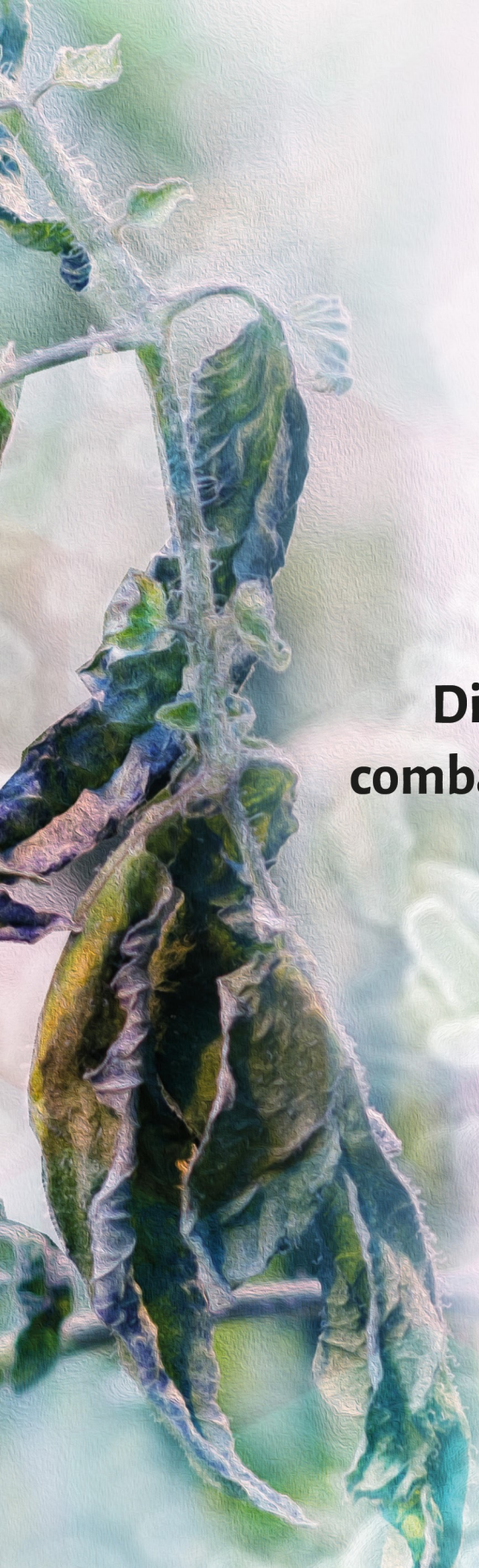

Different approaches of combating bacterial canker in tomato:

in pursuit of resistance

Mas Muniroh Mohd Nadzir

Propositions

1. Only one QTL is necessary for higher tolerance towards *Clavibacter michiganensis* subsp. *michiganensis*.
   (this thesis)

2. There is no *Clavibacter michiganensis* subsp. *michiganensis* free tomato.
   (this thesis)

3. Cultured meat is not a sustainable solution to reduce land use.

4. The fourth industrial revolution will widen social inequalities.

5. Cakes resolve conflicts.

6. Science needs funny people.

Propositions belonging to the thesis entitled

"Different approaches of combating bacterial canker in tomato: in pursuit of resistance"

Mas Muniroh binti Mohd Nadzir
Wageningen, 14 December 2018

Different approaches of combating bacterial canker in tomato:

in pursuit of resistance

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Different approaches of combating bacterial canker in tomato:

in pursuit of resistance

Mas Muniroh binti Mohd Nadzir

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CHAPTER 1

General introduction

The world of Cmm and tomato – possibilities to combat the pathogen

The unwanted guest –*Clavibacter michiganensis* subsp. *michiganensis*

The *Clavibacter michiganensis* (Cm) species belonging to the *Clavibacter* genus is a Gram-positive actinomycete. The *Clavibacter* genus was initially classified as *Corynebacterium* due to its morphological features (non-spore forming, irregular Gram-positive rods). Later on, it was discovered that there is no close relationship with *Corynebacterium* and the genus was classified to a different one (Eichenlaub et al. 2006). Currently, there are seven pathogenic Cm subspecies divided according to their host plant and two additional seed-associated and non-pathogenic subspecies. The pathogenic subspecies are *Clavibacter michiganensis* subsp. *michiganensis* which infects tomato (*Solanum lycopersicum*) (Strider 1969; Davis et al. 1984) and recently found to infect naranjilla (*Solanum quitoense* and *Solanum pectinatum*) (Bolanos-carriel et al. 2017), *Clavibacter michiganensis* subsp. *sepedonicus* which causes ring rot in potato (*Solanum tuberosum*) (Manzer and Genereux 1981), *Clavibacter michiganensis* subsp. *insidiosus* which causes wilting and stunting in alfalfa (*Medicago sativa*) (McCulloch 1925), *Clavibacter michiganensis* subsp. *nebraskensis* which promotes wilt and blight of maize (*Zea mays*) (Schuster et al. 1975), *Clavibacter michiganensis* subsp. *tessellarius* that results in leaf freckles and leaf spots in wheat (*Triticum aestivum*) (Carlson and Vidaver 1982), *Clavibacter michiganensis* subsp. *phaseoli* causing leaf yellowing in bean (*Phaseolus vulgaris* L.) (González and Trapiello 2014) and finally *Clavibacter michiganensis* subsp. *capsici* which causes bacterial canker in sweet pepper (*Capsicum annum*) and bell pepper (*Capsicum frutescens*) (Oh et al. 2016). The non-pathogenic subspecies are *Clavibacter michiganensis* subsp. *californiensis* and *Clavibacter michiganensis* subsp. *chilensis* which are associated with tomato and pepper (Yasuhara-Bell and Alvarez 2014). Recently, reclassification of some of the subspecies into species has been proposed by genomic data and a multi-locus phylogenetic analysis. The presently classified as *Clavibacter michiganensis* subsp. *capsici*, *Clavibacter michiganensis* subsp. *nebraskensis*, *Clavibacter michiganensis* subsp. *insidiosus, Clavibacter michiganensis* subsp. *sepedonicus* and *Clavibacter michiganensis* subsp. *tessellarius* should be reclassified as *Clavibacter capsici* sp. nov., comb. nov., *Clavibacter nebraskensis* comb. nov., *Clavibacter insidiosus* comb. nov., *Clavibacter sepedonicus* comb. nov., and *Clavibacter tessellarius* sp. nov., comb. nov., respectively, (Tambong 2017; Li et al. 2018).

The pathogen of our study is *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). *Clavibacter michiganensis* subsp. *michiganensis* is a mesophilic bacterium with an optimum growth temperature between 25 - 28°C, Cmm does not grow around 35°C and dies at a temperature of 50°C and higher. It grows fast and forms visible colonies three to seven days after plating on a selective agar medium. The optimum pH is

around 7 to 8, but Cmm still can grow as low as pH 5 (Strider 1969). The ability to grow at low pH is essential for Cmm because this allows it to grow in acidic xylem vessels (Eichenlaub et al. 2006).

Mode of entry and disease symptoms

Clavibacter michiganensis subsp. *michiganensis* is a pathogen which can lead to systemic or local infections. The pathogen can enter host plants through openings such as roots, shoots, stomata or hydathodes. The development of disease symptoms depends on the mode of entry, bacterial densities, environmental conditions, plant age at the time of infection, Cmm virulence and tomato genotypes (Sharabani et al. 2013b; Sen et al. 2013; Sharabani et al. 2014; Thapa et al. 2017).

Primary sources of infection are contaminated seeds, diseased seedlings, and infected debris (León et al. 2011; Lamichhane et al. 2011; Tancos et al. 2013). On the soil surface, Cmm can survive up to two years, but shorter periods when they are buried in the soil (Chang et al. 1992a; Gleason et al. 1993). The secondary spread from infected to uninfected plants occurs through agricultural practices such as pruning and watering. Secondary spread arises when Cmm enters non-infected plants through wounds or natural opening such as trichomes, stomas and hydathodes (Sharabani et al. 2013a; Frenkel et al. 2015; Sen et al. 2015; Chalupowicz et al. 2016) (Figure 1).

Systemic infection of Cmm results in stem canker and leaf wilting. Even though the pathogen is known as bacterial canker, the most apparent disease symptom is wilting. After entering the plant, Cmm has a biotrophic lifestyle (Eichenlaub and Gartemann 2011). It spreads in the plants through the vascular vessels resulting in unilateral wilting of leaves and leaflets, necrosis and cankers on stems, petioles and fruit lesions (Eichenlaub et al. 2006; de León et al. 2009). Localized infections in aerial parts of the plants resulting in marginal necrosis of leaflets (Werner et al. 2002), bird's-eye spots on fruits (Medina-Mora et al. 2001; Tancos et al. 2013) and white, small blister-like spots on leaves or stems (León et al. 2011; Chalupowicz et al. 2016). The spreading of Cmm from plant to plant is low if the bacterial density of the primary infection is low. *Clavibacter michiganensis* subsp. *michiganensis* contaminated seeds lead to infected seedlings, but the transmission rate is very variable (0.25 to 85%) (Strider 1969).

However, even a transmission rate as low as 0.01% can initiate a severe outbreak under favourable conditions (Chang 1991). Infection by Cmm does not always lead to plants with wilting symptoms, but these infected plants without symptoms still can be the starting point for secondary infections. Secondary infections can result in serious infections from as few as ten bacteria (Chang et al. 1992a; Frenkel et al. 2015).

Figure 1 | The mode of entry of Cmm. The primary inoculum can be contaminated seeds, seedlings or contaminated debris. a) Cmm can survive up to two years in remaining debris. It can enter through the roots of growing tomato plantlets. b) Latent infected plants may produce contaminated fruits and seeds. The infected seeds will subsequently be the vector and can be the reason for long distance dispersal. Cultural practices such as pruning (c) and watering (d) etc cause secondary spread.

Quarantine status and worldwide distribution

The severe consequences of Cmm infection and its easy spreading make it a quarantine organism listed under the EPPO A2. It can be present in Europe including the Mediterranean region, but Cmm is not commonly dispersed (Grund et al. 1990; Eichenlaub et al. 2006; Eichenlaub and Gartemann 2011; EPPO 2016). The pathogen is found in 16 EU Member States. Except for Greece, the Cmm population in the other EU Member States has a restricted distribution and has low prevalence (EFSA 2014). Due to its quarantine status in Europe, companies need to sell Cmm-free certified seeds and plant lots based on the Good Seed and Plant Practices (GSPP) (Munkvold 2009; GSPP Standard 2017). *Clavibacter michiganensis* subsp. *michiganensis* was first described and presumably originated from North America. The trade of contaminated seeds around the world is the main reason that the pathogen is now found in many continents (León et al. 2011) (Figure 2).

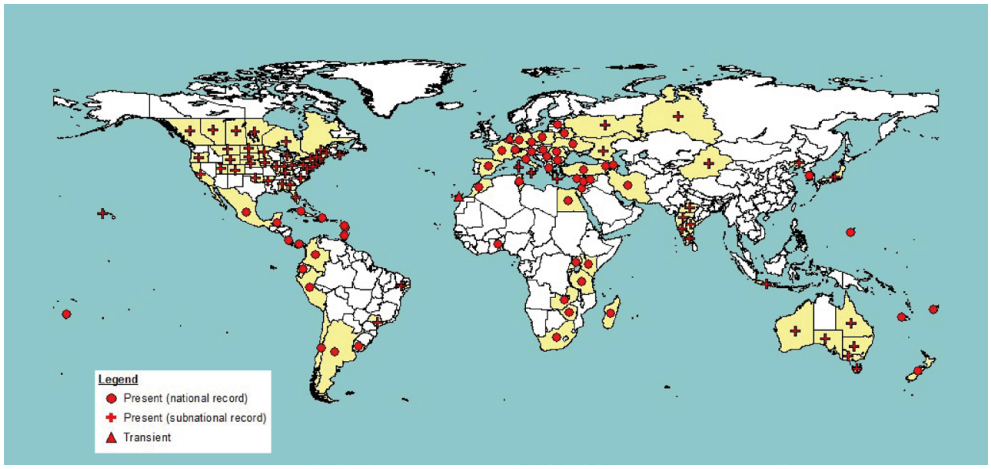

Figure 2 | Distribution of Cmm worldwide. Image extracted from EPPO (2017) PQR - EPPO database on quarantine pests http://www.eppo.int and used with permission.

The interaction between the unwilling host (tomato) and the unwanted guest (Cmm)

Clavibacter michiganensis subsp. *michiganensis* virulence

The first hypothesis why Cmm is virulent in tomato was based on the changes in the production of exopolysaccharides (EPS) (Rai and Strobel 1969). However, mutant Cmm strains with impaired EPS production were still virulent (Bermpohl et al. 1996). Molecular investigations (Eichenlaub et al. 1990; Meletzus and Eichenlaub 1991; Meletzus et al. 1993) showed that the virulent strain Cmm382 (also known as NCPPB382) has two circular plasmids, pCM1 (27 Kb) and pCM2 (70 Kb). Removing the plasmids resulted in the strain Cmm100, a plasmid-free endophytic strain (Meletzus et al. 1993). Both pCM1 and pCM2 carry virulence genes and strains with just a single plasmid resulted in less virulence and wilting. The pCM1 and pCM2 plasmids encode the pathogenicity genes *celA* or *pat*-1, respectively. *celA* encodes an endo-β-1,4-glucanase, and *pat*-1 encodes a serine protease. Later on, it was discovered that a region in the Cmm chromosome together with the plasmids are responsible for disease development. The endophytic strain Cmm100 showed that the Cmm chromosome encodes genes involved in host recognition, infection, suppression and colonization of the host (Dreier et al. 1997; Jahr et al. 2000; Eichenlaub and Gartemann 2011). Both the chromosome and plasmid-encoded genes are essential for proliferation, evasion and virulence.

Cmm382 contains the pathogenicity island (PAI), called the *chp/tomA* region. This region has the lowest GC content (65.5%) in the genome. *Clavibacter michiganensis* subsp. *michiganensis* requires one of the virulence plasmids and the *chp/tomA* region to induce wilting (Chalupowicz et al. 2010). The GFP-tagged Cmm100 (lacking both virulence plasmids) and Cmm27 (lacking the *chp/tomA* region) remained at the vicinity of the inoculation point (Chalupowicz et al. 2012) and are unable to induce wilting. These studies show that Cmm needs at least one of these plasmids' virulence factors and the *chp/tomA* region to effectively suppress the basal defence, to colonize the host and to form cellular aggregates (Chalupowicz et al. 2012; Savidor et al. 2012; Chalupowicz et al. 2016).

The first group of virulence factors encodes serine proteases. Many gene families encode serine proteases in Cmm382 (Gartemann et al. 2008). The first group consists of ten different serine proteases and is named the Chp-family. Seven of these are clustered in the *chp/tomA* region and the other three are found on the pCM2 plasmid (*pat-1*, *phpA* and *phpB*). In this family, *chpC* in the *chp/tomA* region is required for colonization (Stork et al. 2008). The second group of virulence factors encode chymotrypsin-related serine proteases and is named the Ppa-family (Gartemann et al. 2008). This group consists of eleven genes of which ten are located on the chromosome and one on the pCM1 plasmid (*ppaJ*). Six of the ten chromosomal genes (*ppaA* to *ppaE*) are found in the PAI region and the four other genes (*ppaF* to *ppaI*) are located in two different chromosomal loci. The third gene family with three members encodes subtilase proteases. All of the members are located on the chromosome with *sbtA* in the *chp* region of the PAI, while *sbtB* and *sbtC* are located on other parts of the chromosome. The PAI-encoded pectate lyases (PelA1 and PelA2) which are enzymes catalyzing the cleavage of pectin, are an important component of plant primary cell walls (Savidor et al. 2012). Genes on the plasmids are working together with genes on the chromosome. Without the *chp/tomA* region there is a significantly reduced expression of *celA* and *pat-1* and also the expression of serine proteases in the *chp/tomA* region (*chpC* and *ppaA*), decreased in the absence of the virulence plasmids (Chalupowicz et al. 2010). Other virulence sources were found in a recent transcriptomic analysis. Two transcription factors (Vatr1 and Vatr2) were involved in pathogenicity of Cmm (Savidor et al. 2014).

It is interesting to note that a different mode of infection (systemic or local) uses different virulence factors to induce disease symptoms (Chalupowicz et al. 2016). Virulence plasmids, which are essential to induce wilting, are not required for blister formation which appears after local infection. Differential gene expression studies showed that genes encoding serine proteases (*chpC* and *sbtA*), cell wall-degrading enzymes (*pgaA* and *endX/Y*), a transcriptional regulator (*vatr2*), a putative perforin (*perF*) and a putative sortase (*srtA*) are expressed early (8–16 hpi) during blister

formation. On the other hand, during wilting development, these genes are expressed at a later period (24–72 hpi) or are lowly expressed (Chalupowicz et al. 2016).

The molecular work of Cmm has been focusing on Cmm strain382 and mutants derived from it. We cannot rule out the possibility that different Cmm strains use different virulence factors. For example, *Clavibacter michiganensis* subsp. *michiganensis* strains from California do express full virulence in the absence of the pCM2 plasmid for virulence (Thapa et al. 2017). So far, many studies have been done to understand Cmm pathogenicity, but knowledge is still limited for Cmm and its tomato interaction. In general, more studies have been done to understand the Cmm virulence than for understanding the defence mechanism in the plant.

Cmm – tomato interaction model

A good understanding of the Cmm virulence is of utmost importance to understand the interaction with tomato. There are two levels of defence in the plant immune system (Jones and Dangl 2006). The first level of defence (basal defence), are transmembrane pattern recognition receptors (PRRs) which respond to microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs) resulting in PAMP-triggered immunity (PTI) that can stop further colonization. The responses to the first level of defence do not differentiate between pathogenic and endophytic microbes. Induction of pathogenesis-related (PR) proteins can occur to both (Savidor et al. 2012). The second level of defence happens inside the cell. Recognition of an effector (virulence factor) is either direct through NB-LRR (nucleotide-binding and leucine-rich-repeat) or indirect resulting in effector-triggered immunity (ETI). Recognition using the NB-LRR protein products is encoded by resistance (*R*) genes. Gram-negative type III secretion system (T3SS) secrete effectors to surpass both levels of defence (Desveaux et al. 2006; Jones and Dangl 2006). *Clavibacter michiganensis* subsp. *michiganensis* possesses a type II secretion system (T2SS), and so far, no Cmm effectors targeting specific *R*-genes are known. As for that, we hypothesize in the Cmm-tomato interaction, that Cmm only must surpass the first layer of defence.

Clavibacter michiganensis subsp. *michiganensis* senses the plant environment upon infection and transmits a signal for activation of a virulence pathway. Induction of the virulence pathway leads to transcriptional activation of vatr1 and vatr2 and secretion of several hydrolytic enzymes, including serine proteases of the Ppa, Sbt (Gartemann et al. 2008), and Pat-1 families (Burger et al. 2005), the XysA (Borneman and Akin 1990), CelA (Jahr et al. 2000), and NagA glycosyl hydrolases (Henrissat and Callebaut 1995), and other cell wall-degrading enzymes (Figure 3). The host perceives the invading bacterium, mounts a basal defence response and induces lipoxygenase-1 (LOX1) which may contribute to jasmonic acid production and

antimicrobial activity (Gardner 1991). Further pathogenesis-related (PR) proteins which possess antimicrobial activity, contact toxicity, and are involved in defence signalling (van Loon et al. 2006), EDS1 (enhanced disease susceptibility 1) which is vital for nonhost resistance and *R* gene-mediated response, and other defence-related proteins are released. Consequently, Cmm has to overcome the host defence barrier, hydrolyzes and degrades the host tissue, and upregulates tomato ACC-oxidase to promote ethylene biosynthesis. The host ethylene production is not the cause of its susceptibility, but a major signal that regulates disease progression. There was a significant delay with wilt symptoms when the ethylene insensitive *Never ripe* (*Nr*) tomato mutant and tomato mutant with impairment of ethylene production, the *ACD* (bacterial *ACC* deaminase) mutant, were challenged with Cmm (Balaji et al. 2008). During infection, tomato ethylene-synthesizing enzyme ACC-oxidase (ACO) was induced by wild type Cmm382 but not by the Cmm100 strain without plasmids thus showing Cmm-triggered tomato synthesis of ethylene as an important factor in disease symptom development (Savidor et al. 2012). The role of ACO in ethylene production in the host plant is important since it induces wilting, but it does not defend against the presence of Cmm. Cmm382 actively induces ACO in the host, without the plasmids (Cmm100) the induction is no longer obtained. The lack of wilt symptoms in Cmm100 infected plants might be due to the lack of ethylene synthesis. Strikingly, there is no difference between the regulation of the basal defence responses when challenged with Cmm382 or Cmm100 (Savidor et al. 2012).

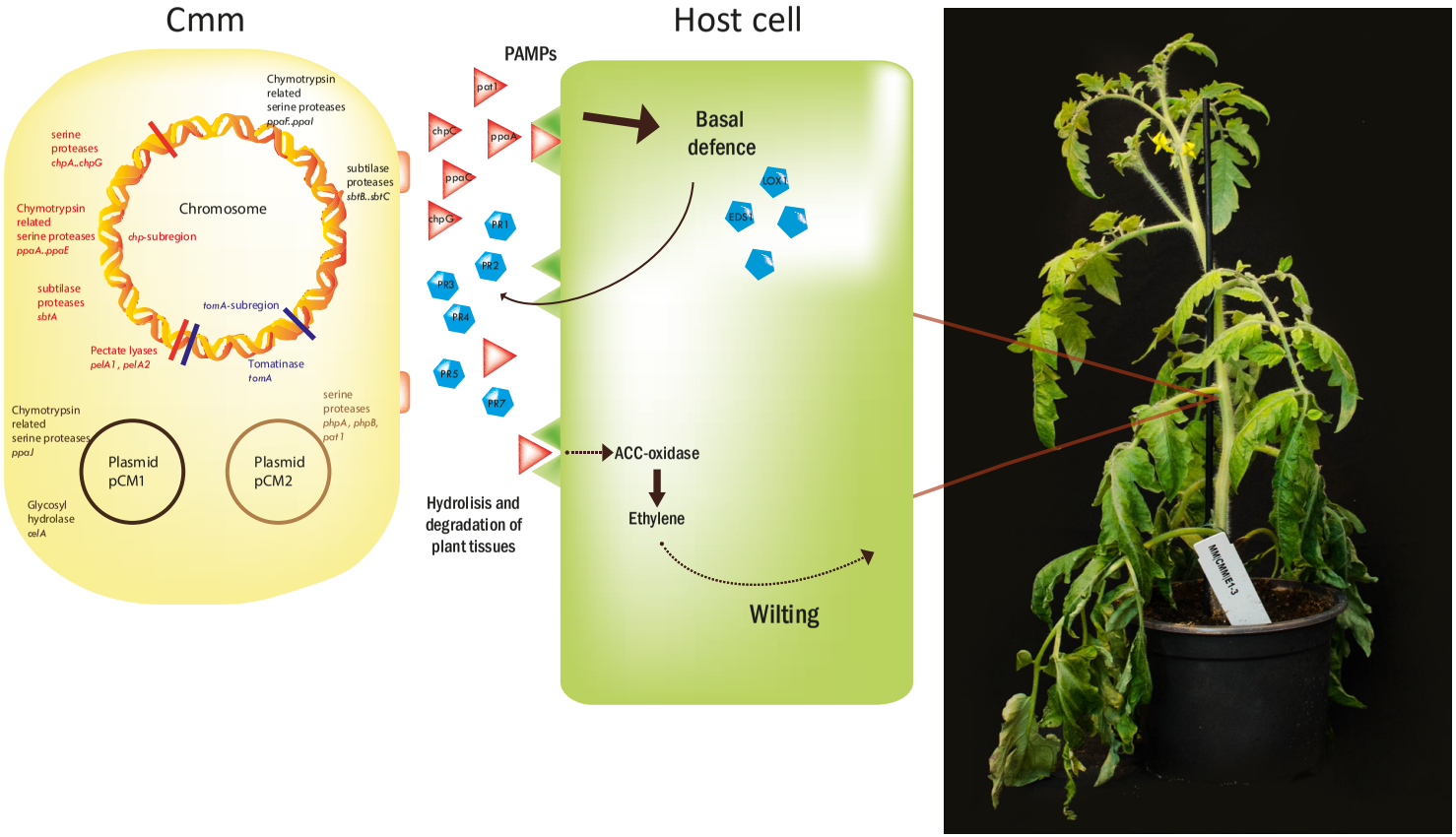

Figure 3 | *Clavibacter michiganensis* subsp. *michiganensis* – proposed tomato interaction model. Model is adapted and modified from Savidor et al. (2012, 2014) and Nandi et al. (2018) with permission.

Source of tomato tolerance to Cmm

Savidor et al. (2012) showed which virulence factors are secreted by Cmm and how the gene products cause wilting after systemic infection. However, the study does not address the potential genes secreted by the host as defence mechanism. There are no *R*-genes against Cmm known to date and all cultivated tomatoes are susceptible (Balaji et al. 2008; Stüwe and Tiedemann 2013). Resistant sources have not been found in wild relatives or cultivated tomatoes, only tolerant sources. The resistance mechanism is the host's ability to inhibit pathogen multiplication, while the tolerance mechanism is the ability of the host to reduce the effect of infection despite the level of pathogen multiplication (Pagan and Garcia-Arenal 2018). Sen et al. (2013) have (re)screened sources of Cmm tolerance in wild relatives of tomato. These include *S. pimpinellifolium* GI.1554, *S. parviflorum* LA735 and *S. parviflorum* LA2072. Other tolerant genotypes are *S. arcanum* LA2157, *S. arcanum* PI127829, *S. arcanum* LA385, *S. habrochaites* LA407 and *S. lycopersicum* cv. IRAT L3. The tolerance mechanism(s) from these wild species is unknown. Other studies looking at quantitative trait loci (QTL) between the wild species and cultivated tomatoes showed that the tolerance is complex, additive and may involve multiple genes (van Heusden et al. 1999; Kabelka et al. 2002).

Differential gene expression of several wild tomato genotypes and cultivated tomato was studied (Lara-Ávila et al. 2011). The study included the susceptible cultivated tomato *S. lycopersicum* cv. Moneymaker and other wild relatives such as *S. arcanum* LA2157 (previously known as *S. peruvianum* LA2157), *S. arcanum* LA2172 (previously known as *S. peruvianum* LA2172), and *S. habrochaites* LA2128. The genes with contrasting regulations in the cultivated tomato compared to the wild relatives include genes involved in the ubiquitin-mediated protein degradation pathway and secretory peroxidase. The group of Lara-Ávila recently silenced (virus-induced gene silencing) the SUMOE2 conjugating enzyme (*SCEI*) which was upregulated in *S. arcanum* LA2172 after infection with Cmm. It encodes an enzyme involved in protein modification through sumoylation, which is a post-translational modification that covalently conjugates the small ubiquitin-like modifier (SUMO) protein to lysines on target proteins. This gene may play a part in the tolerance against Cmm, as silenced *S. arcanum* LA2172 plants are more susceptible to the pathogen (Esparza-Araiza et al. 2015). This finding highlights a potential gene that could play a role in the tolerance against Cmm. However, *S. arcanum* LA2172 was susceptible in our hands to Cmm (Sandbrink et al. 1995). It can be suggested from all findings that there are no real major genes in cultivated or wild species known to give high levels of tolerance against Cmm. Thus, what alternatives can be used to reduce symptom development by Cmm?

How to combat the unwanted guest?

Cmm prevention

Up to now, managing bacterial canker is based on prevention and control. This includes the use of Cmm-free seed, healthy seedlings, cultivation practices including hygiene, crop rotation, and chemical treatments (Gleason et al. 1993; Hausbeck et al. 2000). Once Cmm establishes in greenhouse or field, it is difficult to stop the disease even with the use of bactericides (Hausbeck et al. 2000). Other types of prevention by using biocontrol means have been studied but still a long way lies ahead for a successful application in the field (Yogev et al. 2009; Amkraz et al. 2010; Barda et al. 2015). Successful suppressing the disease at the production site is a challenge for growers and producing Cmm-free seeds is a challenge for the breeders. Genetic aspects of tomato resistance/tolerance, cultural practices and biological control should be considered to manage and reduce Cmm as much as possible (Figure 4).

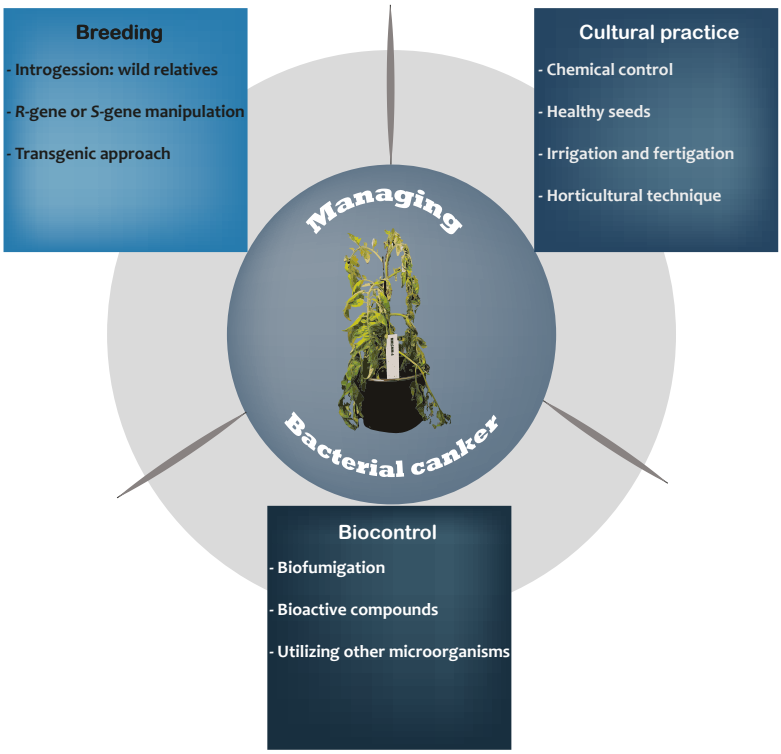

Figure 4 | General scheme of combating Cmm.

Resistance/ tolerance breeding

Developing resistant crops is a major objective for plant breeders. Breeders may use conventional breeding techniques or genetic modification as a breeding strategy. The choice of the strategy depends on the sustainability and acceptance of the consumers. With the help of molecular markers, conventional breeding strategy can be expedited. Introgressing a region from exotic genetic libraries into the background of cultivated crops, enhances the resistance of elite lines (Zamir 2001). As mentioned above, several sources of Cmm tolerance have been found in wild relatives of tomato (Sen et al. 2013). Screening tomato wild relatives for enhancing Cmm resistance/ tolerance has been going on since the 1930's (Cardon 1934) and incorporating the wild relatives in tomato breeding has been tried (Elenkov 1965). Tolerance was found in lines after crossing with *S. pimpinellifolium* (Elenkov 1965; Thyr 1968; Jong and Honma 1976; Thyr 1976; Sen et al. 2013), *S. habrochaites* (Hassan et al. 1968; Vulkova and Sotirova 1993; Sotirova et al. 1994; Francis et al. 2001; Kabelka et al. 2002), and *S. arcanum* (Lindhout and Purimahua 1987; Vulkova and Sotirova 1993; Sandbrink et al. 1995; van Heusden et al. 1999). Inheritance of tolerance in some of the wild relatives is complex and may be controlled by several genes as seen in the F2 population between the cultivated tomato and *S. arcanum* LA2157 (van Heusden et al. 1999; Sen et al. 2015). From the screening of wild relatives, the tolerant individuals still contain a considerable amount of bacteria although lacking symptoms (Sen et al. 2013). Despite that, there is a commercial value for the breeders if cultivated tomato containing the tolerance from the wild species can be developed. The background for the absence of wilt symptoms in the wild relatives might be morphological (Francis et al. 2001; Kabelka et al. 2002), or due to the production of metabolites involved in tolerance (Shinde et al. 2017) or based on the immune system (Jones and Dangl 2006). The pitfalls of introgression breeding can be F1 hybrid sterility, infertility of the progenies, reduced recombination between the chromosomes of the two species, and undesired genes being tightly linked to the trait of interest (linkage drag) (Zamir 2001; Wolters et al. 2015). This is not desirable for breeders, but it can be overcome by doing embryo rescue in the early stage of crossing and backcrossing, and later selecting the lines with small introgression size containing the favourable trait. Our effort of getting nearly isogenic lines with the smallest possible introgression size from the tolerance wild relative *S. arcanum* LA2157 is further discussed in **Chapter 3**.

