Silencing of Host Basal Defense Response-Related Gene Expression Increases Susceptibility of *Nicotiana benthamiana* to *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

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Clavibacter michiganensis subsp. *michiganensis* is an actinomycete, causing bacterial wilt and canker disease of tomato (*Solanum lycopersicum*). We used virus-induced gene silencing (VIGS) to identify genes playing a role in host basal defense response to *C. michiganensis* subsp. *michiganensis* infection using *Nicotiana benthamiana* as a model plant. A preliminary VIGS screen comprising 160 genes from tomato known to be involved in defense-related signaling identified a set of 14 genes whose suppression led to altered host-pathogen interactions. Expression of each

of these genes and three additional targets was then suppressed in larger-scale VIGS experiments and the effect of silencing on development of wilt disease symptoms and bacterial growth during an *N. benthamiana*-*C. michiganensis* subsp. *michiganensis* compatible interaction was determined. Disease susceptibility and in planta bacterial population size were enhanced by silencing genes encoding *N. benthamiana* homologs of ubiquitin activating enzyme, *snakin-2*, extensin-like protein, divinyl ether synthase, 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2, and Pto-like kinase. The identification of genes having a role in the host basal defense response to *C. michiganensis* subsp. *michiganensis* advances our understanding of the plant responses activated by *C. michiganensis* subsp. *michiganensis* and raises possibilities for devising novel and effective molecular strategies to control bacterial canker and wilt in tomato.

The gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* is the causal agent of bacterial canker and wilt disease in tomato (*Solanum lycopersicum*) (11). *C. michiganensis* subsp. *michiganensis* is a quarantine organism under international regulation, and can cause severe economic losses to tomato production worldwide. *C. michiganensis* subsp. *michiganensis* enters the plant from contaminated soil (where it can survive on plant residues up to 3 years) through root or stem wounds and natural openings, colonizes xylem vessels, and becomes systemic (26). *C. michiganensis* subsp. *michiganensis* colonization of xylem vessels leads to the appearance of typical disease symptoms that include unilateral wilting, leaflet necrosis, and lesions on stems. If infection occurs at the later stage of plant development, plants can survive and generate fruit that may develop bird's-eye spots, and seed could be contaminated with *C. michiganensis* subsp. *michiganensis* (18). At present, neither effective resistant tomato cultivars nor effective chemical controls are known. Therefore, disease prevention is mainly through use of pathogen-free certified seed and transplants, and rotation away from tomato for 3 years.

In recent years, important insight into the molecular mechanism of *C. michiganensis* subsp. *michiganensis* pathogenicity has been achieved (17), and the genome sequence of a *C. michiganensis* subsp. *michiganensis* strain has been completed (16). Major *C. michiganensis* subsp. *michiganensis* pathogenicity determinants, such as *CelA* encoding β -1,4-endocellulase (27) and

Pat-1 encoding putative serine protease (13), are plasmid-borne; however, additional genes required for effective *C. michiganensis* subsp. *michiganensis* colonization of tomato plants are located in a chromosomal pathogenicity island of ≈ 125 kb (16). *CelA* and other extracellular enzymes produced by *C. michiganensis* subsp. *michiganensis* were hypothesized to cause degradation of cell walls in xylem vessels, resulting in impairment of water transport and consequent wilting (17,27). The molecular function of *Pat-1* and its possible plant protein targets have yet to be determined. A recent study demonstrated the interdependence of chromosomal and plasmid-located virulence gene expression in *C. michiganensis* subsp. *michiganensis* for a successful infection in tomato (6). Researchers also showed that expression of tomato basal defense-related genes, chitinase class II, and pathogenesis-related (PR) protein-5 isoform was induced only in the absence of a large *chp/tomA* pathogenicity island (PAI) at early stages of *C. michiganensis* subsp. *michiganensis* infection, suggesting that PAI-located genes are involved in suppression of tomato basal defenses.

Host responses to *C. michiganensis* subsp. *michiganensis* infection and the molecular mechanisms associated with the development of disease symptoms caused by this pathogen are largely unknown. Using microarray analysis, we previously identified changes in the expression of 122 tomato genes at 8 days after inoculation with *C. michiganensis* subsp. *michiganensis* (2). During disease progress in this compatible interaction, expression of $\approx 93\%$ of the differentially expressed genes was induced and identities of these genes suggested roles in basal defense for cell wall strengthening, protein degradation, oxidative burst, defense-related hormones and signaling, transcription factors and many PR proteins (2). It was noteworthy that genes related to basal defense response were significantly enriched compared with their estimated frequency in the tomato genome (2), suggesting that the

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host elaborated an active basal defense reaction to counteract pathogen ingress. These genes include several receptor-like kinases and the putative extracellular glycoproteins ethylene-inducing xylanase (EIX1) (34) and tomato protein Ve1 that confers resistance to *Verticillium* wilt (15). In some cases, such as the recognition of bacterial flagellin by the receptor FLS2, pathogen-associated molecular patterns (PAMPs) triggered immunity limits for pathogen growth (43). Enhancing plant basal resistance may be an effective way to protect plants against diseases. It was shown that an early basal resistance inhibits tumor induction by *Agrobacterium tumefaciens* (40). Taken together, identification of genes that play a key role in eliciting basal defense response could help to devise strategies to reduce infection and protect host plants.

