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Differential contribution of *Clavibacter michiganensis* ssp. *michiganensis* virulence factors to systemic and local infection in tomato

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SUMMARY

Clavibacter michiganensis ssp. *michiganensis* (*Cmm*) causes substantial economic losses in tomato production worldwide. The disease symptoms observed in plants infected systemically by *Cmm* are wilting and canker on the stem, whereas blister-like spots develop in locally infected leaves. A wide repertoire of serine proteases and cell wall-degrading enzymes has been implicated in the development of wilt and canker symptoms. However, virulence factors involved in the formation of blister-like spots, which play an important role in *Cmm* secondary spread in tomato nurseries, are largely unknown. Here, we demonstrate that *Cmm* virulence factors play different roles during blister formation relative to wilting. Inoculation with a green fluorescent protein (GFP)-labelled *Cmm*382 indicates that penetration occurs mainly through trichomes. When spray inoculated on tomato leaves, the wild-type *Cmm*382 and *Cmm*100 (lacking plasmids pCM1 and pCM2) strains form blister-like spots on leaves, whereas *Cmm*27 (lacking the *chp*/*tomA* pathogenicity island) is non-pathogenic, indicating that plasmid-borne genes, which have a crucial role in wilting, are not required for blister formation. Conversely, mutations in chromosomal 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*) significantly affect disease incidence and the severity of blister formation. The transcript levels of these genes, as measured by quantitative reverse transcription-polymerase chain reaction, showed that, during blister formation, they are expressed early at 8–16 h after inoculation, whereas, during wilting, they are expressed after 24–72 h or expressed at low levels. Plant gene expression studies suggest that *chpC* is involved in the suppression of host defence.

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Keywords: blister-like spots, cell wall-degrading enzymes, host defence, pathogenicity island, serine proteases.

INTRODUCTION

Bacterial wilt/canker of tomato, caused by the Gram-positive actinomycete *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*), is one of the most devastating bacterial diseases of tomato worldwide (Davis *et al.*, 1984; Eichenlaub and Gartemann, 2011; Strider, 1969). The pathogen causes substantial economic losses by reducing the quantity and quality of the tomato yield (Eichenlaub and Gartemann, 2011; Gartemann *et al.*, 2003; Sen *et al.*, 2015). Infested seeds, contaminated tomato debris, symptomless tomato transplants or leaf surface populations on alternative hosts are common sources of primary *Cmm* inoculum (Chang *et al.*, 1991; Fatmi and Schaad, 2002; Gleason *et al.*, 1991). Disease symptoms on tomato plants vary depending on the type of infection (systemic vs. localized), cultivar susceptibility, host age at infection and nutritional status of the host (Gleason *et al.*, 1993; Sharabani *et al.*, 2013b). In systemic infection, which results from infected seeds or wounds, *Cmm* invades vascular tissues, causing unilateral wilting of leaves and leaflets, followed by wilting of the whole plant, necrosis and cankers on the stems and petioles (de León *et al.*, 2011). Localized infection in aerial parts of the plant causes marginal necrosis of leaflets, bird's-eye spots on fruit and small, white, blister-like spots on leaves or stems (de León *et al.*, 2011; Medina-Mora *et al.*, 2001). *Cmm*, in this case, penetrates the host plant through natural openings, such as stomata and hydathodes, and through broken trichomes (Carlton *et al.*, 1998; Medina-Mora *et al.*, 2001; Strider, 1969).

Diverse plant-pathogenic bacteria can survive and even multiply to various degrees as epiphytes prior to disease initiation under favourable conditions (Hirano and Upper, 1983). *Cmm* has the ability to proliferate epiphytically on leaf surfaces. Thus, epiphytic inoculum may disperse the disease to nearby healthy plants by splashing rain, overhead irrigation or spraying of chemicals

during routine practices in nurseries and glasshouses (Carlton *et al.*, 1998; Chang *et al.*, 1991; Strider, 1969). The entry into leaf tissues is probably a critical first step in the establishment of foliar infection. The significance of hydathodes in the development of marginal leaf necrosis and secondary spread of the pathogen has been documented (Carlton *et al.*, 1998; Sharabani *et al.*, 2013a). Foliar trichomes of tomato are additional favourable sites for the penetration of *Cmm* (Layne, 1967). The susceptibility of leaves is generally correlated with the density of trichomes, and young leaves with the greatest hair density are more vulnerable to disease development than are older leaves (Goto, 2012). Localized leaf symptoms in tomato are often visible within 5–7 days and are characterized by marginal necrosis or discoloured blister-like spots caused by the penetration of *Cmm* through hydathodes or trichomes/stomata, respectively. The importance of leaf surface colonization in the secondary spread of *Cmm* in nurseries has been illustrated recently (Frenkel *et al.*, 2016).

Molecular determinants involved in *Cmm* virulence on tomato have been identified in recent years (Eichenlaub and Gartemann, 2011; Gartemann *et al.*, 2008). The *Cmm* strain NCPPB382 (*Cmm*382) harbours two circular conjugative plasmids, pCM1 and pCM2 (Meletzus and Eichenlaub, 1991; Meletzus *et al.*, 1993), which are required for full induction of wilt in infected tomato plants. pCM1 carries the virulence factor CelA, a secreted cellulase with endo-β-1,4-glucanase activity (Jahr *et al.*, 2000), and pCM2 carries Pat-1, a putative serine protease (Dreier *et al.*, 1997). Loss of either pCM1 or pCM2 results in reduced virulence, whereas curing of both plasmids results in a non-virulent strain (strain *Cmm*100) that retains the ability to grow as an endophyte similar to the wild-type (Gartemann *et al.*, 2003; Meletzus *et al.*, 1993). *Cmm*382 contains a chromosomal pathogenicity island (PAI) of 129 kb, referred to as the *chp/tomA* region, which displays a lower G + C content (65.5%) than the rest of the genome. A *Cmm*382 derivative with a deletion of the complete PAI region, but carrying both plasmids (strain *Cmm*27), is impaired in the ability to induce disease symptoms and to colonize xylem vessels (Chalupowicz *et al.*, 2012).

The presence of different gene families encoding multiple serine proteases is a dominant feature of the genome of *Cmm*382 (Gartemann *et al.*, 2008). The first group (10 members), which includes the virulence gene *pat-1*, was designated as the Chp family. Three members of this family (*pat-1*, *phpA* and *phpB*) are located on the pCM2 plasmid, whereas the other seven are clustered in the *chp/tomA* PAI. Among the members of this family, *chpC* has been shown to be required for *Cmm* colonization (Stork *et al.*, 2008). Genes encoding chymotrypsin-related serine proteases (11 members) represent a second group of virulence factors, which were designated as the Ppa family (Gartemann *et al.*, 2008). With the exception of *ppaJ*, which is located on the pCM1 plasmid, the other family members are chromosomal. Six of them (*ppaA* to *ppaE*) are located in the *chp* region of the *chp/tomA* PAI and the rest (*ppaF* to *ppaI*) in two different chromosomal loci. The third group is the three-member family of subtilase proteases: one gene (*sbtA*) is located on the *chp* region of the PAI, whereas the other two genes (*sbtB* and *sbtC*) are present elsewhere on the chromosome. Most of these serine proteases are secreted at early stages of the infection and are required for effective movement of the pathogen and for the formation of bacterial aggregates in the tomato xylem (Chalupowicz *et al.*, 2012; Savidor *et al.*, 2011).