The most common approach of using introgression breeding is to breed for resistance by using *R*-genes. The downside of using this approach is that *R*-genes target specific effectors. Modulation of the effector changes the recognition of an *R*-gene and breaks the resistance (Jones and Dangl 2006). To overcome this problem, stacking several *R*-genes (also called gene pyramiding) can be done. The justification behind this approach is that the pathogen needs to surpass multiple *R*-genes at the

same time, so it needs to modulate several avirulence (*Avr*) genes to be able to infect the cultivar carrying the stacked *R*-genes. Gene pyramiding in resistance breeding has been applied in rice (Huang et al. 1997; Hittalmani et al. 2000), bean (Haley et al. 1993; Miklas et al. 1993), wheat (Liu et al. 1999; Zheng et al. 2017) and other crops. So far, no *R*-gene has been found against Cmm in tomato.

Manipulating susceptibility (*S*) genes can be an alternative approach for acquiring resistance resp. tolerance. *S*-genes are innate genes in the crop that are used by the pathogen to expedite the infection and support compatibility (van Schie and Takken 2014). The genes facilitate host recognition and penetration, encode negative regulators of immune signalling, and allow post-penetration to sustain compatibility. Thus, loss-of-function of the genes could lead to broad-spectrum resistance. The best example of a *S*-gene is the *MLO* (*mildew resistance locus o*) (Acevedo-garcia et al. 2014). Inactivation of the *MLO* gene through loss-of-function or silencing leads to resistance towards powdery mildew (PM) (Pavan et al. 2009). Resistance is due to the inability of fungi to penetrate host cells (Higgs and Peterson 2005). The benefit of using *S*-genes is its broad-spectrum resistance. Silencing or knocking-out an *S*-gene can confer resistance to multiple pathogens (Sun et al. 2016a). However, the downside of manipulating *S*-genes are the possible, pleiotropic effects. *S*-genes not merely exist to facilitate infection processes and disease by the pathogen. Silencing the genes could lead to pleiotropic effects as they have evolutionary conserved functions in plant processes (van Schie and Takken 2014). The magnitude of adverse pleiotropic effects depends on the environmental conditions and plant species (Pavan et al. 2009; Sun et al. 2016b). Thus, using *S*-gene in breeding is desirable, if silencing or knocking-out the gene does not lead to a pleiotropic effect. The use of *S*-genes is further discussed in **Chapter 4**.

Apart from *R*-gene and *S*-gene approaches, modification of other genes might enhance tolerance. Transgenic tomato plants made to combat Cmm have been studied recently. Tomato plants transformed with the bacteriophage CMP1 endolysin gene (*lys*) exhibited reduced to no disease symptoms in transgenic plants (Wittmann et al. 2015). The gene encodes a peptidase which is highly specific for the hydrolysis of *Clavibacter michiganensis* species' murein type B2γ (Schleifer and Kandler 1972). Overexpression of *snakin-2* (*SN2*) and *extensin-like protein* (*ELP*) genes in tomato plants increased tolerance towards Cmm (Balaji and Smart 2012). SN2 protein is an antimicrobial peptide which plays a role in the innate defence against invading pathogen (López-Solanilla et al. 2003). The ELP protein is transcriptionally upregulated after Cmm infection (Balaji et al. 2008). Transgenic lines with high levels of *SN2* or *ELP* mRNA show a significant delay in the development of wilt symptoms and a reduction in the canker lesion size. Also, the modification of the PAMPs recognition receptors lead to tolerance. Transfer of the *Arabidopsis FLAGELLIN-SENSING 2* (*FLS2*),

BRI1-ASSOCIATED RECEPTOR KINASE1 (*BAK1*) and *EF-Tu RECEPTOR* (EFR) into tomato, reduced the Cmm infection up to 83% (Torre et al. 2016). Although genetic modification in crops is still debatable in many countries, its application might be allowed in a later stage.

Cultural practice

Good cultural practice is important for growers. Knowing the pathogen disease cycles, and the suitable environment to reduce Cmm development will reduce yield loss. Cmm-free seeds are the initial step to combat the disease. However, growing healthy seedlings can only be done when no Cmm is in the soil due to infected plants from the last season(s). Once a plant is infected with Cmm, it can easily spread the bacteria to neighbouring plants through wounds or natural openings of the other plants. In general the dispersion of the pathogen can be reduced with manipulating the fertigation or irrigation system (Sharabani et al. 2013a; Frenkel et al. 2015), using uncontaminated appliances (Kawaguchi et al. 2010) and changing the temperature settings in the greenhouse (Chang et al. 1992b; Sharabani et al. 2014). Asymptomatic plants in combination with diagnostic tests can be used to show that Cmm is present in a field (Yasuhara-Bell et al. 2015; Yasuhara-Bell and Alvarez 2015). Finally rotating tomatoes with other solanaceous crops every 2-3 year (Gleason et al. 1991) in combination with other horticulture techniques will reduce the spread of Cmm.

In greenhouses and nurseries, the epiphytic Cmm-infected plants can spread through guttation and splashing droplets due to overhead irrigation and spraying of chemicals (Strider 1967; Sharabani et al. 2013a). The secondary spread in Israel spreads from diseased plants to healthy plants due to the overhead irrigation system. To avoid this, a sub-irrigation system where water was added into pots for irrigation. This was done twice a day for 15 min followed by draining, this method was used to avoid spread of bacteria by water droplets on the foliage. Sub-irrigation reduces Cmm dispersion, but does not completely prevent Cmm spread (Frenkel et al. 2015). Sub-irrigated plants might be infected through the procedure of spraying pesticides. Growers may avoid pathogen dispersal by avoiding contact with plants during periods when they bear guttation droplets and changing the irrigation system. This practice provides an eco-friendly and easy means for decreasing the spread of the disease.

Using contaminated appliances hasten the bacterial spread like it was shown in a recent outbreak in Japan. Since watering was done by tubes under the plastic mulch the spreading of Cmm was not caused by the irrigation system. The spread in greenhouses in Japan was due to farmers removing buds and leaves by hands and pruning with non-sterilized scissors (Kawaguchi et al. 2010). Consequently, disease spreading can be reduced with strict greenhouse hygiene (Jarvis 1992).

In addition, temperature plays a big role in disease development. In some cases, plants become more susceptible towards fungal diseases at lower temperatures (Balass et al. 1993; Ge et al. 1998), while they become more resistant under other circumstances (Kaul and Shaner 1989; Roderick et al. 2000). In the case of Cmm, the optimal temperature for disease development is between 23-28°C (Forster and Echandi 1973; Gartemann et al. 2003). Disease development and the incubation period lasts longer when the pathogen infects at colder temperatures (Chang et al. 1992b). Recent studies showed that temperatures predominant in the first 48 h after inoculation affect the Cmm density, expression of the virulence genes, and affects the disease development season-long. The time observed for half of the plants to wilt or to die after inoculation (T_{50}) in Israel was two month in spring (21–24°C) and autumn (18–23°C), and three to four months in the winter (15–18°C) and summer (28–31°C) (Sharabani et al. 2014). The aggressiveness of the pathogen in different seasons may differ from one country to another. Nevertheless, good knowledge about which temperature affects the disease development the most, is useful to control the disease.

Growers commonly eradicate infected Cmm plants in the fields and greenhouses to prevent the spreading of the disease. Eradication is usually done on symptomatic plants as the disease development is more obvious. Nonetheless, also asymptomatic plants in the field or greenhouse can result in severe disease spreading. The use of the Cmm ImmunoStrip® (Agdia Inc., Elkhart, IN) assay can facilitate detecting the presence of Cmm on asymptomatic plants, even before symptoms develop. The downside of this test is that it cannot discriminate between pathogenic and non-pathogenic Cmm, or other bacteria such as *Ochrobactrum* and *Microbacterium* spp., which can also be present in tomato plants and seeds (Yasuhara-Bell and Alvarez 2015). The advancement of technology might prevent that problem. A Loop-mediated Amplification (LAMP) assay was recently developed that can differentiate between pathogenic, non-pathogenic Cmm, and other bacteria compared to the ImmunoStrip® (Yasuhara-Bell et al. 2013; Yasuhara-Bell et al. 2015; Yasuhara-Bell and Alvarez 2015). Hence, disease outbreaks can be combatted faster since it is possible in an early stage to identify the presence of low densities of Cmm in the plants or seeds.

Application of other horticultural techniques should be considered to ascertain the reduction of the infection *in planta* against Cmm. Grafting is an agricultural practice that has been used for centuries. It fuses the root system (rootstock) from one plant to shoot (scion) of another. The application of this technique is wide, and one of it is managing disease resistance. Grafting has been used widely as a substitute of methyl bromide (MeBr) to manage soilborne pathogens like *Ralstonia solanacearum* (Peregrine and Ahmad 1982; Grimault and Prior 1994; Lin et al. 2008), *Verticillium*

dahliae (Paplomatas et al. 2002; Liu et al. 2009) and root-knot nematodes (Augustin et al. 2002; Cortada et al. 2008). In addition, grafted vegetables can also show a disease reduction due to foliar pathogens *Cucurbita ficifoli* (Gu et al. 2008) and *Leveillula taurica* (Albert et al. 2017); and viruses (Miguel 2004; Huitrón et al. 2011). The application of grafting and the use of known rootstocks to manage Cmm has never been done. In **Chapter 5**, we will discuss the potential use of grafting to manage bacterial canker in tomato.

Biocontrol measures

Combating Cmm with chemicals is not an efficient way to control the disease. Chemical treatments only reduce the pathogen population on the surface of the plants or on the infected seeds (Hausbeck et al. 2000). Furthermore, repeated use of the same type of pesticides might increase the proportion of resistant Cmm strains. This is not true with the use of biocontrol means as Cmm requires a more complex mode of action to break the resistance/tolerance mechanisms. Even though the use of biocontrol agents to manage the disease has not been applied so far in the field, the possibility of using them in the future is likely. Biocontrol means that have been studied to control Cmm is the application of soil amendments (Zanón and Jordá 2008), antibacterial properties of bioactive compounds (Daferera et al. 2003; Kotan et al. 2014; Nguyen et al. 2017; Pham et al. 2017) and the use of other microorganisms (Boudyach et al. 2001; Lanteigne et al. 2012; Barda et al. 2015; Mora et al. 2015).

In recent years, there has been a demand on the use of organic matter in cultivated soil to improve soil quality especially in organic farming. Soil amendments such as biochar, composts, coffee grounds etc. can have different effects on soilborne plant pathogens (Lazzeri et al. 2003). Biofumigation, which is the use of active plant compounds into the soil by decomposing soil amendments to manage soilborne pathogens, can manage plant diseases as good as conventional pesticides (Bello et al. 2000; Stapleton 2000). The application of soil amendments for integrated pest management systems against Cmm has been studied in Spain. Its utilization is to make use of the farmer's cultural practice of keeping their crop residues on the soil surface. Amended soil containing fresh tomato debris artificially infected with Cmm was treated with two different temperatures (25 °C and 45 °C) to determine the effect of soil amendments with thermal treatment. Cmm was eradicated after four weeks treatment at 45 °C, but still present at 25 °C (Zanón and Jordá 2008). These findings suggest adequate heat treatments on plant debris could be a different and effective soil disinfection technique, and the possibility of soil amendments and heat treatments for biofumigation in the field.

Plant extracts exhibit broad-spectrum inhibition and potent activity against many phytopathogens (Pham et al. 2017). Bioactive compounds in the plants can be potentially utilized for developing plant-derived chemicals that are sustainable for the environment (Copping and Duke 2007; Yoon et al. 2011). *Clavibacter michiganensis* subsp. *michiganensis* is sensitive to oregano, thyme and dictammus essential oils as could be seen by the inhibition of the pathogen's growth *in vitro*. The three essential oils were characterised by the presence of *p*-cymene, *γ*-terpinene, thymol and carvacrol. The main compound in oregano oil is thymol, while it is carvacrol in thyme and dictamnus oils (Daferera et al. 2003). Additionally, coating the tomato seeds with carvacrol from *Origanum onites* increased the germination rate of tomato seeds, decreased the disease severity and improved the seedlings growth (Kotan et al. 2014). The antagonistic or synergistic effect may be due to the major component of particular plant compound, although it is possible that minor percentage of several compounds in combination with other mixtures may affect the pathogen's growth or virulence. Aside using the active components of plant extracts to control Cmm, utlitizing other microorganisms are also able to control the pathogen.

Different types of responses may occur in tomato plants in the presence of Cmm and other microorganisms. During systemic infection, Cmm releases virulence factors and colonizes the xylem vessels. Wilting is due to the induction of ACO in ethylene in the presence of Cmm. Cmm382 (containing both virulence plasmids) actively induced ACO in the host, but not Cmm100 (without virulence plasmids) (Savidor et al. 2012). Furthermore, degradation of cell walls in xylem vessels is hypothesized to occur due to the production of CelA and other extracellular enzymes from Cmm (Jahr et al. 2000; Gartemann et al. 2003). It is possible that when other microorganisms are present in the host, they might compete with Cmm and lead to antagonistic interactions (Utkhede and Koch 2004; Amkraz et al. 2010; Jung et al. 2014), the competition for nutrients (Boudyach et al. 2001), might change the hydraulictic conductivity of the xylem vessel (Romero et al. 2014) or trigger an induced resistance response (Barda et al. 2015). Several microorganisms have been tested and shown to inhibit Cmm: *Pseudomonas* spp. (Boudyach et al. 2001; Ślusarski 2009; Amkraz et al. 2010; Lanteigne et al. 2012; Novinscak et al. 2016), *Bacillus* spp. (Kasselaki et al. 2011; Jung et al. 2014; Mora et al. 2015), *Pseudozyma aphidis* (Barda et al. 2015), *Aureobasidium pullulans* and *Pantoea agglomerans* (El et al. 2017). Considering these studies, it is tempting to speculate that a microbial consortium containing more than one microorganism could enhance the resistance of the host plant. Further work on utilizing microbial consortia against Cmm is discussed in **Chapter 5**.

Aim of this thesis

The focus of this thesis is the application of several approaches to fight Cmm to get symptomless tomato plants with minimal bacterial density. From the overview of Cmm virulence and lifestyle, we can speculate that it is a challenge to combat and manage the pathogen. Relying on tolerant lines is not enough, other aspects of prevention should be applied to avoid Cmm dispersion which can lead to diseased plants. We have developed several means to control the pathogen. We developed an efficient system for the disease assay, which in turn speed up our fine mapping process. We made use of the host gene to work against the uninvited guest. Furthermore, we look at a horticultural practice and use of biological agents that could control the pathogen.

Chapter 2 describes a newly developed disease screening protocol which is efficient and cost effective. The quarantine status of the pathogen and lack of space hurdled the disease screening of large populations. The disease assay was based on the inoculation of *in vitro* seedlings, in which we could discriminate between highly tolerant, middle tolerant and susceptible genotypes. In combination with the use of PathoScreen™ imaging system, we could detect the presence of the GFP-tagged Cmm in whole plantlets. This new disease assay can be applied to many wild tomato relatives, where many of them produced similar symptom scores as with the more laborious greenhouse symptom assay. The use of this protocol speeds up the disease screening process of large populations using minimal space.

Following up from the previous chapter**, Chapter 3** describes the fine mapping of the QTL on chromosome 7 using the *in vitro* disease screening. Fine mapping was done using the KASP (KBiosciences, UK) assay. The disease assay was performed on a selection of nearly isogenic lines using the newly developed disease assay. We could delimit the QTL region to 211 Kb and we have individuals with only a small introgession of *S. arcanum* LA2157 that are highly tolerant to Cmm.

Chapter 4 describes the application of susceptibility (*S*) genes against Cmm. Since no known resistance (*R*) genes have been found against Cmm, utilizing the *S*-genes could be an alternative approach to fight the pathogen. From the VIGS assay, we found a candidate gene (*WAT1*) that confers higher tolerance than the control plants. This finding could be a starting point of utilizing *S*-genes in tolerance breeding against Cmm.

Chapter 5 describes the alternative approaches to combat Cmm: grafting and utilizing biocontrol agents. A grafting method using the rootstock of a highly tolerant genotype (*S. arcanum* LA2157) to the scion of a susceptible (genotype) and vice versa was done to see any difference with the wilting severity. Biocontrol agent

using *Pseudomonas* spp. consortia was applied to enhance the tolerance mechanism in different tomato genotypes. The wilt symptom was compared in the treated plants (with biocontrol agent) to the non-treated (without biocontrol agents) plants.

Finally, **Chapter 6** summarizes the main results obtained in this thesis, gives concluding remarks and future perspectives to combat Cmm.

CHAPTER 2

Development of an *in vitro* protocol to screen *Clavibacter michiganensis* subsp. *michiganensis* pathogenicity in different *Solanum* species

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abstract

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is a quarantine organism in Europe and in many other countries. It is one of the most severe bacterial pathogens affecting tomato. Screening tomato plants for their tolerance level to Cmm requires a large amount of space under quarantine conditions and is therefore costly. This project developed a new inoculation protocol on *in vitro* tomato plants to facilitate a more economic and higher throughput disease screening. A new method using the PathoScreen™ system was tested to localize Green Fluorescent Protein-tagged Cmm *in planta* and to quantify the pathogen based on the percentage of corrected GFP (cGFP%). The system was sensitive in detecting the GFP-tagged Cmm in the shoots, but a high autofluorescence in the roots masked detection and thus sensitivity of the assay. The best inoculation procedure was found by doing direct inoculation method on the fourth leaf stage plants. The *in vitro* protocol was tested on several wild relatives of tomato, which were previously screened in a greenhouse assay. The correlation between wilt symptoms *in vitro* and wilt symptoms in the greenhouse was overall moderate (r = 0.6462). The protocol worked well in differentiating the two parents that we used in our mapping studies. This study shows that the *in vitro* protocol can be efficiently used for resistance/tolerance breeding in many tomato genotypes.

Keywords: *Clavibacter, Cmm, Tomato, Disease screening, in vitro, PathoScreen*

Introduction

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is a Gram-positive bacterium which causes wilting and canker in tomato (*Solanum lycopersicum*) (Davis et al. 1984). It can decrease tomato production worldwide and leads to major economic losses. The severe consequences of an infection with Cmm and its easy spreading make it listed under the EPPO A2, which defines as a quarantine pest present in the European and Mediterranean region but not commonly dispersed, and is regularly administrated (EPPO 2016). *Clavibacter michiganensis* subsp. *michiganensis* can enter the host plant via roots, natural openings and wounds (Chang 1991). Contaminated seeds and infected plant debris in the soil are the main sources of infection (Strider 1969; de León et al. 2009). The symptoms of the bacterial infection depend on several factors: infection pathways, level of resistance of the host, plant age at infection, bacterium density and the physiology of the tomato plant (Gleason et al. 1993; Sharabani et al. 2013b). The common symptom caused by Cmm in infected plants is wilting, other symptoms can be stem canker, firing of the foliage, discoloration of the vascular bundles, adventitious root formation on stems, bacterial sludge from infected parts and bird's eye spots on fruits (Strider 1969). Measures have been used to control Cmm so far are production of pathogen-free seed and good agriculture practices. The use of biocontrol agents might be an alternative approach to manage the pathogen in the future, but the application in the field has not been used so far (Barda et al. 2015). Another effective way to combat Cmm outbreaks will be the development of resistant/tolerant cultivars. No commercial tomato varieties are known that prevent Cmm symptoms even though related wild crossable relatives have been identified without symptoms after infection with Cmm. Examples are *S. pimpinellifolium* (Thyr 1976; Sen et al. 2013), *S. habrochaites* (Vulkova and Sotirova 1993; Francis et al. 2001), *S. lycopersicum* (Elenkov 1965) and *S. arcanum* (Lindhout and Purimahua 1987; Vulkova and Sotirova 1993). To broaden the genetic variation of cultivated varieties parts of the genome of wild tomatoes can be introgressed (Haggard et al. 2015). From our previous studies, the best source of tolerance is *S. arcanum* LA2157 (van Heusden et al. 1999).

To pinpoint the chromosomal region and to identify underlying genes that are responsible for the tolerance it is necessary to test the interaction between the pathogen and the host in bigger populations. However, the strict laws on quarantine organisms in Europe make this very expensive and this hinders the progress of the work. After the plants have been infected they must be grown in small and expensive greenhouse compartments in a special quarantine section. Our group has tested different tomato genotypes in the greenhouse with different inoculation methods to find the best protocol for greenhouse screening (van den Bulk et al. 1991). However,

currently greenhouse screening is costly for big populations. We wanted to develop a new screening method *in vitro* to make high throughput screenings possible.

In vitro methods for determining the level of resistance have been developed in several crops (Svabova et al. 2005). This has been done on plant parts (Borras et al. 2001), on callus cultures (Mangal and Sharma 2002) or on protoplasts (Nyange et al. 1997). For tomato, an *in vitro* inoculation with Cmm on tomato has been done on cells (Kraemer et al. 1988), callus (Sotirova et al. 1999) and seedlings (Lelis et al. 2014). Those studies showed an interaction between the pathogen and plant, and the development of the disease symptoms without the presence of other microorganisms. Disease symptoms, such as wilting, could be clearly seen six days post inoculation (dpi). Other symptoms (yellowing of lower leaves, followed by black spots, decay of the petiole, canker) that were seen in the greenhouse assay were also observed in the *in vitro* inoculated plants (Lelis et al. 2014).

In our study, we tested different tomato genotypes with different inoculation methods for optimising an *in vitro* protocol. The PathoScreen™ assay was used to detect the localization of the Green Fluorescent Protein-tagged pathogen *in planta* and to quantify the bacteria (Lelis et al. 2014). Our protocol showed that *in vitro* plants can be efficiently used in high throughput disease screenings.

Materials and methods

Growth of *in vitro* plants

The susceptible *Solanum lycopersicum* cv. Moneymaker and the highly tolerant *S. arcanum* LA2157 were used for developing an *in vitro* protocol. The seeds were sterilized in 70% ethanol for 2 min, 1% NaOCl (Sigma-Aldrich, St. Louis, MO) for 20 min and washed with sterile water for 5 min. Seeds were sown on a germination medium (GEM) (2.2 g MS salts/L, 10 g sucrose/L, 8 g Daishin agar/L; pH 5.8) and incubated for 3 days at 4 °C and then for approximately 2 weeks in a growth chamber (Technisch Buro I.K.S. B.V, Leerdam, The Netherlands) at 25 °C with a relative humidity of 40–70%, and a 16 h/ 8 h day/ night photoperiod. Cotyledonary explants obtained from seedlings were transferred to rooting medium (MS30B5) (4.3 g MS salts/L, 112 mg Vitamin B5/L, 30 g sucrose/L and 8 g agar/L; pH 5.8) (Lelis et al. 2014).

Inoculation of *in vitro* plants

The GFP-tagged, virulent Cmm strain IPO3525, kanamycin and rifampicin resistant, was used for inoculation (Lelis et al. 2014). Two different plant stages were tested (the second and fourth leaf stage) and two types of inoculation methods (direct

and indirect inoculation). Direct inoculation was done by cutting the first leaf using scissors that were dipped into 10^7 cfu ml^{-1} bacterial suspension and the indirect inoculation was done by drop inoculating 100 µl of 10^7 cfu ml^{-1} bacterial suspension at the interspace between stem base and agar medium. Three replicates were used for each treatment with untreated plants as controls. The inoculated *in* vitro plants were collected at several time points (3, 7, 14 21, 28 and 35 days post inoculation – dpi). Wilting symptoms were recorded based on the following scale: 0 = no symptoms; 1 = 0 to 25% leaf wilting; 2 = 26 to 50% leaf wilting; 3 = 51 to 75% leaf wilting; 4 = 76 to 100 % leaf wilting; 5 = dead plants (Figure 1).

The *in vitro* inoculation method was also tested on twelve wild relatives of tomato (each with ten plantlets): *S. habrochaites* LA407, *S. cheesmanii* LA0166, *S. parviflorum* LA735, *S. pimpinellifolium* GI1554, *S. habrochaites* LYC4, *S. arcanum* LA2157, *S. pennellii* LA716, *S. neorickii* LA1045, *S. arcanum* PI127829, *S. chilense* IVT56140, *S. cheesmanii* LA1409, and *S. arcanum* LA385.

These genotypes were previously tested in a greenhouse assay (Sen et al. 2013). The wild relatives were inoculated using the direct inoculation method at the fourth leaf stage. Wilting symptoms were recorded every week up to the 35 dpi. The symptom scoring was based on the scale above.

Localization in planta and quantification of bacteria of *in vitro* growing plants

The population densities of the bacteria between the different variables *in planta* were measured by collecting samples of the treated and untreated plants on 0, 3, 7, 14, 21, 28 and 35 dpi. The roots and the shoots (stems and leaves) were removed from the medium and sterilized with 70% ethanol. The surface of the plants were blotted dry and placed onto petri dishes (120 mm x 120 mm) and images were captured using the imaging system PathoScreen™ (PhenoVation B.V: https://www.phenovation.com/) under several parameters: absolute and relative area of Cmm-GFP and plants, GFP corrected and red, green, and blue intensities (RGB values).

The plant parts were then placed in extraction bags (Bioreba, Reinach, Switzerland), macerated with hammer, homogenized in Ringer buffer (2 ml g^{-1} of tissue) and analysed by dilution plating. Dilution plating was done by diluting six 10-fold serial dilution of the 10 µl macerated suspension onto 60 mm x 15 mm petri dishes containing TBY medium (10 g tryptone/L, 5 g yeast extract/L, 5 g NaCl/L, pH 7.5 and 15 g agar/L) with final concentration of kanamycin (50 µg ml^{-1}) and rifampicin (25 µg ml^{-1}). Plates were incubated at 28 °C for 4 days. The number of GFP- positive bacterial colonies was determined using epifluorescence stereomicroscopy.

PathoScreen™ and statistical analyses

The localization of GFP-tagged bacteria in the shoots was determined using the PathoScreen™ Seed Data Analysis Software (version 2.4.1). Images of the plants were captured, the region without the presence of chlorophyll was masked, the Cmm-GFP signal in the images of the inoculated plants were normalized against the images of untreated plants, and the percentage of the corrected GFP (cGFP %) signal was determined by the number of pixels detected in a normalized image. The correlation between the *in vitro* symptom scoring and cGFP% was calculated. To determine the difference between the disease index with the age of plants at the time of inoculation (two versus four leaf stage) and inoculation methods (direct versus indirect), the variables were analysed by doing a two-way ANOVA analysis using the SPSS 23.0 statistical software package (SPSS Inc., Chicago, IL,). The difference between the bacterial titre and the two variables were also determined with the same analysis. Data of the cfu number were transformed to log10 after adding a value 1. Effects were significant at P = 0.05. Symptom scoring data of each wild tomato genotype was averaged to determine the correlation of the wilting symptom of the *in vitro* infected plants and the greenhouse infected plants.

Figure 1 | Symptom score scale *in vitro*. 0 = no symptoms; 1 = 0 to 25 % leaf wilting; 2 = 26 to 50 % leaf wilting; 3 = 51 to 75% leaf wilting; 4 = 76 to 100 % leaf wilting; 5 = dead.

Results

In vitro inoculation on *S. lycopersicum* cv. Moneymaker and *S. arcanum* LA2157

For optimising the *in vitro* assay, an indirect and a direct inoculation were compared. Additionally, two different plant ages were tested, the inoculation was done on the plants in the second leaf stage and the fourth leaf stage.

The first symptoms on the susceptible genotype cv. Moneymaker became visible fourteen days past inoculation (dpi). The symptoms were: (1) yellowing of the lower leaves followed by unilateral wilting, (2) black spots on the leaves, (3) necrosis of the midribs and veins, (4) development of adventitious roots and (5) stem canker. *S. arcanum* LA2157 did not show any disease symptoms at fourteen dpi. Later, at 21 dpi, mild wilting symptoms were observed on *S. arcanum* LA2157 but without stem canker development.

On cv. Moneymaker a more clear leaf wilting and also stem cankers were observed after the direct inoculation (cutting first leaves with Cmm-infected scissors) (Figure 2c, e) compared to the indirect inoculation (Figure 2g, i). Indirect inoculation on the second leaf stage of cv. Moneymaker resulted in a wilting score of 1 (Figure 2g), which cannot be explained as the density in the plant is as high as the concentration of directly inoculated cv. Moneymaker with wilting score 5 (Table 1) (Figure 2c). We can conclude that indirect inoculation is not an optimal method to detect wilting symptoms especially when inoculated at the second leaf stage plants. The wilting symptoms on 35 dpi were less severe in *S. arcanum* LA2157 (Table 1) (Figure 2d, f, h, j). Ultimately the direct inoculation method on the second leaf stage resulted in stunted *S. arcanum* LA2157 (Figure 2d) plants and dead cv. Moneymaker plants (Figure 2c). These results confirm that *S. arcanum* LA2157 is more tolerant than cv. Moneymaker. The untreated plants of both genotypes did not show any symptoms (Figure 2a, b).

Based on a two-way ANOVA analysis, the symptom score was significantly different between the two inoculation methods but was not dependent on plant age ($P = 0.05$).

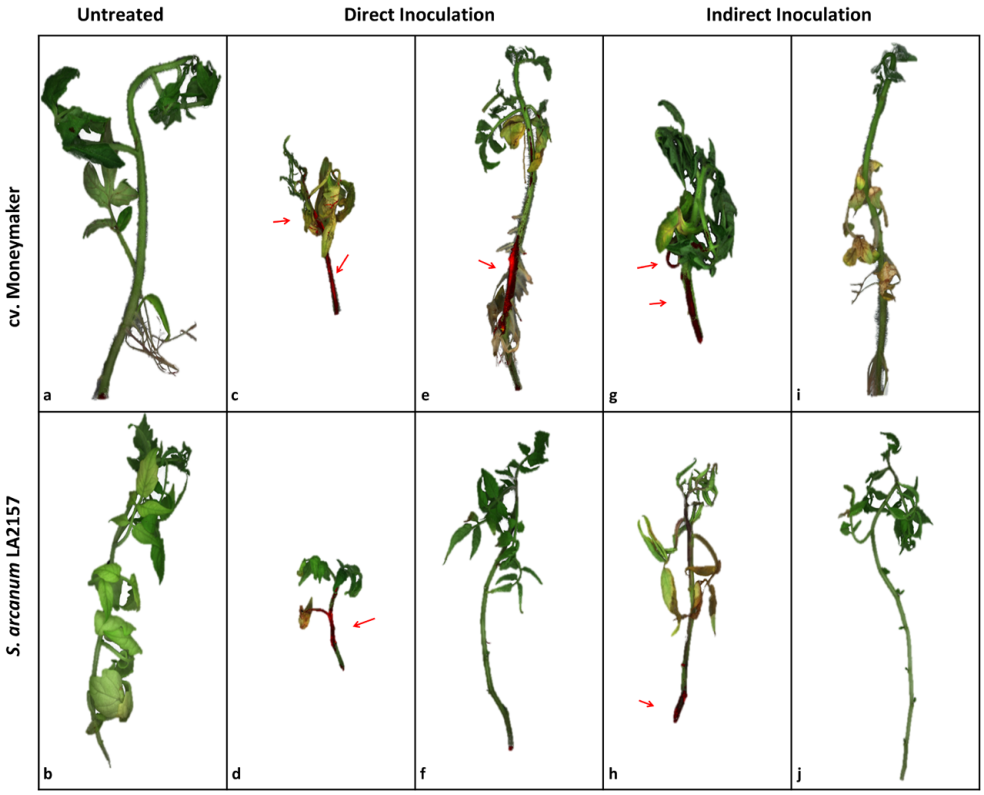

Figure 2 | *In vitro* plants after 35 dpi. Untreated plants (a, b). Plants inoculated during the second leaf stage (c, d, g and h) and fourth leaf stage (e, f, i and j). Plants inoculated with direct inoculation (c, d, e and f) and indirect inoculation (g, i, h and j). Red arrows indicate strong signal of GFP-tagged Cmm. Images were taken with the PathoScreen™ system under the RGB and GFP lights. Analysis of the images and the number of pixels containing GFP were done using the PathoScreen™ analysis software.

