Sequential Expression of Bacterial Virulence and Plant Defense Genes During Infection of Tomato with *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

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The molecular interactions between *Clavibacter michiganensis* subsp. *michiganensis* and tomato plant were studied by following the expression of bacterial virulence and host-defense genes during early stages of infection. The *C. michiganensis* subsp. *michiganensis* genes included the plasmid-borne cellulase (*celA*) and the serine protease (*pat-1*), and the serine proteases *chpC* and *ppaA*, residing on the *chp/tomA* pathogenicity island (PAI). Gene expression was measured following tomato inoculation with *Cmm382* (wild type), *Cmm100* (lacking the plasmids pCM1

and pCM2), and *Cmm27* (lacking the PAI). Transcriptional analysis revealed that *celA* and *pat-1* were significantly induced in *Cmm382* at initial 12 to 72 h, whereas *chpC* and *ppaA* were highly expressed only 96 h after inoculation. Interdependence between the expression of chromosomal and of plasmid-located genes was revealed: expression of *celA* and *pat-1* was substantially reduced in the absence of the *chp/tomA* PAI, whereas *chpC* and *ppaA* expressions were reduced in the absence of the virulence plasmids. Transcription of chromosomal genes involved in cell wall degradation (i.e., *pelA1*, *celB*, *xysA*, and *xysB*), was also induced at early stages of infection. Expression of the host-defense genes, chitinase class II and pathogenesis-related protein-5 isoform was induced in the absence of the PAI at early stages of infection, suggesting that PAI-located genes are involved in suppression of tomato basal defenses.

The plant-pathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* is a gram-positive actinomycete and is the causal agent of tomato (*Solanum lycopersicum*) bacterial wilt, an economically important disease worldwide (8,37). A noticeable early symptom of the disease is a unilateral wilting of leaves, which culminates with the development of canker lesions on tomato stems, and tissue disintegration that leads to plant death (37). The interaction of *C. michiganensis* subsp. *michiganensis* with tomato can be divided into two stages: (i) an endophytic stage that leads to colonization of the tomato xylem by a large *C. michiganensis* subsp. *michiganensis* population, and (ii) a symptom-inducing stage (17). Two plasmids, pCM1 and pCM2, which were originally discovered in the wild-type strain NCPPB382, are essential for virulence (26). Loss of either of these two plasmids reduces virulence, whereas simultaneous loss of both plasmids eliminates virulence but retains the endophytic colonization of the plant by the pathogen (17,26). pCM1 encodes for CelA, a secreted cellulase with endo- β -1,4-glucanase activity (22), and pCM2 encodes for Pat-1, a putative serine protease (10). CelA protein contains catalytic and binding domains that show extensive similarity to other bacterial cellulases, and a unique third domain with a weak similarity to α -expansins from plants

which was also found in CelA of *C. michiganensis* subsp. *sepedonicus* (25). A homologue of CelA lacking the third domain (CelB) is present on the chromosome (16). *C. michiganensis* subsp. *michiganensis* also produces a variety of cell wall-degrading enzymes (e.g., xylanase, pectate lyases, and polygalacturonase), which might be involved in canker production and tissue maceration (16).

A prominent characteristic of *C. michiganensis* subsp. *michiganensis* is the occurrence of numerous extracellular serine proteases belonging to three different families (16). Noteworthy are two homologues of the virulence factor *pat-1* located on the pCM2 plasmid, and seven chromosomal homologues (i.e., *chpA*, *chpG* of which three, *chpA*, *B*, and *D*, are pseudogenes) located on a large pathogenicity island (PAI) of ≈ 129 kb known as the *chp/tomA* region (16). The *chp/tomA* PAI appears to be involved in the pathogenicity of *C. michiganensis* subsp. *michiganensis*: a *C. michiganensis* subsp. *michiganensis* mutant lacking the complete PAI region exhibited impaired virulence and was unable effectively to colonize tomato (16). Moreover, *chpC* was recently shown to be directly implicated in a virulent interaction with the host (36). Although attempts to demonstrate proteolytic activity of *pat-1* with various substrates were unsuccessful, its enzymatic action could be inferred from the elimination of its contribution to virulence by substitution of the serine residue with threonine in its catalytic domain (2). Thus, it is possible that, similarly to CelA, Pat-1 might act directly on a protein located in the host cell wall or membrane, as a substrate-specific protease (2).

During compatible host-pathogen interactions, phytopathogenic bacteria must overcome the immune response of their host plants (23). Two phases of the plant immune system are recog-

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nized: the first, the basal defense phase, uses transmembrane pattern recognition receptors that respond to microbe or pathogen-associated molecular patterns (e.g., MAMPS or PAMPS) such as flagellin or lipopolysaccharide (3); the second phase acts largely inside the cell, using polymorphic resistance genes (7). The basal defense responses do not discriminate between pathogenic and nonpathogenic microbes and include induction of pathogenesis-related (PR) proteins such as chitinases, glucanases, and proteases (30,31). In gram-negative phytopathogenic bacteria both phases of the immune response are effectively suppressed by type III effectors, which are translocated into the host cells via the type III secretion system (T3SS) (6,9). Although *C. michiganensis* subsp. *michiganensis* lacks a T3SS, it might possess virulence effectors that function through targets on the external plant membrane or that are imported into the cell by as yet unknown mechanisms, as was recently shown for oomycete plant pathogens (38).

