Putative Serine Protease Effectors of *Clavibacter michiganensis* Induce a Hypersensitive Response in the Apoplast of *Nicotiana* Species

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Clavibacter michiganensis subsp. *michiganensis* and *sepedonicus* cause diseases on solanaceous crops. The genomes of both subspecies encode members of the *pat-1* family of putative serine proteases known to function in virulence on host plants and induction of hypersensitive responses (HR) on nonhosts. One gene of this family in *C. michiganensis* subsp. *sepedonicus*, *chp-7*, is required for triggering HR in *Nicotiana tabacum*. Here, further investigation revealed that mutation of the putative catalytic serine residue at position 232 to threonine abolished the HR induction activity of Chp-7, suggesting that enzymatic activity is required. Purified Chp-7 triggered an HR in *N. tabacum* leaves in the absence of the pathogen, indicating Chp-7 itself is the HR elicitor from *C. michiganensis* subsp. *sepedonicus*. Ectopic expression of *chp-7* constructs in *N. tabacum* leaves revealed that Chp-7 targeted to the apoplast triggered an HR while cytoplasmic Chp-7 did not, indicating that Chp-7 induces the HR in the apoplast of *N. tabacum* leaves. Chp-7 also induced HR in *N. sylvestris*, a progenitor of *N. tabacum*, but not in other *Nicotiana* species tested. ChpG, a related protein from *C. michiganensis* subsp. *michiganensis*, also triggered HR in *N. tabacum* and *N. sylvestris*. Unlike Chp-7, ChpG triggered HR in *N. clevelandii* and *N. glutinosa*.

Well-adapted plant pathogens deploy effectors to suppress host defense responses, which is crucial for pathogen colonization and disease symptom development (Block et al. 2008). In gram-negative plant pathogens, proteinaceous effectors are mainly delivered into the host cells via the type III secretion system (T3SS) (Alfano and Collmer 2004). Some fungal and oomycete effectors secreted from pathogen haustoria are taken up by host plant cells mediated by an N-terminal RxLR-dEER motif in the effector proteins (Dou et al. 2008; Whisson et al. 2007). Certain pathogen effectors can be recognized directly or indirectly by plant resistance (R) proteins, leading to robust plant resistance, often accompanied by a hypersensitive response (HR), a type of cell death within the infected tissue (Caplan et al. 2008). Recognition may be direct, as exemplified by recognition of AVR-Pita from *Magnaporthe grisea* by the rice resistance (R) protein Pi-ta (Jia et al. 2000). It may also be

indirect, conceptualized as the ‘guard hypothesis’ in which a plant target (the guardee) is monitored by an R protein (the guard) for modifications by a pathogen effector. Conformational changes in or loss of the guardee protein activate the guard R protein (Dangl and Jones 2001). For example, the *Arabidopsis* R protein RPS5 recognizes the *Pseudomonas syringae* type III effector AvrPphB, which is a cysteine protease, by monitoring the associated guardee host protein PBS1, which is cleaved by AvrPphB (DeYoung et al. 2012; Shao et al. 2003). Recognition of effectors by R proteins can occur in intra- or intercellular spaces, though examples of the latter are relatively rare (Martin et al. 2003). Perhaps the best-studied effector–R protein pairs that occur in the apoplast consist of effectors secreted by the fungal pathogen *Cladosporium fulvum* and tomato Cf receptor-like proteins (RLP), such as the fungal apoplastic effector Avr2 and tomato Cf-2 (Rooney et al. 2005). Avr2 inhibits the host apoplastic protease Rcr3, leading to a conformational change that is, in turn, recognized by the R protein Cf-2 (Van’t Klooster et al. 2011).

Clavibacter michiganensis is a diverse species of gram-positive bacteria in family Microbacteriaceae. Most characterized isolates are plant pathogens but nonpathogenic isolates also occur (Zaluga et al. 2013). Currently, there are six subspecies identified within *C. michiganensis*, based on the host plants they colonize (Eichenlaub and Gartemann 2011; González and Trapiello 2014). Although they cause diseases on various major crop species including tomato, potato, maize, alfalfa, wheat, and bean, each of the *C. michiganensis* subspecies only infects a narrow range of plant hosts. For example, *C. michiganensis* subsp. *michiganensis* is the causal agent of bacterial canker disease on tomato (*Solanum lycopersicum*) and can infect the model plant *Nicotiana benthamiana* (Balaji et al. 2011). *C. michiganensis* subsp. *sepedonicus* causes bacterial ring rot disease on cultivated potato (*Solanum tuberosum*) and causes disease symptoms relatively quickly in inoculated eggplant and tomato, often used as artificial hosts for virulence assays (Larson 1944).

In *C. michiganensis*, full virulence on host plants is associated with several virulence factors. Symptom development caused by *C. michiganensis* subsp. *michiganensis* in tomato requires the two native plasmids pCM1 and pCM2. pCM1 carries *celA*, which encodes a β -1,4-endocellulase and is required for full virulence on tomato (Meletzus et al. 1993). *C. michiganensis* subsp. *sepedonicus* contains a gene homologous to *celA* that is also required for disease symptom development on potato (Laine et al. 2000). pCM2 carries *pat-1* (pCM2_0054), which encodes a putative serine protease of