In vitro inoculation of tomato wild relatives

For comparing the *in vitro* and greenhouse screenings (Sen et al. 2013) twelve wild species of tomato and cv. Moneymaker were used. A direct inoculation was done because the wilting symptoms are more obvious and more uniform, and this method is more similar to the greenhouse testing procedure. *In vitro* symptom scoring was done on 7, 14, 21 and 28 days after inoculation. At seven dpi, all plants were still symptomless, wilting started at 14 dpi. A significant difference between genotypes was observed on 14, 21 and 28 dpi ($P < 0.001$ at all-time points). At fourteen dpi, some cv. Moneymaker and *S. neorickii* LA1045 plants developed stem canker. *S. cheesmanii* LA1409 and *S. cheesmanii* LA0166 showed more severe wilting than cv. Moneymaker at 14 and 21 dpi (Figure S1). An overall correlation of 0.6462 was found between the wilting scores in the greenhouse and the *in vitro* conditions

(Figure S2). The somewhat moderate correlation was due to two genotypes with very contrasting scores under the two conditions: *S. habrochaites* LYC4 and *S. pennellii* LA716. Under the greenhouse conditions *S. habrochaites* LYC4 and *S. pennellii* LA716 are susceptible (score 5), but *in vitro* tolerant (score 1 and 2; respectively).

Infected wild relatives were examined with PathoScreen™ and dilution plating to determine the *in planta* colonization and bacterial titre. A strong GFP signal was detected in the plants with severe wilting symptoms. No GFP signal was detected on symptomless and control plants. The bacterial titre based on dilution plating (14, 21 and 28 dpi) showed a highly significant difference between genotypes ($P < 0.001$; Figure S1). Surprisingly, *S. arcanum* LA385, *S. arcanum* PI127829, *S. habrochaites* LYC4 and *S. arcanum* LA2157 have low wilting scores and yet still contained high bacterial titres (Figure S3).

Population dynamics of Cmm in the *in vitro* inoculated plants

The population dynamics of Cmm was examined in the roots and shoots (stems and leaves). The bacterial titre in the plant tissue was related to the timing of the inoculation and which inoculation method was used. The direct inoculation resulted in the beginning in higher concentrations Cmm in shoots and roots of the plants compared to the indirect inoculation. Thirty five days after direct inoculation on the second leaf stage, there was hardly any difference in the bacterial titre of cv. Moneymaker and *S. arcanum* LA2157 in the shoots and roots. However, there was a higher bacterial titre in cv. Moneymaker than in *S. arcanum* LA2157 on the fourth leaf stage inoculation (Table 1). A similar trend was observed after the indirect inoculation. Here, a higher concentration Cmm was detected in the second leaf stage inoculation compared to the fourth leaf stage inoculation in both genotypes. In the fourth leaf stage inoculation at 35 dpi, there was a higher Cmm concentration in the shoot of cv. Moneymaker than in *S. arcanum* LA2157 (Table 1). The indirect inoculation resulted in a higher variation of the bacterial titre throughout the experiment.

Based on the two-way ANOVA analysis, the bacterial titre log (cfu/g+1) was significantly different between the two inoculation methods and the plant age ($P = 0.05$).

Table 1 | Comparison of *S. lycopersicum* cv. Moneymaker and *S. arcanum* LA2157 35 dpi after inoculation.

	Second leaf stage[a]						Fourth leaf stage					
	Direct inoculation[b]			Indirect inoculation			Direct inoculation			Indirect inoculation		
Genotype	**Disease index[c]**	**Plant part**	**Bacterial titre[d] (cfu/g)**	**Disease index**	**Plant part**	**Bacterial titre (cfu/g)**	**Disease index**	**Plant part**	**Bacterial titre (cfu/g)**	**Disease index**	**Plant part**	**Bacterial titre (cfu/g)**
S. lycopersicum cv. Moneymaker	5.0	Shoot	1.70×10^{12}	1.0	Shoot	5.07×10^{11}	4.0	Shoot	3.70×10^{12}	3.3	Shoot	1.55×10^{11}
		Root	4.07×10^{12}		Root	3.27×10^{12}		Root	6.31×10^{11}		Root	8.58×10^{10}
S. arcanum LA2157	1.0	Shoot	6.31×10^{12}	0.0	Shoot	4.17×10^{12}	1.3	Shoot	1.65×10^{11}	1.0	Shoot	1.16×10^{8}
		Root	1.58×10^{12}		Root	2.24×10^{12}		Root	8.25×10^{10}		Root	7.94×10^{10}

[a] The plant stage when the inoculation was done, the second leaf stage or the fourth leaf stage.

[b] The different inoculation methods used. Direct inoculation = cutting the leaves with scissors dipped into 10^7 cfu ml^{-1} bacterial suspension. Indirect inoculation = drop inoculating 100 µl of 10^7 cfu ml^{-1} bacterial suspension at the interspace between stem base and agar medium.

[c] Disease index was determined by averaging the symptom score of each treatment (n = 3). : 0 = no symptoms; 1 = 0 to 25% leaf wilting; 2 = 26 to 50% leaf wilting; 3 = 51 to 75% leaf wilting; 4 = 76 to 100 % leaf wilting; 5 = dead plants. From the two-way ANOVA analysis, the symptom score was significantly different between the two inoculation methods but was not dependent on plant age (P = 0.05).

[d] From the two-way ANOVA analysis, the bacterial titre log (cfu/g+1) was significantly different between the two inoculation methods and the plant age (P = 0.05).

In planta colonization

The PathoScreen™ analysis showed the colonization of the GFP-tagged Cmm *in planta* (Figure 2). Bacterial signals were detected *in planta* as early as 21 dpi with direct inoculation on second leaf stage in both genotypes. The Cmm titre did not correlate with wilting and *in planta* colonization. The corrected GFP normalized against untreated plants (cGFP %) had a low correlation (r = 0.225) with log (cfu+1/g). The wilting symptom was more severe in the cv. Moneymaker than in *S. arcanum* LA2157, but their bacterial titres were almost equal (second leaf stage inoculation) (Figure 2 and Table 1), and surprisingly the GFP signal was hardly detected in *S. arcanum* LA2157 (Figure 2d, h) compared to cv. Moneymaker (Figure 2c, g).

At 35 dpi, a strong GFP signal was detected in different parts of cv. Moneymaker such as the stem, petiole, midvein and lateral vein (Fig 2c, e and g), but the translocation was less in *S. arcanum* LA2157 and the pathogen was detected only in the stem (Figure 2d, h). No GFP signal could be detected in the fourth leaf stage inoculation of the indirect inoculated cv. Moneymaker and *S. arcanum* LA2157 (Figure 2i, j), and direct inoculated *S. arcanum* LA2157 (Figure 2f).

PathoScreen™ could not be used to detect the presence of Cmm in the roots as it could not efficiently differentiate between the autofluorescence presence in the roots and the Cmm-GFP signal.

Discussion

We developed an *in vitro* protocol to evaluate disease symptoms of Cmm in tomato. Two types of variables were tested: inoculation method and plant age. Both direct and indirect inoculation methods resulted in the presence of bacteria and wilting in the plants and the bacterial titre was relatively similar. The indirect inoculation method resulted in less visible wilt symptoms and very variable bacterial titres. Other studies already indicated that a root inoculation method is undesirable due to the disparity with wilt symptoms (Forster and Echandi 1973). The plant age also affects wilting, the best plant age to do *in vitro* inoculation was the fourth leaf stage. An inoculation at this stage, makes it easy to differentiate severity of wilting. The age of the plants plays an important role in disease development (Sharabani et al. 2013b). Younger plants are more susceptible to Cmm compared to older plants. Inoculation done on older plants (more than 16 leaf stage) do not result in wilt symptoms and inoculation on young plants may result in early plant death (Sharabani et al. 2013b). In previous studies done in our group, inoculation done on the fourth leaf stage could more distinctly differentiate between the tolerant genotype Irat L3 and the susceptible genotype cv. Moneymaker than inoculation done on the second leaf

stage (van den Bulk et al. 1991). The result is similar to the results obtained in our *in vitro* screening. Thus, in our system, the best inoculation procedure is a direct inoculation on the fourth leaf stage of the plant. This inoculation procedure resulted in less variation in wilting severity within a genotype, but in clear differences between susceptible and resistant/tolerant genotypes.

In agreement with earlier studies with cv. Moneymaker (Lelis et al. 2014) the disease symptoms developed faster also in the *in vitro* growing wild relatives. Some variation of severity of wilting was found between individual plants of a genotype. The variation could be due to the fact that some genotypes are heterogeneous. Since many of the wild genotypes are self-incompatible, genetic differences can exist within a genotype (Grandillo et al. 2011). The tolerant genotypes from the *in vitro* screening contained considerable amounts of bacterial titre even with low symptom scores (Figure S3). It is similar with what has been observed by Sen et al. (2013). What causes the wild relatives to be tolerant is still unknown. It could be that that tomato wild relatives are nonhost to Cmm (Niks and Marcel 2009) or the metabolic compounds in some wild relatives could inhibit the virulence of Cmm (Shinde et al. 2017). When these tolerant and symptomless plants are grown in the field or greenhouse they could be the source of disease dispersion on the production site, which causes many susceptible plants to severely wilt and wither (Kawaguchi et al. 2010).

The correlation between the symptoms of the *in vitro* screening with the greenhouse experiment was 0.6462 (Figure S2). This moderate score was due to two genotypes, which have high conflicting results between greenhouse scores and *in vitro* scores (*S. habrochaites* LYC4 and *S. pennellii* LA716). Those genotypes are susceptible in the greenhouse (Sen et al. 2013) but were tolerant in our *in vitro* experiment. We hypothesize that the differences in environmental conditions during *in vitro* plant growth and in the glasshouse affected symptom development in these two genotypes more than in the other genotypes. This might be due to differences in the tolerance mechanisms of the wild relatives. Or that specific conditions during the *in vitro* screening might also change the metabolic pathways responsible for tolerance to Cmm. Different metabolites may be synthesized after *in vitro* inoculation compared to greenhouse infection (Schauer et al. 2005). Or that differences in environmental conditions make it more difficult to score the disease severity. For instance, humidity is high (100 %) in the *in vitro* containers and moderately high (60 %) in the greenhouse. Under 100% humidity the *S. pennellii in vitro* plants were small and the growth was stunted. On the small leaves it is difficult to score wilting severity reliably. *S. habrochaites* originates from humid environments (Grandillo et al. 2011). The less high humidity in the greenhouse might result in stress and a relative high susceptibility (Sen et al. 2013). In general, the *in vitro* inoculations work well on most wild relatives. The difference between the two parental lines of our mapping

population (Moneymaker and *S. arcanum* LA2157) was very clear. This makes the *in vitro* method very suitable for recombinant screenings and fine mapping in progeny of these two plants. We used the PathoScreen™ system to detect and quantify the GFP-tagged Cmm *in planta*. This system captures high quality images and quantifies GFP-tagged pathogen based on the pixel number. The PathoScreen™ system is more sensitive and convenient than the epifluorescence stereomicroscope (ESM). The PathoScreen™ system detected the pathogen *in planta* as fast as 21 dpi without the alternative approach of enriching the samples. Using ESM or CLSM, an incubation of plant tissue for 24 to 48 h under selective conditions for growth of the pathogen was required to visualize the bacteria (Lelis et al. 2014). Another advantage is that the pathogen is visualized in the complete *in vitro* plant instead of a visualization in different small parts (Tancos et al. 2013; Lelis et al. 2014). A disadvantage is that with PathoScreen™ the GFP-tagged pathogen cannot be visualized in the roots. This is due to the high autofluorescence in the roots. To visualize the GFP-tagged *in planta*, the PathoScreen™ like ESM and CLSM require a high bacterial titre of at least 10^{12} cfu/ml of GFP-tagged Cmm. However, the PathoScreen™ system is so far the most sensitive and convenient method to detect a bacterial GFP signal. The sensitivity and convenience make the system suitable for detecting GFP-tagged phytopathogens.

In vitro inoculation is a new approach to screen large populations for resistance/tolerance levels. This technique has earlier been used to screen for resistance and to develop new breeding lines (Evans 1986). Tomato callus was screened with Cmm to identify tolerant regenerants (van den Bulk et al. 1991; Zagorska et al. 2004). But a disease screening using callus is not efficient to screen existing tomato genotypes. It is more time effective to use plantlets from seeds for *in vitro* inoculation (Flores et al. 2012).

In conclusion, our *in vitro* inoculation method allows mass screening of many tomato genotypes in an efficient way against a quarantine organism like Cmm. Less space, time and costs are needed for the disease screening. Our work showed that the wilting with the *in vitro* inoculation was similar to the wilt symptoms in a greenhouse assay with only a few exceptions of specific wild relatives. Hence, the protocol could be successfully used to replace greenhouse screenings. This *in vitro* inoculation and screening method will be used to fine map a QTL region originating from *S. arcanum* LA2157 known to harbour a tolerance factor against Cmm.

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Supplementary material

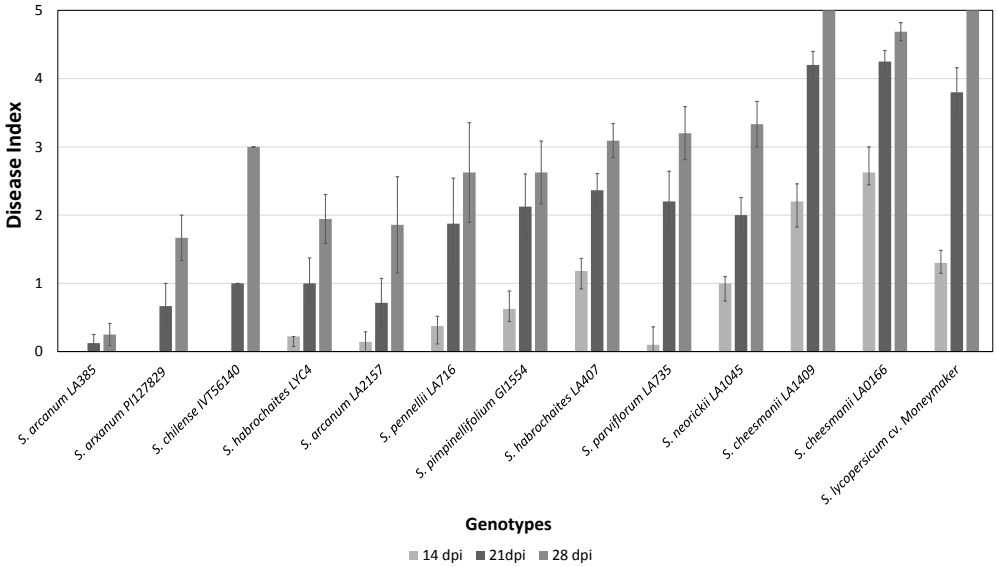

Figure S1 | Disease index of *in vitro* wild tomato genotypes at 14, 21, and 28 dpi. The bars represent the average symptom score of each genotype, vertical lines represent the standard error of the mean (n=10).

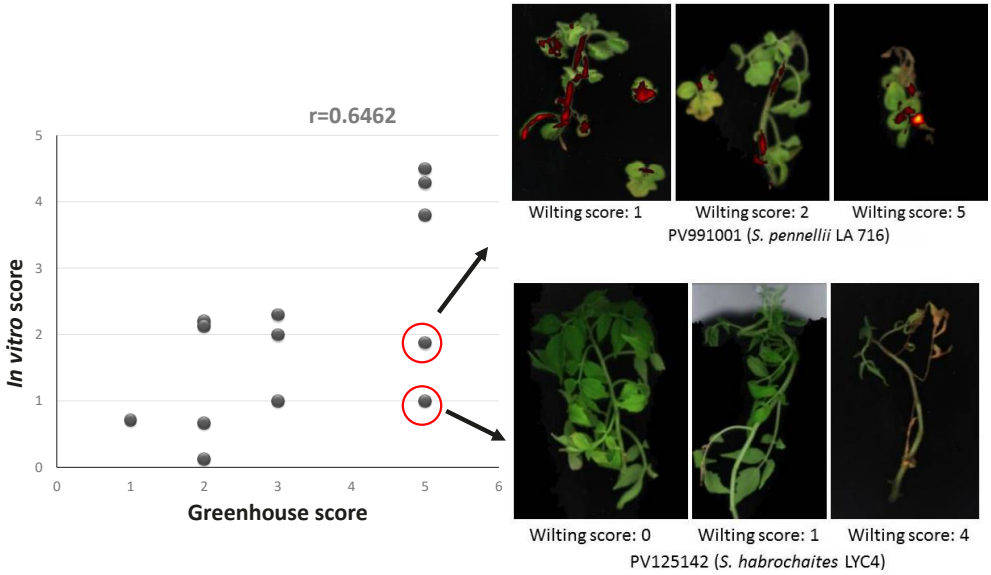

Figure S2 | Relationship between the wilting symptom scores *in vitro* and the greenhouse (Sen et al. 2013). Red circles indicate the genotypes with high conflicting scores between greenhouse and *in vitro* (*S. habrochaites LYC4 and S. pennellii* LA716). Three plants out of ten plants of these genotypes are shown to indicate the variation of disease symptom within each genotype.

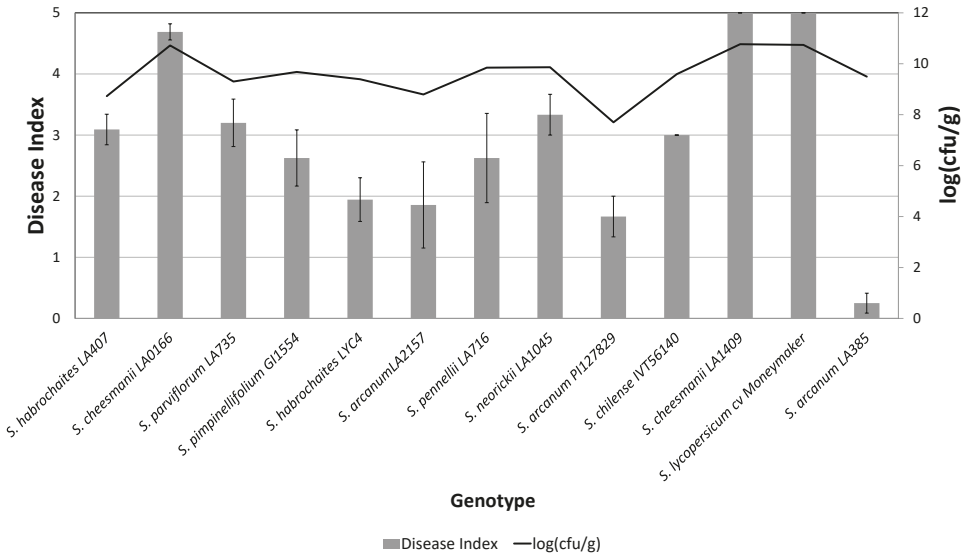

Figure S3 | Disease index and bacterial density of *in vitro* wild tomato genotypes at 28 dpi. The bars represent the average symptom score of each genotype, vertical lines represent the standard error of the mean (n=10). Bacterial titre is represented by the black line.

CHAPTER 3

Fine mapping of a *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) tolerance gene originating from *Solanum arcanum* LA2157

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abstract

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is a quarantine bacterium in Europe and many other countries. Outbreaks cause severe losses in tomato production. Resistance/tolerance breeding is one of the approaches to reduce the effects of Cmm. To understand the resistance mechanism and to identify candidate genes, we fine mapped on chromosome 7 the most important genetic factor(s) involved. This fine mapping was optimised by using a disease screening on *in vitro* plants. The marker analyses were done with Single Nucleotide Polymorphisms (SNPs) and the KASP platform. An in-house prediction tool, the HaploSmasher, was used to predict the gene variants in the fine mapped region of the highly tolerant wild source *Solanum arcanum* LA2157. The region has been fine mapped to 211 Kb which resulted in 15 annotated genes. The results are the first step to understand a tolerance mechanism against Cmm.

Keywords: *Clavibacter, Cmm, Tomato, Disease screening, in vitro, QTL, Fine mapping, KASP*

Introduction

Infection with the Gram-positive pathogenic bacteria, *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), causes wilting and canker in tomato (*Solanum lycopersicum*) (Strider 1969; Davis et al. 1984; Gartemann et al. 2003). Cmm is considered one of the most important bacterial diseases of tomato. The substantial economic losses caused by the pathogen and the difficulty to control the disease made Cmm a quarantine organism under the European Union Plant Health Legislation (CABI and EPPO 1999; Gartemann et al. 2003; Eichenlaub and Gartemann 2011).

Contaminated seeds can be the starting point of major Cmm outbreaks, but in general the production loss in a field or greenhouse is caused by secondary spread of Cmm (León et al. 2011; Lamichhane et al. 2011; Tancos et al. 2013) due to agricultural practices such as the use of contaminated equipment and the spontaneous spread from diseased plants to healthy plants (Sharabani et al. 2013a; Frenkel et al. 2015; Sen et al. 2015; Chalupowicz et al. 2016). Once the pathogen has infected the plant, severe leaf wilting and stem canker can occur. Ultimately the plants with wilting and canker symptoms will die. The severity of the infection is dependent on environmental conditions, the virulence of the strain and the plant genotype (Jahr et al. 1999; Sharabani et al. 2014; Sen et al. 2015). Seed companies and plant multipliers are obliged to sell Cmm-free certified seeds and plantlets based on the Good Seed and Plant Practices (GSPP) (Munkvold 2009; GSPP Standard 2017). For seed companies, resistant/tolerant cultivars should have no seed transmission of Cmm and preferably no Cmm in the plant. For growers, the presence of Cmm should not lead to lower yields or loss of quality of the tomatoes. So far, there are no tomato cultivars in the market that are resistant or medium resistant to Cmm (Thyr 1971; van Steekelenburg 1985; Sen et al. 2013), but several wild relatives of tomato are resistant towards Cmm and show no symptoms (Sen et al. 2013). We investigated how we can transfer the tolerance from the wild species into modern cultivars and which mechanisms and sort of genes play a role in the tolerance.

Screening for Cmm resistance/ tolerance in wild tomato accessions has been going on since the 1930's (Cardon 1934). In tomato breeding, introgression breeding in general started in the sixties of the 20th century (Elenkov 1965). Differences in tolerance were found between individual plants in offspring populations of several crosses between wild tomato species and cultivated tomato. Examples of these wild species are: *S. pimpinellifolium* (Elenkov 1965; Thyr 1968; Jong and Honma 1976; Thyr 1976; Sen et al. 2013), *S. habrochaites* (Hassan et al. 1968; Vulkova and Sotirova 1993; Sotirova et al. 1994; Francis et al. 2001; Kabelka et al. 2002), and *S. arcanum* (Lindhout and Purimahua 1987; Vulkova and Sotirova 1993; Sandbrink et al. 1995; van Heusden et al. 1999). Resistance due to a substantially lower bacterial titre has not yet been

found in the wild relatives. Infected, highly tolerant plants still contain a considerable amount of bacteria (Sen et al. 2013). Nonetheless, these accessions are still valuable as none of them wilt severely after infection and no visible reduction in yield is found. The highest tolerance we identified was in *Solanum arcanum* LA2157 (Sandbrink et al. 1995; van Heusden et al. 1999). Three major Quantitative Trait Loci (QTL) were identified on chromosomes 5, 7 and 9 in the van Heusden study. In an F2 mapping population the QTLs were additive and more than one QTL was needed to have a similar resistance level as the resistant source (van Heusden et al. 1999). The most prominent QTL is located on chromosome 7 and in this study we focused on further fine mapping the gene(s) underlying this QTL. Our fine mapping was successful and reduced the QTL region to 211 Kb. Since the tolerance mechanism is still unknown it is difficult to identify the most likely candidate gene based on their gene sequence only.

Materials and methods

Plant materials and *in vitro* propagation

We used a progeny population of selfed heterozygous Nearly Isogenic Lines (NILs) for fine mapping. The material development is shown in Figure 1.The original F_2 population was a cross between *Solanum lycopersicum* cv. Solentos and *S. arcanum* LA2157 (van Heusden et al. 1999). Progenies containing the introgressed region located in the QTL regions were backcrossed to *S. lycopersicum* cv. Moneymaker to obtain F_2BC_3 NIL lines. A whole background screening was done to identify those NILs with a minimum level of the resistant donor on other chromosomes (Sen 2014). Nearly Isogenic Lines containing different QTLs can be crossed, selfed and screened with markers to find NILs with more than one QTL (combiNILS). Three hundred and seventy seeds of the selfings were sterilized in 70% ethanol for 2 min, 1% NaOCl (Sigma) for 20 min and washed with sterile water for 5 min. Seeds were sown on a GEM medium (2.2 g MS salts/L, 10 g sucrose/L, 8 g Daishin agar/L; pH 5.8) and incubated for 3 days at 4 °C and then for approximately 2 weeks in a growth chamber (Technisch Buro I.K.S. B.V, Leerdam, The Netherlands) at 25 °C, with a relative humidity of 40–70 %, and a 16 h/ 8 h day/ night photoperiod. Cotyledonary explants were transferred to MS30B5 medium (4.3 g MS salts/L, 112 mg vitamin B5/L, 30 g sucrose/L and 8 g agar/L; pH 5.8). Three weeks after transplanting, cuttings were made from the stem of each genotype grown in the MS30B5 medium. Leaf samples were collected and send to VHLGenetics® (Wageningen, The Netherlands) for DNA extraction and genotyping.

Marker development

KASP assays (KBiosciences, UK) were used for genotyping. The nomenclature of the Single Nucleotide Polymorphism (SNP) is based on their physical position on the tomato map (Sol 2.40). Polymorphic markers were designed from the QTL regions of chromosome 7 and 9 (Sen 2014). Other sources of KASP markers were based on results of the SolCAP array (http://solcap.msu.edu/) and the Wageningen UR 150 Tomato Genome Resequencing project (Aflitos et al. 2014). For confirmation of SNPs, some regions were resequenced. The PCR reaction was set up in a final volume of 20 µl (2 µl of PCR buffer (10x), 1 µl 5 mM deoxynucleoside tri-phosphates (dNTPs), 0.1 µl of DreamTaq DNA Polymerase (Thermo Scientific, USA) (5 U/ul), 0.25 µl of forward primer (10 µM), 0.25 µl of reverse primer (10 µM) and 1 µl of DNA (10 µM). Touchdown PCR with 25 cycles 94 °C, 30 s; 60 (-0.4) °C, 30 s; 72 °C, 30 s and 10 cycles 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s. Single band PCR products were send for sequencing (GATC BIOTECH, Germany). Single Nucleotide Polymorphism between the parents were choosen to set up KASP assays (Table S1).

Inoculation on the *in vitro* plants

Three hundred and seventy plantlets of the fine mapping population were screened for the QTL regions on chromosome 7 and 9. After this screening a limited number of plants were *in vitro* screened for Cmm tolerance. Two cuttings per accession were used for the disease assay and the virulent bacterial strain Cmm IPO3356 (Culture Collection of Plant Research International) was used for inoculation. Inoculation was done in plants with four to five leaves, in this stage the petiole of the lower leaf was cut with scissors which had been dipped into 10^8 cfu ml^{-1} bacterial suspension. Plants were scored on 7, 14, 21 and 28 days post inoculation (dpi) by looking at wilting symptoms and the presence of cankers on the stem. Wilting symptoms were scored based on the following scale: 0 = no symptoms; 1 = to 25 % leaf wilting; 2 = 26 to 50 % leaf wilting; 3 = 51 to 75% leaf wilting; 4 = 76 to 100 % leaf wilting; 5 = dead plants. The wilting score combined with the severity of the cankers led to three categories. Plants scored up to 2 were categorised as highly tolerant, plants with wilting score 3 (without stem canker) were categorised as medium tolerant and finally plants scored 3 (with stem canker), 4 and 5 (with/without stem canker) were categorised as susceptible.

Genes in the fine mapped region

Candidate genes in the fine mapped region were mined from the available annotated genes ITAG 3.2 using the Jbrowse browser. Differences in protein sequences between the two parental lines were determined using the in-house HaploSmasher software developed by Plant Breeding, Wageningen University & Research, Wageningen, the Netherlands (http://xapps.plantbreeding.nl:5001). HaploSmasher is a prediction tool which predicts the effects of genetic variants of the annotated genes in tomato. Differences in allelic variations between *S. arcanum* LA2157 and Heinz were determined based on filtering the impact prediction starting from high impact (variants cause disruptive change in the protein), moderate impact (variants cause a non-disruptive change in the protein) to low impact (variants unlikely change protein behaviour) (http://snpeff.sourceforge.net/SnpEff_manual.html).

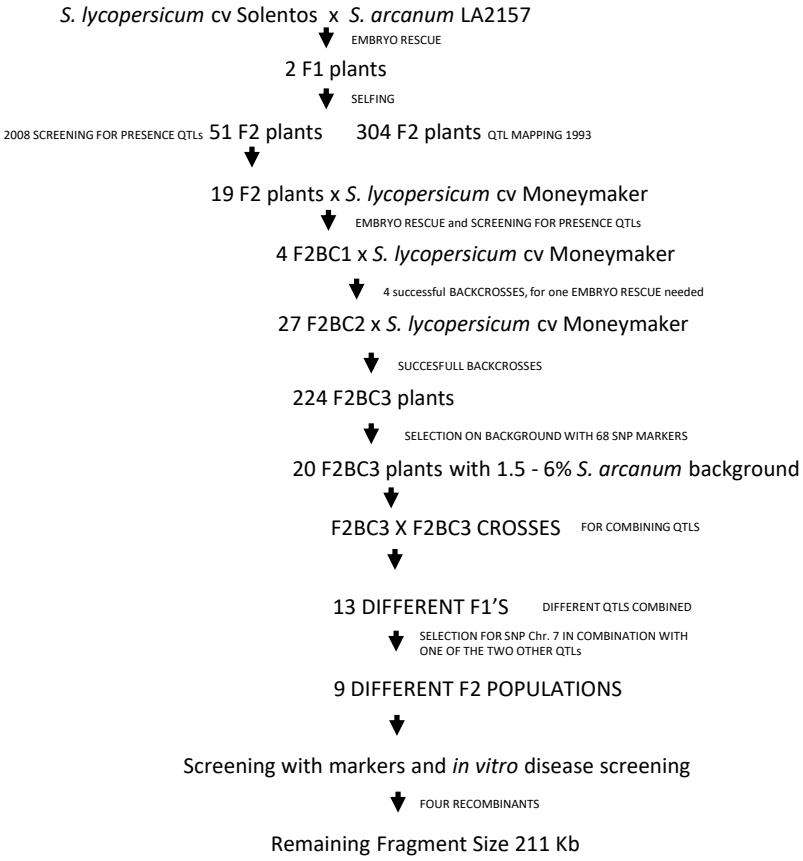

Figure 1 | Material development.

Results

Marker development and mapping

We focused the QTL on chromosome 7 since it is the QTL with the biggest effect. The additional effect of the presence of the whole QTL region on chromosome 9 was also determined. To do this, crosses had to be made between nearly isogenic lines (NILs) containing the QTL region on chromosome 7 and lines with the QTL region on chromosome 9. In our laboratory *S. lycopersicum* cv. Moneymaker and *S. arcanum* LA2157 were resequenced (MM) or de novo sequenced (LA2157) (Aflitos et al. 2014). Twelve KASP assays were designed between the positions of the known markers TG418 and TG61 on chromosome 7 (genetic distance 30 cM; physical region 3.5 Mb) and eight assays between TG254 and TG223 on chromosome 9 (genetic distance 30 cM; physical region 0.5 Mb). Eight out of twelve markers on chromosome 7 and seven out of eight markers on chromosome 9 were indeed polymorphic in the KASP-assay. In total 370 F1 seedlings were genotyped, after screening 127 plants were selected for the *in vitro* screening. Two selection criteria were used: (1) the presence of a recombination between markers SOL07-538753 and SOL07-35387838 (2) chromosome 9 was homozygous *S. arcanum* LA2157 or homozygous cv. Moneymaker between marker SOL09-4822 and SOL09-2840894. The 127 plants were divided into three categories based on their phenotypes (highly tolerant, medium tolerant, and susceptible). More markers were added and some of the F1 plants were genotyped again. In this way, the size of the QTL region on chromosome 7 was delimited to the region between SOL07-1053473 and SOL07-1762987. Plants with no *S. arcanum* LA2157 genome in the QTL region of chromosome 7 but homozygous LA2157 for the QTL region on chromosome 9 were all scored as susceptible (Figure 2a). Plants heterozygous or homozygous LA2157 for both regions were scored as highly tolerant (Figure 2b). Four recombinants in the region of interest on chromosome 7 were found (Figure 2c). The susceptible recombinants PV131855-32 and PV131850-58 delimited the QTL to the region between position 1223013bp and 1762987bp. Another susceptible recombinant (PV131857-29) delimited the QTL to the region between position 1053473bp and 1557551bp. The highly tolerant, double recombinant PV131857-36 points to the region between 1345699bp and 1677474bp. All together this results in a QTL which encompasses the region from 1345699bp to 1557551bp, meaning a reduction to 211 Kb.