The present study was undertaken to investigate the sequential expression of the plasmid-borne genes *celA* and *pat-1* and of additional chromosomal genes encoding serine proteases and cell wall-degrading enzymes during early stages of tomato infection by *C. michiganensis* subsp. *michiganensis*. Moreover, the relationship between expression of these genes and the presence of the virulence plasmids (pCM1 and pCM2) and the *chp/tomA* chromosomal PAI was examined. Finally, the possible suppression of tomato PR proteins by products of putative virulence genes was investigated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *C. michiganensis* subsp. *michiganensis* strains used in the present study were the wild-type pathogenic strain *C. michiganensis* subsp. *michiganensis* NCPPB382 (*Cmm382*) and its derivatives, *Cmm100* (lacking the two virulence plasmids, pCM1 and pCM2), *Cmm101* (lacking pCM2), *Cmm102* (lacking pCM1) (27), and *Cmm27*, a deletion mutant of *Cmm382* lacking the *chp/tomA* region (generated in this study). The *C. michiganensis* subsp. *michiganensis* strains were grown at 25°C for 48 to 72 h on nutrient broth or agar (Difco, Le Pont de Claix, France) or M9 medium (MA) agar plates (Na₂HPO₄ [6 g/liter], KH₂PO₄ [3 g/liter], NH₄Cl [1 g/liter], NaCl [0.5 g/liter], MgSO₄ [1 mM], CaCl₂ [0.01 mM], methionine [200 mg/liter], thiamine [200 mg/liter], nicotinic acid [20 mg/liter], glucose [2 g/liter], and agar [8 g/liter]) or MA medium supplemented with 0.5% carboxyl methyl cellulose (CMC). All strains were maintained as glycerol stocks at -80°C (34).

Construction of *Cmm27*. A deletion mutant lacking the *chp/tomA* region was previously reported (16). However, this mutant lacked pCM2; therefore, a similar mutant containing both plasmids (pCM1 and pCM2) was sought. During construction of a knock-out mutant of *Cmm382* in a peptide ABC transporter (CMM_1960) by insertion of a chloramphenicol (*cmx*) resistance gene cassette, a deletion of the *chp/tomA* region was obtained spontaneously. To confirm the nature of the deletion the following procedures, which were used to analyze *chp/tomA* deletion (16) were employed. A polymerase chain reaction (PCR) analysis with primers DR1a-F and DR1b-R (Table 1) of the left and right direct repeats flanking the *chp/tomA* region was conducted. A 2.7-kb amplified fragment from *Cmm27* confirmed that the PAI region was lost. Pulsed-field gel electrophoresis of *Cmm27* DNA according to Kleitman et al. (24) showed a chromosomal deletion of 140-kb in comparison with *Cmm382* (data not shown). Additionally PCR amplification of *ppaA*, *chpC*, and *tomA*, which are located on the PAI region, was conducted according to Kleitman et al. (24) with template DNA of *Cmm27* and the wild-type strain. Results indicated that these genes were absent in the mutant strain. In order to confirm the presence of pCM1 and pCM2 in *Cmm27* PCR with primers based on the sequences of *celA* and

pat-1 indicated the presence of both plasmids.. The inserted *cmx* cassette into the peptide ABC transporter did not affect *C. michiganensis* subsp. *michiganensis* virulence. A *C. michiganensis* subsp. *michiganensis* mutant with identical *cmx* location, as confirmed by Southern hybridization, but with the complete *chp/tomA* region was subjected to a pathogenicity test on tomato. Wilting symptoms were identical to those of the wild-type strain (data not shown). Furthermore, mutation in the ABC transporter alone did not affect the expression of the genes tested in this study (i.e., *celA*, *pat-1*, *chpC*, and *ppaA*).

The construction of clones for marker exchange and electroporation into *Cmm382* were carried out according to Stork et al. (36). Chloramphenicol was used at a concentration of 10 µg/ml.

Plant inoculation and colonization tests. Seeds of the tomato (*Solanum lycopersicum*) line Rio Grande (32) were germinated in

TABLE 1. Relevant primers used for quantitative reverse transcription-polymerase chain reaction analysis