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subfamily S1A and is also required for full virulence (Dreier et al. 1997). Additional genes encoding putative serine proteases homologous to *pat-1* have been identified in the *C. michiganensis* subsp. *michiganensis* genome and named *chromosomal homology of pat-1 (chp)* and *plasmidal homology of pat-1 (php)* genes (Burger et al. 2005, Gartemann et al. 2008). All the *chp* genes are clustered in a 129-kb pathogenicity island in the *C. michiganensis* subsp. *michiganensis* chromosome with lower than genome average %G+C content, suggesting they were acquired via horizontal gene transfer (Gartemann et al. 2008). Alignment of the amino acid sequences encoded by *pat-1* homologs revealed the conserved trypsin family serine protease motifs that contain the predicted catalytic histidine and serine residues (Stork et al. 2008). A loss-of-function mutation in one of the *chp* genes, *chpC* (CMM_0052), compromised colonization of *C. michiganensis* subsp. *michiganensis* and symptom development on tomato (Stork et al. 2008), and the expression of *chpC* is dependent on the presence of both plasmids in *C. michiganensis* subsp. *michiganensis* (Chalupowicz et al. 2010). In *C. michiganensis* subsp. *sepedonicus*, loci encoding homologs of *pat-1* are also present on the chromosome and plasmids, though they are not clustered within a pathogenicity island, presumably due to extensive genomic rearrangement events (Bentley et al. 2008). One close homolog of *pat-1*, named *chp-7* (CMS_2989), has been identified as an important virulence factor of *C. michiganensis* subsp. *sepedonicus* required for full symptom development in infected potato and eggplant but not for colonization (Nissinen et al. 2009). Like Pat-1, Chp-7 contains a predicted catalytic histidine at position 97 within a TAKHC motif and the predicted nucleophilic serine at position 232 within a GDSGG motif (Nissinen et al. 2009). Though no proteolytic activity has been demonstrated for any of the *pat-1* family serine proteases, the serine protease activity of Pat-1 is thought to be involved in virulence of *C. michiganensis* subsp. *michiganensis* in tomato, as exchanging the putative catalytic serine for threonine in Pat-1 rendered it unable to restore the virulence of the plasmid-free strain CMM100 (Burger et al. 2005).

Both *C. michiganensis* subsp. *michiganensis* and *sepedonicus* are known to secrete one or multiple proteinaceous HR elicitors (Alarcón et al. 1998; Nissinen et al. 1997). It has been reported that a *chpG* (CMM_0059) mutant of *C. michiganensis* subsp. *michiganensis* and a *chp-7* mutant of *C. michiganensis* subsp. *sepedonicus* lost the HR-eliciting ability in *Mirabilis jalapa* and *N. tabacum*, respectively, indicating that ChpG and Chp-7 are required for HR (Stork et al. 2008; Nissinen et al. 2009). However, it is not known if these proteins are the direct HR elicitors.

Gram-positive plant-pathogenic bacteria do not have any secretion system known to be functionally equivalent to the gram-negative T3SS. Most of the studied and suspected *C. michiganensis* virulence factors carry a signal peptide for secretion, suggesting they are secreted. This idea is supported by a recent proteomics study using the *C. michiganensis* subsp. *michiganensis* –tomato pathosystem, in which several of the *pat-1* family serine proteases, *ppa* family serine proteases, CelA, and other hydrolases were detected in both culture supernatants of bacteria grown in tomato xylem sap-supplemented minimal medium and the xylem sap from infected tomato plants (Savitor et al. 2012). Chp-7 contains a signal peptide similar to the one in Pat-1 and is predicted to be a secreted protein (Nissinen et al. 2009). It is not known whether any of the proteins secreted by subspecies *michiganensis* or *sepedonicus* enter plant cells.

In this study, we investigated the Chp-7-triggered HR in *N. tabacum*. We found that the HR-inducing ability requires the predicted catalytic serine residue of Chp-7. Purified Chp-7

triggered HR, indicating that Chp-7 is sufficient for triggering an HR and is the recognized elicitor. The recognition of Chp-7 by *N. tabacum* requires that Chp-7 is present in the apoplast. Both Chp-7 and ChpG are recognized in *N. sylvestris*, one of the two progenitor species of *N. tabacum*. ChpG but not Chp-7 is recognized in *N. clevelandii* and *N. glutinosa* as well, indicating that the two proteins do not have identical activities.

RESULTS

A point mutation in the predicted catalytic serine abolished the HR-inducing ability of Chp-7.

Alignment of the amino acid sequences of all the *pat-1* homologs from subspecies *michiganensis* and *sepedonicus* reveals the highly conserved serine protease motif GDSG(G/A/C)(P/F/A)V containing the predicted catalytic serine residue (Fig. 1A). To test whether the conserved serine residue at position 232 of Chp-7 is required for inducing HR in *N. tabacum* leaves, a complementation assay was carried out by introducing either the wild-type *chp-7* sequence or *chp-7ST* containing a Ser232Thr point mutation (Fig. 1A) into CmsC7X20, a *chp-7* knockout mutant derivative of *C. michiganensis* subsp. *sepedonicus* ATCC33113, previously created by inserting the chloramphenicol resistance cassette *cmx* into the coding region of *chp-7* via homologous recombination (Syverson 2011). Both constructs were expressed from the native promoter of *chp-7* and were fused to a C-terminal FLAG epitope tag. The constructs were cloned into the *Escheria coli*–*Clavibacter* shuttle vector pHN216 (Laine et al. 1996), resulting in pHNC7-F and pHNC7ST-F. The transformed CmsC7X20 strains were then assayed for the ability to induce HR in infiltrated *N. tabacum* leaves. Chp-7-FLAG restored the HR-inducing ability in CmsC7X20, while Chp-7ST-FLAG did not (Fig. 1B). Culture supernatants from CmsC7X20_216 containing the pHN216 empty vector, CmsC7X20_C7-F expressing Chp-7-FLAG, and CmsC7X20_C7ST-F expressing Chp-7ST-FLAG were collected. Immunoblotting showed that both Chp-7-FLAG and Chp-7ST-FLAG were secreted from CmsC7X20 and accumulated to similar levels in culture supernatants (Fig. 1C). These results indicated that the predicted catalytic serine residue in Chp-7 is required for its HR-inducing ability in *N. tabacum*.

