistances to pathogens are dominantly inherited, making the transfer of these genes in elite cultivars relatively easy. As a result, more than a dozen resistance genes are present on modern tomato cultivars [31]. Breeding for resistance is even more challenging, dynamic and relevant in view of reduced pesticide use and our changing climate. Environmental changes strongly affect the emergence of new diseases, and it is predicted that the increase in temperatures will increase the spread of pathogens to new geographic areas, where they can potentially infect novel hosts [34]. At the same time, the need for decreased pesticide use highlights the need for the generation of resistant crops [35].

Thankfully, the development of new technologies and new breeding strategies, such as the use of mutant S genes (**Chapter 2**), can help in the development of tomato cultivars that are resistant to multiple pathogens and pests.

The genus *Clavibacter*

Clavibacter is a genus comprising a number of plant associated bacteria in the *Microbacteriaceae* family. The species of the genus are Gram-positive, aerobic, non-sporulating bacteria. The members of the genus were first classified by Davis et al. in a single species with five subspecies [36-38]. Based on recent genomic data the genus was reclassified and the subspecies were elevated to a species status. The newly reclassified genus is comprised of six officially recognised species, and four likely to be species, each with narrow host specificity [39-41]. Each of the plant pathogenic *Clavibacter* species infects one primary host [36, 37]. Seven members of the genus include species that account for economically important diseases in both dicots and monocots [42]. Namely, *C. sepedonicus* causes bacterial ring rot of potato, *C. insidiosus* causes wilting and stunting in alfalfa, *C. nebraskensis* is the causal agent of wilt and blight of maize, *C. tessellarius* causes bacterial mosaic in wheat, *C. michiganensis* causes bacterial canker in tomato and *C. zhangzhiyongii* which is responsible for bacterial leaf brown spot and decline in barley [39, 41]. In the last decade, four novel likely to be species have been isolated and characterized [41, 43]. These are *C. capsici* that causes bacterial canker in pepper [43], and *C. phaseoli* which causes bacterial leaf yellowing in bean [44]. Two non-pathogenic species were also recently isolated from tomato seeds in California and Chile, namely *C. californiensis* and *C. chilensis* [41].

Clavibacter michiganensis physiology

C. michiganensis (*Cm*), the subject of this thesis, is the causal agent of tomato bacterial canker. Physiologically, *Cm* is a mesophile that optimally grows at 25-28^{o}C, at which generation time is high. Visible colonies form on agar plates three to five days after plating [45]. Due to the presence of carotenoids the colonies formed by several *Cm* strains are pigmented yellow, while the production of exopolysaccharides by the bacterium causes the colonies' mucoid morphology [46]. The maximum growth temperature of the pathogen is 35^{o}C, while the thermal death point is 50^{o}C.

Transmission and symptomatology of *Clavibacter michiganensis*

Cm is a seed-borne pathogen. Infected seeds are a primary source of inoculum and responsible for the long-distance dispersal of the pathogen. Tomato seeds can be infected by *Cm* both systemically through the xylem or externally through lesions on tomato fruits [47]. Infected plant debris that remain in the soil can also act as a primary infection source [48, 49]. Secondary infections can occur via entry of *Cm* through natural openings, like the stomata or hydathodes, through wounds or via cultural practices, such as pruning or clipping (Fig. 2) [48, 50-52] .

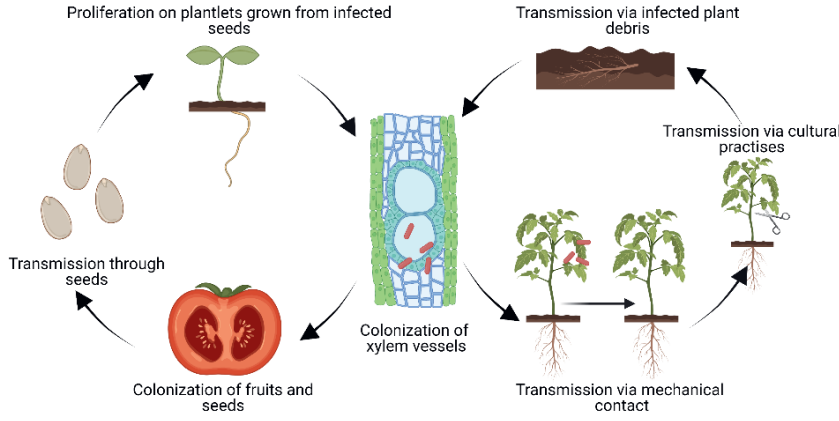

Figure 2| Transmission cycle of *Clavibacter michiganensis*. A primary source of *Cm* inoculum are infected seeds that will grow to become infected plants. After heavy colonization of xylem vessels, *Cm* can colonize fruits and seeds of the developing plants. Another primary source can be infected debris that remain in the soil from previous cultivations. Secondary infections of *Cm* can also occur, mainly through mechanical contact, cultural practices during which hygiene measures are not applied. Figure made with BioRender.

Symptoms on tomato plants vary depending on the infection route, as well as the cultivar and environmental conditions. Systemic infection of greenhouse grown tomatoes, under high temperatures (25-30^{o} C), can lead to interveinal chlorotic water soaked areas that rapidly desiccate [53]. As the disease progresses, systemic infections can lead to the development of cankers on stems and petioles of the infected plants. Xylem discoloration and necrosis are also common symptoms. As a consequence of high temperatures and evapotranspiration stress, the entire infected plant can wilt and desiccate within a short number of days [54]. Localized infection on tomato fruits causes the development of necrotic spots surrounded by

a white halo, known as bird's eye spots [55]. Local infections of the plant aerial parts can cause marginal leaf necrosis, as well as white blister-like spots on the stems and leaves [56] (Fig. 3).

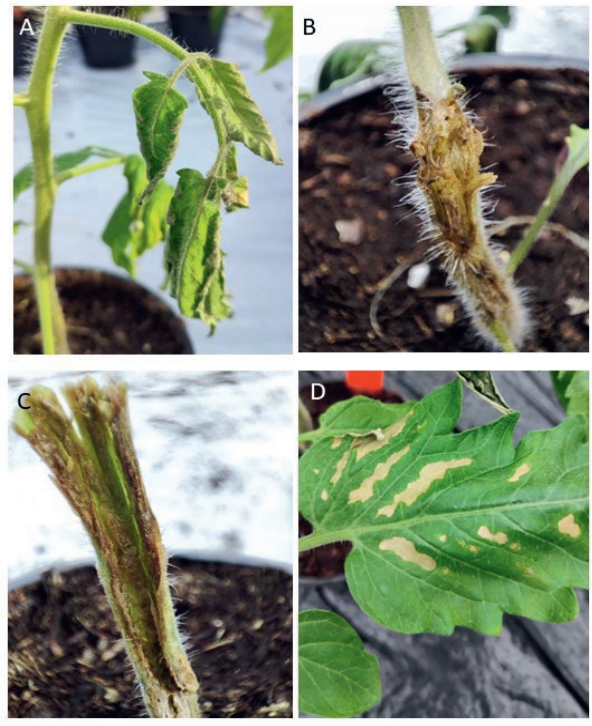

Figure 3| Symptoms caused by *Clavibacter michiganensis* on susceptible tomato plants. A) Wilting of tomato leaflets, B) canker on tomato stem, C) discoloration and necrosis of xylem, D) interveinal necrosis.

Distribution and status of the pathogen

Bacterial canker poses a serious threat for the majority of tomato growing regions worldwide (Fig. 4).The pathogen is considered to cause one of the most destructive bacterial diseases of tomato [36, 42, 46]. Losses between 50-100% caused by *Cm* have been reported in several cases. In Europe the disease is present in 14 EU member countries and while outbreaks are rare, they can be severe [53, 57]. To date, chemical and biological means to control *Cm* are limited [48, 58-60]. Control measures for *Cm* are mainly based on "good seed and plant practise" (GSPP), that include seed treatments and cultivation measures that reduce the risks of introduction and spreading of the pathogen [54].

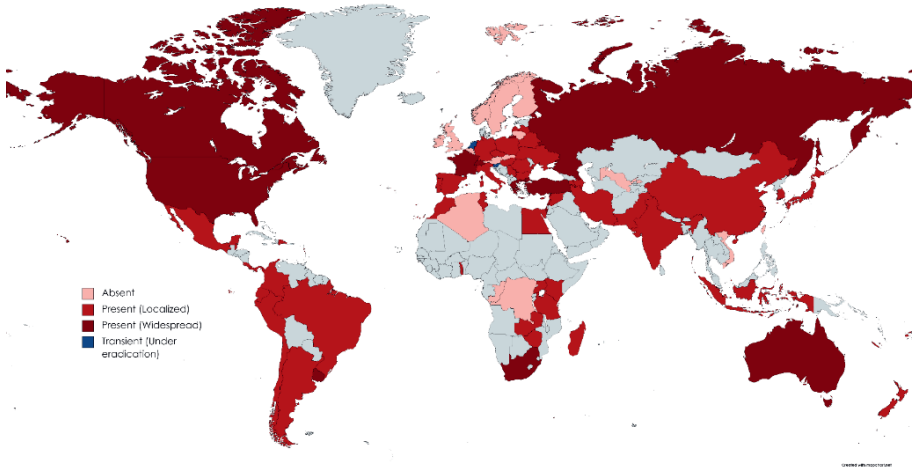

Figure 4| Global distribution of *Clavibacter michiganensis*. Data retrieved from the EPPO Global Database (https://gd.eppo.int/taxon/CORBMI, December 2021). Distribution map created with MapChart (https://www.mapchart.net/).

In an effort to control the destructive consequences of bacterial canker, *Cm* is classified as a Quarantine or A2 pest in multiple countries around the world [36, 57]. Due to the widespread occurrence of *Cm* in the European and Mediterranean region (EPPO), *Cm* has been downgraded from a Quarantine Pest to a Regulated Non Quarantine Pest in December 2019.

Clavibacter michignanenis pathogenomics

The investigation of the molecular basis of *Cm* pathogenicity has been greatly assisted by the sequencing of several *Cm* strains. Pathogenicity studies have mainly been focused on the first sequenced strain NCPPB382 [61, 62]. The genome of strain NCPPB382 consists of a ~3.3 Mb circular chromosome with a 72.6% G+C content. Multiple genomic regions on the chromosome have been associated with pathogenicity, host colonization and suppression of host defences [62]. The largest of these putative pathogenicity islands (PAI) is the *chp/tomA* region (~129 kp), which codes for multiple glycosidases and proteases. The *chp* subregion (79 kb) of the PAI carries only 44 genes, including the serine protease coding genes *chpC*, *chpG*, *ppaA* and *ppaC* [62, 63]. Of these genes, *chpC* was found to be important in colonization of the host, whereas *chpG* has recently been shown to contribute to host specificity (Fig. 5) [64, 65]. In addition, a newly identified gene of unknown function, named

"GeneM", encoded by *Cm* was found to cause necrosis when expressed in tomato and potato plants [66].

The *tomA* subregion contains genes that code for 12 different glycosidases, including the *toma* gene which is involved in the detoxification of α-tomatine, a tomato microbial defence associated alkaloid [61, 62]. Further studies have identified a range of serine proteases, the transcription factors *vatr1* and *vatr2* [56]. Several other genes encoding virulence factors such as carbohydrate active enzymes (CAZymes), including cellulases (*celB*), xylanases (*xysA* and *xysB*), and pectinases (*pelA1* and *pelA2*) have been associated with *Cm* virulence [67].

NCPPB382 harbours two native plasmids, pCM1 (27.4 kb) and pCM2 (70 kb), which both carry important pathogenicity factors. Two of the most essential genes coding for pathogenicity factors are *celA* and *pat-1*, carried by pCM1 and pCM2, respectively. *celA* codes for a β-1,4-endocellulase, while *pat-1* encodes for a putative serine protease that is critical for wilting development on tomato [62, 68, 69]. Loss of either of the plasmids leads to a substantial decrease of the strain's virulence. The loss of both plasmids leads to an endophytic strain (*Cmm*100) that is unable to produce any symptoms on infected tomato plants (Figure 5) [61, 62, 70].

The reference strain NCPPB382 and its derivates have provided an excellent system for the study of *Cm* virulence and genetics. Recent studies into the genetic diversity of *Cm* isolates have, however, described strains that do not require the same genetic makeup as NCPPB382 to induce bacterial canker [61, 71, 72]. Plasmid profiles of recently characterised isolates vary both in size and number, with plasmid content ranging from none to three [61, 71]. In their study, Thapa et al. [61] found that strains isolated from infected tomato plants in California harboured pCM1- and pCM2-like plasmids. While all analysed pathogenic strains harboured a pCM1-like plasmid, strains that lacked a pCM2-like plasmid which codes for pat-1 were found to be equally pathogenic as strains possessing pCM2. This demonstrates that the presence of pCM2-like plasmids is not universally required for pathogenicity [61]. Although many studies have investigated the virulence of *Cm*, knowledge on the different virulence strategies of *Cm* is still limited [73].

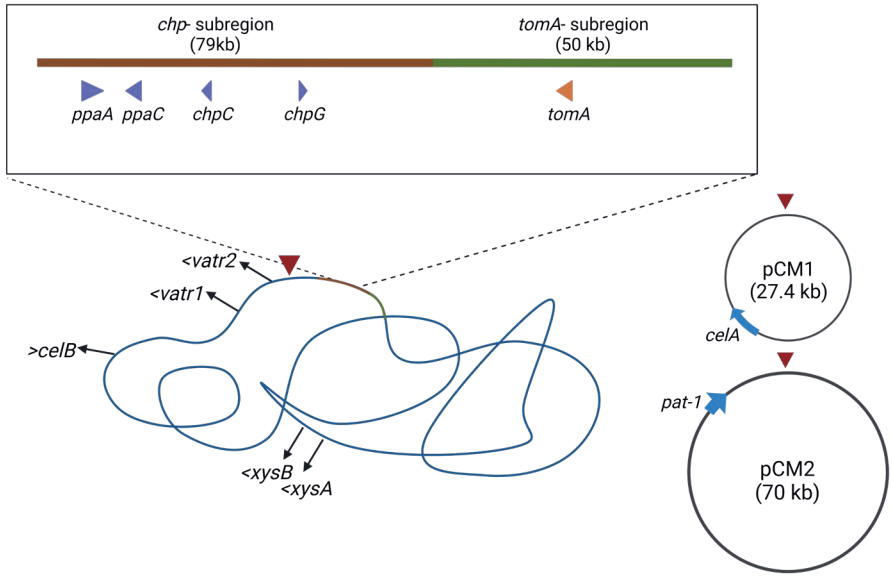

Figure 5| Schematic representation of the genomic structure of *Clavibacter michiganensis*. *Clavibacter michiganensis* has a single circular chromosome that consists of ~3.3 Gb. A pathogenicity island (PAI) on the chromosome with two subregions (chp/tomA) is responsible for coding multiple virulence factors. Other virulence genes are also present on the chromosome of *C. michiganensis*. The reference strain NCPBB382 contains two native plasmids (pCM1 and pCM2) that code for the major virulence factors *celA* and *pat-1*, respectively. Red triangles represent the origins of replication of the chromosome and plasmids. The brown/green part of the chromosome represents the PAI, whereas triangles represent genes present on the region. Other genes on the chromosome are indicated with an arrow and their names. > or < represent the direction of transcription. Figure made with BioRender.

Molecular aspects of the tomato- *Cm* interaction

Despite the considerable importance of *Cm* as a pathogen, little is known about the processes governing the *Cm*-tomato interaction (Figure 6). Transcriptomics and proteomics studies have been carried out in an effort to better understand the molecular basis of the interaction [63, 74-77]. Upon infection of tomato with *Cm*, several components of basal defence are activated in the host. Prominent in the functional categories of *Cm*-responsive genes are genes involved in processes such as protein control and degradation, multiple classes of pathogenesis related proteins, receptor-like kinases and genes involved in R-gene mediated resistance [75, 76]. Notably, *Enhanced Disease Susceptibility1* (*EDS1*), that is required for activation of certain R gene classes, *Pti5* involved in transcriptional activation of

pathogenesis related genes, and members of the *WRKY* transcription family, that are known to be involved in basal defence and plant disease resistance are upregulated in tomato [75]. Together with these genes, genes involved in the biosynthesis and signalling of phytohormones jasmonic acid (JA) and ethylene, such as *Pathogenesis-related protein4* (*Pr4*), *Pr6,* and *1-aminocyclopropane-1-carboxylic acid (ACC) oxidase1 (ACO1)* are also induced (Fig. 6) [75, 76]. Interestingly, a shut-down of a number of genes involved in photosynthesis and lignin biosynthesis has been observed in tomato plants, when *Cm* is well established and the disease has progressed [76].

Tomato genetic factors contributing to susceptibility still remain elusive. Nevertheless, ethylene has been found to be an important host susceptibility factor. To promote wilting development, wild type *Cm* induces the biosynthesis of host derived ethylene, through the specific upregulation of *ACO1*. Symptom development on *Never ripe (Nr)* mutants, impaired in ethylene perception, is delayed [75]. In contrast to the wild type *Cm*, the endophytic strain *Cmm*100 is unable to induce the production of ethylene in the host, further highlighting its importance in wilting development [74].

On a protein level, mounting of basal defence responses through the induction of a wide range of defence associated proteins has been observed. In addition to multiple PR proteins, several proteins involved in hormone biosynthesis are induced in infected plants [74, 76]. An example is lipoxygenase-1 (LOX1) which is involved in the catalysis of antimicrobial oxylipins and metabolites from which JA is derived. In accordance to transcriptomics data, EDS1 and ACO1 proteins, which are involved in the activation of R genes and ethylene synthesis, respectively, are also induced upon *Cm* infection in the host [74]. Contrary to the expected, a number of tomato proteins associated with defence, such as proteins involved in lignin production and wound healing exhibit reduced abundance in *Cm* infected plants. The downregulation of these proteins could potentially suggest that they are actively targeted by *Cm*. *Cm* may inhibit these proteins to interfere with host defence responses [74].

Even though tomato can activate basal defence mechanisms, *Cm* is still able to overcome them and cause disease. To successfully infect tomato *Cm* sequentially expresses virulence associated genes. In the first stages of the disease the expression of plasmid-borne *celA* and *pat-1* is highly induced. Together with the plasmid carried genes, a number of chromosomal genes coding for cell wall-degrading enzymes are upregulated. The expression of pectate lyase *pelA1, xysB* and *celB* is increased in the

first 24 hours post inoculation. At 96 hours post inoculation the expression of all abovementioned genes is decreased. In contrast, the expression of chromosomally encoded serine proteases *chpC* and *paaA* and xylanase *xysA* peaks at 96 hours post inoculation, indicating a role of these genes in later infection stages [78]. Besides the crucial importance of virulence determinants in the development of wilting and canker, Chalupowicz et al. showed that a different set of genes is involved in the development of blisters on aerial parts of infected plants [56]. Serine proteases *chpC*, *sbtA* and the transcription factor *vatr2* are induced in leaves upon spray inoculation with the bacterium. Impairment of genes *pgaA* coding for a polygalacturonase, *endX/Y* encoding two endoglucanases, *perF* and *srtA* coding for a putative perforin and a putative sortase, respectively, has confirmed their involvement in blister development. High induction of these genes has been observed 8-16 hours post inoculation in spray treated plants [56].

Using proteomics approaches several bacterial proteins involved in the interaction have been identified [63, 74]. Many hydrolytic enzymes, including serine proteases, subtilases and cell-wall degrading enzymes are induced during infection [74]. PAI encoded Chp proteins, namely ChpC, ChpE, ChpF and ChpF are expressed and upregulated *in planta*. Confirming its importance in pathogenicity, the pCM1-encoded celA protein has also been identified in the sap of infected tomato plants [74]. Putative proteins, such as polymer degrading enzymes and an RTX toxin are also secreted by *Cm* during infection [63, 74]. In parallel, a large number of ABC transporter proteins are expressed *in planta* to uptake degradation products. In total, 57 ABC transporters involved in the uptake of ions, amino acids, sugars, metals and oligopeptides have been shown to be induced during infection [74]. Among these, sugar transporter subunits were the most common group of transporters [63]. Generally, *Cm* secretes a wide range of degrading enzymes, while at the same time the expression of transporter proteins is upregulated. This suggests that the large number of transporters induced is involved in the uptake of molecules for *Cm* to satisfy its metabolic requirements (Fig. 6) [74].

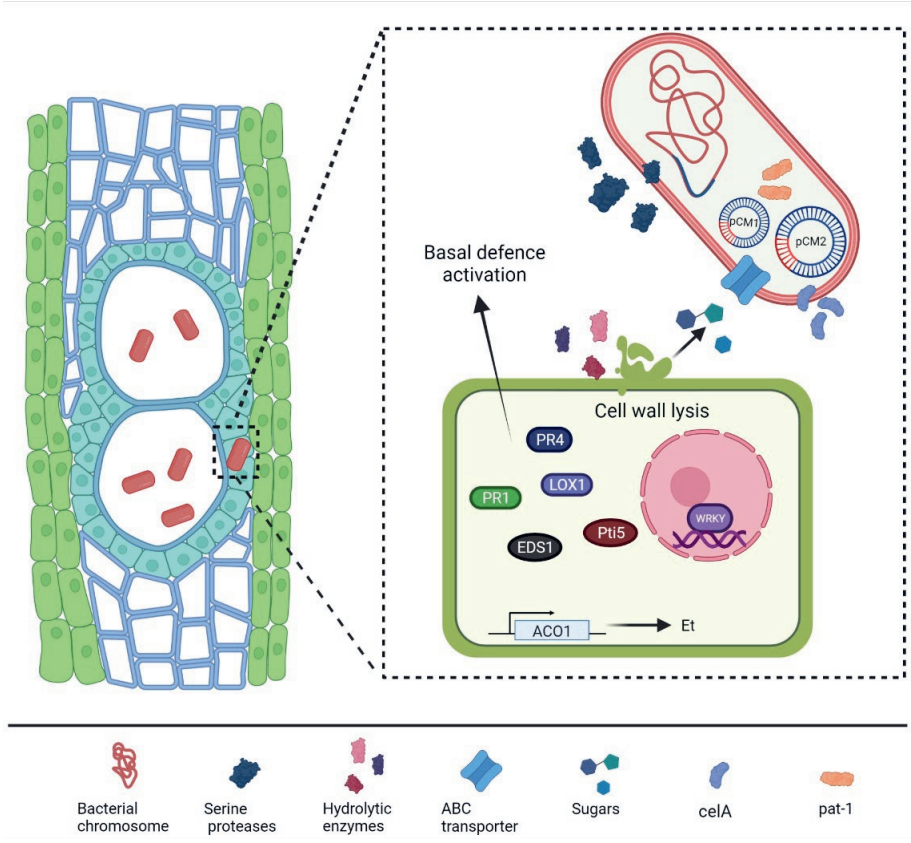

Figure 6| Molecular aspects of the tomato- *Cm* interaction**.** A simplified representation of the molecular components involved in the tomato- *Cm* interaction based on transcriptomics and proteomics data. Infection of tomato by *Cm* results in the activation of basal defences, as it has been highlighted by the upregulation of different classes of pathogenesis related proteins. The pathogen, however, is able to suppress host defences leading to disease development. To cause symptoms on the plants, the pathogen induces the production of ethylene in the host. In parallel, the pathogen produces several hydrolytic enzymes that lead to cell wall lysis. The products of cell degradation are then uptaken by the pathogen through the expression of ABC transporters. Figure made with BioRender.

Tolerance to *Cm*

Despite modest breeding efforts no cultivar with resistance against *Cm* is available on the market. Yet, different tolerant wild accessions have been identified. The first tolerant accession reported was *Solanum pimpinellifolium*, that was used for in the introgression breeding of line Bulgaria 12 [79]. *Solanum habrochaites* (LA)407 and *Solanum arcanum* LA2157 were also found to be highly tolerant to the bacterium

[80, 81]. In a screening of 24 wild species, Sen et al. [82] reported newly identified highly tolerant accessions. These include *S. pimpinellifolium* GI. 1554, *Solanum neorickii* LA 735 and *S. neorickii* LA2072 [82]. Genetic studies have shown that the background of *Cm* resistance is polygenic and complex [48]. The number of genes involved in the tolerance observed in *S. habrochaites* LA407 was estimated to range between one to three genes, with two QTLs on chromosomes 2 and 5 identified [80, 83]. One to four incomplete dominant genes were estimated to confer tolerance in *S. pimpinellifolium* LA407, in *S. arcanum* LA2157 three loci on chromosomes 5, 7 and 9 that are involved in tolerance were identified. These loci are additive and co-dominant, with the major QTL located on chromosome 7 [81]. While these accessions exhibit limited to no wilting symptoms, the growth of the pathogen is not inhibited in any of them [82]. Studies have suggested that the tolerance observed in wild species might be related to vascular morphology [48, 83, 84]. A faster maturation of the vascular tissue in lines carrying the *S. habrochaites* LA407 QTL on chromosome 2 has been observed [83], while in *S. arcanum* LA2157 it was found that the spread of the bacterium is restricted to the protoxylem. This together with the lack of necrotic cankers observed in *S. arcanum* LA2157, were suggested to be a result of the impaired ability of *Cm* to macerate the tissue of some wild species [84].

SCOPE OF THIS THESIS

This thesis aimed at identifying components that lead to susceptibility in the tomato-*Cm* interaction. A large part of this thesis explores the possibility of using mutant alleles of host susceptibility genes to gain resistance to *Cm*. A chapter of this thesis is also dedicated in the identification of the loci underlying the tolerance observed in a previously described cross between tomato and accession *S. arcanum* LA2157.

In **Chapter 2**, we review the current knowledge on plant susceptibility genes, with a special focus on host genes manipulated by bacteria. In this review, we highlight ways to identify and modify S genes. Finally, we propose a new class of S genes involved in the translocation of bacterial effector proteins in host cells.

Chapter 3 describes the role of gene *SlWAT1* in tomato susceptibility to *Cm*. Using knock-down and knock-out tomato lines of the gene we functionally characterised *SlWAT1* as a susceptibility factor to genetically diverse *Cm* strains. Finally, we worked towards understanding the molecular mechanism of the tolerance observed after inactivation of *SlWAT1*. This work provides novel insights into the role of phytohormones in the infection process of *Cm*.

Chapters 4 and **5** aimed at the identification and functional characterization of candidate S genes. Using post-transcriptional silencing through virus-induced gene silencing (VIGS), we selected three candidate genes (*SlWRKY23*, and the putative oxygenases *Solyc09g089680.3* and *Solyc12g005380.2)* for further analysis. For these genes, we generated mutants using CRISPR/Cas9 and studied potential changes in susceptibility and bacterial dynamics of *Cm* in the mutants.

In **Chapter 6**, we set out to identify the genes underlying tolerance on the major QTL on chromosome 7 observed in a previously described cross between the accession *S. arcanum* LA2157 and *S. lycopersicum*. To our surprise, the QTL on chromosome 7 that was previously found to result in high tolerance to *Cm*, did not co-segregate with our observed tolerant phenotypes. Therefore, we employed a bulk segregant analysis (BSA) to identify the causal loci.

This thesis is concluded with **Chapter 7**, in which I summarize and discuss the implications of our main findings in achieving resistance to *Cm*. Finally, I finish the discussion with questions about the molecular aspects of the interaction that remain to be answered.

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CHAPTER 2

Susceptibility reversed: modified plant susceptibility genes for resistance to bacteria

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ABSTRACT

Plants have evolved complex defence mechanisms to avoid invasion of potential pathogens. Despite this, adapted pathogens deploy effector proteins to manipulate host susceptibility (S) genes, rendering plant defences ineffective. The identification and mutation of plant S genes exploited by bacterial pathogens is important for the generation of crops with durable and broad-spectrum resistance. Application of mutant S genes in breeding of resistant crops is limited, due to potential pleiotropy. New genome editing techniques open up new possibilities for the modification of S genes. In this paper, we focus on S genes manipulated by bacteria and propose ways for their identification and precise modification. Finally, we propose that genes coding for transporter proteins represent a new group of S genes.

The fundamentals of plant immunity

Plants are constantly exposed to a multitude of potential pathogens like viruses, fungi and bacteria. For microbes to become pathogenic a high degree of adaptation is required to overcome the layers of defences plants have evolved [1, 2]. Plants possess the ability to fight off the majority of invading microbes, making susceptibility the exception in plant-pathogen interactions [1]. In plants, a two-layered defence system is activated upon interaction of microbial molecules with extracellular and intracellular immune receptors. In the first layer, pattern recognition receptors (PRRs) on the cell surface perceive conserved microbial elicitors called pathogen- associated molecular patterns (PAMPs) leading to PAMP-triggered immunity (PTI). Adapted pathogens can overcome PTI by deploying effector proteins leading to effector-triggered susceptibility (ETS)**.** In the second layer of defence, plants counteract ETS through the evolution of resistance (R) genes. Inside the cell, pathogen effectors are directly or indirectly recognised by the products of corresponding dominant R genes resulting in effector-triggered immunity (ETI) [3, 4]. Effectors can however rapidly evolve to overcome ETI by avoiding recognition of R proteins, leading once again to ETS [3].

To secure a compatible interaction, pathogen effectors target plant factors encoded by susceptibility (S) genes to manipulate host processes to their advantage. Suppression of defences, nutrient acquisition and transport of bacterial proteins in the host cell include some of the processes pathogens use to cause disease (Figure 1) [3-5]. Although S genes are exploited by pathogens to promote disease, their mutation can lead to durable, recessively inherited and potentially broad-spectrum resistance in plants [6].

More than 200 species of bacteria can infect plants and cause diseases [7]. So far, management of bacterial diseases has mainly been based on the use of chemicals and host resistance [8]. For decades, resistance breeding has successfully relied on the introgression of major R genes that recognise microbial effectors and confer resistance in crops. Bacterial effectors are however under strong negative selection when exposed to corresponding R genes resulting in fast break-down of resistance [9, 10]. In addition, bactericide resistance can rapidly evolve through horizontal gene transfer between bacterial species [8, 11].

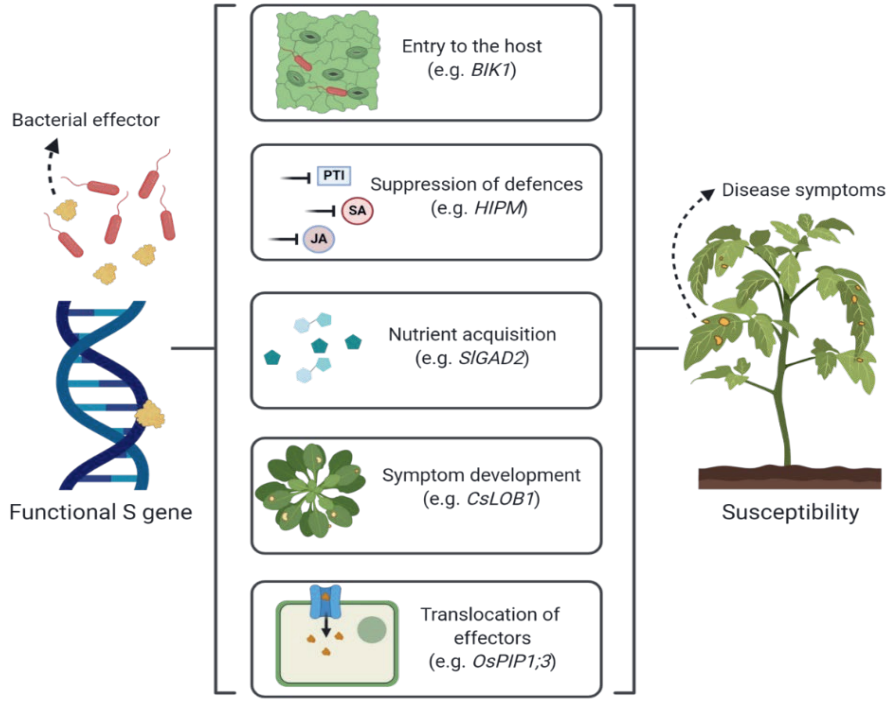

Figure 1| Manipulation of plant susceptibility (S) genes by bacterial pathogens. Plant pathogenic bacteria use effectors proteins to target and manipulate plant S genes. Bacteria use functional S genes in order to complete processes such as entry into their host, suppression of defences, acquisition of nutrients, symptom development and translocation of their effectors into host cells, resulting in plant susceptibility. PTI= PAMP-triggered immunity; SA= salicylic acid, JA= jasmonic acid. Figure made with BioRender.

Therefore, novel breeding strategies, like the use of mutated plant S genes, for the control of bacterial diseases are needed. Here, we review S genes in different pathosystems, but specifically focus on S genes recently shown to be involved in susceptibility to bacteria (Table 1). We propose ways in which S genes can be identified and modified to gain plant resistance to bacteria and suggest that genes coding for transporter proteins represent a new category of S genes.

Susceptibility genes…

…are defined as

Any plant gene that facilitates a compatible interaction with the pathogen can be considered an S gene [12, 13]. S genes belong to diverse gene families and have widely different functions (Figure 1).

Table 1| Identified susceptibility genes manipulated by bacteria, their function and interacting effectors in different plant species.

Susceptibility gene	Effector	Pathogen	Plant species	S gene category	Reference
BIK1	XopR	*Xanthomonas oryzae pv. oryzae (Xoo)*	Arabidopsis	Suppression of defences/ Entry to the host	[38]
TaNCED_5BS, TaNCED_5DS	tal8	*Xanthomonas translucens pv. undulosa (Xtu)*	Wheat	Suppression of defences	[44]
SAM-MT1, SAM-MT2	AvrXccB	*Xanthomonas campestris pv. campestris (Xcc)*	Arabidopsis	Suppression of defences	[41]
HIPM	HrpN	*Erwinia amylovora*	Apple	Suppression of defences	[40, 62]
SWEET11	PthXo1	*Xanthomonas oryzae pv. oryzae (Xoo)*	Rice	Nutrient acquisition	[18, 48]
SWEET13	PthXo2	*Xanthomonas oryzae pv. oryzae (Xoo)*	Rice	Nutrient acquisition	[18, 48]
SWEET14	AvrXa7 PthXo3 TalC TalF	*Xanthomonas oryzae pv. oryzae (Xoo)*	Rice	Nutrient acquisition	[18, 48]
GhSWEET10	Avrb6	*Xanthomonas citri pv. malvacearum (Xcm)*	Cotton	Nutrient acquisition/ Symptom development	[17]
MeSWEET10a	$TAL20_{xam668}$	*Xanthomonas axonopodis pv. manihotis (Xam)*	Cassava	Nutrient acquisition	[49]
CsLOB1, CsLOB2, CsLOB3	Any effector of Xcc and Xfa, PtXa4	*Xanthomonas campestris pv. campestris (Xcc), Xanthomonas fuscans pv. aurantifolii (Xfa)*	Sweet orange, Grapefruit	Symptom development	[53-55]

OsPIP;3	Hpa1	*Xanthomonas oryzae pv. oryzae (Xoo)*	Rice	Translocation of effectors	[57, 59, 60]
OsImpα1a, OsImpα1b	TALEs of Xoo and Xoc	*Xanthomonas oryzae pv. oryzae (Xoo), Xanthomonas oryzae pv. oryzicola (Xoc)*	Rice	Translocation of effectors	[5]
Osaba1	Unknown	*Xanthomonas oryzae pv. oryzae (Xoo), Xanthomonas oryzae pv. oryzicola (Xoc)*	Rice	(Entry to the host) Suppression of defences	[36]
LPT3, LPT4	Unknown	*Pseudomonas syringae*	Arabidopsis	Suppression of defences	[45]
WAT1	Unknown	*Ralstonia solanacearum*	Arabidopsis	Suppression of defences	[25, 26]
Upa20	AvrBs3	*Xanthomonas campestris pv. vesicatoria*	Pepper	Nutrient acquisition/ Symptom development	[51]
CaMLO6	Unknown	*Ralstonia solanacearum*	Pepper	Suppression of defences	[46]
AtGAD1, AtGAD2, AtGAD4, NdGAD4, SlGAD2	RipI	*Ralstonia solanacearum*	Arabidopsis, tobacco, tomato	Nutrient acquisition	[50]

A first categorization of S genes suggests that they largely fall into three categories [12]. The first includes genes that are involved in host entry. A well-known example in susceptibility to powdery mildew is *Mildew Locus O* (*MLO)*. Inactivation of *MLO* prevents fungal penetration in host cells [14]. Genes that act as negative regulators of defences belong in the second category. An example is *Downey Mildew Resistance6* (*DMR6),* a putative 2(OG)-Fe(II) oxygenase that is involved in the catalysis of the defence associated hormone salicylic acid (SA). Loss-of-function of *DMR6* leads to resistance to pathogens via induction of SA levels [15, 16]. The third

category includes genes that allow sustained compatibility with the host, like genes that assist nutrition and metabolic processes of the pathogen. For instance, *Sugars Will Eventually be Exported Transporter (SWEET)* genes, which act as effector targets, are involved in sugar transport to the apoplast where bacteria reside. During infection they are upregulated by transcription activator-like effectors (TALEs) and provide nutrients to the bacteria [17, 18].

As S genes are researched further it is becoming clear that more functional categories of genes are involved in susceptibility. As discussed in the "…to translocate effectors" subsection of this review, it was recently shown that genes coding for transporter proteins are targeted by bacteria for the translocation of their effectors. Thus, transporter proteins represent an important new S gene category.

… are often involved in physiological processes of plants

It might seem counterintuitive that plant genes which promote plant susceptibility to pathogens have been evolutionary retained. Many S genes however are required in physiological processes of plants. Transporter *OsSWEET11* is involved in pollen development and grain filling in rice (*Oryza sativa*). During infection, upregulation of the gene also supports the growth of the bacterium *Xanthomonas oryzae* pv*. oryzae* (*Xoo*) *in planta* [19, 20]. Due to their dual role in physiological processes and susceptibility, inactivation of S genes might lead to resistance along with pleiotropic effects. Yet, the extend of fitness costs is dependent on the plant species and growing conditions. *mlo* barley (*Hordeum vulgare* L.) mutants exhibit broad-spectrum powdery mildew resistance accompanied with autonecrosis and early leaf senescence. In tomato (*Solanum lycopersicum*), the naturally occurring *ol-2* mutant, an ortholog of barley *MLO*, confers broad-spectrum resistance to powdery mildew without any fitness costs observed [21]. Downregulation of the gene *Defence No Death1 (DND1)* results in autonecrosis and severe stunting in tomato. In potato (*Solanum tuberosum*), silencing of the ortholog causes only mild autonecrosis that is dependent on the plant growing conditions [22].

… are often conserved

Orthologs of S genes are often present across species, most probably due to their involvement in biological functions of plants. *SWEET* gene orthologs involved in seed development have been identified in arabidopsis (*Arabidopsis thaliana*), rice and soybean (*Glycine max.*) [20, 23, 24]. The auxin transporter *Walls Are Thin1* (*WAT1),* which is involved in secondary cell wall biosynthesis is a functional S gene to vascular

pathogens in cotton (*Gossypium hirsutum*) and arabidopsis [25, 26]. Since its discovery in barley, *MLO* has been identified in species such as arabidopsis, tomato, pea (*Pisum sativum*), cucumber (*Cucumis sativus* L.), eggplant (*Solanum melongena*), tobacco (*Nicotiana benthamiana),* grapevine (*Vitis vinifera*) and apple (*Malus domestica*) [14, 21, 27-31]. Orthologs of S genes, such as *DMR1*, *DMR6*, *Powdery Mildew Resistance4* (*PMR4*), *PMR6, Cellulose Synthase A catalytic subunit 3* (*CESA3*) and *DND1* have been identified and functionally characterized in arabidopsis, tomato and potato [32-34]. The conservation of S genes is an important feature for their applicability in breeding. In the post-genomics era, discovery of S genes in model species makes the identification and functional characterization of orthologs in crops a relatively easy and rather straightforward task (Figure 2).

S genes are exploited by bacteria…

… to enter the host

Entry to the host is a critical step for bacterial infections. Natural openings like the stomata or hydathodes are important entry portals for bacteria [35]. Stomata closure upon pathogen challenge is a well-known basal defence mechanism that limits pathogen entry [35, 36]. For instance, the negative immune regulator *RPM1-interacting protein4 (RIN4)* acts together with H+ ATPases AHA1 or AHA2 to control stomata re-opening during *Pseudomonas syringae* DC3000 invasion [37]. Similarly, effector XopR of bacterium *Xoo* targets *Botrytis-induced Kinase1 (BIK1)* to suppress PAMP-induced stomata closure [38]. New insights suggest that stomata-regulated transpiration is a novel mechanism restricting bacterial growth and spread. Bacterial pathogen growth and spread appears to be favoured by high humidity and water soaking of leaves. In rice, abscisic acid (ABA) mutant *Osaba1* exhibits broad-spectrum resistance to *Xoo*. The stomata of the mutant plant remain open after infection leading to higher water loss and limited spread of the bacterium [36].

… to supress immune responses

To fight off invading pathogens plants activate complex defence responses, like the generation of reactive oxygen species (ROS), cell wall modifications and the production of antimicrobial compounds [12]. The contribution of these responses to resistance is dependent on the lifestyle of the pathogen. For example, ROS generation and hypersensitive response (HR) limit the growth of (hemi)biotrophs. Inversely, to successfully infect the host necrotrophs stimulate ROS production to induce susceptibility-associated cell death [39]. In apple the necrogenic bacterium *Erwinia amylovora* targets gene *HIPM (HrpN-interacting protein from Malus spp.)* to

stimulate ROS generation and establish infection [40]. In contrast, effector AvrXccB of *Xanthomonas campestris* pv. *campestris* (*Xcc*) targets the putative methyltransferase complex SAM-MT1/SAM-MT2 to suppress ROS production and callose deposition to induce susceptibility [41].

To further modulate immune responses, plants have evolved tightly regulated networks of hormones [42]. The relationships between hormonal pathways can be antagonistic or synergistic [43]. Thus, genes involved in changes in the balance of hormones are prime targets for bacteria. Genes *TaNCED_5BS* and *TaNCED_5DS* which are involved in the catalysis of the hormone abscisic acid (ABA) are upregulated by the pathogen *Xanthomonas translucens* pv. *undulosa* (*Xtu*) in wheat. ABA induced *Lipid Transfer Protein* (*LPT*) genes *LPT3* and *LPT4* are upregulated by *P. syringae* pv. *tomato* in arabidopsis during infection. In both cases, induction of expression of the genes leads to susceptibility through antagonism between the ABA and SA pathways [44, 45]. In arabidopsis, mutation of *WAT1* enhances broad range resistance to vascular pathogens, including *Ralstonia solanacearum*, via altered cross-regulation of auxin and SA pathways [25]. In transgenic pepper (*Capsicum annum*) plants, silencing of gene *CaMLO6* decreases the development of wilting symptoms caused by *R. solanacearum*, possibly due to blocking of SA and JA-dependent signalling [46].

Impairment of S genes involved in the regulation of hormonal pathways targeted by bacteria can lead to resistance to pathogens. Nevertheless, resistance obtained in mutants to a group of pathogens due to antagonistic relationships between hormones can lead to increased susceptibility to pathogen groups with contrasting lifestyles [47].