<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> genes	Sequence (5'→3')	Expressed gene
gyrARTFow gyrARTRev	GTGGTCGGCGAGGTC GCGCGAGCGGGTAG	<i>gyrA</i>
qcrARTFow qcrARTRev	CCGACAAGGACCCGAAGAAGG GATGCCGACGCCGAGGAAC	<i>qcrA</i>
bipARTFow bipARTRev	GCGTTCTGCTTCTTCAGTC CAACATGCGTCAGTCCAC	<i>bipA</i>
celARTFow celARTRev	GGTTCTCCGCATCAAACATATCC TGCTTGCTCGCTCGTCGTC	<i>celA</i>
pat-1RTFow pat-1RTRev	GCTGATTGCGGAGAGGATC GTTCTCGGTTGCTGTGTCCG	<i>pat-1</i>
chpCRTFow chpCRTRev	GACTGCTAATCACTGTGTTG CAATAAACCGTCCGATGG	<i>chpC</i>
ppaARTFow ppaARTRev	AATCGGGCTGGTTCTGGTTT AGATTCTGCGGCATCTGCAT	<i>ppaA</i>
celBFow celBRev	GGAGACCACCAGCGACAAG TGAACGACCAGAACGACGAG	<i>celB</i>
pelA1Fow pelA1Rev	GTGCGTTCTGCGGTAAC GCGGATGGTGATGTGGTC	<i>pelA1</i>
xysAFow xysARev	ACGGGCAACAGCGAGAAC GATCATCGTGAACAGGTCCCTTG	<i>xysA</i>
xysBFow xysBRev	AAGCCCGAGAGCGTCCAG TGCGAGTGCCAGAACAGC	<i>xysB</i>
DR1a-F DR1b-R	GTGCAGCTGCATGGGTC GAGTTGTTGCTACAGCCATCC	<i>chp/tomA</i> region
Tomato plant genes	Sequence (5'→3')	Accession no.
<i>GAPDH</i>	F: CTGGTGCTGACTTCGTTGTTG R: GCTCTGGCTTGTAATTCATTCTCG	U97257
Polygalacturonase inhibitor (PG)	F: CCTTTGTGGCTCTCCCTTG R: TTCATTAGACCCCTGCATACG	L26529
<i>PR-1</i>	F: AACGCTCACAATGAGTTCGTA R: AAGGTCCACCAGAGTGTTCAT	EU589238
<i>PR-5</i>	F: AAACGGTGAATGCCCTGGTTCA R: AGGACCACATGGACCGTGATTA	AJ277064
<i>PR-5</i> isoform	F: ATCGAGGCCAAACATGGGTCAT R: TTGGCATGAACCTCTACCAGCA	AY093595
<i>PR-P69</i> gene subtilisin-like endoprotease	F: ATGTTTGGCAGGTGGTGAGTT R: TGGAATCACATGAGCATCGGCT	X95270
Chitinase class III	F: ATGCTTTCTCTTGGTGGTGGTG R: AATGTTGTGTTGCCCCGCCTTC	Z11563
<i>PR1a</i> (P4)	F: GTGCTGGGGAGGAACCTTGCC R: AACGAGCCCCGACCACAACC	AJ011520
Chitinase class II	F: GCAGGGAGTGCAATAGGTGTGAA R: TTGGCTGTTGTGCTGTCATCCA	U30465

peat-scoria (crushed volcanic stones) for 20 days. The seedlings were then transferred into pots and grown in a growth chamber at 25°C with a photoperiod of 16 h. Stems of 4-week-old tomato seedlings were punctured three times at the junction of the first two leaves, with a needle that had been dipped into a bacterial suspension of 10^8 cells/ml, or into distilled water for mock inoculation, prior to RNA extraction. Inoculum was prepared from *C. michiganensis* subsp. *michiganensis* bacteria grown for 48 h on NA and suspended in distilled water.

Colonization by *C. michiganensis* subsp. *michiganensis* strains in tomato seedlings was carried out as follows: 100 µl of bacterial suspensions of 10^9 and 5×10^5 cells/ml was injected into the stem regions of the first two leaves with a 21-gauge syringe. At various time points (0, 1, 2, 4, 7, 10, and 15 days after inoculation), three seedlings per treatment were independently collected and macerated with 3 ml of 10 mM phosphate buffer (pH 7.0) and serial 10-fold dilutions were plated on CNS plates according to Hadas et al. (18). The numbers of colony-forming units (CFU) per gram fresh weight were determined, and the average of three independent experiments was calculated.

RNA extraction and cDNA synthesis. For RNA isolation from in vitro-cultured bacteria, wild-type strain *Cmm382* was grown on agar plates of MA medium or MA supplemented with CMC for 48 h at 28°C. The cells were then scraped from plates, washed once, and suspended with sterile water to a final OD₆₀₀ of 0.5, which corresponds to a density of approximately 10^9 CFU/ml. Bacterial suspensions were centrifuged and cells pellets were immediately frozen in liquid N₂ and kept at -80°C prior to RNA extraction.

RNA extractions were carried out from in vitro-cultured cells after 48 h and from cells grown in planta at different time intervals. For each time point two separate pools of three to five inoculated stem junction parts (approximately 2 cm long) were cut from independent infected or mock-inoculated plants and used for RNA extraction. To improve bacterial cells lysis, 1 µl of Ready-Lyse Lysozyme Solution (Epicentre, Biotechnologies, Madison, WI) was added to each sample after resuspension in TE buffer following the manufacturer's instructions. The MasterPure RNA Purification Kit (Epicentre) was used to isolate total RNA according to the manufacturer's instructions. RNA quantification was performed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Epsom, Surrey, UK). To remove residual genomic DNA, the isolated RNA was treated with DNase (Turbo DNA-free, Ambion Inc., Austin, TX). The absence of DNA contamination in RNA samples was then validated by quantitative reverse-transcriptase (qRT)-PCR and subsequently used for cDNA synthesis. The quality of DNase-treated samples was verified by running aliquots of purified RNA on a denaturing agarose gel (ribosomal RNA bands are clearly visible with a ratio of approximately 2:1 from bacterial and plant RNA samples). Reverse transcription was carried out from 1 µg of total RNA by using the Verso cDNA Kit (Thermo Scientific, Epsom, UK) with random hexamer primers according to the manufacturer's instructions. Triplicates of cDNA syntheses were performed for 60 min at 53°C. cDNA synthesis for studying the expression of plant genes was carried out at 42°C.