Figure 3a shows that most plants homozygous or heterozygous for the QTL on chromosome 7 but without additional QTLs are highly tolerant (16 out of 18). Plants having both QTLs are always highly tolerant or medium tolerant (Figure 3b) and finally plants with only the QTL on chromosome 9 were evaluated as susceptible (Figure 3c). This clearly shows that the QTL on chromosome 7 is more important and that the introduction of only this QTL most of the times result in tolerant plants.

Annotated genes in the fine mapped region

Annotated genes (ITAG 3.2) have been mined in between marker SOL07-1345699 and SOL07-1557551 using JBrowse software. This resulted in 15 annotated genes. The annotated genes were based on the sequence of Heinz 1706 and the cv. Moneymaker is known to be very similar. Allelic changes and possible mutations of the candidate gene in *S. arcanum* LA2157 were further determined using an in-house variant annotation tool, the HaploSmasher software. Three annotated genes were predicted to have high impact variants which resulted in truncated or non-functional proteins in *S. arcanum* LA2157 (*Solyc07g006620*, *Solyc07g006630*, and *Solyc07g006680*) (Table 1). Nine genes were in the moderate impact category. These genes of *S. arcanum* LA2157 are predicted to have different amino acid changes that might change protein effectiveness but not disrupt the proteins or their function. Another three genes were in the low impact category. These genes have a synonymous mutation or splice variant that most probably will not change the protein behaviour (Table 1). The genes in this region could be the starting point for further studies such as functional analysis or differential gene expression.

a

	Position on Chromosome 7													Position on Chromosome 9							
	1053473	1168873	1189063	1222046	1223013	1345699	1557551	1677474	1699978	1707706	1762987	1783593	1919491	48822	86887	629861	756693	1928437	2144983	2840894	Score
PV131859-09	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	AA	AA	AA	AA	AA	AA	AA	S
PV131859-20	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	AA	AA	AA	AA	AA	AA	AA	S
PV131854-17	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	AA	AA	AA	AA	AA	AA	MM	S
PV131854-02	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	AA	AA	AA	AA	AA	AA	MM	S
PV131855-27	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	AA	AA	AA	AA	AA	AA	MM	S
PV131855-24	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	AA	AA	AA	AA	H	H	MM	S

b

PV131852-06	MM	H	H	H	H	H	H	H	H	H	MM	MM	MM	AA	AA	AA	AA	AA	AA	AA	HT
PV131852-13	MM	H	H	H	H	H	H	H	H	H	MM	MM	MM	AA	AA	AA	AA	H	H	H	HT
PV131852-14	MM	H	H	H	H	H	H	H	H	H	MM	MM	MM	AA	AA	AA	AA	AA	AA	AA	HT
PV131852-15	MM	H	H	H	H	H	H	H	H	H	MM	MM	MM	AA	AA	AA	AA	AA	AA	AA	HT
PV131852-16	MM	H	H	H	H	H	H	H	H	H	MM	MM	MM	AA	AA	AA	AA	AA	AA	AA	HT
PV131852-18	MM	H	H	H	H	H	H	H	H	H	MM	MM	MM	AA	AA	AA	AA	AA	AA	AA	HT
PV131852-19	MM	H	H	H	H	H	H	H	H	H	MM	MM	MM	AA	AA	AA	AA	H	H	H	HT

c

PV131855-32	H	H	H	H	H	MM	MM	MM	MM	MM	MM	MM	MM	AA	AA	AA	AA	AA	AA	MM	S
PV131857-29	MM	MM	MM	MM	MM	MM	H	H	H	H	H	H	H	MM	MM	MM	MM	MM	MM	MM	S
PV131857-36	MM	MM	MM	MM	MM	MM	H	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	HT
PV131850-58	H	H	H	H	H	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	MM	S

Figure 2 | Marker scores of 13 markers on chromosome 7 and seven markers on chromosome 9. MM stands for *S. lycopersicum* cv. Moneymaker, AA for *S. arcanum* LA2157 and H is heterozygous. The disease scores are: S (susceptible), MR (medium tolerant) and HT (highly tolerant). The disease score has been done on two cuttings of the same genotype.

a

	Chr. 7		Chr. 9		
	1345699	1557551	48822	2144983	Score
PV131857-01	H	H	MM	MM	HT
PV131857-08	H	H	MM	MM	HT
PV131857-37	H	H	MM	MM	HT
PV131850-02	AA	AA	MM	MM	HT
PV131850-09	H	H	MM	MM	HT
PV131850-13	H	H	MM	MM	HT
PV131850-17	H	H	MM	MM	HT
PV131850-19	H	H	MM	MM	S
PV131850-21	AA	AA	MM	MM	HT
PV131850-22	AA	AA	MM	MM	S
PV131850-25	H	H	MM	MM	HT
PV131850-43	AA	AA	MM	MM	HT
PV131850-49	H	H	MM	MM	HT
PV131850-51	AA	AA	MM	MM	HT
PV131850-63	H	H	MM	MM	HT
PV131850-66	H	AA	MM	MM	HT
PV131850-69	AA	AA	MM	MM	HT
PV131850-73	AA	AA	MM	MM	HT

b

	Chr. 7		Chr. 9		
	1345699	1557551	48822	2144983	Score
PV131859-03	AA	AA	AA	AA	HT
PV131859-04	AA	AA	AA	H	HT
PV131859-06	H	H	AA	AA	MT
PV131859-07	H	H	AA	H	HT
PV131859-19	H	H	AA	AA	MT
PV131859-21	AA	AA	AA	AA	HT
PV131859-22	H	H	AA	AA	HT
PV131859-23	H	H	AA	AA	HT
PV131859-26	H	H	AA	H	HT
PV131859-27	H	H	AA	H	HT
PV131859-28	H	H	AA	AA	HT
PV131859-29	H	H	AA	AA	HT
PV131859-30	AA	AA	AA	H	HT
PV131859-33	AA	AA	AA	AA	HT
PV131859-34	AA	AA	AA	AA	HT
PV131859-36	H	H	AA	H	HT
PV131854-03	H	H	AA	AA	HT
PV131854-11	H	H	AA	AA	HT
PV131854-13	AA	AA	AA	AA	HT
PV131854-15	H	H	H	H	HT
PV131854-16	AA	AA	AA	AA	HT
PV131854-20	H	H	AA	AA	HT
PV131854-27	H	H	AA	AA	HT

c

	Chr. 7		Chr. 9		
	1345699	1557551	48822	2144983	Score
PV131859-09	MM	MM	AA	AA	S
PV131859-20	MM	MM	AA	AA	S
PV131854-17	MM	MM	AA	AA	S
PV131854-02	MM	MM	AA	AA	S
PV131855-27	MM	MM	AA	AA	S
PV131855-24	MM	MM	AA	H	S

Figure 3 | Marker scores of the flanking markers of the QTL regions on chromosome 7 and 9. MM stands for *S. lycopersicum* cv. Moneymaker, AA for *S. arcanum* LA2157 and H is heterozygous. For disease scores see legend of Figure 2.

Table 1 | Annotated genes in the region of SOL07-1345699...SOL07-1557551.

SGN ITAG 3.2	Position	Description[a]	Ontology term
Solyc07g006550	1370920..1371769	Ribonuclease S-6	
Solyc07g006560	1375314..1372873	Hypersensitive response assisting protein	GO:0033897 – ribonuclease T2 activity
Solyc07g006570	1383245..1385020	Ribonuclease	GO:0033897 – ribonuclease T2 activity
Solyc07g006580	1387336..1406636	Diacylglycerol kinase	GO:0004143 – diacylglycerol kinase activity
Solyc07g006590	1425532..1425798	Kinase superfamily with octicosapeptide/Phox/ Bem1p domain-containing protein	
Solyc07g006600	1448390..1448947	Glutamine dumper 3	
Solyc07g006610	1469104..1474046	Tyrosine kinase family protein	GO:0005515 – protein binding GO:0004674 – protein serine/ threonine kinase activity
Solyc07g006620	1480278..1487817	Receptor-like kinase	GO:0006468 – protein phosphorylation
Solyc07g006630	1496758..1494884	CONSTANS-like protein	GO:0003700 – sequence-specific DNA binding transcription factor activity
Solyc07g006640	1506409..1518012	ADP-ribosylation factor family protein	GO:0005525 – GTP binding GO:0005622 – intracellular
Solyc07g006650	1523093..1516230	Xylose isomerase	GO:0005975 – carbohydrate metabolic process GO:0009045 – xylose isomerase activity
Solyc07g006660	1526859..1525198	Glyoxal oxidase-related protein	
Solyc07g006670	1528452..1529837	HXXXD-type acyl-transferase family protein	GO:0016740 – transferase activity
Solyc07g006680	1533723..1535081	HXXXD-type acyl-transferase family protein	GO:0016740 – transferase activity
Solyc07g006690	1558351..1547717	alpha/beta-Hydrolases superfamily protein	

[a] Annotated genes from the Sol Genomics Network. Genes retrieved from the Wageningen UR 150 Tomato Genome Resequencing Project (https://solgenomics.net/organism/Solanum_lycopersicum/tomato_150).
[b] Impact prediction of the annotated genes of *S. arcanum* LA2157 compared to Heinz using the in-house software, the HaploSmasher. Three impact categories: High, variant has high impact in the gene products causing truncated or loss-of-function protein; Moderate, variant is non-disruptive causing effectiveness of the protein; and Low, variant is harmless and do not change behaviour of the protein. (http://snpeff.sourceforge.net/SnpEff_manual.html).
[c] Type of allelic variation of the annotated gene f *S. arcanum* LA2157 compared to Heinz.
[d] Type of mutation of the annotated gene in *S. arcanum* LA2157.

Functional category	Gene orthologue	Impact prediction[b]	Genetic variation[c]	Type of mutations[d]	References
Nucleic acid-related	RNS6_PYRPY	Low	SNPs	Synonymous mutation	(Kondo et al. 2002)
Defence	Q9SWC6_CAPAN	Moderate	SNPs	Missense mutation	(Tripathi et al. 2010; Tripathi et al. 2017)
Nucleic acid-related	Q41722_ZINVI	Moderate	SNPs	Missense mutation	
Signalling	K4CBC9_SOLLC	Moderate	SNPs	Missense mutation	(Snedden and Blumwald 2000)
Stress	AT3G24715.3	Low	SNP	Synonymous mutation	(Shahzad et al. 2016)
Nucleic acid-related	AT5G57685.1	Moderate	SNPs	Missense mutation	(Pratelli et al. 2010)
Signalling	G7JD53_MEDTR	Moderate	SNPs	Missense mutation	(Lemmon and Schlessinger 2011)
Signalling	G7JD52_MEDTR, AT5G57670	High	Deletion	Frameshift mutation	(Tunc-Ozdemir and Jones 2017)
Transcription	B2MW87_SOLLC, AT5G57660	High	Deletion	In frame mutation	(Riechmann et al. 2000)
Nucleic acid-related	B9IM33_POPTR	Low	SNPs	Synonymous mutation	(Memon 2004)
Carbohydrate metabolism	A0A0V0IEZ8_SOLCH	Moderate	SNP	Missense mutation	(Jaquinod et al. 2007)
Cell wall-related	AT3G57620.1	Moderate	SNPs	Missense mutation	(Kim et al. 2006)
Stress	AT3G26040.1	Moderate	SNPs	Missense mutation	(Černý et al. 2013)
	AT3G26040.1	High	Insertion	Frameshift mutation	
Nucleic acid-related	AT4G25770.1	Moderate	SNPs	Missense mutation	(Dal Bosco et al. 2004)

Discussion

The fine mapping of the two most valuable QTLs was started by Sen (2014) by using more markers in the original F_2 population (van Heusden et al. 1999). Even though more markers help in saturating the linkage map and reducing the QTL region, the region was still large (Sen 2014). To speed up the fine mapping process we used recombinants based on crosses between combi-NILs (both the known QTL region on Chr. 7 and on Chr. 9 heterozygously present). In this way, the QTL regions are present but there is no donor background on the other chromosomes. A previous study had shown that resistance is additive and the combination of more than one QTL results in a better resistance (van Heusden et al. 1999).

We decided to fine map only the QTL on chromosome 7 since it confers the highest resistance level. Fine mapping the QTL region on chromosome 9 was difficult because there was no recombination detected in this QTL region. This was unexpected since this region on the chromosome was expected to have higher levels of recombination. This could be due to the different chromosome structures of the two parents (Yang et al. 2014c) or an inversion of *S. arcanum* LA2157 in certain region(s) of this chromosome (Wolters et al. 2015).

Although we could not test as many plants as we wanted due to technical reasons, the results are clear. The final QTL region on chromosome 7 was only 211 Kb in size and this region was essential to obtain tolerance. Figure 3 shows that the additional effect of the QTL on chromosome 9 is not big. Only two plants were scored susceptible although they were LA2157 in the QTL region on chromosome 7. We are not sure what the reason for this is; maybe an experimental mistake or a special phenotype of these plants. Exceptions were not found when both QTL7 and QTL9 were present (all plants were highly tolerant with two plants being medium tolerant). Our results show that in a relatively easy way the QTL region can even be more delimited. This can be done by extensive genotyping or even sequencing the QTL region of the four recombinants. Depending on the sites of recombination this will delimit the size of the QTL region even more. Phenotyping the offspring which contain the introgression between marker SOL07-1345699 and SOL07-1557551 should confirm the results. Overall, we have shown that in a single nearly isogenic line with only 1.5 – 6% of the wild relative tolerance to Cmm depends on a relatively small region on chromosome 7.

A big challenge that we were facing in the fine mapping was the limited space we have available in our quarantine greenhouse. We could only work in a small quarantine compartment that could barely fit 100 plants. In such a setting, cross-contamination from one plant to another can occur when plants are too close together. Even though we have highly tolerant plants, Cmm can infect the plants

systemically (vascular infection) and externally (local infection). The virulence factors used for Cmm for each type of infections are different (Chalupowicz et al. 2016). Plus, the severity of wilt symptoms depends on many aspects. Previously, the F2 plants that were tested for identifying the QTL was done in a large compartment, and we did vascular infection for the disease test (van Heusden et al. 1999). We do not know whether our NILs are also highly tolerant towards the local infection, so to overcome this problem, we developed a disease screening assay using an *in vitro* approach (**Chapter 2**). This protocol functions very well to distinguish our two parental lines; the *in vitro* screening showed distinct differences in wilt symptoms between cv. Moneymaker and *S. arcanum* LA2157. The scores of the *in vitro* scoring and the greenhouse scoring were comparable for cv. Moneymaker and *S. arcanum* LA2157 using the optimised protocol we developed (**Chapter 2**). The method might need some minor adaptations for different parental combinations and might also give variable results depending on differences between the different resistance mechanisms.

With the *in vitro* screening, up to 1000 screenings can easily be done in one experiment and the screenings become more reliable due the fact that more cuttings can be made of a single genotype. To our knowledge, this is the first time that such a method has been used to screen for Cmm symptoms in a fine mapping study.

The defence mechanisms in tomato plants against Cmm are still unknown, our original QTL analysis and studies done in other populations show that the resistance mechanism look complex (Emmatty and John 1973; Kuriyama and Kuniyasu 1974; van Heusden et al. 1999). It is still unclear what genes are involved in giving resistance. Wilting is not caused by xylem plugging by bacterial cells as Cmm100, cured of the virulent plasmids, still colonized the plants but did not cause wilting symptoms (Meletzus et al. 1993; Savidor et al. 2012). Also in our case the number of the bacterial titre did not correlate with wilting symptoms as tolerant accessions still contain considerable amounts of bacterial titre (Sen et al. 2013). Thus it is highly possible that the tolerance in *S. arcanum* LA2157 might be due to changes in the immune system against Cmm, its morphology or metabolites (Shinde et al. 2017).

Studies on differential gene expression can be done to look at the up- or downregulation of genes in the small fine mapped region. Similar studies have been done but no genes were identified that could be connected directly to resistance in *S. arcanum* LA2157 (Balaji et al. 2008; Lara-Ávila et al. 2011; Savidor et al. 2012). The advancement of technologies like RNA-seq in combination with very well defined nearly isogenic lines might help to overcome this problem. Further

breeding will make it possible to remove all, not tolerant related LA2157 genome in the nearly isogenic lines. In our lines there is still 1.5 – 6% LA2157 present. A functional analysis of genes in the very small fine mapped region can be done to see if silencing, knock-outs or overexpression of the identified genes improve the resistance in susceptible cultivars. We have made a big step in the development of plant material most suited to do further studies. We showed that only a very small region on chromosome 7 of *S. arcanum* LA2157 induces resistance and that the possibilities to clone (the) tolerance factors are promising. Further development and multiplication of material will make it possible to study the effects of this region under greenhouse conditions. This might also give indications how general applicable and durable the resistance is.

Acknowledgements

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Supplementary material

Table S1 | All KASPar markers used. Markers are named based on the physical position of SNP between Heinz and *Solanum arcanum* LA2157.

Marker	Position (bp)	Chr.	Marker used for
SOL07-538753	538753	7	Screening for recombinants
SOL07-681821	681821	7	Screening for recombinants
SOL07-1345699	1345699	7	Screening for recombinants
SOL07-1557551	1557551	7	Screening for recombinants
SOL07-3459000	3459000	7	Screening for recombinants
SOL07-7464234	7464234	7	Screening for recombinants
SOL07-22385907	22385907	7	Screening for recombinants
SOL07-35387838	35387838	7	Screening for recombinants
SOL09-48822	48822	9	Screening for recombinants
SOL09-86887	86887	9	Screening for recombinants
SOL09-629861	629861	9	Screening for recombinants
SOL09-756693	756693	9	Screening for recombinants
SOL09-1928437	1928437	9	Screening for recombinants
SOL09-2144983	2144983	9	Screening for recombinants

Sequence (5′ -> 3′)
GGATCATGCAACAATATCACAGCAGCAATGCTAGTGATCGGTACTCTTACAGCGTTAAGGACACCAGCCAAGACAG TGGATGAGAGGAACACAAC**[T/C]**GCGGTGGCTCCCAACACTCCCAACTGGAAAGTGATAGTCCCCCAAACGATA-ACTGAGTAATACGCGTTTTCCCCACCCTTGAATGTACTTGCCTCG
GGAGACGGCCAACGACTCCTTTATCACCTACCAGTCCATCACCATCAACACCTGTACAGGATACATCTGCAGAAG AATTGTTGTCCAGAAAGATG**[A/G]**CAGGCAATAGGTTGGCAGAATCTTTATGGCCCTCAACAATGAGGAGTCT-GAGTGTTTCTTTTCAGTCTGATTCATTTTCCTTACCTGTTAGTAAGA
TCTCAGTTACATCAGTTCCTCACGAGCTCGTCAGCTCCGCGGATGACACTATCCCATTCAAGCCAATTGAATTCCTG TTCGCTCGACGTGAGATC**[A/C]**AGAAAACAATCAGCAAGAAATTCTCCATGGTCATCGTCGATGACAAGGTCTC-CATCGAAGTTGAAGACGAGATAGTAGACCGGATCCTTGGTGGCT
TTGAGTTTTTTTTCAAAGTCAAAATCAAGATTTGACAATAAGGGTGTCAATAATTGTAGGCTGTTGTGGGTGGAT GAGAAAGGGGAAATGGAATT**[A/G]**CTGAGGAAATTAAGCAGAGGGTGTTTCAGAAATAGTCTTAAGAAGGGT-CAGAAGGTTAAAATTGAGTCAGTTAATGATGGTGAAGATGTGTTTGAT
GCAATCCCAGATATATTCTTCTCCAAAGTCACTAAATCAAGCTCTGGTCGCTCCAAACTCTTCCCAGAAGGGTCACC AATTCGACCCGTAGCACC**[T/C]**CCAATAAGCCCCACAGCATTATGCCCACAACGTAAAAACCAAGAAAGCACAAT-TATACCCAAAAGATTACCAAGGTGTAAGCTTTCAGCAGTCGGG
CTATAATCACCGTAGGATCGATGTATATGAATTGAACTGGGTTAAAACCTTCCTCCATGCCACAAAGTTCCCAGA ACCTCCACCACGATAAGTTCT**[A/G]**GTATCACAGAAGTCGAAGTTGAAGAAATAACTATTTTGGGAGCTTATTG-GAAGTTTTATGAATTGTGATAACCACTTAATGTAAGTTTCTATAA
ATTTCAGCTTTAAAATATTCAACAGCAGTACCAGTGATATTTGTGTCAGCCCTTCAGTATCACGTATTCCCTGACAAGT GGGTTAACCTTTATAG**[A/G]**CCTTTGTGGCTTGTCTCAGCCGTTGTGAACTGTCTCTATTCATTTTATTGGGATCTGA-CAAGAGATTGGGACTTAAGGTGAGTGAATTAAGTTTTA
AAACTTAAAGCTTCTTATAAAGCGACTACTGGTGGAAAATTTTCTGATGCACTTAGACTATTCCTGAGCATCCTT CACACCATTCCTCTGATTGTG**[A/G]**TTGAGTCGAGACGAGAAGTGGATGAAGTAAAGGAATTGATTGTCATAGT-GAAAGAGTATGTTTTGGGTTTGCAGATGGAGCTTAAGAGGAAGGAA
TGGTTCAGTTACACCAGATTTGACCCTGGGAAGGTTGTGGCTGTGGAACACTATGAAGATGAGACCCCAGATGA CACCGAAGATGACGATGAGGG**[T/G]**GGAAAAGAAGCATCTCTTGGGCGTTATTGTGTCTTCTGTAGTAAACTT-GATTTTCAGAAAAATGAAGCAATGCATGATCCAAAATGCACTTGTCAT
GCAAGAAGAAGACCCATGAAGAGTTGGATGACTTACCTCCTTATCAATATATTACACAGGAGTACAACCCGGTCAG TAGGCAAAAGGGAAGTAAG**[A/G]**AGGCCAGCAACCGTACTAGATATGTTTTGCTTCATCTGCTCAAATGTAACTC-TATTTTCATCAACCACGCGTCCTGTCAAGTTTTTCTTATACTCT
TTTCTTTTGTAGGAGAGCTGCCATTCTTCTTTGTTTCTATTATTAAGGTGTATAAAATTCAGCTATGTAGGTTCTCATTA CATTGTACTCAAAAA**[A/G]**GCATAAAGTTCTTGCTCATGAATAAAATTTTACTTTCTATTTCTCTTGAAAGTTCATTT-GCTGGTCTTCTCAAAAGTTCTAAAGTGTATTCTTTTC
TGCTGCAGAATTTGTCTGTTGCAAACAACCAGTTAAGTGGAAAAATTACAGAGGAAGTTGGGTTGATTATGTCATT AGAATTTTTGGATCTTTCC**[A/G]A**GAACATGTTTAGTGGTTCTATACCCTCTAAGCTGACTAGTTTAAAGAACTTAG-TATCTCTTAATCTTTCTTTAAATAGTCTCGATGGAATGGTTC
GAGGATGCTATGAGTGCTCCAATTTTGGAAAGAGAGTACATGCAAGGCATCCTAATCAGTGAGGTTGTCTTAAATG AGCGTTTTTCACCACCATC**[T/C]**GGGAGAGCCAGAAGGAGGCAAAAGAGATTTGTAAGGGAATTAAAGAGAC-CAGGTGAAGCAATCATAAAGGGTCACAGGAGTTATGATTTAATGCTA
AACACCAATATACAGGGTGCATTAATGGCTGTTAGGGATTTAGTTCAGTTCATGGGAAGTATTAAAAGTGGACTATA CAATTCTGTAAGGAGGTA**[T/C]**ATTTGTAAACTTGAGACTCCTAGTTCTGTTCAGGTGACTTTGTCTTCTCAGACA-GATGAGAAGTTACTGATAACTGCAAGTGCAATTACTTTAGCA

Marker	Position (bp)	Chr.	Marker used for
SOL09-2840894	2840894	9	Screening for recombinants
SOL07-778336	778336	7	Fine mapping
SOL07-983528	983528	7	Fine mapping
SOL07-1104812	1104812	7	Fine mapping
SOL07-1223013	1223013	7	Fine mapping
SOL07-1890817	1890817	7	Fine mapping
SOL07-1919491	1919491	7	Fine mapping
SOL07-2087565	2087565	7	Fine mapping
SOL07-2354528	2354528	7	Fine mapping
SOL07-2870461	2870461	7	Fine mapping
SOL07-3078229	3078229	7	Fine mapping
SOL07-3207372	3207372	7	Fine mapping
SOL07-3398993	3398993	7	Fine mapping
SOL07-3360966	3360966	7	Fine mapping
SOL07-1000753	1000753	7	Fine mapping

Sequence (5′ -> 3′)
GCAACAATAGATGATCTTCTCTCCACTGTATATGTTTGGCATGTCCAAAACCCGCATGCTGGTGACGTTTTGGTTGAT TCTATGAAAAGGTATTA**[T/C]**GGAAAGTTACCTGCAGTCGTTGAACTGTTTAGTCAAGTTGGAGCACAGGTCGGA-GATGATTACTATCACATGATGAGAGATGATGTTGTAGTTCCT
TATCAATCGAGTGAGGGCCCTTGAAGTAGCTAAATACTCTATTGCAGGTTCATAGTATGACAAAAGCCACAC ACAAAGGCATGTCTCATAGAGA**[G/A]**GCTGTTTAAAGGAAGAAAATAAAATCAGGAAAAAGAAAGAAA-GATATATTCTTTTGTAATCTAGCTTCAGGAGGTGGTTGATAGGTTGAATGA
AGGACAAAATAACGTCTCTGCCCACAGACCTGAAGATCTCCGAGTAATTTTTTGTTAAGCACAGGAGCAGCTGAG TAAGGCGAGAGCCCTCGC**[A/G]**CACCAGTACAGGCTTCCTGCTCCACTCCTGCACTGGTATTTGTGAAGTTACT-TAATTGCTATTCAGAATCTTCGTAAACTTCACCATCATCAGA
AAACTTATACTAATCAAAACAAAAAGTTACACATTTGATCACTCAGACAAAAATATTTTACTTACAACTACTAGCC AATATACATATACTATATATA**[C/T]**ACTAATTATACAATTGTCGATTTATCTTTTTGGGTGAACGACTATTTAAGTTA-ATTTCTATATTTCATTTTTTTCATTCAAGATTAATTTTTTA
TTAGATAGACTGAGCAACATATGATTTTTCATTGATGGAGGTAAATTTTGTTGTTATCATTTATCACTGAGGTAGTAC CTTTGTCGCGAATGGT**[T/A]**TATTGTTTTTACCTCTCAGAGTGTCACGATAGAGTTACGAATGAGAAATATGTAAA-CATACTATGCGGAGTTACCCAGTATCTTTGTCTGT
CTGTTTCACTTTGGTACCGACTGGAAATTTCTCCTTGTTTTAGGTTGGCGATTTGGGGCTATCCAAAGTGAAATGT CAAACACTTATCTCAGG**[T/C]**GGTGTACGAGGAACTCTTCCCTGGATGGCACCAGAACTTCTCAATGGAAGCAG-CAGTCTTGTCTCTGAGAAGGTATAAAAAAAAATTATTTTGT
GCCAAATTTGCAAGGAGAAGTATTTTCAGGACTAAAGAGAAGTGGATCAAAGAAGTTTAATTGGGCAAAAATG ATTTGGAAATCTCAAGAACA**[A/G]**GATGAATGTTCAATTTGCTTGGATCAATTCAAGATTAGTGATAACTTAATG-CAATTGACATGTGCCCACAAATTCCATTCCAAGTGTTTGGTGC
ACTTAAAGCAGGAACCCCTAGTGCAAGGAACAGCAAGAGCTTAGATGAAGAATTGTGAAGCAATGCACTGTAGA CTTCTGTATACACTGCAGC**[A/G]**CTGAGCCCTCCATAGCCTTTAAGAATCCCAGCAACCGTGCCTCGGCTTAGAG-GAAAGTTTCTCATATTAGTCACAAGCACAGTTGTGCTGAACC
TTGACGTAGTCTCTAAATGATTTTAACCCCGCGAGGTAATTTGACTCTTAATCTTTTCCACTTGCTCTTAGCCAAGG ATGGATGCAAGCTGAG**[T/C]**ACATGTTCTGGCATGAAATCATGCCTTGTAATTATCCCCACAACAGGTATCCTCTG-CAGACAAGAGAACCACAATTTCTTGATTCAGTTCAAGA
GAATAAATGAAGTTGAACATTCATGAATATATAGTACCTCAACAGGAGTTGCCAAGATCAGAAGATACTGGATGGCC TCCACGAAAATTCCAG**[A/G]**TTTGACTTTGGCTAGGCCAACAACGCATATAGCTTGTTCATCGCCACTATATTCAG-GACAGTGACCATCCCTAAACAAAACATGATACCATCAA
GGAATTAAACAACTATAACATAACCCCATTTAAAGTACAATGCTGTTGATATAACATGAAAAATTTCTGGAAATCTCA CAATATGCATCAATC**[T/C]**GAAAGGTTTTCATATAGAAGGCTGTGAAAATGATGCATAGCCTTCTCAACTTGTGGC-GCGTAGCGGCCAGAGCAGTAAGCCGGTGATTCAAGCC
AACAGCCAACTCAACAAGGTATGTCCAACTAGCGCGATCGCGGCTATAACGGCCATAACCATCATCTTGCTTTGGG CTTGAAGAAATTTTTGAATAGGGAAGTTCA**[A/T]**TGCATAGGCAAATAGTTGAGGTATCATCCAAATAGAAAATT-TACCAGCCCATTTTGCTATGTCCATAGGCTGGCCAATGAATAGTAGAATTTGAGTTGCAAATAAG
TCCTCACTTCTTTTTCCCGCAACTACAAAAACTTAACCTAAAACCCTAAATTACTCTTTTAGCAGAGGCGATCAA TGGCTTCCATCGAACAAATCCAAAATGGTTC**[T/G]**CTTGTAACGCATGAATTTGAAGATTCTCAAGTGAATCCG-GAAGAAGCAGACGGAGGTTTGGTGAAAATCGGACAAGAGGTTTCGATATTGAAGCCGAAATGGCCTGG
GCTGGAATAAGTCAATTATAATTCTGACGTAAATTGGAGCAACTTCACCGGCCAGTTAGGGTGATTATCACT TAGGTGAAGAAGAAGTAGTGC**[T/C]**GAACGAATTTGAATTCTGGTCGGATTGTTTCGAACTTGGCTACAAT-GAGTTTGGGCTTGTTGGGGACTTTATAAGGTTCGAATTCTTCTATCCA
AATCACAAAAGAAAAAGGGAAATCTCTTCAAAAACCTGCCTCTGCAAGTAATCGAAACGGAAACTACCAGCT ATCATCCTTATTTATTTTAACATTCTCAACTTCGATT**[T/C]**CAACTCATGTTGATTGGCTTAATGACTTGGGAGAG-TATCTCGATGCAGGAAAGATGTGTGGTACGTGAAATTGGCTCAGCAGTGGACTACAGGAAGTTGACTTCGGTATAG-TATTTAAC

Marker	Position (bp)	Chr.	Marker used for
SOL07-1031074	1031074	7	Fine mapping
SOL07-1047255	1047255	7	Fine mapping
SOL07-1053473	1053473	7	Fine mapping
SOL07-1168873	1168873	7	Fine mapping
SOL07-1189063	1189063	7	Fine mapping
SOL07-1222096	1222096	7	Fine mapping
SOL07-1677474	1677474	7	Fine mapping
SOL07-1699978	1699978	7	Fine mapping
SOL07-1707706	1707706	7	Fine mapping
SOL07-1740467	1740467	7	Fine mapping
SOL07-1762987	1762987	7	Fine mapping
SOL07-1783593	1783593	7	Fine mapping