… to acquire nutrients and cause symptoms

After pathogens have entered the host and suppressed immune responses, they must sustain their compatible interaction. To do so, bacteria utilize host genes to acquire nutrients, proliferate and cause symptoms. Sugar transporters of the *SWEET* family are manipulated by bacteria to fulfil their nutritional needs. *SWEET* genes are upregulated during infection through binding of TALEs to their promoters. Upregulation of their expression increases the efflux of sugars that bacteria use as carbon sources in the apoplast where they reside [17, 18]. In rice, three *SWEET* genes; *OsSWEET11, OsSWEET13* and *OsSWEET14* are targeted by *Xoo* TALEs [18, 48]. Cotton gene *GhSWEET10* is targeted by Avrb6 of *X. citri* pv. *malvacearum* (*Xcm*). Silencing of *GhSWEET10* leads to reduced development of water-soaking symptoms

[17]. Similarly, $TAL20_{Xam668}$ carried by *X. axonopodis* pv. *manihotis* binds and upregulates *MeSWEET10a* in cassava (*Manihot esculenta*) [49].

During infection the highly conserved RipI effector of *R. solanacearum* physically interacts with plant glutamate decarboxylase (GAD) proteins to promote their biochemical activation. In return, *GADs* catalyse the biosynthesis of gamma-aminobutyric acid (GABA), an amino acid used by *R. solanasearum* as a nutrient [50]. Co-immunoprecipitation (co-IP) assays have confirmed the interaction of RipI with GAD proteins in different plant species. In arabidopsis, RipI physically interacts with proteins AtGAD1, AtGAD2 and AtGAD4. In *Nicotiana benthamiana* and tomato, genes *NbGAD4* and *SlGAD2* are targeted by RipI, respectively. Mutation of *AtGAD1* and *AtGAD2* genes in arabidopsis leads to compromised bacterial growth and delayed symptom development. Likewise, downregulation of *SlGAD2* in tomato roots leads to reduced wilting symptoms [50].

In pepper, effector AvrBs3 of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) upregulates the expression of bHLH transcription factor *Upa20* resulting in cell hypertrophy. *Xcv* likely exploits cell hypertrophy for increased nutrient production of enlarged cells [51]. Transcription factor *CsLOB1,* is a major S gene in citrus species [52]. *CsLOB1* is targeted by all the major TALE effectors carried by *X. citri* spp. *citri* (*Xcc*) and *X. fuscans* pv. *aurantifolii* (*Xfa*) strains that cause citrus canker [53]. Upregulation of *CsLOB1* in citrus species promotes bacterial growth and pustule formation [53, 54]. Additionally, two more homologues of *CsLOB1; CsLOB2* and *CsLOB3* are targeted by TALEs and have been shown to contribute to pustule formation in citrus [55].

… to translocate effectors

Many aspects of plant physiology are dependent on membrane transport processes. Transporter proteins such as channels, pumps and other carriers, are involved in important processes like nutrient acquisition, osmoregulation and stress responses [56]. Recent reports have identified new roles of such genes in plant immunity. As an example, aquaporins that act as intracellular channels for the transport of water and small substrates across membranes [57], have been involved in both resistance and susceptibility to bacteria. In arabidopsis, aquaporin *AtPIP1;4* transports pathogen induced H_2O_2 (a core component of ROS) to the cytoplasm for the activation of PTI pathways [58]. By contrast, aquaporin *OsPIP1;3* in rice is an S gene to bacterium *Xoo.* To deliver effector proteins Gram-negative bacteria use a type III translocon, that is assumed to be assembled by interacting bacterial translocators

and eukaryotic proteins [59]. The Hpa1 translocator of *Xoo* physically interacts with *OsPIP1;3* to deliver effector PthXo1 into the cytoplasm. Inactivation of *OsPIP1;3* leads to resistance to *Xoo* through disruption of the translocation of the effector into the cytosol during infection [57, 59, 60]. Exciting examples of genes that can confer broad spectrum resistance to TALEs carrying bacteria are *OsImpα1a* and *OsImpα1b* in rice. These two genes code for nucleocytoplasmic transporters of the importin family. In their study Hui et al. [5] identified five conserved amino acids on the nuclear localization signal (NLS) of all TALEs of the *Xoo* and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strains they studied, that interact with the OsImpα1a/ OsImpα1b proteins. Downregulation of *OsImpα1a* and *OsImpα1b* disables the translocation of TALEs in the nucleus where they target *SWEET* genes, leading to broad-spectrum resistance [5]. Hence, genes involved in transport processes might represent a new category of S genes. Depending on the conservation of the interacting sites of the proteins, suppression of such genes may provide a new strategy to gain broad-spectrum resistance to bacteria.

S genes …

… can be identified

Most S genes have been identified through forward genetic studies. Screenings of wild germplasm or mutagenized populations have yielded a number of recessive alleles that confer resistance [13]. Here, we explore alternative options for the identification of S genes (Fig. 2).

A recurrent feature of S genes is their conservation among species. Several S genes have been identified in the model species *Arabidopsis thaliana*. The abundance of available sequencing and transcriptomics data of crops greatly eases the identification of crop S genes through phylogenetic analyses [12]. After identification of a crop ortholog, functional analyses should follow to confirm the function of the gene as a susceptibility factor.

Bacterial pathogens secrete effectors that can induce susceptibility. Knowledge on the range of effectors carried by pathogens can enable their use as molecular tools for the discovery of S genes [61]. Physical interaction between effectors and S gene proteins has been demonstrated multiple times [5, 57, 62]. The use of protein-protein interaction assays, such as Yeast 2 Hybrid, co-immunoprecipitation (co-IP) or

proximity labelling (PL) using the effector as a probe, can assist the identification of novel S genes [61].

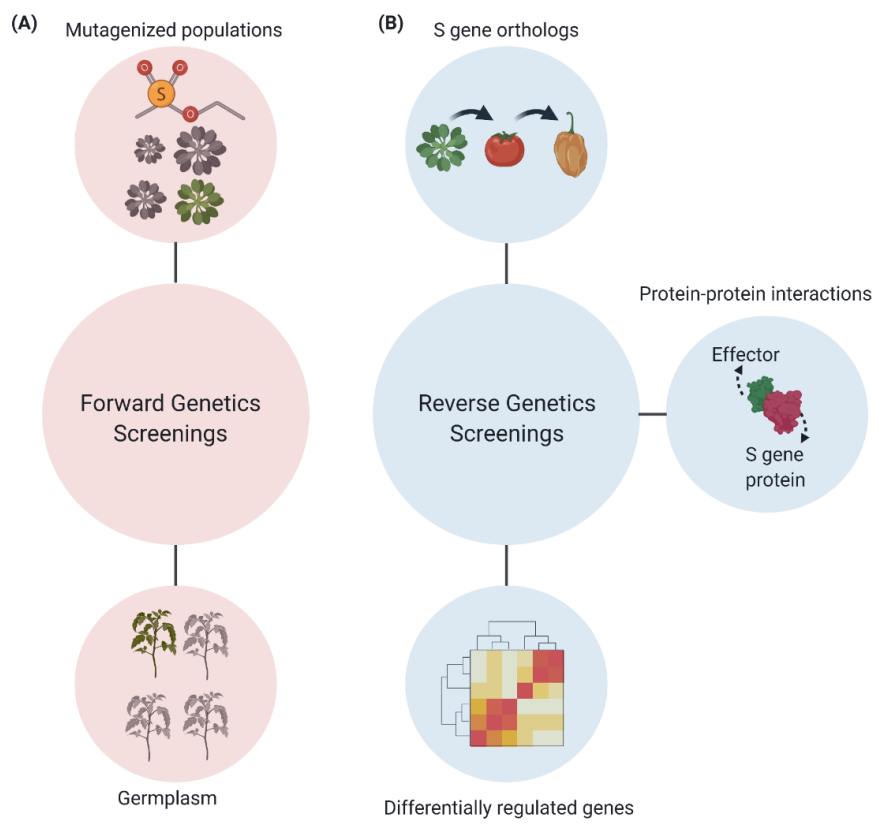

Figure 2| Identification of plant susceptibility (S) genes. A number of plant S genes has been identified through forward genetics screenings, such as screening of mutagenized populations or screening of germplasm (panel A). Here, we propose reverse genetics screens for the identification of S genes (panel B). A characteristic of S genes is their conservation between species. Phylogenetic analyses of known S genes between species can lead to the mining of S gene orthologs in different plant species. Physical interaction between bacterial effectors and proteins encoded by S genes has been demonstrated multiple times. The use of protein-protein interaction assays, using bacterial effectors as molecular probes can lead to the identification of novel S genes. A common pathogen strategy seems to be the upregulation of S genes. The generation of transcriptomics data can aid in the identification of differentially expressed genes between infected and mock treated plants. Differentially regulated genes might represent candidate S genes. Figure made with BioRender.

Upregulation of S genes is a common pathogen strategy. *PME3*, a gene involved in susceptibility of arabidopsis to nematode *Heterodera schachtii*, is upregulated upon

pathogen challenge [63]. Similarly, *MLO* homologs in grapevine, cucumber and tomato are upregulated during powdery mildew infection [29, 31, 64]. Bacterial pathogens use effectors to upregulate corresponding S genes [51, 65, 66]. The generation of transcriptomics data using high throughput techniques, like RNA-seq, can be a valuable tool in the identification of classes of differentially regulated genes. Differentially upregulated genes between infected and mock-treated plants may represent S genes. Using this approach three *CsLOB* homologues were identified in citrus [67].

… can be modified

Non-host resistance (NHR) is the resistance exhibited by the entirety of a plant species against non-adapted pathogens [68, 69]. Hallmarks of NHR are its durability and broad-spectrum [70]. The inability of a pathogen to infect a non-host plant has been largely proposed to be based on host resistance, with both PTI and ETI being involved [71, 72]. To successfully infect a host plant, pathogens use effector proteins to target host genes coding for susceptibility factors [12]. Failure of effectors to successfully manipulate their host target could lead to NHR [72]. In our view, the absence of either an evolved or functional host target or an effector evolved to manipulate the host target could maintain NHR of a plant species. Loss-of-function of S genes, such as *MLO* and *Eukaryotic Translation Initiation Factor 4E* (*EIF4E*), provide durable and broad-spectrum resistance, that exhibits all characteristics of NHR against powdery mildew and potyviruses, respectively [12, 70]. This highlights the potential of using mutant S genes to achieve NHR-like resistance.

The genetic diversity of S genes is understudied. However, a number of natural mutant alleles in crops have been identified [21, 28, 73]. Introgression of natural mutant alleles into elite cultivars or the generation of EMS populations are options for the breeding of resistant genotypes. However, both approaches are time-consuming and may introduce unwanted changes into the elite background. Here we propose options for the breeding of resistant cultivars based on precise genome editing techniques (Figure 3).

Uncoupling of adverse pleiotropic effects and resistance is the main challenge in the application of mutant S genes in breeding [6, 12]. Nevertheless, the development of new genetic engineering tools is offering new possibilities to breeders. The discovery of the CRISPR/Cas9 genome editing technology sparked a revolution in biology [74]. The CRISPR/Cas9 system has already been employed in the study of multiple S genes [48, 53, 54, 75].

Recently, base editors combined with the CRISPR/Cas9 system were used for the generation of single base changes in plants [76, 77]. In our opinion, the use of base editors for the generation of SNPs in S genes is an attractive option. Identification of interacting sites between bacterial and plant proteins (or genes) can localize the target region for the introduction of a SNP [76]. In this way, fitness costs may be avoided by introducing SNPs without altering the catalytic domains of proteins.

Changes in the promoters of S genes targeted by TALEs has shown to be a fitness cost free strategy. Induced susceptibility by TALEs is highly modifiable, due to the predictable nature of effector binding elements (EBEs). Prediction of TALEs and manipulation of their cognate EBEs can lead to fitness costs free resistance. Additionally, simultaneous mutations of EBEs through multiplex-CRISPR can lead to broad-spectrum resistance [48].

Where natural mutants are available in the germplasm, they can be used to directly replace functional S genes in cultivars. Where the germplasm is limited, synthetically generated alleles containing crucial mutations can be used instead. Introduction of loss-of-function alleles in the susceptible background can be achieved through homology directed repair (HDR), as recently was demonstrated by the efficient replacement of salinity tolerance *HKT1;2* (*High-affinity K^+ Transporter 1;2*) allele through HDR in tomato, via the use of a CRISPR/LbCas12a complex [78].

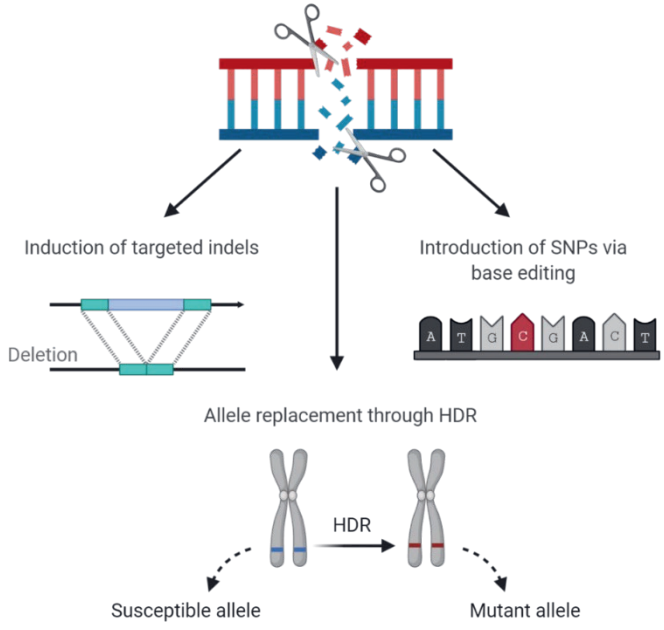

Figure 3| Modification of plant susceptibility (S) genes via precise genome editing. The potential pleiotropic effects observed upon mutation of a plant S gene may hinder their use in the breeding of resistant crops. However, the use of precise genome editing tools is offering new possibilities for generating desired mutations in S genes. The use of CRISPR/Cas systems allows the induction of targeted changes in S genes. Indels in target genes can easily be generated through CRISPR/Cas9. Further, coupling of CRISPR/Cas9 with base editors allows the generation of targeted single nucleotide polymorphisms (SNPs) in genes. Recently, allele replacement through homology directed repair (HDR) in plants using a CRISPR/LbCas12 system was reported [78]. Replacement of functional S alleles with natural or synthetically generated loss-of-function alleles through HDR can help bypass time-consuming procedures by direct replacement of alleles. Figure made with BioRender.

Outstanding questions

- Are there protein domains encoded by S genes that specifically interact with bacterial proteins? Can modification of such domains lead to fitness cost free resistance?
- To what extend are S genes identified in model species functionally conserved across crops? Does impairment of conserved S genes lead to the same pleiotropic defects in different crops?
- Is the molecular mechanism of broad range resistance conferred by mutant S genes the same for different pathogens?
- Are there resistant natural loss-of-function variants in the germplasm that do not exhibit pleiotropic defects? Can we introduce the same variants in susceptible genotypes using precise gene editing?
- What is the link between non-host resistance and susceptibility genes?
- Is there crosstalk between biotic and abiotic stresses governed by S genes? Could additional abiotic stresses complement the resistant phenotype back to a susceptible one? Multiple mutant S genes have been screened for their ability to provide broad range resistance against biotic factors, but knowledge on combination of stresses is lacking.

Concluding remarks

The very nature of bacteria makes the management of the diseases they cause a challenge. Their ability to reach population sizes that favour epidemics in a short period of time, the rapid evolution of their effectors and the development of resistance to antibiotics require the development of novel breeding strategies that can lead to durable resistance. In this paper, we highlighted the potential of using mutated S genes in breeding for plant resistance to bacteria. Furthermore, we propose that transporter proteins represent a new important category of S genes. As more S genes are identified, our knowledge into plant susceptibility and how S genes can be edited will further expand (**Outstanding questions**). Although the use

of mutated S genes in breeding remains a challenge, because of adverse pleiotropic effects, we are confident that the development of new genetic engineering tools will soon solve some of these problems. In different parts of the world the use of such technologies is now becoming a reality. Whether these technical solutions will be freely used in Europe without GM associated regulations remains to be seen.

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CHAPTER 3

Inactivation of tomato *WAT1* leads to reduced susceptibility to *Clavibacter michiganensis* through downregulation of bacterial virulence factors

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ABSTRACT

Bacterial canker of tomato caused by the vascular pathogen *Clavibacter michiganensis*, is considered to be one of the most destructive bacterial diseases of cultivated tomato worldwide. While several molecular studies have identified bacterial factors involved in disease development, the plant genes and mechanisms associated with susceptibility of tomato to the bacterium remain largely unknown. In our study, we set out to identify host susceptibility (S) genes involved in the interaction. We show that inactivation of the tomato gene *Walls Are Thin1* (*SlWAT1*) through RNAi and CRISPR/Cas9 led to high tolerance to genetically diverse *C. michiganensis* strains, without suppression of bacterial growth. Although a full knock-out of the *SlWAT1* gene led to severe fitness costs, downregulation of the gene by RNAi resulted in high tolerance of transgenic plants to the disease without severe pleiotropic effects. Furthermore, our study suggests that the observed tolerance through inactivation of *SlWAT1* is the result of downregulation of bacterial virulence factors, possibly through the reduction of auxin content.

INTRODUCTION

Plant disease resistance is genetically controlled, mostly by dominantly inherited, race specific resistance (R) genes. In the presence of corresponding pathogen-derived effectors many R genes confer resistance through effector-triggered immunity (ETI) [1]. In plant-microbe interactions, resistance is a common outcome. In fact, a high degree of adaptation is required for microbes to become pathogenic [2]. During their co-evolution pathogens have found ways to target and manipulate plant genes, referred to as susceptibility (S) genes, to promote disease development [3-8]. S genes are important for biological functions of plants, which appears to be a significant factor in their retainment across species [2]. This is exemplified by the *Mildew Locus O* (*MLO*) gene family that has been identified in plant species such as barley, tomato, Arabidopsis, grape, apple and cucumber [9-11]. In contrast to dominant R genes, loss-of-function of S genes can potentially lead to recessively inherited, broad-spectrum, and durable resistance [4, 8, 12-16]. For example, loss-of-function of genes in the glutamate decarboxylases (*GAD*s) family provide enhanced resistance against the vascular bacterium *Ralstonia solanacearum* in Arabidopsis and tomato (*Solanum lycopersicum*) [17]. In addition, mutation of *Sugars Will Eventually be Exported Transporter* (*SWEET)* genes in multiple plant species has been demonstrated to be an effective strategy to obtain resistance to *Xanthomonas spp*. [9-11].

Bacterial canker of tomato caused by the Gram-positive bacterium *Clavibacter michiganensis* (*Cm*), is considered to be one of the most important seed-borne diseases of tomato worldwide [18-20]. The pathogen colonizes the vasculature of plants leading to systemic infections that result in wilting of leaves, vascular tissue necrosis and formation of cankers on the stems and petioles of plants, eventually leading to plant death [21-23].

On the molecular level of the tomato- *Cm* interaction, several bacterial factors involved in virulence are known. Full virulence of the *Cm* reference strain NCPBB382 requires the presence of two native plasmids, pCM1 and pCM2, where the major virulence factors *celA* and *pat-1* are located [19, 24]. Loss of either of the plasmids leads to reduced virulence and loss of both results in an endophytic nonvirulent strain [25, 26]. Several other proteins are encoded by genes located on the circular chromosome of *Cm* that are involved in the colonization of plants and induction of disease symptoms [19, 24, 26, 27]. Such genes, include the transcriptional factors *virulence associated transcriptional regulator1* (*vatr1*) and *vatr2*, which act in the

regulation of several other virulence genes. *vatr1* and *vatr2* are involved in the regulation of virulence factors, such as the endo- beta- 1,4- glucanase *celA*, subtilase proteinase *SbtC* and the serine protease *pat-1* and *PhpA*, both on the chromosome and plasmids of *Cm* [24].

While several molecular studies have identified bacterial factors involved in disease development, the mechanisms associated with susceptibility of tomato to the bacterium remain largely unknown. The only experimentally confirmed plant factor involved in disease development is the phytohormone ethylene [24, 26, 28]. During infection *Cm* promotes the production of host-derived ethylene by specifically upregulating the ethylene biosynthetic gene *ACO1*. Mutant *Never ripe* (*Nr*) tomato plants with impaired ethylene perception display significant *Cm* symptom development delay [28]. The observation that the nonvirulent *Cmm100* strain lacks the ability to induce the production of host derived ethylene further highlights the importance of ethylene in *Cm* symptom development [26].

Despite extensive screenings of wild germplasm, resistance to the pathogen has not been identified yet [29]. In our study, we hypothesized that impairment of S genes involved in the tomato- *Cm* interaction might result in loss-of-susceptibility. Therefore, we set out to identify tomato susceptibility genes potentially involved in the interaction.

Recently, the tomato ortholog of *Walls Are Thin1* (*SlWAT1*) was identified and inactivated through RNAi and CRISPR/Cas9 in our group [30]. CRISPR/Cas9 mediated knock-out of the gene led to resistance to the vascular fungi *Verticillium dahliae*, *V. albo-altrum* and *Fusarium oxysporum* f. sp. *lycopersici* [30]. The Arabidopsis *WAT1* gene encodes for a tonoplast localized plant-specific protein. *WAT1* has been shown to be involved in vacuolar auxin transport and secondary cell wall biosynthesis [31, 32]. In Arabidopsis loss-of-function of the gene leads to enhanced resistance to a broad range of vascular pathogens, including the bacterium *Ralstonia solanacearum* [33]. In cotton (*Gosypium hirsutum*) three *WAT* homologs have been identified. Simultaneous transient silencing of the cotton genes enhanced resistance to the vascular fungus *V. dahliae* [34]. In both species resistance involves the repression of indole metabolism and altered contents of indole-3-acetic acid (IAA) and salicylic acid (SA) [33, 34]. In addition, local lignin deposition was associated with *V. dahliae* resistance in cotton [34]. In this study, we show that impairment of *SlWAT1* through RNAi and CRISPR/Cas9 leads to broad-spectrum reduced susceptibility to genetically different *Cm* strains. Next to this, we show that downregulation of the gene reduces

auxin content in tomato stems and leads to downregulation of bacterial virulence factors.

RESULTS

Down-regulation of *SlWAT1* leads to broad-spectrum reduced susceptibility to *Cm*

To study the role of *SlWAT1* in susceptibility of tomato to *Cm*, homozygous T_3 progeny of two RNAi lines (RNAi::SlWAT1_1 (TV181036) and RNAi::SlWAT1_2 (TV181034)) derived from two independent transformants in cv. Moneymaker (cv. MM) background were used [30]. Expression analysis of the T_2 parental lines, revealed that the relative residual expression of lines RNAi::SlWAT1_1 and RNAi::SlWAT1_2 was on average 20% and 54%, respectively [30]. To evaluate the spectrum of resistance conferred by silencing of *SlWAT1*, lines RNAi::SlWAT1_1 and RNAi::SlWAT1_2 were challenged with four genetically diverse *Cm* strains [36]. The infected lines were used in disease assays, during which wilting symptoms on the plants were recorded up to 20 dpi. Severe wilting symptoms caused by all four genetically distinct *Cm* stains were observed on cv. MM plants. Aggressiveness of the four strains differed, with NCPBB382 being the most aggressive and CFBP5843 being the least aggressive strain (Supplementary Figure 1). Both transgenic lines used in the disease assays exhibited significant reduction of wilting symptoms to all tested strains (Figure 1). Mild wilting symptoms were observed on RNAi::SlWAT1_1 transgenic plants when inoculated with the most aggressive NCPBB382 strain. For RNAi::SlWAT1_2 mild symptoms were observed for strains NCPBB382 and IPO3356. We attribute the difference observed between the two lines to the higher residual expression of the target gene in RNAi::SlWAT1_2.

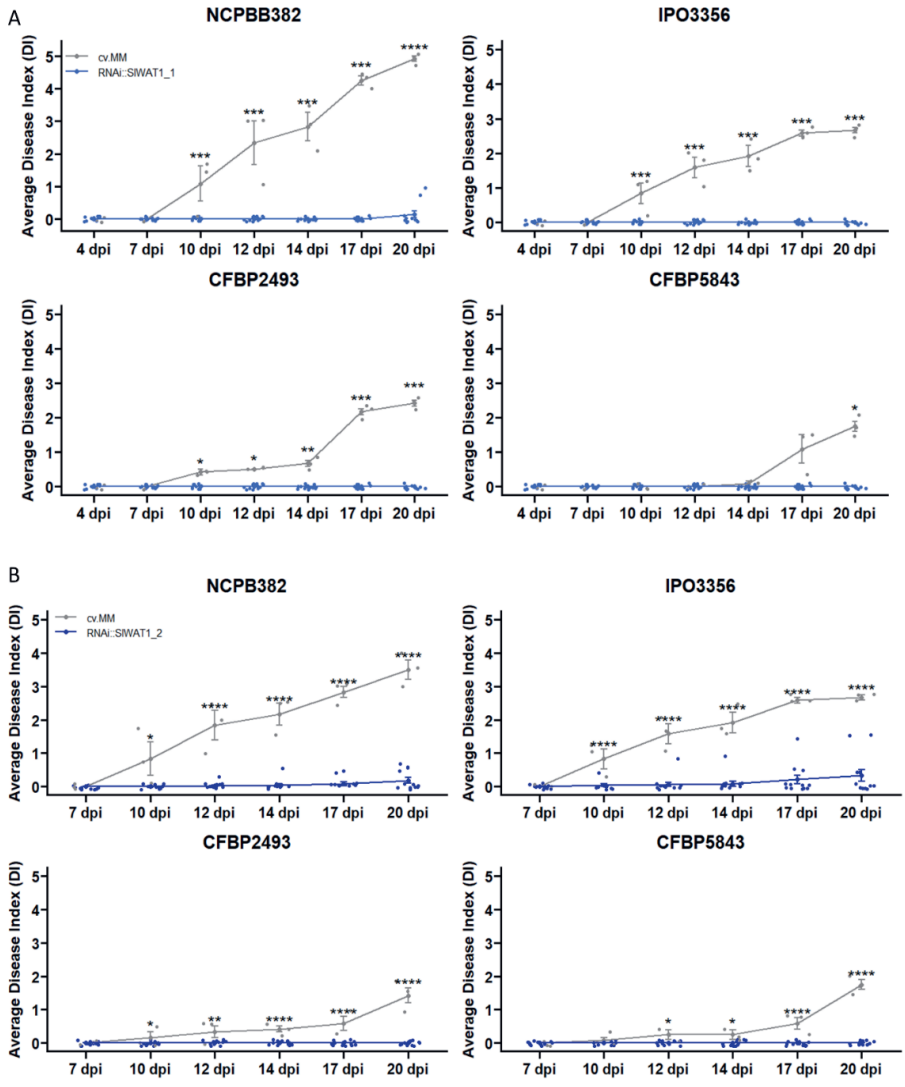

Figure 1| Disease index of *SlWAT1* RNAi silenced lines inoculated with genetically diverse *Cm* strains. Wilting symptom development of A) RNAi::SlWAT1_1 and B) RNAi::SlWAT1_2 lines compared to the background donor susceptible control cv. MM from 7 dpi to 20 dpi. Means of both RNAi::SlWAT1 lines were significantly different from the cv.MM controls, for all strains used in the disease assay (n=12). Bars indicate the standard errors. Asterisks indicate significant differences (Student's t-test, * p≤0.05; ***p≤0.001,****p≤0.0001).

Bacterial growth is not suppressed in RNAi::SlWAT1_1 transgenic plants

To determine whether the reduction in observable wilting symptoms in the transgenic plants was correlated with changes in the bacterial growth *in planta*, the population dynamics of the four different strains were quantified at three time points (4 dpi, 7 dpi and 14 dpi). An estimated $5x10^5$ colony forming units (cfu)/mL was used for the inoculation of the plants. Over the course of infection, all *Cm* strains reached high population densities (~10^9 log_{10}(cfu+1/ g fresh stem tissue)). No significant statistical differences in population densities were observed between the susceptible cv. MM and transgenic plants for strains NCPBB382, IPO3356, CFBP5843 and CFBPB2493 (Fig. 2).

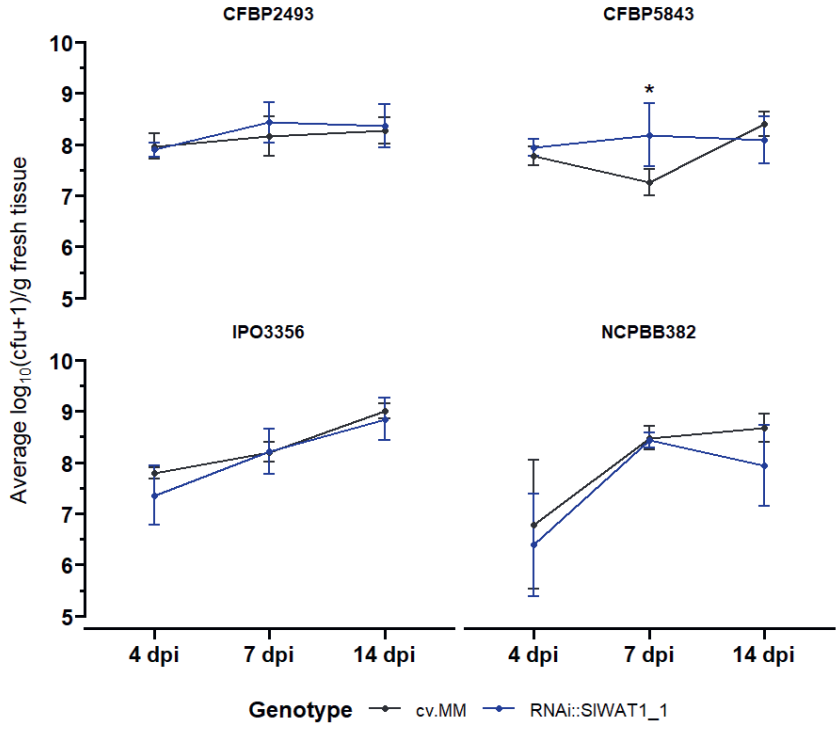

Figure 2| *Clavibacter michiganensis* population dynamics in cv. MM and RNAi::SlWAT1_1 transgenic plants. Bacterial titres of the four bacterial strains used in the experiments were quantified at 4, 7 and 14 dpi. Five biological replicates (n=5) were used per time point and bacterial strain. Lines represent the average log_{10}(cfu+1/ g fresh tissue) ± stdev. The experiments were repeated independently at least twice with similar results. Asterisks indicate statistical differences (Student's t-test, * p≤0.05).

CRISPR/Cas9- mediated knock-out of *SlWAT1* leads to loss-of-susceptibility to *Cm* without suppression of bacterial growth

To exclude the possibility of interference of the residual expression of *SlWAT1* in the RNAi lines with the phenotype observed and to confirm our previous results, we decided to include a CRISPR/Cas9 mutant line in the experiments. Silencing of *SlWAT1* through RNAi did not lead to any observable adverse pleiotropic effects (Fig. 3H). However, for the gene edited mutant line *slwat1,* severe growth retardation was observed, as previously described (Fig. 3) [30]. Besides the severe growth retardation, lack of chlorophyll and strong accumulation of anthocyanins at the abaxial side of developing leaves at early developmental stages were also observed. The latter phenotypic abnormalities were alleviated as the plants grew older.

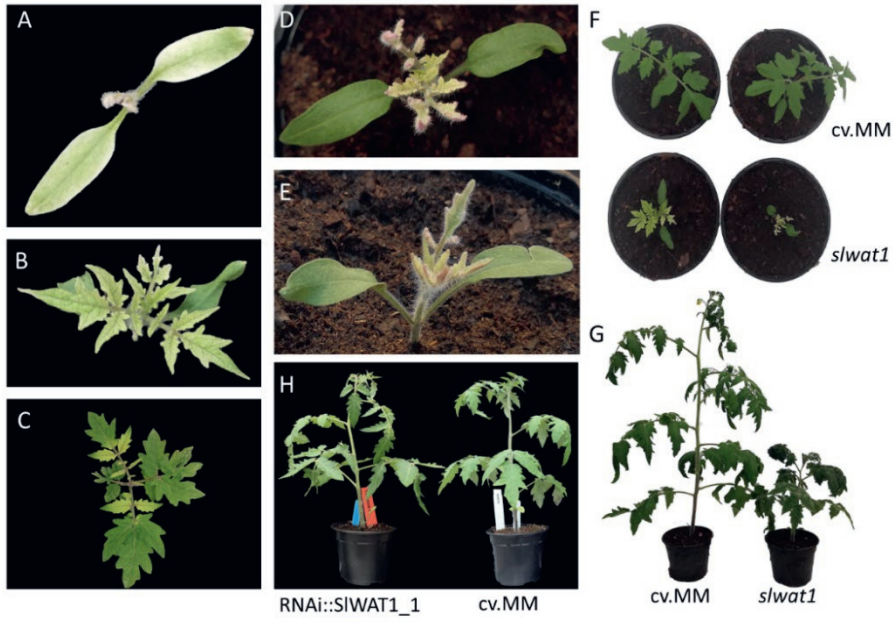

Figure 3| Pleiotropic phenotypes of *slwat1* knock-out mutant plants. A), B), C) Lack of chlorophyll observed in developing *slwat1* mutants. D), E) Anthocyanin accumulation in the abaxial side of leaves of developing mutants. Severe growth retardation in four weeks old F) and ten weeks old G) *slwat1* mutants compared to the cv.MM (*WT*). H) phenotype of 6 weeks old RNAi::SlWAT1_1 transgenic plants compared to cv.MM.

Changes of tomato susceptibility in response to *Cm* due to different developmental stages have previously been reported. Generally, the severity of disease decreases and the incubation period becomes longer with inoculations at later developmental stages [40, 41]. Therefore, for the inoculation of the plants we decided to use control plants at the same developmental stage as the mutants (4^{th} leaf stage). To achieve synchroneity in the developmental stages of our two genotypes control plants were sown every week. When the control plants and the *slwat1* mutants were at the same developmental stage we challenged them with the hypervirulent strain NCPBB382. At 20 days post inoculation (dpi), severe wilting symptoms were observed in the susceptible background cv. MM. No symptoms were observed in the *slwat1* mutants, confirming our previous results (Fig. 4, Fig. S2A). Finally, to eliminate the possibility that the residual gene expression in the RNAi lines resulted in sustained bacterial growth, we quantified the *in planta* bacterial titres recovered from *slwat1* mutants. Similarly to the transgenic plants, no significant statistical changes were found between the susceptible cv. MM and *slwat1* mutants (Fig. S2B).

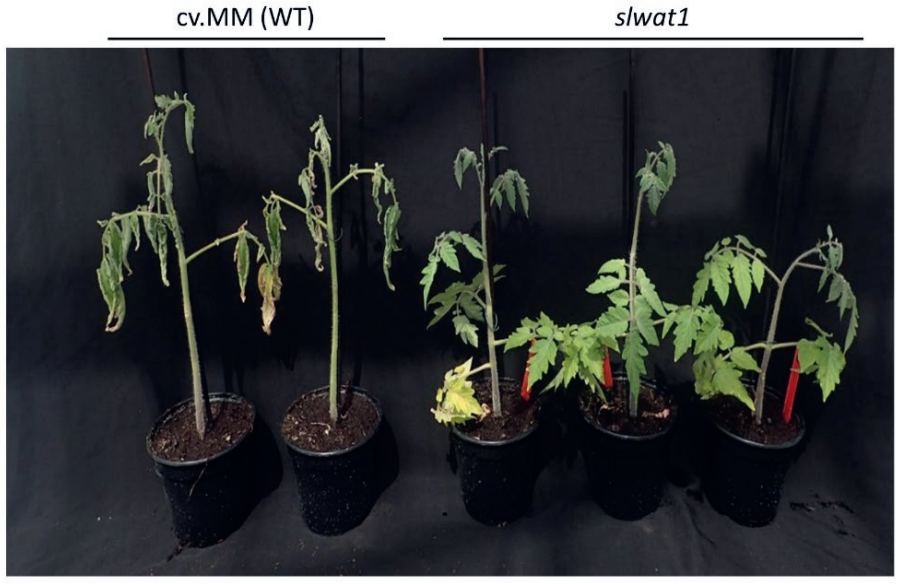

Figure 4| Symptom development of mutants *slwat1* in comparison to the susceptible background cv.MM inoculated with strain NCPBB382 at 20 dpi.

Silencing of *SlWAT1* reduces auxin content and affects the expression of auxin related genes

Repression of indole metabolism and transcriptional changes of auxin related genes have been reported in Arabidopsis *wat1* mutants and cotton *WATs* silenced plants [33, 34]. In our experiments, we monitored the expression of genes involved in auxin transport and auxin responses at different infection time points (Fig. 5). Firstly, we quantified the expression of *SlWAT1*. As expected, the gene was significantly downregulated in RNAi::SlWAT1_1 plants compared to the cv. MM background (Fig. 5A, E). Even though for the rest of the genes we studied no statistically significant differences were found, we did observe differences in the regulation of the genes between cv. MM and transgenic plants (Fig. 5). At 1 dpi, we found that genes *SlPIN1* and *IAA19* were upregulated in mock inoculated transgenic plants compared to cv.MM. Upon inoculation, we observed that the expression of the two genes was downregulated in transgenic plants (Fig. 5B, D). The same pattern of expression can be observed for gene *IAA19* also at 7 dpi (Fig. 5H). At 1 dpi, we observed that the expression of the auxin importer *LAX4* was upregulated in transgenic plants inoculated with *Cm* (Fig. 5C), while at 7 dpi the expression of the gene was only slightly upregulated after inoculation of transgenic plants (Fig. 5G). In contrast to 1 dpi, the expression of auxin efflux transporter *SlPIN1* at 7 dpi was upregulated in infected transgenic plants compared to the susceptible cv.MM plants (Fig. 5F).

Finally, we quantified the levels of auxin in different parts of tomato stems through LC-MS/MS. Free IAA content was quantified at the apical parts of the stem and hypocotyls of transgenic and cv. MM plants that were mock treated or inoculated (7 dpi). Our results confirm that silencing of *SlWAT1* significantly reduces free IAA levels in tomato stems. Consistent with the basipetal auxin transport from source to sink, we also observed a gradient in auxin concentration between the apical meristems and hypocotyls in both genotypes (Fig. 6).

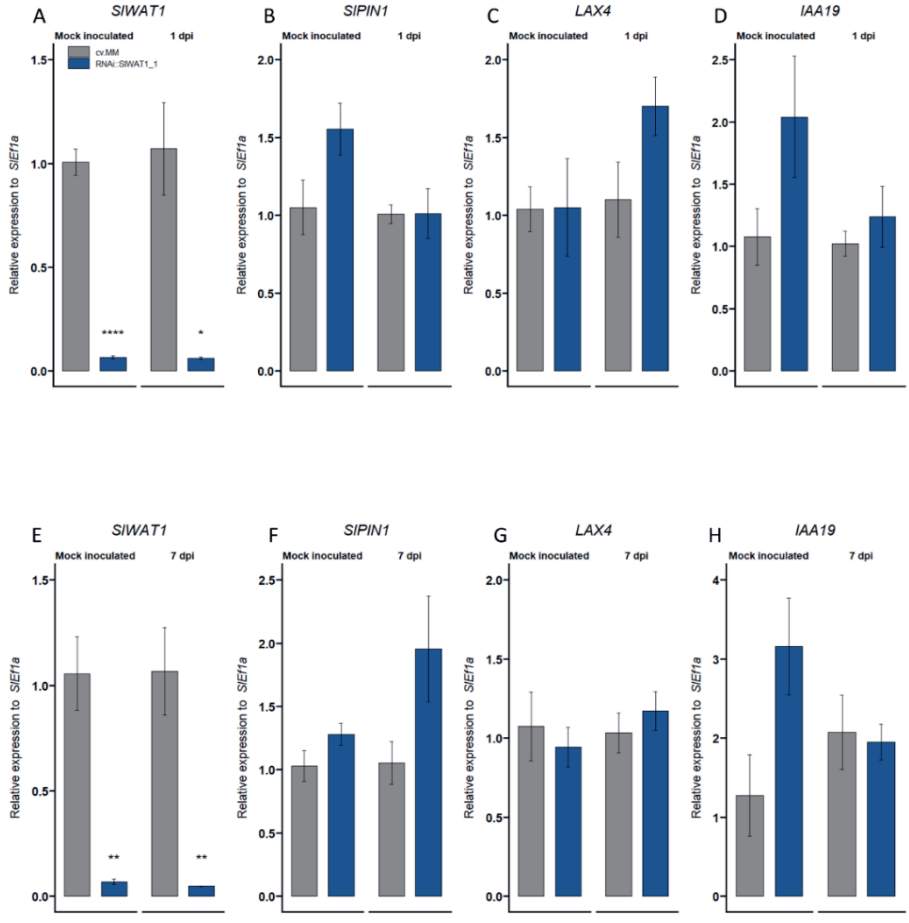

Figure 5| Expression of auxin transporter/ signalling genes is reprogrammed in RNAi::SlWAT1 plants. Relative expression of genes A), E) *SlWAT1*, B), F) Sl*PIN1*, C), G) *LAX4* and D), H)*IAA19* in mock treated and *Cm* inoculated plants at 1 dpi and 7 dpi. Fold changes were normalised relative to expression of the *SlEf1α* in cv.MM plants. Bars represent the average fold change over five independent biological replicates (n=5). Error bars indicate standard errors of the mean.

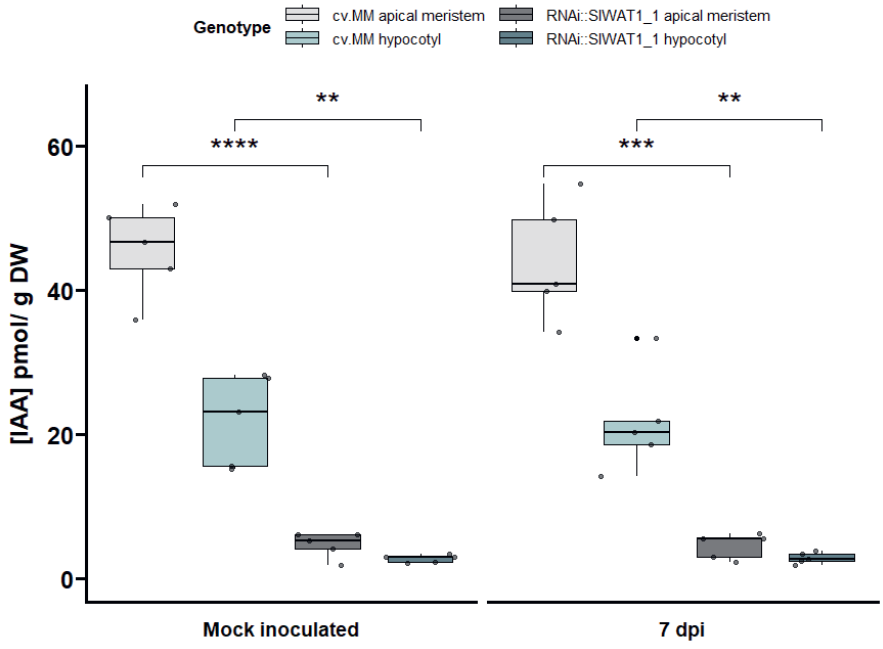

Figure 6| Free IAA content in tomato stems. IAA content in different stem parts of cv. MM and RNAi::SlWAT1_1 plants mock inoculated and 7 dpi. Boxplots of IAA concentration (pmol/ g DW) in apical meristems and hypocotyls of the two genotypes. Lower and upper box boundaries represent the 25th and 75th percentiles, respectively. Lines in the boxes represent medians of five biological replicates (n=5). (Student's t-test,**p≤0.01; ***p≤0.001,****p≤0.0001).