Expression analysis by qRT-PCR. Specific primers for real-time PCR were designed by using the AlleleID program (Premier BioSoft International, Palo Alto, CA) according to the sequence of the corresponding genes (Table 1). The expected size of the amplicons was approximately 85 bp. PCR efficiencies for each primer pair were calculated from the slope of standard curves based on the amplification of four out of seven cDNA dilutions (33). Three *C. michiganensis* subsp. *michiganensis* genes, *gyrA* (CMM_0007), *bipA* (CMM_2179), and *qcrA* (CMM_1836), were tested for use as suitable reference genes. Real-time PCR was performed for each gene in MA medium culture and in inoculated plants. The C_T values of these genes was used as raw data for

analysis of gene expression stability (M) by using the freely available geNORM software (40). Lowest and similar M value (most stable) were obtained for the three genes and *gyrA* and *bipA* were further used as reference *C. michiganensis* subsp. *michiganensis* genes.

The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) from tomato were used as internal normalization control based on a previous study described by Balaji et al. (1).

Real-time PCR reactions were performed in triplicate and contained 4 µl of the diluted cDNA, 200 nM of each primer, and Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in a final volume of 15 µl. Real-time detection was performed with a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). The cycling program consisted of an initial denaturation of 10 min at 95°C, followed by 40 cycles of 5 s at 95°C, 15 s at 60°C, and 20 s at 72°C. Fluorescence was measured at the end of each cycle. Three negative controls and a tenfold dilution series of a mixture of all cDNAs were used as template to construct calibration curves designed for each pair of primers. The absence of nonspecific products and primers dimers was confirmed by analysis of melting curves. Relative quantification was calculated on the basis of $\Delta\Delta C_T$. The ΔC_T value was determined by subtracting the C_T values of the target gene from that of the reference gene and then normalizing as suggested by Rotor-Gene ($\Delta\Delta C_T$). The final relative quantification value is $2^{-\Delta\Delta C_T}$, which represent the level of expression of the gene in relation to the control (cultured cells after 48 h, *C. michiganensis* subsp. *michiganensis* or mock-inoculated plants). Each experiment was conducted three times and statistical analysis was performed with the Excel software (Microsoft, Seattle, WA) and JMP V7 (SAS Institute, Cary, NC).

RESULTS

The plasmid-borne *celA* and *pat-1* are extensively transcribed by *C. michiganensis* subsp. *michiganensis* during early stages of tomato infection. Expression levels of *celA* and *pat-1* were measured with the qRT-PCR at various time points following inoculation of tomato with *Cmm382*, and were compared with those obtained in culture media. cDNA samples were generated from total RNA extracts as described in Materials and Methods. The transcript level of *celA* was slightly induced (twofold) in culture grown on M9 medium (MA) supplemented with carboxymethyl cellulose (CMC) compared with the unsupplemented medium (Fig. 1A). In contrast to the in vitro conditions, significant elevation of *celA* relative expression was observed in planta. Thus, the transcription level of *celA* increased 8- and 14-fold during the initial 12 and 24 h postinoculation (hpi), respectively, reached a peak of 23-fold at 72 hpi, and then declined to 16.8- and 10.4-fold at 96 and 168 hpi, respectively (Fig. 1A).

Relative expression of *pat-1* was greatly induced in *C. michiganensis* subsp. *michiganensis*-infected plants compared with that in MA medium. Transcripts were increased approximately 71-fold at 12 hpi, reached levels of 170- and 160-fold at 24 and 72 hpi, respectively, and decreased to 75- and 50-fold after 96 and 168 hpi, respectively (Fig. 1B).

Deletion of *chp/tomA* region in *C. michiganensis* subsp. *michiganensis* reduces expressions of *celA* and *pat-1* during tomato infection. The mutant *Cmm27*, which lacks the ~129 kb *chp/tomA* PAI and is resistant to chloramphenicol was generated as described in Materials and Methods. The relative transcription levels of *celA* and *pat-1* in *Cmm382* and *Cmm27* were compared by means of qRT-PCR. Transcript levels of *celA* in the mutant *Cmm27* were reduced by factors of 3.5, 2.8, and 1.96 at 12, 24, and 96 hpi, respectively, compared with those in the wild-type strain *Cmm382* (Fig. 2A). A similar reduction was observed for *pat-1*. Thus, *pat-1* expression in *Cmm27* was reduced by factors of 2.5, 1.9, and 1.8 at 12, 24, and 96 hpi, respectively (Fig. 2B).

Expression of serine proteases located on the *chp/tomA* PAI is highly affected by the presence of the virulence plasmids pCM1 and pCM2. As illustrated in Figure 2, expression of *celA* and *pat-1* was significantly reduced in the absence of the *chp/tomA* PAI. Therefore, it was of interest to examine whether this relationship reciprocated, i.e., whether the absence of the virulence plasmids affected transcription of the PAI-encoded genes *chpC* and *ppaA*, which represent two different families of serine proteases (16). The importance of the *chp/tomA* region, and particularly of *chpC*, for virulence has been previously reported (16,36). Relative expression levels of *chpC* and *ppaA* in planta were measured in *Cmm382* and compared with those of *Cmm100* that lacked the two plasmids. In contrast to the early and high expression level of *pat-1* in *Cmm382* (Fig. 1), *chpC* and *ppaA* were only marginally expressed in the wild-type strain *Cmm382* at 12 and 24 hpi (Fig. 3). However, at 96 hpi, elevated relative

expression of both genes (seven- and tenfold, respectively) was observed in the wild-type strain. In the plasmid-free *Cmm100*, transcription of *chpC* could not be detected up to 24 hpi, and at 96 hpi only a very low level of expression compared with that in the wild type was observed (Fig. 3A). Also, expression of *ppaA* in *Cmm100* was very low at 12, 24 hpi, and remained low at 96 hpi (Fig. 3B). The results obtained suggest that *chpC* and *ppaA* were transcribed at later stages of infection and that their expression levels were strongly affected by the presence of the plasmids pCM1 and pCM2.