The development of high-throughput virus-induced gene silencing (VIGS) methods for *Nicotiana benthamiana* enables assessment of the role of a large number of candidate genes in host-pathogen interactions (3,29). An advantage of VIGS, as previously noted, is the ability to assess function for genes whose mutation (or knockout) might be lethal in sexually propagated plants, or for genes that have functionally redundant family members (3). In this study, we evaluated whether the model host for plant pathology studies, *N. benthamiana*, was susceptible to *C. michiganensis* subsp. *michiganensis*. We subsequently used VIGS to investigate the role of basal defense response-related genes in the molecular compatible interaction between *C. michiganensis* subsp. *michiganensis* and *N. benthamiana* by assessing *C. michiganensis* subsp. *michiganensis* multiplication and wilt symptom appearance in the silenced plants. The possible functions of six genes identified by VIGS analyses in controlling *C. michiganensis* subsp. *michiganensis*-induced disease are discussed.

MATERIALS AND METHODS

Bacterial strains, plant material, and growth conditions.

The bacterial strains GV2260 of *A. tumefaciens* and 04101 of *C. michiganensis* subsp. *michiganensis* (a New York field isolate) were used throughout this study. *N. benthamiana* seed were germinated in plastic pots (10 cm in diameter) containing potting mixture made of Cornell mix (a soilless peat mixture), vermiculite, and perlite in a ratio of 4:1:1. Two-week-old seedlings were transplanted to pots containing the above-mentioned mix, with one plant per pot. Plants were grown in a green house at 25 ± 2°C and 70% relative humidity with a 16- and 8-h regime of light and darkness, respectively. Three-week-old plants at a four-leaf-stage were used in the silencing experiments.

***N. benthamiana* as a host for *C. michiganensis* subsp. *michiganensis*.** In all, 45 7-week-old *Tobacco rattle virus* (TRV)-only infected nonsilenced plants were inoculated by injecting a suspension of *C. michiganensis* subsp. *michiganensis* bacteria (1×10^8 CFU/ml) into the stem region at the third leaf from the bottom. The protocol for TRV inoculation is described below. Over a period of 20 days, 30 plants were monitored for development of canker and wilt symptoms while the remaining 15 plants were sacrificed for bacterial CFU estimation. In planta bacterial growth was measured five times (4, 8, 12, 16, and 20 days postinoculation [dpi]) by harvesting stem pieces from three independent plants (1 cm in length, one stem per plant). Tissue was ground in 10 mM MgCl₂ and serially diluted samples of each stem extract were cultured on D2ANX medium plates containing nalidixic acid, polymyxin sulfate, and cycloheximide at 28, 10, and 100 mg/liter, respectively (9). Each stem piece was derived from an independent plant and was cut at 1 cm above the *C. michiganensis* subsp. *michiganensis* inoculation site. After incubation of the plates at 28°C for 3 to 4 days, the number of CFU per gram of tissue was determined for each sample. Mock-inoculated plants were included as a negative control. The entire experiment was performed twice.

TRV vectors and plasmid constructs. TRV-based pTRV1 and pTRV2 VIGS vectors (29) were used in this study. In total, 160 tomato VIGS clones in a pTRV2 vector obtained from Drs. Sophia Ekengren and Gregory Martin (Boyce Thompson Institute, Ithaca, NY) were used for a preliminary VIGS screen in *N. benthamiana* to identify those genes that played a role in host basal defense response to *C. michiganensis* subsp. *michiganensis* and to determine the effect of silencing on *C. michiganensis* subsp. *michiganensis* multiplication and disease symptom development. Fourteen of these clones were selected for further study and sequenced in both directions using forward primer 5'-TTGTG TGTCACAAAGATGG-3' and reverse primer 5'-GAACCTAAA ACTTCAGACACG-3' specific for pTRV2 plasmid. The insert sequences in the 14 clones were identified by blast analysis against the tomato expressed sequence tag (EST) databases in The Institute for Genomic Research (TIGR) and Solanaceae Genomics Network.

Three additional silencing targets—*snakin-2*, extensin-like protein (*ELP*), and divinyl ether synthase (*DES*)—were amplified by reverse-transcription polymerase chain reaction (RT-PCR) on tomato first-strand complementary DNA (cDNA). Total RNA was extracted from stem tissues of tomato plants as described by the supplier's instructions (Qiagen Inc., Valencia, CA) and treated with RNase-free DNase. RNA (2.5 µg) was reverse transcribed for 50 min at 42°C in a 20-µl reaction volume containing 100 U of SuperScript II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA), 10 mM dithiothreitol, 1× reverse transcriptase buffer (Invitrogen), 40 U of RNase inhibitor (Invitrogen), 500 µM each dNTP, and 500 ng of oligo (dT) 18-24 primer. The reaction was stopped by incubation at 70°C for 15 min. RT reaction mixture (1 µl) was used for PCR in a 25-µl reaction volume containing 1 unit of Taq DNA polymerase (New England Biolabs, Ipswich, MA), 200 µM each dNTP, and 300 nM forward and reverse primers for each gene. A 309-bp fragment for *snakin-2* was amplified using forward primer 5'-CGCGGATCCTGGAACCT GTTGTGCTAGA-3' and reverse primer 5'-GCTCTAGACCAG AACAAATATCACACC-3'. A 383-bp fragment of tomato *ELP* (2) was amplified using forward primer 5'-GCTCTAGATGTA GTCTCCATCAAGTGGTCA-3' and reverse primer 5'-CGC GGATCCGCAGCTTAATTTTACTCCTTGG-3'. A 554-bp fragment of *DES* was amplified using forward primer 5'-CGC GGATCCGTGAAATTCCAGGGGACTATG-3' and reverse primer 5'-GCTCTAGAAAGCGCCAAGAAGT GTATCAGA-3'. The cDNA fragments were double digested with *Bam*HI/*Xba*I and cloned into the corresponding sites of pTRV2. The identity of these constructs was confirmed by restriction analysis and nucleotide sequencing. The pTRV2 derivatives were introduced into *A. tumefaciens* strain GV2260 by electroporation and the agro-transformants were confirmed by restriction analysis of the introduced plasmid.