Ethylene biosynthesis is downregulated in transgenic plants

Upregulation of ethylene biosynthesis through gene *ACO1* has been shown to contribute to the development of wilting symptoms in *Cm* infected plants [28]. Based on our previous observations that silencing of *SlWAT1* reduces symptom development on tomato plants, we hypothesized that silencing of the gene will have an effect on ethylene biosynthesis. Therefore, we examined the expression of gene *ACO1* in the transgenic plants. We found that the *ACO1* gene is constitutively downregulated in the transgenic plants compared to the cv. MM background, suggesting that ethylene biosynthesis is reduced in *SlWAT1* tomato silenced plants (Fig. 7, Fig. S3).

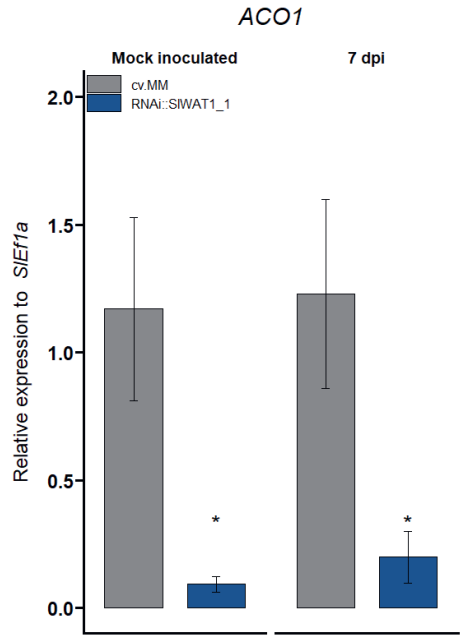

Figure 7| Expression of ethylene biosynthetic gene *ACO1* is constitutively downregulated in RNAi::SlWAT1_1 transgenic plants. Relative expression of gene *ACO1* in mock treated and *Cm* inoculated plants at 7 dpi. Fold changes were normalised relative to expression of the gene in cv.MM plants. Bars represent the average fold change over five independent biological replicates (n=5). Error bars indicate standard errors of the mean. Asterisks indicate significant differences to the expression prior to inoculation (Student's T test, *p≤ 0.05).

Inactivation of *SlWAT1* leads to downregulation of pathogen virulence factors

Recent studies in the interaction between *Pseudomonas syringae* DC3000 and *Arabidopsis*, have revealed a role of IAA in the expression of bacterial virulence factors *in planta* and *in vitro* [42]. Based on our data that free IAA content in the transgenic plants was significantly lower than in the susceptible cv. MM, we sought to investigate if silencing of *SlWAT1* has an effect on the regulation of *Cm* virulence factors. Susceptible cv. MM and transgenic RNAi::SlWAT1_1 were inoculated with *Cm* strain NCPBB382 and stems parts were collected at 1 and 7 dpi. Total RNA from infected plants was isolated and was used to monitor the expression of bacterial virulence genes *celA*, *pat-1*, *vatr2* and *phpA*. At 1 dpi, no amplification of bacterial transcripts was possible (data not shown). No differences were found in the expression of genes *celA* and *pat-1* on cv. MM and transgenic plants at 7 dpi (Fig. 8A,

B). Expression of transcription factor *vatr2* and its target *phpA*, however, was found to be downregulated at 7 dpi, with *phpA* being significantly downregulated (Fig. 8C, D). Overall, our results indicate that silencing of *SlWAT1* can regulate the expression of virulence related genes in *Cm* during bacterial growth *in planta*, possibly through reduced IAA content.

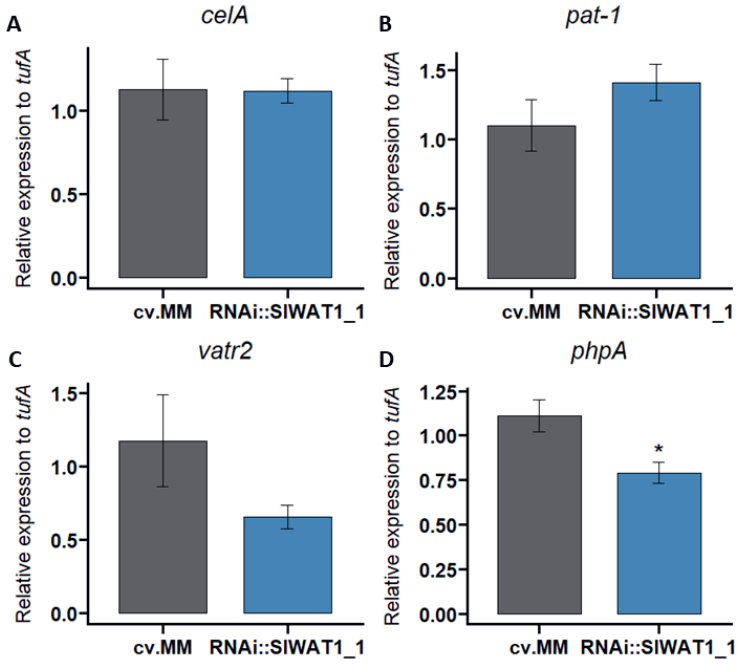

Figure 8| Expression of bacterial virulence genes is downregulated by inactivation of *SlWAT1 in planta*. Relative expression of genes A) *celA*, B) *pat-1*, C) *vatr2* and D) *phpA* on susceptible cv. MM and RNAi::SlWAT1_1 tolerant plants inoculated with *Cm* strain NCPBB382 at 7 dpi. Fold changes were normalised relative to expression of the genes in cv.MM plants. Bars represent the average fold change over five independent biological replicates (n=5). Error bars indicate standard errors of the mean. Asterisks indicate significant differences to the expression of the genes in the different genotypes (Student's T test, *p≤ 0.05).

DISCUSSION

Knowledge of plant susceptibility factors is important to better understand the strategies pathogens use to cause disease. During their co-evolution with plants many pathogens have evolved the ability to manipulate host S genes to establish a compatible interaction [43]. Loss-of-function of host S genes can possibly alter a

compatible interaction into a non-compatible one, leading to pathogen resistance [13, 43, 44]. Here, we report that the loss-of-function of S gene *WAT1* in tomato leads to high tolerance to genetically distinct strains of the bacterial pathogen *Clavibacter michiganensis* (Fig. 1).

WAT1 acts as an S gene that enables the infection process of vascular pathogens [30, 33, 34]. *WAT1* is a tonoplast localized vacuolar auxin transporter, that was first described as a susceptibility gene in *Arabidopsis thaliana* [31]. The arabidopsis *wat1-1* mutant was found to be resistant to a broad range of vascular pathogens, including the bacterium *Ralstonia solanacearum* and the fungus *V. dahliae.* Its function as an S gene to fungal vascular wilts has also been reported in cotton and tomato [30, 34]. Recently, our laboratory reported that CRISPR/Cas9 mediated knock out of tomato *SlWAT1* results in resistance to the fungal wilts *V. dahliae, V. album-altrum* and *Fusarium oxysporum* f. sp. *lycopercsici* [30]. In this study, we show that inactivation of tomato homolog *SlWAT1* results in strong reduction of symptom development caused by genetically distinct *Cm* strains (Fig. 1). These findings suggest the function of *WAT1* as an S gene is possibly conserved across plant species and that its loss-of-function can provide broad-spectrum resistance to vascular pathogens. This is an important trait that has been described for several other S genes [7, 11, 12, 16, 45].

Despite the strong reduction of wilting symptoms, growth of *Cm* was not suppressed by inactivation of *SlWAT1*, in contrast to what it has been reported for other pathogens (Fig. 2, Fig. S2B) [30, 34]. According to our initial hypothesis, the residual expression of *SlWAT1* in the RNAi lines was possibly responsible for the mild symptoms observed and the sustained growth of the pathogen. To confirm the results we obtained from the disease assays and to study the effect of a full knock-out in the growth of the pathogen, we included a CRISPR/Cas9 mutant line in our experiments. In accordance with our previous results, we observed strong symptom reduction in *slwat1* mutant tomato plants at 20 dpi (Fig. 4, Fig. S2A). Further, we did not detect any significant differences in the *Cm* bacterial titres recovered from the *slwat1* mutants and the susceptible background. This led us to hypothesize that *WAT1* is involved in symptom development, rather than sustainment of *Cm*.

A major drawback in the use of mutant S genes to gain resistance to pathogens is the possibility of adverse pleiotropy [46], as also observed in the case of tomato *SlWAT1.* Although a full knock-out of the gene led to severe growth defects (Fig. 3), downregulation of the gene in the RNAi transgenic lines resulted in similar tolerance levels to the pathogen. Alterations in *cis*-regulatory regions of the gene to change its

expression [47], might provide a cost-free strategy to gain tolerance to *Cm*. Alternatively, the exploration of allelic variation in tomato germplasm may lead to the identification of natural variants that disrupt the compatible host-pathogen interaction without fitness costs, as it was done in the case of gene *ROD1* in rice [48].

Changes in hormonal homeostasis is a common strategy used by pathogens to promote disease. While upregulation of host derived ethylene has been found to promote wilting development by *Cm*, the role of other hormones in the infection process remains unclear [28]. Resistance conferred by inactivation of *WAT1* has been associated with altered crosstalk between auxin and SA in Arabidopsis and cotton [33, 34]. According to our findings ethylene biosynthesis and auxin content were reduced in *SlWAT1* inactivated plants. We found that the ethylene biosynthetic gene *ACO1*, that is specifically upregulated by *Cm* to promote wilting symptoms was constitutively downregulated in RNAi::SlWAT1_1 transgenic plants (Fig. 4, Fig. S3). This is also in accordance with previous studies that found that symptom development, but not *Cm* bacterial growth was inhibited on *Nr* ethylene insensitive plants [28].

We also found that the content of free IAA in stem tissues of transgenic plants was significantly lower than in cv. MM (Fig. 5). In addition, expression of auxin related genes was altered in *SlWAT1* impaired plants upon *Cm* inoculation (Fig. 6). At 1 dpi, we found that the expression of auxin influx gene *LAX4* was upregulated in *Cm* inoculated transgenic plants, while the expression of auxin efflux gene *SlPIN1* was upregulated in mock inoculated transgenic plants. These changes in the expression of auxin transporter genes might be induced by *Cm* in an attempt to increase auxin influx around the inoculation point. The lower expression of *IAA19* in infected plants (at 1 and 7 dpi) compared to mock inoculated transgenic plants could also suggest that the auxin contents around the inoculation site are indeed increased during infection, since *IAA19* is upregulated in the absence of auxin [49]. Finally, the upregulation of *SlPIN1* in *Cm* infected *SlWAT1* inactivated plants at 7 dpi, might act as a late compensatory mechanism for the absence of *SlWAT1*, which also facilitates auxin efflux.

Higher contents of SA have been reported for arabidopsis and cotton *WAT1* impaired plants [33, 34]. This could be a direct consequence of the reduction of free IAA content, as the SA and auxin hormonal pathways are mutually antagonistic [50]. Although SA is a known regulator of defences against pathogens, knowledge on its role in resistance against *Cm* is limited. Recently, it was shown that exogenous

application of SA reduces the bacterial populations on tomato cotyledons [51]. The infection of *NahG* transgenic tomato plants with impaired SA accumulation, however, did not result in higher susceptibility to the pathogen [52].

Growing evidence suggests that host derived auxin is an important signalling molecule involved in plant-bacteria interactions [33, 53-55]. Recent studies have reported a direct effect of auxin in the regulation of *Pseudomonas syringae* DC3000 bacterial genes involved in virulence [54, 56]. Elevated IAA content in Arabidopsis quadruple mutant *tir1 afb1 afb4 afb5*, as well as the addition of IAA in *P. syringae* DC3000 cultures led to the repression of genes involved in the production of T3SS at early timepoints. The expression of genes involved in late infection stages, however, was significantly upregulated by elevated IAA contents [54]. Additionally, auxin produced by bacteria itself can act as a virulence factor [53]. Based on our observations of the significant reduction of symptom development and the significantly lower free IAA content in RNAi::SlWAT1_1 transgenic plants, we hypothesized that auxin might play a role in the regulation of *Cm* virulence genes. Therefore, we monitored the transcript levels of virulence factors *celA*, *pat-1*, *vatr2* and *phpA in planta.* No detection of bacterial transcripts was possible at 1 dpi, possibly due to the low proportion of bacterial mRNA in the total isolated RNA. Interestingly, we observed that transcription factor *vatr2* and its target *phpA* were downregulated at 7 dpi. This suggests that inactivation of *SlWAT1* leads to downregulation of *Cm* virulence genes, possibly through the reduced contents of free IAA in the stems of transgenic plants. Previous, transcriptomics analysis has shown that the virulence factors *celA* and *pat-1* reach the peak of their expression between 24-72 hpi and gene expression is reduced after that point [57]. This might be the reason why we did not detect a difference in the expression of *celA* and *pat-1* isolated from cv.MM and RNAi::SlWAT1_1 plants at 7 dpi. To definitely conclude, however, that auxin directly affects the expression of bacterial genes, their expression after supplementation of cultures with IAA could be monitored. Moreover, meta-transcriptomics analysis through RNA-seq on infected mutant plants and their susceptible background could be deployed in different experimental timepoints, in order to elucidate the complete pathways involved in the molecular interaction of the organisms [58]. Finally, future studies on how *Cm* responds to IAA, as well as the production of IAA non-responsive *Cm* mutants, could allow us to fully study and understand the role of auxin as a signalling molecule in the pathosystem.

MATERIALS AND METHODS

Plant materials

The present study included the susceptible *Solanum lycopersicum* cv. Moneymaker MM as a control, T_3 progeny of two independent stable transformants (RNAi::SlWAT1_1, RNAi::SlWAT1_2) in which the *SlWAT1* gene was silenced through RNAi in cv. MM background and T_2 progeny of a bi-allelic heterozygous CRISPR/Cas9 generated *slwat1* mutant line [30]. Prior to infection, transgenic plantlets were screened for the presence of the RNAi silencing construct based on the presence of the 35S and NPTII markers. *slwat1* mutants were screened for the presence of mutant alleles through PCR based genotyping and sequencing.

Plants were grown in a climate regulated greenhouse compartment at 24^{o}C/18^{o}C under a 12h/12h day/night regime. Relative humidity in the compartment was kept to ~60%.

DNA isolation and genotyping

For the genotyping of the RNAi transgenic and *slwat1* mutant plants genomic DNA was isolated using a modified protocol for cetyl trimethylammonium bromide (CTAB) extraction method [35]. PCR was performed with DreamTaq DNA polymerase (Thermo Scientific) and target specific primers (Table S1). The PCR products of the RNAi transgenic plants were visualized on 1% agarose gel for the screening of the presence of NPTII and 35S transgene markers. PCR products of mutant plants were sequenced through Illumina sequencing (Macrogen Europe, Amsterdam).

Bacterial strains and growth conditions

Four genetically diverse *Cm* strains were used in the experiments, i.e. *Cm* strains NCPBB382, IPO3356 (rifampicin resistant mutant), CFBP2493 and CFBP5843 were used in the experiments [36]. Prior to plant inoculation the strains were grown for two days at 25^{o} C on TBY plates (10 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 5 gL^{-1} sodium chloride, 15 gL^{-1} bacteriological agar). Plates were supplemented with appropriate antibiotics when needed (25 µl/mL rifampicin).

Disease assay

Tomato plants at the fourth true leaf stage were inoculated by a petiole clipping off method. The petioles of the first two fully expanded leaves were clipped off with razor blades immersed in the bacterial inoculum and 5 µl of the bacterial inoculum were directly pipetted on the lowest wound. Bacterial inocula of the four bacterial

strains were prepared by re-suspending cells in Ringer's buffer to a final concentration of ~10^8 cfu/ml (OD_{600}=0.1). Prior to re-suspension the *Cm* strains were streaked on TBY plates, supplemented with appropriate antibiotics when needed, and incubated at 25°C for two days. Symptom development (wilting) was monitored up to 20 days post inoculation (dpi). A disease index (DI) scale based on the development of wilting symptoms on the leaves was used (0; no symptoms- 5; all leaves wilting). Per strain, 12 transgenic T_3 plants and three susceptible cv. MM control plants were used. The same procedure was used for the disease assays of *slwat1* mutants. At least five biological replicates of *slwat1* mutants were used in the experiments. Four biological replicates of the susceptible cv. MM were used as controls.

Bacterial quantification

Bacterial quantification was done through serial dilution plating. Stems collected ~1 cm above the lowest inoculation point were sampled at three time points; 4 dpi, 7 dpi and 14 dpi. Stems were pulverized and homogenized in Ringer's solution (Sigma Aldrich). 50 µl of serial dilutions of the homogenate (10^1-10^6) were plated on SCM-F selective plates (Duchefa Biochemie). The medium was supplemented with 1.9 g L^{-1} yeast extract, 20 µL L^{-1} nalidixic acid (100 mg mL^{-1}), 8 mL L^{-1} trimetroprim in MetOH 100% (10 mg/mL), 1 ml L^{-1} cyclohexamide in MetOH 100% (100 mg mL^{-1}), 1 mL potassium tellurite (1%), 50 ml L^{-1} nicotinic acid (2 mg/ mL). Plates were supplemented with appropriate antibiotics when necessary (25 µl/mL rifampicin). Plates were incubated at 25° C for 7 days. Colonies on the plates were counted 7 days post plating and the $\log_{10}$(cfu+1/ g fresh tissue) per plate was calculated. Five biological replicates of RNAi::SlWAT1_1 plants and the susceptible cv. MM were used per time point. Two technical replicates per sample were plated. The same procedure was used for the quantification of *in planta* bacterial titres of strain NCPBB382 in *slwat1* mutants. Five biological replicates per time point were used.

RNA extraction/ cDNA synthesis

Stem samples of ~2 cm in length were collected above the inoculation point. The stems were processed using a Precellys Evolution tissue homogenizer (Bertin Technologies) at 7000 RPM for two rounds of 15 sec, with the cryolysis option on. RNA extraction and on column DNase treatment were done using the RNeasy Mini Kit (Qiagen) and RNase-Free DNase Set (Qiagen) following the manufacturer's instructions. 500 ng of first strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-rad).

Gene expression analysis

Expression levels of tomato genes *SlWAT1*, *ACO1*, *IAA19*, *SlPIN1*, *LAX4* on mock treated and *Cm* infected control and RNAi::SlWAT1_1 transgenic plants were monitored through RT-qPCR at 1 and 7 dpi using specific gene primers (Table S1). The expression of bacterial virulence genes *celA*, *pat-1, vatr2* and *phpA* on infected cv. MM and RNAi::SlWAT1_1 was also assessed at 1 and 7 dpi using target specific primers (Table S1). 5 and 25 ng of cDNA were used as a template for the reactions for the quantification of plant and bacterial transcripts, respectively. Reactions were done in duplicates. At least four biological samples were used per treatment and time point. RT-qPCR was done on a CFX96 Touch Deep Well Real Time PCR Detection system (Bio-Rad).

Prior to cDNA synthesis for the expression analysis of the bacterial virulence genes, the total RNA isolated was run on 1% agarose gel to confirm the absence of genomic DNA from the samples. The Livak $2^{-\Delta\Delta Ct}$ method was used to normalize and calibrate transcript values relative to the endogenous *SlEf1α* for tomato and gene *tufA* for the bacterial genes.

Auxin quantification

Auxin was quantified through Liquid Chromatography- Mass Spectrometry (LC-MS/MS). Plant stem parts were collected and flash frozen in liquid nitrogen. The collected tissue was processed using a Precellys Evolution tissue homogenizer (Bertin Technologies) at 7000 RPM for two rounds of 15 sec, with the cryolysis option on. ~25 mg of tissue were used for the auxin extraction. Ground stem samples were extracted with 1 mL of cold methanol containing [phenyl $^{13}C_6$]-IAA (0.1 nmol/mL) as an internal standard in a 2-mL eppendorf tube and purified as previously described [37, 38]. Samples were filtered through a 0.45 µm Minisart SRP4 filter (Sartorius) and measured on the same day. Auxin was analyzed on a Waters Xevo TQs tandem quadruple mass spectrometer as previously described [37, 39].

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SUPPLEMENTARY MATERIAL

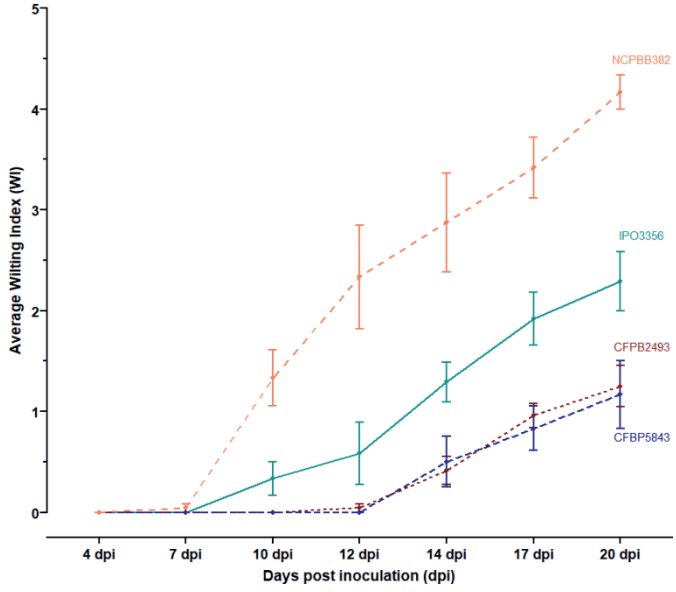

Figure S1| Strain aggressiveness**.** Four genetically diverse strain were used for the inoculation of cv.MM plants to assess their aggressiveness. Symptom development was monitored up to 20 dpi. Mean values of three biological replicates (n=3). Bars represent standard errors.

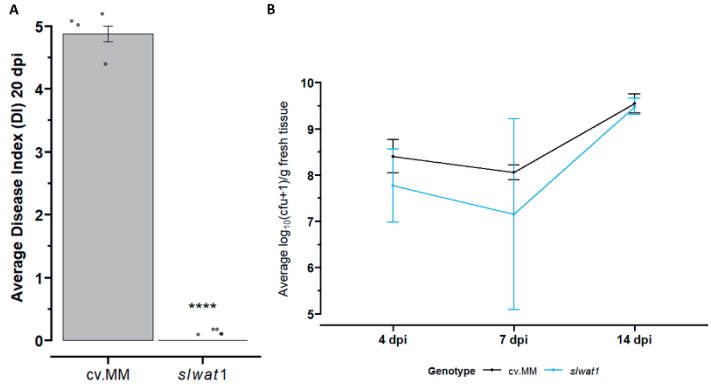

Figure S2| Symptom development and bacterial dynamics on *slwat1* mutants. A) Wilting symptom development of *slwat1* mutants compared to the background donor susceptible control cv. MM at 20 dpi. Mean values of the *slwat1* mutant were significantly different from the cv.MM controls (n=4). B) *Clavibacter michiganensis* population dynamics in cv.MM and *slwat1* mutants. Bacterial titres of bacterial

strain NCPBB382 used in the experiments were quantified at 4, 7 and 14 dpi. Three biological replicates (n=3) were used per time point. Lines represent the average $\log_{10}$(cfu+1/ g fresh stem tissue) ± stdev. The experiments were repeated independently at least twice with similar results. Asterisks indicate significant differences (Student's t-test, ****p≤0.0001).

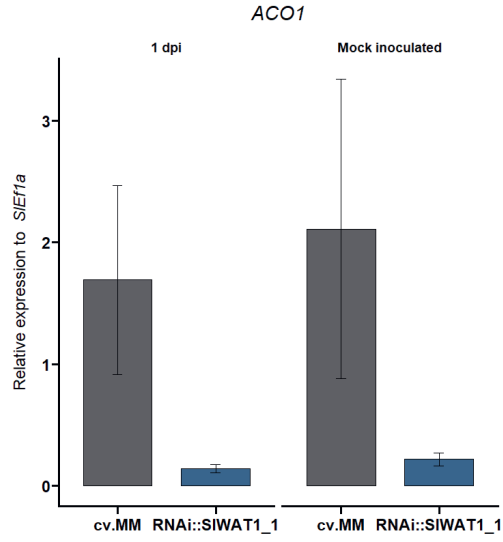

Figure S3| Expression of ethylene biosynthetic gene *ACO1*. Relative expression of gene *ACO1* in mock treated and *Cm* inoculated plants at 1 dpi. Fold changes were normalised relative to expression of the gene in cv.MM plants. Bars represent the average fold change over five independent biological replicates (n=5). Error bars indicate standard errors of the mean.

Table S1| List of primers used in this study.

Gene	Primer sequence (5'-3')	Description
NPTII	GAAGGGACTGGCTGCTATT AATATCACGGGTAGCCAAC	Genotyping of transgenic plants
35S	TACAAAGGCGGCAACAAAC AGCAAGCCTTGAATCGTCC	Genotyping of transgenic plants
SlWAT1	CACCGGCCCAACAATTTACAGCCC GAACTAGCCAAGCCTGAGGG	Genotyping of mutants
SlWAT1	GGGGGTCCAGTTTTTGTTGC CTCCGATTATCCCGCCCAAG	RT-qPCR
SlEf1a	ATTGGAAACGGATATGCCCCT TCCTTACCTGAACGCCTGTCA	RT-qPCR
ACO1	ATGGATCGATGTTCCTCCCATG ATTCGTGTCCCGTCTGTTTG	RT-qPCR

IAA19	AGTGATCGAAACAGCAGCAG CCAGAGCAGGCTTTTGACAC	RT-qPCR
SlPIN1	CCAAGGATCATAGCATGTGG AGACCAACAGCAATGGAAGC	RT-qPCR
LAX4	ATGCTGAGAAGCAAGCAGAG CCAGAGCAGGCTTTTGACAC	RT-qPCR
celA	CCTCTTCACCACGACTCACC GCAACGTACATCGGTCTGC	RT-qPCR
pat-1	TGTCGCGCATAAACAGGATA AACGAAACACGGGCTATACG	RT-qPCR
vatr2	GCACATCCTCGAGATCATGG GTCGATGAAGAAGAGCTTCGTGAC	RT-qPCR
phpA	CCAATTGCACATGAGTCCAG GAGTCATCCGTGCCAGTAGC	RT-qPCR
tufA	CAGGAGCCCGCAGTTCT GTCCCACCGTCAAGACC	RT-qPCR

CHAPTER 4

The tomato *SlWRKY23* gene: a candidate susceptibility gene for *Clavibacter michiganensis*

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ABSTRACT

Traditional breeding for resistance has mainly been based on the introduction of dominant resistance (R) genes into elite cultivars. In the tomato- *Clavibacter michiganensis* (*Cm*) pathosystem, however, no R genes conferring resistance have been identified yet. Alternatively to the use of R genes, the identification and mutation of evolutionary retained plant susceptibility (S) genes can provide novel sources of resistance. In this study, we set out to functionally characterize the tomato *SlWRKY23* gene, a homolog of the Arabidopsis S gene *WRKY27*, as a potential susceptibility factor to *Cm*. For the initial characterization of the gene virus induced gene silencing (VIGS) was used. Silencing of *SlWRKY23* repeatedly resulted in reduction of symptom development caused by *Cm* on tomato plants. Subsequently, we employed CRISPR/Cas9 for the generation of *SlWRKY23* for further functional studies. Bioassays including disease assays and *in planta* bacterial quantification were performed using the generated mutants. Surprisingly, contrary to what was observed during the VIGS assay, the mutants were found to be fully susceptible to *Cm*. This apparent contradiction is discussed.

INTRODUCTION

In the absence of an adaptive immune system, plants have evolved an intricate network of immune responses. The current view of plant immunity suggests the activation of two interconnected branches of an innate immune system upon pathogen attack [1-3]. The first extracellular branch uses immune receptors called pattern-recognition receptors (PRRs) to detect conserved pathogen-associated molecular patterns (PAMPs), leading to the activation of PAMP-triggered immunity (PTI) [1]. For example, the bacterial flagellin is one of the most well studied PAMPs, that triggers the immune system of both plants and animals. In *Arabidopsis thaliana* the *Flagellin Sensitive2* (*FLS2*) receptor recognises the conserved 22 amino acid peptide of flagellin, flg22, activating PTI [4]. To suppress basal PTI, pathogens secrete effector proteins to interfere with host processes, resulting in effector-triggered susceptibility (ETS). In the second branch, plants evolve resistance (R) proteins to directly or indirectly recognise corresponding pathogen effectors in the cell, leading to a robust immune response termed effector-triggered immunity (ETI). For instance, recognition of the *Ralstonia solanacearum* type III effector PopP2 by the protein of R gene *Ralstonia Resistance1-R* (*RRS1-R*) results in ETI [5]. The selective pressure put by R proteins to pathogens drives them to diversify their effectors in order to avoid recognition by R proteins or to suppress ETI [1]. In addition to suppressing or evading immunity, pathogens can render plant defences ineffective by manipulating evolutionarily retained host susceptibility (S) genes through ETS pathways. S genes code for host proteins that pathogens take advantage of to complete several of their processes [6].

Responses to pathogens require large scale reprogramming of gene transcription both in a temporal and environmental context. The transcriptional changes associated with the activation of PTI and ETI are controlled through interacting networks of transcriptional regulators from several protein families [7]. One of these families is the superfamily of WRKY transcription factors (TFs). Multiple studies have demonstrated roles for WRKY TFs in both branches of the plant innate immune system [1, 8, 9]. WRKY TFs constitute one of the largest families of transcriptional regulators in plants [8, 10]. More than 100 members of the family have been identified in higher plant species such as rice (*Oryza sativa*) and soybean (*Glycine max*). In the model species *Arabidopsis thaliana* 72 members of the family are found, whereas 83 are present in tomato (*Solanum lycopersicum*) [11, 12]. WRKYs are defined by the presence of the highly conserved heptapeptide WRKYGQK in a 60 amino acid region at their N-terminus, followed by a Cys_2His_2 or $Cys_2HisCys$ zinc-

finger motif at their C-terminus [9, 12, 13]. As transcriptional regulators, WRKYs interact with specific recognition sites of cis-regulatory elements of genes. WRKYs bind to W-boxes, with the consensus sequence (C/T)TGAC(T/C), on gene promoters to induce or suppress the expression of downstream genes [14, 15].

Loss-of-function or gain-of-function studies have determined the roles of several *WRKY* genes as positive or negative regulators of plant defences to pathogens. In Arabidopsis 49 WRKY genes are differentially regulated after inoculation with a strain of *Pseudomonas syringae* expressing the avirulence gene *avrRpt2* or after salicylic acid (SA) treatment, indicating a role of this gene family in defence related processes [16]. In rice, overexpression of *OsWRKY80* enhanced resistance to the fungal pathogen *Rhizoctonia solani* [17]. Tomato *SlWRKY8* inhibited the growth of bacterium *P. syringae* DC3000 on transgenic plants overexpressing the gene, possibly due to upregulation of SA pathogenesis-related (PR) proteins. Moreover, overexpression of *SlWRKY8* resulted in higher tolerance of transgenic plants to drought or salt stress through the regulation of ROS-scavenging enzymes [18]. Soybean *GmWRKY40* was found to act as a positive regulator of plant defences to the oomycete *Phytophthora sojae*. RNAi-mediated suppression of the gene enhanced susceptibility of transgenic plants to the pathogen, through modulation of H_20_2 accumulation. Furthermore, the gene was found to physically interact with JAZ proteins that are thought to suppress JA-defence signalling [19].

In contrast to these positive regulators, many *WRKY* genes have been found to act as suppressors of plant defences, and thus act as S genes. For example, genes *CaWRKY1* and *CaWRKY40b* act as negative regulators of immunity associated genes in pepper. Silencing of *CaWRKY1* and *CaWRKY40b* was shown to enhance resistance to the bacterial pathogens *Xanthomonas axonopodis* pv. *vesicatoria* race 1 and *Ralstonia solanacearum*, respectively [20, 21]. In tomato, formation of giant cells during infection by the nematode *Meloidogyne javanica* is increased in roots of transgenic plants overexpressing *SlWRKY45* [22]. The partially redundant *WRKY18*, *WRKY40* and *WRKY60* TFs of Arabidopsis physically and functionally interact with each other in a network that controls differential responses to plant pathogens [23]. Simultaneous loss-of-function of the three genes inhibits bacterial growth of hemibiotrophic *P. syringae* DC3000 through modulation of SA pathways [23].

Bacterial canker of tomato caused by the vascular bacterium *Clavibacter michiganensis* (*Cm*) is considered to be one of the most important bacterial diseases of tomato [24, 25]. The pathogen invades the xylem of tomato plants leading to

systemic infections that result in severe wilting and eventually plant death [26-28]. Despite the importance of the pathogen, little is known about the host factors that lead to susceptibility. Host derived ethylene has been shown to promote wilting, as *Never ripe* (*Nr*) mutants impaired in ethylene perception exhibited delayed symptom development [29]. Several components of defence, including WRKY TFs, are activated upon infection of plants by *Cm* [30]. Nevertheless, *Cm* is still able to infect tomato and as yet all cultivars on the market are susceptible to the bacterium [25, 29].

Of special interest to us was Arabidopsis *WRKY27*, as inactivation of the gene conferred enhanced tolerance to the vascular bacterial pathogen *Ralstonia solanacearum* [31]. Similarities between the lifestyle of the two bacterial pathogens, *R. solanacearum* and *C. michiganensis*, prompted us to identify and functionally characterize potential tomato homologs. In their study, Mukhtar et al. (2008) were able to show that lack of a functional *WRKY27* resulted in delayed symptom development to the vascular bacterium *R. solanacearum*. Even though there was a significant delay in symptom development, no inhibition of bacterial growth was observed in *WRKY27* silenced plants. Further functional analysis of the gene suggested that Arabidopsis *WRKY27* was involved in the regulation of genes involved in the ethylene signalling pathway, as the mutant failed to activate ethylene responsive genes. Finally, spatial expression data suggested that *WRKY27* possibly influences symptom development by *R. solanacearum* by affecting signalling or trafficking of components between the phloem and the xylem [31].

Given that several S genes are functionally retained across species, we exploited the available genomic information to identify the ortholog of *WRKY27* in tomato. To further characterize the functional role of the gene in the tomato- *Cm* interaction, we used transient silencing through virus induced gene silencing (VIGS) and genome editing through CRISPR/Cas9 to inactivate the identified homologs.

RESULTS

Tomato *SlWRKY23* is the homolog of Arabidopsis *WRKY27*

To assess the potential involvement of the tomato *WRKY27* homolog in susceptibility to *Cm*, putative *WRKY27* tomato homologs were identified by searching the Sol Genomics Network (SGN) database. Generally, low protein identity between WRKY27 and its tomato homologs was found. In total four tomato genes with the highest protein identity (id% ≥ 40%) to the Arabidopsis *WRKY27* and its

three close homologs (*WRKY29*, *WRKY22* and *WRKY25*) were selected for the phylogenetic analysis. The additional Arabidopsis homologs were also included in the phylogenetic analysis to better estimate the relationship of the genes (Figure 1A). A protein coded for by a gene annotated as *Solyc08g081610 (SlWRKY23)* [12] on tomato chromosome 8 clustered the closest to the Arabidopsis *WRKY27* gene. Even though *Solyc08g081610* closely clustered to *WRKY29*, it was the only gene clustering close to *WRKY27*, therefore it was considered to be the ortholog of *WRKY27* in tomato and hereafter referred as *SlWRKY23*. *SlWRKY23* is 2,091 bp in length and it is encompassed of three exons and two introns, which encode a 304 amino acid protein. A single functional domain of the WRKY family was predicted by the InterPro domain database on the amino acid sequence encoded by *SlWRKY23* (Figure 1B).

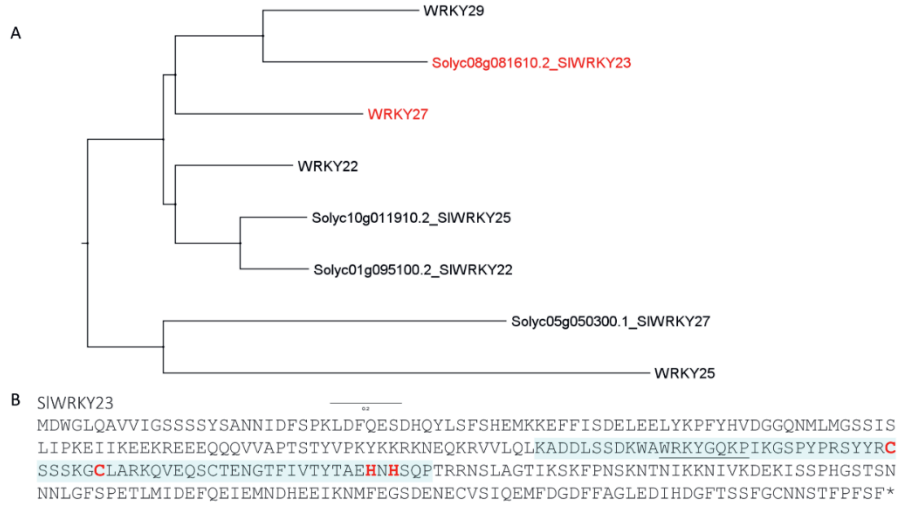

Figure 1| A) Neighbor joining based phylogenetic tree of the WRKY27 protein and its homologs in tomato. The amino acid sequences of the proteins were used for construction of the tree. The Kimura protein method was used for correction of distances. Based on the phylogenetic analysis gene *Solyc08g081610* located on tomato chromosome 8 was considered to be the ortholog in tomato. B) Protein produced by the *SlWRKY23* gene. The WRKY domain is highlighted in blue. The seven underlined amino acids represent the highly conserved heptapeptide WRKYGQKP present in WRKY domains. Amino acids in red indicate the positions of the conserved cystine and histidine residues in the DNA binding zinc-finger motif.

Transient silencing of *SlWRKY23* reduces tomato susceptibility to *Cm*

To study the potential involvement of *SlWRKY23* in susceptibility of tomato to *Cm*, a modified TRV viral vector carrying a 200 bp gene specific fragment was used to transiently silence the gene (Figure 2A). Susceptible cv. MoneyMaker (cv. MM) seedlings were agroinfiltrated with the TRV::SlWRKY23 construct. A construct targeting the exogenous β-glucuronidase gene (GUS) was used as a negative control in the assays. Silencing of *SlWRKY23* did not lead to any observable pleiotropic defects. Upon inoculation of the transformed plants with *Cm* strain NCPBB382, a statistically significant reduction in wilting was observed in TRV::SlWRKY23 compared to TRV::GUS agroinfiltrated plants at 34 and 20 dpi. This was observed repeatedly (Figure 2B). Therefore, we considered *SlWRKY23* to be a candidate S gene.

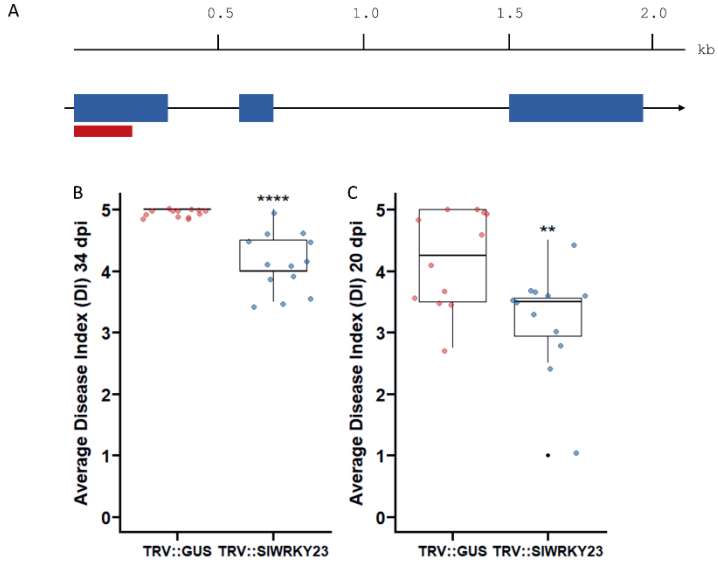

Figure 2| A) Graphical representation of *SlWRKY23* and the silencing fragment used in the construction of the VIGS vector. Blue boxes represent exons, black lines represent introns. Red box represents the exonic region amplified for the construction of the VIGS vector for the transient silencing of the gene. Symptom development of plants agroinfiltrated with the TRV::SlWRKY23 silencing construct compared to the TRV::GUS negative control at B) 34 dpi (days post inoculation) and C) 20 dpi. The 34 dpi disease assay in B was conducted to investigate if disease symptoms would significantly advance after 20 dpi. Figures B) and C) represent two independent experiments. Centre lines show medians, the box limits indicate the 25th and 75th percentiles (Student's t-test, **p≤0.01;****p≤0.0001).

Characterization of mutations induced by CRISPR/Cas9

To further study the role of *SlWRKY23*, CRISPR/Cas9 was employed to introduce targeted mutations in its coding sequence. Susceptible cv. MM seedlings were transformed with a CRISPR/Cas9 vector carrying four sgRNAs (Supplementary Figure 1). The target sequences of the sgRNAs spanned exons two and three of the gene, which code for the functional WRKY domain. Due to contamination and rooting problems which occurred during transformation, only four primary transformants were obtained. Sequencing of the region targeted by the sgRNAs revealed mutations in two of the four transformants. Both mutants carried bi-allelic heterozygous mutations (Figure 3, Fig. S2). Allele 1 (*slwrky23_1_1*) of mutant *slwrky23_1* carries a 31 bp deletion between the target sequences of sgRNA1 and sgRNA2. Furthermore, a 5 bp deletion in the target sequence of sgRNA3 and the insertion of a T in the target sequence of sgRNA4 are also present. Allele 2 (*slwrky23_1_2*) carries a large 845 bp deletion between the target sequences of sgRNA2 and sgRNA3 and the insertion of a T in the target sequence of sgRNA4. Both mutant alleles of mutant *slwrky23_2* carry small indels in the target sequences of sgRNA2, sgRNA3 and sgRNA4. Mutant allele 1 (*slwrky23_2_1*) carries a 12 bp deletion in the target sequence of sgRNA2, a 9 bp deletion in the target sequence of sgRNA3 and the insertion of a G in the target region of sgRNA4. Allele 2 (*slwrky23_2_2*) carries a 2 bp deletion in the target sequence of sgRNA2, a 5 bp mutation in the target sequence of sgRNA3 and a 6 bp deletion in the target sequence of sgRNA4. The mutations induced by CRISPR/Cas9 led to frame shifts in the functional WRKY protein domain and truncation of the resulting proteins in both mutants.