Because *Cmm100* lacks both plasmids, it was of interest to examine which of the plasmids, pCM1 or pCM2, was responsible for the low expression levels of *chpC* and *ppaA*. Therefore, the relative expression levels of these genes were measured in *Cmm101* (lacking pCM2) and *Cmm102* (lacking pCM1). The results (Fig. 4A) demonstrated that the expression of *chpC* and

Fig. 1. Transcript levels of **A**, *celA* and **B**, *pat-1* determined by quantitative reverse-transcriptase-polymerase chain reaction (qRT-PCR) at various time points after inoculation of tomato plant with the wild-type *Cmm382*. Template cDNAs were generated from total RNA extracts of 4-week-old tomato seedlings line Rio Grande and from bacteria grown on M9 medium (MA) and MA supplemented with carboxymethyl cellulose (CMC) for *celA*. Gene expressions in *Clavibacter michiganensis* subsp. *michiganensis*-infected plants were compared with those obtained from bacteria grown on MA and were normalized with *gyrA* and *bipA* that were used as endogenous references. Results represent the means of three different experiments with standard deviation. *celA* and *pat-1* expression in *C. michiganensis* subsp. *michiganensis*-infected plants were statistically different ($P < 0.05$) than in MA according to Student's *t* test.

Fig. 2. Effect of deletion of the *chp/tomA* PAI on transcript levels of *celA* and *pat-1*, as determined by quantitative reverse-transcriptase-polymerase chain reaction. Expression levels of **A**, *celA* and **B**, *pat-1* were measured in the mutant *Cmm27* and compared for each time point with those in the wild-type strain *Cmm382* after inoculation of tomato plants. Ct values were normalized with *gyrA* and *bipA* as endogenous references. Results represent the means of three different experiments with standard deviation. *celA* and *pat-1* expression in *Cmm27*, at each time point, were statistically different ($P < 0.05$) than in *Cmm382* according to Student's *t* test.

ppaA at 96 hpi were similar to those obtained for *Cmm100*, indicating that each of the two plasmids had the same effect of reducing the expression levels of the chromosomal serine proteases *chpC* and *ppaA*.

Expression of *C. michiganensis* subsp. *michiganensis* chromosomal genes encoding cell wall-degrading enzymes during tomato infection. Based on the recently annotated genome of *Cmm382* (16), four genes encoding cell-wall degrading enzymes, namely, *pelA1* (encoding a pectate lyase), *celB* (encoding a cellulase), and *xysA* and *xysB* (encoding xylanases), were chosen for monitoring their transcriptional activity during infection of tomato by *Cmm382*, *Cmm100*, and *Cmm27*. All these genes (except *pelA1*) are located on the chromosome outside of the *chpI* to *MA* PAI. In *Cmm382*, transcription of *pelA1*, which resides on the PAI, was increased fourfold and sevenfold at 12 and 24 hpi, respectively, but sharply reduced to almost twice the initial level at 96 hpi (Fig. 5). Relative expression of *pelA1* in *Cmm100* increased more than in *Cmm382*; it reached sixfold and eightfold induction at 12 and 24 hpi, respectively, and then declined sharply by 96 hpi. These results may indicate that pCM1 and pCM2 exerted a slight but significant negative effect on expression of *pelA1*. A similar effect was observed in the case of *celB*: substantial increases in its transcription levels were recorded in *Cmm100* compared with those in *Cmm382*, reaching 9.5-fold and 5-fold at 12 and 24 hpi, respectively (Fig. 5). After 96 hpi the expression levels of *celB* in both *Cmm382* and *Cmm100* were reduced. The transcription level of *celB* in *Cmm27*, which lacks the *chpI* to *MA* region, was similar to *Cmm382* (Fig. 5). In order to find out which of the two plasmids was responsible for the higher expression levels of *celB* in *Cmm100* the transcription was measured in *Cmm101* and *Cmm102* after 12 hpi (Fig. 4B). Results clearly demonstrated that after 12 hpi expression of *celB* in strains lacking *celA* (i.e., *Cmm100* and *Cmm102*) was higher by 26- and 14-fold, respectively, than in strains containing *celA* (i.e., *Cmm382* and *Cmm101*).

Transcription of *xysA* and *xysB* was also induced following tomato infection with *Cmm382*, *Cmm100*, and *Cmm27*, but always to a lesser extent than that observed for *celB* and *pelA1* genes (Fig. 5). Relative expression of *xysA* increased after 96 hpi in *Cmm382* and *Cmm27*, whereas that of *xysB* was higher at 12 and 24 hpi, and decreased at 96 hpi.