Virus infection by *Agrobacterium* spp.-mediated infiltration. Agroinoculations for VIGS were performed using the leaf infiltration method as previously described (29). GV2260 containing pTRV1 or pTRV2 and its derivatives were mixed in a 1:1 ratio and inoculated into the lower leaves of 3-week-old *N. benthamiana* plants by leaf infiltration using a needle-less syringe. For the preliminary screen with 160 clones, 4-week-old *N. benthamiana* plants were used. Plants were left in the greenhouse for growth and viral spread.

High through-put preliminary screens. For the preliminary screen with 160 clones, four plants per clone were used, and inoculated with TRV as described above. These plants were inoculated by infiltrating two leaves per plant with a *C. michiganensis* subsp. *michiganensis* suspension (1×10^8 CFU/ml). The infiltration site was monitored daily for 10 days following inoculation.

Seventeen clones were included in the next experiment to further examine the developmental phenotype due to silencing of these potential basal defense-related genes (Table 1). In this experiment, 14 3-week-old plants were individually inoculated

with pTRV1 and the appropriate pTRV2-EST fragment as described above. Treatments were assigned in a completely random design. The constructs pTRV2:*SIPDS* (*phytoene desaturase* from *S. lycopersicum*) and pTRV2 were introduced as positive and negative controls, respectively. Plants were observed daily for 2 weeks to identify silencing-associated phenotypes.

Determination of host susceptibility in silenced plants. Eleven genes (Table 1, genes not in bold) were included in these VIGS studies to determine the possible role of each gene in basal defense to *C. michiganensis* subsp. *michiganensis* by silencing 12 3-week-old plants for each clone. Treatments were assigned in a completely random design. Two weeks after virus infection, silenced plants were inoculated with a suspension of *C. michiganensis* subsp. *michiganensis* at 1×10^8 CFU/ml in the stem region at the third leaf from the bottom and monitored over a period of 20 days for the appearance and progress of wilt symptoms. Mock-inoculated control plants were included for each clone, and empty-vector *C. michiganensis* subsp. *michiganensis*-inoculated plants were used as positive controls. The entire experiment was performed twice. To get a quantitative estimation of the silencing effect on *C. michiganensis* subsp. *michiganensis* multiplication and of the timing of wilt symptom onset, we applied a parameter known as wilting index (WI), which has been previously defined as the number of days after infection at which 50% of the plants display the first wilt symptoms (2,31).

Host susceptibility and bacterial multiplication in plants silenced for six genes involved in basal defense. Two independent biological repeats were performed for six genes found to be involved in basal defense against *C. michiganensis* subsp. *michiganensis*. In each repeat, for each of the six target genes plus the vector-only control, 60 plants were silenced and inoculated with *C. michiganensis* subsp. *michiganensis* as described above. Treatments were assigned in a completely random design. Thirty of the plants were monitored daily for 20 days for the development of wilt symptoms and used to calculate the WI as described above. The other 30 plants were used to determine the in planta *C. michiganensis* subsp. *michiganensis* population size and to extract RNA for transcript analysis (described below). The entire experiment was repeated twice. WI data for each replicate were combined and subjected to analysis of variance (ANOVA) using the Mixed Models (MIXED) procedure of SAS (version

9.2; SAS Institute Inc., Cary, NC). Treatment effects were considered statistically significant at $\alpha = 0.05$.

In planta bacterial growth was measured at two time points (4 and 8 dpi) by grinding three stem pieces (1 cm in length, one stem per plant) in 10 mM MgCl₂ and plating serially diluted samples of each stem extract on D2ANX medium (9). Each stem piece was derived from an independent silenced plant and was cut at 1 cm above the *C. michiganensis* subsp. *michiganensis* inoculation site. After incubation of the plates at 28°C for 3 to 4 days, the number of CFU per gram of tissue was determined for each sample. Data were analyzed using Student's *t* test, and treatment effects were considered statistically significant at $\alpha = 0.05$.

Semiquantitative RT-PCR. Total RNA was isolated from TRV-only infected plants and those silenced for the six target genes with the RNeasy Plant Mini Kit (Qiagen Inc.) according to the manufacturer's instructions and treated with RNase-free DNase. For each treatment, a pool of eight 1-cm-long stem pieces, cut from eight independent plants (one stem per plant) at the third leaf from the bottom were used for RNA extraction. Stem tissues were harvested from plants 2 weeks post-VIGS. Total RNA (2.5 µg) was reverse transcribed as previously described. PCR was performed in 100-µl reactions using 3 µl of reverse transcription mix containing the first-strand cDNA, 1× PCR buffer containing 1.5 mM MgCl₂, 200 µM each dNTP, 300 nM of each gene-specific primer (Supplementary Table S2), and 2 U of *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). To ensure that equal amounts of cDNA were used for silenced and nonsilenced plants, parallel reactions were run with *N. benthamiana* elongation factor α (a house keeping gene) (*NbEF- α* ; AY206004) primer as control. Each PCR reaction included an initial denaturation at 94°C for 2 min, followed by cycling conditions of 94°C for 30 s, annealing for 30 s (temperature specified in Supplementary Table S2 for each gene), and an extension at 72°C for 1 min. A 10-µl aliquot was removed from each reaction after 20, 25, 30, 35, 40, 45, and 50 cycles, respectively. The aliquots were analyzed on a 1.2% agarose gel stained with ethidium bromide. The intensity of PCR products corresponding to target genes was quantified at 30 and 50 cycles in TRV-only infected control and silenced plants using a Quantity One Version 4.6.9 software (Bio-Rad Laboratories, Hercules, CA). The entire experiment was repeated.