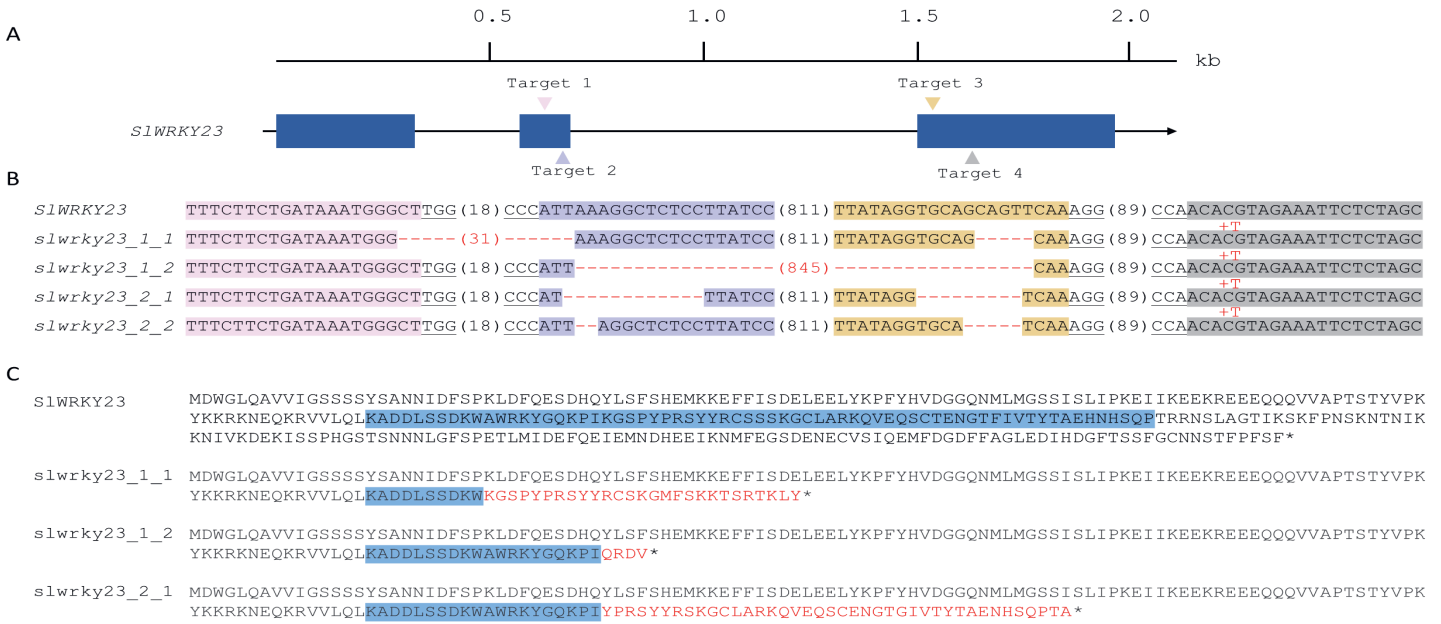

Figure 3| A) Graphical representation of *SlWRKY23* and the target regions of the four sgRNAs carried by the CRISPR/Cas9 vector. Blue boxes represent exons, black lines represent introns. Triangles indicate the position of the target of each sgRNA. B) Mutations identified in T_1 mutant plants. Highlighted sequences represent the 20 nt target sequences of the sgRNAs. Each colour corresponds to the triangle indicating the position of each target in Figure 3 A. The underlined sequences represent the PAM motifs of each sgRNA. Black numbers in parentheses represent missing nucleotides. Red dashes and red numbers in parentheses indicate deleted bases. A "+" symbol followed by a base indicate the positions of insertions. C) Proteins encoded by the WT and mutant alleles. Highlighted in blue are the amino acids present in the WRKY domain. Amino acids in red indicate frame shifts. Asterisks indicate stop codons.

Targeted deletion of *SlWRKY23* does not alter tomato susceptibility to *Cm*

Based on our observations during the transient silencing assays, we hypothesized that a full knock-out of gene *SlWRKY23* would lead to loss-of-susceptibility to *Cm*. T_2 progeny of the two generated mutant primary transformants were used in further bioassays. Prior to the bioassays the T_2 progeny were genotyped in order to confirm the presence of the segregating mutant alleles (Fig. S3). We did not observe any negative pleiotropic effects in the mutant T_2 progeny. The mutant plants were comparable in size and appearance to the wild-type cv. MM.

Disease assays were carried out by challenging the mutant plants (T_2) with *Cm* strain NCPBB382. The susceptible background cv. MM was used as a control. Regardless of the plant genotype, severe wilting symptoms were observed on the tomato plants. No statistically significant differences were found between cv. MM and mutant *slwrky23_2* at 7, 17 and 20 dpi. In the case of *slwrky23_1*, symptom development was statistically significantly higher at the same time points. No significant differences were found for the rest of time points used in the disease assay (Fig. 4). Even though, in early stages of the first disease assay we performed (7 dpi), a statistically significant reduction in symptom development was observed for *slwrky23_1* (Fig. S4) these latter results could not be repeated in other experiments.

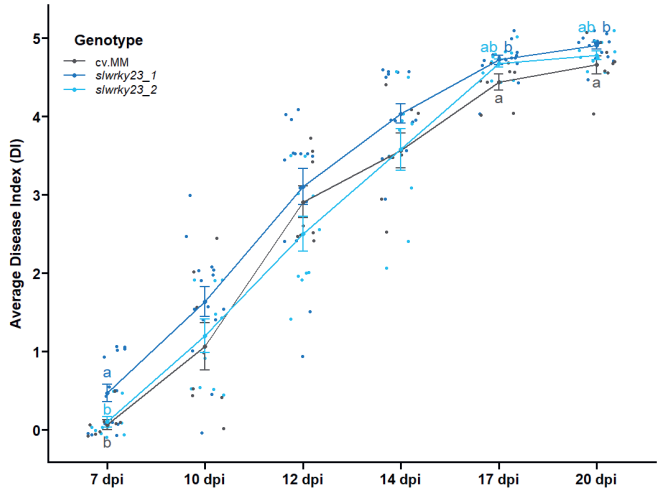

Figure 4| Symptom development on two independent T_2 *slwrky_23* mutant lines compared to the susceptible background (cv.MM). Error bars indicate standard errors. Letters indicate significant differences (ANOVA, Tukey's post hoc).

In parallel with the disease assays the population dynamics of strain NCPBB382 were monitored *in planta*. An estimated 5x10^5 colony forming units (cfu)/mL were used for the inoculation of plants. The bacterial growth in tomato stems was monitored at three different time points (4, 7 and 14 dpi) through serial dilution plating. During the course of infection *Cm* reached high densities (~$10^{9.5}$ cfu+1/g fresh tissue) *in planta*. At 4 dpi, the number of bacteria recovered from mutants *slwrky23_1* and *slwrky23_2* were statistically significantly higher than the susceptible cv.MM control plants. Over the course of the assay, however, the differences between the different genotypes decreased (7 dpi) and at the end of the experiment they were no longer significant (14 dpi) (Fig. 5).

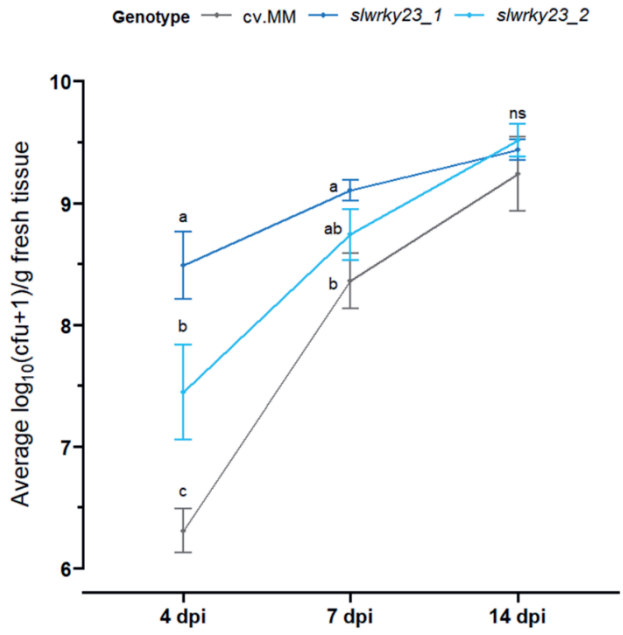

Figure 5| Bacterial titres of bacterial strain NCPB3382 recovered by mutant plants and their susceptible background were quantified at 4, 7 and 14 dpi. Three biological replicates (n=3) were used per time point. Lines represent the average log_{10}(cfu+1/ g fresh tissue) ± stdev. The experiments were repeated independently at least twice with similar results. Letters indicate statistical differences (ANOVA, Tukey's post hoc, ns= not significant).

DISCUSSION

Extensive breeding efforts have so far failed at identifying tomato genes conferring qualitative resistance to *Clavibacter michiganensis* [25]. In the absence of dominant R genes, the identification and inactivation of susceptibility genes involved in the interaction may provide a novel source of monogenic resistance [6, 32-34]. A considerable number of S genes have been shown to be functionally retained as susceptibility factors in different plant species [35-41]. In our study, we set out to identify and characterize host genes involved in tomato susceptibility to *Cm*.

By combining phylogenetic analyses and reverse genetics we were able to identify and functionally characterize the tomato ortholog (*SlWRKY23*) of Arabidopsis *WRKY27*. During the first functional characterization experiments of *SlWRKY23*, we transiently silenced the gene through VIGS. We repeatedly observed that transgenic plants infiltrated with the TRV::SlWRKY23 silencing construct exhibited reduced wilting symptoms upon inoculation with *Cm*, compared to the control TRV::GUS infiltrated plants. In addition, we generated CRISPR/Cas9 mutants to further evaluate the gene in terms of potential pleiotropy and confirm its functional role as an S gene. Although a reduction of symptoms was observed for *slwrky23_1* in the very early stages of the first experiment, no such effect was visible at the final phase of our experiments. Alongside the disease assays, we also monitored the bacterial growth of *Cm* in the stems of *slwky23* mutant plants. Consistently to what has been described for *R. solanacearum* after inactivation of *WRKY27* [31], *Cm* reached high densities *in planta*. This, however, comes as no surprise, since the *slwrky23* mutant plants were found to be fully susceptible. The loss-of-function of TF *WRKY27* in Arabidopsis was found to result in delayed wilting symptoms due to the inability of the *wrky27* mutant to activate the expression of ethylene responsive genes [31]. The significance of ethylene signalling in the development of wilting symptoms has previously been described for both *R. solanacearum* and *Cm*. The tomato *nr* and Arabidopsis *ein2-1* ethylene insensitive mutants exhibited delayed symptom development when infected with *Cm* and *R. solanacearum*, respectively [29, 42]. For both pathogens, however, there was no correlation between delayed wilting development and bacterial growth, suggesting that ethylene is involved in tolerance, rather than resistance to these pathogenic bacteria [29, 31]. Analysis of the expression of ethylene related genes or the content of ethylene in plants with a non-functional *SlWRKY23* could provide further insights in the possible role of *SlWRKY23* in regulation of ethylene synthesis and perception.

The discrepancies between the wilting symptoms observed after knock-down and knock-out of the gene could be attributed to several reasons. VIGS is a technology that uses recombinant viruses to inhibit transcription of endogenous genes via the complementarity based RNA interference (RNAi) pathway [43]. Initial studies reported that a small interfering RNA (siRNA) guide with identity of 19 continuous or more nucleotides to the endogenous target is needed for efficient gene silencing [44]. Efficient and wide-spread silencing of off-targets, however, has been found even when mismatches between the target gene and the guide siRNA exist [45]. Even though care was taken to avoid possible off-targets during the design of the VIGS construct used in the experiments, we cannot eliminate the possibility of downregulation of genes other than our target (Table S2). To verify the specificity and efficiency of our silencing construct, quantification of *SlWRKY23* transcripts and other possible off-target could be done.

Delivery of silencing fragments through viral vectors has been found to be prone to transitivity. RNA transitivity is a mechanism that results in the production of siRNAs from a transcript targeted by primary small RNAs (sRNAs). The production of secondary siRNAs can drive the expansion of the silencing signal to additional sequences of the transcript [46]. WRKY TFs belong in large families of genes that interact in complex networks with possible functional redundancy. Considering the sequence similarity between WRKY genes, we can hypothesize that the production of secondary siRNAs with high identity to other transcripts of WRKY genes could lead to the simultaneous silencing of multiple homologs. Therefore, it would be possible that the phenotype we observed in the transiently silenced plants was the result of the silencing of redundant genes. In addition, even though we considered *SlWRKY23* to be the ortholog of the Arabidopsis *WRKY27* gene, the tomato ortholog also closely clustered with the Arabidopsis homolog *WRKY29* (Fig. 1). Therefore, if we consider *WRKY29*, which has been found to be associated with PTI [47], to be the true functional ortholog of *SlWRKY23* instead of *WRKY27*, one would potentially expect that targeted mutagenesis of *SlWRKY23* would not lead to changes in susceptibility.

Studies in a number of model species, such as Arabidopsis, zebrafish and mouse have also revealed phenotypic differences between knock-down and knock-out mutants [48]. An alternative to off-target effects during post-transcriptional knock-down of genes that could explain the observed differences between the phenotypes is genetic compensation response (GCR). Recent studies in zebrafish and mice revealed the activation of compensatory gene expression to buffer the effects of possible

deleterious mutations, that was not observed after transcriptional knock-down of target genes [49, 50]. In particular, it was found that knock-down of zebrafish *Egfl7* led to severe vascular deformities, whereas a complete knock-out of the gene mediated by CRISPR/Cas9 did not lead to any observable changes in the mutant embryos. After transcriptomics analysis Rossi et al. (2015) were able to show that the phenotype in the knock-out mutants was rescued by the upregulation or genes whose proteins contained key functional domains also found in the target gene [50]. Similarly, knock-out of the *Bag3* gene, which leads to cardiovascular disease in zebrafish and humans, did not lead to aberrant phenotypes. In contrast, knock-down of the gene led to cardiomyopathy in zebrafish embryos. Proteomics analysis revealed that knock-out of *Bag3* results in upregulation of the related *Bag2* gene, that can rescue the diseased phenotype [50]. Even though the mechanisms governing GCR are still poorly understood, it is suggested that the nonsense-mediated mRNA decay (NMD) pathway is involved in GCR. NMD is activated by the presence of premature stop codons in mRNA. The RNA fragments generated during the degradation of aberrant mRNA through this pathway could be what triggers the compensatory response, by activating mRNA surveillance pathways that function to regulate gene expression [48].

The recent examples of GCR highlight the importance of thoroughly studying the function of genes through both knock-down and knock-out experiments. In our case, further genome-wide transcriptomics or proteomics studies could help us understand the discrepancies observed between the phenotypes. In addition, the possible implication of *SlWRKY23* in the regulation of ethylene related genes could be investigated in both knock-down and knock-out plants. Finally, these studies may lead to the identification of additional genes that could be compensating for the function of knocked-out genes. Identification of such genes could lead to novel potential targets to be engineered.

MATERIALS AND METHODS

Phylogenetic analysis

The *Arabidopsis thaliana WRKY27* (*At5g52830*) amino acid sequence was used as query on the Sol Genomics Network (SGN) database BLAST tool (https://solgenomics.net/tools/blast/) to search for homologous sequences in the ITAG 2.4 tomato genome release. Together with *WRKY27* its close homologs *WRKY22* (*At4g01250*), *WRKY25* (*At2g30250*) and *WRKY29* (*At4g23550*) were included in the phylogenetic analysis. The tomato sequences with the highest protein identity to the *A. thaliana* WRKY27 protein were aligned and used for the construction of a neighbor-joining phylogenetic tree using the CLC Sequence Viewer 8.0.0 software. For the correction of distances, the Kimura Protein method was used. The sequence clustering the closest to the Arabidopsis *WRKY27* was considered the ortholog in tomato.

Plant materials

The present study included the susceptible *Solanum lycopersicum* cv. Moneymaker as a control and T_2 progeny of two independent CRISPR/Cas9 induced mutants (*slwrky23_1* and *slwrky23_2*) in cv. Moneymaker background. Plants were grown at Unifarm (WUR) in a climate regulated greenhouse compartment at 24°C/18°C under a 12h/12h day/night regime. Relative humidity was kept at ~60% in the greenhouse compartment.

Generation of VIGS construct

To study the role of gene *SlWRKY23* (*Solyc08g081610.2*) in susceptibility of tomato to *Cm*, tobacco rattle virus (TRV) based VIGS was performed on the susceptible tomato cv. Moneymaker (Lie et al 2002). For the generation of the silencing construct, a 200 bp gene specific exonic fragment was amplified from the cDNA of *SlWRKY23*. The fragment was amplified with Phusion High-Fidelity DNA polymerase (New England Biolabs) and was directionally ligated into the entry vector pENTR/D-TOPO (Invitrogen). Subsequently, the entry vector was transformed into *Escherichia coli* DH5α chemically competent cells (Invitrogen). Recombinant plasmids of the entry vector were isolated using a MiniPrep Isolation Kit (Qiagen) and sequenced (Macrogen Europe, Amsterdam) to confirm the ligation of the correct amplicon. Gateway Technology cloning was used for the subsequent transfer of the silencing fragment into destination vector TRV2 [51]. Recombinant TRV2 plasmids were

isolated, sequenced for the confirmation of the correct insert and transformed into *Agrobacterium tumefaciens* GV3101 electrocompetent cells.

CRISPR/Cas9 vector design and construction

Exonic sequences of gene *Solyc08g081610.2* were imported onto the CRISPOR web tool (http://crispor.tefor.net/) for the design of the sgRNAs spacers [52]. The spacers with the highest specificity scores and highest predicted efficiencies were selected for the assembly of the CRISPR/Cas9 vector. Four spacers targeting exons two and three of gene *SlWRKY23* were selected. Golden Gate based modular cloning (MoClo) was used to assemble the sgRNAs and binary vectors, as it has previously been described [53, 54]. The CRISPR-PINK system (TSL Norwich, Synbio) was used for the generation of the CRISPR/Cas9 vector. Level 1 constructs of each sgRNA fused to the *Arabidopsis thaliana* U6-26 promoter were built by using plasmids pICH47761-pU6-26-CRISPR-Pink (position 4), pICH47772-pU6-26-CRISPR-Pink (position 5), pICH47781-pU6-26-CRISPR-Pink (position 6), pICH47791-pU6-26-CRISPR-Pink (position 7) based on their position on the final vector. The final level 2 vector pICSL4723 was assembled by using the level 1 constructs of each sgRNA, level 1 constructs pICH47742-p2x35s-spCas9-tNOS, pICH47751-pOLE1-OLE1cds-tagRFP-tOLE1 and linker pICH47791link-pU6-26-CRISPR-Pink. The final level 2 vector was transformed into *Agrobacterium tumefaciens* AGL1+virG electrocompetent cells under chloramphenicol, carbenicillin and kanamycin selection. The presence of plasmids was confirmed by colony PCR.

Bacterial strains and growth conditions

Cm strain NCPBB382 was used in the bioassays. Prior to plant inoculation the strain was grown at 25°C on TBY plates (10 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 5 gL^{-1} sodium chloride, 15 gL^{-1} bacteriological agar) for two days. For the preparation of the bacterial inoculum bacterial cells were resuspended in Ringer's buffer to a final concentration of ~10^8 cfu/mL (OD_{600}=0.1).

Disease assay

Tomato plants at the fourth true leaf stage were inoculated by a petiole clipping off method. The petioles of the first two fully expanded leaves were clipped off with razor blades immersed in the bacterial inoculum and 5 µl of the bacterial inoculum were directly pipetted on the lowest wound. Symptom development was monitored in a varying number of days in different experiments, but at least up to 20 days post inoculation (dpi). The longer disease assay for the first VIGS assay was conducted to

investigate if disease symptoms would significantly advance after 20 dpi. A disease index (DI) scale based on the development of wilting symptoms on the leaves was used (0; no symptoms, 1; one leaf wilting, 2; <2/3 of leaves wilting, 3; 2/3 of leaves wilting, 4; 3/4 of leaves wilting, 5; all leaves wilting).

Generation of tomato mutants

For the generation of CRISPR/Cas9 mutants stable tomato transformations were carried out as previously described, using the susceptible tomato cultivar Moneymaker (cv. MM) [35]. The regenerated primary transformants were moved to the greenhouse, where they were acclimated prior to selfing for the production of seeds.

Identification of CRISPR/Cas9 induced mutations

Genomic DNA (gDNA) from leaves of the primary transformants (T_1) was isolated using a standard CTAB method. The target region of transformed plants was amplified and visualized on 1% agarose gel for the detection of visible indels. All PCR products were ligated into the pGEM-T easy vector (Promega). The ligation products were transformed into *E. coli* DH10β chemically competent cells. Recombinant plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions and were sequenced for the identification of CRISPR/Cas9 induced mutations.

To confirm the presence of the expected mutations in the T_2 progeny gDNA was isolated from young leaves using the CTAB method. Because of the large expected mutation in the *slwrky23_1* mutants the target region of the four sgRNAs was amplified and visualized on 1% agarose gel. Due to the small size of indels present in the alleles of the progeny derived from *slwrky23_2*, High Resolution Melting (HRM) analysis was used to determine the alleles present in the mutants. PCR amplifications containing LC Green were done. The HRM genotyping was performed on a Light Scanner instrument with continuous melting curve acquisition (10 acquisitions per ^{o}C) during a 0.1^{o} C/s ramp from 40-95^{o}C. The retrieved data were analysed using the Light Scanner software.

Bacterial quantification

Bacterial quantification of strain *Cm* NCPBB382 was done through serial dilution plating. Stems collected ~1 cm above the lowest inoculation point were sampled at

three time points; 4 dpi, 7 dpi and 14 dpi. Stems were pulverized and homogenized in Ringer's solution (Sigma Aldrich). 50 µl of serial dilutions of the homogenate (10^1 -10^6) were plated on SCM-F selective plates (Duchefa Biochemie). The medium was supplemented with 1.9 g L^{-1} yeast extract, 20 µL L^{-1} nalidixic acid (100 mg mL^{-1}), 8 mL L^{-1} trimetroprim in MetOH 100% (10 mg/mL), 1 ml L^{-1} cyclohexamide in MetOH 100% (100 mg mL^{-1}), 1 mL potassium tellurite (1%), 50 ml L^{-1} nicotinic acid (2 mg/ mL). Plates were incubated at 25° C for 7 days. Colonies on the plates were counted 7 days post plating and the $\log_{10}$(cfu+1/ g fresh tissue) per plate was calculated. Three biological replicates were used per time point. Two technical replicates per sample were plated.

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SUPPLEMENTARY MATERIAL

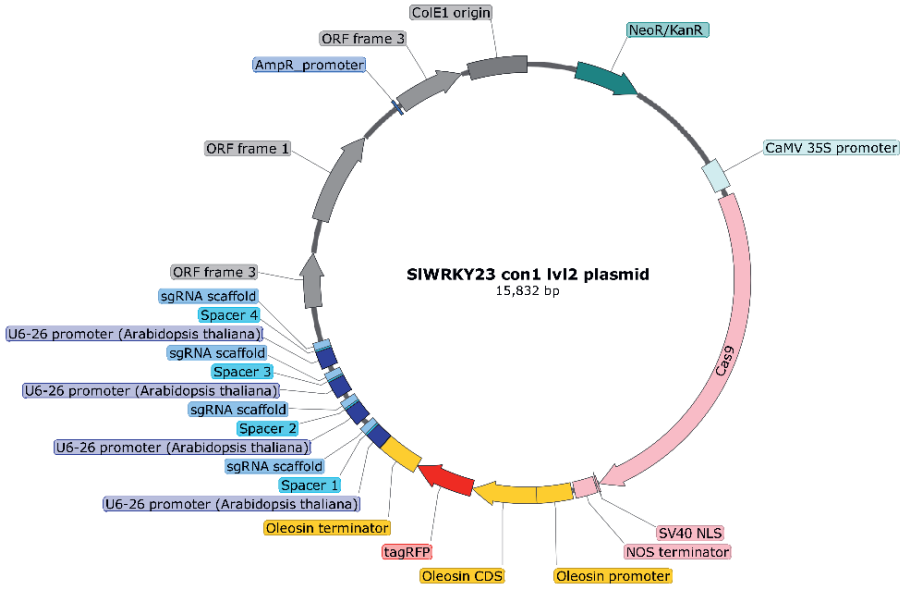

Figure S1| Level 2 CRISPR/Cas9 vector for the targeted mutagenesis of *SlWRKY23.*

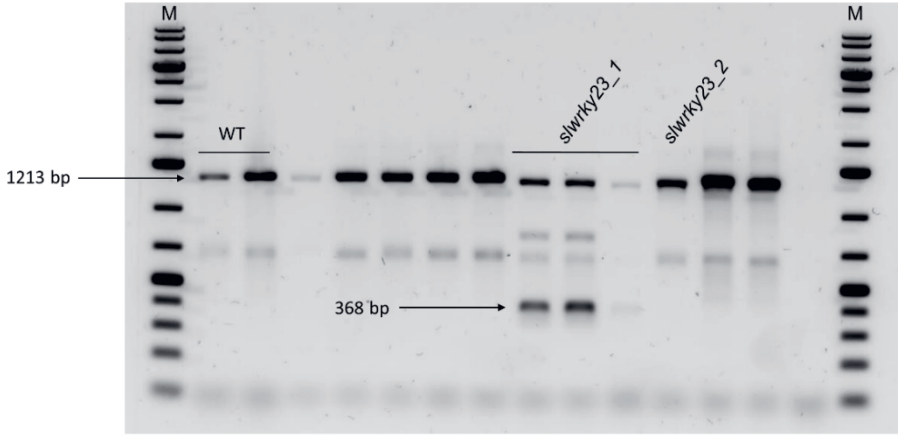

Figure S2| Visualization of amplification products of primary tomato transformants on 1% agarose. Numbers on the left of bands indicate their size. M; DNA marker, WT; WildType background. Generated mutants are referred to as *slwrky23_1* and *slwrky23_2*

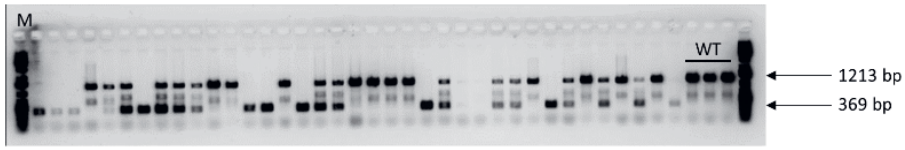

Figure S3| Visualization of amplification products of T_2 progeny of the primary tomato transformants of *slwrky23_1* on 1% agarose. Numbers on the right of bands indicate their size. M; DNA marker, WT; WildType background.

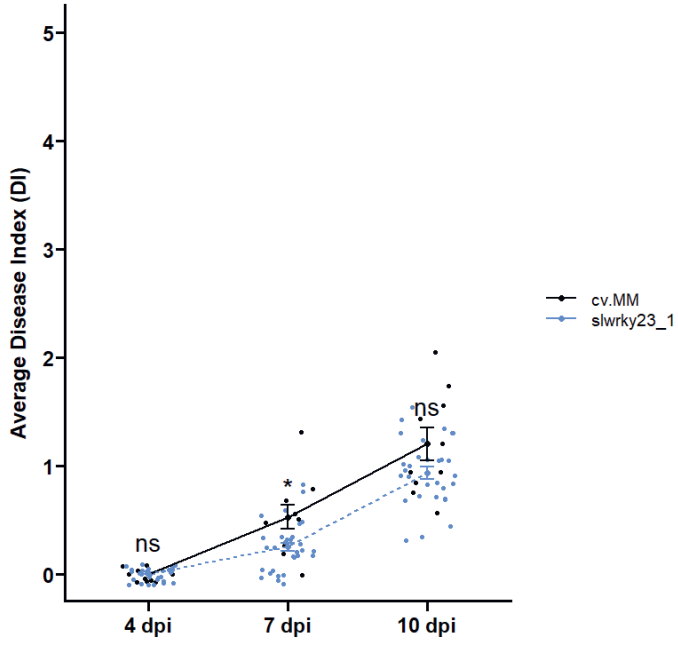

Figure S4| Progression of symptom development on cv.MM and *slwrky23_1* between 4 and 10 dpi. Asterisks indicate significant differences (*p≤0.05), ns= not significant.

Table S1| List of primers used in this study. The underlined "cacc" was used as an overhang for the directional cloning of the silencing fragments into the pENTR vector.

Target gene	Forward primer (5'-3')	Description
Solyc04g008094.2	caccGATTGGGGTCTTCAAGCTGT TTTTGTCCACCATCAACATGA	VIGS silencing fragment
Solyc04g008094.2	TTTCTTCTGATAAATGGGCT	Spacer 1
Solyc04g008094.2	GGATAAGGAGAGCCTTTAAT	Spacer 2
Solyc04g008094.2	TTATAGGTGCAGCAGTTCAA	Spacer 3
Solyc04g008094.2	GCTAGAGAATTTCTACGTGT	Spacer 4
Solyc04g008094.2	ATTTGGTTGAATATATGTGTGATG ATTATTTGATGTTGAACCATGTG	Genotyping

Table S2| List of potential VIGS off-targets. Dashes and nucleotides in bold indicate the position of mitchmatches between the off-target and target sequence.

Target sequence	Off-target sequence	Off-target	Id%
TGAGATGAAAAAAGAGTTTTTT	TGAGATGAAAAAAGAG**A**TTTTT	Solyc12g062940.1	95%
CCATTTTATCATGTTGATG	CCATTTTATCATGTTGATG	Solyc04g078860.2	100%
CTTTTTCACATGAGATGAAAA	CTTTTTCACATGA**T**ATGAAAA	Solyc12g019950.1	95%
GATTGGGGTCTTCAAGCTGTT	GATTGGG**A**TCTTCAAGCTGTT	Solyc07g006540.2	95%
TATTAGTGATGAATTAGAAGAACTT	TATTAGTGATGAATTA**C**A-AACTT	Solyc02g086040.1	92%
AGATGAAAAAAGAGTTTTTTATTA	AGATGAAAAAAGA**A**TTTTTTTTTA	Solyc01g111180.2	92%
AGATGAAAAAAGAGTTTTTTATTA	AGATGAAAAAAGA**A**TTTTTT**T**TTA	Solyc01g110700.2	92%
TTTTCACATGAGATGAAAAAAGAGTTTT	TTTTCACAA**G**AG-T-AAAGAGTTTT	Solyc01g005620.2	89%

Table S3|. List of potential CRISPR/Cas9 off-targets. Asterisks indicate the position of mismatches between off-targets and the 20 nt spacer sequences.

Spacer	Off-target sequence	Mismatch position	Off-target
2	TGATAAAGAGAGCTTTTAATTGG	*.....*.....*.....	intron:mRNA:Solyc03g025820.2.1
2	GGATAAGGGGAACCTTTGATGGG	........*..*.....*..	exon:mRNA:Solyc02g072190.3.1
2	GGGTAAGGGGAGCCTTTGATTGG	..*.....*........*..	exon:mRNA:Solyc02g021680.3.1
2	GGGTAAGGGGATCCTTTAATGGG	..*.....*..*........	exon:mRNA:Solyc01g079360.3.1
2	GGAAAAGGCAAGCCTTTAATGAG	...*....**..........	intergenic:mRNA:Solyc07g066220.3.1-mRNA:Solyc07g066230.3.1

2	GGTTAAGGATTGCCTTTAATGAG	..*.....**.....	intergenic:mRNA:Solyc03g064040.1.1-mRNA:Solyc03g064043.1.1
2	GGATATGGAGAGCCTTTGATGGG	.....*.....*..	exon:mRNA:Solyc01g095100.3.1
2	GGATAAGGTGATCCTTTAATTGG	.....*..*.....	exon:mRNA:Solyc07g055280.3.1
2	GGATAAGGTGATCCTTTAATTGG	.....*..*.....	exon:mRNA:Solyc10g007970.2.1
3	CTACAGATGCAGCAGTTCAAAGG	*..*..*.....	exon:mRNA:Solyc01g079360.3.1
3	TTTTAGGTGCAGCAATTCCAGGG	..*.....*...*.	exon:mRNA:Solyc11g010430.2.1
3	CTATAGGTGCAGTAGTTCAAAGG	*.....*.....	exon:mRNA:Solyc02g021680.3.1
4	CCTAGAGAAATTCTACGTTTCAG	*.....*.....*.	intergenic:mRNA:Solyc04g028565.1.1-mRNA:Solyc04g028470.2.1

CHAPTER 5

Expression based selection of candidate susceptibility genes for functional analysis against *Clavibacter michiganensis*

Eleni Koseoglou*, Arcadio Garcia Pérez*, Jan M. van der Wolf, Richard G.F. Visser, Yuling Bai

*These authors contributed equally to this work.

ABSTRACT

Bacterial canker of tomato caused by the bacterial pathogen *Clavibacter michiganensis* (*Cm*), is considered to be one of the most devastating diseases of tomato. To date, no qualitative resistance to the pathogen has been identified. To find novel sources of resistance to the pathogen we set out to identify plant genes used by the bacterium to cause disease, referred to as susceptibility (S) genes. By taking advantage of a publicly available microarray dataset, nine differentially upregulated genes upon *Cm* infection were selected as potential S gene candidates for further functional analysis. Virus induced gene silencing (VIGS) was used as a fast functional analysis and results showed that simultaneous silencing of two homologs *Solyc09g089680.3* and *Solyc12g005380.2* (both encoding 2-oxoglutarate-dependent dioxygenases) led to reduced symptom development on infected tomato plants. In order to confirm these results, CRISPR/Cas9 was employed for the generation of double mutants of genes *Solyc09g089680.3* and *Solyc12g005380.2*. Bioassays including disease assays and *in planta* bacterial quantification were performed using the double mutants. Remarkably, the use of the double mutants in our bioassays led to different results. Whereas we observed increased resistance through our VIGS experiments, we could not confirm these initial results with the double knock outs. We discuss the potential reasons for these different observations below.

INTRODUCTION

The bacterial phytopathogen *Clavibacter michiganensis* (*Cm*) causes what is considered to be one of the most destructive diseases of cultivated tomato (*Solanum lycopersicum*) [1, 2]. *Cm* invades the xylem vessels of tomato plants leading to systemic infections that can cause severe disease. Commonly observed symptoms of the disease include the wilting of leaves and leaflets, interveinal chlorosis and necrosis of leaves, cankers on the stems and petioles of plants and the formation of necrotic spots on tomato fruits. As the disease progresses, the whole plant wilts and eventually dies [2-4]. Bacterial canker poses a great threat to the tomato production sector worldwide. No resistance against the pathogen is available and chemical and biological means of control do not result in satisfactory levels of protection [4].

To generate disease resistant crops, traditional breeding has mainly focused on the introgression of single, dominantly inherited resistance (R) genes from wild species to elite cultivars [5]. The intracellular receptors encoded by R genes directly or indirectly recognise corresponding pathogen effectors, resulting in a gene for gene interaction that leads to resistance [6, 7]. Even though resistance conferred by recognition of effectors by R genes is highly effective, deployment of R genes can put high selective pressure on pathogen populations. This selective pressure can result in the emergence of new virulent strains that are no longer detected by R genes, ultimately leading to break down of resistance [6]. An alternative approach to gain resistance against pathogens is the use of loss-of-function alleles of susceptibility (S) genes [5, 8, 9]. S genes encode host factors that are manipulated by pathogens to establish a compatible interaction with the plant [9-11]. S genes have widely different functions. Pathogens use S genes to complete processes that can range from the uptake of nutrients to the translocation of their effector proteins into plant cells [9, 12, 13]. In many cases, it has been shown that S genes are upregulated by pathogens, as induction of their expression may support pathogen growth or suppress host responses during infection [13-15].

In contrast to dominantly inherited R genes, S genes are inherited in a recessive way, as resistance is conferred by their mutant alleles. As several studies on mutant S genes suggest, the use of mutant S gene alleles in breeding can lead to broad-spectrum and durable resistance [13, 16-18]. Thus, the identification and mutation of S genes can provide novel sources of plant resistance.

For the identification of S genes different approaches may be used. Forward and reverse genetics methods have both been used in the identification of several S genes. For example genes *Downey Mildew Resistance6* (*DMR6*) and *Powdery Mildew Resistance6* (*PMR6*) have both been identified through screenings of mutant arabidopsis populations [19, 20], whereas the function of genes such as *PMR4* and *Defence No Death1* (*DND1*) as susceptibility genes in tomato has been assessed through reverse genetic approaches [21, 22]. Upregulation of S genes upon pathogen challenge has been recurrently observed [15, 23-25]. Upregulation of the *Pectin Methylesterase3* (*PM3*) gene in *Arabidopsis* after infection by the nematode *Heterodera schachtii* correlates with enhanced susceptibility to the pathogen [15]. Similarly, bacterial species in the *Xanthomonas* genus make use of their transcription activator like effectors (TALEs) to induce the expression of host genes [26-28]. The use of transcriptomics data for the identification of differentially expressed genes during pathogen infection has led to the identification of several S genes, including three *CsLOB* homologs in citrus [14]. Therefore, the analysis of transcriptomics data can aid in the identification of S genes.

In the case of *Cm*, no resistance is available. Genetic studies have, however, identified sources of tolerance to the bacterium [29]. Nevertheless, the tolerance conferred by these sources is polygenic, and with a complex genetic background [29-31]. In an effort to identify new sources of tomato resistance against *Cm* we decided to investigate the possibility of identifying and modifying S genes. To do so, we took advantage of a publicly available microarray study, in which differentially upregulated genes upon tomato infection by *Cm* were identified [32]. In our study, we used gene expression based prediction of potential S genes. Based on the 122 differentially upregulated genes that were reported by Balaji *et al*. (2008) [32], we selected genes with a high differential induction upon *Cm* infection. Thereafter, for the functional confirmation of the selected genes as susceptibility factors, we used a combination of virus induced gene silencing (VIGS) and genome editing through CRISPR/Cas9.

RESULTS

Expression-based selection and transient silencing of candidate S genes

To identify potential S gene candidates, we used a publicly available dataset of differentially regulated tomato genes upon infection with *Cm*. Of the 9,254 genes present on the array used in the study, 122 genes were found to be differentially upregulated [32]. The GenBank Accession number for each differentially upregulated

gene was converted to the equivalent SolycIDs (Supplementary Table 1). After filtering of the 122 genes for general defence related genes that are most likely involved in basal immunity (e.g. *SlPR1*), the genes with the highest fold-ratio expression change at 4 and 8 dpi were selected for further functional analyses. Eight GenBank Accession numbers corresponding to nine SolycIDs were selected (Table 1).

For the functional analysis of the selected candidate genes, TRV (Tobacco Rattle Virus) based virus induced gene silencing (VIGS) vectors carrying gene specific exonic fragments were constructed. For genes that multiple specific exonic sequences were identified two constructs were generated. For the rest of the genes one construct per gene was generated. Due to the high nucleotide and protein identity of the close homologs *Solyc09g89680.3* and *Solyc12g006380.2*, we decided to construct a VIGS vector targeting both homologs, to account for potential functional redundancy. An additional specific vector targeting *Solyc12g006380.2* was generated and used in silencing assays. In total 11 constructs for nine selected genes were used in the screening for potential candidate S genes.

Table 1| List of differentially upregulated genes selected for functional analysis.

GenBank Accession	Gene Solyc ID	Fold Ratio Change		Gene function	Construct
		4 dpi	8 dpi		
BT012691	*Solyc09g089680.3* *Solyc12g006380.2*	3	22	2-oxoglutarate-dependent dioxygenase 2-oxoglutarate-dependent dioxygenase	TRV::A/ TRV::B
U89256	*Solyc02g077370.1*	21	163	Ethylene Response Factor C.5	TRV::C1/ TRV::C2
X85138	*Solyc01g107810.2*	8	66	Glycosyltransferase	TRV:D1/ TRV::D2
BI205190	*Solyc12g098590.2*	1	59	Glycosyltransferase	TRV::E
BI204920	*Solyc03g117860.3*	7	70	RING/U-box superfamily protein	TRV::F
BI210305	*Solyc10g055740.2*	5	27	Amino acid transporter	TRV::G
AJ831935	*Solyc03g095770.3*	3	36	WRKY transcription factor 80	TRV::H
BI206504	*Solyc09g015770.3*	8	31	WRKY transcription factor 81	TRV::I

cv. Moneymaker (cv. MM) tomato seedlings were agroinfiltrated with the 11 constructs by infiltrating the abaxial side of the cotyledons until saturation. As a negative control a TRV::GUS construct was used in the assays. Additionally, a construct targeting the endogenous tomato *Phytoene desaturase* (*PDS*) gene was used as a visual control of gene silencing. Approximately, two weeks after agroinfiltration the plants were inoculated with the hypervirulent *Cm* strain NCPBB382. Of the 11 constructs used in the assay, a small but significant reduction in wilting symptom development (at 20 dpi) was repeatedly observed in plants transformed with the vector targeting homologs *Solyc09g89680.3* and *Solyc12g006380.2* (Construct TRV::B). A statistically significant increase of wilting symptoms was observed when gene *Solyc10g055740.2* was silenced (Construct TRV::G). No significant reduction of wilting symptoms was observed for any of the other constructs used in the silencing assays. Genes *Solyc09g89680.3* and *Solyc12g006380.2* were selected for further functional analysis through CRISPR/Cas9 targeted mutagenesis (Figure 1).

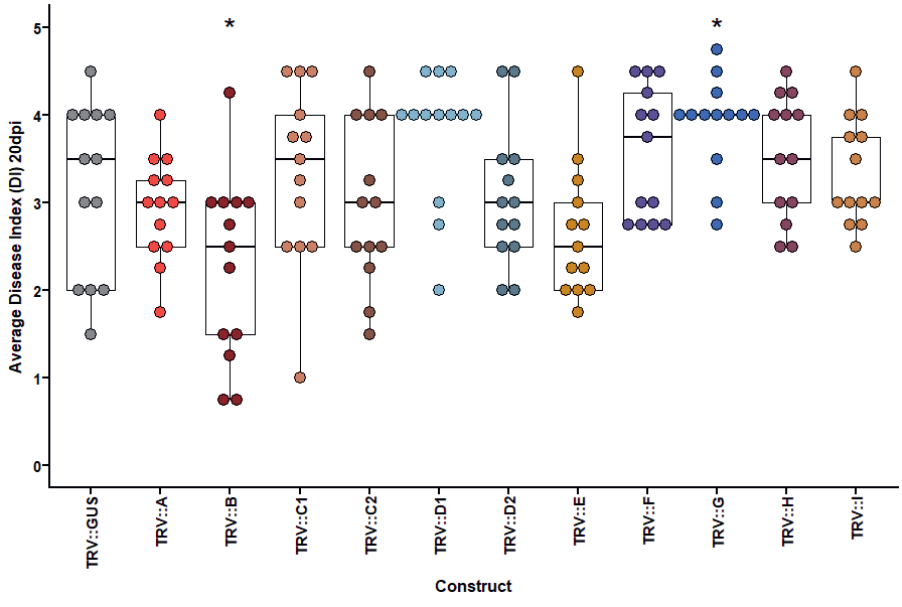

Figure 1.|VIGS assay of selected genes. Wilting symptom development on tomato plants (cv Moneymaker) agroinfiltrated with the VIGS silencing vectors compared to the negative control TRV::GUS at 20 dpi. Centre lines show medians, the box limits indicate the 25th and 75th percentiles. (Student's t-test, *p≤0.05).

Characterization of mutations

Our initial data supported the hypothesis that simultaneous silencing of close homologs *Solyc09g089680.2* and *Solyc12g006380.2*, encoding putative 2-oxoglutarate Fe(II) dependent-oxygenases reduces susceptibility of tomato to *Cm*. Therefore, we decided to generate double loss-of-function mutants of the genes, in order to study the full effect of inactivation of the genes. Double knock-out mutants were obtained by employing CRISPR/Cas9. Cotyledons of susceptible cv. MM plants were transformed with a CRISPR/Cas9 vector carrying eight sgRNAs; each homolog was targeted by four sgRNAs (Supplementary Figure 2, Supplementary Table 3). Two double mutants were identified after stable transformations and genotyping of the primary transformants. Gene *Solyc09g89680.2* of mutant 1, denominated as *doxy_1 (Chr9),* carries two mutant bi-allelic homozygous alleles. In both alleles, a 112 bp deletion was identified between the target regions of sgRNA1 and sgRNA2. An additional 62 bp deletion was induced between sgRNA3 and sgRNA4. In the second mutant (*doxy_2 (Chr9)*) multiple deletions were identified for homolog *Solyc09g089680.2*. On both mutant alleles deletions exceeding the targeted region of sgRNA1 and sgRNA2 were found (Fig. S1). A 47 bp deletion between positions 1566-1612 bp and a 386 bp deletion between positions 1621-2007 bp, flanking sequence 5' AAAGAAATG 3', are present on both mutant alleles (Fig. S1). Finally, a 9 bp deletion and a T>G substitution in the seed sequence of sgRNA4 were identified in mutant alleles 1 and 2, respectively. All mutations led to frame shifts and premature termination of translation (Fig. 2).