Sequence (5′ -> 3′)
CACACCTAAAATTGGAAAAAAGAAAAAACAGTACATCATCATTTTATAAGAATCCTTATGTTTACTGGAAGGAA ACACAGTACTACAGGAATGTACCAAGTGCATAAAAGCTAA**[G/A]**GCACAAATGTCAACTAACAAAAACACT-TACTTGTCACGATCAACTCCAGGAAGGTCAGCAGCAATAAGAGCCATTACTCCCATACGGTACATGTGATCAGC-TATAGACTCAGG
CATAAAACACAAACCAAACATAATTCCTTAAAACACAAATCACAATGATCAAACTAGTAGTACAAAGTTACCTTA-CATTAAGACTTCACCATAATATTTATTACTTAATATTTT**[C/T]**TTGAGGTAGTGAATTTTTTTTCACTAGTGAAATAT-GACATGCATGCATGGATTTAGTTGCCACCATTTCCTCCACCATACCCACTACCCTGTCCACCTCCATATCCACTAC-CACTGGA
CATTTCAGGAACCAAGTTAGGTGGATATGGAAGTTTGATGAACTAGATTATCCTATTGATGGCATAGATGAGGTTGTT AGACCTAACAGTTTTGCTAATGCTGCACCATTTTGTC**[G/A]**AATGATGTATGTTAAAATTTTGACGATTGTATAAACT-GCAGATTAGATTCCAAGTCCATAATGTTAGTTATCCATCAATGCCACTGGAGCAAGATAAAGATTCAAAACCATTTG
GCCGATGGTCAATTGACATGAATCTACTTCCATATCGTCACCAGTACATAAACCCTTAAGATGAAATGTCATTATAAT GCTAAATCGATGAATGAGCATCAACTAGACCAATAT**[A/T]**TGCTGAACTGGACTCACCTAGCAATCTTCACTATACT-CATAAGAGGGAGGCATAAGAATGGTGAAGGAAGGGGACGTGGTTGTTTTTCTGGACGAGTACTTAAAATCTCCTC-CA
AATCAGCAATTATCCCTTCTTCAACCACCTCCACCCCCATTTTCTACTCATGATTCTAGTGGTGGTGGTATTTTC AATTTGAATAACAAGGTTAGTCCAAGTATACTTCTAGT**[A/C]**ATCATTATTCTTGCTATTATCTTTTTTATATCTG-GTTTGCTTCATTTAGCTGTAAGATGTCTATTAAGGCCATCAAATAGAGATCCAGATGATTTAGATAATGTAA-CAGCCCTTCAAGG
CTTCCACCGACGTCATCGACGTCGCCACCACCATCGATTTCCGGCGACCCCAACGTCATCGTTTGATTTGCCTTGT GTTAGTTATTGTGGGTCACAGCAATCTG**[C/T]**TAGGGATTGTGCGATTTGCTTGGAGGGATTTAAGGATGGAGA-AATTTGTAGGAAATTACCTGATTGTGGACACCTTTTTCATGTGAAATGTGTGGATTCTTGG
CAGTCAGAAAAAGAAATTGAACCCCATGAAAAAGGTCACAGCTCCTTTTTTTCTTGCAGTGTTTCTGTGGAGCC TTTTGGTTCAAAACAACCTTCAGGACACCTGGAT**[T/A]**TTTCATTATCAGAAGGAAGATATCACCCTCAGCTTTT-GAGACTAGAGTCTCGCTTGAATTCAGACAAGCAAAAGAGTACAGATACTCCGAAGGATGGAGACACTGATGAAAT
TGAAGTGTATTTCATTTAATTGTGGTTCTTACCTTTTGATAAAGAATGTGCCAGCTCTTATATGGACTTTAAATGTTCTT GTAGGTATACGTAGATTCTTTGGTCAAGAAGGC**[A/G]**TATGATAATTGGAATCAAGTCGTTGAATATGATGGCAAGT-CATTTCTGAACATCAAGCAAAATCAAAATCCAAGCTCTTCTAGGAACGAGCTTCCTGTTGGGCCAGTGGATTACCC
TGATGAACAAATTTCCTCCAGGTTTTCGATTTCATCCTACGGATTATGAGCTAATTAAACATTATTTGGAGAGGAAGC TTGCTAATTTGCCTTTGCATCC**[C/T]**AATAAGATCTATGAGTTGAATATTTACAAGTATGAGCCCGACACGATTGCTG-GTACGTATAAATTTTGAATCCGTCTCTTTGATTATATTCATGAGTTTTGTT
AAATTATTTTAATTTCATAAAGTAGGAAAATGAACATGACTTGTTTAGTTAAAGTTAAAAGCACATGTTCCTAGTCA AATTATAACTAAAAAATGGAAATAATTAATTAAAAT**[A/G]**ATAACTATTAAACATGACATATATATATATATATATAT-TATATTATGGTACTATAAATTAGGGGCAAGGTGCACATAGAAAATGAAACATGTTTAGGACTACATGGTACAACAT-GAGTT
GAGACAACAACGATGAATATTTTGTCGATTTTGATGGTGTAGACAAGGAAAGGATGGAGAGAGCATTAAGAATA GCCATTATCAACCAGCTACAGACATAATG[**C/T]**AGGAAAGAGAGGAAAATTGGATAGTTAAATCCGACTGTAA-CAAGAACCTGGAAGAGAAAAGAGGAAAGCAAATGTTACGATCTCTAAAAGATTTAATATCTACTTT
CAAGCATGGTTGAGTCCATTGGTGTTTTTAACTTGTTGATCCTCCGAAACGATCTTCTTCCAGAACCAAT-GCTTTTCCCAAAGACGCATCATCTCTTCAATCGGAACTCCCTT**[C/T]**GTTTCAGGCAAGAACAGATAGAT-GAATATCGTCATGACAGCAATCCAACAGGAGAAAAACAGGAAAATCCCGAACCTCATAGCACATAAAAGTGAGA-GGAAAGACTGTGCTATC

CHAPTER 4

Exploiting the potential use of susceptibility genes against *Clavibacter michiganensis* subsp. *michiganensis* symptoms in tomato

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This chapter will be incorporated in a more extensive paper about S-genes and Cmm resistance

4

abstract

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is the causal agent of bacterial canker in tomato. Cmm occurs worldwide, can lead to economic losses and can be transmitted via seeds. For these reasons, it has been classified as a quarantine organism in Europe and many other countries. No resistant/tolerant cultivars against Cmm are available on the market. A new alternative for breeding cultivars with enhanced levels of resistance/tolerance is based on silencing susceptibility genes (*S*-genes). Susceptibility genes are plant genes, whose impairment leads to durable, broad-spectrum resistance. Four candidate *S*-genes, *CESA3*, *CESA4, PMR6* and *WAT1*, were tested using the virus-induced gene silencing (VIGS) assay to see the effects on the infection of Cmm. The results showed that silencing the *WAT1* orthologue in tomato leads to higher tolerance against the bacterium.

Keywords: *Cmm, Gram-positive bacterium, Solanum arcanum, VIGS, WAT1*

Introduction

Canker of tomato caused by the xylem-invading Gram-positive bacterium, *Clavibacter michiganensis* subsp. *michiganensis,* is considered to be one of the most economically important bacterial diseases of tomato, as yield losses can be severe (Eichenlaub et al. 2006; Sen et al. 2015). In some years up to 70% yield reduction has been reported in North Carolina (CABI and EPPO 1999). To regulate the spread of this harmful organism, Cmm has been classified as a quarantine organism in the EU and other countries (EPPO 2016). The bacterium was first reported in the USA in 1910 and since then it has spread throughout the world causing important losses to both glasshouse and open field tomato crops (Strider 1970; Kawaguchi et al. 2010). The most prominent symptoms of the disease are unilateral wilting of leaflets, formation of cankers on stems and petioles and bird-eye like spots on the fruits. Ultimately, the whole plant wilts and dies (Gartemann et al. 2003).

Levels of tolerance to Cmm have been found in several wild, crossable species of tomato (van Heusden et al. 1999; Francis et al. 2001; Coaker and Francis 2004; Sen et al. 2013). According to previous studies done in our group, the best source of resistance originates from the accession *S. arcanum* LA2157. A quantitative trait loci (QTL) analysis showed that the resistance is both polygenic and additive. The QTL on chromosome 7 was most prominent (van Heusden et al. 1999). So far, no *R*-genes against Cmm have been identified and no resistant/tolerant cultivars are on the market.

Traditional resistance breeding is based on the introgression of *R*-genes from wild species into elite cultivars (Gawehns et al. 2013). These *R*-genes typically encode for intracellular receptors of the nucleotide-binding leucine-rich-repeats (NB-LRR) family, which recognize specific products of the avirulence (Avr) genes of the pathogen. This recognition leads to a complex signalling cascade resulting in resistance, mostly exhibited as a hypersensitive response (HR) (Jones and Dangl 2006). However, the resistance can easily be overcome by the pathogen due to the highly race specific resistance that *R*-genes confer (Pavan et al. 2009).

An alternative approach to breed for resistance is based on the impairment of susceptibility genes (Pavan et al. 2009). Susceptibility genes are plant genes used by the pathogen for its proliferation and promotion of the disease symptoms (Gawehns et al. 2013; van Schie and Takken 2014). Loss-of-function of susceptibility genes is expected to lead to a more durable, broad-spectrum resistance (Pavan et al. 2009; Sun et al. 2016b). However, a major drawback of using *S*-genes in breeding is that these genes do not merely exist for the pathogen only, but they have evolutionary conserved functions in plant development. Therefore, impairment of such genes could potentially lead to adverse pleiotropic effects (Pavan et al. 2009). Nevertheless,

successful applications of impaired *S*-genes in breeding without severe pleiotropic effects are known. The most well-known example is the *MLO* gene in barley (Buschges et al. 1997). Since its discovery in barley, *MLO* has been identified and characterised in several other species (Consonni et al. 2006; Feechan et al. 2009; Zheng et al. 2013; Pessina et al. 2014; Acevedo-Garcia et al. 2017), enhancing the hypothesis that *S*-genes are conserved across plant species. The natural loss-of-function mlo allele has been used in breeding for the last 40 years. It provided broad-spectrum and durable resistance against all known isolates of barley powdery mildew (Jørgensen 1992; Buschges et al. 1997).

Due to the complex genetic background of resistance to Cmm, the use of traditional breeding approaches to obtain high levels of resistance is very challenging. In this paper, we studied the potential use of silenced *S*-genes against Cmm. *Arabidopsis S*-gene orthologues in tomato, *Cellulose synthase 3* (*CESA3*), *Cellulose synthase 4* (*CESA4*), *Powdery Mildew Resistance 6* (*PMR6*) and *Walls Are Thin 1* (*WAT1*), were identified through an *in silico* analysis and virus-induced gene silencing (VIGS) was used to silence the identified genes and to study the effects of silencing on the tomato-Cmm interaction.

Materials and methods

Identification of candidate *S*-genes

The identification of candidate *S*-genes potentially involved in tomato-Cmm interactions was done in two ways. Firstly, candidate *S*-genes located in the QTL region of chromosome 7 and chromosome 9 were chosen (van Heusden et al. 1999) and secondly *S*-genes were chosen based on a literature study (van Schie and Takken 2014). These genes are *CESA3* and *PMR6* (Table 1). The genes from the literature study were filtered based on two criteria: (a) genes known to be involved in interactions with vascular pathogens in *Arabidopsis thaliana* and in other Solanaceae (b) the protein variation between *Solanum lycopersicum* cv. Moneymaker and *S. arcanum* LA2157 (referred to as Moneymaker resp. LA2157). The genes for this category are *CESA4* and *WAT1*. In order to identify the orthologues and homologs, the *Arabidopsis* protein sequences, obtained from TAIR (https://www.arabidopsis.org/Blast/), were used as a query on the Solanum Genomics Network (SGN) database (https://solgenomics.net/tools/blast/). For each of the *A. thaliana* genes, the homolog with the highest score and lowest evalue was selected from the list of homologs provided by SGN. Protein variation between Moneymaker and LA2157 was determined using the in-house HaploSmasher software developed by Plant Breeding, Wageningen University & Research, Wageningen, the Netherlands (http://xapps.plantbreeding.nl:5001).

HaploSmasher is a prediction tool which predicts the effects of genetic variants of the annotated genes in tomato. Differences in allelic variations between LA2157 and Heinz 1706 (with which Moneymaker has high similarity) were determined based on filtering the impact prediction starting from High impact (variant causes disruptive change in the protein), Moderate impact (variant causes a non-disruptive change in the protein) to Low impact (variant unlikely changes protein behaviour) (http://snpeff.sourceforge.net/SnpEff_manual.html). Finally, the Wageningen UR 150 Tomato Genome Resequencing project (Aflitos et al. 2014) provided the sequences and JBrowse was used to identify SNPs and other mutations, such as deletions and insertions, between Moneymaker and LA2157. Additionally, to find a possible trend of gene expression in different tissues in Moneymaker an *in silico* transcriptomic analysis was performed by accessing the Tomato Functional Genomics Database (http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi) where the expression levels of the candidate genes in cv. Heinz are stored.

Virus-induced gene silencing (VIGS) and Cmm inoculation

The best target region for the VIGS constructs (*CESA3*: *Solyc07g005840.2*, *CESA4*: *Solyc09g072820.2*, *PMR6*: *Solyc09g008380.2* and *WAT1*: *Solyc04g080940.2*) were identified using the SGN VIGS tool (http://vigs.solgenomics.net/). The target region is in the coding region (CDS), with at least 21 basepair (bp) unique to a specific gene which is used to identify the targeted region. Primers were designed to amplify a 150-200 bp region of the candidate genes (Table 2). The PCR reaction was set up to a final volume of 50 µl: 10 µl of HF buffer (5x), 2 µl 5 mM deoxynucleoside triphosphates (dNTPs), 0.15 µl of DreamTaq DNA Polymerase (Thermo Scientific, USA) (5 U/ul), 0.38 µl of Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, USA), 1.25 µl of forward primer (10 µM), 1.25 µl of reverse primer (10 µM) and 2.5 µl of cv. Moneymaker cDNA (10 µM). Touchdown PCR with 25 cycles 98 °C, 30 s; 60 (-0.4) °C, 25 s; 72 °C, 30 s and 10 cycles 98 °C, 30 s; 55 °C, 25 s; 72 °C, 30 s. Fragments targeting the candidate genes for silencing were amplified and cloned into pENTR-TOPO (Thermo Scientific, USA), sequenced for confirmation and subsequently cloned into TRV2 vector (Liu et al. 2002) using the Gateway system. Plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. TRV infection was done through *Agrobacterium*-mediated infiltration on cotyledons of 10-day old Moneymaker seedlings using needleless syringes (ten plants per treatment). The TRV2 vector, containing the β-glucuronidase gene (GUS), a gene that has no homology with any endogenous gene in tomato, was used as control (TRV2::GUS plants).

Inoculation with Cmm was done approximately two weeks after agroinfiltration of the plants (third to fourth leaf stage). The Cmm strain IPO3356 (Culture Collection of Wageningen Plant Research), is highly virulent and rifampicin resistant (Rif+). The

petiole of the first and second leaf of the tomato plants were cut with knives and dipped into 10^8 cfu/ml bacterial suspension. The leaves were collected for a gene expression analysis. Disease index (DI) was scored based on the following scale: 0 = no symptoms; 1 = up to 25 % leaf wilting; 2 = 26 to 50 % leaf wilting; 3 = 51 to 75% leaf wilting; 4 = 76 to 100 % leaf wilting; 5 = dead plants (Sen et al. 2013). Scoring of the plants was done at several time points post inoculation (dpi). The agroinfiltration and inoculation were replicated. The first experiment was performed using four silencing constructs of four different genes, and any successful silencing was repeated. The second experiment consisted of two silencing constructs of the *WAT1* gene designed to target different exons; namely exon 1 (TRV2::WAT1) and exon 4 (TRV2::93).

Gene expression analysis

The first two true leaves from each plant were sampled for RNA isolation as described above. Total RNA isolation was done using the MagMax™ 96 Total RNA Isolation Kit (QIAGEN) in combination with a KingFisher processor, and cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad) using 1 µg RNA. For the gene expression analysis qPCR was performed using a CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A). Primer pairs were designed in an intron-exon junction region to avoid amplification of any remaining genomic DNA in the samples, and to specifically amplify the target genes (Table 3). The APT tomato was used as reference gene (GenBank: BT012816) (Expósito-Rodríguez et al. 2008).

Total bacterial DNA isolation and quantification

Stem pieces were collected above the inoculation point from all the plants. The stems were placed in BIOREBA™, crashed with a hammer and suspended with Ringer (three times the weight of the stem). The suspension was used for total bacterial DNA isolation. DNA isolation was done using a BioSprint® One-For- All Vet Kit (384) Kit (QIAGEN) in combination with a Kingfisher processor. Bacterial quantification was done using a TaqMan assay as described by Sen et al. (2013).

Table 1 | Summary of *S*- genes used for the VIGS assay against *Clavibacter michiganensis* subsp. *michiganensis*.

S-gene	Solyc ID	Sequence identity (%)[a]	Function[b]	Susceptibility mechanism[b]	Pleiotropic effect[c]	Haplosmasher[d]	Genetic Variation[e]	Located in the QTL[f]	References
CESA3	*Solyc07g005840.2*	67	Cellulose synthase	Probable DAMP induced defence suppression. Mutants have increased JA/Eth	Dwarfed plants	Moderate	SNPs	Yes	(Ellis and Turner 2001; Ellis et al. 2002)
CESA4	*Solyc09g072820.2*	80	Cellulose synthase	Probable DAMP induced defence suppression. Mutants have increased ABA)/ JA/ Eth	-	Moderate	SNPs	No	(Hernandez-Blanco et al. 2007)
PMR6	*Solyc09g008380.2*	66	Pectate lyase	Pectin accumulation in the extrahaustorial matrix possibly resulting in decreased nutrient availability to the pathogen	-	Moderate	SNPs	Yes	(Vogel et al. 2002; Chandran et al. 2013)
WAT1	*Solyc04g080940.2*	75	Auxin transporter (tonoplast), high expression in stems, tissues with secondary cell walls.	Defence suppression SA	-	Moderate	SNPs	No	(Ranocha et al. 2010; Denancé et al. 2013; Ranocha et al. 2013)

[a] Overall amino acid identity (percentage) between the tomato and *Arabidopsis* protein.
[b] Gene function and susceptibility mechanism in *Arabidopsis* according to van Schie and Takken (2014) and literature.
[c] Mutant phenotype observed on the VIGS tomato plants.
[d] Impact prediction of the annotated genes of *S. arcanum* LA2157 compared to cv. Heinz using the in-house software, the HaploSmasher. Three impact categories: High, variant has high impact in the gene products causing truncated or loss-of-function protein; Moderate, variant is non-disruptive causing effectiveness of the protein; and Low, variant is harmless and do not change behaviour of the protein. (http://snpeff.sourceforge.net/SnpEff_manual.html).
[e] Type of allelic variation of the annotated gene f *S. arcanum* LA2157 compared to cv. Moneymaker.
[f] *S*-gene in the previously described QTL region (van Heusden et al. 1999).

Table 2 | Primer pairs designed for the amplification of the four candidate *S*- genes.

Gene	Annotated gene	Primer name	Sequence (5′ → 3′)
CESA4	*Solyc09g072820.2*	CESA4F1	caccCCTGTCGAGAAGGTTAGCTGTT
		CESA4R1	TCGGGTGCTCTAGGCTCTAC
WAT1	*Solyc04g080940.2*	WAT1F1	caccATGGCAGATACTAGTGGTTCATCC
		WAT1R1	TGCAGCTCTAGAGACAACATGA
		KH_093_Fw[a]	caccGGCCCAACAATTTACAGCCC
		KH_093_Rv	CCCTCAGGCTTGGCTAGTTC
CESA3	*Solyc07g005840.2*	CESA4MF1	caccCGTCTTAAAGGGAGTCCAAGG
		CESA4MR1	AGGAGGGTACTGAGCGGAAT
PMR6	*Solyc09g008380.2*	PMR6F2	caccTACTCTTCCTGGTCAACATCCTG
		PMR6R2	GGTCACATCGCCAACAATCA

[a] The construct from this primer pair was used when we repeated our experiment.

Table 3 | The qPCR primers of the target genes and the housekeeping gene to check the silencing level in the VIGS plants.

Gene	Primer name	Sequence (5′ → 3′)
PMR6	qAtPMR6F2	TAAAGAGGTAACAAAAAGAG
	qAtPMR6R2	AATTTCTCCATTTTCCTTCA
CESA3	qCESA4MF1	GGGTTCCACCTTCGTCGAG
	qCESA4MR1	ATCCATCCCAGCTCCAAACC
CESA4	qCESA4F2	GCTTTGGTTAGCGAGTAGCT
	qCESA4R2	CATGGCTTCTCTAACTGCCT
WAT1	qWAT1F1	GGGGGTCCAGTTTTTGTTGC
	qWAT1R1	CTCCGATTATCCCGCCCAAG
APT	qAPTF	CCATGAGGAAACCCAAGAAGT
	qAPTR	CCTCCAGTCGCAATTAGATCAT

Results

Potential use of *S*-genes against Cmm

The concept of using *S*-genes in tomato to lower the effects of an infestation of Cmm has never been tested before. This is the first study to investigate the possibilities of using impaired *S*-genes as source of tolerance towards Cmm. The genes tested were *CESA3* (*Solyc07g005840.2*), *CESA4* (*Solyc09g072820.2*), *PMR6* (*Solyc09g008380.2*) and *WAT1* (*Solyc04g080940.2*) (Figure S1). The tomato CESA3 protein has 67% homology and PMR6 has 66% homology with their *Arabidopsis* orthologues. *CESA3* and *PMR6* were chosen because they are in the QTL regions of chromosome 7 and 9, respectively. The differences in protein sequences between Moneymaker and LA2157 were predicted using the HaploSmasher software. The impact variant is moderate, meaning that there is an amino acid difference in LA2157 that may affect the protein, but it is non-disruptive. The other two genes, *CESA4* and *WAT1*, have 80% and 75% protein homology with *Arabidopsis* orthologues. Impairment of *CESA4* gene in *Arabidopsis* leads to resistance against *Ralstonia solanacearum* (Hernandez-Blanco et al. 2007), and the *Arabidopsis wat1* mutant exhibits broad-spectrum resistance to several vascular pathogens (*R. solanacearum, Verticillium dahliae* and *V. albo-altrum*) (Denancé et al. 2013). The impact variant of the amino acid sequence of these proteins in LA2157 is moderate (Table 1).

Out of the four genes, the experiment with the *WAT1* gene resulted in less wilting towards Cmm (Figure 1a). The disease index of the *WAT1* plants are lower and significantly different (P = 0.05) than other treatments (Figure 1b). The silencing level of individual *WAT1* plants was checked by comparing it with the level of the GUS control plants (Figure 1c). Six of the silenced plants had low DI scores and a non-silenced plant had a high DI score (Figure 1c). There are three silenced plants with high DI score which might be due to the patchiness of the silencing level.

No reduced susceptibility was found in *CESA3, CESA4* and *PMR6* inoculated plants. The DI scores in these cases were similar to the GUS control from 12 dpi (first wilting symptoms) till 19 dpi (harvest) (Figure 1b). No association was found between the silencing level and DI scores in *CESA4* and *PMR6* silenced plants. No enhanced resistance was found in *CESA3, CESA4* and *PMR6* inoculated plants. The DI scores in these cases were like the GUS control from 12 dpi (first wilting symptoms) till 19 dpi (harvest) (Figure 1b). The gene expression analysis was not performed on *CESA3* silenced plants due to the adverse pleiotropic effects (Figure 1a).

There was no significant difference between the bacterial titres in all silenced and control plants. A Pearson's correlation test gave no significant correlation between the symptom scoring and the Cmm DNA bacterial titre (r = 0.015).

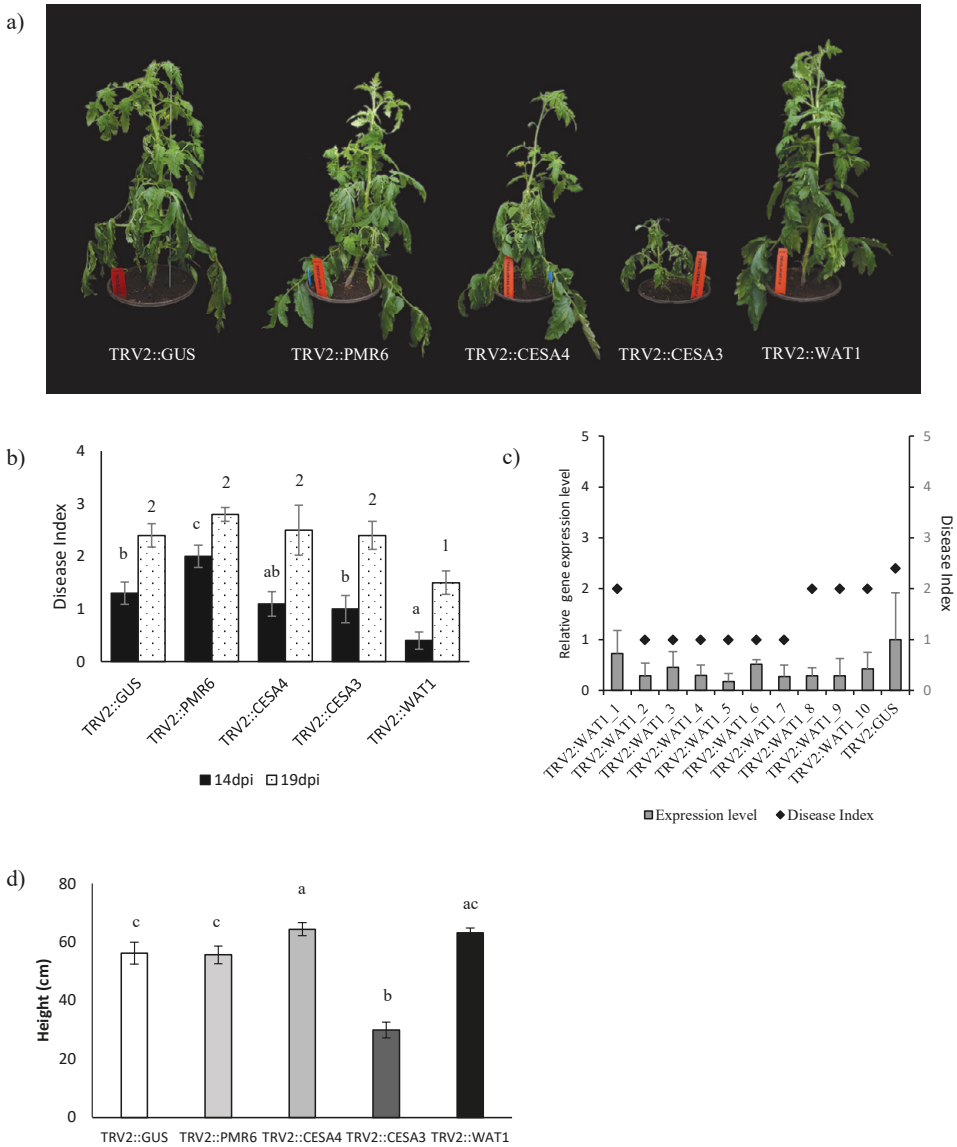


Figure 1 | Screening of the tomato VIGS plants. a) Wilting observed on the control (TRV2::GUS) and other TRV2 constructs (TRV2::PMR6, TRV2::CESA4, TRV2::CESA3, TRV2::WAT1). b) Disease index on the 14 and 19 dpi. The bars represent average score of each genotype (n = 10) and vertical lines represent standard errors. Within each chart, bars sharing the same letter or numbers are not significantly different (P = 0.05). c) Relative gene expression and DI (19 dpi) of the 10 individual TRV2::WAT1 plants. Each column represents the average relative gene expression of two technical replicates and vertical lines represent standard errors. Diamond shaped markers represent the DI score. The average relative gene expression and average DI score of ten biological replicates of GUS control plants are given in comparison to the other plants. d) Average height (cm) of the TRV2 constructs plants. Bars represent average score of each genotype (n = 10) and vertical lines represent standard errors. Within each chart, bars sharing the same letter are not significantly different (P = 0.05).

Pleiotropic effect

Silencing a gene may affect the plant phenotype. In this experiment a strong pleiotropic effect was observed in *CESA3* silenced plants. The *CESA3* plants were stunted in growth and the average height of *CESA3* inoculated plants was significantly lower ($P < 0.001$) than the other silenced plants. Similar pleiotropic effects were not observed in other silenced plants (Figure 1d). The *CESA4* and *WAT1* plants are even somewhat higher than the control.

WAT1 as a potential *S*-gene against Cmm

To confer that silencing the *WAT1* gene leads to a higher tolerance to Cmm, the experiment was repeated. Additionally, a new construct (TRV2::93), which was designed at another location of the *WAT1* gene, was used as well (Figure S2). Repeated analysis (expression level and disease test) confirmed the tolerance of *WAT1* plants compared to GUS control plants (Figure S3a). TRV2::WAT1 plants have a lower average disease index which is significantly different ($P = 0.05$) than TRV2::93 and TRV2::GUS plants. Average disease index of TRV2::93 plants was not as low as TRV2::WAT1 plants, but lower than TRV2::GUS plants. The silencing level of each individual plant was checked by comparing it with the level of the GUS control plants (Figure S3b, c). Seven of the TRV2::WAT1 silenced plants had lower DI scores and three silenced plants had a higher DI score (Figure S3b). There were four silenced TRV2::93 plants with variation in DI and six non-silenced plants (Figure S3c). No pleiotropic effects were observed.

Discussion

The coding sequence of the four chosen tomato *S*-genes (*CESA3* (*Solyc07g005840.2*), *CESA4* (*Solyc09g072820.2*), *PMR6* (*Solyc09g008380.2*) and *WAT1* (*Solyc04g080940.2*) showed several SNPs between Moneymaker and LA2157 (Table 1). The differences in the nucleotide or protein sequences between these orthologues might be responsible for an enhanced tolerance towards Cmm. To find the right genes all potential *S*-genes have to be silenced one by one in a susceptible cultivar and after pathogen challenge be evaluated. A VIGS assay can be used for this purpose as it provides rapid and high throughput screening of many genes (Liu et al. 2002). However, VIGS is never complete and partial and patchy tissue distributions are to be expected (Lu et al. 2003; Orzaez et al. 2009). Furthermore, it is possible that sometimes a silenced phenotype is not observed because the function of the target gene is still supported by the residual low level of mRNA in the virus vector-infected plants or other homologs of the gene are still functional (Lu et al. 2003). The results of VIGS provide only an indication about the gene function and not a proof (Lu et al.

2003). Stable mutants generated through RNAi or CRISPR/Cas9 techniques should be obtained for further confirmation.

In parallel to these loss-of-function approaches, overexpression of the genes in a tolerant genotype can be used (Prelich 2012). Expression of a candidate *S*-gene can verify if susceptibility can be restored in a resistant mutant. This can be done by generating transgenic plants overexpressing the candidate *S*-gene from the susceptible cultivar (Berg et al. 2015). Assuming that the differences between these orthologues are responsible for the resistance in genotype LA2157, overexpression of the genes would restore the susceptibility in LA2157.

From previous studies, tolerance towards Cmm was found in LA2157. *S*-gene(s) localized in the detected QTL region may play a role in tolerance to Cmm. Examples of this have been found in other studies (Fukino et al. 2013; Berg et al. 2015). We used two *S*-genes located in the QTL regions (*CESA3* and *PMR6*) but *CESA3* and *PMR6* silenced plants did not have higher levels of tolerance to Cmm.