A variety of cell wall-degrading enzymes (i.e. cellulase, xylanase, pectate lyase and polygalacturonase) are also secreted by *Cmm*. A gene homologous to the plasmid-borne *celA*, but lacking the α-expansin-like domain, is present on the chromosome. Canker and tissue maceration have been hypothesized to result from the active degradation of different components of the plant cell wall at late stages of infection (Gartemann *et al.*, 2003), although some genes encoding cell wall-degrading enzymes have been found to be induced at early stages of the disease (Chalupowicz *et al.*, 2010). Finally, a recent transcriptomic analysis identified two transcription factors (Vatr1 and Vatr2) with key contributions to *Cmm* virulence in tomato (Savidor *et al.*, 2014).

It has been speculated that endophytic colonization by *Cmm* is facilitated by the suppression of plant defences (Chalupowicz *et al.*, 2010). This premise was inferred from the observation that tomato plants infected with *Cmm*27 showed a significant increase in the relative expression of two genes encoding pathogenesis-related (PR) proteins (chitinase II and PR5 isoform), when compared with plants infected with *Cmm*382 or *Cmm*100 containing the intact *chp/tomA* PAI (Chalupowicz *et al.*, 2010). Thus, serine proteases or other genes residing on this PAI could function directly or indirectly as suppressors of plant defences. This possibility has not been studied previously using specific gene mutations and is addressed in the present study.

Most of the molecular studies on *Cmm* virulence have been carried out with systemic infection leading to wilt and canker symptoms. In contrast, the molecular mechanisms mediating blister formation on leaves remain unknown. In the present study, we show that pCM1 and pCM2 plasmids, which are critical for systemic infection and wilting symptoms, are not required for the localized formation of blister-like spots. We further demonstrate that serine proteases and cell wall-degrading enzymes are expressed differentially during the development of blisters when compared with wilting symptoms, and *chpC*, a serine protease residing on the PAI, is involved in the suppression of host defence.

RESULTS

Blister formation on tomato leaves by *Cmm*

To mimic the secondary spread of *Cmm* in nurseries, the aerial parts of tomato plants were spray inoculated with a suspension

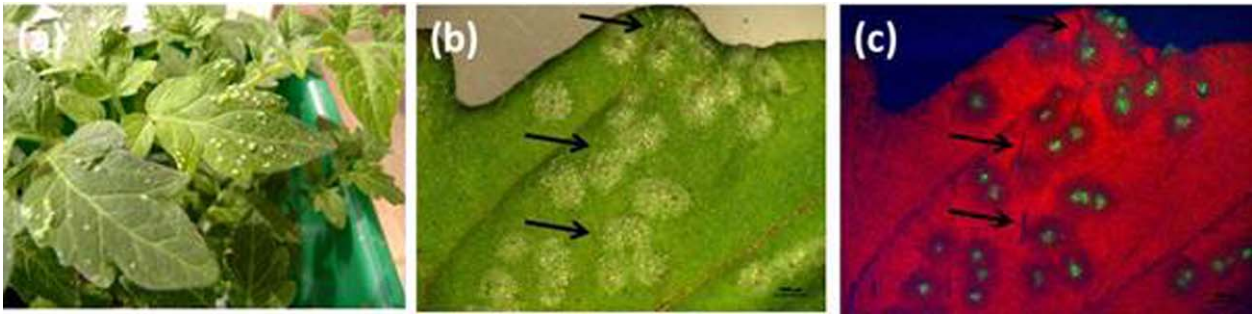

Fig. 1 Blisters formed by *Cmm*382 on tomato leaves. The foliage of tomato seedlings was spray inoculated with bacterial suspension at a titre of 10^8 cells/mL. (a) Tomato leaflets with blisters at 7 days post-inoculation. Close-up images (magnification, ×5) of a leaflet inoculated with green fluorescent protein (GFP)-labelled *Cmm*382 visualized by light (b) and fluorescent binocular (c) microscopy. Arrows point to trichomes. *Cmm*, *Clavibacter michiganensis* ssp. *michiganensis*.

(10^8 cells/mL) of *Cmm*382 and monitored for the appearance of disease symptoms. Blister-like lesions were visible on infected leaves at 7 days post-inoculation (dpi) (Fig. 1a). The lesions ranged in size from 0.5 to 3 mm in diameter, depending on the density with which they developed on the adaxial surface of the leaflets. The lesions were white, circular, with a raised pimple appearance (Fig. 1a,b). Images of leaves infected with green fluorescent protein (GFP)-labelled bacteria, visualized with a fluorescent binocular microscope, revealed extensive multiplication and aggregates of the pathogen at the centre of the blisters (Fig. 1c). Generally, blisters developed near trichomes that were probably damaged during spray inoculation of the leaves with the pathogen (Fig. 1b,c).

To determine the genetic determinants of *Cmm*382 that might affect blister formation, two mutants, *Cmm*100 (lacking the plasmids pCM1 and pCM2) and *Cmm*27 (lacking the *chp/tomA* PAI), were spray inoculated onto tomato leaves. *Cmm*100 caused blisters similar to the wild-type *Cmm*382, whereas *Cmm*27 did not cause any symptoms (Fig. 2). When these plants were examined at 30 dpi, 80% of the plants infected with *Cmm*382 had developed wilting symptoms or died, whereas the plants infected with *Cmm*100 or *Cmm*27 were asymptomatic.

The determination of endophytic populations of *Cmm* mutants in leaves revealed that, 3 days after spray inoculation, *Cmm*100 colonized the leaves at a titre of *c.* 10^8 colony-forming units (CFU)/g, whereas *Cmm*27 reached a titre of only 10^4 CFU/g (Fig. S1a, see Supporting Information). Examination of the movement of the mutants from the leaves to the stem revealed that the *Cmm*100 population in the stem at 7 dpi was similar to that of the wild-type (*c.* 10^5 CFU/g), whereas *Cmm*27 could not be detected in the stems even after 21 dpi (Fig. S1b).