Both mutants of *Solyc12g006380.2* carried bi-allelic homozygous mutations. Both alleles of *doxy_1 (Chr12)* carried a 929 bp mutation between the target regions of sgRNA1 and sgRNA3. The deletions carried by both alleles of *doxy_2 (Chr12)* exceeding the target regions of sgRNA1 and sgRNA2, as also observed for *doxy_2 (Chr9)*. A 966 bp deletion is present on *doxy_2 (Chr12)* (Fig. S1). Additionally, the insertion of at T was found in the seed sequence of sgRNA3 (Fig. 3). The mutations led to frameshift and premature termination of translation.

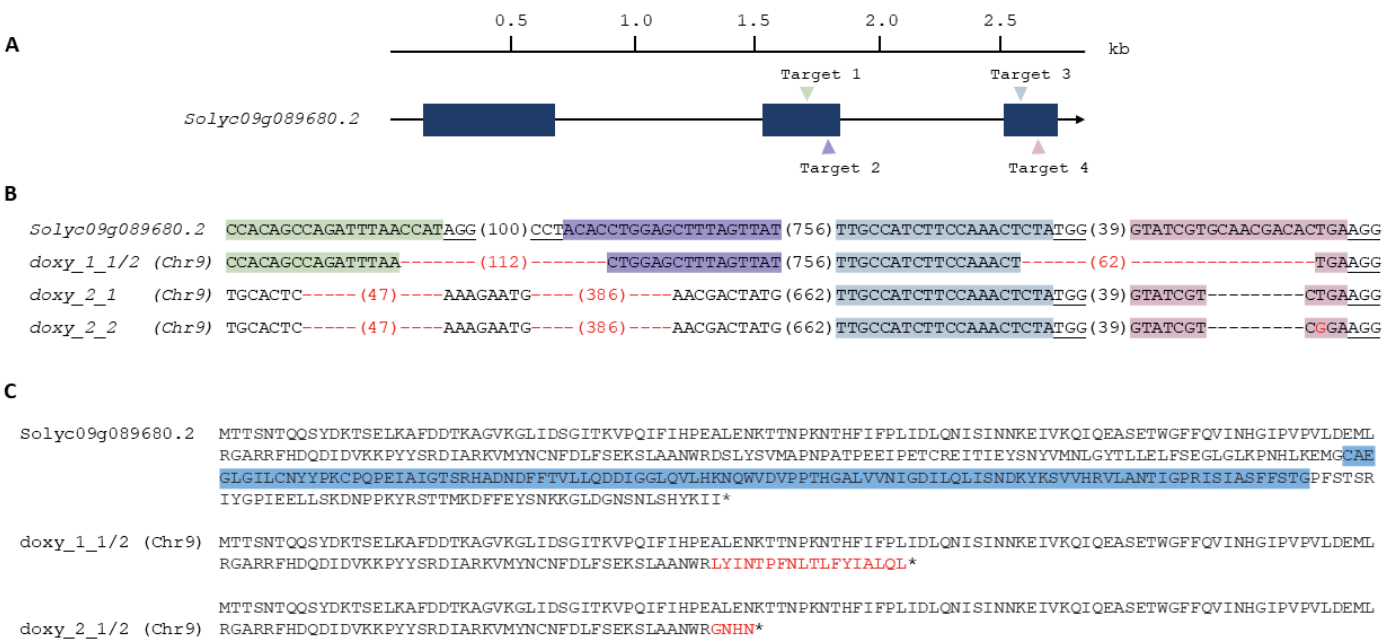

Figure 2| Graphical representation of *Solyc09g8960.2*, coding for putative 2-oxoglutarate-dependent dioxygenase and the target regions of the four sgRNAs carried by the CRISPR/Cas9 vector. A) Blue boxes represent exons, black lines represent introns. Triangles indicate the position of the target by each sgRNA. B) Mutations identified in T1 mutant plants. Highlighted sequences represent the 20 nt target sequences of the sgRNAs. Each colour corresponds to the triangle indicating the position of each target in Figure 2 A. The underlined sequences represent the PAM motifs of each sgRNA. Black numbers in parentheses represent missing nucleotides. Red dashes and red numbers in parentheses indicate deleted bases. Bases in red represent substitutions. C) Protein encoded by the WT and mutant alleles. Highlighted in blue are the amino acids present in the functional oxygenase domain. Amino acids in red indicate frame shifts. Asterisks indicate stop codons.

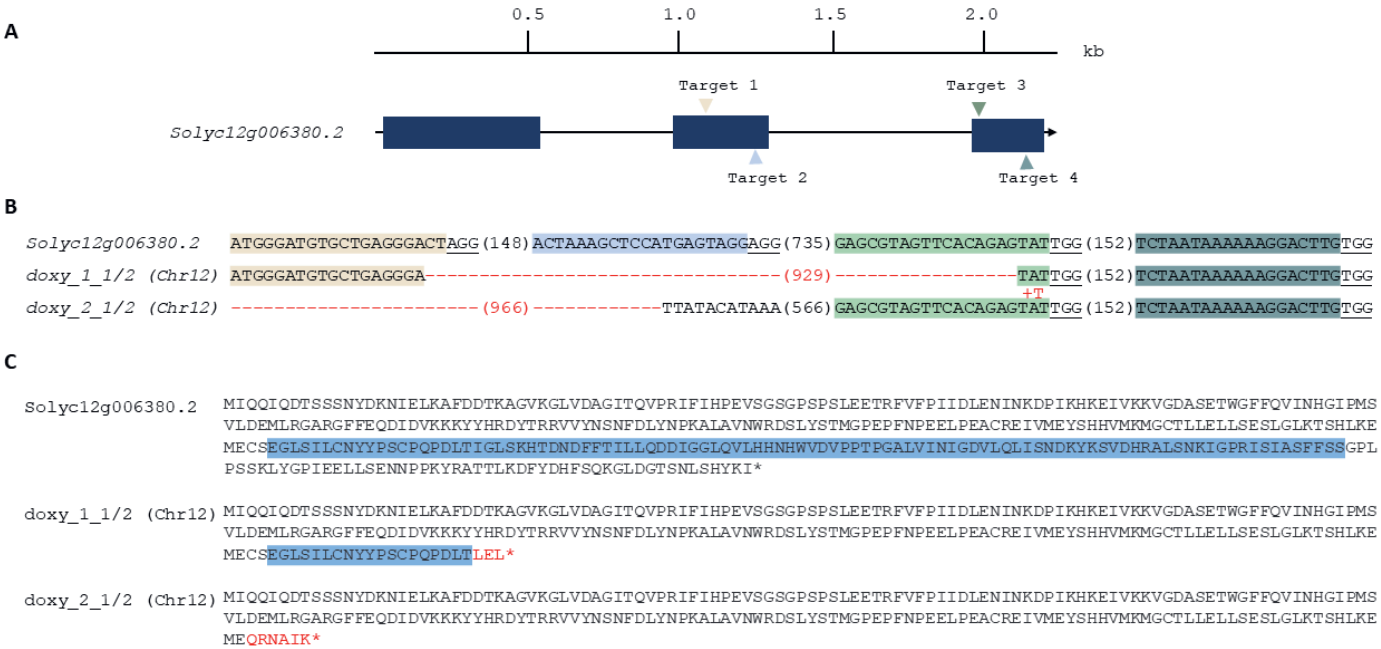

Figure 3|Graphical representation of *Solyc09g8960.1* , coding for putative 2-oxoglutarate-dependent dioxygenase, and the target regions of the four sgRNAs carried by the CRISPR/Cas9 vector. A) Blue boxes represent exons, black lines represent introns. Triangles indicate the position of the target by each sgRNA. B) Mutations identified in T1 mutant plants. Highlighted sequences represent the 20 nt target sequences of the sgRNAs. Each colour corresponds to the triangle indicating the position of each target in Figure 2 A. The underlined sequences represent the PAM motifs of each sgRNA. Black numbers in parentheses represent missing nucleotides. Red dashes and red numbers in parentheses indicate deleted bases. Bases in red represent substitutions. C) Protein encoded by the WT and mutant alleles. Highlighted in blue are the amino acids present in the functional oxygenase domain. Amino acids in red indicate frame shifts. Asterisks indicate stop codons.

Double knockout mutation of *doxy* genes does not alter susceptibility to *Cm*

Based on our initial observations that simultaneous downregulation of genes *Solyc09g089680.2* and *Solyc12g006380.3* through VIGS reduced susceptibility to *Cm*, we hypothesized that complete loss-of-function of both genes would lead to loss-of-susceptibility to *Cm*. T_2 progeny of the double mutants *doxy_1* and *doxy_2* were used in our bioassays. T_2 progeny were genotyped through PCR before bioassays, to confirm the presence of the expected mutations. Our results confirmed the presence of the expected mutant alleles in the progeny (Fig. S3).

Four weeks old mutant plants (T_2) were inoculated with *Cm* strain NCPBB382. The susceptible background cv. MM was used as a negative control in the disease assays. Regardless of plant genotype severe wilting symptoms developed on the plants. Both double mutants *doxy_1* and *doxy_2* were found to be fully susceptible to *Cm*. No statistically significant differences in the disease index were found between *doxy_1* and cv. MM, while the disease index of mutant *doxy_2* was significantly higher than the susceptible cv.MM background (Fig. 4A).

Together with the disease assays we also quantified the bacterial titres of strain NCBPP382 *in planta* at different infection time points. An estimated $5x10^5$ cfu/mL were used for the inoculation of plants. The population dynamics of strain NCBPP382 in tomato stems were monitored at 4, 7 and 14 dpi, through serial dilution plating. During the course of infection *Cm* reached high densities (~$10^{9.5}$ cfu+1/g fresh stem tissue) *in planta*. For mutant *doxy_1* no statistically significant differences were found compared to the susceptible background cv.MM at any of the selected time points. At 4 dpi, the bacterial titres recovered from *doxy_2* were significantly higher than the susceptible cv. MM plants. During the course of infection, however, no further differences were observed (Figure 4B, C).

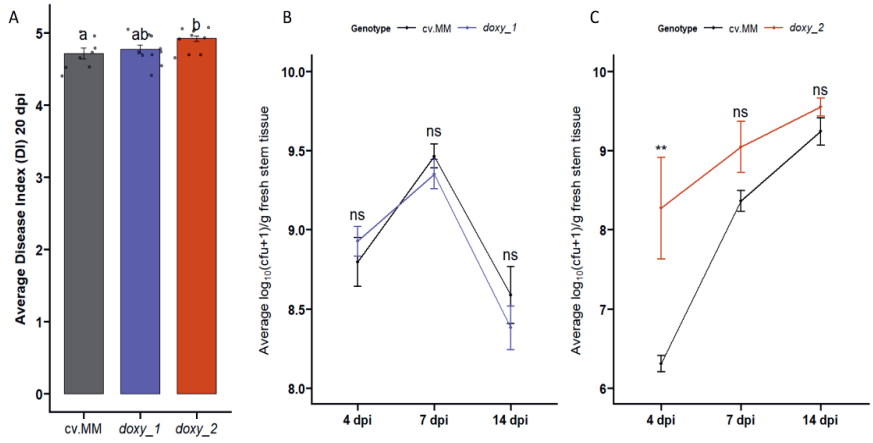

Figure 4| A) Symptom development on two independent T_2 doxy mutants compared to the susceptible background (cv.MM). Errors bars indicate standard errors. Letters indicate significant differences (ANOVA, Tukey's post hoc). Bacterial titres of bacterial strain NCPBB382 recovered from mutant B) *doxy_1* and C) *doxy_2* plants and their susceptible background were quantified at 4, 7 and 14 dpi. Three biological replicates (n=3) were used per time point. Lines represent the average $\log_{10}$(cfu+1/ g fresh stem tissue) ± stdev. The experiments were repeated independently at least twice with similar results. (Student's t-test, **p≤0.01).

DISCUSSION

The durable and broad-spectrum nature of resistance conferred by mutant S genes, makes the use of their loss-of-function alleles highly desirable in resistance breeding [5]. This is an especially attractive alternative for pathogens for which qualitative resistance is lacking or is very limited. To date, no resistance to *Cm* has been identified. Screening of wild tomato accessions has, however, led to the identification of sources of tolerance [29, 31, 33]. Tolerance to *Cm* has been shown to be polygenic and complex [29-31, 34], therefore the identification and mutation of S genes may provide a novel, simpler form of resistance. This study aimed at identifying and functionally characterizing candidate S genes, through the combination of analysing transcriptomics data previously generated [32] and reverse genetic approaches.

Out of the nine silenced genes, simultaneous post-transcriptional silencing via VIGS of the close homologs *Solyc09g089680.3* and *Solyc12g006380.2,* repeatedly led to reduced susceptibility to *Cm*, when compared to the TRV::GUS negative control. The two candidate genes code for putative 2-oxoglutarate (2OG) and Fe(II) dependent dioxygenase (2OGD) proteins. 2OGDs are widespread among bacteria, fungi,

vertebrates and plants [35]. 2OGDs are biochemically active enzymes that most commonly catalyse hydroxylations. They constitute the second largest family of enzymes in plants, with 131 2OGDs divided in seven clades present in tomato [36]. In plants, 2OGDs participate in biological processes, including DNA demethylation, plant hormone and specialized metabolite biosynthesis [37-39]. 2OGDs play significant roles in the catabolism of plant defence hormones, such as auxin, ethylene, jasmonic acid and salicylic acid [40] Multiple 2OGDs in different plant species have been demonstrated to be involved in the hydrolysis of phytohormones, leading in some of the cases to suppression of plant defences [19, 40-45].

Our observation that the simultaneous silencing of the two homologs reduced susceptibility to *Cm*, while silencing of only one homolog did not, suggested the possibility of functional redundancy between the two genes. In the case of 2OGDs involved in susceptibility to pathogens, functional redundancy has been reported between the pathogen-inducible *DMR6* and *DMR6-Like Oxygenase1* (*DLO1*) in arabidopsis. The two genes act in coordination to tightly regulate defence related developmental trade-offs, due to the hyperaccumulation of SA [32].

Following the post-transcriptional silencing assays, we generated double knock-out mutants of *Solyc09g089680.3* and *Solyc12g006380.2* to further study their involvement in susceptibility of tomato to *Cm*. During genotyping of the mutants, we discovered that mutant *doxy_2* contained large deletion, which exceeded the regions targeted by the sgRNAs. Cells have evolved different mechanisms to restore chromosomal integrity after double stranded breaks (DSB) [46]. Non- homologous end joining (NHEJ) is thought to be the predominant repair mechanism in cells [47]. In plants two NHEJ mechanisms have been described so far. The canonical-NHEJ (c-NHEJ), which leads to small indels at the breakpoint junctions and the alternative-NHEJ (a-NHEJ) pathway [46, 48]. In contrast to the c-NHEJ pathway that produces small indels, the a-NHEJ pathway is highly mutagenic and can cause large deletions leading to loss of genetic information [48]. We attributed the large deletions observed in the possible involvement of a-NHEJ in the repair of the DSB caused by Cas9.

Although during our VIGS assays, a small but significant reduction in wilting was observed by simultaneously silencing of *Solyc09g089680.3* and *Solyc12g006380.2,* no such reduction in symptom development was observed for the *doxy_1* and *doxy_2* double mutants. In accordance with the fully susceptible phenotype of the mutants, *Cm* strain NCPBB382 reached high population densities *in planta* after stem

inoculations. By the end of the experiment, no significant differences in population dynamics were observed between the susceptible cv. MM and the double mutants.

The observed differences in wilting phenotypes between the knock-down and knock-out of the candidate genes can be attributed to multiple factors. As already discussed in **Chapter 4,** VIGS is a technology that takes advantage of the homology directed RNA interference (RNAi) pathway, leading to post-transcriptional silencing of genes [49]. Even though initial studies suggested that at least a 19-nt identity between the target and the small interfering RNA (siRNA) guide is needed for silencing of the endogenous target, recent studies have reported efficient silencing of targets even when mismatches are present [50, 51]. 2OGDs belong in a large family of highly homologous genes. Despite the care that was taken during the design of the VIGS constructs to minimize off-targets, off-targets with nucleotide identity less that 19-nt were still present (Table S2). Therefore, we cannot eliminate the chance of silencing of genes other that our endogenous target. Quantification of transcripts of the primary targets and potential off-targets could verify the specificity and efficiency of the silencing constructs.

The development and widespread use of new genome editing tools have revealed significant phenotypic discrepancies caused through knock-down and knock-out assays [52]. To buffer the effect of possible deleterious mutations organisms may respond through transcriptional adaptation [53, 54]. This transcriptional adaptation has been attributed a phenomenon called genetic compensation response (GCR). Recent studies in mice, zebrafish and even human genetic studies, suggest that GCR is activated in response to severely mutated alleles [53, 54]. Even though the mechanisms of GCR still remain largely unknown, it has been suggested that mRNA surveillance pathways function to compensate gene expression in the presence of mRNAs, that contain premature stop codons, through the non-sense mediated mRNA decay pathway [52]. To further elucidate the reasons for the differences observed in our assays, the use of wide-genome transcriptomics studies could help in the identification of genes, that may be compensating for the function of the knocked-out targets. In addition, identification of genes that are compensating the phenotype could be assessed as potential candidate S genes.

MATERIALS AND METHODS

Expression based selection of candidate genes

For the primary selection of candidate genes, we used a publicly available microarray dataset [32]. Of the 9,254 tomato genes present on the microarray, 122 were found to be differentially induced upon *Cm* challenge [32]. The GenBank accession number of each gene was converted to the Solyc ID, based on the SGN database assembly 2.40. For the conversion of the numbers, a phylogenetic tree based on amino acid sequences was built per gene. The Solyc ID proteins with the highest identity to the GenBank protein sequences were aligned and used for the construction of phylogenetic neighbour-joining phylogenetic trees. For the correction of distances the Kimura Protein method was used. The phylogenetic trees were generated using the CLC Sequence Viewer 8.0.0 software. The sequences clustering the closest to the GenBank accession numbers were considered the equivalent SolycIDs (Supplementary Table 1). Genes with the highest expression fold ratio change were selected for further functional analysis.

Generation of VIGS vectors and VIGS assays

For the initial functional characterization of the genes, gene specific VIGS vectors or VIGS vectors targeting multiple genes were constructed. Tobacco rattle virus (TRV) based VIGS was performed on the susceptible cv. Moneymaker (cv. MM). For the generation of the silencing fragments, gene specific exonic fragments were amplified from the cDNA of each target gene. The fragments were amplified with Phusion High-Fidelity DNA polymeras (New England Biolabs) and were directionally ligated into the vector pENTR/D-TOPO (Invitrogen). The entry vectors were subsequently transformed into *Escherichia coli* DH10b chemically competent cells. Recombinant plasmids of the entry vectors were isolated and sequenced (Macrogen, Amsterdam) to confirm the ligation of the correct silencing fragments. Gateway cloning was used for the subsequent transfer of the silencing amplicon into the destination vector TRV2. Recombinant TRV2 plasmids were isolated, sequenced and transformed into *Agrobacterium tumefaciens* AGL1+virG electrocompetent cells.

CRISPR/Cas9 vector design and construction

A CRISPR/Cas9 vector carrying eight sgRNAs was constructed for the simultaneous mutation of homologs *Solyc09g089680.3* and *Solyc12g006380.2*. Exonic sequences of genes *Solyc09g089680.3* and *Solyc12g006380.2* were imported onto the CRISPOR web tool (http://crispor.tefor.net/) for the design of the sgRNAs spacers [55]. The

spacers with the highest specificity scores and highest predicted efficiencies were selected for the assembly of sgRNAs (Table S3). Four spacers per gene were selected. Golden Gate based modular cloning (MoClo) was used to assemble the sgRNAs and binary vectors, as it has previously been described [56, 57]. The CRISPR-PINK system (TSL Norwich, Synbio) was used for the generation of the CRISPR/Cas9 vector. Level 1 constructs of each sgRNA fused to the *Arabidopsis thaliana* U6-26 promoter were built using plasmids pICH47772-pU6-26-CRISPR-Pink (position 5), pICH47781-pU6-26-CRISPR-Pink (position 6), pICH47791-pU6-26-CRISPR-Pink (position 7), pICH47732-pU6-26-CRISPR-Pink (position 8), pICH47742-pU6-26-CRISPR-Pink (position 9), pICH47751-pU6-26-CRISPR-Pink (position 10) and pICH47761-pU6-26-CRISPR-Pink (position 11) based on their position on the final vector. The final level 2i vector pICSL4723 was assembled using the level 1 constructs of each sgRNA, level 1 constructs pICH47742-p2x35s-spCas9-tNOS, pICH47751-pOLE1-OLE1cds-tagRFP-tOLE1 and linker pICH41800. The final level 2i vector was transferred into *Agrobacterium tumefaciens* AGL1+virG electrocompetent cells for the transformation of tomato plants.

Generation of tomato mutants

For the generation of CRISPR/Cas9 mutants stable transformations of tomato cotyledons were carried out as previously described, using the susceptible tomato cultivar cv. MM [21].

Identification CRISPR/Cas9 induced mutations

Genomic DNA (gDNA) from leaves of the primary transformants (T_1) was isolated using a standard CTAB method. The target region of transformed plants was amplified and visualized on 1% agarose gel for the detection of visible indels. Due to the high A-T content of gene *Solyc12g006380.2* amplification of the region flanking the area targeted by the sgRNAs was not possible using standard PCR conditions. Amplification of the region was achieved through an optimized 2-step PCR with a lower than usual extension temperature [58]. An extension temperature of 65^{o}C and the addition of 3.5 mM $MgCl_2$ in each reaction were crucial for the amplification of the region. Phusion Taq polymerase (New England Biolabs) was used for the amplification of the desired region. PCR products were ran on 1% agarose gel for the detection of possible large deletions. A-tailing of blunt end Phusion Taq polymerase amplified fragments of gene *Solyc12g006380.2* was done using DreamTaq polymerase (ThermoFisher Scientific) prior to ligation of the fragments into vector

pGEM T-Easy (Promega). The ligation products were transformed into *E. coli* DH10β chemically competent cells. Recombinant plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions and were sequenced for the identification of CRISPR/Cas9 induced mutations.

Bacterial strain and growth conditions

Cm strain NCPBB382 was used in the bioassays. Prior to plant inoculation the strain was grown at 25o C on TBY plates (10 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 5 gL^{-1} sodium chloride, 15 gL^{-1} bacteriological agar) for two days. For the preparation of the bacterial inoculum bacterial cells were harvested and resuspended in Ringer's buffer to a final concentration of ~10^8 cfu/mL (OD_{600}=0.1).

Disease assay & bacterial quantification

Tomato plants at the fourth true leaf stage were inoculated by a petiole clipping off method. The petioles of the first two fully expanded leaves were clipped off with razor blades immersed in the bacterial inoculum and 5 µl of the bacterial inoculum were directly pipetted on the lowest wound. Symptom development was monitored up to 20 days post inoculation (dpi). A disease index (DI) scale based on the development of wilting symptoms on the leaves was used (0; no symptoms- 5; all leaves wilting).

Bacterial quantification

Bacterial quantification of *Cm* strain NCPBB382 was done through serial dilution plating. Stems collected ~1 cm above the lowest inoculation point were sampled at three time points; 4 dpi, 7 dpi and 14 dpi. Stems were pulverized and homogenized in Ringer's solution (Sigma Aldrich). 50 µl of serial dilutions of the homogenate (10^1 -10^6) were plated on SCM-F selective plates (Duchefa Biochemie). The medium was supplemented with 1.9 g L^{-1} yeast extract, 20 µL L^{-1} nalidixic acid (100 mg mL^{-1}), 8 mL L^{-1} trimetroprim in MetOH 100% (10 mg/mL), 1 ml L^{-1} cyclohexamide in MetOH 100% (100 mg mL^{-1}), 1 mL pottasium tellurite (1%) and 50 ml L^{-1} nicotinic acid (2 mg/ mL). Plates were incubated at 25° C for 7 days. Colonies on the plates were counted 7 days post plating and the $\log_{10}$(cfu+1/ g fresh tissue) per plate was calculated. Three biological replicates were used per time point. Two technical replicates per sample were plated.

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SUPPLEMENTARY MATERIAL

Table S1| List of genes differentially upregulated upon *Cm* inoculation.

GenBank accession number	SolycID	Gene function
BG628643	*Solyc09g008670.3*	threonine deaminase
CN384809	*Solyc07g049530.2*	1-aminocyclopropane-1-carboxylate oxidase 1
BT013271	*Solyc06g073080.3*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
BI207493	*Solyc04g009860.3*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
	Solyc04g009850.3	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
BI210054	*Solyc04g009860.3*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
	Solyc04g009850.3	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
BT012691	*Solyc09g089680.3*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
	Solyc12g006380.2	2-oxoglutarate-dependent dioxygenase
BI930800	*Solyc09g061840.3*	3-ketoacyl-CoA thiolase
AW096548	*Solyc09g061840.3*	3-ketoacyl-CoA thiolase
AI781985	*Solyc09g061840.3*	3-ketoacyl-CoA thiolase
BG630484	*Solyc09g061840.3*	3-ketoacyl-CoA thiolase
AI781985	*Solyc07g045350.3*	Acetoacetyl-CoA thiolase
	Solyc05g017760.3	Acetoacetyl-CoA thiolase
BE353179	*Solyc09g082240.3*	Acetyltransferase (GNAT) domain protein
	Solyc09g082250.2	Acetyltransferase (GNAT) domain protein
BG629612	*Solyc05g050130.3*	Acidic endochitinase
Z15141	*Solyc02g082920.3*	acidic extracellular 26 kD chitinase
	Solyc02g082930.3	acidic extracellular 27 kD chitinase
X92855	*Solyc11g011330.2*	Alcohol dehydrogenase, putative
AJ271093	*Solyc04g079730.1*	allene oxide synthase
AY034148	*Solyc08g075540.4*	alternative oxidase 1au
BI210305	*Solyc10g055740.2*	Amino acid transporter, putative
CK574960	*Solyc09g009420.1*	AMP-dependent synthetase and ligase family protein
BT013554	*Solyc10g084560.2*	Ankyrin repeat family protein
AY656838	*Solyc01g091170.3*	arginase 2
AF332960	*Solyc06g062920.3*	auxin-regulated dual specificity cytosolic kinase
AF416289	*Solyc06g075690.3*	auxin-regulated protein AF416289
BG631274	*Solyc07g049660.3*	Benzyl alcohol O-benzoyltransferase
CN385420	*Solyc01g058720.3*	Calcium-binding EF-hand
CN385704	*Solyc03g115930.2*	Calcium-binding EF-hand family protein
BI922302	*Solyc02g094000.1*	Calcium-binding protein
AW621230	*Solyc03g119250.3*	Calmodulin binding protein-like, putative
AI898214	*Solyc04g048900.3*	Calreticulin
BE354113	*Solyc04g048900.3*	Calreticulin

BG630825	*Solyc04g048900.3*	Calreticulin
AI776170	*Solyc02g077050.3*	Cathepsin B-like cysteine proteinase
U30465	*Solyc02g082960.3*	Chitinase
	Solyc02g061770.3	chitinase 2
BG627176	*Solyc03g114890.3*	COBRA-like protein
	Solyc03g114880.2	COBRA-like protein
BG127578	*Solyc08g083110.3*	Cystathionine gamma-synthase, putative
BI936016	*Solyc08g083110.3*	Cystathionine gamma-synthase, putative
AI776392	*Solyc05g005460.3*	Cysteine/Histidine-rich C1 domain family protein
AW224087	*Solyc02g093700.3*	Cystinosin like
BT012820	*Solyc03g122350.3*	Cytochrome P450
	Solyc03g122360.3	Cytochrome P450
AI489456	*Solyc04g078290.3*	Cytochrome P450
	Solyc04g078270.3	Cytochrome P450 family protein
BM536079	*Solyc09g005120.3*	DnaJ domain-containing protein
AY359965	*Solyc07g008620.1*	EIX receptor 1
BT012812	*Solyc11g069700.2*	Elongation factor 1-alpha
U89256	*Solyc02g077370.1*	Ethylene Response Factor C.5
CN385772	*Solyc01g090300.2*	Ethylene-responsive transcription factor, putative
	Solyc01g090320.3	Ethylene-responsive transcription factor, putative
	Solyc01g090340.2	Ethylene-responsive transcription factor, putative
AJ133600	*Solyc01g006390.2*	Extensin-like protein
BG130169	*Solyc01g006400.3*	Extensin-like protein
BT013354	*Solyc03g007920.3*	Glutaminyl-peptide cyclotransferase
AY007560	*Solyc01g081310.3*	glutathione S-transferase T3
BG629373	*Solyc10g079930.1*	Glycosyltransferase
	Solyc10g079940.1	Glycosyltransferase
X85138	*Solyc01g107810.2*	Glycosyltransferase
BI205190	*Solyc09g092500.1*	Glycosyltransferase
	Solyc12g098590.2	Glycosyltransferase
BT014414	*Solyc12g042600.2*	Glycosyltransferase
BG123740	*Solyc06g036310.3*	Heavy metal transport/detoxification superfamily protein
AI780536	*Solyc07g056200.3*	Heavy metal transport/detoxification superfamily protein, putative
AW032581	*Solyc12g010410.2*	Homeobox protein knotted-1, putative
AW625293	*Solyc02g093710.1*	Hop-interacting protein THI031
AI487223	*Solyc12g010980.2*	HXXXD-type acyl-transferase family protein
M69247	*Solyc09g007020.1*	Induced by ET; pathogenesis-related protein
BT013249	*Solyc07g064600.3*	Inducible plastid-lipid associated protein
AF332960	*Solyc12g049360.2*	Kinase family protein
AW033860	*Solyc08g066320.3*	Kinase family with leucine-rich repeat domain-containing protein
	Solyc08g066270.2	Kinase family with leucine-rich repeat domain-containing protein
CN384480	*Solyc01g105070.3*	LECEVI16G peroxidase precursor
X79337	*Solyc05g007950.3*	LERNALE L.esculentum ribonuclease le

BT013501	*Solyc06g071810.1*	Leucine-rich repeat receptor-like kinase
U09026	*Solyc08g014000.3*	lipoxygenase A
BG630282	*Solyc00g009090.3*	LRR receptor-like kinase
CK716210	*Solyc05g052130.3*	Metacaspase 1
	Solyc01g105320.3	Metacaspase 1, putative
	Solyc01g105310.3	Metacaspase 2
AJ277944	*Solyc10g052470.1*	Myb family transcription factor family protein
BT014403	*Solyc10g055760.2*	NAC domain protein NAC6
AW622607	*Solyc11g013810.2*	Nitrate reductase
Y10149	*Solyc08g079860.2*	P69C protein
BI921701	*Solyc10g085010.2*	PAR1 protein
	Solyc10g086710.2	PAR1 protein
AI777697	*Solyc03g025670.3*	PAR1 protein
	Solyc03g025680.3	PAR1 protein
CN385925	*Solyc02g090490.3*	Patatin
	Solyc03g044710.3	Patatin
M69248	*Solyc09g007010.1*	Pathogenesis-related protein 1
BT013355	*Solyc01g097240.3*	Pathogenesis-related protein PR-4
CN385367	*Solyc04g071900.3*	Peroxidase
	Solyc04g071890.3	Peroxidase
AW647641	*Solyc05g046010.3*	Peroxidase
	Solyc05g046020.3	Peroxidase
	Solyc05g046030.3	Peroxidase
BE435725	*Solyc09g064940.2*	Phenazine biosynthesis PhzC/PhzF family protein
BT014164	*Solyc01g098590.3*	Phosphoglycerate mutase family protein
AI776170	*Solyc02g077040.4*	phytophthora-inhibited protease 1
BI923152	*Solyc06g034370.1*	Plant invertase/pectin methylesterase inhibitor superfamily protein
BI208311	*Solyc02g087530.1*	P-loop containing nucleoside triphosphate hydrolases superfamily protein
AW032318	*Solyc03g033840.3*	P-loop containing nucleoside triphosphate hydrolases superfamily protein
	Solyc03g033790.3	P-loop containing nucleoside triphosphate hydrolases superfamily protein
BF176599	*Solyc02g078100.3*	Pollen Ole e 1 allergen/extensin
BM956714	*Solyc01g109460.3*	Polyol monosaccharide transporter 4
M69248	*Solyc00g174340.2*	PR1
AY093595	*Solyc08g080620.1*	PR-5x
BT014226	*Solyc05g056400.3*	Protein disulfide-isomerase
	Solyc05g056385.1	Protein disulfide-isomerase
BT013507	*Solyc06g071280.3*	Protein EDS1
AY007559	*Solyc09g011630.3*	Putative glutathione S-transferase T2
	Solyc09g011640.4	Putative glutathione S-transferase T2
AW033860	*Solyc08g066310.2*	Receptor-like protein kinase
BG630902	*Solyc11g007400.1*	Receptor-like protein kinase

BI208131	*Solyc04g076010.3*	RING/FYVE/PHD zinc finger superfamily protein
BF051105	*Solyc10g008400.1*	RING/U-box superfamily protein
BI204920	*Solyc03g117860.3*	RING/U-box superfamily protein
AI897122	*Solyc03g115920.3*	RING/U-box superfamily protein
Y10403	*Solyc05g007510.3*	RNA-directed RNA polymerase
M69247	*Solyc09g007010.1*	SA pathway marker; Pathogenesis-related protein 1
BT012932	*Solyc05g014120.1*	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
BI422442	*Solyc04g040180.3*	S-adenosylmethionine-dependent methyltransferase, putative
BT013999	*Solyc12g099160.2*	Serine carboxypeptidase family protein
BI422862	*Solyc02g030300.3*	Serine/threonine-protein kinase
AW219676	*Solyc04g007750.3*	Sn-1 protein
	Solyc04g007760.3	Sn-1 protein
Y10149	*Solyc08g079870.3*	Subtilisin
	Solyc08g079840.2	Subtilisin-like endoprotease
BT013554	*Solyc10g084325.1*	Subtilisin-like protease
	Solyc10g084320.2	Subtilisin-like protease
BT013533	*Solyc06g008620.1*	tolB protein-like protein
X85138	*Solyc01g107820.2*	TOMATO WOUND-INDUCED 1/ UDP-glucosyltransferase family 1 protein
AI895800	*Solyc03g120900.2*	Transducin/WD40 repeat-like superfamily protein
BG130949	*Solyc03g120900.2*	Transducin/WD40 repeat-like superfamily protein
CN385231	*Solyc02g088130.1*	Transmembrane protein
U20592	*Solyc11g022590.1*	Trypsin inhibitor-like protein precursor
AF272366	*Solyc09g005080.1*	Verticillium wilt disease resistance 2
CN385590	*Solyc03g116890.3*	WRKY transcription factor 39
AJ831935	*Solyc03g095770.3*	WRKY transcription factor 80
BI206504	*Solyc09g015770.3*	WRKY transcription factor 81
BI923438	*Solyc08g006470.3*	Zinc finger protein
AI781951	*Solyc08g006470.3*	Zinc finger protein
CN384672	*Solyc06g075780.2*	Zinc finger, C2H2
BG630902	*Solyc11g007420.1*	Receptor-like protein kinase

Table S2| List of potential VIGS off-targets for construct *Solyc09g089680.3/Solyc12g0063080.2*. Dashes and nucleotides in bold indicate the position of mismatches between the off-target and target sequence.

Target sequence	Off-target sequence	Id%
CGTCCTAGACGAAATGTTGCGTGGGGC ACGACGTTTTCACGATCAAGATATCGA CGTTAAAAAGCCATACTATAGTCGAGA TATTGCAAGGAAAGTTATGTACAATTG CAATTTTGATCTATTTAGTGAGAAATC TCTTGCA	CGTCCTAGACGAAATGTT**A**CGTGG**AA**C ACGACGTTTTCACGA**G**CAAGATATC**A**A CGTTAAAAA**A**CCATACT**G**TAGTCGAGA T**G**TT**A**C**G**AG**A**AA**G**GT**C**ATGTACAATTG **T**AATTTTGA**G**TT**G**TTTAGTGAGAAATC T**T**TTGCA	87%
GCAGCAAATTGGAGAGACTCACTTTAC TCTGTC- ATGGCTCCTAATCCTGCTACTCCCGAG GAAATCCCCGAGA-CATGCAGGGAAAT	GC**T**GCAAATTGGAGAGACTC**CT**TTT**G**C T**T**T- **T**C**A**ATGGCTCCTAATCCT**C**C**C**AG**T**CC**A** GA**A**GAA**TT**TCC**A**- AGA**C**CATGCAGGGAAAT	81%
ATGTTGCGTGGGGCACGA- CGTTTTCACGATCAAGATATCGACGTT AAAAAGCCATACTATAGTCGAGATATT GCAAGGAAAGTTATGTACAATTGCAAT TTTGATCTATTTAGTGAG- AAATCTCTT--- GCAGCAAATTGGAGAGACTCACTTTAC TCTGTCATGGCTCCTAATCCTGCTACT CCCGAGGAAATCCC- CGAGACATGCAGGGAAA	ATGTTGC**AA**GG**AA**CACGA**AA**GTTTT- **TT**GA**G**CAAGATAT**T**GA**G**GTTAA**G**AA**T**C **AG**TA**T**TA**C**A**C**TCGAGATATT**A**C**G**A**AA**A AAGT**GG**T**T**TA**TTC**TTGCAATTTTGAT- T-T**G**TA-T- AG**CCCT**TCT**G**TT**CCA**GC**T**GCAAATTGG AGAGAC**A**CACTTT**T**CT**G**T**T**A**T**GGCT CCTAATCCT**C**CTA**G**TCC**A**GA**A**GAA**G**T**T** CC**AAC**AG-CATGCAG**C**GAAA	73%
CGAGATATTGCAAGGAAAGTTATGTAC AATTGCAATTTTGATCTATTTAGTGAG AAATCTCTTGCAGCAAATTGGAGAGAC TCACTTTACTCTGTCATGGCTCCTAAT CCTGCTACTCCCGAGGAAATCCCC	CG**T**GAT**G**TT**A**CAAGGAA**G**GT**C**A**CT**TAC AAT**A**GCAATTTTGATCTA**C**T**C**------ AAATC**G**C**CAA**CAGC**T**AATTGGAG**G**GAC **A**C**C**CTTTACT**G**TGTCATGG**A**TCCTAAT CCT**C**CT**GA**TCC**T**GA**A**GAAAT**T**CCC	76%
AATTGGAGAGACTCACTTTACTCTGTC ATGGCTCCTAATCCTGCTACTCCCGAG GAAATCCC-CGAGACATGCAGGGAAAT	AATTGGAGAGACTCA**A**TTT**T**CT**G**T**T**A ATGGCTCC**G**AATC**A**T**C**CTA**G**TCC**A**GAG GAA**T**T**G**CC**AAC**AG-CATGCAGGGAAAT	80%
GCAGCAAATTGGAGAGACTCACTTTAC TCTGTCATGGCTCCTAATCCTGCTACT CC	GC**T**GCAA**G**TTGGAGAGACTCA**A**TTT**T**C T**T**T**T**TCATGGCTCC**G**AATCCT**C**CTA**G**T CC	84%
CACGAC- GTTTTCACGATCAAGATATCGACGTTA AAAAGCCATACTATAGTCGAGATATTG	CACGAC**A**GTTTT- **T**CGA**G**CAAGATA**C**CGA**GA**T**C**AA**G**AAA**C** **A**ATA**T**TA**C**A**C**TCGAGATATTG	77%
GCAGCAAATTGGAGAGACTCACTTTAC TCTGTCATGGCTCCTAATCCT	GC**T**GCAAATTGGAGAGAC**A**CACTTTT**C** T**GT**T**T**AATGGCTCCT**G**ATCCT	85%
GATCAAGATATCGACGTTAAAAAGCCA TACTATAGTCGAGATATTGCAAGGAAA GTTATGTACA-A- TTGCAATTTTGATCTATTTAGTGAG- AAATC--TC- TTGCAGCAAATTGGAGAGACTCACTTT ACTCTGTC-ATGGCTCCTAATCCT	GA**G**CAAGATATCGA**G**GTTAA**G**AAGC**AG** TA**T**TA**C**A**C**TCGAGATAT**GA**C**G**AGG- AAGGT- **G**GT**T**CA**T**A**G**T**A**GCAATTTTGAT-T- T**G**TA-T- AG**CCCT**TC**TG**TC**ACA**GC**T**GCAAATTGG AGAGACTC**CG**TTT**TA**TT- TC**A**ATGGCTCCTAATCCT	73%

GCAAATTGGAGAGACTCACTTTA-C-TCTGTCATGGCTCCTAATCCTGCTACT	GCAAATTGGAG**G**GA-T-AC**A**TTA**G**C**T**T**G**T**C**TCATGGCTCCTAAT CCT**C**CTACT	83%
GCAGCAAATTGGAGAGACTCACTTTAC TCTG—TCATGGCTCCTAATCCTGCTACTCCCG AGGAA	GCA**A**C**T**AATTGGAG**G**GA-T-ACTTT**T**TTCTG**CA**T**T**ATGGCTCCTAAT CCT**C**CTA**G**TCC**T**GA**A**GAA	78%
AATTGGAGAGACTCACTTTA-C-TCTGTCATGGCTCCTAATCCTGCTACT	AATTGGAG**G**GA-T-A**G**TTTA**G**C**T**T**G**TGT**T**ATGGCTCCTAAT CC**AC**CTACT	80%

Table S3| List of primers used in this study. The underlined "cacc" was used as an overhang for the directional cloning of the silencing fragments into the pENTR.

Target gene	Primer sequence (5'-3')	Description
Solyc12g006380.2	caccGACATGCAGGGAAATCACAA CCTCAGCACATCCCATTTCT	VIGS silencing fragment
Solyc09g089680.3/ Solyc12g0063080.2	caccATCATGGTATCCCCGTCCCC ATTTCCCTGCATGTCTCGGG	VIGS silencing fragment
Solyc02g077370.1	caccTGGTTCCAACTCCTCAAAGTG GCACCATGTCTAGCCGAATC	VIGS silencing fragment
Solyc02g077370.1	caccTTCCTTATTTACCCCAACGAAA AACGCAGCTTCTTCAGCAGT	VIGS silencing fragment
Solyc01g107810.2	caccGAACGCAATTGTCTCCGTTT GCCCAATAGCCCATGATTTA	VIGS silencing fragment
Solyc01g107810.2	caccAAAGAGTGTCGTCCCGATTG CGTTCTGGTCAGCCTGATTT	VIGS silencing fragment
Solyc12g098590.2	caccGAGGTCGCACGTGAAGTCAA AGTGCAACTCTAAGGCTTCCT	VIGS silencing fragment
Solyc03g117860.3	caccAATTGGCAGAGGAATTGCAG TCATCAGGATCATGGCTCAG	VIGS silencing fragment
Solyc10g055740.2	caccGTCGATCGTATTGAGCGATG CCAACAGCGGAAGAAAAGAG	VIGS silencing fragment
Solyc03g095770.3	caccCCTTCTTCTCCCTCTGCTCA CCAAGAACATAGCCGAAGGT	VIGS silencing fragment
Solyc09g015770.3	caccTCATTGAAATCCCCAATAAAGC ACGCGCACTAGTCGAATCTT	VIGS silencing fragment
Solyc09g089680.2	CCACAGCCAGATTTAACCAT	Spacer 1 (Chr9)
Solyc09g089680.2	ATAACTAAAGCTCCAGGTGT	Spacer 2 (Chr9)
Solyc09g089680.2	TTGCCATCTTCCAAACTCTA	Spacer 3 (Chr9)
Solyc09g089680.2	GTATCGTGCAACGACACTGA	Spacer 4 (Chr9)
Solyc12g006380.1	ATGGGATGTGCTGAGGGACT	Spacer 1 (Chr12)
Solyc12g006380.1	ACTAAAGCTCCATGAGTAGG	Spacer 2 (Chr12)
Solyc12g006380.1	GAGCGTAGTTCACAGAGTAT	Spacer 3 (Chr12)
Solyc12g006380.1	TCTAATAAAAAAGGACTTGA	Spacer 4 (Chr12)
Solyc12g006380.1 (doxy_1/2)	GTCGAGATATTGCAAGGAAAGTT GGGACCATGGGATATTTTCTT	Genotyping
Solyc09g089680.3 (doxy_1)	GGATGCACTCTGCTTGAGTT ACATAACTAGAATGTGAACCAAACGA	Genotyping
Solyc09g089680.3 (doxy_2)	TTAAGTTGGTTGACCCTCGT TTTTACACCCTCCTTATCACTTGTC	Genotyping

Table S4|. List of potential CRISPR/Cas9 off-targets up to 3 mismatches. Asterisks indicate the position of mismatches between off-targets and the 20 nt spacer sequences.