Silencing of the *CESA3* homolog leads to severe pleiotropic effects in tomato. Silencing this gene in *Arabidopsis* leads also to reduced growth, due to the constitutive activation of the jasmonic acid (JA) signalling pathway (Ellis and Turner 2001). Constitutive activation of JA defences represents an important example of "growth-defence trade-off" in plants, as it severely restricts plant growth (Ellis and Turner 2001; Yang et al. 2012; Vos et al. 2013; Huot et al. 2014). That an induction of defence responses can be associated with plant fitness shows that simply breeding for constitutively active defences is not always a feasible option (Huot et al. 2014). *CESA3* is obviously not a suitable gene for *S*-gene breeding as silenced plants exhibited strong pleiotropic effects and no reduced susceptibility.

The other two genes, *WAT1* and *CESA4,* are not located in the QTL, nevertheless their impairment could enhance resistance. Silencing the gene *WAT1* conferred a higher tolerance to Cmm. The *Arabidopsis WAT1* (*Walls Are Thin 1*) is a gene involved in secondary cell wall formation (Ranocha et al. 2010). In *wat1 Arabidopsis* mutants, a severe reduction in secondary wall thickness, but not xylem vessels has been observed (Miedes et al. 2014). Knocking-out/down *WAT1* leads to specific, broad-spectrum resistance to vascular pathogens in *Arabidopsis* including *R. solanacearum*, *Verticillium dahliae* and *V. albo-altrum,* but not to non-vascular pathogens (Denancé et al. 2013). One would assume that *wat1* mediated resistance must be caused by alterations of the strength of the cell walls, but it has been shown that the resistance conferred by a mutated *WAT1* involves the activation of immune responses, mainly localized in the vascular system of the plants (Miedes et al. 2014). In the case of *wat1* mutants, it is suggested that salicylic acid (SA) is responsible for the resistance to vascular pathogens. This is further supported by the fact that *wat1* roots contain constitutively

higher amounts of SA, compared to the Col-0 *Arabidopsis* plants. Furthermore, it is shown that the introduction of *NahG*, a bacterial gene whose product converts SA to catechol, in *wat1* plants leads to full susceptibility of the mutant plants (Denancé et al. 2013).

In our experiment, Moneymaker plants infiltrated with the construct targeting the *WAT1* gene showed a significantly lower susceptibility to Cmm than the control plants. This result suggests that the molecular mechanisms contributing to the tolerance against Cmm are affected by the silencing of *WAT1*. The fact that silencing of the gene did not lead to any severe pleiotropic effects further increases the possibility of using *WAT1* in *S*-gene breeding. It is interesting that despite being tolerant to Cmm, the silenced *WAT1* plants still contain a high bacterial density. Wilting symptoms and bacterial titre are not correlated in the Cmm-tomato interaction. A recent study indicates that highly tolerant wild tomato relatives contain considerable amounts of bacteria (Sen et al. 2013), which is in line with what we have observed in our experiment. The virulence factors of Cmm can cause wilting in tomato plants (Eichenlaub and Gartemann 2011; Savidor et al. 2012). We speculate that silencing the *WAT1* gene does not inhibit the bacterial growth, but in fact reduces the Cmm virulence factors. Our results showed that it is highly possible that SA is involved in resistance against Cmm. It has been long established that plant hormones such as SA, JA and ethylene (ET) are involved in the regulation of plant defence responses (Ton et al. 2002). The signalling pathways of these hormones are interconnected in complex networks, allowing plants to rapidly adapt to their biotic environments, in order to grow and survive in a cost-efficient way (Pieterse et al. 2012). Silencing of *WAT1* has been shown to lead to accumulation of SA (Denancé et al. 2013). Furthermore, *CESA3* and *CESA4* mutants have been shown to activate the JA/ ET and ABA pathways, respectively. Mutations of the *PMR6* gene lead to resistance against pathogens through a JA, SA or ET independent pathway (Vogel et al. 2002).

In the case of the Cmm-tomato interaction, no studies are known about the involvement of SA, JA or ABA in the resistance mechanisms. It has been shown that there is an elevated ethylene production in tomato plants infected with Cmm (Savidor et al. 2012). The ACD mutant in tomato had an impairment (up to 90% reduction) of the ethylene production. When the ACD mutant was challenged with Cmm they exhibited delayed wilting. Delayed wilting is also observed on the tomato ethylene insensitive *Never ripe* (*Nr*) mutant (Balaji et al. 2008). Ethylene production in tomato is a major signal that regulates disease progression and is not used by Cmm for virulence or proliferation. Time course experiments specifically designed to study the involvement of signalling hormones in Cmm -tomato interactions might shed light in this. The expression of marker genes in different signalling pathways at different time points could be studied, to elucidate the effect of hormones on tolerance against Cmm.

Acknowledgements

We would like to thank Dr. Henk Schouten (Plant Breeding, Wageningen University & Research) for supplying the list of candidate *S*-genes mapped on the tomato genome and Katharina Hanika MSc (Plant Breeding, Wageningen University & Research) for TRV2::93 construct and the map of the *WAT1* gene. Nunhems, vegetable seeds of BASF, the Netherlands; Universiti Putra Malaysia and the Ministry of Higher Education Malaysia, Malaysia are thanked for their financial support.

Supplementary material

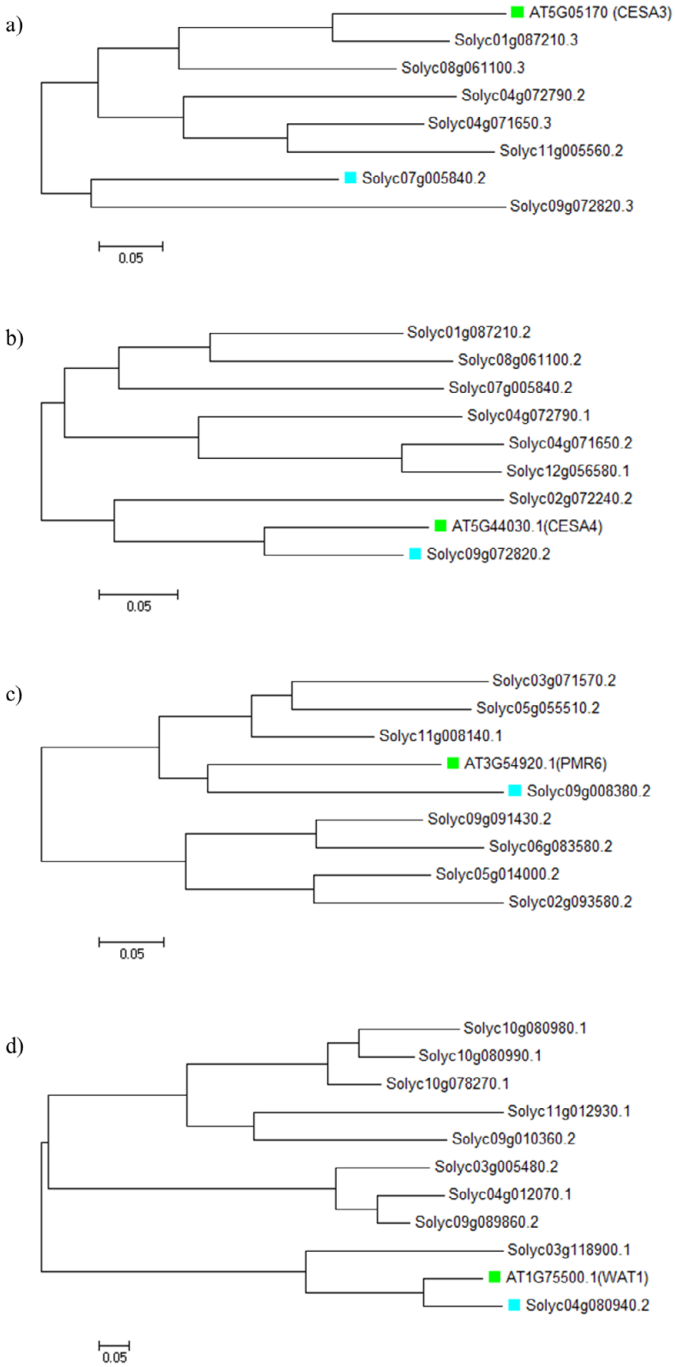


Figure S1 | Neighbour-joining phylogenetic tree of the *Arabidopsis* protein sequences (green squares) and their tomato orthologues. The constructs we designed are indicated with blue squares. a) CESA3, b) CESA4, c) PMR6, and d) WAT1. The scale bar shows 0.05 amino acid substitutions per site.

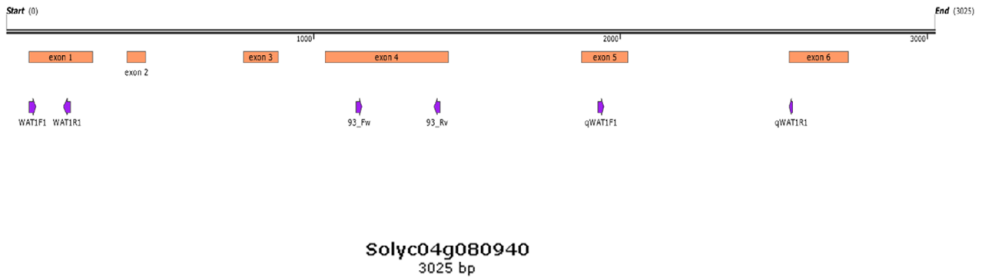
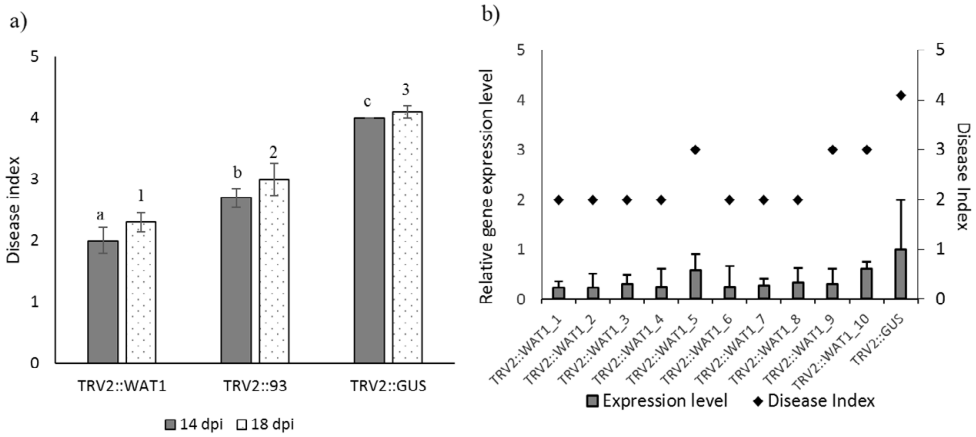


Figure S2 | Schematic diagram of the *WAT1* gene and the primers designed (purple arrows) to produce the TRV2 constructs (TRV2::WAT1 and TRV3::93) and to check the relative gene expression.



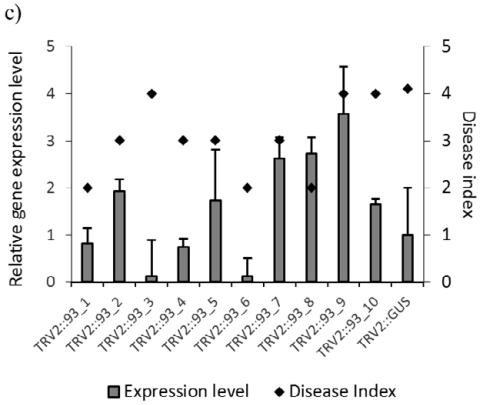


Figure S3 | Screening of *WAT1* plants (n = 10) of the repeated experiment. a) Symptom score on 14 and 18 dpi. The bars represent average scores of each genotype (n = 10) and vertical lines represent standard errors. Within each chart, bars sharing the same letter or numbers are not significantly different (P = 0.05). Relative gene expression and DI (18 dpi) b) TRV2::WAT1 construct from the first experiment located on exon 1, and new c) TRV2::93 construct located on exon 4. Each column represents the average relative gene expression of two technical replicates and vertical lines represent standard errors. Diamond shaped markers represent the DI score. The average relative gene expression and average DI score of ten biological replicates of GUS control plants are given in comparison to the other plants.

CHAPTER 5

Alternative ways to reduce *Clavibacter michiganensis* subsp. *michiganensis* disease symptoms in tomato

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To develop an integrated strategy for minimizing effects after infection with *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in tomato, we studied the effect of grafting using the highly tolerant *Solanum arcanum* LA2157 and the use of *Pseudomonas* spp. consortia as a biocontrol agent. Two types of Cmm inoculation were tested; root inoculation and stem inoculation. After a root infection, a significant reduction of wilting (by 25 %) was seen on reciprocal grafted *Solanum lycopersicum* cv. Moneymaker on *S. arcanum* LA2157 rootstock but not on self-grafted cv. Moneymaker. After stem inoculation, no reduction of wilting symptoms was observed. A comparable observation was made with plants of cv. Moneymaker treated with *Pseudomonas* spp. consortia. Two types of consortia were used throughout the experiment, enhancer 1 (E1) contained eight *Pseudomonas* strains and enhancer 5 (E5) contained six *Pseudomonas* strains. A reduction in wilting (up to 50 %) symptoms was found between treated and non-treated plants but only when Cmm was root inoculated. Only a small reduction of leaf wilting was seen on cv. Moneymaker plants after infection via the stem when the *Pseudomonas* spp. consortia were added weekly to the root.

Keywords: *Clavibacter, Cmm, Tomato, Grafting, Biocontrol*

Introduction

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is one of the most threatening bacteria infecting tomato and it is a quarantine organism in Europe (Grund et al. 1990; Eichenlaub et al. 2006; Eichenlaub and Gartemann 2011; EPPO 2016). This Gram-positive bacterium causes wilting and canker on its host (*Solanum lycopersicum*) (Davis et al. 1984). It can enter the host plant via roots, natural openings and wounds (Chang 1991; Carlton et al. 1994; Sharabani et al. 2013a). Contaminated seeds and infected plant debris in the soil are the main sources of infection (Strider 1969; de León et al. 2009; Vega and Romero 2016).

The quarantine status and difficulty to manage the pathogen make it problematic to many parties. Seed companies need to sell Cmm-free seeds, plant growers need to supply Cmm-free tomato seedlings and farmers would like to have plants that are preferably not infected by the pathogen. Measures to control Cmm so far are Cmm-free seed/plants and good agricultural practices. Currently there are no commercial tomatoes that are resistant/ highly tolerant against Cmm. In our group, *Solanum arcanum* LA2157 was used as tolerance source (van Heusden et al. 1999). The genotype is highly tolerant towards Cmm; no disease symptoms occurred but considerably high bacterial density still present. We still do not know which genes underlie some of the identified tolerances.

The use of wild resistant/tolerant genotypes for grafting could be an alternative approach to reduce symptom development by Cmm. Grafting is an old horticultural method that connects the root system (rootstock) of one plant to the shoot (scion) of another (Warschefsky et al. 2016). The introduction of vegetable grafting took place at the turn of the 20th century to manage soilborne pathogens and it still is used frequently (Sakata et al. 2007; Louws et al. 2010). It can also be used to limit the effects of foliar pathogens (Sakata et al. 2006; Albert et al. 2017).

Another alternative to potentially reduce infections and symptom expression is the use of microorganisms against Cmm. Its application is attractive since it can substitute the use of environmentally unfriendly copper-based chemical treatments to control Cmm. (Werner et al. 2002; Hausbeck 2017). Furthermore, chemical treatments fail to control the pathogen (Hausbeck et al. 2000; Jiang et al. 2015). Our study focuses on the use of *Pseudomonas* spp. provided by BioscienZ: Inventers of the patent WO2017178529A1 (de Laat et al. 2017). The *Pseudomonas* species used are *P. protegens, P. brassicacearum*, two *P. putida* strains, two *P. moraviensis* strains, *P. reinekei* and *P. extremaustralis*. The species were initially tested *in vitro* to see if culturing a single *Pseudomonas* species or several species (consortia) together with Cmm can inhibit the pathogen growth. Consortia that gave the highest effects *in vitro* were tested *in planta* in this study.

From literature, many of the species have been tested with different pathogens and they resulted in disease suppression in the plants. *Pseudomonas* species have been shown to promote plant growth, reduce disease incidence by changing the host phenolic profile, by secreting secondary metabolites, and/or production of antibiotics. *Pseudomonas protegens* produces the antimicrobial secondary metabolite 2,4-diacetylphloroglucinol (DAPG) that plays an important role in the biocontrol of plant diseases (Ramette et al. 2011). Similarly, *P. brassicacearum* LBUM300 produces DAPG and hydrogen cyanide (HCN) and have shown antagonistic activity against the plant pathogens *Verticillium dahliae*, *Phytophthora cactorum*, and Cmm (Paulin et al. 2009; Lanteigne et al. 2012; Novinscak et al. 2016; Paulin et al. 2017). *Pseudomonas putida* induces chlorogenic acid, caffeic acid, catechin and rutin in tomato infected by Cmm (Park et al. 1988; Aksoy et al. 2017; Sun et al. 2017). *Pseudomonas moraviensis* produces important secondary metabolite to have antifungal properties like phenazine-1- carboxylic acid, phenazine-1-carboxamide, pyoluteorin and pyrrolnitrin (Ait Bahadou et al. 2018). *Pseudomonas reinekei* has been shown to reduce the percentage of sclerotia on potato tubers caused by *Rhizoctonia solani* (Mrabet et al. 2013). Finally, *P. extremaustralis* has been shown to promote growth of wheat plants (*Triticum durum*) (Kudoyarova et al. 2017).

In this study two strategies for reduction of Cmm symptoms were explored. Firstly, grafted tomato plants were tested, in which a tolerant rootstock is combined with a susceptible scion. To our knowledge, grafting has never been tested against Cmm before. Secondly, the efficacy of *Pseudomonas* consortia as biocontrol agents was tested against Cmm after root- and stem inoculations.

Materials and methods

Plant materials

The susceptible *Solanum lycopersicum* cv. Moneymaker (MM) and the highly tolerant *S. arcanum* LA2157 (LA) were used for the grafting experiment. To see the effect of *Pseudomonas* consortia on different tomato genotypes, cv. Moneymaker (MM), and the near isogenic line (NIL) containing a small introgression with the main QTL of *S. arcanum* LA2157 were used.

Grafting procedures

Four grafting treatments were performed (root:shoot); reciprocal grafted plants (LA:MM and MM:LA) and self-grafted plants (MM:MM and LA:LA) acted as controls. Plants were grown in the greenhouse (12 h day light, 25 °C day and 18 °C night temperature). Seedlings were grown in plug trays and grafting was performed on three-leaves stage plants. A rootstock and scion of similar size were selected. Cotyledons and first leaves were removed, stems were cut with a blade approximately at a 45° angle above the cotyledons. After cutting, the scion was attached to the rootstock with a grafting clip. Grafted plants were then covered for about two weeks with plastic (90% relative humidity) to promote the formation of the graft union. Stem or root inoculation were performed afterward.

Inoculations of Cmm

The virulent rifampicin resistant Cmm strain IPO3356 (Culture Collection of Plant Research International) was used for inoculation (Lelis et al. 2014). Two types of inoculations were performed on the grafting and biocontrol agents experiments; the root inoculation was done by removing the soil from the roots of the seedlings grown in plug trays and roots were dipped into 50 ml of 10^8 cfu ml^{-1} bacterial suspension for 30 min and the stem inoculation was done by cutting the petioles of the first two leaves of fourth leaf stage seedlings with knives dipped into a bacterial suspension (10^8 cfu ml^{-1}). Ten plants were used for each treatment with water inoculated plants as controls. Experiments were done once for each study. Wilting symptoms were recorded based on the following scale on several days: 0 = no symptoms; 1 = 0 to 25% leaf wilting; 2 = 26 to 50%; 3 = 51 to 75%; 4 = 76 to 100 %; 5 = dead plants.

Supplementing the plants with *Pseudomonas* consortia

Freeze-dried biocontrol consortia were provided by BioscienZ (Breda, The Netherlands). Two types of consortia were used throughout the experiment, enhancer 1 (E1) which contained *P. protegens* PR01, *P. brassicacearum* BR01, two *P. putida* strains (#27 and #87), two *P. moraviensis* strains (#11 and #17), *P. reinekei* #55 and *P. extremaustralis* #29 and enhancer 5 (E5) which contained all of the above except the two *P. putida* strains. Plants were grown in the greenhouse (12 h day light, 25 °C day and 18 °C night temperature). Plants not treated with consortia acted as controls. Two experiments were performed: 1) To test the effect of consortia with a weekly application on cv. Moneymaker. Plants were supplemented four weeks with consortia by diluting the freeze-dried cells with demi water (2 g/l), 10% of the pot volume. A week after the fourth application, a stem or root inoculation was done with Cmm. One day after inoculation with Cmm, plants were treated with consortia

for another two weeks. 2) To test the effect of minimal and direct application of consortia on cv. Moneymaker and NILs, plant roots were dipped in the consortia by diluting the freeze-dried cells with demi water (2 g/l) or water (control) for an hour during the first leaf stage before transplanting to bigger pots and the plants were sprayed with consortia one week later. A week after the treatment, stem inoculation was performed.

Total bacterial DNA isolation and quantification

Plants parts were collected for quantifying Cmm in the grafting experiment. The plant parts were placed in extraction bags with a synthetic intermediate layer (Bioreba, Switzerland), crashed with a hammer and suspended with Ringers solution (Sigma) three times the weight of the plant part. The suspension was used for total bacterial DNA isolation. DNA isolation was done using a BioSprint® One-For- All Vet Kit (384) Kit (QIAGEN) in combination with a Kingfisher processor. Bacterial quantification (DNA copy number) was done using a TaqMan assay as described by Sen et al. (2013).

Detecting the presence of *Pseudomonas* consortia in *in vitro* plants

Moneymaker (MM) seeds were sown and grown *in vitro*. The seeds were sterilized in 70% ethanol for 2 min, 1% NaOCl (Sigma-Aldrich, St. Louis, MO) for 20 min and washed with sterile water for 5 min. Seeds were arranged in petri dishes on sterile and wet filter paper. Seeds were incubated for 3 days at 4 °C and then for approximately 2 weeks in a growth chamber (Technisch Buro I.K.S. B.V, Leerdam, The Netherlands) at 25 °C with a relative humidity of 40–70%, and a 16 h/ 8 h day/ night photoperiod. Germinated seedlings were transferred to tubes containing sterile vermiculite (1.5 g) and rooting medium (MS30B5) (4.3 g MS salts/L, 112 mg Vitamin B5/L, 30 g sucrose/L; pH 5.8). *Pseudomonas* consortia (E1 and E5) were supplied to the *in vitro* plantlets weekly for two weeks with consortia by diluting the freeze-dried cells with demi water (2 g/l), 10% of the vermiculite volume. Untreated *in vitro* plants served as control.

To check the presence of *Pseudomonas* consortia in the *in vitro* plants, five treated and untreated plants were collected 21 days after the first application of enhancers. The roots were removed and the shoots (stems and leaves) were sterilized with 70% ethanol. The surface of shoots was blotted dry and placed in extraction bags (Bioreba, Reinach, Switzerland), macerated with hammer, homogenized in 100 µl Ringer buffer (2 ml g^{-1} of tissue). 20 µl of the plant suspension were plated on Cetrimide agar (20 g gelatine peptone/L, 1.4 g magnesium chloride/L, 10 g potassium sulphate/L, 0.3 g cetrimide/L, 13.6 g agar/L). Plates were incubated at 35 °C for two days.

Statistical analyses

A Kruskal-Wallis analysis using the SPSS 23.0 statistical software package (SPSS Inc., Chicago, IL) was used to determine the difference between the disease index of different graft treatments. The sameanalysis was also used to see the difference between the treated and non-treated plants with the biocontrol consortia. Effects were significant at P = 0.05.

Results

Effect of grafting against Cmm

The effect of different rootstocks with different inoculation methods was studied to determine if grafting enhanced tolerance on the susceptible cv. Moneymaker (root:shoot; LA:MM and MM:MM) against Cmm symptoms after stem respectively root inoculation. MM:LA plants were used to see if grafting the tolerant scion on susceptible rootstock will affect the wilting symptoms.

When grafted plants were stem inoculated with Cmm, the first wilting symptoms were observed on 11 dpi on MM:MM and on LA:MM. On 29 dpi, there was a significant difference in the wilting symptoms among MM:MM, LA:LA, MM:LA and LA:MM $\chi2$ (3) = 19.90, p = 0. 000, but there was no significant difference in wilting symptoms between LA:MM and MM:MM (Table S1, Figure 1b, d). No severe wilting symptoms were observed on LA:LA and MM:LA (Figure 1a, c).

Symptom expression after root infection developed slower than after stem inoculation. The first wilting symptoms were observed on MM:MM at 21 dpi. On 36 dpi, a significant difference in wilting symptom among MM:MM, LA:LA, MM:LA and LA:MM was present $\chi2$ (3) = 29.51, p = 0. 000, and there was a significant difference in wilting between LA:MM (M rank 26.10, average score 2) and MM:MM (M rank 33.10, average score 3) (Table S2, Figure 1f, h). On 36 dpi no severe wilting symptom were observed on LA:LA and MM:LA (Figure 1e, g).

Bacterial density measurements (TaqMan assay) showed differences between roots and shoots of root inoculated grafted plants. There is a significant difference in Cmm DNA copy number $\chi2$ (3) = 21.037, p = 0. 000 of the root samples between MM:MM and MM:LA. The roots of MM:MM (1.18×10^6) contained higher bacterial density than MM:LA (2.97×10^3). The bacterial density of the roots of LA:LA and LA:MM could not reliably be determined because they were below the detection limit ($< 10^3$) (Table S3).

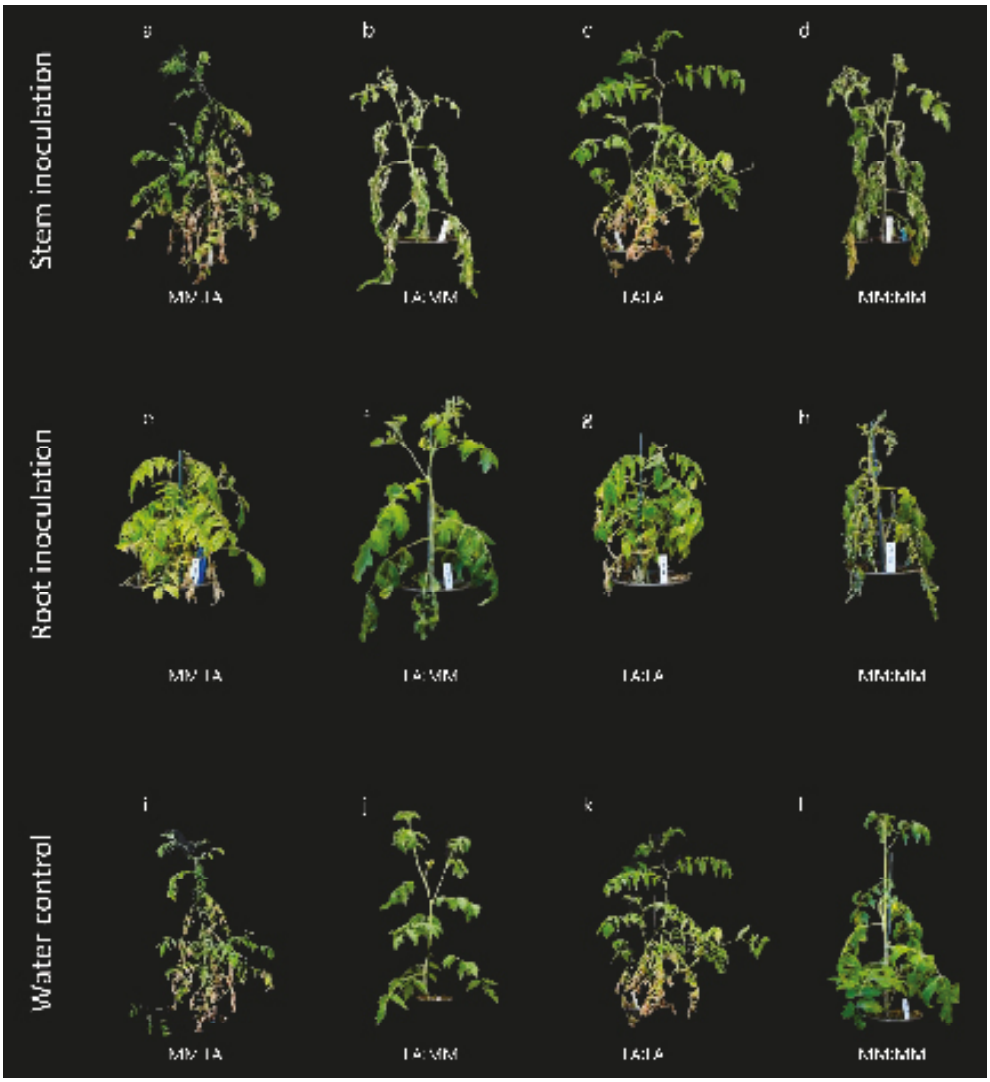

Figure 1 | Wilting symptom after different treatments (root:shoot) of *Solanum lycopersicum* cv. *Moneymaker* (MM) and *Solanum arcanum* LA2157 (LA) with stem inoculation (a, b, c, d), root inoculation (e, f, g, h) and water control (i, j, k, l).

For the shoots, there was a significant difference $\chi 2$ (3) = 14.61, p = 0. 003 in bacterial density among MM:LA, LA:MM and MM:MM. The shoots of LA:MM (2.45 x 10^3) and MM:LA (2.89 x 10^3) had a lower bacterial density and were significantly different from the density in MM:MM (1.68 x 10^6). The density of Cmm in LA:LA shoots could not be determined.

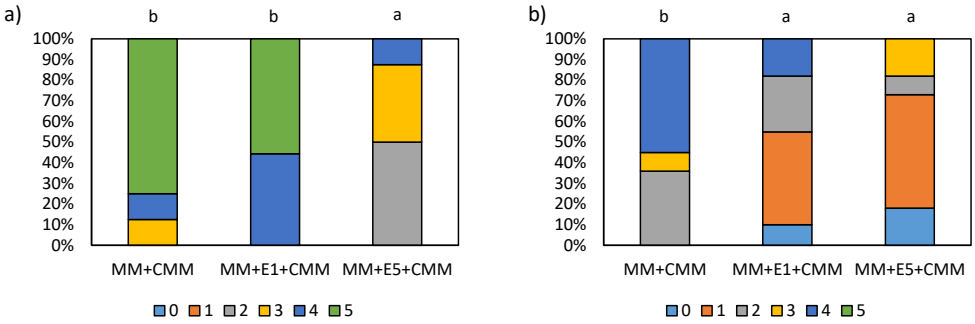

Figure 2 | The effect of applying *Pseudomonas sp.* consortia (E1 and E5) on cv. Moneymaker (MM). Plants were treated by supplying 10% of the pot volume with E1, E5 or water for four weeks. Inoculation was performed a week after and plants were subsequently supplied with the different treatments for two more weeks. Coloured bars show the percentage of plants for each disease index. Disease index is 0 = no symptoms; 1 = 0 to 25% leaf wilting; 2 = 26 to 50%; 3 = 51 to 75% ; 4 = 76 to 100 %; 5 = dead plants. a) stem inoculation with Cmm during the fourth leaf stage on 28 dpi, b) root inoculation with Cmm during the fourth leaf stage on 35 dpi. Within each chart,bars sharing the same letter are not significantly different with Kruskal-Wallis at P = 0.05 using stepwise step-down analysis.

Effect of supplying the Pseudomonas consortia weekly to plants

The effect of different *Pseudomonas* consortia (E1 and E5) was tested to see if application of the consortia could enhance the tolerance of the susceptible cv. Moneymaker after stem or root infections. The first wilting symptoms were observed 14 dpi.

On 28 dpi (the last day of the experiment) we evaluated the stem inoculated plants and found a significant difference $\chi2$ (2) = 15.155, p = 0. 001 in wilting between the control plants and plants that had been in contact with consortia (MM+E1+CMM and MM+E5+CMM) (Figure 2a). For every treatment variation in wilting severity was present and the percentage of plants within each disease index scale was calculated. Half of the plants that were treated with E5 (MM+E5+CMM) wilted up to 50 %, the remaining plants were more severely wilted. All plants that were not treated (MM+CMM) or treated with E1 (MM+E1+CMM) were severely wilted (score 3 and above) at the end of the experiment.