Chp-7 triggers an HR in *N. tabacum* leaves in the absence of pathogen.

As Chp-7 is a secreted protein, it may be sufficient to trigger HR in *N. tabacum* leaves. To test this hypothesis, an epitope-tagged construct, *chp-7-6xHis*, driven by an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible promoter, was synthesized and introduced into *E. coli* for expression. Tandem histidine-tagged proteins from cell lysates of *E. coli* cultures induced with IPTG or uninduced were purified with cobalt-based affinity resin. The eluted proteins were analyzed by gel electrophoresis and immunoblotting. Chp-7-6xHis was detected as a band at the predicted mass of 28 kDa present in the IPTG-induced culture but not in uninduced cultures or cultures lacking the recombinant construct (Fig. 2B). Several contaminating *E. coli* proteins were present in eluates from all cultures. The eluted proteins were infiltrated into *N. tabacum* leaves following a dilution series. HR was observed in the areas infiltrated with the diluted eluate from the IPTG-induced *E. coli* culture but not in areas infiltrated with eluate from the uninduced culture, even at the undiluted level (Fig. 2A). The intensity of the cell death decreased as the Chp-7-6xHis concentration was reduced. This showed that Chp-7 itself is the HR elicitor and can trigger HR in *N. tabacum* leaves in the absence of *C. michiganensis* subsp. *sepedonicus*.

Fig. 1. Complementation of the hypersensitive response-inducing ability of a *Clavibacter michiganensis* subsp. *sepedonicus* Δ *chp-7* mutant in *Nicotiana tabacum* with wild-type *chp-7* or *chp-7ST*. **A**, Alignment of the amino acid sequences of *pat-1* homolog genes in *C. michiganensis* subsp. *michiganensis* and *sepedonicus*. Shading highlights highly conserved positions. An asterisk depicts the serine residue substituted to threonine in Chp-7ST. **B**, Bacterial suspensions in potassium phosphate buffer were infiltrated into the intercellular spaces of the leaf with needleless syringes. Wild type = ATCC 33113; Δ *chp-7* = CmsC7X20, a *chp-7* knockout mutant; Δ *chp-7/ΔEV* = CmsC7X20_216, CmsC7X20 transformed with empty vector pHN216; Δ *chp-7/chp-7+* = CmsC7X20_C7, CmsC7X20 transformed with pHNC7 carrying *chp-7*; Δ *chp-7/chp-7ST* = CmsC7X20_C7ST, CmsC7X20 transformed with pHNC7ST carrying *chp-7ST*. The infiltrated leaves were photographed at 48 h post-inoculation. Similar results were observed in three independent experiments. **C**, Immunoblot of the supernatants of bacterial cultures in YGM broth (De Boer and Copeman 1980) with anti-FLAG antibody. Sample inputs EV, C7-FLAG, and C7ST-FLAG are the culture supernatants of CmsC7X20_216, CmsC7X20_C7-F, and CmsC7X20_C7ST-F, respectively.

Chp-7 is not translocated into plant cells.

The genome of *C. michiganensis* doesn't contain genes for a secretion system known to deliver bacterial proteins into plant cells. That does not rule out the possibility that *C. michiganensis* is capable of translocating proteins into host cells through an as-yet-unknown mechanism. We were interested to determine whether Chp-7 is translocated into plant cells following secretion by *C. michiganensis* subsp. *sepedonicus*. To measure Chp-7 translocation, we used an adenylate cyclase (Cya) fusion assay that has been widely used to detect translocation of pathogen effectors into plant cells (Casper-Lindley et al. 2002; Schechter et al. 2004). Candidate translocated proteins are fused to the catalytic domain of adenylate

Fig. 2. Chp-7-6xHis protein purified from *Escherichia coli* is sufficient to trigger the hypersensitive responses (HR). **A**, *Nicotiana tabacum* leaves infiltrated with eluent containing purified Chp-7-6xHis protein diluted to various extents with potassium phosphate buffer (pH 6.5). A similar undiluted protein extract from an uninduced *E. coli* culture was used as a negative control. The infiltrated leaves were photographed at 48 h post-inoculation. The experiment was repeated twice and similar results were observed. **B**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluates from cell lysates from *E. coli* BL21(DE3) or its derivative transformed with pJEC7H and containing the *chp-7-6xHis* construct, with or without β -D-1-thiogalactopyranoside (IPTG) induction. The protein bands were visualized with Coomassie brilliant blue G-250 staining. The predicted mass of Chp-7-6xHis is 28 kDa. Asterisks indicate contaminating proteins from *E. coli*.

cyclase of the cyclolysin from *Bordetella pertussis*. Cya is activated in a calmodulin-dependent manner and catalyzes the conversion of ATP into 3',5'-cyclic AMP (Sory and Cornelis 1994). Detection of cAMP in infiltrated leaves serves as the measurement of candidate-Cya translocation. Accordingly, we constructed a *chp-7-cya* fusion. The activity of the Chp-7-Cya fusion protein was first tested in *N. tabacum* leaves by infiltrating the leaves with CmsC7X20_C7Cya expressing the Chp-7-Cya fusion. The Chp-7-Cya fusion protein triggered HR to a similar extent as the wild-type Chp-7, indicating Chp-7-Cya is active for HR induction (Fig. 3A). Because the onset of HR might interfere with cAMP production, the measurement of cAMP was carried out using infiltrated *N. benthamiana* leaves, as Chp-7 does not induce HR in this species. An effector-to-host analyzer (EtHAn) strain (Thomas et al. 2009) carrying the Cya construct with a T3SS signal (T3sp-Cya) was used as a positive control. Leaf samples from infiltrated areas were collected at 0, 10, and 24 h postinoculation (hpi). The leaf samples infiltrated with EtHAn:Cya showed approximately a thousandfold increase in cAMP concentration at 10 and 24 hpi compared with 0 hpi. The leaf samples infiltrated with CmsC7X20_C7Cya did not show increased cAMP production during the same time course (Fig. 3B), which suggests Chp-7-Cya is not translocated into plant cells following secretion from *C. michiganensis* subsp. *sepedonicus*.