Spacer	Off-target sequence	Mismatch position	Off-target
2 (Chr12)	ACTAAAGCTCCAGGTGTAGGAGG	...........*.*.....	exon:mRNA:Solyc09g089680.3.1
2 (Chr12)	ACTAAAGCTAAATGAATAGGAGG	.........**....*....	intergenic:mRNA:Solyc10g047080.1.1-mRNA:Solyc10g047083.1.1
2 (Chr12)	TCTAGAGGTCCATGAGTAGGAGG	*...*..*.....	intergenic:mRNA:Solyc08g065190.3.1-mRNA:Solyc08g065197.1.1
3 (Chr12)	TAGTGTAGTTCACAGAGTCTGGG	*..*.....*.	intron:mRNA:Solyc07g009140.3.1
4 (Chr12)	TCTAATAAAAAAGGAGTAGAGGG	.....*.*..	intergenic:mRNA:Solyc12g035360.2.1-mRNA:Solyc12g035362.1.1
4 (Chr12)	TGTAATAAAAAAGTACTTGATGG	.*.....*.....	intergenic:mRNA:Solyc10g049992.1.1-mRNA:Solyc10g049996.1.1
4 (Chr12)	TCAAATGAAAAAGGAATTGAAGG	..*...*.....*....	intergenic:mRNA:Solyc01g014360.1.1-mRNA:Solyc01g014370.1.1
4 (Chr12)	TCTACTATATAAGGACTTGAAGG	....*..*.*.....	intergenic:mRNA:Solyc07g018090.3.1-mRNA:Solyc07g018130.2.1
4 (Chr12)	AGTAATAAAAGAGGACTTGAAAG	**.....*.....	intergenic:mRNA:Solyc07g051890.1.1-mRNA:Solyc07g051920.1.1
4 (Chr12)	TCTAATATAATAGGACTAGAGGG	.....*..*.....*..	intergenic:mRNA:Solyc09g074760.1.1-mRNA:Solyc09g074765.1.1
4 (Chr12)	TGTAATTAAAAAGTACTTGATGG	.*....*.....*.....	intergenic:mRNA:Solyc08g015810.2.1-mRNA:Solyc08g015820.1.1
4 (Chr12)	TCTAATTAACAAGTACTTGAAGG	.....*..*...*.....	intergenic:mRNA:Solyc06g033780.1.1-mRNA:Solyc06g033784.1.1
4 (Chr12)	TCCAAAAGAAAAGGACTTGACGA	..*..*.*.....	intergenic:mRNA:Solyc06g043210.1.1-mRNA:Solyc06g043170.3.1/mRNA:Solyc06g043175.1.1

2 (Chr9)	ATAACTAAAGCACCAGGAATGGG	..........*.....**.	exon:mRNA:Solyc11g045520.2.1
2 (Chr9)	AAAGATAAAGCTCCAGGTGTGGA	.*.**..............	intergenic:mRNA:Solyc07g038170.1.1-mRNA:Solyc07g038190.3.1
2 (Chr9)	ACAACTAAAGCTCCATGAGTAGG	.*.............*.*..	exon:mRNA:Solyc12g006380.2.1
3 (Chr9)	TTGCCATTTTACAAACTCTAGGA	.......*..*.........	intergenic:mRNA:Solyc07g021670.2.1-mRNA:Solyc07g021700.3.1
3 (Chr9)	ATGCCATCTTCCAAGCTTTATGG	*.............*..*..	exon:mRNA:Solyc09g089750.1.1
3 (Chr9)	GTGCCATCTTCCAATCTTTATGG	*.............*..*..	exon:mRNA:Solyc09g089720.2.1
3 (Chr9)	TTGGCATCTTACAAACTCAAGGG	...*......*.......*.	intergenic:mRNA:Solyc09g059910.2.1-mRNA:Solyc09g059914.1.1
3 (Chr9)	TTGGCATCTTACAAACTCAAAGG	...*......*.......*.	intergenic:mRNA:Solyc08g059710.3.1-mRNA:Solyc08g059730.1.1
3 (Chr9)	TTACCATCTTCCAAGCTGTATGG	..*...........*..*..	exon:mRNA:Solyc09g089710.3.1
4 (Chr9)	GTATCGTGCAACCACAGTGAAGG	............*...*...	exon:mRNA:Solyc09g089690.3.1
4 (Chr9)	GTGTCATGCAACGACAATGATGG	..*..*.........*...	intergenic:mRNA:Solyc06g016680.1.1-mRNA:Solyc06g016690.1.1
4 (Chr9)	ATATCGTGCAACCACAGTGACGG	*...........*...*...	exon:mRNA:Solyc09g089730.3.1

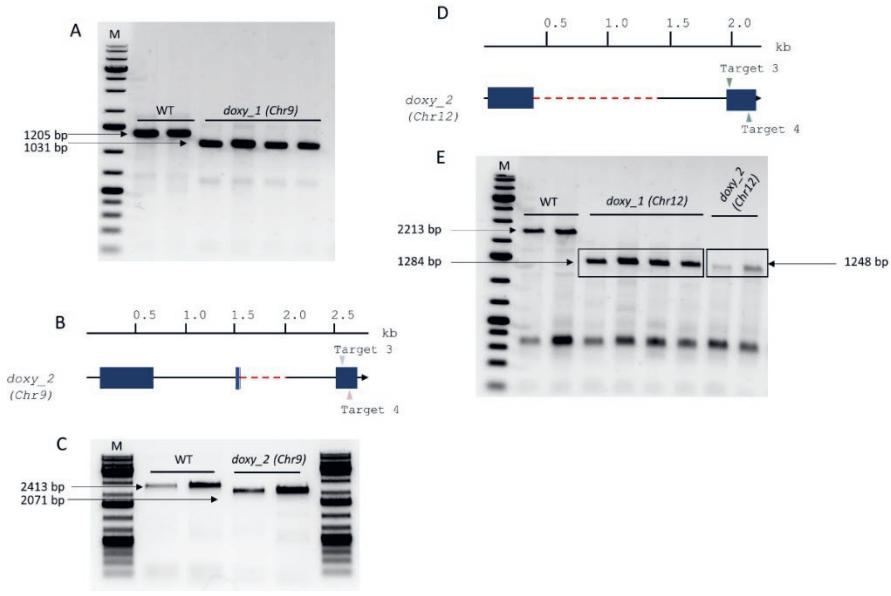

Figure S1| Overview of double mutants. A),C),E) Visualization of amplification products of primary tomato transformants on 1% agarose. Numbers on the left of bands indicate their size. M; DNA marker, WT; WildType background. B), D) Schematic representation of the deletions detected in *doxy_2 (Chr9)* and *doxy_2 (Chr12)*.

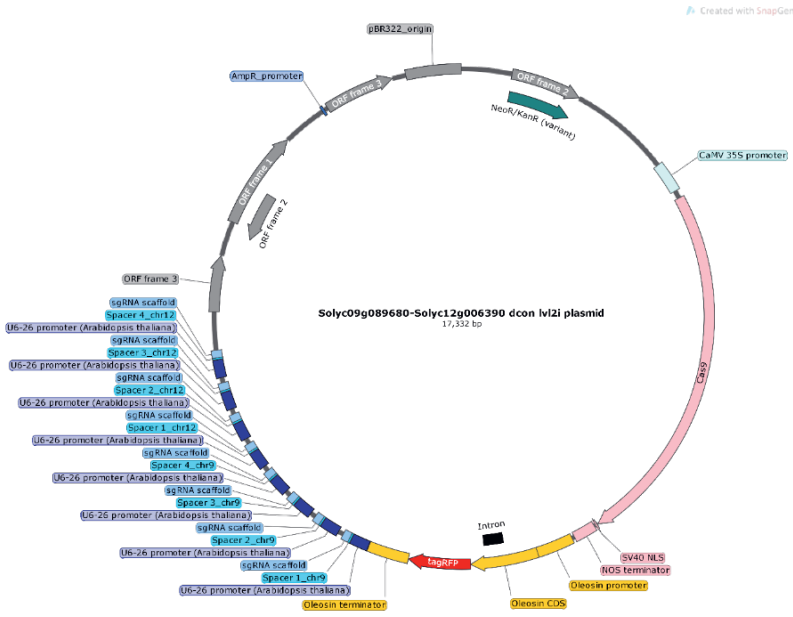

Figure S2| Level 2i CRISPR/Cas9 vector for the generation of the double knock-out mutants of genes *Solyc09g089680.3/Solyc12g0063080.2*.

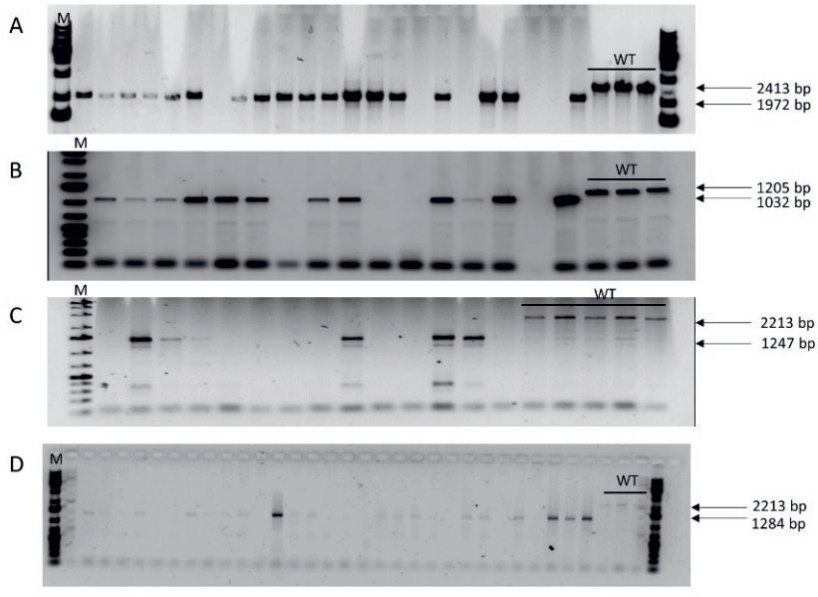

Figure S3| Visualization of amplification products of progeny of the primary tomato transformants on 1% agarose. Numbers on the right of bands indicate their size. M; DNA marker, WT; WildType background. Presence of mutant alleles present in A) progeny of *doxy_2 Chr9*, B) progeny of *doxy_1 Chr9*, C) progeny of *doxy_2 Chr12*, D) progeny of *doxy_1 Chr12*.

CHAPTER 6

Identification of two novel loci underlying tolerance originating from *Solanum arcanum* LA2157

Eleni Koseoglou, Derek Mudadirwa, Matthijs Brouwer, Jan M. van der Wolf, Richard G.F. Visser, Yuling Bai

ABSTRACT

Clavibacter michiganensis (*Cm*) is a Gram-positive phytopathogenic bacterium of tomato. Outbreaks of *Cm* can result in severe yield and economic losses, as to date control of the pathogen is limited to the "good seed and plant practice" (GSPP) protocol. Screening of wild material has resulted in the identification of several sources of tolerance to *Cm*. The genetic background of tolerance provided by these sources is polygenic and complex. Based on previous results from an advanced line of a cross between *Solanum arcanum* LA2157 and *S. lycopersicum* it was concluded that a single introgression on chromosome 7 of tomato is enough to confer high levels of tolerance to *Cm*. We set out to further functionally characterize this locus in an effort to identify the gene(s) underlying the observed tolerance. Testing of near isogenic lines (NILs) containing a fixed introgression on chromosome 7 did not lead to the expected results, as segregation in *Cm* tolerance was observed in NILs homozygous for the *S. arcanum* LA2157 allele. Therefore, we employed whole genome sequencing in combination with a bulk segregant analysis to identify loci involved in the observed tolerant phenotype. Our results suggest that two additional loci on chromosomes 2 and 4 together with the QTL on chromosome 7 are needed for tolerance to *Cm*.

INTRODUCTION

Resistance and tolerance represent the two major mechanisms of plant defences to pathogens [1]. Even though both resistance and tolerance aim at the survival and reproduction of the host, the two mechanisms act in distinct ways [2]. Resistance limits the multiplication of the pathogen in an infected plant, while tolerance reduces the effects of infection regardless of the pathogen population size in an infected plant [1, 2].

The gram-positive phytopathogenic bacterium *Clavibacter michiganensis* (*Cm*) is responsible for bacterial canker of tomato (*Solanum lycopersicum*), one of the most destructive diseases of cultivated tomato [3, 4]. The spread of the pathogen over long distances is primarily facilitated by contaminated seeds, while cultural practices can lead to a rapid spread of the pathogen in infected crops, resulting in severe disease outbreaks [5-8].

Cm colonizes the vasculature of tomato plants, leading to systemic infections. Severity of the disease depends on several factors, including the route of infection, environmental conditions, the tomato genotype, the developmental stage of the plant at the time of infection, and the virulence of the infecting strain [8-11]. The most commonly observed symptoms of the disease are wilting of leaves and leaflets, the development of cankers on the stems and petioles of infected plants, as well as discoloration and necrosis of the xylem [4, 12]. Localized infections of tomato fruits can lead to the development of necrotic spots, known as bird's eye spots, while local infections of aerial parts can result in marginal leaf necrosis, and white blister-like spots on the stems and leaves of plants [13, 14]. Control of the pathogen is currently limited to the "good seed and plant practice" (GSPP) protocol, which aims at decreasing the risks of introduction and spread of the pathogen [15]. Chemical and biological agents for the control of *Cm* do not provide satisfactory levels of protection, while resistance to the pathogen has yet to be identified.

Early studies claimed several sources of resistance to the pathogen. Nevertheless, pathogen populations in these early studies were not assessed [16-22]. Recent research that included the quantification of bacterial populations in wild accessions has only reported sources of tolerance [11, 23, 24]. Based on these recent results we cannot definitely conclude that the reported resistance was indeed resistance. For the purposes of this chapter we will refer to resistance and tolerance based on the terminology used in the original papers.

In most cases, the reported resistance/tolerance conferred by wild accessions was found to be polygenic and complex (Table 1). An (unreported) accession of *Solanum pimpinellifolium*, which was used for the development of line "Bulgaria 12" (or PI 330727), was the first wild species reported to be resistant to *Cm* [16]. Interspecific crosses between *S. pimpinellifolium* accessions (A129 and A134) with *S. lycopersicum* suggested that several genes (between four and eleven) with additive effects are involved in the resistance observed in *S. pimpinellifolium*. However, no map positions of these genes were reported [16]. *S. habrochaites* LA407 is one of the most well described sources conferring resistance to *Cm*. Initial genetic studies in crosses of *S. habrochaites* LA407 with *S. lycopersicum* resulted in one to three genetic loci [18]. Further studies of the crosses could map two QTLs on chromosomes 2 and 5 with an epistatic effect [17, 19].

The polygenic nature of resistance to *Cm* was further demonstrated between inter- and intraspecific crosses of *S. arcanum* and *S. lycopersicum* [11, 21, 22]. The interspecific cross between *S. arcanum* LA2157 and *S. lycopersicum* cv. Solentos yielded three QTLs located on tomato chromosomes 5, 7 and 9 related to resistance. The three QTLs were found to be additive, with the QTL on chromosome 7 having the biggest contribution to resistance [21]. Further fine-mapping of backcrosses between *S. arcanum* LA2157 and *S. lycopersicum* cv.MM, with a higher number of genetic markers per chromosome, reduced the size of the previously identified QTLs on chromosomes 5 and 7. In addition, two novel QTLs on tomato chromosomes 6 and 11 were identified [23]. Subsequent fine-mapping of the QTL on chromosome 7 concluded that a single ~211 kb introgression on chromosome 7 is enough to confer high tolerance to *Cm*. Based on the tomato reference genome 15 genes were reported to be present on the ~211 kb introgression [24]. Finally, an intraspecific backcross population between the resistant *S. arcanum* LA2157 and susceptible *S. arcanum* LA2172 resulted in the identification of five QTLs on chromosomes 1,6,7,8, and 10 [22]. In parallel to the fine-mapping of the backcrosses between *S. arcanum* LA2157 and *S. lycopersicum* cv.Moneymaker (cv.MM), bacterial enumeration in these crosses concluded that the bacterial titres were not different from the susceptible parent cv.MM [23]. Therefore, the observed lack of symptoms was due to tolerance, rather than the previously reported resistance.

In contrast to most studies reporting multiple loci involved in resistance/tolerance to *Cm*, a dominant locus derived from *S. arcanum* var. "humifusum" linked to resistance has been reported on chromosome 4. Even though it was suggested that

a single dominant gene was responsible for the observed resistance, the authors concluded that the resistance level was dependent on the presence of other modifier genes [25]. Therefore, in our view, this source should also be considered as polygenic.

In an effort to identify novel sources of tomato resistance to *Cm*, 24 wild species were screened by our laboratory (Plant Breeding, WUR). The screen led to the report of three previously undescribed highly tolerant accessions, namely *S. pimpinellifolium* G1.1554, *S. neorickii* LA735 and *S. neorickii* LA2072 [11]. Further mapping studies of recombinant inbred lines (RIL) derived from crosses between *S. pimpinellifolium* GI.1554 and cv.MM, resulted in the identification of five QTLs on tomato chromosomes 1,2,7,8 and 12 associated with tolerance to *Cm*. The QTL on chromosome 7 was found to have a major contribution to the observed tolerance [26].

Table 1| Overview of loci associated with tolerance to *Cm* derived from wild accessions.

Tolerance source	Susceptible parent	Population	Tolerance/ Resistance type	Reference
S. habrochaites LA407	*S. lycopersicum* cv. Ohio 86120	F2	QTLs Rcm 2.0 (Chr2) and Rcm 5.1 (Chr5)	[14]
S. arcanum LA2157	*S. lycopersicum* cv. Solentos	F2	QTLs on Chr5, Chr7 and Chr9	[21]
S. arcanum LA2157	*S. lycopersicum* cv. MM	Recombinant inbred lines (RILs)	QTLs on Chr5, Chr7, Chr6 and, Chr11	[23]
S. arcanum LA2157	*S. lycopersicum* cv. MM	Recombinant inbred lines (RILs)	~211 kb introgression on Chr7	[24]
S. pimpinellifolium GI. 1554	*S. lycopersicum* cv. MM	RILs	QTLs on chromosomes 1, 2, 7, 8, and 12	[26]
S. arcanum LA2157	*S.arcanum* LA2172	Backcross (BC) of intraspecific cross	QTLs on chromosomes 1, 6, 7, 8, and 10	[22]
S. arcanum var. "humifusum"	*S. lycopersicum x S. chilense LA460*	F_2BC population of three genome hybrid *S. lycopersicum* line Cm 180	Dominant gene on Chr4 (with modifier genes)	[25]

In this study, we decided to functionally characterize the 15 genes previously reported to be present on the ~211 kb introgression in the *S. arcanum* LA2157 and cv.MM crosses, with the intention of identifying the gene(s) underlying the observed

tolerance [24]. In our study, we used a BC_3S_6 line and its selfing with a fixed introgression on chromosome 7. Surprisingly, during our disease assays we could not confirm the results previously reported. Therefore, we employed marker analysis, as well as whole genome sequencing in combination with bulk segregant analysis (BSA) to identify loci involved in the observed tolerant phenotypes.

RESULTS

Phenotypic and genotypic evaluation of lines PV175536 and PV185517

In an effort to confirm previous results, we used one BC_3S_6 line (PV175536) and its selfing (PV185517) derived from an initial cross between *S. arcanum* LA2157 and *S. lycopersicum* cv. Solentos, which carry the QTL on chromosome 7 homozygously in the susceptible cv. MM background. When previously tested, plants homozygously containing the introgression on chromosome 7 were found to be highly tolerant to *Cm* [24].

To validate that the expected introgression from *S. arcanum* LA2157 on chromosome 7 was present in the plants, we developed cleaved amplified polymorphic sequence (CAPS) markers (Q7M1 to Q7M9, Supplementary Table 1) flanking the previously reported region in the line. Marker analysis confirmed that the expected 697 kb introgression (physical position; SOL07-1060331 to SOL07-1784948) from *S. arcanum* LA2157 was present homozygously in the two introgression lines (Table 2).

Table 2| CAPS markers analysis of the QTL on chromosome 7 in line PV175136**.** In the table a number of plants with segregating phenotypes is given. A= homozygous for *Solanum arcanum* LA2157 allele, M=homozygous for *S. lycopersicum* cv.MM, S= susceptible, T= tolerant.

PV175136 line	Q7M1	Q7M7	Q7M2	Q7M3	Q7M4	Q7M5	Q7M9	Disease index/ Phenotype
PV175136_5	M	A	A	A	A	M	M	4 / S
PV175136_13	M	A	A	A	A	M	M	5 / S
PV175136_20	M	A	A	A	A	M	M	2.5/ S
PV175136_29	M	A	A	A	A	M	M	0/ T
PV175136_4	M	A	A	A	A	M	M	0.25/ T
PV175136_27	M	A	A	A	A	M	M	0.25/ T

In our first experiment, the PV175136 line was inoculated with *Cm* and symptom development was monitored. On average a significant reduction of wilting symptoms was observed in line PV175536 compared to the susceptible control cv.MM at 20 days post inoculation (dpi). While on average the plants were

significantly more tolerant than cv.MM plants, we also recorded two plants that were highly susceptible (DI ≥ 2.5) in the PV175136 line (Figure 1).

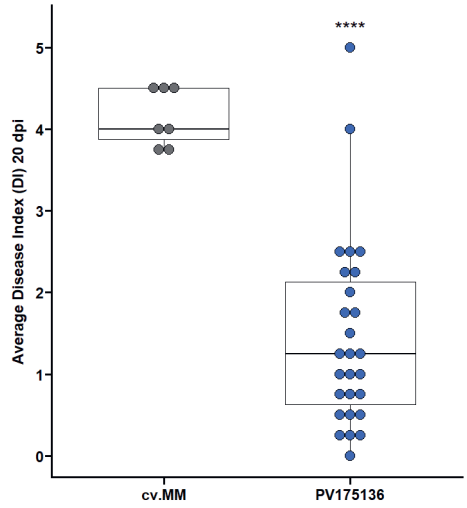

Figure 1| Phenotypic evaluation of line PV175136. Wilting symptoms on the introgression line PV175536 and the susceptible background cv.MM at 20 days after inoculation with *Clavibacter michiganensis* NCPBB382. Centre lines indicate medians, the box limits indicate the 25th and 75th percentiles. (Student's t-test, ****p≤0.00).

To confirm these results, we decided to repeat the bioassays on a line derived from the selfing of PV175536-8. Prior to the infection of the plants, we confirmed that the PV185517 line carried the introgression on chromosome 7 homozygously. Two independent experiments were done with line PV185517. Our results were in accordance with what we previously observed for line PV175536. On average the symptom development of line PV185517 was significantly lower than the susceptible cv.MM at 20 dpi (Fig. 2 A, B). Nevertheless, we observed highly tolerant (0 ≤ DI < 2.5) and highly susceptible (2.5 ≤ DI ≤ 5) plants in the PV185517 family (Fig. 2 C-D). The plant phenotypes of the line, however, did not co-segregate with the QTL on chromosome 7 (Table 3).

Table 3| CAPS markers analysis of the QTL on chromosome 7 in line PV185517**.** In the table a number of plants with segregating phenotypes is given. A= homozygous for *Solanum arcanum* LA2157 allele, M=homozygous for *S. lycopersicum* cv.MM, S= susceptible, T= tolerant.

PV185517 line	Q7M1	Q7M7	Q7M2	Q7M3	Q7M4	Q7M5	Q7M9	Disease index/ Phenotype
PV185517_14	M	A	A	A	A	M	M	5/ S
PV185517_18	M	A	A	A	A	M	M	3.75/ S
PV185517_31	M	A	A	A	A	M	M	5/ S
PV185517_37	M	A	A	A	A	M	M	0/ T
PV185517_39	M	A	A	A	A	M	M	0/ T
PV185517_40	M	A	A	A	A	M	M	0/ T

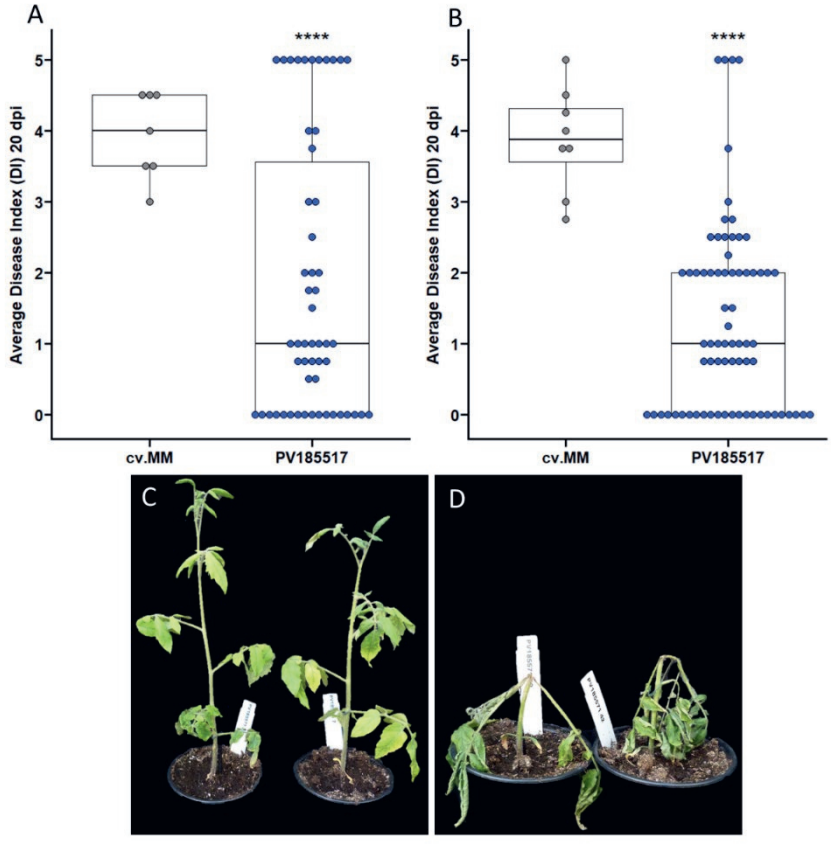

Figure 2| Phenotypic evaluation of line PV185517. A), B) Wilting symptoms on introgression line PV185517 and the susceptible background cv. Moneymaker (cv.MM) at 20 days post inoculation (dpi) inoculated with *Clavibacter michiganensis* NCPBB382. A) and B) represent two independent experiments.

Phenotype of C) tolerant plants and D) susceptible plants in the PV185517 family. Centre lines indicate medians, the box limits indicate the 25th and 75th percentiles. (Student's t-test, ****p≤0.00).

Additional QTLs on chromosomes 5 and 9 do not contribute to the observed tolerance

Based on our results that the observed tolerance was not co-segregating with the QTL on chromosome 7, we speculated that another previously reported QTL from the initial cross, on either chromosome 5 or onchromosome 9 was still segregating in the tested line [23]. To investigate this possibility, markers were run along the previously described genomic regions on chromosomes 5 (physical position; 39792518..61792631) and 9 (physical position; 52411..4698709). Eight markers were run along each region on chromosomes 5 and 9. Only one single nucleotide polymorphism (SNP) (52533C>G) was identified between cv.MM and line PV185517. However, no segregation of the SNP was found between the plants of line PV185517, suggesting that this SNP was not responsible for the observed phenotypic segregation in the line.

Tolerance to *Cm* requires QTL7 in combination with two additional loci on chromosomes 2 and 4

To identify sequence variants linked to *Cm* tolerance in the PV185517 family, we combined whole genome sequencing (WGS) with bulk segregant analysis (BSA). We selected 14 fully resistant and 14 susceptible plants of the line PV185517 from the two independent experiments to compose the resistant bulk (R-bulk) and the susceptible bulk (S-bulk), respectively. Two peaks with different k-mer frequencies were observed for the R-bulk. The highest peak was observed between positions 43262484.. 48143527 (6.98 Mb) on tomato chromosome 2. We also observed a peak between positions 63165755..63767930 (602 kb) on chromosome 4 (Fig. 3). Several lower k-mer peaks were observed on other genomic regions (Fig. 3). These lower k-mer peaks could be mapped due to lack of coverage in the S bulk, a hypothesis that still requires validation. On the mapped loci on chromosomes 2 and 4 only two genes were found to be associated with tolerance. These two genes contained k-mers that were specific to the R-bulk (Table S2). These were genes *Solyc02g084740.4* (*DWARF/CYP85A1*) and *Solyc04g081190.3* (*vsf-1*) on tomato chromosomes 2 and 4, respectively. Allele frequencies in the R-bulk for genes *Solyc02g084740.4 and Solyc04g081190.3* were estimated to be 0.57 and 0.6, respectively. Further inspection of the sequencing data revealed that both of the sequences of the genes mapped on the R-bulk were identical to *the S. arcanum* LA2157 allele. Several S-bulk

specific k-mers were also linked to genes specifically present in the S-bulk (Table S3, Fig. S1). Further analysis of the sequencing data revealed that the S-bulk specific k-mers were identical to the susceptible cv.MM sequences.

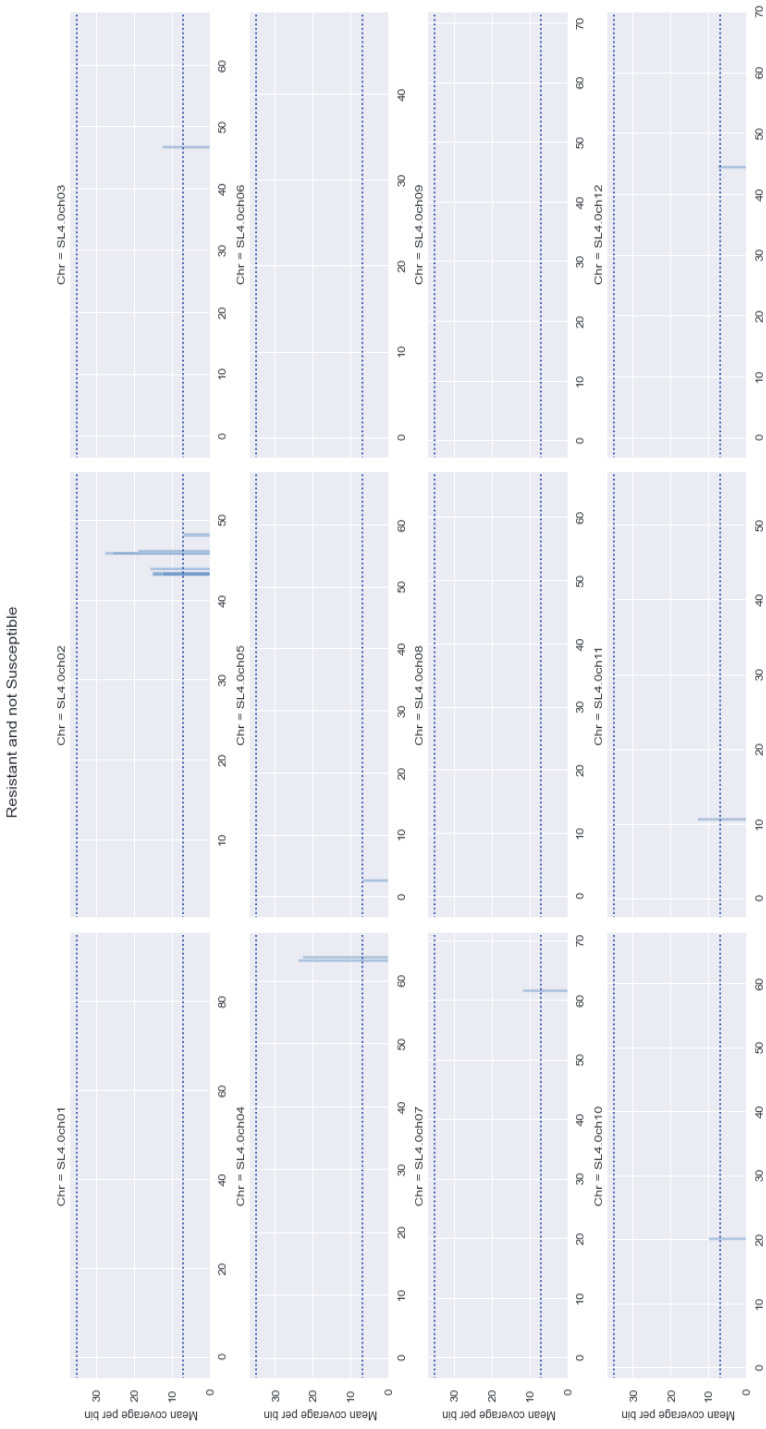

Figure 3 | Loci linked to tolerance to Cm mapped on chromosomes 2 and 4 of tomato. Density graphs of unique k-mers mapped in 1 Mb bins on the tomato (*S. lycopersicum*) reference genome ITAG 4.0. Blue vertical lines indicate loci associated with tolerance.

Changes in the amino acid sequence of the *S. arcanum* LA2157 allele

To detect for potential protein interactions between our new mapped genes on chromosomes 2 and 4 and the 15 genes mapped on QTL on chromosome 7, we searched the STRING database for functional protein association networks. No interactions were detected on the database. The database, however, only allowed for the use of the sequences of the *S. lycopersicum* reference genome. We therefore decided to predict the protein sequences produced by the *S. arcanum* LA2157 alleles of the two candidate genes. Due to gaps in the sequencing the prediction of the protein of the *S. arcanum* LA2157 *Solyc02g084740.4* allelic variant was not possible. Nevertheless, we could predict the *S. arcanum* LA2157 produced protein for gene *Solyc04g081190.3.* After alignment of the predicted *S. arcanum* and LA2157 and the *S. lycopersicum* protein sequences we detected 8 amino acid changes (Fig. 4).

```
                                    20                  40                  60
S.lycopersicum_vsf-1 MAQSNSKPPM SSQNFGVGAV SHVRSLSQSS IFSNSCLPPL SPFPPSEPGM VSGHSSLKDI  60
S.arcanumLA2157_vsf-1 MAQSNSKPPM SSQNFGVGAV SHVRSLSQSS IFSNSCLPPL SPFPPSEPGM VSGHSILKDI  60
          Consensus MAQSNSKPPM SSQNFGVGAV SHVRSLSQSS IFSNSCLPPL SPFPPSEPGM VSGHSXLKDI
                                    80                  100                 120
S.lycopersicum_vsf-1 SMEENDVNSQ GVGVVSSFTR DGLPPRKGHR RSNSDVPLGF SAMIQSSPQL MPISGQKVLG 120
S.arcanumLA2157_vsf-1 SMEEVDVNSQ GVGVVSSFTR DGLPPRKGHR RSNSDVPLGF SAMIQSSPQL MPISGQKVLG 120
          Consensus SMEEXDVNSQ GVGVVSSFTR DGLPPRKGHR RSNSDVPLGF SAMIQSSPQL MPISGQKVLG
                                    140                 160                 180
S.lycopersicum_vsf-1 RAVSLGDSNG KIDERKPKGE VTDELLFSYM NLENIETLNG SGTKDRDKDS IVSGTKVTGS 180
S.arcanumLA2157_vsf-1 RAVSLGDSNG KIDERKSKGE VTDELLFSYM NLENIETLNG SGTEDRDKDS IVSGTKVSGS 180
          Consensus RAVSLGDSNG KIDERKXKGE VTDELLFSYM NLENIETLNG SGTXDRDKDS IVSGTKVXGS
                                    200                 220                 240
S.lycopersicum_vsf-1 ESSKNEAESV MKGNNVSIQP INLREGTKRS ADANIAPAAR HFRSLSMDSA IGNFHYGDES 240
S.arcanumLA2157_vsf-1 ESSKNEAESV MKGNTVSIQP INLREGTKRS ADANIAPAAR HFRSLSMDSA IGNFHYGDES 240
          Consensus ESSKNEAESV MKGNXVSIQP INLREGTKRS ADANIAPAAR HFRSLSMDSA IGNFHYGDES
                                    260                 280                 300
S.lycopersicum_vsf-1 PNLPTSLMMR SGQLSPSNSG NESSSKHNLD FGNSEFSEAE MKKIMADERL AEIAVLDPKR 300
S.arcanumLA2157_vsf-1 PNVPTSLMMR SGQLSPSNSG NESSSKHNLD FGNSEFSEAE MKKIMADERL AEIAVLDPKR 300
          Consensus PNXPTSLMMR SGQLSPSNSG NESSSKHNLD FGNSEFSEAE MKKIMADERL AEIAVLDPKR
                                    320                 340                 360
S.lycopersicum_vsf-1 AKRILANRLS AARSKERKTR YISELEHKVQ KLQTETTTLS TQVTILQKNF VEISSLNSEL 360
S.arcanumLA2157_vsf-1 AKRILANRLS AARSKERKTR YISELEHKVQ KLQTETTTLS TQVTILQKNF VEISSLNSEL 360
          Consensus AKRILANRLS AARSKERKTR YISELEHKVQ KLQTETTTLS TQVTILQKNF VEISSLNSEL
                                    380                 400                 420
S.lycopersicum_vsf-1 KFRIQAMEQQ AQLRDALHEA LTAEVQRLKL AAGEHREEGR LPNNMTQQTP VKHNTFQMQR 420
S.arcanumLA2157_vsf-1 KFRIQAMEQQ AQLRDALHEA LTAEVQRLKL AAGEHREEGR LPNNMTQQTP VKHNMFQMQR 420
          Consensus KFRIQAMEQQ AQLRDALHEA LTAEVQRLKL AAGEHREEGR LPNNMTQQTP VKHNXFQMQR
                                    440
S.lycopersicum_vsf-1 QQPSQMQQLS VGKASAASAT PASA  444
S.arcanumLA2157_vsf-1 QQPSQMQQLS VGKASAASAT PASA  444
          Consensus QQPSQMQQLS VGKASAASAT PASA
```

Figure 4| Alignment of the *S. lycopersicum* vsf-1 protein against the predicted protein of the *S. arcanum* LA2157 allelic variant. Amino acids in red indicate changes between the two proteins.

Of the eight amino acid changes we predicted in the *S. arcanum* LA2157 protein, three were predicted to result in changes in the amino acids charge. The p.S56I change results in a polar to non-polar amino acid substitution. The p.P137S

substitution results in a non-polar to polar amino acid change, while the p.K164E substitution results in a change from a basic to an acidic amino acid.

DISCUSSION

Wild *Solanum* species harbour genetic diversity that can be used as a valuable source of disease resistance. Screenings of wild tomato accessions have resulted in the identification of several sources of resistance/tolerance to *Cm* [11, 19, 21, 26]. Loci or markers closely linked to resistance/tolerance to *Cm* have been mapped on most of the tomato chromosomes (Fig. 5). Colocalization of QTLs on chromosomes 7 and 9 between studies have been reported for the QTLs mapped from crosses between *S. arcanum LA2157* and *S. lycopersicum* [20, 23, 24]. Of the mapped QTLs, introgressions derived from *S. arcanum* LA2157 and *S. pimpinellifolium* GI. 1554 on chromosome 7 of tomato have been reported to have a major effect in tolerance [21, 23].

In our lab, efforts to identify the genes underlying the tolerance observed in crosses between *S. arcanum* LA2157 and *S. lycopersicum*, resulted in a list of 15 genes in a ~211 kb introgression on the major QTL on chromosome 7. Data also suggested that the introgression on chromosome 7 alone was enough to confer high tolerance to *Cm* [24].

In this study, we set out to functionally characterize these 15 genes in the described region on chromosome 7. As a first step, we decided to confirm that the QTL on chromosome 7 alone is enough to confer high tolerance to *Cm*, as previously reported. During our diseases assays, we indeed recorded a significant reduction in wilting symptoms on the introgression lines homozygous for the *S. arcanum* LA2157 alleles of the QTL on chromosome 7. Nonetheless, we repeatedly observed phenotypic segregation between the plants in the line, with plants being highly tolerant or highly susceptible to the pathogen (Fig. 1, Fig. 2).

Previously, it was reported that the combination of the QTL on chromosome 7 with either of the QTLs on chromosomes 5 or 9 leads to high levels of tolerance [20]. After marker analysis on the previously reported regions on chromosomes 5, 7 and 9 of the line we could not detect co-segregation of the QTLs with the observed segregating phenotypes (Table 1, Table 2, Fig. 1, Fig. 2).

Based on our results, we hypothesized that together with the QTL on chromosome 7 additional loci might be responsible for the observed tolerance. To identify any potential underlying loci we combined whole genome sequencing with a bulk

segregant analysis. Using this approach, we were able to link two loci on tomato chromosomes 2 (6.98 Mb) and 4 (602 kb) with the observed tolerance (Fig. 3). Two genes with distinguishing k-mers on the loci on chromosomes 2 and 4 were mapped. Those were genes *Solyc02g084740.4* and *Solyc04g081190.3*, coding for DWARF/CYP85A1 and transcription factor VASCULAR SPECIFIC FACTOR-1 (vsf-1), respectively. Other loci with lower peaks were also observed. These peaks might represent minor effect loci or be present due to lack of coverage on the S-bulk, a hypothesis that needs to be validated. Interestingly, a single dominant gene originating from *S. arcanum* var. "humifusum" was also reported on tomato chromosome 4 [25]. The position of the gene, however, was not mapped and therefore we cannot conclude if it co-localizes with our mapped locus on chromosome 4.

It has long been speculated that morphological differences in the vascular system of wild tomato accessions might be responsible for the described tolerance to *Cm* [11, 19, 28]. Interestingly, both genes we mapped on chromosomes 2 and 4 could be related to vascular morphology. Gene *Solyc02g084740.4* (*DWARF/CYP85A1*), belongs to the cytochrome P450 family. The gene was identified in tomato through transposon mutagenesis, and it was found that its mutation causes severe dwarfism in tomato plants [29]. Later on, it was reported that the DWARF/CYP85A1 protein encodes a C-6 oxidase involved in brassinosteroid (BR) biosynthesis [30]. BRs have been found to be important in several developmental processes of plants, including cell elongation, cell diving and vascular differentiation [31]. Loss-of-function *CYP85A* homologs in different species, including Arabidopsis, rice, cucumber and barley results in dwarf phenotypes and aberrant development of vascular tissues [32-36].It is interesting to mention that the dwarf tomato cv. Micro-Tom (MT) carries a mutant *DWARF* gene that results in the production of a truncated protein [37]. An ethyl methanesulfonate (EMS) mutant cv. MT population has been developed in our laboratory for the discovery of tomato mutants resistant to different diseases [38, 39]. As a side project, in an effort to identify tomato mutants resistant to *Cm* we decided to test this mutant population. As a first step for the experiment we infected wildtype (WT) cv.MT plants with *Cm*. Four weeks after infection, however, no symptoms were observable on the WT plants (data not shown). At that moment, we attributed the lack of observable symptoms to the changed morphology of the cv.MT leaves or the fast life cycle of the cultivar.