Differential expression of PR proteins in tomato infected by *Cmm382*, *Cmm27*, and *Cmm100*. It was hypothesized that the

expression of host genes associated with defense might be differentially altered in response to tomato infection by virulent and nonvirulent *C. michiganensis* subsp. *michiganensis* strains. Therefore, relative expressions level of nine genes encoding PR proteins were measured in tomato seedlings inoculated with *Cmm382*, *Cmm27*, and *Cmm100* compared with mock inoculation. Transcripts levels were measured at 12 hpi, using qRT-PCR with specific primers listed in Table 1. Three genes—chitinase class II, polygalacturonase (PG) inhibitor and PR-5 isoform—out of nine that were tested showed different levels of expression during the early stages of infection (Fig. 6). Chitinase II and PG inhibitor showed the highest levels of expression compared with PR-5 isoform. However, the expression levels of chitinase II and PR-5 isoform in *Cmm27*-inoculated plants were significantly higher than in those inoculated with the wild-type strain or *Cmm100*, which suggests that genes residing on the PAI might be responsible for suppression of some PR proteins.

Fig. 4. Relative expression of chromosomal genes *chpC*, *ppaA*, and *celB* during infection of tomato plants with *Cmm382*, *Cmm100*, *Cmm101*, and *Cmm102*. Total RNAs were extracted from infected plants collected at A, 96 h and B, 12 h after inoculation, and were used to analyze gene expression by quantitative reverse-transcriptase-polymerase chain reaction. Relative expressions were obtained by comparing the transcript levels in *Clavibacter michiganensis* subsp. *michiganensis*-infected plants with those in MA medium and were normalized with *gyrA* and *bipA*. Results represent the means of three different experiments with standard deviation. *chpC* and *ppaA* expression in *Cmm100*, *Cmm101*, and *Cmm102* were statistically different ($P < 0.05$) than in *Cmm382*. *celB* expression in *Cmm382* and *Cmm101* were statistically different than in *Cmm100* and *Cmm102* according to Student's *t* test.

Fig. 3. Transcript levels of A, *chpC* and B, *ppaA* determined by quantitative reverse-transcriptase-polymerase chain reaction at various time points after infection of tomato plants with the wild-type *Cmm382* and *Cmm100* lacking both plasmids. Relative expression levels of *chpC* and *ppaA* in *Clavibacter michiganensis* subsp. *michiganensis*-infected plants were normalized with *gyrA* and *bipA* and bacteria grown in MA medium as control. Results represent the means of three different experiments with standard deviation. *chpC* and *ppaA* expression in *Cmm100*, at 96 h postinoculation, were statistically different ($P < 0.05$) than in *Cmm382* according to Student's *t* test.

Colonization of tomato plants by *Cmm382*, *Cmm100*, and *Cmm27*. Tomato colonization by the nonvirulent strains *Cmm100* and *Cmm27* was compared with that by the virulent wild-type strain *Cmm382*. Tomato seedlings were inoculated by injecting the *C. michiganensis* subsp. *michiganensis* suspension into the stems of the first three leaves, and the bacterial population was monitored after 1, 2, 4, 7, 10, and 15 days. Inoculations were with high (10^9 cells/ml) and low (5×10^5 cells/ml) bacterial suspensions. With high inoculum, the multiplication of *Cmm382* and *Cmm100* were similar and increased by approximately 3.8 orders of magnitude during the first 7 days (Fig. 7A). Initial disease symptoms appeared after 10 days in plants inoculated with the wild-type strain. Fifteen days after inoculation the population of *Cmm382* was slightly lower than that of *Cmm100*, probably because of the death of cells as disease symptoms advanced. In contrast, the population of *Cmm27* was significantly lower than that of the wild type during the entire experiment (Fig. 7A). At lower inoculum, the titer of *Cmm382* was slightly higher than that of *Cmm100* during the initial 7 days after inoculation, but reached the same population level after 10 days (Fig. 7A). Disease symp-

toms appeared after 12 days on plants inoculated with *Cmm382*. The population of *Cmm27* was 2 to 3 orders of magnitude smaller than those of *Cmm382* and *Cmm100* during the entire experimental period. Figure 7B shows plants infected with high concentrations of *Cmm382*, *Cmm100*, and *Cmm27* 3 weeks after inoculation. Disease symptoms were elicited only by *Cmm382*. The same results were obtained when the inoculum level was 5×10^5 cells/ml (data not shown).

DISCUSSION

In this study we have followed transcriptional changes in plants of genes encoding confirmed and putative *C. michiganensis* subsp. *michiganensis* virulence factors during the initial infection period as well as the transcription of selected tomato genes that might be associated with defense. A notable finding was the early relative expression of the plasmid-borne genes *celA* of pCM1 and *pat-1* of pCM2, which were proven essential for *C. michiganensis* subsp. *michiganensis* virulence (10,22). These genes were relatively highly expressed within the first 12 hpi, reaching peaks at

Fig. 5. Transcript levels of *pelA1*, *celB*, *xysA*, and *xysB* during infection of tomato with the wild-type *Cmm382*, *Cmm100*, and *Cmm27*. Gene expressions were determined in *Clavibacter michiganensis* subsp. *michiganensis*-infected plants by quantitative reverse-transcriptase-polymerase chain reaction compared with those obtained in MA medium and were normalized with *gyrA* and *bipA*. Experiments were repeated three times with three replicates for each strain. Errors bars represent the standard deviations and asterisks denote significant differences ($P < 0.05$) in relative expression of the mutants compared to *Cmm382* at each time point, according to Student's *t* test.