Transient expression of *chp-7* secreted into the apoplast triggers HR in *N. tabacum* leaves.

To determine in which subcellular plant compartment Chp-7 acts to trigger an HR, three different *chp-7* constructs with or without a plant secretion signal were made for transient expression in *N. tabacum* (Fig. 4A). The *chp-7_{Apop}* and *chp-7ST_{Apop}* constructs contain an N-terminal plant signal peptide from tobacco PR1a (amino acids 1 to 31) known to target protein accumulation into the apoplast (van Esse et al. 2006; Wulff et al. 2004). The *chp-7_{Apop}* construct has the wild-type *chp-7* sequence, while *chp-7ST_{Apop}* contains a Ser₂₃₂Thr point mutation in the sequence. The *chp-7_{Cyto}* construct differs from *chp-7_{Apop}* only in that it omits the signal peptide sequence from PR1a and should direct Chp-7 accumulation to the cytoplasm of plant cells. All three constructs contain the intron PIV2 from the potato *ST-LS1* gene, to prevent expression in *Agrobacterium tumefaciens*, and a C-terminal hemagglutinin (HA)-prescission-biotin (HPB) tandem epitope tag (Qi and Katagiri 2009). The recombinant constructs were inserted into binary vector pMDC32 expressed from a *Cauliflower mosaic virus* (CaMV) 35S promoter for expression in *N. tabacum*. Following infiltration of *N. tabacum* leaves with *A. tumefaciens* strains carrying the recombinant constructs, apoplastic Chp-7 triggered HR at 2 days postinoculation (dpi), while neither secreted Chp-7^{S232T} nor cytoplasmic Chp-7 did (Fig. 4B). Amounts of all three versions of recombinant Chp-7 proteins were visualized by immunodetection with anti-HA antibody. Total proteins were extracted from the infiltrated leaves at 12 hpi, just before the HR triggered by apoplastic Chp-7 became visible. Proteins produced by *chp-7ST_{Apop}* or *chp-7_{Cyto}* accumulated to a much higher level compared with the level of apoplastic Chp-7 (Fig. 4C). The expression of *chp-7_{Apop}* is likely suppressed by the onset of HR, explaining its low accumulation. Biotinylation occurs predominantly in the cytosol and, to a lesser extent, in mitochondria and chloroplasts (Chapman-Smith and Cronan 1999). Proteins exported to the apoplast via the endoplasmic reticulum Golgi-mediated secretory routes are thus not biotinylated. Cytoplasmic Chp-7 was biotinylated while apoplastic Chp-7ST was not (Supplementary Fig. S2), which is consistent with their localization in the cytoplasm or apoplast, respectively. Therefore, the transient

expression of constructs including the PR1a signal peptide did deliver Chp-7 proteins into the apoplast, while the transient expression of *chp-7_{Cyto}* led to protein accumulation in the cytoplasm. Evidently, Chp-7 is only effective in inducing HR when it is present in the apoplast. Taken together, these experiments strongly suggest Chp-7 is recognized in the apoplast of *N. tabacum* leaves.

Fig. 3. Cya assay of Chp-7 translocation. **A**, Hypersensitive responses in *Nicotiana tabacum* elicited by Chp-7-Cya secreted by *Clavibacter michiganensis* subsp. *sepedonicus*. Wild type = ATCC 33113; Δ *chp-7* = CmsC7X20, a *chp-7* knockout mutant; Δ *chp-7/*EV = CmsC7X20_216, CmsC7X20 transformed with the empty vector pHN216; Δ *chp-7/pHNC7Cya* = CmsC7X20_C7Cya, CmsC7X20 transformed with pHNC7Cya carrying a *chp-7-cya* fusion. The infiltrated leaves were photographed at 48 h postinoculation (hpi). **B**, cAMP measurement in *N. benthamiana* leaf samples infiltrated with *C. michiganensis* subsp. *sepedonicus* expressing *chp-7* or *chp-7-cya*, or EtHAn expressing T3sp-cya at 0, 10, and 24 hpi. Means and standard errors were estimated from three replicates using a mixed-effect linear model. Different letters indicate significant differences ($P < 0.05$).

ChpG from *C. michiganensis* subsp. *michiganensis* is also recognized by *N. tabacum* in the apoplast.