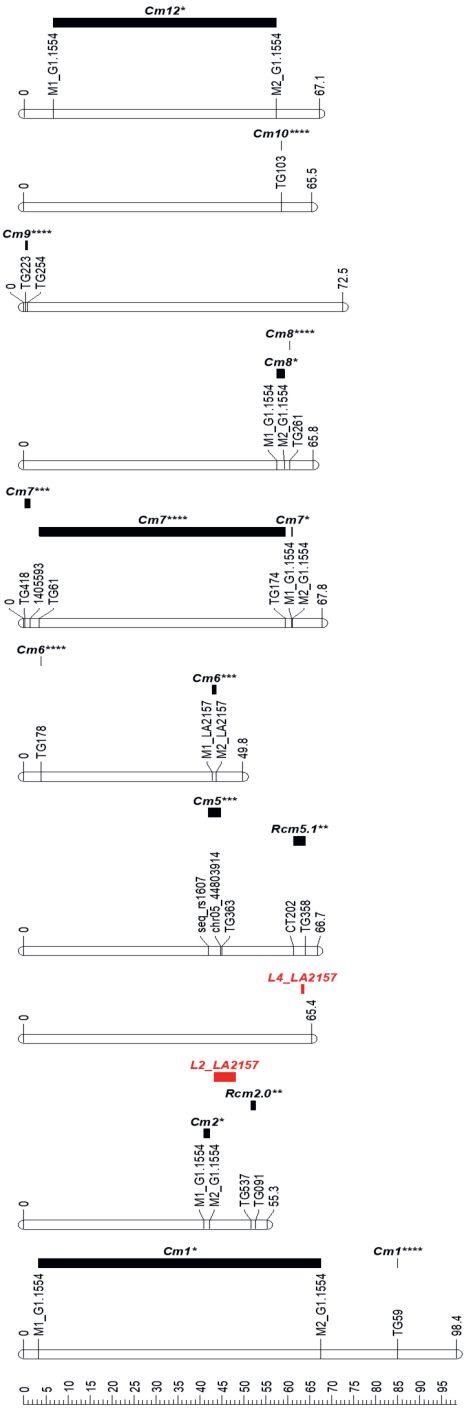

Figure 5 | Overview of mapped QTLs reported to be linked to tolerance to *Cm*. Physical positions (Mb) of previously described QTLs linked to tolerance to *Cm* mapped on the *Solanum lycopersicum* genome. Bars indicate the QTL intervals determined by two flanking markers or one closely linked marker. Red bars indicate the newly mapped loci linked to tolerance on chromosomes 2 and 4 of tomato. References: *[26], **[19], ***[27], ****[20], *****[24].

In light of our new data, it could be possible that the lack of symptoms was a consequence of the *DWARF* mutation in the cv.MT background. In case that this mutation is responsible for the lack of symptoms, the DWARF gene might represent a novel susceptibility (S) gene to *Cm*. This hypothesis remains to be tested.

VSF-1 is a development related member of the bZIP family of transcription factors, and is expressed in vascular tissues [40]. Analysis of interactors of VSF-1 has revealed a strong interaction with the promoter of structural glycine rich cell wall protein GRP1.8, which is specifically deposited on protoxylem and metaxylem cells [41, 42]. Functional analysis of the rice homolog of *VSF-1* (*RF2a*) reported that mutation of *RF2a* results in non-uniform lignification of the xylem, as well as alteration in phloem development [43].

Upon inspection of the sequencing data of the two genes mapped on the R-specific bulk, we could confirm that the SNPs present on the k-mers mapped to the genes were identical to the *S. arcanum* LA2157 allelic variant. We were able to show that differences in the coding sequence of the *S. arcanum* LA2157 allele of gene *Solyc04g081190.3* result in the production of an altered protein (Fig. 4). Amino acid substitutions in the proteins produced may influence protein-protein interactions [44]. Therefore, it is likely that changed interactions between the newly mapped loci on chromosomes 2 and 4 with the QTL on chromosome 7 result in tolerance. Based on the loci that we have mapped, molecular markers can be developed. Marker analysis of susceptible and resistant plants of the PV185517 line can confirm the involvement of the loci in tolerance. The use of molecular markers can also verify if the lower peaks mapped are minor loci or due to lack of sequencing coverage. As a next step, functional analysis of genes present in the regions important for tolerance can further aid in the identification of the genes underlying the tolerant phenotype. In addition, morphological studies of the vascular systems of tolerant and susceptible plants might uncover differences that support the hypothesis that vascular changes are responsible for the observed tolerance.

MATERIALS AND METHODS

Plant materials

In this study, we used an BC_3S_6 near isogenic line (NIL) PV175136 and its selfing PV185517. The material was developed from the original F_2 population between the tolerant accession *Solanum arcanum* LA2157 and the susceptible *Solanum lycopersicum* cv. Solentos [20]. Shortly, progeny containing the identified QTLs described by van Heusden et. al (1999) [20] were backcrossed to *Solanum lycopersicum* cv. Moneymaker (cv.MM) to obtain BC_3S_6 NILs. Selfing of BC_3S_6 NIL PV175136 gave rise to PV185571, that were used in this study. This study also included the susceptible *S. lycopersicum* cv. MM as a susceptible control.

Bacterial strains and growth conditions

Cm strain NCPBB382 was used in the bioassays. Prior to plant inoculation the strain was grown at 25°C on TBY plates (10 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 5 gL^{-1} sodium chloride, 15 gL^{-1} bacteriological agar) for two days. For the preparation of the bacterial inoculum bacterial cells were resuspended in Ringer's buffer to a final concentration of ~10^8 cfu/mL (OD_{600}=0.1).

Disease assays

Tomato plants at the fourth true leaf stage were inoculated by a petiole clipping off method. The petioles of the first two fully expanded leaves were clipped off with razor blades immersed in the bacterial inoculum and 5 µl of the bacterial inoculum were directly pipetted on the lowest wound. Symptom development was monitored for up to 20 days post inoculation (dpi). A disease index (DI) scale based on the development of wilting symptoms on the leaves was used (0; no symptoms, 1; one leaf wilting, 2; <2/3 of leaves wilting, 3; 2/3 of leaves wilting, 4; 3/4 of leaves wilting, 5; all leaves wilting).

Development of cleaved amplified polymorphic sequences (CAPS) markers

In previous research, it was described that NIL PV175136 contained a fixed 697 kb introgression on chromosome 7 [24]. To confirm the introgression size on chromosome 7, six in-gene CAPS markers flanking the reported introgression region (physical position SOL07-1060331 to SOL07-1784948) were designed. Genes in the region were mined from the available annotated ITAG3.2 genes on Jbrowser. Single nucleotide polymorphisms (SNPs) between *S. arcanum* LA2157 and *S. lycopersicum* cv.MM were identified, based on the *de novo* genome sequence of *S. arcanum*

LA2157 [45] and the tomato genome ITAG 2.4 (SolGenomics Network). Polymorphic CAPS markers were developed based on the identified SNPs.

Genomic DNA isolation and genotypic

Genomic DNA (gDNA) was isolated from cotyledons of young tomato plants using a modified cetyl trimethylammonium bromide (CTAB) extraction method [46]. Gene specific primers were designed for the amplification of the allelic variants. Following amplification, the amplification products were incubated with the appropriate restriction enzyme at 37^{o}C overnight. The digested products were visualized on 2% agarose gel for the detection of the alleles present in each sample.

DNA isolation and pooling

Whole-genome sequencing (WGS) of a susceptible and a resistant pool of 14 plants each was done to identify loci involved in the observed phenotypes. Genomic DNA of plants was extracted from leaves that were flash frozen in liquid nitrogen and stored at -80^{o}C, using a modified (CTAB) extraction method [46]. DNA concentration of each sample was assessed using a Qubit Fluorometer (Invitrogen). Samples were cleaned with the Genomic DNA & Concentrator™-10 kit (Zymo Research). For each bulk, 28.57 ng of gDNA of each individual were pooled. For the WGS experiment, 400 ng of (pooled) gDNA were used for the library preparation and sequencing. Sequencing was done on an Illumina Novaseq 6000 platform producing 151 bp paired end reads at a 35x depth (Novogene Europe, Cambridge, United Kingdom).

Comparative Subsequence Sets Analysis (CoSSA)

A modified version of the CoSSA workflow was used for the identification of bulk-specific k-mers [47]. Reads of the S- and R- bulks were mapped against the tomato reference genome SL4.0. k-mers were built for each of the sequenced bulks by using the KMC database. As a compromise between sequence uniqueness and sequence correctness a k-mer size of 31 nucleotides was used. To obtain k-mers specifically present in the R-bulk, the S-bulk k-mers were subtracted from the R-bulk. S- bulk specific k-mers were obtained using the same approach. Reads were filtered out using the previously computed “resistant and not susceptible” and “susceptible and not resistant” k-mers. Reads containing at least 15 of the k-mers were filtered out. To account for sequencing errors, k-mers that were observed in the dataset only once were removed. To identify the genomic regions harbouring the traits of interest, the R- and S-bulk specific k-mers were mapped on the tomato reference genome SL4.0. Additionally, the fraction of “only resistant” reads was plotted against

the resistant read coverage and the fraction of “only susceptible” reads against the susceptible read coverage was plotted. Further filtering was applied by plotting the fraction of “only resistant” reads against the resistant read coverage, while the fraction of “only susceptible” reads against the susceptible read coverage. Finally, it was required that more than k/3= 10 k-mers were uniquely mapped to the k-size bin. These restrictions applied to the distinctive reads, resulted in the filtered coverage of the 12 tomato chromosomes for both the R- and S-bulks.

Protein-protein interaction network prediction

For the prediction of potential protein-protein interactions between the two newly mapped loci on chromosomes 2 and 4 of tomato and the 15 mapped genes on the QTL on chromosome 7, we used the reference sequences of genes *Solyc02g084740.4* and *Solyc04g081190.3* as queries on the STRING database for functional protein association networks (https://string-db.org/) using the default settings.

Protein prediction of *S. arcanum* LA2157 alleles

To predict the protein sequences produced by the *S. arcanum* LA2157 allelic variants the coding sequence (CDS) of the genes was predicted based on the CDS of the *S. lycopersicum* allele. The CDS was then used on the expasy translate tool (https://web.expasy.org/translate/) to predict the open reading frames and the protein sequences. The default settings were used in the prediction of the proteins.

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Table S1| Details of CAPS markers used in this study**.** In the table, the physical position of the expected sequence fragment is given. The primers and the polymorphic SNP on which the marker was designed are also given in the table

CAPS marker	Location	Primer sequence (5'-3')	SNP
Q7M1	SL3.0ch07:1055917..1060714	CTATGGGAGACTCAACGTAT ATCCTCTCCAAGTTAAACG	GGAACAGGAG[T/C]TCCACCTCC
Q7M2	SL3.0ch07:1506409..1518012	ACCAGTTCTACTTGAAAAGAA ACGATTAAGGTAGAGGCCAAGG	TCTACGAAAT[T/A]TTATGTGCT
Q7M3	SL3.0ch07:1574953..1577763	CACAAGACATTGAGTGTTCATTGAT TGCAACATGTCCTTAAGTGGA	GTTATTGGTA[T/C]TAGCAGGAC
Q7M4	SL3.0ch07: 1624160..1636142	TTGTATTGTTTTCTAGGATTGA GCATGAAGAAAGTAAGGGGACT	GAAGTGTAGC[T/A]TTCCCCGGT
Q7M5	SL3.0ch07:1754891..1758020	TGGCTTTACAACCAAAGATCA TCCAAATATAACCCTGCCAAA	CGCGTAAGTA[T/C]TTTCATCGT
Q7M7	SL3.0ch07:1266878..1270553	AACGTGAGATCACACGAAC CGCGAAAAACAACAATTTA	GTTACCATAGT[G/A]CATATCTT
Q7M9	SL3.0ch07:1783293..1787487	ATAGGTGGTTGGTAGTACGTA TTATTTCCTTGTTCGATTAAGC	GAAACTTTTT[T/A]AAGATAAAT

Table S2| Locations on the *S. lycopersicum* ITAG4.0 reference genome with resistant specific coverage.

Chromosome	Start	End	Gene	Gene function
SL4.0ch02	43262484	43262515		
SL4.0ch02	43262515	43262546		
SL4.0ch02	43262546	43262577		
SL4.0ch02	43899410	43899441		
SL4.0ch02	45861090	45861121	*Solyc02g084740*.4	Cytochrome P450
SL4.0ch02	45861121	45861152	*Solyc02g084740.4*	Cytochrome P450
SL4.0ch02	46120963	46120994		
SL4.0ch02	48143496	48143527		
SL4.0ch03	46778783	46778814		
SL4.0ch04	63165755	63165786	*Solyc04g081190.3*	Vsf-1 transcription factor
SL4.0ch04	63767899	63767930		
SL4.0ch05	2574426	2574457		
SL4.0ch07	61619289	61619320		
SL4.0ch10	20068811	20068842		
SL4.0ch11	10668123	10668154		
SL4.0ch12	44524339	44524370		

Table S3| Locations on the *S. lycopersicum* ITAG4.0 reference genome with susceptible specific coverage.

Chromosome	Start	End	Gene	Gene function
SL4.0ch01	1850483	1850514		
SL4.0ch01	51678519	51678550		
SL4.0ch01	69201641	69201672		
SL4.0ch02	29817877	29817908		
SL4.0ch02	29817939	29817970		
SL4.0ch02	33193901	33193932		
SL4.0ch02	42609345	42609376		
SL4.0ch02	42727858	42727889		
SL4.0ch02	42789083	42789114	*Solyc02g080620.3*	Prephenate dehydratase family protein
SL4.0ch02	42852137	42852168		
SL4.0ch02	42930195	42930226	*Solyc02g080820.3*	Peroxisomal membrane protein PMP22
SL4.0ch02	42936178	42936209		
SL4.0ch02	42936209	42936240		
SL4.0ch02	42936240	42936271		
SL4.0ch02	43090062	43090093		
SL4.0ch02	43090093	43090124		
SL4.0ch02	43600787	43600818		
SL4.0ch02	44461967	44461998	*Solyc02g082810.3*	CCA-adding enzyme
SL4.0ch02	44461998	44462029	*Solyc02g082810.3*	CCA-adding enzyme

SL4.0ch02	44491634	44491665	*Solyc02g082840.3*	Protein GRIP
SL4.0ch02	45195985	45196016		
SL4.0ch02	45527778	45527809		
SL4.0ch02	45695488	45695519		
SL4.0ch02	45939117	45939148		
SL4.0ch02	46164487	46164518		
SL4.0ch02	46277296	46277327		
SL4.0ch02	46325687	46325718	*Solyc02g085390.4*	Uncharacterized ATP-dependent helicase C25A8.01c
SL4.0ch02	46378883	46378914		
SL4.0ch02	46424050	46424081		
SL4.0ch02	46478455	46478486		
SL4.0ch02	46542377	46542408		
SL4.0ch02	46579360	46579391	*Solyc02g085790.3*	T-complex protein 1 subunit zeta
SL4.0ch02	46579391	46579422	*Solyc02g085790.3*	T-complex protein 1 subunit zeta
SL4.0ch02	46672298	46672329		
SL4.0ch02	46672329	46672360		
SL4.0ch02	46672360	46672391		
SL4.0ch02	46888988	46889019		
SL4.0ch02	46889019	46889050		
SL4.0ch02	46999596	46999627	*Solyc02g086270.4*	Receptor-like kinase
SL4.0ch02	47230236	47230267	*Solyc02g086550.3*	Os06g0661900 protein
SL4.0ch02	47230267	47230298	*Solyc02g086550.3*	Os06g0661900 protein
SL4.0ch02	47405696	47405727		
SL4.0ch02	48085154	48085185		
SL4.0ch02	48085216	48085247		
SL4.0ch02	48143496	48143527		
SL4.0ch02	48193778	48193809		
SL4.0ch02	48380336	48380367		
SL4.0ch02	48655120	48655151		
SL4.0ch02	48829898	48829929		
SL4.0ch02	49070210	49070241	*Solyc02g089150.4*	MAPprotein kinase-like protein
SL4.0ch02	49282281	49282312		
SL4.0ch02	49286497	49286528		
SL4.0ch02	49770159	49770190		
SL4.0ch02	49770190	49770221		
SL4.0ch03	16906501	16906532		
SL4.0ch03	50748116	50748147		
SL4.0ch04	4253913	4253944		
SL4.0ch04	27998921	27998952		
SL4.0ch04	31211575	31211606		

SL4.0ch04	51481266	51481297		
SL4.0ch04	53085919	53085950		
SL4.0ch04	61408241	61408272		
SL4.0ch04	61614174	61614205	*Solyc04g079040.4*	Serine carboxypeptidase 1
SL4.0ch04	61614205	61614236	*Solyc04g079040.4*	Serine carboxypeptidase 1
SL4.0ch04	61614236	61614267	*Solyc04g079040.4*	Serine carboxypeptidase 1
SL4.0ch04	61624590	61624621	*Solyc04g079060.3*	
SL4.0ch04	61638416	61638447		
SL4.0ch04	61638447	61638478		
SL4.0ch04	61647313	61647344		
SL4.0ch04	61718582	61718613	*Solyc04g079170.4*	Unknown Protein
SL4.0ch04	61718613	61718644	*Solyc04g079170.4*	Unknown Protein
SL4.0ch04	61746451	61746482		
SL4.0ch04	61829748	61829779		
SL4.0ch04	62233833	62233864		
SL4.0ch04	62257207	62257238		
SL4.0ch04	62257238	62257269		
SL4.0ch04	62257269	62257300		
SL4.0ch04	62257858	62257889		
SL4.0ch04	62365583	62365614		
SL4.0ch04	62513856	62513887		
SL4.0ch04	62513887	62513918		
SL4.0ch04	62631005	62631036		
SL4.0ch04	62651465	62651496		
SL4.0ch04	62774163	62774194	*Solyc04g080710.4*	BHLH transcription factor-like protein
SL4.0ch04	62819516	62819547		
SL4.0ch04	62819547	62819578		
SL4.0ch04	62819578	62819609		
SL4.0ch04	62826677	62826708		
SL4.0ch04	62826708	62826739		
SL4.0ch04	62835791	62835822	*Solyc04g080770.3*	GTP-binding protein hflX
SL4.0ch04	62941253	62941284	*Solyc04g080915.1*	
SL4.0ch04	62944632	62944663		
SL4.0ch04	62944663	62944694		
SL4.0ch04	62944694	62944725		
SL4.0ch04	62944787	62944818		
SL4.0ch04	62955358	62955389		
SL4.0ch04	63026286	63026317	*Solyc04g080990.2*	C4-dicarboxylate transporter/malic acid transport family
SL4.0ch04	63026317	63026348	*Solyc04g080990.2*	C4-dicarboxylate transporter/malic acid transport family
SL4.0ch04	63141730	63141761		

SL4.0ch04	63141761	63141792		
SL4.0ch04	63216595	63216626		
SL4.0ch04	63230669	63230700		
SL4.0ch04	63230948	63230979		
SL4.0ch04	63267032	63267063		
SL4.0ch04	63267063	63267094		
SL4.0ch04	63267094	63267125		
SL4.0ch04	63269605	63269636		
SL4.0ch04	63303519	63303550	*Solyc04g081290.3*	Lysine decarboxylase-like protein
SL4.0ch04	63314214	63314245		
SL4.0ch04	63363845	63363876		
SL4.0ch04	63363876	63363907		
SL4.0ch04	63363907	63363938		
SL4.0ch04	63394411	63394442	*Solyc04g081450.3*	Unknown Protein
SL4.0ch04	63432479	63432510		
SL4.0ch04	63432510	63432541		
SL4.0ch04	63451265	63451296		
SL4.0ch04	63451296	63451327		
SL4.0ch04	63625113	63625144		
SL4.0ch04	63655276	63655307		
SL4.0ch04	63655307	63655338		
SL4.0ch04	63655338	63655369		
SL4.0ch04	63733799	63733830		
SL4.0ch04	63746075	63746106	*Solyc04g081910.4*	Calcium-dependent protein kinase
SL4.0ch04	63803518	63803549		
SL4.0ch04	63803580	63803611		
SL4.0ch04	63812632	63812663		
SL4.0ch04	63872307	63872338		
SL4.0ch04	63872338	63872369		
SL4.0ch04	63932199	63932230		
SL4.0ch04	63932230	63932261		
SL4.0ch04	63955759	63955790	*Solyc04g082260.4*	Receptor protein kinase-like protein
SL4.0ch04	63955790	63955821	*Solyc04g082260.4*	Receptor protein kinase-like protein
SL4.0ch04	64087075	64087106		
SL4.0ch04	64128150	64128181		
SL4.0ch04	64154779	64154810	*Solyc04g082540.2*	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1
SL4.0ch04	64154810	64154841	*Solyc04g082540.2*	Dolichyl-diphosphooligosaccharide-

				protein glycosyltransferase subunit 1
SL4.0ch04	64227009	64227040		
SL4.0ch04	64227040	64227071		
SL4.0ch04	64227071	64227102		
SL4.0ch04	64241052	64241083	*Solyc04g082670.4*	SWI/SNF complex subunit SMARCC1
SL4.0ch04	64253607	64253638		
SL4.0ch04	64289629	64289660	*Solyc04g082760.3*	SWI/SNF complex subunit SMARCC1
SL4.0ch04	64289660	64289691	*Solyc04g082760.3*	SWI/SNF complex subunit SMARCC1
SL4.0ch04	64298867	64298898	*Solyc04g082780.3*	Cinnamoyl CoA reductase-like 1
SL4.0ch04	64317405	64317436	*Solyc04g082810.4*	AT-hook DNA-binding protein
SL4.0ch05	8538268	8538299		
SL4.0ch05	23630401	23630432		
SL4.0ch05	28133275	28133306		
SL4.0ch05	56643851	56643882		
SL4.0ch06	2570458	2570489		
SL4.0ch06	25828611	25828642		
SL4.0ch07	29571675	29571706		
SL4.0ch07	29571706	29571737		
SL4.0ch07	32874601	32874632	*Solyc07g026930.3*	Tetracycline transporter
SL4.0ch07	33550773	33550804		
SL4.0ch07	33550804	33550835		
SL4.0ch07	33550835	33550866		
SL4.0ch07	33550866	33550897		
SL4.0ch07	33550897	33550928		
SL4.0ch07	33550928	33550959		
SL4.0ch07	33553656	33553687		
SL4.0ch08	1507902	1507933	*Solyc08g006930.3*	Photosystem I reaction center subunit X psaK
SL4.0ch09	9828240	9828271		
SL4.0ch09	19395026	19395057		
SL4.0ch09	61438280	61438311		
SL4.0ch09	62162223	62162254		
SL4.0ch09	66761042	66761073		
SL4.0ch11	47942554	47942585		
SL4.0ch11	52125818	52125849		

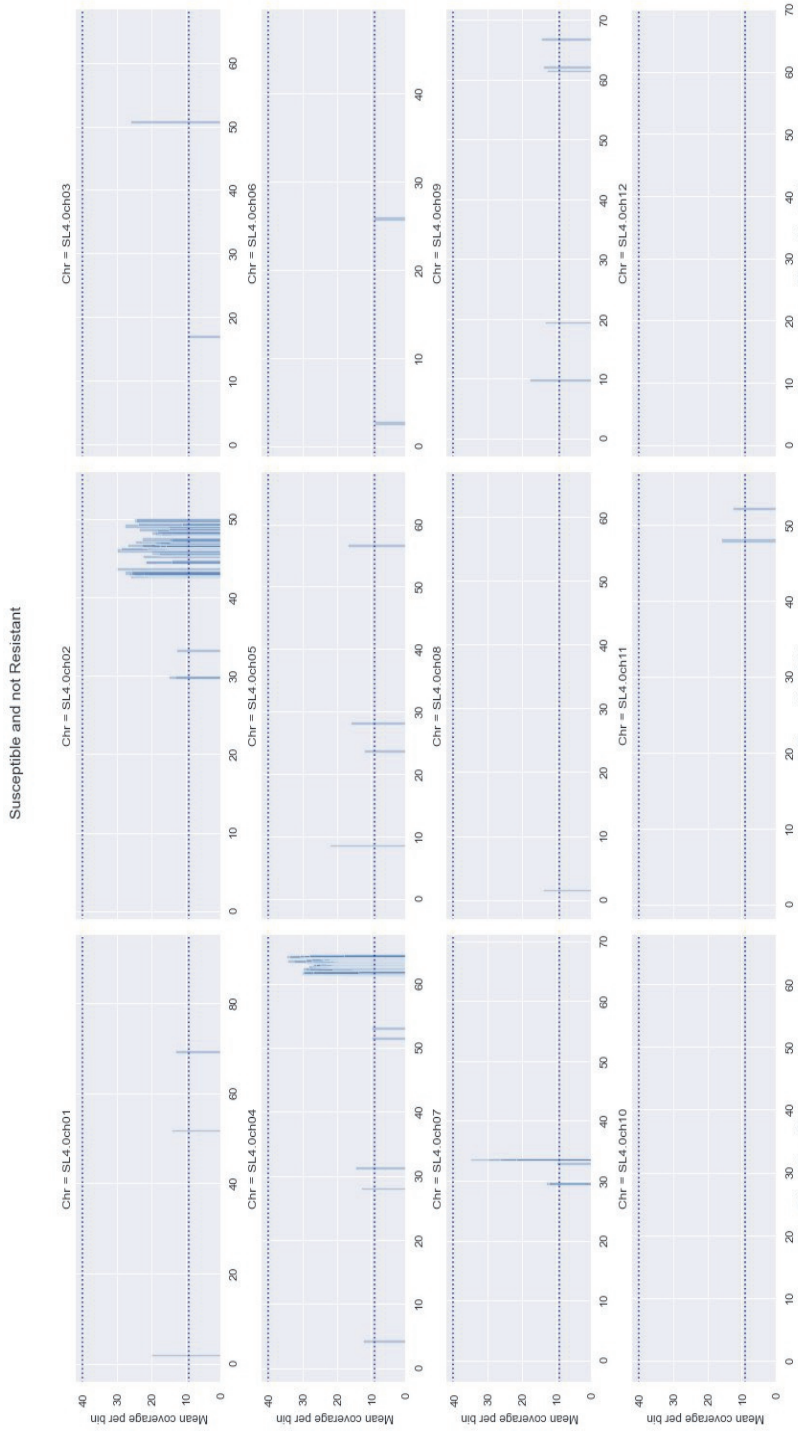

Figure S1| Density graphs of unique k-mers mapped in 1 Mb bins on the tomato (*S. lycopersicum*) reference genome ITAG 4.0. Blue lines indicate loci associated with susceptibility.

CHAPTER 7

General discussion

In early 2020, the pandemic caused by the emergence of the novel coronavirus COVID-19 brought the world to a halt. But, what about outbreaks of plant diseases? Even though susceptibility and disease are exceptions in plant pathogen interactions, pathogens can lead to major problems for agriculture [1, 2]. Historically, the emergence of plant pathogens has caused catastrophic epidemics [3, 4]. The outbreak of the oomycete *Phytophtora infestans*, which caused the infamous Irish famine, led to the death of more than one million people in Ireland alone and at least the same number all over Europe [3]. The *Fusarium oxysporum* f.sp. *cubense* (*Foc*) emergence in the 1900s decimated the "Gros Michel" banana industry, while the emergence of the *Foc* isolate TR4 and the lack of resistant germplasm are once again threatening the global banana industry [5, 6]. In 2013, the bacterium *Xylella fastidiosa* emerged as a pathogen of global importance, after its destructive consequences on olive trees in Italy [7, 8]. The severity and emergence of plant diseases has exponentially increased in the last 200 years [9-11]. Increase in the global trade and shifts in geographic distribution of pathogens in response to climate change are expected to make the emergence of plant diseases even more frequent in the coming decades [12, 13].

Pathogens and their hosts share long co-evolutionary histories, through which plants have evolved a complex immune system to avoid pathogens [14, 15]. The reduction of losses caused by plant pathogens has mainly been managed by the introgression of dominant resistance (R) genes from wild germplasm to elite cultivars [2, 14, 16]. Pathogens, however, due to their short generation times and population sizes are generally in an evolutionary advantage over plants. Within only a few (pathogen) generations beneficial mutations can accumulate rendering pathogen perception by the host's immune system ineffective [17]. This is especially true for the introduction of pathogens in novel areas, that can result in the disruption of the co-evolution of host-pathogen interactions. In the absence of evolved host resistance, emergent pathogens can cause serious disease outbreaks [18]. During co-evolution pathogens have also acquired the ability to suppress host immunity by the manipulation of host susceptibility (S) genes (**Chapter 2**) [19-21]. Although, S genes may represent a "weak spot" in plant immunity, research has shown that their loss-of-function can result in broad-spectrum and potentially durable resistance [19, 22, 23].

In this thesis I largely focused on the identification, impairment and functional characterization of S genes potentially involved in the molecular interaction between tomato (*Solanum lycopersicum*) and the bacterium *Clavibacter michiganensis* (*Cm*)

(**Chapters 3, 4** and **5**). In addition, in **Chapter 6** we set out to identify the loci underlying tolerance to *Cm* in an advanced population of a *S. arcanum* LA2157 and *S. lycopersicum* cross. During the process of completing this thesis several questions arose, which I will try to address in this chapter. Finally, I will present a novel model for the *Cm*-tomato interaction which was based on my results in combination with current information derived from literature. This model provides exciting new opportunities for the continuation of research on the molecular interactions in the tomato- *Cm* pathosystem.

How can we identify S genes?

Searching for an S gene in a crop genome can be a daunting task. Taking into account the functional diversity of S genes, any gene in a crop genome could potentially encode a susceptibility factor. Several S genes have been identified through forward genetic screens in mutagenized populations or wild germplasm [24-28]. The identification of S genes through forward genetics, however, can be hindered by the difficulty of performing these screens on a large scale, as well as the polyploidy of many crops [19]. While S genes might be widely diverse, they also share several common characteristics, which may help in their identification through reverse genetic screens (**Chapter 2**). In this thesis, we took advantage of these common characteristics to identify S genes involved in the tomato- *Cm* pathosystem by combining different reverse genetics approaches.

An important feature of S genes is their retainment across species [19]. The model plant Arabidopsis and the rapidly increasing availability of crop genomes have been instrumental in the identification of S genes in several crops [29]. Forward genetics screens for the identification of loss-of-susceptibility mutants have yielded a number of S genes in Arabidopsis [24, 27, 30]. Once an S gene has been identified in Arabidopsis, phylogenetic analyses based on the crop genome of interest can be used for the discovery of candidate crop homologs (**Chapter 2**). This approach has been used in the identification of S gene homologs in several crops, including *Powdery Mildew Resistance4 (PMR4), Downey Mildew Resistance6* (*DMR6*) in tomato, *Defence No Death1* (*DND1*) in potato and multiple *Mildew Locus O* (*MLO*) homologs in several crops [31-35]. In the same manner, we identified and functionally characterized candidate S genes *SlWAT1* (**Chapter 3**) and *SlWRKY23* (**Chapter 4**).

Another important characteristic of S genes is that their transcription can potentially be altered upon pathogen challenge. Pathogen induced upregulation of S genes has

been frequently observed [36-39]. As an example, S genes in the *Glutamate Decarboxylases* (*GAD*) family that contribute to *Ralstonia solanacearum* proliferation during compatible interactions, have been found to be upregulated in crops such as Arabidopsis, tomato, peanut and wild potato [37]. In **Chapter 5**, we took advantage of available transcriptomics data of *Cm* infected tomato to identify candidate S genes, that are upregulated upon inoculation [40]. As a result, we selected two highly homologous putative 2-oxoglutarate-dependent dioxygenases after initial functional analysis as candidate S genes (**Chapter 5**).

Adapted pathogens secrete effector proteins that interfere with host processes [14]. Several effectors have been reported to interact with host susceptibility factors [41-43]. In **Chapter 2**, we describe how pathogen effectors can be used to identify S genes through protein-protein interactions. Knowledge on the significance of effectors in pathogen fitness can aid in the selection of the most suitable ones for interaction assays. For example, effector DspA/E of bacterium *Erwinia amylovora*, which belongs to a highly conserved family of Gram-negative phytopathogenic bacteria effectors is crucial for bacterial multiplication [44]. In addition, the effector was found to inhibit salicylic acid (SA) basal defences leading to susceptibility of its hosts and to associate with conserved genes in several of its hosts [45, 46]. The disruption of host targets of such effectors could potentially result in durable resistance. Moreover, the conserved nature of their targets is important in the identification of homologs in multiple crop species.

To conclude that an identified gene(s) indeed acts as a susceptibility factor(s), functional assays should follow the selection of candidate gene(s). In our case, we used post-transcriptional silencing through VIGS for the initial functional screening of the selected genes (**Chapters 4 & 5**). Even though VIGS offers the advantage of quickly assessing the function of several genes and the possibility to silence multiple homologs at once, concerns about its use in the analysis of S genes emerged during the experiments. One of the most important limiting factors of using VIGS to identify S genes, is that VIGS does not always lead to uniform silencing of target genes. As a result large numbers of plants should be included in the analysis [47]. In the case of S genes, partial silencing of a gene might lead to the erroneous conclusion that the gene is not involved in the interaction, if a high degree of silencing is needed to observe resistance, and wrongly discarded from further analysis, because their partial silencing did not lead to significant changes in susceptibility. The use of viral vectors for silencing can in many cases lead to the development of viral symptoms

on gene-silenced plants [47-49]. During our experiments, we occasionally observed symptoms caused by the Tobacco Rattle Virus (TRV) vector we used for silencing. Such symptoms in many cases made the phenotyping of plants difficult, which could also result in prematurely discarding potential S genes from analysis. For these reasons, it is important to consider the limitations of methods used for functional analyses of S genes. If possible, combination of methods should be used to confirm the obtained results.

Is CRISPR/Cas9 the best tool to study S genes?

The discovery of the CRISPR/Cas9 system has enabled scientists to precisely and rapidly modify traits of agronomical interest in several plant species [50, 51]. The flexibility and ongoing advances of CRISPR/Cas systems have allowed plant scientists to create mutations ranging from single base substitutions to large pericentric inversions in crops [52-55]. CRISPR/Cas systems have also been widely applied in the functional study of plant S genes (**Chapter 2**) [33, 34, 56-58].

In **Chapters 4** and **5**, we employed post-transcriptional silencing through VIGS and CRISPR/Cas9 to study the function of candidate S genes *SlWRKY23* (**Chapter 4**) and two highly homologous 2-oxoglutarate-dependent dioxygenases (**Chapter 5**). Our initial results suggested that post-transcriptional downregulation of the candidate genes resulted in reduced symptom development on tomato plants. The next step was to generate knock-out mutants of the genes to further investigate the role of the genes in susceptibility of tomato to *Cm*. To our surprise, in contrast to our initial data the mutants we generated were fully susceptible to *Cm*.

One may assume, that the full knock-out of a gene will lead to higher phenotypic "resolution" than post-transcriptional silencing during functional studies of genes- but is that true? Recent studies in mice, zebrafish, the nematode *Caenorhabditis elegans,* the bacterium *Streptomyces scabies* and the insect *Helicoverpa armigera*, demonstrated that is not always the case [59-65]. Knock-down and knock-out of the same gene have been reported to lead to differences in the observed phenotypes. Such a phenomenon has been described for several genes in zebrafish. As an example, downregulation of the cardiovascular associated *Bag3* in zebrafish resulted in severe cardio-myopathy in zebrafish embryos. In contrast, targeted knock-out of the gene through CRISPR/Cas9 did not provoke any cardio-myopathy related dysfunctions to the embryos [59]. These data suggest that targeted mutations that lead to loss-of-function alleles may not always result in phenotypic changes.

To maintain genetic robustness, organisms require mechanisms to buffer the effects of deleterious mutations. One such mechanism that has received attention with the advent of genome editing technologies is genetic compensation response (GCR) [66]. According to the model for GCR, mutation of a gene can lead to compensatory overexpression of related genes, which can overtake the function of the mutant gene [62, 63]. Even though the mechanism of GCR is still poorly understood, new research suggests that the presence and degradation of mutant mRNA through the non-sense mediated mRNA decay (NMD) pathway is required for the activation of the mechanism [59, 63]. To eliminate aberrant mRNA that could otherwise result in errors during protein synthesis, eukaryotic cells have evolved surveillance pathways. NMD was recently reported to be involved in the activation of GCR, as inactivation of NMD factor *Upf1* in mutant zebrafish resulted in the reduction in the degradation of mutant RNA and abolished the transcriptional activation of compensating genes. These results illustrate the importance of degrading mRNA in the GCR mechanism, as it has been hypothesized that the production of long-non coding RNAs (lncRNA) during decay of mutant RNA is responsible for the transcriptional changes observed [63, 66]. Even though the literature on GCR is mainly based on observations in zebrafish, limited examples in other organisms also exist [60, 65]. Considering the importance of genetic robustness in the viability of organisms [61], it would not be farfetched to hypothesize that GCR is conserved across kingdoms.

Sequencing of our generated mutants revealed the presence of premature stop codons in all the generated mutant alleles (**Chapter 4** and **5**). It would, therefore be tempting to assume that the differences in the observed phenotypes between knocked-out and knocked-down genes were due to GCR. Nonetheless, since we did not perform any transcriptional analysis of the target genes or potential off-targets, we cannot exclude that the observed reduced susceptibility during the VIGS assays was a result of off-target activity. When phenotypic differences are observed between knock-down and knock-out experiments during functional characterization of potential S genes, a combination of downregulation, CRISPR/Cas9 methods and transcriptomics can be used to elucidate the reason behind these differences. In the absence of phenotypic changes in knock-out mutants, transcriptional studies may shade light into potential GCR. As described above, GCR is connected to the NMD pathway and requires the presence of mutant mRNA for its activation. Therefore, alternatively to introducing mutations in the coding sequences of genes, the promoter regions or the whole gene could be deleted through CRISPR/Cas9. This

would eliminate the transcription of the gene and abolishing the production of mutant mRNA that could activate GCR [63].

Are pleiotropic effects caused by mutant S genes avoidable?

Possibly the biggest concern when working with S genes is if the loss-of-function of the candidate gene will lead to pleiotropic defects. Contrary to what the phrase "susceptibility gene" suggests, the primary function of S genes is not their involvement in susceptibility [19]. The retainment of S genes across plant species suggests that they are primarily involved in important biological processes of plants, such as embryo development [67, 68]. Thus, the loss-of-function of S genes can potentially lead to pleiotropy that adversely influences plant development [32].

The experimental part of this thesis begins with such an example. In **Chapter 3**, we functionally characterized the tomato *Walls Are Thin1* (*SlWAT1*) homolog as a potential S gene to *Cm*. The targeted mutation of the gene led to loss-of-susceptibility to the pathogen, however the mutant plants exhibited severe growth defects.

The co-evolution of plants and pathogens can result in the manipulation of host S genes by the pathogen. However, one can assume that when there is high pathogen pressure, mutant alleles of S genes that do not result in pleiotropic defects will be positively selected. As an example, the natural loss-of-function mutant of *Resistance Of Rice to Disease1* (*ROD1)* gene results in broad spectrum resistance to fungal and bacterial pathogens in rice. This natural mutant, however, performs worse than the wild type in terms of yield. Through investigation of the allelic variation of *ROD1* in rice germplasm, a variant that confers resistance in the absence of yield penalties was discovered. This variant was widespread in varieties grown in regions where pathogen pressure is high, suggesting a specific adaptation to the pathogen [43]. Therefore, the identification of such allelic variants and their subsequent introgression into elite cultivars could reduce potential pleiotropic effects. When such alleles are not available in nature, gene editing and potentially mutagenesis can be used to generate and introduce these allelic variants in the desired cultivars [69].

As previously discussed in **Chapter 2**, more and more recent studies report that S genes (proteins) physically interact with pathogen effectors [37, 70-73]. Possibly the most well-known example of such interaction is the interaction of *Xanthomonas* spp. TALEs with promoters of *Sugars Will Eventually be Transported* (*SWEET*) genes [42, 74, 75]. Introduction of SNPs in the effector binding element on the promoter of

SWEET genes has been proven to be an effective strategy to gain cost-free resistance to TALEs-carrying bacteria [41]. Even though for most S genes the functional domains of the produced proteins can in most cases be easily predicted, it remains unclear if pathogen effectors interact with these domains or other parts of the encoded proteins. Identification of interacting sites of host and pathogen proteins can be used as a guide for the introduction of mutations. Thereafter, the use of gene editing for the generation of mutations in interacting sites can be useful in dissecting negative pleiotropic effects and resistance. Therefore, knowledge on the repertoire of effectors carried by pathogens is of outmost importance, as they can be used as "tools" in the identification of interacting S genes [76].

Although a full knock-out of *SlWAT1* resulted in developmental defects, RNAi silencing of the gene was enough to confer similar levels of tolerance without observable pleiotropic defects (**Chapter 3**). This observation led to another question- is it always necessary to fully disrupt the functionality of proteins encoded by S genes? Recessive *MLO* mutant alleles provide strong resistance against powdery mildew pathogens in several plant species [25, 77-80]. The observed resistance, however, is in most cases accompanied by deleterious effects [81]. In the case of *MLO*, weak allelic variants that support reduced protein functionality, which results in significant disease reduction uncoupled of pleiotropic effects have been identified in barley [82]. Identification of weak alleles could constitute a compromise between the level of resistance and pleiotropic effects, when other sources of resistance are lacking. Alternatively, modification of *cis*-regulatory elements of S genes could lead to alteration of expression [83], that could result in desirable cost-free resistance.

How can S genes be deployed to achieve resistance?

This thesis mainly focused on the identification and modification of S genes that are involved in the tomato- *Cm* interaction. From our findings, some guidelines of how S genes can be employed in crops to achieve potential resistance can be derived.

The first step in employing mutant S genes in breeding programs is the identification of a candidate gene. In **Chapter 2**, we propose multiple ways to identify candidate S genes [21]. Once a candidate S gene has been selected, functional studies to confirm its role in susceptibility of the studied pathosystem should follow [31, 56, 84]. Depending on whether or not reduced susceptibility is observed after impairment of candidate S genes, different approaches can be used to assess if a candidate S gene can be used to gain resistance. When no reduced susceptibility is observed and the possibility of GCR or functional redundancy are ruled out [63], one may conclude

that the candidate S gene is possibly not involved in the interaction of the studied pathosystem (**Chapters 4 and 5**). If reduced susceptibility after impairment of a candidate gene is observed, one should carefully assess the mutants for any possible adverse pleiotropic effects (**Chapter 3**). If no pleiotropic defects are observed, the mutant allele of the gene can be introduced into the desirable crop to achieve resistance. When pleiotropic defects are observed different approaches based on genome editing or the mining of natural mutants in the germplasm, that do not display pleiotropic defects, can be used to avoid them [41, 43].