TABLE 1. Supporting information for the genes targeted by virus-induced gene silencing in *Nicotiana benthamiana*^a

Gene	Fragment length (bp) ^b	TC no. ^c	Nucleotide identity (%) ^d	Length (nt) ^e
Elongation factor	689	TC14415	79	41
Heat shock protein 90^f	1,691	NP880339	85	73
Eukaryotic initiation factor	996	TC13235	80	50
β-Tubulin	752	No hits found	...	...
60S ribosomal protein	813	TC12372	91	41
40S ribosomal protein	737	TC16301	78	26
Coumarate-CoA ligase	1,892	CK299019	86	31
Uridine diphospho-xylose synthase	2,109	NP864107	89	44
Unknown protein	1,175	No hits found	...	...
DNA J	525	CK285070	89	61
Ribulose-1 5-bisphosphate carboxylase small subunit precursor ^f	463	TC15331	96	154
Pto-like serine/threonine kinase	2,368	TC16220	82	27
Ubiquitin activating enzyme	447	EH367417	86	39
3-Hydroxy-3-methylglutaryl-coenzyme A reductase 2 ^f	420	TC11881	99	220
Snakin-2	309	TC14694	82	24
Extensin-like protein	383	TC13329	75	27
Divinyl ether synthase	554	CK293631	84	27

^a Genes in bold had a lethal phenotype when silenced in 3-week-old plants. cDNA fragments used for *Tobacco rattle virus* (TRV)-mediated silencing in *N. benthamiana* were isolated from tomato.

^b Length of the cDNA fragments cloned in the TRV2 vector used for silencing.

^c Identity of the most similar *N. benthamiana* contigs retrieved when the tomato cDNA sequence was used in a BLAST search against The Institute for Genomic Research *N. benthamiana* gene index (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi>).

^d Overall sequence identity (percentage) between the tomato and *N. benthamiana* genes.

^e Length of the longest continuous stretch (nt = nucleotides) of sequence identity between the tomato and *N. benthamiana* genes, where the sequences are identical between the two genes.

^f Genes with additional homologs that could be affected by co-silencing.

RESULTS

N. benthamiana as a model host for *C. michiganensis* subsp. *michiganensis*. Though a VIGS system has been established for tomato (14), it is not as robust as VIGS in *N. benthamiana*, which

Fig. 1. Disease symptoms and bacterial growth in *Nicotiana benthamiana* plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis*. Five-week-old plants were inoculated with a suspension of *C. michiganensis* subsp. *michiganensis* bacteria at a titer of 10^8 CFU/ml in the stem at the third leaf from the bottom stem or mock inoculated. **A**, Leaves with wilt symptoms (indicated by arrows) and the plant stem showing canker symptoms (encircled areas) were photographed at 4, 8, 12, 16, and 20 days postinoculation (dpi). **B**, Bacterial population size determined from stem samples harvested at different time points during a period of 20 dpi. Data represent the mean $\pm$ standard deviation ($n = 3$).

is highly efficient, uniform, and used routinely for large-scale screening of candidate genes in host–pathogen interactions. There are no previous studies where *N. benthamiana* has been used a model host for *C. michiganensis* subsp. *michiganensis*. Thus, the high susceptibility of *N. benthamiana* to VIGS combined with the fact that tomato ESTs can effectively knock down gene expression in *N. benthamiana* (12) prompted us to deploy it as a model host for *C. michiganensis* subsp. *michiganensis*. To this end, an experimental system was developed to achieve coordinated and reproducible infection of *N. benthamiana* plants by *C. michiganensis* subsp. *michiganensis*. At 4 dpi, canker lesions (Fig. 1A) were visible at the inoculation site in the stem of 50% of a set of 30 *C. michiganensis* subsp. *michiganensis*-inoculated plants, whereas wilt symptoms were not yet apparent. At 8 dpi, 30% of the *C. michiganensis* subsp. *michiganensis*-inoculated plants showed initial wilt symptoms (Fig. 1A), and the canker lesions started to enlarge by spreading in both directions from the inoculation site and appeared on the stems of all *C. michiganensis* subsp. *michiganensis*-inoculated plants. At 12 dpi, 45% of the inoculated plants showed typical wilt symptoms. At 16 dpi, 60% of the inoculated plants exhibited severe wilt symptoms. By 20 dpi, 70% of the inoculated plants exhibited clear and advanced wilt symptoms. A mock-inoculation treatment was also included as a control and the mock-inoculated plants remained symptom-free throughout the course of the experiment (Fig. 1A). To establish a correlation between the appearance of disease symptoms and bacterial CFU, *C. michiganensis* subsp. *michiganensis* growth in stem tissues above the inoculation site was monitored over the 20-day period. Bacterial multiplication was exponential during the first 10 days postinfection and approached saturation at 12 dpi (Fig. 1B), with the stem tissue colonized with *C. michiganensis* subsp. *michiganensis* at a titer of 10^{10} to 10^{11} CFU/g. The experiment was repeated with consistent results (data not shown). Taken together, these results showed that, in our experimental system, initial canker lesions appeared when *C. michiganensis* subsp. *michiganensis* was in the exponential phase and still multiplying in infected stems, whereas advanced leaf wilting and formation of enlarged cankers was observed when the bacterial titer approached saturation.

VIGS in *N. benthamiana* using tomato orthologs involved in plant defense responses affects *C. michiganensis* subsp. *michiganensis* infection. To identify host genes affecting *C. michiganensis* subsp. *michiganensis* multiplication and appearance of disease symptoms, the TRV-based VIGS vector system was used to silence candidate genes in 4-week-old *N. benthamiana*. Nearly 160 genes identified to be involved in a resistance response of tomato to *Pseudomonas syringae* pv. *tomato* (speck disease pathogen) (32) were tested in a preliminary screen. Of these, 14 resulted in an altered response wherein the silenced plants developed rapid necrotic lesions in the *C. michiganensis* subsp. *michiganensis* infiltrated zone. In the modified interaction, silenced plants responded to the *C. michiganensis* subsp. *michiganensis* inoculation ≈ 24 h earlier than control TRV-only nonsilenced plants (data not shown). For further VIGS analysis, three additional targets (*ELP*, *snakin-2*, and *DES*) were selected.