C. michiganensis subsp. *michiganensis* secretes one or multiple proteins that can trigger HR in *Mirabilis jalapa* and *N. tabacum* (Alarcón et al. 1998). So far only *chpG* was

Fig. 4. Hypersensitive responses elicited by transient expression of *chp-7* targeted to the apoplast of *Nicotiana tabacum*. **A**, Schematics of the *chp-7* transient expression constructs. 35S = *Cauliflower mosaic virus* 35S promoter; sp = signal peptide region of the tobacco *PR1a* gene; piv2 = second intron of the potato *LS-ST1* gene; HPB = hemagglutinin (HA)-prescission-biotin tag. **B**, *N. tabacum* leaf infiltrated with *Agrobacterium tumefaciens* GV3101 or its derivatives GV_C7HPB_Apop, carrying 35S::chp-7_{Apop}; GV_C7STHPB_Apop, carrying 35S::chp-7ST_{Apop}; or GV_C7HPB_Cyto, carrying 35S::chp-7_{Cyto}. The infiltrated leaves were photographed at 48 h postinoculation (hpi). Similar results were observed in three independent experiments. **C**, Immunoblot with monoclonal anti-HA antibody of total protein extracts from the *N. tabacum* infiltrated leaf areas at 12 hpi. Two independent samples were loaded for chp-7ST_{Apop} and chp-7_{Cyto}. The predicted mass of Chp-7_{Cyto} is 41 kDa. Ponceau S staining was used as a loading control.

reported to be required for *C. michiganensis* subsp. *michiganensis* to trigger an HR in *M. jalapa* (Stork et al. 2008), however, the identity of one or more elicitors in *N. tabacum* remains unknown. The genome of *C. michiganensis* subsp. *michiganensis* contains seven intact *pat-1* homolog genes (Gartemann et al. 2008). Among these, the pCM2 plasmid-encoded *pat-1*, *phpA*, and *phpB* have the highest amino acid sequence similarity to *chp-7* (Supplementary Fig. S1). Therefore, we tested the idea that one or more of these genes functions in a similar way as *chp-7*. The Cm15-2.0sm strain (*C. michiganensis* subsp. *michiganensis* wild-type), the CMM100 strain (*C. michiganensis* subsp. *michiganensis* Δ pCM1 Δ pCM2) and the CMM101chpG β (*C. michiganensis* subsp. *michiganensis* Δ chpG Δ pCM2) strain were infiltrated in *N. tabacum* leaves and responses were examined at 2 dpi. While HR occurred in the area infiltrated with Cm15-2.0sm or CMM100, it was not observed in the leaf area infiltrated with CMM101chpG β , while some chlorosis developed (Fig. 5A). CMM100 lacks *pat-1*, *phpA*, and *phpB*, as these genes are carried on pCM2. Therefore, none of these genes are required for HR in *N. tabacum*. CMM101chpG β lacks *chpG* as well as *pat-1*, *phpA*, and *phpB*. Its failure to induce HR indicates that *chpG* is required. Consistently, full-length *pat-1*, *phpA*, or *phpB* expressed from the *chp-7* promoter all failed to restore the HR-triggering phenotype of CmsC7X20 in a complementation assay (Supplementary Fig. S3). To test if apoplastic accumulation of ChpG is sufficient to trigger an HR in *N. tabacum*, a recombinant construct encoding the tobacco *PR1a* signal peptide fused to ChpG and expressed from the CaMV 35S promoter was delivered into *N. tabacum* leaves through *Agrobacterium*-mediated transformation. A similar construct of ChpC was made and expressed. HR cell death was observed following transient expression of the *PR1a*-ChpG construct but not in the leaf area transiently expressing the *PR1a*-ChpC fusion protein. These results show that ChpG is the HR elicitor of *C. michiganensis* subsp. *michiganensis* in *N. tabacum* and that ChpG, like Chp-7, is recognized in the apoplast.

The ability to recognize Chp-7 in *Nicotiana* species arises from the *Sylvestres* section.

N. tabacum is an allotetraploid plant whose S and T genomes are derived from two wild progenitors, *N. sylvestris* and *N. tomentosiformis*, respectively (Kenton et al. 1993). To estimate the origin of one or more genes required for recognizing Chp-7 and ChpG in *N. tabacum*, wild-type and mutant strains of *C. michiganensis* subsp. *michiganensis* and *sepedonicus* were infiltrated into leaves of *N. sylvestris* and *N. tomentosiformis*. *N. sylvestris* behaved like *N. tabacum*, as HR was triggered by ATCC33113 (subsp. *sepedonicus* wild-type), Cm15-2.0sm (subsp. *michiganensis* wild-type), and CMM100 (subsp. *michiganensis* Δ pCM1 Δ pCM2), but not by CmsC7X20 (subsp. *sepedonicus* Δ chp-7) or CMM101chpG β (subsp. *michiganensis* Δ chpG Δ pCM2). Transient expression of apoplastic Chp-7 and ChpG both triggered HR in *N. sylvestris*, demonstrating that these proteins are sufficient for triggering HR in this species, as well. In contrast, none of the strains or transiently expressed proteins triggered HR in *N. tomentosiformis* (Fig. 5B and C). Interestingly, all the *C. michiganensis* subsp. *michiganensis* strains induced some chlorosis in both *N. sylvestris* and *N. tomentosiformis*, while *C. michiganensis* subsp. *sepedonicus* strains did not. These results show that the ability of *N. tabacum* to respond to Chp-7 and ChpG is encoded in the S genome.

To test whether the ability to recognize Chp-7 and ChpG is a common trait among *Nicotiana* species, strains of *C. michiganensis* subsp. *sepedonicus* and *michiganensis* were infiltrated into leaves of *N. glutinosa*, *N. attenuata*, *N. rustica*, *N. clevelandii*, *N. benthamiana*, and *N. glauca*, each representing

a section in the *Nicotiana* phylogeny. *N. clevelandii* and *N. glutinosa* both developed a *chpG*-dependent HR in response to *C. michiganensis* subsp. *michiganensis*, though in *N. glutinosa*, the ChpG-triggered HR took more than 2 days to become visible (Fig. 6). In both species, transient expression of the *PR1a-ChpG* recombinant construct was sufficient to trigger HR. However, neither of the two species recognized *C. michiganensis* subsp. *sepedonicus* or Chp-7. The foliar responses at 3 dpi of all the *Nicotiana* species tested are summarized in Table 1. As some species recognize ChpG but not Chp-7, the activities of these proteins must be different, even though they induce similar responses in *N. tabacum*.