72 and 24 hpi for *celA* and *pat-1*, respectively, after which they declined steadily but, nevertheless, maintained significant expression levels at least up to 168 hpi (Fig. 1A and B). The transcription of *pat-1* and *celA* in planta was extensively induced in comparison with that in minimal medium, which suggests that host signals are involved in their full activation (13,31). Pat-1 and CelA are known to affect wilting symptoms but not the colonization of *C. michiganensis* subsp. *michiganensis* (12) (Fig. 7), which suggests that they might be associated with mechanisms that trigger the appearance of symptoms. The observation that curing of either pCM1 or pCM2 only reduced virulence whereas loss of both plasmids resulted in a nonpathogenic strain (12) suggests that they act through independent mechanisms.

The current view of molecular plant-pathogen interactions is that pathogens secrete effectors into the apoplast and/or cytoplasm of host plants to suppress host defenses, facilitate colonization and trigger symptoms production (3,23). Effectors may include pathogen enzymes whose plant products act as elicitors which affect virulence (20). The early relative expression of *celA* in the present study supports the hypothesis that it might be involved in release of elicitors from tomato cell walls. The presence in CelA of the α -expansin domain (22), which is assumed to facilitate binding to native cell walls, strengthens this hypothesis. It has long been established that some oligosaccharides, designated as oligosaccharins (15), can induce signal transduction pathways, thereby initiating a variety of plant responses, including growth, development and defense (4). It was also previously demonstrated that short-chain oligogalacturonides may elicit rapid changes in membrane permeability (19) as well as ethylene production (5,35). Disruption of membrane permeability is an important cause of wilting symptoms (39). Moreover, we have previously reported that *C. michiganensis* subsp. *michiganensis* inoculations of tomato *never ripe* mutant impaired in ethylene perception, or of transgenic tomato plants with reduced ethylene synthesis, showed significant delay in appearance of wilting symptoms compared with wild-type plants (1). Thus, symptom development may be partially attributed to oligosaccharins generated by CelA that impair membrane permeability and conceivably lead to the production of ethylene. The possibility that some other cell wall-degrading enzymes of *C. michiganensis* subsp. *michiganensis* such as *pelA1*, *celB*, *xysA*, or *xysB* participate in the processing that lead to the production of oligosaccharins cannot be excluded, because they, too, are significantly

expressed during the early incubation period of the disease (Fig. 5). However, they appear not to be involved in the production of primary elicitors because they do not trigger wilting symptoms in *Cmm100* lacking CelA and Pat-1. It is noteworthy to mention that the plasmid-borne CelA of *C. michiganensis* subsp. *sepedonicus*, the causal agent of wilt and ring rot of potato, which is structurally and functionally highly similar to CelA of *C. michiganensis* subsp. *michiganensis*, is also upregulated during early stages of infection, as some of the Pat-1 homologues of *C. michiganensis* subsp. *sepedonicus* (21).

The high induction of *pat-1* expression in planta (Fig. 1B) could support the hypothesis that a peptide generated by Pat-1 from a specific plant protein could be formed. Signaling peptides are known to play major roles in animals or yeasts, and a growing number of signal peptides are being isolated and characterized in plants (14). In recent years some type III effectors of gram-negative phytopathogenic *Xanthomonas campestris* and *Pseudomonas syringae*, including XopD, AvrPphB, and AvrRpt2, were characterized as cysteine proteases, and their proteolysis of specific host substrates was shown to be an important strategy used by the pathogen to alter host physiology (28). However, serine proteases have not yet been demonstrated to enter and function within the plant cell, therefore, their mode of action might be different. It was recently reported that the serine protease *chp-7* was involved in *C. michiganensis* subsp. *sepedonicus* virulence on potato (29).

In contrast to the early transcription of the plasmid-borne Pat-1, the relative expression levels of the serine protease genes *chpC* and *ppaA*, which are located on the *chp/tomA* PAI (16), increased significantly only after 96 hpi (Fig. 3). The titer of the *chpC* mutant in planta has been shown to be drastically reduced, from 9.9×10^9 to 2×10^7 CFU/g after 28 days, and the reduced population was correlated with very weak disease symptoms (36). Additionally, it was demonstrated that a bacterial population greater than 10^8 bacteria/g in plant homogenate was necessary for the development of disease symptoms (36) (Fig. 7A). However, reaching a population of 10^7 CFU/g, as in the case of *Cmm27* (Fig. 7A) might also require an external supply of nutrients from the plant by an as yet unknown mechanism. In this connection it should be stressed that *C. michiganensis* subsp. *michiganensis*, as well as the other phytopathogenic subspecies or nonpathogenic *C. michiganensis* strains are known to survive as endophytes in plants other than the hosts on which they are pathogenic (12). These findings may suggest that the role of ChpC and possibly also of other serine proteases located on the PAI is to provide the increment in the *C. michiganensis* subsp. *michiganensis* population that is necessary for converting the endophyte into a pathogen. The late increase in expression of *chpC* might be in accordance to the latter function.