Because the TRV clones were not derived from *N. benthamiana* and effective silencing by VIGS requires a 21- to 23-bp minimal region of nucleotide identity (39), we performed BLAST analyses with the sequences of each of the 17 tomato cDNA fragments against the TIGR database of *N. benthamiana* ESTs. For 14 of the 17 genes, a single *N. benthamiana* ortholog with a highly significant sequence similarity was identified (Table 1). One shortcoming of VIGS and other silencing technologies is the possibility that closely related nontarget genes are co-silenced (4,25). Three genes in this study had multiple *N. benthamiana* homologs with a 21-bp or longer region identical to the tomato gene sequence (Table 2). Therefore, it is possible that these homologs could be silenced as well.

Developmental phenotypes revealed by silencing tomato ESTs in *N. benthamiana*. For each of the 17 clones identified in the preliminary screen, VIGS was performed on 14 3-week-old *N. benthamiana* plants, and plant development was monitored for 4 weeks. The constructs pTRV2:*SIPDS* and pTRV2 were introduced as positive and negative control, respectively. Silencing of the endogenous *PDS* gene, which causes photobleaching, was used as a control for VIGS efficiency. In two independent experiments, even at 2 weeks post-agroinfiltration, the leaves and stem exhibited a complete photobleach phenotype in *N. benthamiana* plants silenced for the *PDS* gene (data not shown). Based on these results, 2-week-old silenced plants were used for all *C. michiganensis* subsp. *michiganensis* bioassays. For 12 of the 17 clones, silencing of the *N. benthamiana* genes resulted in a developmental phenotype, the most frequent of which was severe stunting followed by death of the silenced plants (Supplementary Figure S1A; Supplementary Table S1). The silencing phenotypes were apparent at 2 weeks post-agroinfiltration. The stunting and death phenotype associated with silencing of these six genes (Supplementary Figure S1A) was also observed in the preliminary VIGS screen but it occurred much later because, in those preliminary experiments, the treated plants were older (4-week-old plants were used for VIGS), which allowed leaf infiltration with *C. michiganensis* subsp. *michiganensis* before the plants started to decay. The six clones that induced this lethal phenotype were excluded from further analyses.

Silencing of some genes led to developmental alterations that might result from a defective meristem functioning. Reduction in levels of the coumarate-CoA ligase, uridine diphospho-xylose synthase (*UDP-XS*), unknown protein (*UNP*), and *DNA J* resulted in an attenuation of plant growth and severe stunting (Supplementary Figure S1B). Another phenotype observed was the mild deformation (crinkling) of the upper, presumably silenced, leaves, as shown for 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 (*HMGR*) and ubiquitin activating enzyme (*UAE*) (Supplementary Figure S1B). Suppression of Pto-like kinase (*PLK*), *snakin-2*, *ELP*, ribulose-1,5-bisphosphate carboxylase small subunit precursor (*RBCS*), and *DES* did not lead to any visible morphological changes (Supplementary Figure S1C).

Determination of host susceptibility in VIGS plants. We next evaluated the possible role of each of the remaining 11 genes in basal defense responses to *C. michiganensis* subsp. *michiganensis* infection. Silenced plants were inoculated with *C. michiganensis* subsp. *michiganensis* and monitored over a period of 20 days for the appearance and progress of wilt symptoms. Mock-inoculated control plants remained symptomless throughout the course of the experiment, and *C. michiganensis* subsp. *michiganensis*-inoculated TRV empty-vector control plants were used as positive controls. The WI was determined for each of the 11 silenced

genes and the controls. Whereas the WI for control *C. michiganensis* subsp. *michiganensis*-inoculated plants was 19 days, plants silenced for *N. benthamiana* homologs of *snakin-2*, *ELP*, *DES*, *UAE*, *HMGR*, and *PLK* had a WI of 6 to 14 days, demonstrating a significantly earlier onset of wilting. Plants silenced for the other five genes (coumarate-CoA ligase, *UDP-XS*, *UNP*, *DNA J*, and *RBCS*) developed wilt symptoms much later than the *C. michiganensis* subsp. *michiganensis*-inoculated control plants. These five VIGS clones were initially identified from the preliminary VIGS screen, where they responded 24 h earlier than control plants to *C. michiganensis* subsp. *michiganensis* infiltration in the leaves, exhibiting an early necrotic response. However, in this large-scale analysis, a quantitative scoring method was applied by using the WI to determine the effect of silencing on the development of characteristic wilt symptoms. The quantitative analysis revealed that silencing of these five genes delayed wilting when compared with the nonsilenced

Fig. 2. Effect of silencing on development of wilt symptoms in *Nicotiana benthamiana* plants infected with *Clavibacter michiganensis* subsp. *michiganensis*. Two weeks post-silencing, plants were inoculated with a *C. michiganensis* subsp. *michiganensis* suspension (10^8 CFU/ml) and examined for development of wilt symptoms during a 20-day period. Percentage of plants showing wilt symptoms was calculated in a group of 30 plants for each of the silenced genes and the *Tobacco rattle virus* (TRV)-only infected control. Data shown are representative of two independent experiments for each silenced gene. Wilting index (WI), defined as the number of days after inoculation at which 50% of the total plants show first wilting symptoms, were as follows: TRV, 16; Pto-like kinase (*PLK*), 9; ubiquitin activating enzyme (*UAE*), 9; 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2 (*HMGR*), 8; *snakin-2*, 7; extensin-like protein (*ELP*), 6; and divinyl ether synthase (*DES*), 8.