Contribution of virulence genes to the development of blister and wilt symptoms

Several *Cmm*382 mutants of candidate virulence genes were tested for their effect on blister formation compared with wilting. The genes were selected based on known or suspected involvement in *Cmm* virulence (Table 1). These included genes encoding serine proteases from three different families located on the *chp/tomA* PAI (*chpC*, *chpG*, *chpE*, *chpF*, *ppaA*, *ppaC*, *sbtA*), serine proteases located on the chromosome (*sbtB* and *sbtC*) and genes encoding cell wall-degrading enzymes (*xysA* and *xysB* encoding xylanases, *celB* encoding a cellulase, *pgaA* encoding a polygalacturonase and *endX/Y* encoding two endoglucanases). Additional genes examined included: *gmdA* and *manB* (involved in exopolysaccharide synthesis), *vatr2* (encoding a transcriptional regulator), *perF* (encoding a protein possibly involved in cytolysis processes, CMM_2382) and *srtA* (encoding a putative sortase enzyme, CMM_0013, that anchors surface proteins) (Table 1). Tomato seedlings were either spray or puncture inoculated with wild-type *Cmm* and its mutants, monitored for the appearance of blisters and wilting, and the data obtained were used to calculate disease incidence and severity, as described in Experimental procedures. In general, all the mutants lowered the incidence and severity of blister formation compared with *Cmm*382 and *Cmm*100 (Fig. 3a). However, the highest reduction in disease incidence and severity was observed for mutants in seven genes: *chpC*, *sbtA*, *pgaA*, *endX/Y*, *vatr2*, *perF* and *srtA*. The *chpC* mutant produced few blisters. The rates of disease incidence of plants inoculated with the mutants in *sbtA* and *endX/Y* were 20% and 10%, respectively, with low severities of 15% and 6%, respectively (Fig. 3a). Mutations in *pgaA*, *vatr2*, *perF* and *srtA* reduced the disease incidence to 8%, 8%, 15% and 10%, respectively, and showed only low disease severity.

When the mutants were tested for their ability to cause wilting, only *chpC*, *sbtA* and *vatr2* reduced disease symptoms significantly (Fig. 3b). The disease incidence in plants inoculated with the *chpC* and *vatr2* mutants was 5% and displayed low disease severity, and that of *sbtA* was 25% with low disease severity of 20%. Disease incidence rates in plants inoculated with *pgaA* and *endX/Y* mutants were 90% and 93% for wilting, relative to 8% and 10% for blister formation, respectively (Fig. 3b).

Fig. 2 Blister development on tomato seedlings at 7 days after spray inoculation with *Cmm*382 (wild-type), *Cmm*100 (lacking plasmids pCM1 and pCM2) and *Cmm*27 (with a deletion of the *chp/tomA* pathogenicity island). The foliage of tomato seedlings was spray inoculated with bacterial suspension at a titre of 10^8 cells/mL. *Cmm*, *Clavibacter michiganensis* ssp. *michiganensis*.

Differential expression of putative virulence genes during blister formation compared with wilting

To further strengthen the results obtained with the different *Cmm*382 mutants, we measured the expression levels of chromosomal genes (*chpC*, *sbtA*, *pgaA*, *endX/Y*, *vatr2*, *perF* and *srtA*) that significantly affected blister formation (Fig. 3a) and plasmid-borne genes (*celA* and *pat-1*) crucial for wilting in the wild-type. Following spray or puncture inoculation, total RNA was extracted from *Cmm*382-infected leaves or stems, respectively, at 0, 8, 16, 24, 48 and 72 h post-inoculation (hpi), and the expression level was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The results presented in Figs 2 and 3a show that blister formation caused by the *Cmm*100 mutant was similar to that of the wild-type, whereas wilting was completely impaired (Fig. 3b and Chalupowicz *et al.*, 2010). These results suggest that *celA* and *pat-1*, two virulence determinants residing on pCM1 and pCM2, respectively, might not be expressed during blister formation. Measurement of the expression level of these genes in the wild-type, when spray inoculated, indeed corroborated this assumption (Fig. 4a), suggesting that, at the early stages of infection, these virulence factors are not required for blister formation.

Inactivation of *chpC*, *sbtA* and *vatr2* reduced both blister formation and wilting (Fig. 3). However, these genes displayed different expression patterns following leaf or stem inoculation. *chpC*, *sbtA* and *vatr2* transcripts could be measured as early as 8–16 h in *Cmm*-treated leaves, whereas, in infected stems, *chpC* and *vatr2* transcripts accumulated later, after 72 h and 24 h, respectively, and that of *sbtA* remained unchanged (Fig. 4b).

Inactivation of *pgaA*, *endX/Y*, *perF* and *srtA* genes significantly reduced blister formation, but not wilting (Fig. 3). The expression levels of these genes in the wild-type following spray inoculation were highly induced early (at 8–16 h), whereas these genes were barely expressed following puncture inoculation (Fig. 4c). The relative expression of these genes was further examined following spray inoculation with the *chpC* mutant. The transcript levels of *pgaA* and *endX/Y* were reduced by 7.5- and 4.5-fold in the *chpC* mutant compared with the wild-type at 16 h and 24 hpi, respectively. The expression levels of *perF* and *srtA* in the *chpC* mutant were similar to those of the wild-type (results not shown).

Expression of plant defence-related genes in response to infection by *Cmm*382 and *chpC* mutant

The serine protease ChpC was found to be crucial for the development of both blister formation and wilting (Fig. 3). It has been suggested previously that PAI-located genes are involved in the suppression of tomato basal defences (Chalupowicz *et al.*, 2010). To further support this premise, the expression levels of different putative defence-related genes were measured after leaf and stem inoculation of tomato plants with the wild-type and *chpC* mutant. The plant genes were selected based on proteomic analysis of *Cmm*-inoculated tomato plants (Savidor *et al.*, 2011). Of the 10 genes measured (Fig. 5), three [*ACO1* (1-aminocyclopropane-1-carboxylic acid (ACC) oxidase 1), *PR4* (pathogenesis-related protein 4) and *AMSP* (auxin-mediated signal pathway)] were induced significantly during blister formation, when leaves were infected with the *chpC* mutant relative to the wild-type (Fig. 5a). The increases in transcript levels of *ACO1*, *PR4* and *AMSP* were four-, three- and nine-fold, respectively. The transcript level of *PR5*-Iso (pathogenesis-related protein 5 isoform) was reduced by 1.6-fold compared with the wild-type. During wilting, the transcript levels of six genes were increased in plants inoculated with the *chpC* mutant compared with the wild-type (Fig. 5b). Thus, the relative expression levels of *ACO1*, *PR3* (pathogenesis-related protein 3), *PR4*, *PR5*-Iso, *PR7* (pathogenesis-related protein 7) and *SBT* (subtilase) were 3.6-, two-, 1.6-, 1.7-, nine- and six-fold higher, respectively, in plants inoculated with the *chpC* mutant compared with *Cmm*382. *AMSP* was not expressed during wilting (Fig. 5b). In

Table 1 Mutants of *Clavibacter michiganensis* ssp. *michiganensis* strain NCPPB382 used in this study.