A most interesting outcome of the present study was the demonstration of interdependence between chromosomal and plasmid-located genes in achieving optimal expression. Thus, the transcription of the plasmid-borne genes *celA* and *pat-1* was reduced by 50 to 80% in *Cmm27* lacking the chromosomal *chp/tomA* region (Fig. 2), whereas relative expression levels of the serine protease homologues *chpC* and *ppaA* were reduced by 70 to 80% in *Cmm100* lacking pCM1 and pCM2 (Fig. 3). Previous studies (reviewed in 12) suggested that the evolution of pathogenicity in *C. michiganensis* subsp. *michiganensis* was achieved by the acquisition of pCM1 and pCM2 via horizontal gene transfer into endophytic *C. michiganensis* subsp. *michiganensis* strains. These two plasmids are conjugative and could be readily transferred into endophytic *C. michiganensis* subsp. *michiganensis* strains, thereby rendering them pathogenic. Similarly, pathogenic strains might be cured of these plasmids and thereby become endophytic. Thus, loss and gain of these virulence plasmids appears to be an ongoing process in *C. michiganensis* subsp. *michiganensis*-infected tomato. In addition to the plasmids, the chromosomal

Fig. 6. Expression of PR proteins in response to tomato infection by *Cmm382*, *Cmm100*, and *Cmm27*, as measured by quantitative reverse-transcriptase-polymerase chain reaction. The transcript levels of Chitinase class II, PG-inhibitor and PR-5 isoform were examined in *Clavibacter michiganensis* subsp. *michiganensis*-infected plants 12 h after inoculation. Relative levels of the monitored genes were normalized with *GAPDH* used as an internal reference and mock-inoculated plants as control. Results represent the means of three different experiments with standard deviation. Asterisks denote significant differences ($P < 0.05$) in gene expression in *Cmm27* compared with *Cmm382* according to Student's *t* test.

PAI, *chp/tomA* region, which is crucial for increased colonization and virulence on tomato, might also have been acquired by *C. michiganensis* subsp. *michiganensis* via horizontal gene transfer (16). The mechanisms involved in the cross-talk between the virulence plasmids and the *chp/tomA* PAI in planta are not yet clear. It might be speculated that common regulatory mechanisms are involved in this phenomenon, as exemplified in the interdependence between *celA* and *celB*. Thus, the absence of pCM1 harboring the *celA* could explain the significant increase (three-fold) in relative expression of the chromosomal *celB* (Figs. 4B and 5). In addition, the presence of the two plasmids elevated the relative expression of the PAI's serine proteases, *chpC* and *ppaA*. Thus, both plasmids were necessary for full expression of *chpC* and *ppaA* (Fig. 4A). This could be attributed to the presence of serine proteases on both plasmids (*pat-1* on pCM2 and *ppaJ* on pCM1) (16). Nevertheless, the possibility that other genes residing on the plasmids might affect the relative expression of cellulases and serine proteases cannot be excluded.

The PR proteins are a group of plant-encoded proteins induced by various stress stimuli (11). They are believed to play an

important role in plant defense, in pathogenic constraints, and in general adaptation to stressful environments. Additionally, some PR proteins such as chitinases or glucanases, exhibit enzymatic activity that might be involved in degradation of pathogen cell walls. However, in spite of numerous investigations into the induction of PR proteins, their precise function in plant defenses remained mostly elusive (11). An acceptable paradigm is that pathogens have to suppress/evade the plant basal defenses, which are also represented by the PR proteins, during early stages of infection, to enable in planta growth of the pathogen and subsequent disease induction (23). The possibility that the *chp/tomA* PAI could be involved in suppression of the basal defense in tomato is inferred from the results presented in Figure 6. Thus, deletion of the PAI in *Cmm27* significantly increased the relative expression of chitinase II and PR-5 isoform during the initial 12 hpi compared with that in strains harboring the PAI (*Cmm382* and *Cmm100*). Although the expression of PG inhibitor was significantly induced by *Cmm27* compared with that in the wild type, its induction by *Cmm100* was similar to that by *Cmm27*. This may suggest that the induction of PG inhibitor might be

Fig. 7. A, Colonization and **B,** symptoms development produced by *Cmm382*, *Cmm100*, and *Cmm27* in infected tomato plants. **A,** Stems of 4-week-old tomato seedlings (line Rio Grande) were inoculated with bacterial suspensions of 10^9 and 5×10^5 cells/ml, and populations of *Cmm382*, *Cmm100*, and *Cmm27* were measured as described in Materials and Methods. Results presented are averages of three independent experiments, with five replicates for each time point. Bars indicate standard deviations. **B,** Tomato plants infected with *Clavibacter michiganensis* subsp. *michiganensis* strains 3 weeks after inoculation with bacterial suspension of 10^9 cells/ml.

associated with the presence of the virulence plasmids. It is noteworthy that a microarray study of tomato genes that were upregulated 4 days postinfection by *C. michiganensis* subsp. *michiganensis* revealed that 21% could be associated with defense responses (1). In the present study only two out of nine tested tomato genes encoding defense proteins were expressed after 12 hpi (Fig. 6). This observation could imply that additional defense proteins are induced during the early stages of infection.

The results of the present study strengthen the hypothesis that pathogenicity of *C. michiganensis* subsp. *michiganensis* was acquired by horizontal gene transfer of plasmids, on the one hand, and of chromosomal PAI, on the other hand. A unique mutual interaction between these two elements was demonstrated, which might be involved in the adaptation of endophytic *C. michiganensis* to pathogenicity on tomato. The fact that additional genes are also induced during early stages of infection might indicate that these chromosomal genes also participate in the virulence process.

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