DISCUSSION

Extensive research on gram-negative phytopathogenic bacteria such as *Pseudomonas syringae* has provided insights into pathogen virulence strategies and host defense responses. However, much less is known about their gram-positive plant pathogenic counterparts in the family Microbacteriaceae, such as *Clavibacter michiganensis*. Gram-positive bacteria lack outer membranes and most have a thick cell wall comprised of peptidoglycan. These distinct structural differences relative to gram-negative bacteria necessitated evolution of different strategies for interacting with plant hosts. Lacking a secretion system equivalent to gram-negative T3SS and T4SS makes the apoplastic space the primary interface for the interactions between *C. michiganensis* and plants. In this study, we showed that effector proteins secreted by *C. michiganensis* subsp. *sepedonicus* and *michiganensis* are recognized in the apoplast in *N. tabacum*.

Gene-for-gene type resistance is mediated by plant R proteins and specifically recognized pathogen effectors. The only group of R proteins known to recognize extracellular effectors are the RLP class, including tomato Cf and Ve proteins, which contain extracellular leucine-rich repeat (eLRR) domains (van Ooijen et al. 2007). The R protein Cf-2 initiates an HR upon

detecting conformational changes in the extracellular cysteine protease Rcr3 induced by binding of *Cladosporium fulvum* Avr2 as an uncompetitive inhibitor (Van't Klooster et al. 2011). The recognition of Chp-7 and ChpG in the apoplast may be mediated by one or more R proteins from *N. tabacum* that contain an eLRR domain. As the substrates of Chp-7 and ChpG remain unidentified at this point, it is equally possible that the recognition occurs by Chp-7 or ChpG interacting with one or more guarder proteins in the apoplast monitored by an R protein or by direct interaction with one or more R proteins as its substrate, though to our knowledge there is no known example for the latter scenario.

The *C. michiganensis* subsp. *michiganensis* genome has 10 *pat-1* homologs (among them *chpA*, *chpB*, and *chpD* are pseudogenes), and the *C. michiganensis* subsp. *sepedonicus* genome encodes 11 *pat-1* homologs (only *chp-1* is a pseudogene) (Bentley et al. 2008; Gartemann et al. 2008). Single mutations in *pat-1* homolog genes, including *pat-1*, *chpC*, and *chp-7*, resulted in compromised symptom development or in planta colonization (Dreier et al. 1997; Nissinen et al. 2009; Stork et al. 2008), indicating the *pat-1* family of putative serine proteases plays crucial and nonredundant roles for pathogen virulence. In addition, the observation that *N. clevelandii* and *N. glutinosa* can recognize ChpG but not Chp-7 indicates different plant proteins are involved. The lack of redundancy suggests that they function independently, perhaps by interacting with different host targets.

Interestingly, we observed chlorosis symptoms in the leaves of multiple *Nicotiana* species infiltrated with *C. michiganensis* subsp. *michiganensis* while subsp. *sepedonicus* induced chlorosis only in *N. rustica* (Table 1). The chlorosis likely occurred independently of ChpG, as the intensity of chlorosis induced by CMM101chpG β was indistinguishable from those by other *C. michiganensis* subsp. *michiganensis* strains (Fig. 5C). In the *Nicotiana* species that can recognize ChpG, such as *N. tabacum*, this *C. michiganensis* subsp. *michiganensis*-induced chlorosis

Fig. 5. Recognition of Chp-7 and ChpG in *Nicotiana tabacum* and one of its progenitor species, *N. sylvestris*. *Clavibacter michiganensis* suspensions in potassium phosphate buffer or *Agrobacterium tumefaciens* suspensions in MES buffer (10 mM MES, 10 mM MgSO₄, pH 5.6) were infiltrated into leaves of **A**, *N. tabacum*, **B**, *N. sylvestris*, and **C**, *N. tomentosiformis*. The bacterial inocula used were, from *C. michiganensis* subsp. *michiganensis* (Cmm), wild type (WT) = Cmm15-2.0sm; Δ chpG Δ pCM2 = CMM101chpG β ; and Δ pCM1 Δ pCM2 = CMM100; from *C. michiganensis* subsp. *sepedonicus* (Cms) WT = ATCC 33113 and Δ chp-7 = CmsC7X20; and 35S::chp-7_{Apop} = GV_C7HPB_Apop; 35S::chpG_{Apop} = GV_chpG_Apop; and 35S::chpC_{Apop} = GV_chpC_Apop. The infiltrated leaves were photographed at 48 h postinoculation. Similar results were observed in at least six leaves for each experiment. Scale bars = 160 mm.

symptom was likely masked by HR. The cause of the chlorosis and whether it associates with virulence of *C. michiganensis* subsp. *michiganensis* remain to be elucidated in further investigations.

The expression of most of the studied virulence factors secreted by *C. michiganensis*, including Pat-1, CelA, and other cell wall-degrading enzymes are inducible upon entering host tissues or upon addition of xylem sap into culture media (Chalupowicz et al. 2010). Moreover, the induced expression of virulence genes located on plasmids or within the pathogenicity island on the chromosome, called the PAI region, is interdependent. Deletion of either plasmid of *C. michiganensis*

subsp. *michiganensis* resulted in dramatic reductions in expression of genes within the PAI region, and deletion of the PAI region also led to reduced induction of *pat-1* and *celA* expression (Chalupowicz et al. 2010). However, *chp-7* is not inducible during infection of potato leaves (Holtmark et al. 2008) and the protein extract precipitated from culture supernatant of *C. michiganensis* subsp. *sepedonicus* triggered HR (Nissinen et al. 1997). In this study, Chp-7-FLAG was detected in the supernatant of YGM broth cultures (Fig. 1C) (De Boer and Copeman 1980), suggesting it is constitutively expressed in culture in rich defined medium, which is consistent with the previous observations.