Strain/mutant	Relevant characteristics	Gene location	Reference or source
*Cmm*382	NCPPB382, wild-type		NCPPB
GFP-labelled *Cmm*382	NCPPB382 transformed with pK2-22 harbouring the *gfp* gene		Chalupowicz *et al.* (2012)
*Cmm*100	Lacking plasmids pCM1 and pCM2		Meletzus and Eichenlaub (1991)
*Cmm*27	Lacking the pathogenicity island (PAI) (*chp/tom*)		Chalupowicz *et al.* (2010)
chpC$^-$	Chl^r, marker exchange mutant of *chpC*, encoding a serine protease of the Chp family	*chp/tomA* PAI	Stork et al. (2008)
chpG$^-$	Chl^r, marker exchange mutant of *chpG*, encoding a serine protease of the Chp family	*chp/tomA* PAI	Stork et al. (2008)
chpE$^-$	Chl^r, marker exchange mutant of *chpE*, encoding a serine protease of the Chp family	*chp/tomA* PAI	Sessa laboratory
chpF$^-$	Chl^r, marker exchange mutant of *chpF*, encoding a serine protease of the Chp family	*chp/tomA* PAI	Sessa laboratory
ppaA$^-$	Chl^r, marker exchange mutant of *ppaA*, encoding a serine protease of the Ppa family	*chp/tomA* PAI	Eichenlaub laboratory
ppaC$^-$	Chl^r, marker exchange mutant of *ppaC*, encoding a serine protease of the Ppa family	*chp/tomA* PAI	Eichenlaub laboratory
sbtA$^-$	Chl^r, marker exchange mutant of *sbtA*, encoding a serine protease of the subtilase family	*chp/tomA* PAI	Eichenlaub laboratory
sbtB$^-$	Chl^r, marker exchange mutant of *sbtB*, encoding a serine protease of the subtilase family	Chromosome	Eichenlaub laboratory
sbtC$^-$	Chl^r, marker exchange mutant of *sbtC*, encoding a serine protease of the subtilase family	Chromosome	Eichenlaub laboratory
celB$^-$	Chl^r, marker exchange mutant of *celB*, encoding a cellulase	Chromosome	Eichenlaub laboratory
xysA$^-$	Chl^r, marker exchange mutant of *xysA*, encoding xylanase A	Chromosome	Eichenlaub laboratory
xysB$^-$	Chl^r, marker exchange mutant of *xysB*, encoding xylanase B	Chromosome	Eichenlaub laboratory
endX/Y$^-$	Chl^r, marker exchange mutant of *endX/Y*, an encoding endoglucanase	Chromosome	Eichenlaub laboratory
pgaA$^-$	Chl^r, marker exchange mutant of *pgaA*, encoding polygalacturonase	Chromosome	Eichenlaub laboratory
gmdA$^-$	Chl^r, marker exchange mutant of *gmdA*, involved in exopolysaccharide synthesis	Chromosome	Eichenlaub laboratory
manB$^-$	Chl^r, marker exchange mutant of *manB*, involved in exopolysaccharide synthesis	Chromosome	Eichenlaub laboratory
vatr2$^-$	Chl^r, marker exchange mutant of *vatr2*, encoding a transcriptional regulator	Chromosome	Savidor *et al.* (2014)
perF$^-$	Chl^r, marker exchange mutant of the gene encoding perforin	Chromosome	Eichenlaub laboratory
srtA$^-$	Chl^r, marker exchange mutant of the gene encoding sortase	Chromosome	Sessa laboratory

general, the relative expression of the plant genes was higher during wilting than during blister formation.

DISCUSSION

Clavibacter michiganensis ssp. *michiganensis* can penetrate its host through different routes (de León *et al.*, 2011). When foliar infection occurs, the epiphytic pathogen can enter through natural openings, causing blisters or marginal necrosis. Blisters on tomato leaves are usually observed in nurseries as a result of overhead irrigation and favourable conditions. When unnoticed, this secondary spread of the pathogen may be transferred to the field and growth net-houses (Frenkel *et al.*, 2016). In this study, microscopic observation of blisters formed on tomato leaves by the GFP-labelled *Cmm*382 strain (Fig. 1b,c) indicated that trichomes are one of the entrance sites for infection. Goto (2012) pointed out that *Cmm* commonly enters its host through various morphologically distinct types of trichome that are distributed on the surface of the leaves and stems of tomato plants. Layne (1967) showed that infection occurs primarily on the upper leaf surfaces with younger leaves being more susceptible than older ones. Injuries at the time of inoculation greatly increase the overall incidence and severity of infection (Kontaxis, 1962). The presence of trichomes is not essential for infection by *Cmm*, because it can also invade plants via stomata and wounds (Goto, 2012). Studies by Carlton *et al.* (1998) indicated that *Cmm* can invade the leaf tissue through hydathodes and can be transported into the intercellular space when guttation droplets containing bacterial cells are drawn back into the leaf. It has been demonstrated recently that exudation of *Cmm* in guttation fluid of infected tomato plants constitutes the main source for secondary disease spread in tomato glasshouses (Sharabani *et al.*, 2013a). However, when such infections occur, the common symptom formed on the leaf surface is marginal necrosis of the leaflets rather than blisters (Carlton *et al.*, 1998; de León *et al.*, 2011).

Most of the molecular studies on the interaction of *Cmm* with tomato have been carried out with systemic infection through the stem, roots or seeds. In the present study, we have demonstrated that foliar infection leading to local symptoms requires different bacterial (e.g. *pgaA* and *endX/Y*) and host genes from those required for systemic infection. Thus, the plasmids pCM1 and pCM2, carrying the virulence factors Pat-1 and CelA, respectively, which are instrumental for the development of wilt and canker symptoms (Eichenlaub and Gartemann, 2011), are not required for the initial stages of blister formation (Figs 2 and 3). *Cmm*100, which is considered to be a good colonizer without causing wilting symptoms, formed blisters on leaves comparable with the wild-type (Fig. 3). In addition, it can multiply and move from the leaf surface to the leaflet petiole and the stem at 7 dpi (Fig. S1). This finding implies that the genes responsible for penetration and blister formation reside on the chromosome. The question of which genes are involved can be partially answered by results obtained with *Cmm*27, which lacks the *chp/tomA* PAI. This mutant did not form blisters on the leaves (Figs 2 and 3) and did not move to the stem (Fig. S1). The *chp/tomA* PAI accommodates several serine proteases of three different families (Gartemann *et al.*,