For plants inoculated with Cmm through the roots the first wilting symptoms were spotted 14 dpi, the progress of the wilting severity was slower and the final score was done 35 dpi. There was a significant difference $\chi2$ (2) = 9.330, p = 0. 009 in wilting of the plants treated with consortia and non-treated plants. The treated plants with consortia (MM+E1+CMM and MM+E5+CMM) wilted significantly less than non-treated plants MM (MM+CMM) (Figure 2b). Only 18% of MM+E1+CMM and MM+E5+CMM and 64% of MM+CMM plants were severely wilted (score 3 and above). All plants that were not inoculated with Cmm remained healthy (data not shown).

Effect of dipping the roots and spraying the shoots with *Pseudomonas* consortia

The effect of applying *Pseudomonas* consortia (E1 and E5) was tested to see if a minimal and direct application on the roots and shoots would enhance tolerance of cv. Moneymaker against Cmm after stem infection. The highly tolerant NIL was included to see if there was any complementary effect of the *Pseudomonas* consortia.

On 28 dpi, there was no significant difference in wilting symptoms of stem inoculated plants which were treated with consortia and non-treated MM plants. More than half of the plants were severely wilted (score 3 or higher) for each treatment (Figure 3a). The final scoring for the NILs was 35 dpi. All NIL plants except one were still healthy (score 0). All plants that were not inoculated with Cmm remained also healthy.

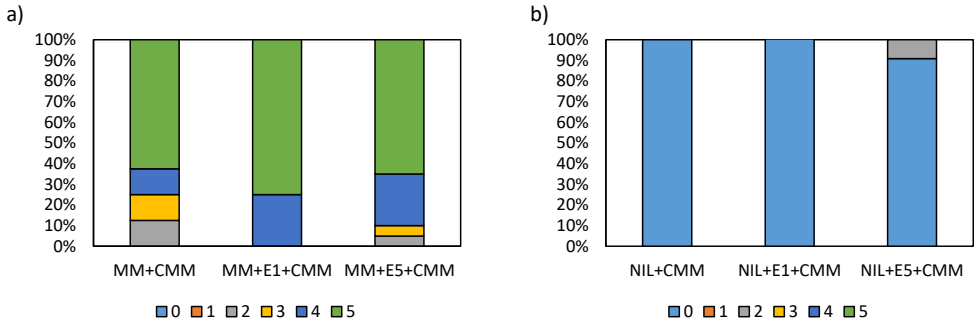

Figure 3 | The effect of applying *Pseudomonas sp.* consortia (E1 and E5) on cv. Moneymaker (MM) and Near-isogenic line (NIL). Plant roots were dipped in E1, E5 or water for 30 minutes before transplanting, a week after the shoots were sprayed with the treatments. Stem inoculation with Cmm was performed during the fourth leaf stage. Coloured bars show the percentage of plants for each disease index. Disease index is 0 = no symptoms; 1 = 0 to 25% leaf wilting; 2 = 26 to 50%; 3 = 51 to 75% ; 4 = 76 to 100 %; 5 = dead plants. a) disease index of MM at 28 dpi b) disease index of MM at 35 dpi.

Presence of *Pseudomonas* consortia in the *in vitro* plants

To determine if the *Pseudomonas* consortia present in the tomato stems and leaves, E1 and E5 were supplemented to *in vitro* growing tomato seedlings. This avoids the potential presence of other *Pseudomonas* species. We observed growth of *Pseudomonas* on Cetrimide agar containing suspension of *in vitro* plantlets supplemented by E1 and E5. No *Pseudomonas* growth was observed on untreated plants.

Discussion

In this study, we tested two approaches that might be useful in an integrated strategy to reduce symptom expression of Cmm in tomato. These techniques were grafting and the use of a biocontrol agent.

Solanum arcanum LA2157 was used as rootstock as it has been shown to be the most tolerant genotype found so far with limited leaf wilting after infection with Cmm (van Heusden et al. 1999; Sen et al. 2013). This genotype has been used in our breeding program to identify a major QTL conferring tolerance to Cmm. Furthermore, *S. arcanum* LA2157 was shown to be resistant against early blight (*Alternaria solani*) (Chaerani et al. 2007; Shinde et al. 2017). We tested the effect of grafting to control Cmm symptoms because of the success of grafting against soil and foliar pathogens (Sakata et al. 2006; Louws et al. 2010) and it is a fast way to exploit the resistance/tolerance for available commercial cultivars. The genetic and biochemical mechanisms behind this resistance mechanism in grafting is unclear. Our results showed that grafting reduces wilting when the pathogen enters through the root. Previous studies showed that there can be an exchange of genetic material between the scion and the rootstock of grafted plants. Some proteins and RNAs can translocate over the graft junctions (Stegemann and Bock 2009). However, this translocation is restricted to the graft site and no translocation to distal plant tissue was found (Stegemann and Bock 2009). Stem inoculation was done at the petiole of first and second leaves, above the graft union site. Conceivably, the effect conferring tolerance in the rootstock of *S. arcanum* LA2157 did not translocate to the cv. Moneymaker scion above the graft junction which explained the appearance of disease symptoms on cv. Moneymaker. Recently, graft-transmitted resistance of cherry pepper to powdery mildew (*Leveillula taurica*) was shown to be due to elevated accumulation of reactive oxygen species (ROS), NADPH oxidase and pathogenesis-related (PR) gene expression (Albert et al. 2017). Their enhancement is likely due to an unknown graft-transmissible signal. If this signal occurred in our situation, it did not prevent Cmm proliferation and virulence.

If we look at another bacterial vascular pathogen of tomato, grafting is widely used to avoid disease symptoms caused by *Ralstonia solanacearum*. The success of grafting against *R. solanacearum* in tomato may be due to restriction of bacterial root colonization in the resistant cultivar (Prior 1993; Caldwell et al. 2017). Resistant roots of cv. Hawaii 7996, delay colonization in the root vasculature. Once the pathogen invasion occurs, it is spatially confined to a smaller area within the root vascular cylinder. Bacterial colonization is not substantial in larger meta-xylem and cell wall deterioration is not conspicuous. The delay in colonization of the root vascular cylinder may facilitate the activation of defence response faster in the resistant

cultivar (Caldwell et al. 2017). Other defence mechanisms may also play a role to suppress bacterial wilt. As observed in another resistant cultivar *S. lycopersicum* var. *cerasiforme* cv. CRA66, *R. solanacearum* bacteria colonized large meta-xylem vessels, and in this genotype the pathogen spread within the vasculature was not inhibited. Resistance in different resistant genotypes may be due to different genes and mechanisms. (Caldwell et al. 2017). The reason why there is a reduction of wilting after root inoculation in a grafted Moneymaker shoot on a *S. arcanum* LA2157 rootstock is unclear. It could be a tolerance factor of *S. arcanum* LA2157 or its xylem vessels structure hinders the progression of Cmm to the scion. Previous histological studies showed that some tolerant genotypes like Irat L3 and Hawaii 7998, have more and bigger tyloses than susceptible genotypes like Moneymaker and Lyconorma. (Stüwe and Tiedemann 2013). Tyloses are protuberances on parenchyma cells of xylem vessels that can block the spread of pathogens (Brodersen and McElrone 2013). Tyloses are a common defence mechanism in xylem vessels against several vascular pathogens, for example *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici* (Hutson and Smith 1980; Yadeta and Thomma 2013). We do not know whether tylose formation plays a role in *S. arcanum* LA2157. Microscopical studies should be performed to support this hypothesis.

There are many studies about the possibilities of antagonistic microorganisms against Cmm, but there is no report on its practical application yet. Bottlenecks can be reproducibility, the costs of producing the antagonistic microorganisms on a large scale, and the best way to apply them on tomato plants. Many studies characterised the interaction of Cmm and antagonistic bacteria *in vitro* and subsequently tested them *in planta*. Inoculation *in planta* was through combining antagonistic bacteria and Cmm (Boudyach et al. 2001; Amkraz et al. 2010; El et al. 2017) or inoculating Cmm right after/before the treatment with antagonistic bacteria (Utkhede and Koch 2004; Lanteigne et al. 2012). This type of inoculation mimics the situation that happens in petri dishes where Cmm and other microorganisms are both present. In nature, Cmm does not cohabit with antagonistic bacteria thus the pathogen could enter the host through primary and secondary spread at any moment. Hence, we think that inoculating Cmm together with a biocontrol agent together is biased and does not depict what really happens in nature. As for that, in our study we have a "rest" period in which a week after the application of the biocontrol agents, the inoculation of Cmm was done. In the few studies that did the experiments in a similar way, the disease symptoms of treated plants remained high (Ślusarski 2009; Romero et al. 2014; Aksoy et al. 2017). Only plants that have been treated with *Pseudozyma aphidis*, a fungal biocontrol agent, show less Cmm disease symptoms. Plants were sprayed with the fungus on the shoot and three days later inoculation of Cmm was done by petiole clipping (Barda et al. 2015). In that study, *Pseudozyma aphidis* triggered

pathogenesis-related genes and activated a resistance response in a salicylic-acid-independent manner (Barda et al. 2015).

Our results show that application of *Pseudomonas* spp. consortia resulted in higher wilting reduction on the root inoculated plants that have been weekly treated with the biocontrol agents. This might be due to the antagonistic interaction of *Pseudomonas* spp. consortia and Cmm. Some of the *Pseudomonas* species used in this study are shown to have antimicrobial activity that could prevent pathogen growth (Ramette et al. 2011; Lanteigne et al. 2012; Novinscak et al. 2016; Paulin et al. 2017; Sun et al. 2017). Thus, the direct contact of Cmm and *Pseudomonas* spp. consortia in the rhizosphere or root may delay the pathogen to colonize the xylem vasculature. There is little to no effect on the stem inoculated plants if they were weekly treated with *Pseudomonas* spp. consortia and there is no effect on stem inoculated cv. Moneymaker with minimal application of *Pseudomonas* spp. consortia. We hypothesized that even if the *Pseudomonas* spp. consortia presented on the shoots or in the xylem vessels, their population would not be enough to inhibit Cmm proliferation. Applying the consortia on the foliar weekly (for about six weeks) may increase the *Pseudomonas* spp. populations in the shoot and reduce the disease symptoms (Kritzman 2014). To determine the effectiveness of applying the *Pseudomonas* spp. against Cmm in the rhizosphere, a longer scoring time, bigger tomato populations and studies on population dynamics of the biocontrol agents are needed.

Combination of different *Pseudomonas* species can improve effectiveness against many plant pathogens because of the combination of different disease-suppressive mechanisms (de Boer et al. 2003; Bakker et al. 2007). It is interesting to note that enhancer 5 (E5) could reduce wilting symptoms even without two *P. putida* strains. Different consortia combinations of the available *Pseudomonas* spp. could be tested *in planta* to see the synergistic effect against Cmm. Induced systemic resistance (ISR), is an essential component for resistance of beneficial microbes in the rhizosphere by priming the whole plant body against a broad-spectrum of pathogens and insect herbivores (Pieterse et al. 2014). We hypothesized that Cmm is not affected by ISR as the resistance mechanism is effective against necrotrophic pathogens and insects that are sensitive to jasmonic acid (JA) and ethylene (ET) dependent defences (van Wees et al. 2008). *Clavibacter michiganensis* susbp. *michiganensis* is mainly a biotrophic pathogen although it can develop into a necrotrophic lifestyle at a later stage (Eichenlaub et al. 2006). Our NIL plants did not exhibit severe wilting symptom with or without the treatment with *Pseudomonas* spp. consortia showing that the tolerance mechanism from *S. arcanumum* LA2157 is very effective to fight Cmm. So far, we still do not know what causes the NIL to be highly tolerant towards the pathogen.

Our study shows the use of grafting and biological control agents in reducing wilting symptoms but only if Cmm enters trough the root. In the future, more rootstocks could be tested and combining both grafting and biological control agents together could be done to see if it will give a stronger effect towards reducing disease symptoms caused by Cmm.

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Supplementary material

Table S1 | Effect of stem inoculation with *Clavibacter michiganensis* subsp. *michiganensis* of grafted plants with different combinations of rootstocks and scions between *S. lycopersicum* cv. Moneymaker (MM) and *S. arcanum* LA2157 (LA) on the disease effect

Treatment[x]	Disease index[y]			
	14 dpi	19 dpi	24 dpi	29 dpi
MM:LA	0.0 a[z]	0.9 a	1.9 a	2.2 a
LA:MM	1.1 c	2.4 b	4.3 b	4.3 b
LA:LA	0.0 ab	1.0 ab	2.0 a	2.0 a
MM:MM	0.9 bc	2.5 b	3.9 b	4.1 b

[x]Treatment (rootstock:scion)
[y]Average symptom score of each treatment (n = 10).
[z] Mean separation within column by Kruskal-Wallis at P = 0.05 using stepwise step-down analysis. Within each column, disease score sharing the same letter are not significantly different.

Table S2 | Effect of root inoculation with *Clavibacter michiganensis* subsp. *michiganensis* of grafted plants with different combinations of rootstocks and scions between *S. lycopersicum* cv. Moneymaker (MM) and *S. arcanum* LA2157 (LA) on the disease effect.

Treatment[x]	Disease index[z]			
	21 dpi	26 dpi	31 dpi	36 dpi
MM:LA	0.0	0.0 ab[y]	0.1 a	0.3 a
LA:MM	0.0	0.3 bc	0.9 b	2.0 b
LA:LA	0.0	0.0 a	0.0 a	0.1 a
MM:MM	0.1	1.0 c	1.8 b	3.0 c

[x]Treatment (rootstock:scion).
[y]Average symptom score of each treatment (n = 10).
[z] Mean separation within column by Kruskal-Wallis at P = 0.05 using stepwise step-down analysis. Within each column, disease score sharing the same letter are not significantly different.

Table S3 | DNA copy number of *Clavibacter michiganensis* subsp. *michiganensis* of root inoculated grafted plants on 36 dpi.

Treatment[x]	Disease index[y]	Plant part	Bacterial titre
MM:LA	0.3	Shoot	2.89×10^3
		Root	2.97×10^3
LA:MM	2.0	Shoot	2.45×10^3
		Root	n/a
LA:LA	0.1	Shoot	n/a
		Root	n/a
MM:MM	3.0	Shoot	1.68×10^6
		Root	1.18×10^6

[x] Treatment (rootstock:scion)
[y]Average symptom score of each treatment (n = 10).

CHAPTER 6

General discussion

In pursuit of symptomless tomato plants after infection with *Clavibacter michiganensis* subsp. *michiganensis*

Our group started to search for resistance against bacterial canker in tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in the 1980's (van Steekelenburg 1985), and in the 1990's a mapping study was executed and three Quantitative Trait Loci (QTL) were found (van Heusden et al. 1999). *Solanum arcanum* LA2157 was the source of resistance. During these studies it became clear that our source of resistance is highly tolerant towards Cmm. That means even though wilting and canker symptoms do not appear, bacterial density in the infected plant is still considerably high (Sen et al. 2013). This makes breeding companies reluctant to use this type of resistance since the bacteria can spread unnoticed from symptomless tolerant plants to susceptible cultivars. Nevertheless, highly tolerant tomato plants that remain symptomless after Cmm infection for a long period are valuable for farmers.

In our pursuit of developing tomato varieties that stay symptomless after Cmm infection, we came closer to the genes that are involved and we found out that also *S*-genes can add to tolerance. Furthermore, the use of grafting and biological control reduced wilting symptoms if Cmm infection came via the roots. In the following topics, we will discuss in details the potentiality of the approaches that we used in this thesis to get symptomless resp. reduced symptom tomato plants. Afterwards we will discuss other possibilities of getting tolerant tomato plants and the future perspectives.

In vitro assay

To screen for resistance resp. tolerance requires a lot of plants and a large space, which is a challenge when it comes to Cmm. Already for a long time Cmm is a quarantine organism and the regulations to work with it became stricter. In the past, screening of tomato plants against Cmm could be carefully done in a normal greenhouse and after cleaning this greenhouse it could be used for other purposes. More space makes it possible to screen larger populations for extended periods making the distinction between highly tolerant, middle tolerant and susceptible more clear. For our experiments we could only use a small quarantine compartment. This made it impossible to screen many plants at the same time, partly because cross-contamination can occur if too many plants are in a small compartment. In our search for an alternative method we developed an *in vitro* assay where in small containers individual plantlets can be screened in a climate room. This *in vitro* assay makes it possible to screen larger populations in one experiment (**Chapter 2**).

Screening for resistant genotypes using *in vitro* assays is not new (Svabova et al. 2005). In the Cmm-tomato interaction, it has been used to generate resistant/tolerant somaclones (van den Bulk et al. 1991) and to screen somaclones with Cmm *in vitro* (Sotirova et al. 1999). Other studies that use *in vitro* assays are listed in Table 1. Those studies used the dual culture of the host and pathogen *in vitro* to avoid cross-contamination of the pathogen (O'Herlihy et al. 2012), the easiness to see the symptoms on the *in vitro* plants (Barlass et al. 1986; Russo and Slack 1998; van Vuuren and Woodward 2001; Mazier et al. 2004; Winterhagen et al. 2007; Rodríguez-Moreno et al. 2008; Al Abdallat et al. 2010; Miazzi et al. 2010; Hanus-Fajerska et al. 2014; Xu et al. 2015; Azadmanesh et al. 2016), and the speed of the screening system (Loreti et al. 2008; Sedlák et al. 2016). However, some drawbacks can be present (Table 1). Nonetheless, an *in vitro* screening method is an efficient method to screen large sample numbers using limited experimental space.

Table 1 | *In vitro* methods used for screening for resistance in several crops.

Crop	Pathogen	Remark
Tomato (*Solanum lycopersicum*)	*Tomato yellow leaf curl virus* (TYLCV)	Development of *in vitro* method for TYLCV inoculation of tomato
	Ralstonia solanacearum	Dual culture of 5000 mutants with *R. solanacearum in vitro*
	Meloidogyne incognita, *M. javanica* and *M. arenaria*	Development of *in vitro* culture of tomato with nematodes
Lettuce (*Lactuca sativa* L.)	*Lettuce mosaic virus* (LMV)	Development of *in vitro* method for LMV inoculation of lettuce
Grapevine (*Vitis vinifera*)	*Plasmopara viticola*	Dual culture of grapevine with downy mildew for *in vitro* screening
	Grapevine fanleaf virus (GFLV)	*In vitro* screening method for detecting Grapevine fanleaf virus (GFLV) from ectoparasitic vector nematode *Xiphinema index* (Longidoridae)
	Erysiphe necator	*In vitro* screening to evaluate the susceptibility of different grapevine cultivars to powdery mildew
Daphne	*Thielaviopsis basicola*	*In vitro* root culture to screen different *Daphne* genotypes against fungal pathogen
Cassava (*Manihot esculenta* Crantz)	*Meloidogyne javanica*	*In vitro* screening method for root-knot nematodes resistance in different cassava cultivars
Water spinach (*Ipomoea aquatica*)	*Meloidogyne incognita*, *M. javanica* and *M. arenaria*	Development of *in vitro* culture of water spinach roots with nematode
Potato (*Solanum tuberosum*)	*Potato virus Y* (PVY)	Development of *in vitro* inoculation for screening PVY resistant transgenic potato plants
	Pectobacterium carotovorum	*In vitro* screening method to evaluate 46 potato genotypes for resistance to bacterial soft rot
Apple (*Malus pumila*)	*Erwinia amylovora*	Development of *in vitro* inoculation for testing apple resistance to fire blight
Olive (*Olea europaea*)	*Pseudomonas savastanoi* pv. *savastanoi* and *P. savastanoi* pv. *nerii*	Pathogenicity test of *in vitro* olive plants on *P. savastanoi* pv. *savastanoi* and *P. savastanoi* pv. *nerii*
Pear (*Pyrus communis*)	*Erwinia amylovora*	*In vitro* protocol to evaluate the resistance of pear cultivars against *Erwinia amylovora*

[a] The disadvantage of the *in vitro* screening as reported. n/a = not available.

Bottleneck[a]	Reference
n/a	(Al Abdallat et al. 2010)
n/a	(O'Herlihy et al. 2012)
n/a	(Xu et al. 2015)
n/a	(Mazier et al. 2004)
• Differences between different degrees of resistance were not apparent • Takes a long time to establish sufficient plant cultures	(Barlass et al. 1986)
• Infection rate is lower due to shorter incubation time	(Winterhagen et al. 2007)
n/a	(Miazzi et al. 2010)
n/a	(Hanus-Fajerska et al. 2014)
• Limited development of root-knot nematodes on MS (Murashige & Skoog) medium	(van Vuuren and Woodward 2001)
• Populations of *M. javanica* and *M. arenaria* are less than *M. incognita* on the water spinach roots. Could be due to host preference of the species	(Xu et al. 2015)
n/a	(Russo and Slack 1998)
• Some discrepancies with the symptoms observed between different inoculation methods	(Azadmanesh et al. 2016)
n/a	(Sedlák et al. 2016)
n/a	(Rodríguez-Moreno et al. 2008)
n/a	(Loreti et al. 2008)

Genetic analysis of tolerance against Cmm

Several studies have been done to understand the nature of tolerance in tomato against Cmm and different types of interactions were found: additive (van Heusden et al. 1999; Coaker and Francis 2004), polygenic (Thyr 1972), incomplete dominance (Thyr 1976) and dominant (Vulkova and Sotirova 1993). For our group, we aimed for resistance and in principle now that we found out that the bacterial density is still high in the symptomless plants, tolerance instead of resistance is a better term to use. Previously an intraspecific backcross population between the susceptible *S. arcanum* LA2172 and the highly tolerant *S. arcanum* LA2157 was used to map loci putatively involved in tolerance (Sandbrink et al. 1995), and five regions were identified (on chromosomes 1, 6, 7, 8 and 10). The QTL mapping of the interspecific F2 population between *S. lycopersicum* cv Solentos and *S. arcanum* LA2157 revealed QTL on chromosomes 5, 7 and 9. The tolerance loci were additive and co-dominant, and the QTL on chromosome 7 conferred the largest part of the variation (van Heusden et al. 1999).

About fifteen years later fine mapping studies were done by adding more markers in the QTL regions. The different QTL regions were reduced to 28 Mb on chromosome 5, 1.2 Mb on chromosome 7 and the QTL region on chromosome 9 was not reduced (Sen 2014). Our current fine mapping effort has reduced the QTL on chromosome 7 to 211 kb and it was shown that a Nearly Isogenic Line (NIL) containing this QTL is highly tolerant (**Chapter 3**). This was not expected based on the F2 mapping results where at least one additional QTL besides the QTL on chromosome 7 was needed. This newly fine mapped region can be further delimited by adding more markers or an extra fine mapping effort. An alternative to find the genes underlying the tolerance is to do functional studies: 1) virus-induced gene silencing (VIGS) assays in our Nearly Isogenic Lines (NILs); silencing the right genes will lead to susceptibility (Balaji et al. 2011; Esparza-Araiza et al. 2015), 2) stable silencing of the candidate genes in the NILs by genetic transformation and 3) overexpression of the candidate genes in susceptible tomatoes to see whether overexpression can lead to symptomless plants (Balaji and Smart 2012).

Another source that confers tolerance is *S. habrochaites* LA407. *S. habrochaites* LA407 was used due to the fact that it can be easily crossed to cultivated tomato (Francis et al. 2001). Mapping studies using an inbred backcross population (IBC) resulted in the identification of loci on chromosomes 2 (Rcm 2.0) and 5 (Rcm 5.1) (Kabelka et al. 2002). Follow up studies from the same group were done to fine map and to identify the genetic effects. Fine mapping narrowed Rcm 2.0 to a 4.4 cM interval and Rcm 5.1 to a 2.2 cM interval. The two loci exhibit additive gene action and interact epistatically (Coaker and Francis 2004). In another experiment a

QTL, controlling stem morphology, originated from *S. habrochaites* LA407 was also mapped on chromosome 2. It was speculated that this QTL might be involved in tolerance against Cmm (Coaker et al. 2002). However, no proof could be found that the Rcm 2.0 and the vascular morphology QTL were controlled by the same gene(s).

Susceptibility gene(s) as source of tolerance

Additionally, we tried to introduce tolerance by using susceptibility genes (*S*-genes). Combining the tolerance factor(s) with non-functional *S*-genes might make the plant more and more durable tolerant. Exploiting *S*-genes for resistance is not new, but it was never done for the tomato-Cmm interaction (**Chapter 4**). We choose two *S*-genes located in the QTL of chromosomes 7 and 9 to see whether their localization in the QTL is coincidental or that they are involved in conferring tolerance. We hypothesized that *S*-genes, involved in Cmm tolerance, are non-functional in *S. arcanum* LA2157. This approach was successful to find the *S*-gene causing resistance to powdery mildew in cucumber (Berg et al. 2015).

In our study, virus-induced gene silencing (VIGS) assays in tomato with the two *S*-genes, located in the QTL regions, did not result in different levels of tolerance. Two other *S*-genes were selected based on previous studies in host plants challenged with vascular pathogens (van Schie and Takken 2014). One of these, the *WAT1* gene, looked promising as the silenced tomato plants have a higher tolerance to Cmm than the control plants. We do not know the mechanism in which *wat1* functions in preventing wilting and canker symptoms. One of the limiting factors is the VIGS assay, even though the application of a VIGS assay can expedite the screening of many candidate genes, the patchiness of the silencing level in the different parts of the plants affects the score of the tolerance. After identifying the best candidate genes with VIGS, stable transformants must be made using gene silencing techniques like RNAi, or knocking-out genes using the CRISPR-Cas9 system. Stable transformants can then be used to determine the role of *WAT1* in preventing symptoms (Arora and Narula 2017). Currently, our group is testing more *S*-genes to see if there are other genes that could lead to reduced susceptibility.

Utilizing both tolerance factors and *S*-genes might improve the durability of tolerance against Cmm. The region of the *WAT1* gene was not found in our mapping studies but this might be due to a functional *WAT1* gene was in both tomato as well as in *S. arcanum* LA2157. A combination of the QTL on chromosome 7 and an impaired *WAT1* gene in a single plant might enhance the tolerance.

Alternative ways to manage the spread of the pathogen

In the process of looking at preventing Cmm outbreaks, *R*-genes and *S*-genes can play an important role but a more holistic approach should also include agronomic considerations like cultural practises and the use of biocontrol agents. The use of grafting techniques and biocontrol agents (*Pseudomonas* species) were tested to see if they could be used as an alternative or complementary way to prevent symptom development (**Chapter 5**). For the experiment involving grafting, we hypothesized that there could be a translocation of genes involving tolerance or signals from the rootstock of *S. arcanum* LA2157 to the scion of cv. Moneymaker that could lead to tolerance. Unfortunately, that was not the case. Reduction of wilting symptoms only occurred on the scion of cv. Moneymaker grafted on *S. arcanum* LA2157 rootstock when Cmm entered through the root but not stem. The reason behind reduction of wilting symptoms is unclear. It could be that the tolerance factor of *S. arcanum* LA2157 or the structure of its xylem vessels hinder the progression of Cmm to the scion. Further microscopy studies could be done to confirm this hypothesis. Even though we did not see reduction of wilting symptom in the stem inoculated of cv. Moneymaker (scion) grafted on *S. arcanum* LA2157 (rootstock), we cannot rule out the possibility of grafting as an alternative method to reduce Cmm symptoms. More rootstocks that could reduce Cmm symptoms, prevent other pathogens and improve plant vigour and yield can be tested.

For our study on biocontrol agents, instead of focusing on one species, a consortium of different species, was used to study the antagonistic effect, or the enhancement of plant immunity. We observed reduction of wilting symptoms only after root-, but not after stem inoculation. The fact that less wilting was observed from root entry but not stem entry might be due to antagonistic behaviour and not due to induced systemic resistance (ISR). *Pseudomonas* spp. may hinder the pathogen to colonize the xylem vasculature in the rhizosphere or root as seen for some pseudomonas species (Ramette et al. 2011; Lanteigne et al. 2012; Ait Bahadou et al. 2018). Even though beneficial microbes are known to induce systemic resistance (ISR), we hypothesized that Cmm is not affected by it. Induced systemic resistance is effective against necrotrophic pathogens and insects that are sensitive to jasmonic acid (JA) and ethylene (ET) dependent defences (van Wees et al. 2008). *Clavibacter michiganensis* susbp. *michiganensis* is mainly a biotrophic pathogen although it can evolve into a necrotrophic lifestyle at a later stage (Eichenlaub et al. 2006). Screening of more biocontrol agents could be tested with the available *Pseudomonas* species to test their synergistic effect to reduce Cmm disease symptoms.

Tomato tolerance against Cmm

The mystery of *S. arcanum* LA2157

In our group we are focusing on *S. arcanum* LA2157 as a resistance source. In our search for the best donor, we have screened solely on wilting symptoms (van Steekelenburg 1985; van Heusden et al. 1999), and the resistance was thus characterised by a lack of wilt symptoms. Recently we found that *Solanum arcanum* LA2157 is not really resistant but rather tolerant to Cmm. It is possible that Cmm interacts differently with *S. arcanum* LA2157 than with cultivated tomatoes and wilting could not be seen clearly because of the morphology of the leaves. Previous studies (De Jong and Honma 1976) state that the criteria to determine non-resistance should include wilting, stunting and the presence of canker. In certain genotypes and environments, wilting alone is not the best observation to determine susceptibility against Cmm. One should examine other traits as well if wilting is not obvious enough to determine susceptibility. Neither stem discoloration nor canker was seen on infected *S. arcanum* LA2157 plant, but minor stunting did occur on some plants. In **Chapters 2 & 3**, we included both leaf wilting and stem canker as phenotyping criteria. We observed that many of our *in vitro* plantlets developed canker which is rarely seen in our greenhouse plants. It is likely that the high humidity in the *in vitro* conditions is affecting the canker development on susceptible plants. Our plants from the NIL population do not have wilting symptoms (at the most only a few leaflets) and do not stunt or have stem discolorations or cankers

Up to now, the underlying tolerance mechanism in *S. arcanum* LA2157 is still a mystery. Some innate genes in the QTL region of *S. arcanum* LA2157 might differ from those of tomato or Cmm is non-adapted to *S. arcanum* LA2157. Taking these two aspects into account, the tolerance in *S. arcanum* LA2157 might be a nonhost resistance (Senthil-Kumar and Mysore 2013; Gill et al. 2015). There are few points to support this hypothesis. Firstly, the *C. michiganensis* subspecies specifically infects one type of host and the common host of Cmm is tomato (*Solanum lycopersicum*) (Eichenlaub et al. 2006). The pathogen is thought to be a relatively new pathogen which developed from endophytic bacterial species and not many genetic differences were found between different Cmm strains (Eichenlaub and Gartemann 2011; Thapa et al. 2017; Sen et al. 2018). *Solanum arcanum* LA2157 is a distinct relative of cultivated tomato, it might be that Cmm has not adapted to be virulent on this *Solanum* species (Grandillo et al. 2011). Other *S. arcanum* accessions are also highly tolerant (Sen et al. 2013), with the exception *S. arcanum* LA2172 (Sandbrink et al. 1995). However, *S. arcanum* LA2172 was considered tolerant by another group (Lara-Ávila et al. 2011). Despite the ambiguity in these findings, Cmm may not yet adapted to the *S. arcanum* LA2157. Previous QTL analysis suggested, that several genes were associated with tolerance pointing in the direction of nonhost resistance (van Heusden et al. 1999; Senthil-Kumar and Mysore 2013).

There are three layers of defence for nonhost resistance (Senthil-Kumar and Mysore 2013). The first one restricts the entry of the pathogen (physical barrier) and usually is already present or it can be induced. The second layer of defence acts as soon as the pathogen reaches the apoplastic region. This step involves both constitutive as induced defences. The constitutive layer of defence could be due to the antimicrobial compounds produced by the host coupled with overall apoplastic physiological incompatibility. The last layer involves inducible defence responses triggered in the plant cytoplasmic region and executed in the cell wall region or apoplast. The induced plant defence responses described above require perception of pathogen-associated molecular patterns (PAMPs), virulence factors etc. In the *S. arcanum* LA2157 Cmm interaction, it is possible that after infection, the small xylem vessel and the cell wall composition act as preformed defence barriers and restrict Cmm spreading (Romero et al. 2014; Esparza-Araiza et al. 2015). Induced defences may occur from tylose formation as observed in a previous study (Stüwe and Tiedemann 2013). The high accumulation of antimicrobial α-tomatine in *S. arcanum* LA2157, may act as a preformed defence in the second step against tomatinase, an enzyme which is encoded by the *tomA* gene from Cmm. A high level of α-tomatine in *S. arcanum* LA2157 makes it resistant against *Alternaria solani* (Shinde et al. 2017). Whether *tomA* is required for Cmm virulence is not clear (Eichenlaub and Gartemann 2011). *tomA* mutants cause wilting and have similar bacterial densities as the controls (Kaup et al. 2005). This may be due to the low α-tomatine in Moneymaker which does not affect the growth of the pathogen (Kaup et al. 2005). Despite this, we cannot rule out the possibility that a high α-tomatine concentration in *S. arcanum* LA2157 inhibits Cmm growth or colonization.