TABLE 2. Comparison of the most closely related paralogs of three silenced genes

<i>Nicotiana benthamiana</i> ortholog ^a	<i>N. benthamiana</i> paralogs or off-targets ^b	TC no. ^c	Nucleotide identity (%) ^d	Length (nt) ^e
<i>HSP90</i>	<i>HSP90b</i>	TC16336	82	71
<i>RBCS</i>	<i>RBCSb</i>	CN748449	87	59
	<i>RBCSc</i>	CN748824	82	40
<i>HMGR</i>	<i>CA</i> ^f	NP375379	94	39
	<i>40S-RP</i> ^f	EC277789	90	29
	<i>EIF-4A</i> ^f	EC277784	72	39

^a *HSP90*, heat shock protein 90; *RBCS*, ribulose-1 5-bisphosphate carboxylase small subunit precursor; and *HMGR*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase 2. See Table 1 for contig numbers for *N. benthamiana* ortholog.

^b Shown is the *N. benthamiana* paralog or off-target with the second largest stretch of nucleotide identity found from a BLAST search of The Institute for Genomic Research *N. benthamiana* gene index.

^c Gene identity of the closest paralog or off-target.

^d Sequence identity (in percentage) between the tomato and *N. benthamiana* paralog or off-target.

^e Longest continuous stretch (nt = nucleotides) where the sequences are identical between the tomato and *N. benthamiana* paralog or off-target.

^f *CA* = chloroplast carbonic anhydrase; *40S RP* = 40S ribosomal protein; and *EIF-4A* = eukaryotic translation initiation factor. These off-targets showed 100% identity in a single continuous nucleotide stretch of >21 bp. Percent sequence identity scored based on the matching nucleotide numbers are 48/51 nucleotides (nt) (94%), 50/55 nt (90%), and 84/116 nt (72%) for *CA*, *40S RP*, and *EIF-4A*, respectively.

control plants. These silenced plants did not have a WI during a disease monitoring period of 20 days when control plants had a WI of 19 days. We observed a similar pattern for these five genes in an independent experiment where the silenced plants did not show a WI and the control nonsilenced TRV-only infected plants showed a WI of 17 days. Because these genes were not involved in host basal defense, they were not carried on in this study.

Silencing of six genes enhances *C. michiganensis* subsp. *michiganensis* susceptibility in *N. benthamiana*. With the six remaining genes, two independent large-scale biological repeats were carried out. In each repeat, for each of the six target genes and the TRV vector-only control, 60 plants were silenced, half of which were allotted to monitor the development of wilt symptoms and to calculate the WI. The other half was used to determine the in planta *C. michiganensis* subsp. *michiganensis* population size and to extract RNA for transcript analysis. In these experiments, the inoculated control plants showed a WI of 16 days (Fig. 2). In both repeats, plants that had been silenced for one of the six genes showed wilt symptoms sooner than the control plants, with a WI of 7 to 12 days, depending on the silenced gene. The WI of all VIGS treatments were significantly different from the control TRV-only plants at $\alpha = 0.05$.

Increased disease susceptibility is correlated with VIGS-mediated suppression of target gene expression. Two weeks after TRV inoculation, for each of the six genes, total RNA was isolated from a pool of stem tissues harvested from eight independently silenced plants and the relative abundance of the target gene transcripts was determined by semiquantitative RT-PCR

using primers designed against the *N. benthamiana* orthologs (Supplementary Table S2). A clear reduction was observed in the transcript levels of each of the genes targeted by VIGS in the silenced plants when compared with the control plants (Fig. 3). To evaluate the silencing efficiency, we quantified the intensity of the ethidium bromide-stained band corresponding to the target gene in silenced and TRV-only infected control plants after 30 cycles in the RT-PCR reaction. VIGS efficiency, represented as percent reduction, was 70 to 99% depending on the gene silenced (*PLK*, 70%; *UAE*, 81%; *DES*, 84%; *ELP*, 92%; *HMGR*, 96%; and *snakin-2*, 99%). Reduction in the transcript level in silenced plants was also evident upon quantification of band intensities after 50 cycles (data not shown). These data were confirmed in an independent biological repeat (data not shown). These experiments demonstrate that the tomato sequences were effective in silencing the *N. benthamiana* orthologs.

In the case of *HMGR* silencing, we found three potential off-targets that had a single region of ≥ 21 bp with perfect sequence identity (Table 2). Because this sequence identity might allow these off-target genes to be silenced by the tomato cDNA fragment, we developed gene-specific primers for each of them and examined their transcript abundance in *HMGR*-silenced plants and control plants. In each case, in spite of the nucleotide identity over a >21-bp region, we found no decrease in transcript abundance for these off-targets (Fig. 3), which was further confirmed by amplicon intensity quantification analysis. Thus, although we cannot exclude the silencing of very closely related paralogous genes in all cases, these data support a high degree of silencing specificity for the *N. benthamiana* orthologs of our tomato gene set.