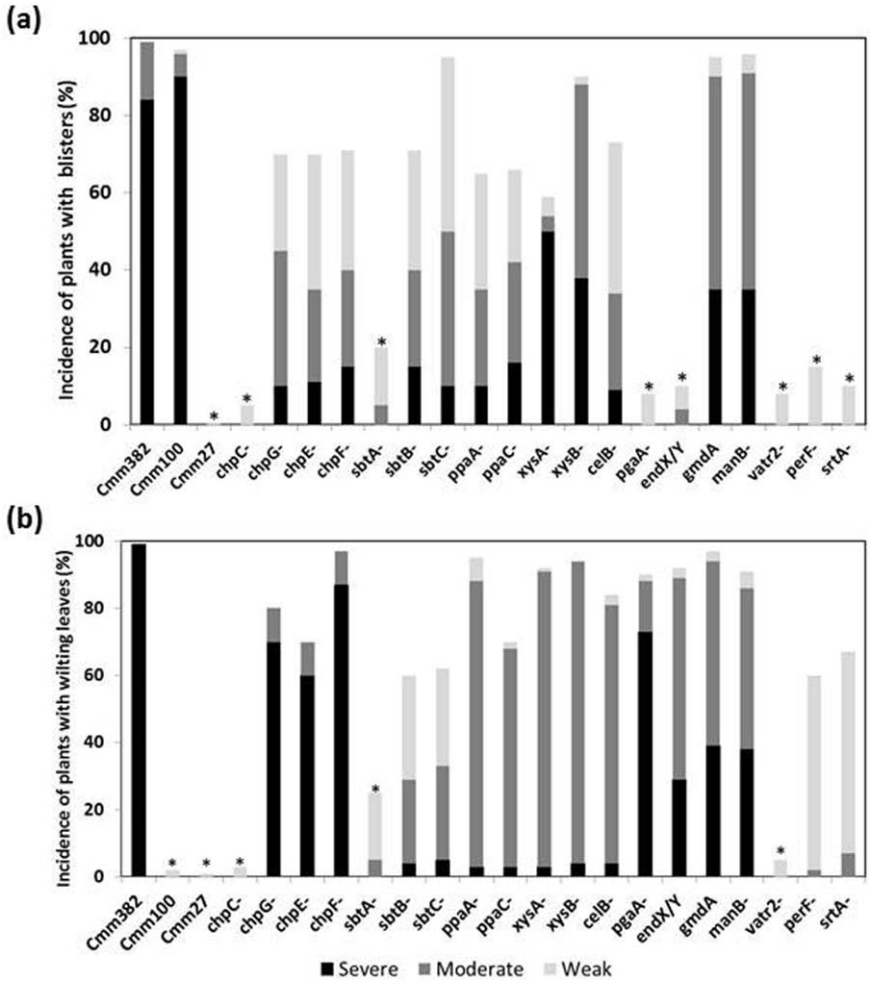

Fig. 3 Disease incidence and severity of *Clavibacter michiganensis* ssp. *michiganensis* mutants on tomato plants. Tomato seedlings were either spray inoculated (a) or puncture inoculated (b) with the wild-type *Cmm*382 and its mutants, and monitored for blister formation or wilting at 7 and 15 days post-inoculation (dpi), respectively. The different mutants are listed in Table 1. The grades for disease severity were as follows: for blister formation: weak (1–10 blisters per plant), moderate (10–50 blisters per plant) and severe (above 50 blisters per plant); for wilting: weak (one wilted leaf per plant), moderate (two to four wilted leaves per plant) and severe (above four wilted leaves per plant or dead plant). *Statistically significant difference ($P < 0.05$) in disease incidence compared with *Cmm*382, according to Student's *t*-test.

2008). When seven serine protease mutants (Table 1) were tested for their ability to form blisters on leaves or wilting, only the mutants in *chpC* and *sbtA* significantly reduced symptom development (Fig. 3). We have demonstrated here that ChpC, which has been shown previously to be important for wilting and colonization (Stork *et al.*, 2008), is also crucial for blister formation (Fig. 3). However, its expression, as measured by qRT-PCR, differs: during wilting, the expression peak was at 72 hpi, whereas, in the case of blister formation, it was expressed very early at 8 hpi (Fig. 4b). The *chpC* mutant, unlike *Cmm*27, could multiply endophytically in the mesophyll and move down to the stem (Fig. S1), suggesting that additional genes located on the PAI are required for movement of the pathogen.

Of the three members of the subtilase family, only *sbtA*, which resides on the PAI, significantly affected disease incidence and severity (Fig. 3a,b). However, it was expressed at low levels during wilting, whereas it reached an expression peak during blister formation at 16 hpi (Fig. 4). The two other tested proteases of the Ppa family (*ppaA* and *ppaC*) did not affect disease symptoms. During wilting, they were implicated in colonization of the host (Gartemann *et al.*, 2008), and *ppaA* transcripts were found to accumulate at 96 hpi (Chalupowicz *et al.*, 2010), but their role in blister formation is apparently additive.

The proteases of the Chp, Ppa and subtilase families tested showed, in general, a reduction in disease incidence and/or disease severity during blister formation and wilting (Fig. 3). These proteases are predicted to be secreted (Eichenlaub and Gartemann, 2011), but their targets are still unknown. It is possible that they act synergistically during penetration and movement in the host. Many plant pathogens that produce proteases have been studied, but, in most cases, their function in pathogenesis is still not clear. Progress in elucidating the function of cysteine proteases has been achieved with Gram-negative phytopathogenic bacteria (Mudgett, 2005). In these bacteria, cysteine proteases act as effectors that are introduced into the host cell by the type III secretion system (T3SS). *Cmm* does not have a T3SS or any other secretion system known to transfer proteins into the host cell (Gartemann *et al.*, 2008). Although it has not been demonstrated that serine proteases act as effectors of *Cmm*, or of the potato pathogen *Clavibacter michiganensis* ssp. *sepedonicus* (*Cms*), a