Fig. 6. *Clavibacter michiganensis* subsp. *michiganensis* elicits *chpG*-dependent hypersensitive responses in *Nicotiana clevelandii* and *N. glutinosa*. *C. michiganensis* suspensions in potassium phosphate buffer or *Agrobacterium tumefaciens* suspensions in MES buffer (10 mM MES, 10 mM MgSO₄, pH 5.6) were infiltrated into leaves of **A**, *N. clevelandii* and **B**, *N. glutinosa*. The bacterial inocula used were *C. michiganensis* subsp. *michiganensis* (*Cmm*) WT = Cmm15-2.0sm; Δ *chpG* Δ *pCM2* = CMM101*chpG* Δ ; and Δ *pCM1* Δ *pCM2* = CMM100; *C. michiganensis* subsp. *sepedonicus* (*Cms*) WT = ATCC 33113 and Δ *chp-7* = CmsC7X20; and 35S::*chp-7*_{Apop} = GV_C7HPB_Apop; 35S::*chpG*_{Apop} = GV_35S::*chpG*_Apop; 35S::*chpC*_{Apop} = GV_35S::*chpC*_Apop. The infiltrated leaves of *N. clevelandii* and *N. glutinosa* were photographed at 48 and 72 h postinoculation, respectively. Both experiments were repeated three times with similar results. Scale bars = 160 mm.

Table 1. Foliar responses of *Nicotiana* species infiltrated with *Clavibacter michiganensis* subsp. *sepedonicus* and *michiganensis*^a

Plant species	<i>C. michiganensis</i> subsp. <i>sepedonicus</i>			<i>C. michiganensis</i> subsp. <i>michiganensis</i>				
	WT	Δ <i>chp-7</i>	35S:: <i>chp-7</i>	WT	Δ <i>chpG</i> Δ <i>pCM2</i>	Δ <i>pCM1</i> Δ <i>pCM2</i>	35S:: <i>chpG</i>	35S:: <i>chpC</i>
<i>N. tabacum</i>	HR	ND	HR	HR	Chlorosis	HR	HR	ND
<i>N. sylvestris</i>	HR	ND	HR	HR	Chlorosis	HR	HR	ND
<i>N. tomentosiformis</i>	ND	ND	ND	Chlorosis	Chlorosis	Chlorosis	ND	ND
<i>N. benthamiana</i>	ND	–	–	Chlorosis	Chlorosis	Chlorosis	–	–
<i>N. glutinosa</i>	ND	ND	ND	HR	Chlorosis	HR	HR	ND
<i>N. rustica</i>	Chlorosis	Chlorosis	–	Chlorosis	Chlorosis	Chlorosis	–	–
<i>N. glauca</i>	ND	–	–	ND	–	–	–	–
<i>N. clevelandii</i>	ND	–	ND	HR	ND	HR	HR	ND
<i>N. attenuata</i>	ND	–	–	ND	–	–	–	–

^a Plant foliar responses were recorded at 3 days postinoculation. WT = wild type, HR = hypersensitive response, ND = none detected, –, not tested.

Pat-1 and Chp-7 both contain an LPGSG motif in the C-terminal region, which resembles the LPxTG sortase-processing motif for sortase-mediated covalent attachment to the cell wall of gram-positive bacteria (Burger et al. 2005; Nissinen et al. 2009). However, the detection of Chp-7-FLAG in the culture supernatant by immunoblot with monoclonal anti-FLAG antibody suggests the C-terminal region downstream of the LPGSG motif was not cleaved by the *C. michiganensis* subsp. *sepedonicus* sortase. Therefore, the LPGSG motif in Chp-7 is either not a bona fide sortase-processing motif or at least a subpool of Chp-7 proteins are not anchored to the *C. michiganensis* subsp. *sepedonicus* cell wall and HR induction can be independent of cell-wall anchoring of Chp-7.

The substrates for the putative serine proteases involved in pathogenicity or induction of HR by *C. michiganensis* remain unknown. There is no detectable proteolytic activity in *C. michiganensis* subsp. *michiganensis* culture supernatants assayed with various generally used substrates (Burger et al. 2005) or using purified Chp-7-6xHis from *E. coli* assayed with azocoll (this study; data not shown). Although the conserved motifs in the sequences of *pat-1* family genes resemble trypsin family serine proteases, they may also be some other kind of hydrolase with a nonprotein substrate. If they are proteases, they may have strict substrate specificity. Furthermore, though the amino acid sequences of Chp-7 and Pat-1 share 78% identity, Pat-1 cannot restore the HR induction in the *chp-7* mutant strain, suggesting the variable residues dictate interactions with their specific substrates. Nevertheless, the point mutation replacing the serine at position 232 with threonine in Chp-7 abolished its HR-inducing ability in *N. tabacum* (Figs. 1B and 4B), indicating the serine residue predicted to function

as a nucleophile is required in this context and the behavior of Chp-7 is recognized by *N. tabacum*. The substrate of Chp-7 is likely of plant origin, as the purified Chp-7-6xHis is able to trigger HR in the absence of *C. michiganensis* subsp. *sepedonicus* (Fig. 2).