It is unclear if there is any induced defence occurring in *S. arcanum* LA2157 against Cmm in the third layer of defence. For Proteobacteria, the virulence factors/effectors enter the plant cell through the type 3 secretion system (T3SS). Effectors (avirulence factors) are recognized directly through NB-LRR (nucleotide binding and leucine-rich-repeats) or indirectly in resistant plants by a hypersensitive reaction (HR) preventing the spreading of the pathogen in the plant tissue (Jones and Dangl 2006). However, Cmm has a type 2 secretion system (T2SS) but not a T3SS , and no genes encoding effector proteins resembling those identified in Gram-negative bacterial pathogens were found in Cmm (Gartemann et al. 2008). Thus, just like the tomato-Cmm interaction model discussed in the general introduction, *S. arcanum* LA2157 may not have resistance genes leading to effector-triggered immunity (ETI) to Cmm.

To identify what kind of genes underlie the tolerance in *S. arcanum* LA2157, we delimited our QTL on chromosome 7. This led to a region of 211 Kb containing 15 annotated genes. We speculated that the gene(s) in the region might have different expression levels in *S. arcanum* LA2157 compared to tomato and therefore make it more tolerant. So far there is only one study that compares the differential gene expression between

Solanum arcanum LA2157 and tomato when infected with Cmm. That study revealed protein transport protein Sec23 is expressed ten times more in *S. arcanum* LA2157 (8 dpi) compared to *S. lycopersicum* after infection. This protein is expressed five times more in *S. arcanum* LA2157 than in *S. lycopersicum* without Cmm infection (Lara-Ávila et al. 2011). This protein is not in our fine mapped region, but it would be interesting to see if this gene contributes to tolerance against Cmm. The Sec23 protein is a component of the coat protein complex II (COPII) that promotes the formation of transport vesicles from the endoplasmic reticulum (ER) to the Golgi apparatus (Zeng et al. 2015). In recent studies in Arabidopsis, it was found that two components of the *Sec23* homologs, the *AtSec23A* and *AtSec23D*, play essential roles in pollen wall formation as the mutant plants had an impaired exine pattern (Aboulela et al. 2018). So far, no study has shown the potential use of this protein under biotic stress and it is not clear whether this protein is involved in tolerance against Cmm. A VIGS assay or stable knock-down/out transformation of the *Sec23* gene in *S. arcanum* LA2157 will show whether silencing/knocking-out the gene leads to susceptibility (Esparza-Araiza et al. 2015). If it does, overexpression of the gene in a susceptible tomato could enhance tolerance.

Different metabolites and differentially expressed genes might be the reason why *S. arcanum* LA2157 is highly tolerant towards Cmm. However, comparing metabolites and gene expression between *S. arcanum* LA2157 and *S. lycopersicum* will lead to many differences. To focus on genes involved in tolerance, it is better to do comparative metabolomic studies and differential gene expression studies between the NIL containing the small introgression of *S. arcanum* LA2157 in a background of Moneymaker. A combination of these studies with the knowledge of the genes identified in the fine mapping experiment will pinpoint the gene products or metabolites that are responsible for the tolerance mechanism of this specific QTL.

Other ways to get tolerance in *S. lycopersicum*

The group of phytopathogenic *Clavibacter michiganensis* subspecies is considered as recently developed from endophytic bacterial strains (Eichenlaub and Gartemann 2011). The divergence between Cmm and *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) was calculated to have occurred less than 1.2 million years ago (Bentley et al. 2008). It is hypothesized that Cmm acquired its virulence factors via horizontal gene transfer (Gartemann et al. 2008; Chalupowicz et al. 2010). Recently, it was found that different Cmm strains use different virulence factors thus there is a need of breeding tomato cultivars that can withstand multiple strains (Thapa et al. 2017). Other ways to get tolerance can also be achieved by manipulating the genes of *S. lycopersicum* (Pavan et al. 2009; Balaji and Smart 2012).

Balaji et al. (2008) describe gene expression profiles in tomato of Cmm-infected stem tissue during the endophytic stage (4 dpi) and in a later stage (8 dpi). One hundred and twenty-two genes were differentially expressed in at least one time point which represents 1.3% of the genes analysed. More genes are likely to be differently expressed during the interaction as the array used in the study contained approximately one-fourth (9,254 genes) of the total estimated tomato genes (35,000 genes). Table 2 summarizes part of the differentially expressed genes. Genes in the table are more than 15 times upregulated in the tomato cultivar Rio Grande after inoculation with Cmm.

Based on the *S*-gene concept, we could use innate genes of the susceptible genotype for tolerance against Cmm (Sun et al. 2014; van Schie and Takken 2014; Sun et al. 2016a). According to the *S*-gene theory, Cmm uses tomato gene products to facilitate susceptibility and to contribute to the infection process. These susceptibility genes might be upregulated after Cmm infection. For instance, the *Mildew Locus O* (*MLO*) gene is upregulated upon pathogen infection (Berg et al. 2015; Pessina et al. 2016). Upon inoculation with cucumber powdery mildew pathogen, *Podosphaera xanthii*, there is transcriptionally upregulated expression of *CsaMLO8* in the cucumber hypocotyl (Berg et al. 2015). The same observation was seen in grapevine as *VvMLO7*, *11* and *13* are upregulated during grape powdery mildew (*Erysiphe necator*) infection (Pessina et al. 2016).

However, not all upregulated genes are *S*-genes. A good example is that upon Cmm infection, a subset of tomato genes involved in ethylene biosynthesis and response was induced (Balaji et al. 2008). The ACD mutant of tomato causes an ethylene reduction up to 90%. After inoculation with Cmm the ACD mutant plants had a delayed wilting. Delayed wilting is also observed on the tomato ethylene insensitive *Never ripe* (*Nr*) mutant. Ethylene production in tomato is a major signal that regulates disease progression, and is not used by Cmm for virulence or proliferation. Two other upregulated proteins, the RING/U-box superfamily protein and extensin-like protein, are also not *S*-genes. When these genes were silenced, the plants exhibited wilting symptoms after Cmm inoculation (Balaji et al. 2011).

Additionally, not all known *S*-genes in Table 2 that cause enhancement of resistance against other pathogens, can potentially be used against Cmm. The *DMR6* gene encodes 2-oxoglutarate (2OG) and Fe (II) oxygenase protein. It is shown that the Arabidopsis *dmr6* mutant confers resistance to the downy mildew pathogen *Hyaloperonospora arabidopsis/parasitica* (van Damme et al. 2005), *Pseudomonas syringae* and *Phytophthora capsici* (Zeilmaker et al. 2015) and recent studies in silencing the gene in potato led to resistance against *Phytophthora infestans* (Sun et al. 2016b). *DMR6* gene acts as a suppressor of plant immunity and silencing it activates the plant defence responses by increasing the level of salicylic acid (SA) (van Damme et al. 2008; Zeilmaker et al. 2015). We tested the tomato *dmr6* RNAi line mutant against Cmm, and did not see

enhancement of resistance (data not shown in the thesis). Another *S*-gene that enhances resistance against pathogens is the PROTEIN DISULFIDE ISOMERASE (PDI) gene family. It plays a role as chaperone which helps in the arrangement of disulfide bonds for correct protein folding (Houston et al. 2005). In barley (*Hordeum vulgare*), the naturally recessive resistance against Bymoviruses, the barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV), is found in locus *rym11*. The susceptibility factor in this locus is the *PROTEIN DISULFIDE ISOMERASE LIKE 5–1* (*HvPDIL5-1*). Inhibition of PDI activity in the host suppressed the Bymovirus replication or infection in barley (Yang et al. 2014b). It is not known whether inhibiting this protein could also work against Cmm due to the different life cycle. Nevertheless, to determine if this gene or other upregulated genes shown in Table 2 can be potentially used against Cmm by using the *S*-gene concept, a VIGS assay could first be done. Virus-induced gene silencing can be tested on the susceptible genotype for a faster screening process. Follow up experiments should then include making stable transformation of the genes that not only confer resistance/tolerance in the VIGS screening but ideally also show no pleiotropic effects. This will confirm that inhibition of the gene(s) will lead to resistance/tolerance to Cmm.

A complementary approach of making use of the upregulated genes in Table 2 is to do overexpression studies. If silenced plants from the VIGS screening exhibit severe wilting, the genes that have been silenced might be important to fight the pathogen. Thus, overexpressing those genes may lead to resistance/tolerance just like the tomato mutant overexpressing the extensin-like protein exhibited a higher tolerance towards Cmm (Balaji and Smart 2012). Other overexpressed genes in Table 2 that enhance resistance against other pathogens are *WRKY80, CAT1* and *NAC6*. Just like the expression pattern in tomato, the rice *WRKY80* gene (*OsWRKY80*) is highly upregulated after infection of rice sheath blight disease (*Rhizoctonia solani*). The strong induction of *OsWRKY80* expression by exogenous application of JA,ET and pathogen inoculation suggests that this gene may be involved in JA/ET-dependent defence signalling pathways. Overexpression of the gene in rice significantly enhanced disease resistance to the rice sheath blight disease (Peng et al. 2016). The same goes for *CAT1* as overexpression of *CAT1* in Arabidopsis resulted in a better resistance against *P. syringae*. This resistance might be due to activation of SA (Yang et al. 2014a). Furthermore, overexpression of the NAC transcription factors, the *HvNAC6*, increases the number of penetration resistant cells against *Blumeria graminis* f.sp. *hordei* (Bgh). *HvNAC6* may act downstream of abscisic acid (ABA) biosynthesis, mediate early non-specific biotic stress sensing signals and influence stomata movement (Jensen et al. 2007). The genes mentioned above could be potential candidates to fight Cmm. It would be interesting to see whether overexpression of these genes in tomato could also enhance resistance/tolerance against Cmm.

Table 2 | Differentially expressed tomato genes in response to infection by Cmm. The table is based on and modified from Balaji et al. (2008)[a].

GenBank Accession Number	SGN ITAG 3.2	Description[b]	Expression ratio		VIGS[c]	
			4 dpi	8 dpi	Pleitropic effect	Wilt symptom
U89256	*Solyc02g077370.1*	Ethylene Response Factor C.5	21.6	163.2	No phenotype	Susceptible
BT013271	*Solyc06g073080.3*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	20.9	94.8		
AF272366	*Solyc09g005080.1*	verticillium wilt disease resistance 2	50.2	87.5		
AI776170	*Solyc02g077040.4*	phytophthora-inhibited protease 1	32	80.8		
BI204920	*Solyc03g117860.3*	RING/U-box superfamily protein	7.2	70.3	Mild crinkling	Susceptible
X85138	*Solyc01g107820.2*	TOMATO WOUND-INDUCED 1	8.5	66.2		
BI205190	*Solyc09g092500.1*	Glycosyltransferase	1.1	59.2		
BG629612	*Solyc05g050130.3*	Acidic endochitinase	19	55.4		
BT014226	*Solyc05g056400.3*	Protein disulfide isomerase	15.5	52		
AW032318	*Solyc03g033840.3*	P-loop containing nucleoside triphosphate hydrolases superfamily protein	8.1	38.1		
AJ831935	*Solyc03g095770.3*	WRKY transcription factor 80	3.5	36.4		
AI898214	*Solyc04g048900.3*	Calreticulin	6.5	35.6		

Over-expression[d]	Remarks	References
	• *DMR6* gene catalyzes 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase • *Arabidopsis thaliana dmr6* mutants exhibits reduced susceptibility against *Hyaloperonospora arabidopsis/parasitica*, *Pseudomonas syringae* and *Phytophthora capsici*.. • Silencing the gene leads to resistance against potato late blight (*Phytophthora infestans*) • However, tomato *dmr6* RNAiline plants were susceptible against Cmm when tested by our group (results not shown in the thesis)	(van Damme et al. 2005; van Damme et al. 2008; Zeilmaker et al. 2015; Sun et al. 2016b)
	• Resistance against *Verticillium* wilt	(Kawchuk et al. 2001)
	• A pathogenesis-related (PR) protein. • It is closely related to tomato apoplastic Cys protease, the Rcr3. It functions in fungal resistance and targeted by the protease inhibitor Avr2 of *Cladosporium fulvum*	(Tian et al. 2006)
	• Defence-related gene which responds rapidly to wound and pathogen-related signals.	(O'Donnell et al. 1998)
	• Effector-triggered immunity (ETI) marker gene. • It exhibits high induction in tomato only during ETI *Pseudomonas syringae* pv. Tomato	(Pombo et al. 2014; Pombo et al. 2017)
	• Pathogenesis-related protein 11	(Andolfo et al. 2014; Manzo et al. 2016)
	• It catalyzes the correct folding of proteins and prevents the aggregation of unfolded or partially folded precursors. • Suppression of the protein delays replication to plant viruses. • Susceptibility factor to Bymoviruses • Natural loss-of- function alleles of *HvPDIL5-1* in barley confer resistance to Bymoviruses	(Houston et al. 2005; Yang et al. 2014b)
	• Upregulated gene upon infection (24 hpi) against *Phytophthora infestans and Botrytis cinerea*	(Rezzonico et al. 2017)
	• Overexpression of *Oryza sativa* WRKY80 gene (*OsWRKY80*) significantly enhanced disease resistance to *Rhizoctonia solani*	(Peng et al. 2016)
	• Upregulated gene in *P. infestans* infected tissue. • Potential gene belonging to major resistance (*R*) gene	(Rezzonico et al. 2017)
	• The Arabidopsis *AtCRT1/2* and *AtCRT3* are involved in regulating plant defence against *Pseudomonas syringae*	(Qiu et al. 2012)
	• ACE35 protein is required for nonhost resistance to *Xanthomonas oryzae* pv. *Oryzae* but not required for the Cf-4/Avr4-dependent HR.	(Li et al. 2012)

GenBank Accession Number	SGN ITAG 3.2	Description[b]	Expression ratio		VIGS[c]	
			4 dpi	8 dpi	Pleitropic effect	Wilt symptom
AI780536	*Solyc07g056200.3*	Heavy metal transport/ detoxification superfamily protein	9.3	33.9		
Y10149	*Solyc08g079870.3*	subtilisin	11.6	33.4		
BI206504	*Solyc09g015770.3*	WRKY transcription factor 81	8.5	31.8		
BE354113	*Solyc04g048900.3*	Calreticulin	8.3	30.3		
CN385704	*Solyc03g115930.2*	Calcium-binding EF-hand family protein	7.6	28.7		
BG630825	*Solyc04g048900.3*	Calreticulin	8.3	27.7		
BI210305	*Solyc10g055740.2*	Amino acid transporter	5.1	27.1		
CN385590	*Solyc03g116890.3*	WRKY transcription factor 39	2.4	24.7		
K03291	*Solyc03g020080.3*	Pin-II type proteinase inhibitor 69	18.1	24		
BT012691	*Solyc12g006380.2*	2-oxoglutarate-dependent dioxygenase	3.9	22.5		
BG627176	*Solyc03g114890.3*	COBRA-like protein	4.3	19.9		
AJ133600	*Solyc01g006390.2*	Extensin-like protein	11.6	19.5	No phenotype	Susceptible
BT014403	*Solyc10g055760.2*	NAC domain protein NAC6	4.5	17.4		
BT013533	*Solyc06g008620.1*	tolB protein-like protein	2.3	16.8		
M69247	*Solyc09g007010.1*	Pathogenesis-related protein 1	1.8	16.2		
AW033860	*Solyc08g066310.2*	Receptor-like protein kinase	2.6	15.6		
X79337	*Solyc05g007950.3*	LERNALE L.esculentum ribonuclease le	10.6	15.5		

[a] Part of the upregulated genes from the 122 upregulated genes from Balaji et al. (2008). The genes chosen in this table are more than 15 times differentially regulated in cv Rio Grande inoculated with Cmm. Genes are in descending order of fold ratio at 8 dpi.
[b] Annotated genes from the Sol Genomics Network ITAG 3.2.
[c] Genes tested for virus-induced gene silencing (VIGS) assay on *Nicotiana benthamiana*, pleiotropic effect observed (if any), and wilt symptom after challenged with Cmm (Balaji et al. 2011).
[d] Overexpression of the gene in cv Mountain Fresh and wilt symptom observed upon Cmm infection (Balaji and Smart 2012).

Over-expression[d]	Remarks	References
	• The Arabidopsis subtilase gene, the *SBT3.3*, may be linked to pathogen recognition and activation of signalling processes	(Ramírez et al. 2013)
	• EPI1 protein of *P. infestans* inhibits and interacts with pathogenesis-related protein P69B subtilase	(Tian et al. 2005)
	• Overexpression of the Arabidopsis CATIONIC AMINO ACID TRANSPORTER1 (CAT1) improved disease resistance against *P. syringae*	(Yang et al. 2014a)
	• Common jasmonic acid (JA) marker	(Li 2002; Ataide et al. 2016)
	• Regulating the orientation of cell expansion. • Arabidopsis *COBRA* mutants have reduced level of crystalline cellulose microfibils which suggested a role of the gene in crystallization or cellulose deposition.	(Schindelman et al. 2001)
Tolerance		
	• Positive regulator of penetration resistance. • Overexpression of the gene in barley leads to resistance towards powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (Bgh)	(Jensen et al. 2007)
	• Marker gene for the systemic acquired resistance (SAR) response. • Expression of PR1 is salicylic acid (SA) responsive	(López-Gresa et al. 2016)
	• Large superfamily of proteins with similar structure. Involved in broad array of plants responses which include resistance to pathogens	(Goff and Ramonell 2007)

The future of Cmm tolerance

The quarantine status of the pathogen in Europe does not make it easy to screen big populations of tomato plants for resistance/tolerance against Cmm. Furthermore, the tight regulations on Cmm in the Netherlands are necessary but hinder the research on tolerance and the tolerance mechanism of tomato against Cmm. Different strategies have to be executed to by-pass this challenge. New protocols using *in vitro* plantlets as described in **Chapter 2** help with the screening process. It reduces the space and cost to accommodate big populations and also the time period to monitor the symptoms. This application has helped with our fine mapping process in **Chapter 3**. However, the relation between results of *in vitro* and greenhouse studies depends on the tomato genotypes. A confirmation of the wilt symptoms of the *in vitro* technique with the greenhouse or field data should be done. The two parental genotypes that we were using for our breeding program exhibited similar disease symptoms *in vitro* and greenhouse assay. In our ongoing study, a modification of this protocol will be used to screen a large number of *in vitro* tomato seedlings with different *Pseudomonas* consortia against Cmm (**Chapter 5**). This helps us to identify the best combination of different *Pseudomonas* strains to fight Cmm using minimal space.

The use of next-generation sequencing enabled us to partly elucidate the mystery behind the tolerance against Cmm. From a genomic perspective, many tomato genotypes have been resequenced or *de novo* sequenced (Víquez-Zamora et al. 2013; Aflitos et al. 2014). This included the parental genotypes that we have been using for our study (cv Moneymaker and *S. arcanum* LA2157). The available information expedited marker development for the fine mapping process. With the use of the RNA-sequencing method, we can look at the genes that play a role in tolerance. As discussed earlier, differential gene expression between cv. Moneymaker and the NIL can be an approach to see how the genes in the fine mapped region (211 Kb) behave. Using our NIL collection, we could delimit the size by looking at the differential gene expression of the NIL by comparing the transcriptomic data from cv. Moneymaker and the NIL. This shows also whether the not annotated genes in the region might play a role. In parallel to this work, the genes that are upregulated or unique in the susceptible genotypes could be potential candidate *S*-genes. Testing these genes by doing VIGS assays, followed by RNAi approaches or Crispr/Cas9 genome editing strategies might be interesting to see if there are novel *S*-genes that can be used against Cmm. Using the RNA-seq technology to find new candidate *S*-genes to fight Cmm is currently ongoing in our group.

Take-home messages

The tolerance against Cmm has good sides: the tolerance is good for the tomato to combat Cmm and for Cmm to grow and multiply. Without pathogenicity Cmm is just a bacterium like so many other naturally harmless bacteria. The problem is that worldwide all tomato cultivars are sensitive to Cmm. In the dynamic world of seed companies, it might be possible to introduce more and more tolerant varieties. But it is not yet unravelled what the best and most direct method to make tolerant or even resistant varieties. The strict regulations on Cmm slow down the research progress and testing in field conditions must be done in countries where it is allowed. Our newly developed disease screening will help in research. With our fine mapping work, we reduced the region responsible for resistance/tolerance conferred by *S. arcanum* LA2157, but we still do not know what the genes are responsible for it. Whether this is needed for the development of tolerant cultivars is questionable. The use of the next-generation sequencing could help with speeding up answering the mechanism behind the tolerance and whether the tolerance level can be optimised. Maybe it is needed to optimise the tolerance level by introducing susceptibility genes in tomato. This will make breeding more complicated because *S*-genes are recessive. If we do not have resistant cultivars we should also investigate other ways of prevention. We have tested two alternative methods, but there are more ways that could be used to prevent the spread of Cmm. In the future we hope to understand the interaction between tomato and Cmm better and that this will allow us to make truly resistant varieties.

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Summary

Clavibacter michiganensis subsp. *michiganensis* (Cmm) is one of the most important bacterial pathogens affecting tomatoes. It is a quarantine organism in Europe and other countries. The pathogen can infect tomatoes through multiple ways and can be transmitted via seeds. The quarantine status and the devastating disease symptoms caused by Cmm are problematic for many parties. Even though resistance/tolerance breeding is one of the most important and successful ways to manage diseases, other paths can also be walked to manage disease symptoms. A combination of several methods might successfully limit the disease symptoms even more. Our goal is to get symptomless tomato plants with minimal bacterial density. In the present thesis, we developed a new and an efficient way to screen big tomato populations *in vitro*. Furthermore, we also studied different approaches to get symptomless tomato plants after infection with Cmm.

Chapter 1 presents an introduction about the pathogen, the pathogen-host interaction and the different techniques that can be used to manage Cmm. In **Chapter 2**, we look at the development of a new disease screening method on *in vitro* tomato seedlings. This system is efficient and cost effective to screen big populations in one experiment. Two different inoculation methods were tested in combination with two different plant ages. Based on the inoculation procedure that gave uniform wilting symptoms, we proceeded to test this protocol on several wild relatives of tomato. The disease scoring of the tomato wild relatives was compared to the scoring on the same accessions in the greenhouse. The majority produced similar symptom scores. This new protocol speeds up the disease screening process of large populations using minimal space. In **Chapter 3** we used the *in vitro* disease screening protocol to fine map the QTL on chromosome 7. The marker analyses were done with Single Nucleotide Polymorphisms (SNPs) and the KASP platform (KBiosciences, UK). To predict the gene variants in the fine mapped region of the highly tolerant wild source *Solanum arcanum* LA2157, we used an in-house prediction tool, the HaploSmasher. The QTL region has been fine mapped to 211 Kb which resulted in 15 annotated genes. The results are the first step to understand a tolerance mechanism against Cmm. In **Chapter 4** several susceptibility (*S*) genes were used to test effectiveness against Cmm. Susceptibility genes are plant genes, whose impairment leads to durable, broad-spectrum resistance. No resistance (*R*) genes have been found against Cmm, thus exploiting the *S*-genes could be an alternative approach to fight the pathogen. Four candidate *S*-genes, *CESA3*, *CESA4*, *PMR6* and *WAT1*, were tested using the virus-induced gene silencing (VIGS) assay to see the effects on the infection of Cmm. From the VIGS assay, we found *WAT1* confers higher tolerance than the control plants. This finding could be a starting point of applying *S*-genes in tolerance breeding

against Cmm. In **Chapter 5** alternative approaches were applied to reduce Cmm disease symptoms by applying grafting and utilizing biocontrol agents. Reciprocal and self-grafting were used with a highly tolerant genotype (*S. arcanum* LA2157) and a susceptible genotype (cv. Moneymaker). For the biocontrol agent experiment, *Pseudomonas* spp. consortia were applied to enhance the tolerance mechanism in different tomato genotypes. Different Cmm inoculation methods were tested to see the effectiveness of grafting and biocontrol agent against the pathogen from different entries. From these two methods, we found reduced wilting symptoms only when the pathogen enters from the roots. Finally, **Chapter 6** presents the general discussion of the thesis. In conclusion, we developed a new protocol that could ease disease screening using minimal space and time, fine mapped the QTL on chromosome 7 to a smaller region, found a potential new tolerance against Cmm by manipulating the *WAT1* gene and tested two alternative approaches to minimize wilting symptom.

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Thank you! Bedankt! Danke! Terima kasih!

-Mas-

About the author

Mas Muniroh binti Mohd Nadzir (Mas) was born on the 28th of May 1985 in Kuala Lumpur, Malaysia. After finishing her high school, she received a scholarship from the Malaysian government to do a bachelor in Biotechnology at the University of California, Davis (UCD) and graduated in 2004. She continued her MSc in Agrotechnology at the Universiti Putra Malaysia (UPM) in which Dr Adriaan W. van Heusden (Sjaak) was one of her supervisors. The (fun) encounter she had with Sjaak brought her to continue her PhD with him together with the bacteria expert, Dr Jan van der Wolf. She started her PhD program in Plant Breeding (PBR) at Wageningen University and Research in June 2014 where she worked with a "challenging" pathogen Cmm. She enjoyed working on her PhD project while socialising with her PBR colleagues and friends (and baking lots of cakes) ever since.

Education Statement of the Graduate School Experimental Plant Sciences

Issued to:	**Mas Muniroh binti Mohd Nadzir**
Date:	**14 December 2018**
Group:	**Laboratory of Plant Breeding**
University:	**Wageningen University & Research**

The Graduate School EXPERIMENTAL PLANT SCIENCES

1) Start-Up Phase	*date*
► **First presentation of your project**	
Proposal presentation at Breeding for Resistance Group	17 Feb 2015
► **Writing or rewriting a project proposal**	
Resistance to bacterial canker in tomato	28 Nov 2014
► **Writing a review or book chapter**	
► **MSc courses**	
Advanced Statistics (MAT-20306)	01 Sep - 20 Oct 2014
Genomics (ABG-30306)	27 Oct - 20 Dec 2015
► **Laboratory use of isotopes**	
Subtotal Start-Up Phase	*13.5 **

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD students days 'Get2Gether', Soest, the Netherlands	29-30 Jan 2015
EPS PhD students days 'Get2Gether', Soest, the Netherlands	09-10 Feb 2017
► **EPS theme symposia**	
EPS Theme 2 Symposium 'Interactions between plants and biotic agents' & Willie Commelin Scholten Day, Utrecht, the Netherlands	20 Feb 2015
EPS Theme 2 Symposium 'Interactions between plants and biotic agents' & Willie Commelin Scholten Day, Leiden, the Netherlands	22 Jan 2016
EPS Theme 2 Symposium 'Interactions between plants and biotic agents' & Willie Commelin Scholten Day, Wageningen, the Netherlands	23 Jan 2017
► **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	10-11 Apr 2017
Solanaceae Symposium 'Solanaceae genetic resources in research and breeding', Nijmegen, the Netherlands	26 Apr 2017
► **Seminars (series), workshops and symposia**	
Symposium: Plant Breeding - Research Day, Wageningen, the Netherlands	24 Sep 2014
Symposium: Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding, Wageningen, the Netherlands	11 Dec 2014

Symposium: 2nd Wageningen PhD Symposium 'Connecting Ideas, Combining Forces' , Wageningen, the Netherlands	06 May 2015
Symposium: Plant Breeding - Research Day, Wageningen, the Netherlands	29 Sep 2015
Symposium: The Wageningen Plant Microbiome Network Kick-off meeting, Wageningen, the Netherlands	29 Jun 2016
Mini-symposium: Rewriting our genes?, Wageningen, the Netherlands	30 Sep 2016
Seminar: Dr. Hideki Takahashi - 'Intron-mediated enhancement of disease resistance gene expression in *Arabidopsis thaliana*'	10 Mar 2015
Seminar: Dr. Siobhan Brady - 'Regulation of root morphogenesis in tomato species in the face of a changing environment'	09 Sep 2015
Seminar: Dr. Jane Parker - 'Plant intracellular immunity: evolutionary and molecular underpinnings'	21 Jan 2016
Seminar: Dr. Asaf Levy - 'Bacteria and the future of agriculture: from sequence to function'	22 Feb 2018
Seminar: Dr. Margaret Frank - 'Grafting-induced vigor in crops: new approaches to understanding an ancient practice'	19 Jun 2018
Seminar: Dr. Mary C. Wildermuth - 'Salicylic acid and cell cycle control of plant-microbe interactions'	25 Jun 2018
► **Seminar plus**	
► **International symposia and congresses**	
12th Solanaceae Conference, Bordeaux, France	25-29 Oct 2015
5th International Symposium on Tomato Diseases, Málaga, Spain	13-16 Jun 2016
20th Eucarpia General Congress, Zürich, Switzerland	29 Aug - 01 Sep 2016
14th Solanaceae and 3rd Cucurbitaceae Joint Conference, Valencia, Spain	03-06 Sep 2017
19th Eucarpia Meeting of the Tomato Working Group, Naples, Italy	02-04 May 2018
► **Presentations**	
Poster: 12th Solanaceae Conference, Bordeaux, France	25-29 Oct 2015
Poster: 5th International Symposium on Tomato Diseases, Málaga, Spain	13-16 Jun 2016
Poster: 20th Eucarpia General Congress, Zürich, Switzerland	29 Aug - 01 Sep 2016
Poster: 14th Solanaceae and 3rd Cucurbitaceae Joint Conference, Valencia, Spain	03-06 Sep 2017
Talk: 19th Eucarpia Meeting of the Tomato Working Group, Naples, Italy	02-04 May 2018
► **IAB interview**	
► **Excursions**	
Visit to the company Keygene, Wageningen, the Netherlands	12 Oct 2017
Visit to Flower Trials 2018 and to the company Dümmen Orange, De Lier, the Netherlands	15 Jun 2018

Subtotal Scientific Exposure *17.6 **

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Postgraduate course 'Genome Assembly', Wageningen, the Netherlands	28-29 Apr 2015
Postgraduate course 'Basic Statistics', Wageningen, the Netherlands	20-27 May 2015
Postgraduate course 'Transcription Factors and Transcriptional Regulation', Wageningen, the Netherlands	12-14 Dec 2016
Postgraduate course 'Introduction to R for Statistical Analysis', Wageningen, the Netherlands	17-18 May 2018
Postgraduate course 'The Power of RNA-Seq', Wageningen, the Netherlands	11-13 Jun 2018
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	*4.6 **

4) Personal Development	date
► **Skill training courses**	
EPS Introduction Course, Wageningen, the Netherlands	20 Jan 2015
PhD Competence Assesment, Wageningen, the Netherlands	03 & 18 Feb 2015
Course 'Project and Time Management', Wageningen, the Netherlands	Mar - May 2015
Course 'Information Literacy PhD including EndNote Introduction', Wageningen, the Netherlands	15-16 Apr 2015
Wageningen Graduate Schools PhD Workshop Carousel, Wageningen, the Netherlands	17 Apr 2015
Course 'Presenting with Impact', Wageningen, the Netherlands	May - Jun 2015
Wageningen Graduate Schools PhD Workshop Carousel, Wageningen, the Netherlands	18 Apr 2016
Course 'Scientific Writing', Wageningen, the Netherlands	Jan - Mar 2017
Course 'Scientific Artwork - Vector graphics and images', Wageningen, the Netherlands	02-03 Oct 2017
Workshop 'Last Stretch of the PhD Programme', Wageningen, the Netherlands	22 Sep 2017
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.6 **

TOTAL NUMBER OF CREDIT POINTS	***42.3 ****

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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