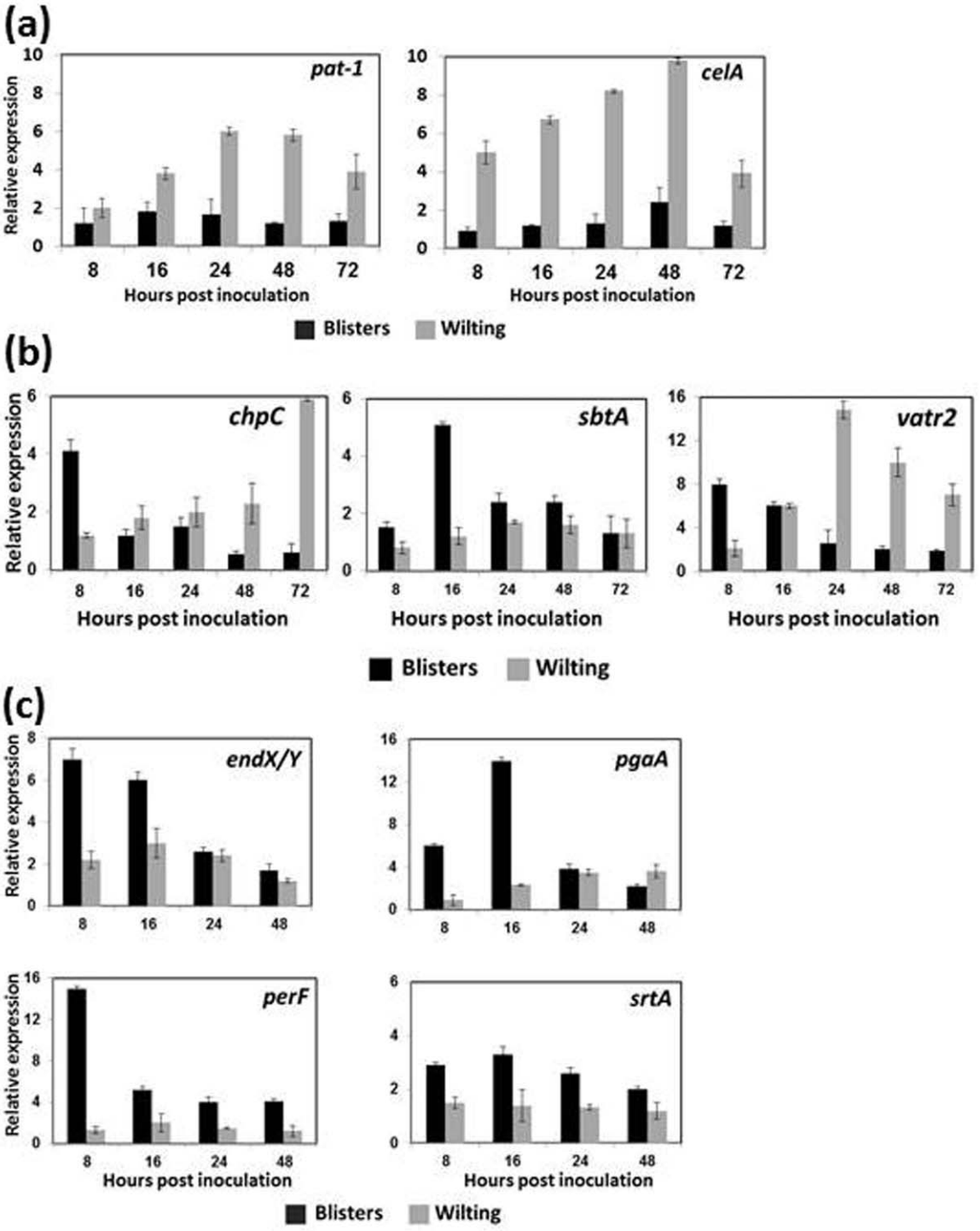

Fig. 4 Relative expression of *Cmm*382 virulence genes during blister formation and wilting. Total RNA was extracted from *Cmm*382-infected leaves and stems, and the expression level was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (a) Transcript levels of *pat-1* and *celA* residing on pCM2 and pCM1, respectively. (b) The serine proteases *chpC* and *sbtA* residing on the *chp/tomA* pathogenicity island (PAI) and *vatr2*, a transcriptional regulator. (c) The cell wall-degrading enzymes *endX/Y* and *pgaA*, perforin (*perF*) and sortase (*srtA*). Relative expression was normalized with *gyrA* and *bipA* as internal references. For each gene, expression at different time points is shown relative to that at time zero. Results represent the mean of three different experiments with three replicates for each time point. Vertical bars represent the standard error. *Cmm*, *Clavibacter michiganensis* ssp. *michiganensis*.

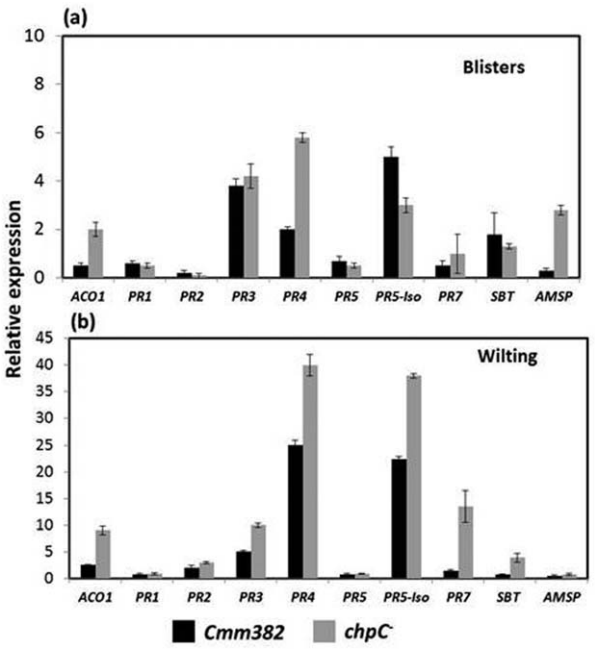

Fig. 5 Relative expression of plant defence-related genes in response to infection by *Cmm*382 and *chpC* mutant. Total RNA was extracted from infected leaves (a) and stems (b) at 24 h post-inoculation and the expression level was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Transcripts of the tested genes were normalized using *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) as an internal reference and mock-inoculated plants as control. Results represent the mean of three different experiments with three replicates for each time point. Vertical bars represent the standard error. *Cmm*, *Clavibacter michiganensis* ssp. *michiganensis*.

recent study has reported that Chp-7, a serine protease of *Cms*, induces a hypersensitive response (HR) in the apoplast of *Nicotiana tabacum* (Lu *et al.*, 2015). In addition, it is also possible that several serine proteases have overlapping substrate specificities, thus masking phenotypes in single mutations.

Cell wall-degrading enzymes (i.e. polygalacturonase, pectate lyases, xylanases and endoglucanases) may be responsible for tissue maceration and canker development during tomato infection by *Cmm* (Gartemann *et al.*, 2008). The expression of *celA*, *celB*, *pelA1*, *xysA* and *xysB* at 96 hpi of tomato seedlings has been demonstrated by qRT-PCR (Chalupowicz *et al.*, 2010), and the proteins have been shown to be secreted into the xylem sap by proteomic analysis (Savidor *et al.*, 2011). In our study, mutation in *pgaA* (encoding polygalacturonase) and *endX/Y* (encoding endoglucanase) reduced blister formation in infected tomato leaves, but did not affect wilting (Fig. 3). In addition, these genes were expressed early after foliar, but not stem, inoculation (Fig. 4c). Cell wall-degrading enzymes have been speculated to be involved in the elicitation of oligosaccharides, which, in turn, cause changes in membrane permeability and symptom development of *Cmm* (Hahn *et al.*, 1989). It can be hypothesized that entry through natural openings, such as trichomes or stomata, requires the expression of cell wall-degrading enzymes, whereas entry through wounds, which provides the nutritional components necessary for initial proliferation within the plant, does not. In addition, the relative expression of *pgaA* and *endX/Y* during foliar inoculation was reduced in the *chpC* mutant compared with the wild-type, suggesting that the expression of *pgaA* and *endX/Y* is under the control of *chpC*.