Resistance conferred by single R genes is highly specific and in most cases easily broken, as a single mutation in the corresponding pathogen effector can abolish perception by the plant's immunity [14]. Therefore, gene stacking of several R genes or the combination of R genes and QTLs can confer a broader spectrum and more durable resistance [85]. This approach, however, requires knowledge on the specificity of R genes [86]. The dispensable nature or the scarcity of R genes in some pathosystems makes the use of mutant S genes a particularly attractive alternative for breeding. Especially in pathosystems in which resistance has not been identified yet, research and deployment of S genes should be prioritized. Besides their use as a sole source of resistance S genes should also be deployed as an additional source of resistance. Generally, pathogens are dependent on susceptibility factors to complete specific processes. That means that for the pathogen to overcome the resistance conferred by mutant S genes it must acquire a new function, which is much more difficult than the loss or mutation of single effectors [19]. Therefore, the use of mutant S genes together with other sources of resistance can act as an additional layer of resistance.

What is the role of auxin in the tomato- *Cm* interaction?

Plant pathogens have evolved multiple strategies to promote disease in their hosts. These strategies include the secretion of effectors and the production of phytohormones or their analogues, that interfere with host processes [14, 87]. Several pathogens possess the ability to produce the phytohormone auxin, and in many cases pathogen-derived auxin has been implicated in pathogen virulence [88-90]. Even though the role of auxin in *Cm* virulence has not been described in literature, based on our results in **Chapter 3** we hypothesized that auxin might be involved in the tomato- *Cm* interaction.

The observed resistance in *WAT1* mutants has been associated with altered contents of auxin (IAA) and its precursor tryptophan (Trp) [30, 84]. Based on the observed

functional conservation of *WAT1* between Arabidopsis and cotton, we hypothesized that impairment of *SlWAT1* would also lead to IAA related changes in tomato. Our results indicate that the loss-of-function of *SlWAT1* indeed affects the contents of free IAA and expression of auxin related genes in tomato (**Chapter 3**).

One of the most exciting recent findings has been that auxin can act as a microbial signal to modulate bacterial gene expression [87, 89]. Recent studies in the auxin producing *Pseudomonas syringae pv. tomato* (*Pst*) DC3000 have reported that auxin acts as a microbial signal both *in planta* and in cultures that regulates the expression of virulence associated genes [89]. The reduced levels of free IAA in *SlWAT1* impaired tomato stems and the lack of symptoms prompted us to analyse the expression of *Cm* associated virulence genes. In **Chapter 3**, we found that the expression of specific *Cm* virulence factors was downregulated on *SlWAT1* impaired plants. These results suggest that IAA might act as a microbial signal in the *Cm*-tomato interaction to regulate the expression of bacterial genes.

Apart from its function as a signalling molecule during plant pathogen interactions bacterial-derived auxin can also act as a virulence factor, as it was shown that *Pst* auxin mutants are significantly less virulent than the wildtype [89]. Trp is the major precursor of IAA and five pathways for the production of IAA through Trp have been described in bacteria [87]. Taking into consideration the observed functional conservation of *WAT1* between species, one could assume that Trp levels will also be reduced in tomato *SlWAT1* impaired plants. Accordingly, we hypothesized that *Cm* is potentially able to produce auxin using Trp and that the reduction in levels of plant Trp could lead to reduced IAA production by the bacterium. In turn, the reduced bacterial derived auxin could have an effect on the observed symptoms.

Although the potential production of auxin by *Cm* has not been addressed in literature, the closely related pathogen *Rhodococcus fascians* uses the indole-3-pyruvate (IPyA) pathway to produce auxin from Trp [91]. Thus, we hypothesized that *Cm* could possibly use the same pathway if it is able to produce auxin. On the basis of this assumption, we used a phylogenetic analysis to detect any homologs of genes known to participate in the pathway in the genome of *Cm*. Our analysis resulted in the identification of homologs for each of the three steps of the pathway. That further enhanced our hypothesis that *Cm* can produce auxin. All the identified genes were present on the circular chromosome of *Cm,* indicating that the pathway is chromosomally encoded (Fig. 2A & 2B). To confirm the hypothesis of auxin production, we subsequently used high performance liquid chromatography to

detect the presence of auxin in *Cm* cultures rich in tryptophan. Our preliminary results confirmed the presence of IAA both in lysed *Cm* cells and the supernatant of the cultures (Fig. 2C). The detection of IAA in the supernatant of cultures suggests that *Cm* is able to produce and secrete auxin.

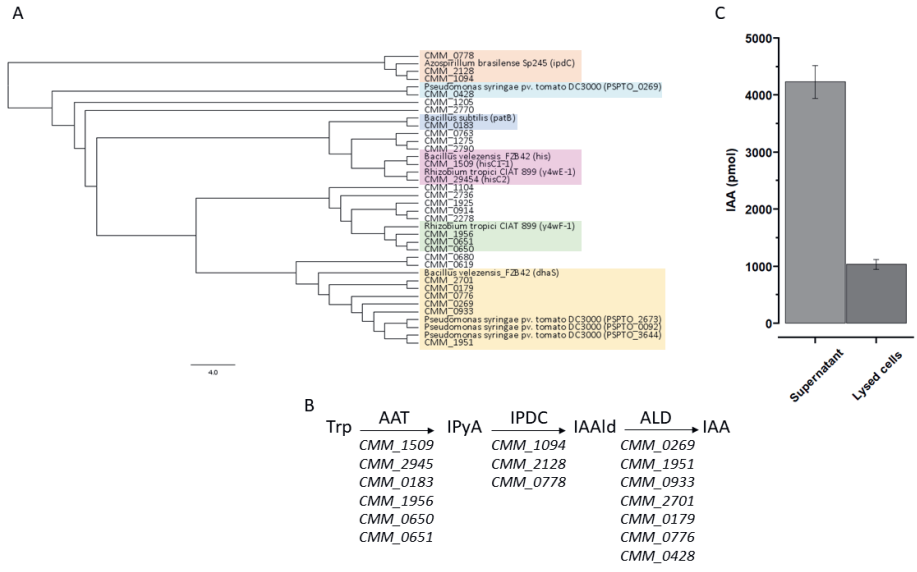

Figure 1| Auxin production by *Clavibacter michiganensis*. A) Phylogenetic analysis of orthologs involved in the production of auxin in the IPyA pathway. Clades in different colours highlight the *Cm* homologs and their orthologs in other auxin producing bacteria. B) Proposed *Cm* pathway for the production of auxin. The IPyA pathway is a three step pathway. The first step involves the conversion of Trp into IPyA by amino acid amino transferases (AAT). In the second step, IPyA is catalysed by IPyA decarboxylases (IPDC) into indole-3-acetaldehyde (IAAld). Finally, IAAld is converted into IAA by IAAld dehydrogenases (ALD). For each of the steps the identified homologs are given below the arrows. C) Average IAA amount (pmol) quantified through chromatography in the supernatant and lysed *Cm* cells. Bars indicate error bars (n=5).

Based on these results, I formulated a model for the role of auxin in the tomato- *Cm* pathosystem. This model proposes a dual role for auxin in the interaction (Fig. 3). Firstly, plant-derived auxin is used as a signalling molecule to regulate the expression of *Cm* virulence factors. Secondly, the pathogen produces auxin that enhances its virulence. Auxin is involved in multiple developmental processes in plants, including cell elongation and expansion through the loosening of cell walls [92]. Hence, in our model we assumed that the bacterial derived auxin is used in accessory with plant derived auxin by *Cm* to loosen up the cell walls of tomato during infection. Loosening of cell walls would allow for easier degradation of the cells by the hydrolytic enzymes

produced by *Cm*. Sequentially, the degradation products could serve as a source of nutrients for the pathogen. In addition, loosening of cells walls by bacterial derived auxin may potentially contribute to the development of necrotic symptoms by toxins produced by *Cm*.

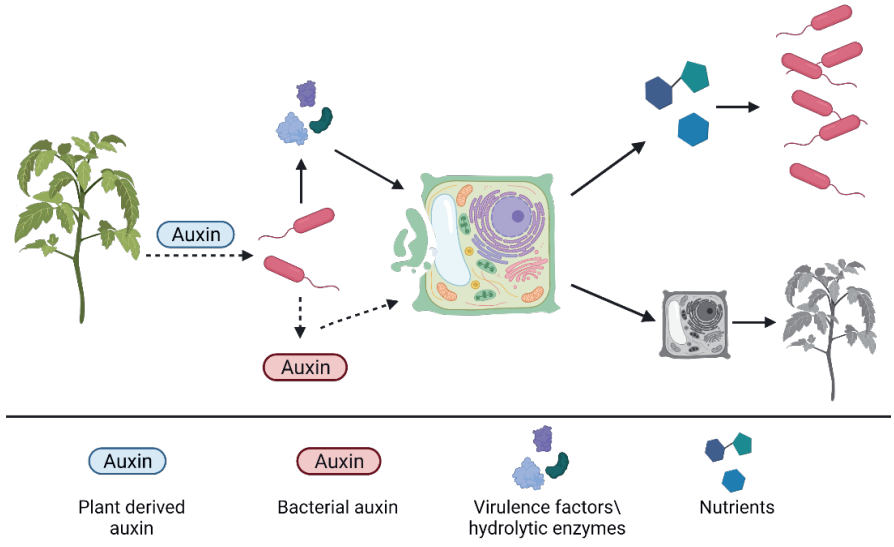

Figure 2| Hypothetical model for the role of auxin in the tomato- *Cm* interaction. Plant derived auxin is used by the pathogen for the expression of its virulence factors. In parallel, the pathogen produces auxin that is involved in loosening and lysis of tomato cell walls. The lysis of the cells could be used by the pathogen for either the acquisition of nutrients that lead to the growth of the bacteria or the death of cells that leads to necrotic symptoms. Figure made with BioRender.

Future studies concerning the possible role of auxin in the interaction are needed to confirm our hypothesis. The use of tomato mutants impaired in auxin synthesis or perception, such as the auxin resistant *Diageotropica* mutant [93], could allow for further confirmation for the role of auxin in the interaction. Furthermore, functional analyses of the candidate genes of *Cm*, we identified through phylogenetic analyses, are crucial in determining which genes are involved in the biosynthetic pathway of auxin. To this end, we developed a CRISPR/Cas9 system that allows the rapid genetic manipulation of the pathogen for the production of mutants (data not shown). These mutants can be used both in the elucidation of the pathway, as well as determining which is the effect of bacterial-derived auxin on *Cm* virulence. Finally, we assumed that *Cm* uses the IPyA pathway to convert Trp to IAA, however, the presence of other

biosynthetic pathways should also be assessed. We could assume that in the presence of multiple IAA biosynthetic pathways in the pathogen production of IAA *in planta* may require additional pathways that are not expressed in culture [90]. Phylogenetic studies based on the presence of homologs from other pathways, together with expression analysis of the potential homologs may provide further insights into the biosynthesis of auxin. Lastly, one should also take into account that the pathogen potentially produces other phytohormones that may be important for its virulence. Ethylene has been found to be an essential phytohormone involved in the tomato- *Cm* interaction, as the induction of its levels in the host contributes to the development of wilting symptoms. Our results (**Chapter 3**) suggest that ethylene is downregulated in RNAi::SlWAT1 transgenic plants and that could contribute to the reduction of observed wilting symptoms. Therefore, the potential of *Cm* to produce ethylene should also be assessed.

Why is tomato only tolerant to *Cm*?

Efforts to produce tomato cultivars that are resistant to *Cm* started almost 90 years ago [94, 95]. So far, however, only tolerant sources with a highly complex genetic background have been identified in wild tomato germplasm [94, 96, 97]. In **Chapter 6,** we studied the progeny of a cross between tomato (*S. lycopersicum*) and the highly tolerant *S. arcanum* LA2157, in an effort to identify the genes underlying the observed tolerance conferred by a QTL on chromosome 7. Instead of simplifying the genetic basis of the observed tolerance, our results suggested that two additional loci that were not previously described are required for tolerance to *Cm*. This once again highlighted the genetic complexity of tolerance to *Cm*. In **Chapters 3, 4 and 5**, we attempted to introduce tomato resistance to *Cm* by the mutation of host S genes. Of the candidate S genes we studied, loss-of-function of *SlWAT1* resulted in loss-of-susceptibility to *Cm*. Nonetheless, mutation of the gene only led to tolerance to the pathogen, as the bacterium reached high population densities *in planta* (**Chapter 3**).

Even though vascular bacterial pathogens are among the most aggressive phytopathogens, R genes have been identified only for a small number of xylem-colonizing bacterial pathogens [98, 99]. One of the questions regarding xylem-pathogens is if the plant is able to sense them, as the xylem is predominantly composed of dead cells [98]. The limited contact of xylem pathogens with living cells has been proposed as an explanation for the scarcity of resistance genes for this group of pathogens [99].

Although largely speculative, the possibility that tomato has not yet evolved resistance against the pathogen cannot be excluded. It has been suggested that the *Clavibacter* species have only recently emerged as pathogens, which developed from endophytic bacteria. Pathogenicity of *Clavibacter* species was speculated to have possibly evolved through the horizontal gene transfer of virulence genes [100]. According to the zig-zag model of the evolution of the plant immune system, pathogen pressure drives the evolution of resistance genes in plants [14]. *Cm* is classified as a quarantine or a regulated pathogen in several countries around the world [101]. Taking into account the fact that its quarantine status limits its association with the host and that the bacterium is considered to have become pathogenic quite recently, one could hypothesize that tomato did not have the time or pathogen pressure required for the evolution of R genes [102]. Additionally, in the genome of *Cm* no genes coding for typical type III bacterial effectors, present in Gram-negative bacteria, that are injected into host cells exist. It has therefore been proposed that a classical gene-for-gene interaction resulting in resistance, based on the perception of effectors by R genes, does not exist in the pathosystem [103].

Is plant death a population resistance response to *Cm*?

Cell death is thought to have a central role in plant immune responses to biotrophs. Successful perception of pathogens by resistance proteins typically results in a hypersensitive response (HR), a form of rapid localized cell death at the site of attempted pathogen penetration [104]. HR is a widespread phenomenon in plants and it is usually associated with highly effective resistance to (hemi)biotrophic pathogens [14, 104, 105].

Contrary to this localized cell death, extensive tissue necrosis and plant death are commonly associated with susceptibility of hosts to pathogens. Especially in the case of necrotrophs cell death can prove beneficial to the pathogens, as they require dead tissue to acquire nutrients [104]. Even though plant death is considered to be a failure of host resistance, a new study suggested that plant death may function as a resistance mechanism at the population level for viral infections [106]. According to the model proposed by this study, death of infected plants may restrict the transmission of the pathogen to healthy plants that are in close proximity. This “suicidal pathogen elimination” might save closely related organisms from infection, therefore contributing to resistance at a population level [106]. This population resistance mechanism has been attributed to insufficient induction of plant defences. In addition, it was hypothesized that resistance of this type is generally

most efficient for pathogens with a narrow host range [106]. Another study in bacterial populations infected with bacteriophages also concluded that this "suicidal pathogen elimination" strategy may prevent pathogen spread to nearby bacterial cells. Additionally, the same study suggested that the induction of high pathogen virulence could be a host strategy, that results in host death and eventual restriction of pathogen dispersal [107].

Even though infection of plants by *Cm* results in the upregulation of genes associated with basal defence in the host, the upregulation of these genes is not sufficient for the induction of resistance [40, 108]. As a consequence, infection of tomato plants by *Cm* will in most cases lead to plant death. Based on the aforementioned proposed model, we wondered if plant death could be a population resistance mechanism to *Cm*. Considering the mode of *Cm* spread and the fact that the pathogen has a very narrow host range, we speculated that such a population resistance mechanism might be beneficial to the host [109, 110].

Spread of *Cm* is facilitated both by infected seeds and infected plant debris that remain in the soil from previous cultivations [111]. Even though *Cm* can persist on both sources for relatively long periods of time, the pathogen is able to survive on seeds for several years [112, 113]. The dispersal of tomato seeds by animals or by trade is responsible for the spread of *Cm* over long distances [112]. Thus, plant death stops the production of seeds by infected plants and may eliminate the long distance spread of the pathogen. In turn, the limited spread of the pathogen may decrease the occurrence of disease in new areas, giving the host an advantage over the bacterium.

Is resistance against *Cm* an achievable goal?

Attempts for the identification of resistance to *Cm* in wild accessions have so far only yielded QTLs linked to tolerance [94, 96, 97, 114]. In this thesis we set out to identify S genes, the mutant alleles of which would potentially confer resistance to *Cm* (**Chapter 3**, **4** and **5**). Our efforts, however, once again resulted in the identification of high tolerance to the pathogen (**Chapter 3** and **6**).

Tolerance and resistance represent two major mechanisms of plant defences against pathogens [115, 116]. Both mechanisms aim at the survival and reproduction of the host despite infection [117]. While resistance limits pathogen multiplication, host tolerance aims at the reduction of the effects of infection on the host regardless the size of pathogen population [116]. The selection imposed by the two mechanisms on

the pathogen, however, lead to distinct outcomes. While evolution of host resistance results in the reduction of pathogen prevalence in the host population, tolerance eventually leads to increased prevalence of the pathogen [116]. The quarantine or restricted status of *Cm* in many countries around the world makes the discovery of resistance against the pathogen an essential goal.

Even though, our efforts to introduce resistance to *Cm* in tomato by identifying and mutating S genes did not result in resistance, that does not necessarily mean that mutant S genes cannot lead to resistance. Identification of S genes that are potentially involved in the sustainment of the pathogen or that facilitate its entry into the host, might represent gene targets that could lead to resistance [19]. For instance, some vascular pathogens, including *Cm*, are able to enter their host through hydathodes [118-120]. The role of hydathodes in immunity against pathogens is still poorly understood. Nevertheless, genes involved in PAMP-triggered immunity (PTI) and reactive oxygen species (ROS) production have been found to be expressed at the hydathodes [121]. One could assume that adapted pathogens have found ways to suppress defence responses at the hydathodes to enter their hosts. Therefore, identification and mutation of genes that are manipulated by bacteria to enter their hosts through hydathodes, could inhibit pathogen entry and result in resistance.

Nonhost resistance (NHR) is defined as a broad-spectrum plant defence, which confers immunity to an entire plant species to all isolates of a microbe that is pathogenic on other species [122]. NHR is usually both broad-spectrum and durable, making it highly desirable for crop improvement [123]. For that reason, the identification and introduction of NHR components host species can result in resistance. Transgenic approaches have already succeeded in introducing NHR components from nonhost to host species [124, 125]. For instance, expression of the *Bs2* pepper gene in tomato results in resistance to the bacterial pathogen *Xanthomonas campestris* pv. *vesicatoria*, through recognition of the corresponding avrBs2 effector [126]. Another example of a nonhost gene successfully conferring resistance to a host species is maize gene RXO1. Introduction of the gene in rice results in resistance to the rice pathogen *X. oryzae* pv. *oryzicola* [127, 128]. Induction of nonhost defences upon infection of non-adapted pathogens usually results in HR cell death [122]. It has already been demonstrated that *Cm* proteins can elicit an HR responses on nonhost species. The ChpG and Chp-7 serine proteases secreted by *Cm* can elicit HR responses in the nonhost eggplant and *Nicotiana* species, respectively

[129, 130]. Identification of the nonhost genes underlying the HR response to these serine proteases, and their introduction in tomato might be a way to gain resistance against *Cm*.

Another possibility to introduce tomato resistance to *Cm* is the identification of host effector targets and their use to engineer NLR receptors [131]. Several *Cm* proteins that are crucial for its virulence have been identified [132-134]. Nevertheless, the targets of these proteins have not yet been explored. Some effector proteins in host cells can be identified by intracellular immune receptors in host cells [14]. Most of these intracellular receptors belong to the nucleotide-binding leucine-rich repeat (NLR) family and can induce defence signalling pathways upon perception of a corresponding effector protein [131]. In fact, the majority of cloned R genes encode intracellular receptors that belong to NLRs [135]. These NLR receptors generally consist of a coiled-coil (CC) or Toll/IL-1 receptor (TIR) domain, a central nucleotide binding domain, and a leucine rich repeat domain [136]. A subset of these NLRs contains an additional unconventional integrated domain (ID). Integration of such domains has been found to be frequent and widespread in plants and they can resemble the effector host target [131, 135]. Recent studies have demonstrated that engineering of such IDs can expand the resistance conferred by NLRs. The introduction of IDs that are targets of *Cm* virulence proteins in endogenous tomato R genes might be a possible way to gain resistance against the pathogen. In rice, the *Mangaporthe oryzae* effector AVR-Pik is able to bind and stabilize heavy-metal associated (HMA) proteins OsHIP19 and OsHIPP20 [137]. The corresponding NLR receptor Pik-1 confers resistance to *M. oryzae* by recognition of AVR-Pik, through binding of the effector to a HMA integrated domain [131]. Exchange of the Pik-1 HMA with the OsHIP19 HMA resulted in the extension of binding capability of Pik-1 leading to responses to AVR-Pik variants that were previously unrecognised [131]. In addition, introduction of mutations in the Pik-1 HMA domain also resulted in extended binding capacity of the NLR [131]. Interestingly, the *OsHIPP20* gene that is bound by the AVR-Pik effector was shown to be an S gene [137].

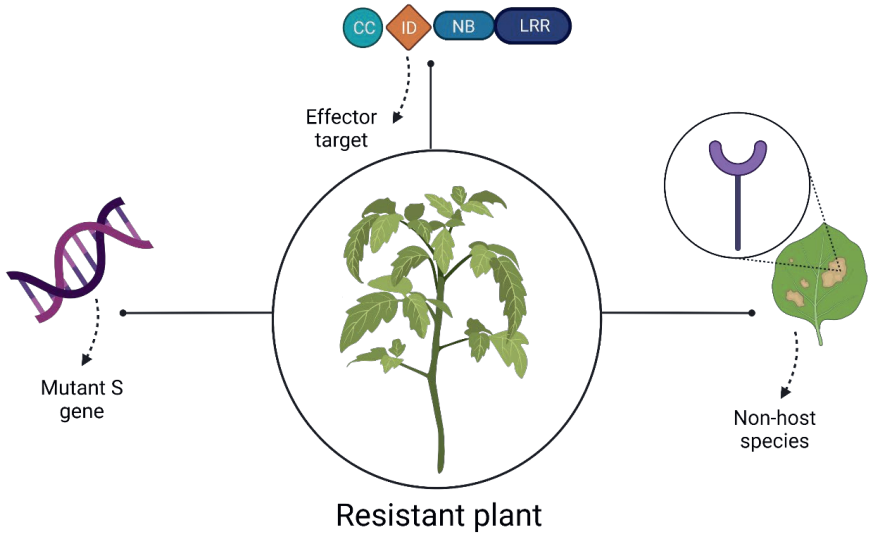

Figure 3| Proposed ways to gain tomato resistance to *Cm*. The mutation of S genes involved in the replication or entry of *Cm* to the host could provide resistance to the pathogen. Additionally, the identification of effector targets could be used to engineer R genes that recognize the effector and trigger immune responses. Finally, the identification and introduction of receptors that lead to resistance to *Cm* in nonhost species into tomato could be another possibility. Figure made with BioRender.

Concluding remarks

In this thesis I have worked on the identification of determinants of susceptibility and the study of their role in the interaction between tomato and the bacterial pathogen *Clavibacter michiganensis*. Through the results described in this thesis I have tried to shed light into new aspects of the interaction. An aspect that still remains unexplored in the interaction is the determination of the host targets of *Clavibacter michiganensis* proteins. Host targets of these proteins might function as S genes in the interaction providing us with novel sources of resistance. Identification of such targets may provide exciting opportunities to continue studying the pathosystem in the future. Ultimately, better understanding of the interaction will provide us with new ways to control the pathogen. I am certain that the accumulating knowledge on plant S and R genes combined with the advances in genome editing will provide powerful solutions to gain resistance against different pathogens, including *Clavibacter michiganensis*.

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APPENDICES

Summary

Samenvatting

Acknowledgments

About the author

Education statement

SUMMARY

SUMMARY

Bacterial canker of tomato (*Solanum lycopersicum*), caused by the phytopathogenic Gram-positive bacterium *Clavibacter michiganensis* (*Cm*), is considered to be one of the most destructive diseases of tomato. Outbreaks of the disease can result in substantial yield and economic losses of both field and greenhouse grown tomatoes. Infected seeds, unhygienic cultural practices, contaminated plant debris and mechanical contact between infected and non-infected plants all contribute in the dissemination of the pathogen. *Cm* colonizes the xylem vessels of the plants and can cause symptoms systemically. Depending on the type of infection, symptoms may vary. Systemic infections typically cause wilting of leaves, cankers on the stems and petioles of tomato plants and stem discoloration and necrosis. Localized infections cause marginal necrosis and bird's eye spots on fruits.

Despite extensive breeding efforts, no resistance against the bacterium has been identified yet. Biological and chemical measures to control *Cm* are limited. Control of the pathogen is mainly based on the "good seed and plant practice" protocol that aims to reduce the risks of introduction and spreading of the pathogen. In an effort to control the consequences of bacterial canker, *Cm* is classified as a quarantine or A2 regulated non-quarantintine pest in multiple countries around the world. No cultivar with resistance against *Cm* is available on the market. Yet, different tolerant wild accessions have been identified. The background of tolerance conferred by these accessions, however, is complex and polygenic.

This thesis aimed at identifying determinants of susceptibility in the tomato- *Cm* interaction, in an effort to bypass the problem of the complex genetics of tolerance and to identify resistance to the pathogen. A large part of this thesis explores the possibility of using mutant alleles of host susceptibility (S) genes to gain resistance to *Cm*. A chapter of this thesis is also dedicated to the identification of the loci underlying the tolerance observed in a previously described cross between tomato and the accession *S. arcanum* LA2157.

In **Chapter 2**, we review the current knowledge on plant S genes, with a special focus on known host genes manipulated by bacteria. In this review, we highlight ways to identify and modify S genes to gain resistance to pathogens. Finally, we propose a new class of S genes involved in the translocation of bacterial effector proteins in host cells.

Chapter 3 describes the role of gene *SlWAT1* in tomato susceptibility to *Cm*. Using knock-down and knock-out tomato lines we functionally characterised *SlWAT1* as a susceptibility factor to genetically diverse *Cm* strains. Further, we worked towards understanding the molecular mechanism of the tolerance observed after inactivation of *SlWAT1*. Our results suggest a potential role for auxin in the interaction. Finally, we show that silencing of *SlWAT1* leads to the downregulation of specific bacterial virulence factors, which possibly contributes to the reduction of symptom development on transgenic plants.

Chapters 4 and **5** aimed at the identification and functional characterization of candidate S genes. Using post-transcriptional silencing through virus-induced gene silencing (VIGS), we selected three candidate genes (*SlWRKY23*, and the putative oxygenases *Solyc09g089680.3* and *Solyc12g005380.2)* that led to reduced symptom development upon silencing for further analysis. For these genes, we generated mutants using CRISPR/Cas9 and studied potential changes in susceptibility and bacterial dynamics of *Cm* in the mutants. While silencing of the genes resulted in a reduction in susceptibility of tomato plants to *Cm*, knock out of mutants of the genes through CRISPR/Cas9 did not exhibit any changes in susceptibility. We hypothesized that the differences we observed in susceptibility between the knock-out and knock-downs of the genes was due to an active genetic compensation response.

In **Chapter 6**, we set out to identify the genes underlying tolerance of the major QTL on chromosome 7 observed in a cross between the accession *S. arcanum* LA2157 and *S. lycopersicum*. To our surprise, the QTL on chromosome 7 that was previously found to result in high tolerance to *Cm*, did not co-segregate with our observed tolerant phenotypes. Therefore, we employed a bulk segregant analysis (BSA) strategy to identify the causal loci for the observed tolerance. Two loci linked to tolerance on chromosomes 2 and 4 of tomato were mapped. In these loci two genes, namely DWARF and vsf-1, contained resistance specific k-mers. Sequence analysis revealed that the alleles for both of the mapped genes in the resistant bulk were identical to the *S. arcanum* LA2157 alleles. Finally, we hypothesized that these two loci in combination with the previously reported QTL on chromosome 7 contribute to a high level of tolerance to the bacterium.

This thesis is concluded with **Chapter 7**, in which I summarize and discuss the implications of our main findings in achieving resistance to *Cm*. Several questions that arose during the completion of this thesis are discussed in this chapter. Finally, I propose a new direction for the study of this challenging pathosystem.

SAMENVATTING

SAMENVATTING

Bacteriekanker in tomaat (*Solanum lycopersicum*), veroorzaakt door de fytopathogene Gram-positieve bacterie *Clavibacter michiganensis* (*Cm*), wordt beschouwd als een van de meest destructieve ziekten van tomaat. Uitbraken van de ziekte kunnen leiden tot aanzienlijke opbrengst- en economische verliezen van zowel vollegrond- als kastomaten. Geïnfecteerde zaden, onhygiënische praktijken, besmette plantenresten, en direct contact tussen geïnfecteerde en niet-geïnfecteerde planten dragen allemaal bij aan de verspreiding van de ziekteverwekker. *Cm* koloniseert de xyleemvaten van de plant en kan systemisch symptomen veroorzaken. Afhankelijk van het type infectie kunnen de symptomen variëren. Systemische infecties veroorzaken meestal verwelking van bladeren, kankers op de stengels en bladstelen van tomatenplanten, en stengelverkleuring en necrose. Gelokaliseerde infecties veroorzaken verwelking en necrose van bladranden en topjes en vogeloogvlekken op fruit.

Ondanks uitgebreide veredelingsinspanningen is er nog geen resistentie tegen de bacterie vastgesteld. Biologische en chemische maatregelen voor de bestrijding van *Cm* zijn beperkt. De bestrijding van de ziekteverwekker is voornamelijk gebaseerd op het “good seed and plant practice”-protocol dat als doel heeft de risico's van introductie en verspreiding van de ziekteverwekker te verminderen. Als poging om de gevolgen van Bacteriekanker onder controle te houden, is *Cm* in meerdere landen over de hele wereld geclassificeerd als een quarantaine- of A2-gereguleerde niet-quarantaineplaag. Tot nu toe is er geen gewas met resistentie tegen *Cm* op de markt. Toch zijn er verschillende tolerante wilde accessies geïdentificeerd. De achtergrond van tolerantie die door deze toetredingen wordt verleend, is echter complex en polygeen.

Dit proefschrift was gericht op het identificeren van determinanten de gevoeligheid in de tomaat-*Cm*-interactie, in een poging het probleem van de complexe genetica van tolerantie te omzeilen en resistentie tegen de ziekteverwekker te identificeren. Een groot deel van dit proefschrift onderzoekt de mogelijkheid om mutant allelen van gastheergevoeligheid (S) genen te gebruiken om resistentie tegen *Cm* te krijgen. Een hoofdstuk van dit proefschrift is ook gewijd aan de identificatie van de loci die ten grondslag liggen aan de tolerantie die wordt waargenomen in een eerder beschreven kruising tussen tomaat en de accessie *S. arcanum* LA2157.

In **Hoofdstuk 2** bespreken we de huidige kennis over S-genen van planten, met uitgebreide aandacht voor bekende gastheergenen die door bacteriën worden gemanipuleerd. In deze review belichten we manieren om S-genen te identificeren en te wijzigen om resistentie tegen pathogenen te krijgen. Ten slotte stellen we een nieuwe klasse S-genen voor die betrokken zijn bij de translocatie van bacteriële effectoreiwitten in gastheercellen.

Hoofdstuk 3 beschrijft de rol van het gen *SlWAT1* in de gevoeligheid van tomaten voor *Cm*. Met behulp van knock-down en knock-out tomatenlijnen hebben we *SlWAT1* functioneel gekarakteriseerd als een gevoeligheidsfactor voor genetisch diverse *Cm*-stammen. Verder hebben we gewerkt aan het begrijpen van het moleculaire mechanisme van de tolerantie die wordt waargenomen na inactivering van *SlWAT1*. Onze resultaten suggereren een mogelijke rol voor auxine in deze interactie. Ten slotte laten we zien dat silencing van *SlWAT1* leidt tot de neerwaartse regulatie van specifieke bacteriële virulentiefactoren, wat mogelijk bijdraagt aan de vermindering van symptoomontwikkeling op transgene planten.

Hoofdstukken 4 en **5** waren gericht op de identificatie en functionele karakterisering van kandidaat-S-genen. Met behulp van post-transcriptionele silencing door middel van virus-geïnduceerde gen silencing (VIGS), selecteerden we drie kandidaat-genen (*SlWRKY23* en de vermeende oxygenasen *Solyc09g089680.3* en *Solyc12g005380.2*) die leidden tot verminderde symptoomontwikkeling na silencing voor verdere analyse. Voor deze genen hebben we mutanten gegenereerd met CRISPR/Cas9 en mogelijke veranderingen in gevoeligheid en bacteriële dynamiek van *Cm* in de mutanten bestudeerd. Terwijl het uitschakelen van de genen resulteerde in een vermindering van de gevoeligheid van tomatenplanten voor *Cm*, vertoonde knock-out van mutanten van de genen via CRISPR/Cas9 geen veranderingen in gevoeligheid. We veronderstelden dat de verschillen die we waarnamen in gevoeligheid tussen de knock-out en knock-downs van de genen te wijten waren aan een actieve genetische compensatierespons.

In **Hoofdstuk 6** zijn we begonnen met het identificeren van de genen die ten grondslag liggen aan tolerantie van de belangrijkste QTL op chromosoom 7, waargenomen in een kruising tussen de toetreding *S. arcanum* LA2157 en *S. lycopersicum*. Tot onze verbazing, de QTL op chromosoom 7 die eerder werd vastgesteld als reden van de hoge tolerantie voor *Cm* co-segregeerde niet samen

met onze waargenomen tolerante fenotypes. Daarom hebben we een bulk segregant-analyse (BSA)-strategie gebruikt om de causale loci voor de waargenomen tolerantie te identificeren. Twee loci gekoppeld aan tolerantie op chromosomen 2 en 4 van tomaat werden in kaart gebracht. Twee genen liggen bij deze loci, namelijk DWARF en vsf-1, die specifieke k-meren bevatten die betrokken zijn bij resistentie. Sequentieanalyse onthulde dat de allelen voor beide in kaart gebrachte genen in de resistente bulk identiek waren aan de *S. arcanum* LA2157-allelen. Ten slotte veronderstelden we dat deze twee loci in combinatie met de eerder gerapporteerde QTL op chromosoom 7 bijdragen aan een hoge mate van tolerantie voor de bacterie.

Dit proefschrift wordt afgesloten met **Hoofdstuk 7**, waarin ik de implicaties van onze belangrijkste bevindingen voor het bereiken van resistentie tegen *Cm* samenvat en bespreek. In dit hoofdstuk worden een aantal vragen besproken die tijdens het afronden van dit proefschrift zijn opgekomen. Ten slotte stel ik een nieuwe richting voor de studie van dit uitdagende pathosysteem voor.

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ABOUT THE AUTHOR

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Eleni Koseoglou was born on 5 October 1992 in Katerini, Greece. In 2010 she was admitted to the School of Agricultural Sciences at University of Thessaly, from where she graduated with an integrated MSc in Crop Production and Rural Environment in 2015. In 2017, she obtained an MSc in Plant Sciences with specialization in Plant Breeding and Genetic Resources from Wageningen University & Research. In October 2017, she received a personal grant from the Dutch Research Council (NWO) for her PhD proposal on plant susceptibility genes. In January 2018, she started a PhD on the topic in the Laboratory of Plant Breeding at Wageningen University and Research.

EDUCATION STATEMENT

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Eleni Koseoglou**
Date: **18 November 2022**
Group: **Plant Breeding**
University: **Wageningen University & Research**

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project** "The other side of resistance: Loss-of-function of susceptibility genes in tomato for resistance against *Clavibacter michiganensis* subsp. *michiganensis*"	10 Jul 2018	1.5
► **Writing or rewriting a project proposal**		
► **MSc courses**		
	Subtotal Start-Up Phase	1.5

2) Scientific Exposure	*date*	*cp*
▶ **EPS PhD student days**		
EPS PhD Get2Gether, Soest (NL)	11-12 Feb 2019	0.6
EPS PhD Get2Gether, online	1-2 Feb 2021	0.4
▶ **EPS theme symposia**		
EPS theme 2 symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents', Amsterdam (NL)	24 Jan 2018	0.3
EPS theme 2 symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents', Wageningen (NL)	1 Feb 2019	0.3
EPS theme 2 symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents', Utrecht (NL)	4 Feb 2020	0.3
EPS theme 2 symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents', online	9 Feb 2021	0.2
EPS theme 2 symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents', online	8 Feb 2022	0.2
EPS theme 3 'Adaptation and metabolism', Wageningen (NL)	5 Nov 2021	0.3
▶ **Lunteren Days and other national platforms**		
Annual Meeting "Experimental Plant Sciences", Lunteren (NL)	9-10 Apr 2018	0.6
Annual Meeting "Experimental Plant Sciences", Lunteren (NL)	8-9 Apr 2019	0.6
Annual Meeting "Experimental Plant Sciences", online	13-14 Apr 2021	0.5
Annual Meeting "Experimental Plant Sciences", Lunteren (NL)	11-12 Apr 2022	0.6
▶ **Seminars (series), workshops and symposia**		

Studium Generale: Plant Philosophy - The Green Darwin about Dates and Dinner, Wageningen (NL)	30 Jan 2018	0.1
Symposium: CRISPR-Cas: From Evolution to Revolution, Wageningen (NL)	8 Mar 2018	0.2
IS-MPMI workshop: Taking MPMI Discoveries to the Field, online	2 Dec 2020	0.2
IS-MPMI Seminar: Dual Role of Auxin in Regulating Plant Defense and Bacterial Virulence Gene Expression During Pseudomonas syringae PtoDC3000 Pathogenesis, online	13 Apr 2021	0.1
WEES seminar: Daniel Croll-"Drivers and breaks of pathogen emergence"	18 Feb 2021	0.1
WEES seminar: Eske Willerslev- "The hunt for our molecular past"	17 Mar 2021	0.1
WEES seminar: Cyrus A. Mallon- "The patterns and mechanisms of microbial invasions"	23 Sep 2021	0.1
WEES seminar: Simon Griffith- "The zebra finch: lessons from the outback into a 'model' system"	21 Oct 2021	0.1
WEES seminar: Annette Becker- "Short history of carpel development and evolution"	16 Dec 2021	0.1
WEES seminar: Charissa de Bekker- "Connecting parasite and host genomes with behavioral phenomes using zombie ants as a model"	24 Feb 2022	0.1
EPS CRISPR/Cas workshop, Wageningen (NL)	13-14 Sep 2021	0.6
▶ **Seminar plus**		
▶ **International symposia and congresses**		
SOL International Online Meeting 2020	9-11 Nov 2020	0.6
21st Eucarpia General Congress, online	23-26 Aug 2021	1.2
14th International Conference on Plant Pathogenic Bacteria (ICPPB), Assisi (IT)	3-8 Jul 2022	1.4
▶ **Presentations**		
Talk: "The other side of resistance: Loss-of-function of susceptibility genes for resistance to *Clavibacter michiganensis* subsp. *michiganensis*", Consortium kick-off meeting	13 Jun 2018	1.0
Talk: "Identification of candidate suceptibility genes (S) genes against *Clavibacter michiganensis* subsp. *michiganensis*", Biointeractions & Plant Health Business Unit meeting	27 Feb 2020	1.0
Talk: "Identification and impairment of susceptibility genes involved in the tomato-*Clavibacter michiganensis* interaction", EPS PhD Get2Gether 2021	2 Feb 2021	1.0
Talk: "Inactivation of *WALLS ARE THIN1* in tomato reduces susceptibility to the vascular bacterium *Clavibacter michiganensis*", EPS theme 2 & Willie Commelin Scholten Day "Interactions between plants and biotic agents"	9 Feb 2021	1.0
Talk: "Inactivation of tomato *WAT1* results in auxin-dependent tolerance to genetically diverse *Clavibacter michiganensis* strains", ICPPB 2022, Assisi (IT)	7 Jul 2021	1.0
Talk:"Final consortium presentation"	20 Apr 2022	1.0
Poster: "Impairement of *SlWAT1* leads to reduced susceptibility to *Clavibacter michiganensis*", SOL international meeting 2020	10-12 Nov 2020	1.0
Poster: "Inactivation of WAT1 in tomato reduces suscepibility to *Clavibacter michiganenis* through transcriptional reprogramming of auxin related genes", Annual Meeting "Experimental Plant Sciences"	13-14 Apr 2021	1.0

▶ **3rd year interview**		
▶ **Excursions**		
EPS company visit to Koppert Biological Systems, Rotterdam (NL)	26 Oct 2018	0.2
Subtotal Scientific Exposure		18.1

3) In-Depth Studies	*date*	*cp*
▶ **Advanced scientific courses & workshops**		
EPS course "The power of RNA-seq", Wageningen (NL)	11-13 Jun 2018	0.9
EPS course "Transcription factors and transcriptional regulation", Wageningen (NL)	10-12 Dec 2018	1.0
SLU-WUR course "Plant Breeding and Biotechnology", Wageningen (NL)	11-13 Jun 2019	1.6
HarvardX course "Statistics and R" by HarvardX (edX course), online	Apr 2020	0.6
G2P-SOL Advanced Training School II "Using Genetic Resource Data for Breeding", online	11-13 Oct 2021	0.6
▶ **Journal club**		
Member of the Plant Breeding PhD and Post-doc literature discussion club	Jun 2019 – May 2020	0.6
Member of the IdeaS Plant Breeding discussion group	Nov 2021 – Jul 2022	0.3
▶ **Individual research training**		
Molecular Biology/Plant Developmental Biology, WUR - High Performance Liquid Chromatography training	27 Jan/ 1 Apr 2022	0.6
Subtotal In-Depth Studies		6.2

4) Personal Development	*date*	*cp*
▶ **General skill training courses**		
Participation in the Plant Breeding PhD discussion club (peer consultation), Wageningen (NL)	Jan 2018 - May 2019	0.6
EPS Introduction course, Wageningen (NL)	27 Mar 2018	0.3
WGS PhD competence assessment, Wageningen (NL)	25-27 Nov 2018	0.3
WGS course "Supervising BSc and MSc thesis students", Wageningen (NL)	5-6 Mar 2020	0.6
YoungWUR workshop "Data Visualization - The information is beautiful WAY", online	14 Nov 2020	0.3
EPS workshop "Becoming a mindful scientist", online	10 Sep 2021	0.1
▶ **Organisation of meetings, PhD courses or outreach activities**		
Organizer & host of the "Plant Breeding Monday Seminars"	Jan 2019 - Jan 2021	1.5
▶ **Membership of EPS PhD Council**		
Subtotal Personal Development		3.7

5) Teaching & Supervision Duties	*date*	*cp*

▶ **Courses**		
▶ **Supervision of BSc/MSc students**		
"Functional Characterization of Candidate Susceptibility Genes Potentially Involved in the Tomato- *Clavibacter michiganensis* subsp. *michiganensis* interaction"- Melanie Smith, MSc thesis	Sep 2018 – Mar 2019	1.0
"Mapping of a major *Clavibacter michiganensis* subsp. *michiganensis* resistance QTL in tomato"- Derek Mudadirwa, MSc thesis	Jul 2019 – Jan 2020	1.0
"Analysis of candidate genes involved in the susceptibility of *S. lycopersicum* towards *Clavibacter michiganensis*"- Arcadio Garcia Pérez, MSc minor thesis	Mar-Jul 2021	1.0
	Subtotal Teaching & Supervision Duties	3.0

TOTAL NUMBER OF CREDIT POINTS*	**32.5**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.	
** A credit represents a normative study load of 28 hours of study.*	

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