Enhanced disease susceptibility is associated with increased *C. michiganensis* subsp. *michiganensis* titers. To gain insight into the basis for increased disease susceptibility, we analyzed *C. michiganensis* subsp. *michiganensis* titers in individual stems of infected plants silenced for each of the six genes and of control plants at two time points: at 4 dpi, when bacterial growth in inoculated stems of control plants is logarithmic and canker lesions are the only visible disease symptom; and at 8 dpi, when distinctive wilt and canker symptoms are visible on leaves and stems, respectively (Fig. 1). Although, at 4 dpi, no significant differences could be noted, at 8 dpi, in all cases, the increase in the bacterial populations was much larger and significant in the silenced plants compared with that in the control plants (Fig. 4). In the control plants, the increase in the *C. michiganensis* subsp. *michiganensis* population between 4 and 8 dpi was 70-fold. In the silenced plants, the largest increases in CFU were noted for *UAE* and *snakin-2* (4,000- and 3,000-fold), followed by *HMGR* and *DES* (1,500- and 800-fold), *ELP* (500-fold) and, finally, the smallest increase was in *PLK*-silenced plants (130-fold) (Fig. 4). A similar pattern of increase in bacterial titer in silenced plants versus control was observed in an independent biological repeat, with the smallest increase in CFU observed for *PLK*-silenced plants when compared with other target genes (Supplementary Figure S2). Based on these results, it seems that the WI (Fig. 2) and bacterial titer (Fig. 4) are not strictly inversely proportional. However, it should be noted that the WI was calculated from a set of 30 plants and bacterial titer was determined from a group of three representative plants for each silencing treatment. It is likely that a variation in the bacterial titer between the independent silenced plants in a group of 30 plants contributes to the final WI. Overall, silenced plants had significantly higher bacterial populations and developed wilt symptoms much earlier than control, nonsilenced plants.

DISCUSSION

N. benthamiana is susceptible to a wide range of plant-pathogenic agents such as viruses, bacteria, and fungi, making

Fig. 3. Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) to demonstrate virus-induced gene silencing-mediated suppression of specific RNA transcripts in *Nicotiana benthamiana*. Total RNA was isolated from *Tobacco rattle virus* (TRV)-only infected plants or plants silenced for specific genes (listed on right) and was used to generate first-strand complementary DNA (cDNA). cDNA was used in an RT-PCR using primers specific to the target gene. Off-targets for gene *NbHMGR* with one ≥ 22 -bp stretch of 100% identity is shown in brackets. To compare the abundance of transcripts between silenced and nonsilenced plants, PCR products were sampled from each PCR cycle number indicated. RT-PCR products were separated on a 1% agarose gel and visualized after staining with ethidium bromide. Bottom panel in the figure shows the amount of elongation factor α (*NbEF- α*) transcripts in TRV-only infected plants as well as plants silenced for divinyl ether synthase (*NbDES*). This control was performed for every gene-silenced plant with similar results (data not shown).

this species a widely used model for host–pathogen research, particularly in the context of innate immunity and defense signaling. The development of plant virus-based vectors quickly led to the discovery of VIGS technology (39) that transformed *N. benthamiana* into a powerful reverse-genetics system (12,29). Although a VIGS system has been established for tomato, the efficiency of silencing and level of desired transcript suppression is still low and less uniform compared with *N. benthamiana* (35). For this study, *N. benthamiana* was selected because it is a well-established host for high-throughput VIGS and enables assessment of the role of a large number of candidate genes in host–pathogen interactions. Importantly, *N. benthamiana* ESTs are generally highly homologous to those of agriculturally relevant solanaceous crops, such as tomato, potato, and pepper. Thus, functional genomics projects concerned with host–pathogen interactions conducted in *N. benthamiana* are believed to yield a framework of genes that play similar roles in agronomically important crops. However, we cannot rule out the fact that, though *N. benthamiana* ESTs are generally highly homologous to tomato, there is likelihood for variation or a totally different disease phenotype in silenced tomato (natural host of *C. michiganensis* subsp. *michiganensis*) when compared with our results on *N. benthamiana*. This possibility will be verified in future studies.

Although *N. benthamiana* is a host of many plant-pathogenic agents, it had not been shown to be susceptible to *C. michiganensis* subsp. *michiganensis*. This study demonstrates for the first time the induction of wilt and canker symptoms in *N. benthamiana* characteristic of *C. michiganensis* subsp. *michiganensis* infection in its natural host, tomato. We took advantage of the accessibility of *N. benthamiana* to the VIGS methodology to screen tomato genes known to be involved in the host defense response for their putative role during the interaction with *C. michiganensis* subsp. *michiganensis*. It was particularly interesting that the silencing of six genes increased susceptibility to *C. michiganensis* subsp. *michiganensis*. Although the level of susceptibility conferred varied among the genes tested, as shown by the difference in the bacterial titer and WI, it is possible that different degrees of silencing for each of the genes contributed to the different levels of observed susceptibility.

Our data indicate that *N. benthamiana* homologs of tomato *ELP*, *snakin-2*, *UAE*, *DES*, *HMGR*, and *PLK* play a role in host basal defense response to *C. michiganensis* subsp. *michiganensis*. Research on a number of host–pathogen systems has shown that extensin levels increase in response to pathogen ingress and proliferation (10,24). A recent study convincingly demonstrated a functional linkage between extensin synthesis and plant resistance to pathogen attack (41). They showed that *EXT1* extensin-over-expressing transgenic *Arabidopsis* limits pathogen invasiveness and were resistant to *P. syringae* pv. *tomato* DC3000. More recently, our work on transcriptome analysis of the tomato–*C. michiganensis* subsp. *michiganensis* compatible interaction revealed that the tomato *ELP* encoding cDNA was upregulated 12- to 20-fold at 4 and 8 dpi after *C. michiganensis* subsp. *michiganensis* infection (2). The data reported here clearly indicate that silencing an *N. benthamiana* *ELP* enhanced susceptibility to *C. michiganensis* subsp. *michiganensis* infection and induced early wilting.