The mutants in *perF* (encoding a putative perforin) and *srtA* (a predicted sortase), which reside on the chromosome, were also found to be essential for blister formation, but not for wilting (Figs 3 and 4). Although the role of these proteins in *Cmm* is not known, there are indications suggesting a possible role in virulence. For example, pore formation is utilized by bacterial and mammalian cells to exert a pathogenic effect and to induce cell lysis (Hotze and Tweten, 2012; Keyel *et al.*, 2013). Perforins were identified in the pathogenic bacteria *Chlamydia* spp. and *Photorhabdus luminescens* (Ponting, 1999; Rosado *et al.*, 2007). Analysis of the protein MACPF (membrane attack complex/perforin) domain of *Photorhabdus luminescens* revealed structural similarity with pore-forming cholesterol-dependent cytolysins (CDCs) of Gram-positive bacteria (Rosado *et al.*, 2007). The pores generated by the cytolysin streptolysin O mediate the translocation of a bacterial protein into the host cell cytoplasm in *Streptococcus pyogenes* (Madden *et al.*, 2001). Sequence analysis of the *Cmm* putative perforin shows a MACPF domain and, although it shares only limited sequence similarity to streptolysin O (the CDCs), three-dimensional folding shows high similarity to pore-forming proteins. The latter suggests that *perF* might contribute to *Cmm* virulence by the translocation of proteinaceous effectors into the host cells during penetration through leaves and, to a lesser degree, when entry occurs through wounds or seeds.

Surface proteins play a fundamental role in microbial physiology and are frequently virulence factors (Spirig *et al.*, 2011). Sortase enzymes in Gram-positive bacteria function as cysteine transpeptidases, which can bind proteins to the cell surface by covalently joining them to the cell wall (Hendrickx *et al.*, 2011; Scott and Barnett, 2006). Although they are not essential for bacterial viability when cells are grown in rich medium, sortases can be important virulence factors, as they display surface proteins that mediate bacterial adhesion to host tissues, host cell entry, evasion and suppression of the immune response, and acquisition of essential nutrients. Our observation that the mutant in *srtA* significantly affected blister formation suggests its possible involvement in *Cmm* attachment to the leaf surface, which could promote successful proliferation and penetration into the mesophyll tissues.

The transcriptional regulator Vatr2, which is required for blister formation and wilting, was expressed at 8–16 h after spray

inoculation and at 24 h after puncture inoculation. It is possible that early activation of Vatr2 is required to trigger the virulence signal transduction pathway and to suppress the plant defence response for extensive colonization in the plants.

Previous transcriptomic and proteomic studies have demonstrated that a wide array of PR proteins from different families, as well as ethylene synthesis, are induced during *Cmm* infection (Balaji *et al.*, 2008; Savidor *et al.*, 2011). It was speculated that *chpC* is involved in the suppression of host defences. In our study, several host defence-related genes were found to be activated during blister formation and wilting in plants inoculated with the *chpC* mutant relative to the wild-type (Fig. 5). The gene encoding the *ACO1* enzyme, which catalyses the final step of ethylene biosynthesis, and the pathogenesis-related gene *PR4* were significantly induced in *chpC* mutant-infected plants. This suggests that *chpC* suppresses the expression of *ACO1* and *PR4*. Thus, the lack of disease symptoms during infection with the *chpC* mutant may be attributed in part to impaired suppression of the host defence response.

An interesting observation is the significant induction of *AMSP* in plants infected with the *chpC* mutant by spray relative to puncture inoculation. Various pathogens manipulate phytohormone signalling pathways by altering the essential developmental features of plant hosts to facilitate pathogen entry (Kazan and Lyons, 2014; Melotto *et al.*, 2008). It might be speculated that ChpC suppresses the auxin signalling-mediated pathway, thereby manipulating the entry of *Cmm* through natural openings to promote blister formation in leaves.

The present study suggests that the wide repertoire of virulence genes is differentially utilized by *Cmm* according to the plant entry route.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Clavibacter michiganensis ssp. *michiganensis* strain NCPPB382 and its mutants used in this study are listed in Table 1. Bacteria were grown in Luria–Bertani (LB) broth and agar (Difco, le Pont de Claix, France) or on C. nebraskense selective medium (CNS) agar plates (Gross and Vidaver, 1979) at 25°C for 48 h. *Cmm* mutants were grown in LB or CNS medium supplemented with 10 μg/mL chloramphenicol (Chl).

Generation of mutants

Knockout mutants of *Cmm*382 were generated by gene replacement via homologous recombination, according to Stork *et al.* (2008). Briefly, the *Escherichia coli* plasmid pSMART (Lucigen, Middleton, WI, USA), which is unable to replicate in *Cmm*, was used to construct the suicidal vector. For mutants generated in the Eichenlaub laboratory (Table 1), pSMART clones carrying the target genes were selected from the *Cmm*382 pSMART library and used for the construction of mutagenesis vectors. In mutants generated in the Sessa laboratory (Table 1), the DNA of the target genes was amplified by PCR with specific primers and the products were cloned into pBlueScriptII KS(+) (Agilent Technologies, Santa Clara, CA, USA) or pSMART plasmids, as described by Savidor *et al.* (2014). The target genes were disrupted by the insertion of a Chl resistance gene cassette excised from plasmid pOKU9 or pEC70. The plasmids containing the mutated gene were transformed into the non-methylating *E. coli* strain GM110. Following plasmid extraction, the DNA was either alkaline or heat denatured and electroporated into competent *Cmm*382 cells, as described by Kirchner *et al.* (2001). Transformants growing in Sorbitol Broth medium (Stork *et al.*, 2008) supplemented with Chl were further screened for double-crossover events and integration of the Chl cassette into the correct site of the target gene by PCR with specific primers or DNA hybridization.

The mutants constructed in the Eichenlaub and Sessa laboratories were complemented using the vector pHN216 (Laine *et al.*, 1996). A DNA fragment containing the intact target gene was cloned into this vector, which is able to replicate in *Cmm*382 and *E. coli*. The virulence of the complemented mutants was tested by puncture inoculation of the stem, as described below.

Plant inoculation and colonization tests

Tomato plants (*Solanum lycopersicum*) of the susceptible cultivar 1125 were obtained from a commercial nursery (Hishtil Ltd., Moshav Nehalim, Israel). The plants were maintained using standard glasshouse conditions: temperature of 26–28°C, photoperiod of 12 h of light, daily irrigation and fertilization three times a week.

Three-week-old plants (with two true leaves) were inoculated by one of two procedures: (i) spraying the foliage with a *Cmm* suspension (100 mL) using a hand air-pressure sprayer; or (ii) puncturing the stem at the intersection of the first two leaves with a needle, followed by placing 10 μL of bacterial suspension on the wound site. Inoculum was prepared from *Cmm* cells grown for 48 h on LB agar plates. The cells were collected and suspended in 2 mL of sterilized distilled water, adjusted to an optical density at 595 nm (OD_{595}) of 0.5, and diluted to a final concentration of 10^8 cells/mL. A mock spray or puncture inoculation treatment with sterile water served as a control.

To study the colonization and movement of *Cmm* from leaf to stem at the early stages of blister formation, the endophytic bacterial population was determined at various time points (3, 7, 15 and 21 days) after spray inoculation of tomato leaves with the wild-type and its mutants. Samples were taken from two sites: the first two leaves, and a 1-cm section of the stem located 1 cm above the cotyledons. Plant samples were disinfected with 1% (v/v) sodium hypochlorite for 30 s, washed twice with sterile water, and transferred to plastic bags for homogenization with 1 mL of sterile water. Serial 10-fold dilutions of the obtained suspensions were plated on CNS plates. The number of CFU per gram was determined and the mean of three plants for each time point was calculated. The experiment was repeated three times.

Disease assessment and data analysis

Disease incidence of blister formation or wilting was monitored 7 and 15 days after spray or puncture inoculation, respectively. Thirty plants were used for each inoculation method. Disease severity of blister formation was evaluated on leaves of individual plants according to a four-grade scale as follows: symptomless plant (plant looks healthy), weak (1–10

Table 2 Sequence of primers used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
Cmm genes		
gyrA	TTCCTCGACTACGCGATG	GGCCGACTTGAAGAAGGAG
bipA	GCTCGGTGATCTTCACGTTCTT	CCTGCTGCGCGTCTTCA
pat-1	GCTGATTCGCGAGAGGATC	GTTCTCGGTTGCTGTGTCGC
celA	GCGTCTGTGGAAGTGGTTTTC	AACGCAAGGAGAAGGACGAG
chpC	TCAACGTGTCACGAGATTG	GCACGATGGTGTATATCAAGC
sbtA	TGATCCGGTAGGAGGTAGC	ATTGTTCGACATCACAGTACC
vatr2	GGACATCCTCGAGATCATGG	GTCGATGAAGAGCTTCGTGAC
endX/Y	CCGATCATCATCGCGGAGAC	AACCAGACGAAGGCGACGAC
pgaA	GCCAGGGGGTACTTCACCT	GTGGCATCCGCATCAAGAGC
perF	GGATCCCGAAGCTGATGAAC	ACGGTGTTGCCGTAGATGGA
srtA	CGTCCTCATCATGCTGTTCCT	CGCTGCTCACGACGATGT
Tomato genes		
GAPDH	GCTGGTGCTGACTTCGTTGTTG	TAGCAAAGGTGCAAGGCAGTT
AMSP	AGGTCCATTGTGGGTGCAA	TTCTGGCCTTTCAGCTCTCTTC
ACO1	TGAGGTGATCACTAACGGGAAGT	TCGTGTCCCGTCTGTTTGTG
PR1	GGCATCCCGAGCACAAAA	CCCAGCACCAGAATGAATCA
PR2	TAGGATTACTTGTCGCCACCAA	GGCAAGTTGTTCCCCATCAT
PR3	ACCCGTTGCACTGTCTTGTCT	AATGGAACCGGCATTTTGTG
PR4	GGCCGCAGCGCAAAG	CCAGTTTATGTTTTGCGGATTG
PR5	ATCGAGGCCAAACATGGGTCAT	TTGGCATGAACCTCTACCAGCA
PR5-Iso	ATCGAGGCCAAACATGGGTCAT	TTGGCATGAACCTCTACCAGCA
PR7	CCTGCTAAGTGGAAAGGAGTTTG	CCTGCTAAGTGGAAAGGAGTTTG
SBT	CAGCAGTCTTCTCATCAATCTTA	CCCGTTACAGAGAAGAAGCTGA

blisters per plant), moderate (10–50 blisters per plant) and severe (more than 50 blisters per plant). The severity of wilting was evaluated by a three-grade scale: weak (one wilted leaf per plant), moderate (two to four wilted leaves per plant) and severe (more than four wilted leaves per plant or dead plant). The experiment was repeated three times. Data were used to calculate the incidence (percentage) of plants with blisters or wilting leaves for each mutant.

RNA extraction and expression analysis by qRT-PCR

The procedure used for qRT-PCR analysis was conducted as described previously (Chalupowicz *et al.*, 2010). RNA was isolated from *Cmm*-inoculated and control plants at different time points after inoculation. Plants were inoculated as described above. For each time point, two separate pools of three spray-inoculated leaves or three puncture-inoculated sites (1-cm sections of the stem) were cut and used for RNA isolation with the MasterPure RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). RNA quantification was performed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Nucleic acids were treated with DNAse (Turbo DNA-*free*, Ambion Inc., Austin, TX, USA) to remove residual genomic DNA. The DNA-free RNA (1 μg) served as a template for cDNA synthesis using the qScript™ cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD, USA).

The synthesized cDNA was used as template in qRT-PCRs with Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μL, according to the manufacturer's instructions. Specific primers for qRT-PCR were designed by the Primer Express 3.0 program (Applied Biosystems) and are listed in Table 2. Two *Cmm* genes, *gyrA* and *bipA*, and one from tomato, *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*), were used as internal normalization control genes in bacterial and plant gene expression studies, respectively. Negative control samples were included in each qRT-PCR experiment to verify the absence of any significant amount of genomic DNA contamination in the cDNA samples. Real-time reactions were performed in triplicate with a 7300 real-time PCR system (Applied Biosystems). Relative quantification and statistical analysis were performed with the Real-time PCR Data Analysis program of the real-time PCR software (Applied Biosystems). The results represent the mean of three different experiments with three replicates for each time point.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Endophytic growth of *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) at the early stages of blister formation. Three-week-old tomato plants were spray inoculated with bacterial suspensions (10^8 cells/mL) of *Cmm*382, *Cmm*100, *Cmm*27 and *chpC* mutant (*CmmchpC*). Endophytic populations were measured in the first two leaves (a) and stem (b) at different time points after inoculation. The results presented are the average of three plants for each time point. The experiment was repeated three times with similar results. CFU, colony-forming unit.