Our findings also demonstrate that the silencing of *snakin-2* enhanced susceptibility to *C. michiganensis* subsp. *michiganensis* and advanced wilt symptom development. Furthermore, our data confirm the previous observations on the antimicrobial role of snakin-1 and snakin-2 peptides in defense response to bacterial and fungal pathogens (5,28,37). It is interesting that purified snakin-2 peptide from potato tubers caused rapid aggregation and inhibited growth of *C. michiganensis* subsp. *sepedonicus*, a species closely related to *C. michiganensis* subsp. *michiganensis* and the causative agent of potato ring rot disease (5). Recently, it was shown that overexpression of the *snakin-1* gene enhanced

resistance in transgenic potato plants to *Erwinia carotovora* and *Rhizoctonia solani* (1). Considering its role as an antimicrobial peptide and based on our results reported here, it is tempting to speculate that *snakin-2* could serve as a promising candidate to reduce *C. michiganensis* subsp. *michiganensis* infection and disease development.

Increased susceptibility to *C. michiganensis* subsp. *michiganensis* was also evident in plants silenced for *UAE* and *DES*. *UAE* is the first in the multi-enzymatic system that operates ubiquitination, which has been shown to be important in plant defense (30,33). A recent study (22) identified a *modifier of sncl 5 (mos5)* mutant in *Arabidopsis*, which carries a mutation in ubiquitin-activating enzyme 1 that suppresses *suppressor of npr1-1 constitutive 1 (sncl)*-mediated constitutive resistance and exhibits enhanced susceptibility to virulent pathogen and is impaired in response to infection with avirulent bacteria. The *mos5* gene encodes an essential component of the ubiquitin pathway and implicates a requirement for ubiquitination in resistance-protein signaling (22). It is interesting to note that *UAE* was upregulated by 70-fold in our microarray analysis of the tomato–*C. michiganensis* subsp. *michiganensis* compatible interaction (2). The *mos5* results (22) along with the *UAE* results from this study suggest a conserved role for *UAE* in basal defense. It has been shown that divinyl ethers colneleic acid and colnelenic acid derived by the sequential action of a 9-LOX (lipoxygenase) and a 9-DES accumulate in tobacco or potato in response to elicitor treatment or pathogen ingress (19,21,36). 9-LOX-silenced potato plants show decreased levels of 9-LOX-derived oxylipins in leaves inoculated with the incompatible pathogen *P. syringae* pv. *maculicola* (20). Genes encoding allene oxide synthase and LOX were induced four- to sevenfold in our previous microarray analysis (2), suggesting a role for oxylipins in the basal defense reaction to *C. michiganensis* subsp. *michiganensis* attack. Those data, combined with the increased susceptibility that we observed

Fig. 4. Growth of *Clavibacter michiganensis* subsp. *michiganensis* in silenced stem tissue of *Nicotiana benthamiana* plants. Two weeks after silencing, plants were inoculated with a suspension of *C. michiganensis* subsp. *michiganensis* at 10^8 CFU/ml into the stem at the third leaf from the bottom and bacterial populations were estimated in stems of the silenced and nonsilenced *Tobacco rattle virus* (TRV)-inoculated control plants harvested at 4 and 8 days postinoculation (dpi). Gene targeted by virus-induced gene silencing is indicated under each pair of bars. Each bar represents the average and standard deviation (SD) derived from sampling three stems from three independently silenced plants (one stem per plant). Data represent the mean $\pm$ SD ($n = 3$) and are representative of two independent experiments. Asterisks denote significant differences ($P < 0.05$) in the bacterial titer in silenced plants compared with TRV-only infected nonsilenced control plants at each time point, according to Student's *t* test.

in plants silenced for the *DES* gene, may indicate a role for DES-derived divinyl ethers in combination with other oxylipins in the basal defense reaction to *C. michiganensis* subsp. *michiganensis*.

HMGR-silenced plants inoculated with *C. michiganensis* subsp. *michiganensis* developed wilt symptoms much earlier and harbored more bacteria when compared with nonsilenced plants, suggesting a role for *HMGR* in the basal defense to *C. michiganensis* subsp. *michiganensis* infection. In potato tubers, both enzyme activity and mRNA levels of *HMGR* were shown to increase in response to pathogen infection and elicitor treatment (7,8,38,42). Direct manipulations of tuber *HMGR* levels by pretreatment with defense elicitors (arachidonic acid) or specific inhibitors of *HMGR* (mevinolin) significantly impacted disease responses to the soft-rotting bacterium, *E. carotovora* (42), suggesting that *HMGR* regulation plays a key role in mediating disease resistance.

A set of tomato transcription factors—Pti4, Pti5, and Pti6 (Pti:Pto-interacting)—were shown to activate defense responses when expressed in *Arabidopsis* (23). A *Pto*-responsive gene and Pti5, in particular, were induced 6- and 163-fold, respectively, in tomato inoculated with *C. michiganensis* subsp. *michiganensis* (2). Results reported here showed that susceptibility to disease and bacterial growth increased in plants silenced for the *PLK* gene. However, the bacterial titer increase in *PLK*-silenced plants was smaller when compared with plants silenced for the other five genes. This observation was consistent in both experimental repeats (Fig. 4; Supplementary Figure S2). Despite the smaller increase in *C. michiganensis* subsp. *michiganensis* titer, the fact that *PLK*-silenced plants showed advanced wilt symptoms indicates that other factors besides bacterial multiplication contribute to disease establishment and severity.

This study has identified six genes that play a significant role in the host basal defense-related reaction to *C. michiganensis* subsp. *michiganensis*. Utilizing *N. benthamiana* as an experimental host of *C. michiganensis* subsp. *michiganensis* adds to the growing set of tools that can be used to study this pathogen. In combination with transcript expression profiling experiments, SAGE, and other gene discovery methods, VIGS will be a powerful and effective approach to characterize the host–pathogen interaction and for identifying new target genes linked to plant tolerance or resistance to *C. michiganensis* subsp. *michiganensis*.

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