We demonstrated that Chp-7 is sufficient to trigger an HR in the apoplast of *N. tabacum* leaves. By analogy to the Cf system in tomato, we hypothesize that Chp-7 cleaves a plant-derived apoplastic target, leading to conformational changes in the target, which, in turn, activate an eLRR domain-containing R protein and HR ensues. Investigation of the virulence functions of Chp-7 and identification of one or more Chp-7 substrates in *N. tabacum* and the cognate R protein would allow this hypothesis to be tested.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table 2. The *C. michiganensis* wild-type strain was cultured on YGM medium, CmsC7X20 was cultured on YGM medium supplemented with 10 µg of chloramphenicol per milliliter, and CmsC7X20 strains transformed with constructs in the pHN216 vector were cultured on YGM medium supplemented with chloramphenicol at 10 µg/ml and kanamycin at 25 µg/ml. The *chp-7* knock-out mutant CmsC7X20 was generously provided by R. L. Syverson. It was created by disrupting the *chp-7* coding region in the genome of *C. michiganensis* subsp. *sepedonicus* with a chloramphenicol resistance cassette, *cmx*, through homologous recombination with a suicide vector pRLSTC7X2 (Syverson 2011). CMM100 (Meletzus and Eichenlaub 1991)

Table 2. Bacterial strains used in this study^a

Strain	Description	Source/reference
<i>Clavibacter michiganensis</i> subsp. <i>sepedonicus</i>		
ATCC 33113	Type strain, wild type, virulent and HR ⁺	ATCC; Bentley et al. 2008
CmsC7X20	ATCC 33113 <i>chp-7</i> (CMS_2989):: <i>cmx</i> , knockout of <i>chp-7</i> created by homologous recombination with pRLSTC7X2; Cm ^r	Syverson 2011
CmsC7X20_216	CmsC7X20 transformed with pHN216; Cm ^r , Km ^r	This study
CmsC7X20_C7	CmsC7X20 transformed with pHNC7; Cm ^r , Km ^r	This study
CmsC7X20_C7ST	CmsC7X20 transformed with pHNC7ST; Cm ^r , Km ^r	This study
CmsC7X20_C7-F	CmsC7X20 transformed with pHNC7-F; Cm ^r , Km ^r	This study
CmsC7X20_C7ST-F	CmsC7X20 transformed with pHNC7ST-F; Cm ^r , Km ^r	This study
CmsC7X20_C7Cya	CmsC7X20 transformed with pHNC7Cya; Cm ^r , Km ^r	This study
CmsC7X20_Pat1	CmsC7X20 transformed with pHNC7pro_pat1; Cm ^r , Km ^r	This study
CmsC7X20_PhpA	CmsC7X20 transformed with pHNC7pro_phpA; Cm ^r , Km ^r	This study
CmsC7X20_PhpB	CmsC7X20 transformed with pHNC7pro_phpB; Cm ^r , Km ^r	This study
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>		
Cm15-2.0sm	Representative wild-type isolate, spontaneous streptomycin resistant, virulent and HR ⁺ ; Sm ^r	A. K. Vidaver
CMM100	Cured derivative of wild-type <i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB 382 strain, lacking both native plasmids pCM1 and pCM2, avirulent	Meletzus and Eichenlaub 1991
CMM101chpGβ	Mutant strain created by homologous recombination knock-out <i>chpG</i> (CMM_0059) in CMM101 background, lacking native plasmid pCM2; Cm ^r	Stork et al. 2008
<i>Agrobacterium tumefaciens</i>		
GV3101	GV3101(pMP90); Rf ^r , Gm ^r	Koncz and Schnell 1986
GV_C7HPB_Cyto	GV3101 transformed with pMDC-C7HPB; Rf ^r , Gm ^r , Km ^r	This study
GV_C7HPB_Apop	GV3101 transformed with pMDC-spC7HPB; Rf ^r , Gm ^r , Km ^r	This study
GV_C7STHPB_Apop	GV3101 transformed with pMDC-spC7STHPB; Rf ^r , Gm ^r , Km ^r	This study
GV_chpG_Apop	GV3101 transformed with pEG100-spG; Rf ^r , Gm ^r , Km ^r	This study
GV_chpC_Apop	GV3101 transformed with pEG100-spC; Rf ^r , Gm ^r , Km ^r	This study
<i>Escherichia coli</i>		
BL21(DE3)	Strain BL21 (DE3) carrying pLysS; Cm ^r	Invitrogen
BL_C7H	BL21(DE3) transformed with pJEC7H; Cm ^r , Km ^r	This study

^a HR = hypersensitive response, ATCC = American Type Culture Collection, Cm = chloramphenicol, Km = kanamycin, Gm = gentamicin, Rf = rifampicin, and Sm = streptomycin.

and CMM101chpG β (Stork et al. 2008) have been described. All *C. michiganensis* cultures were started from glycerol stocks at -80°C and grown at 25.5°C for 4 days before use. *Escherichia coli* BL21 (DE3) pLysS (Invitrogen) and its derivatives were grown on Luria-Bertani (LB) medium at 37°C and supplemented with chloramphenicol at $34\text{ }\mu\text{g/ml}$ and kanamycin at $50\text{ }\mu\text{g/ml}$ as necessary. *Agrobacterium tumefaciens* GV3101 (Koncz and Schell 1986) and its derivatives were grown on LB medium at 28°C and were supplemented with gentamycin at $20\text{ }\mu\text{g/ml}$ and kanamycin at $25\text{ }\mu\text{g/ml}$ as necessary.

Cloning and site-specific mutagenesis of *chp-7*.

For complementation studies, CmsC7X20 was transformed with pHNC7 or pHNC7ST created by inserting wild-type *chp-7* or *chp-7*^{S232T} into shuttle vector pHN216 (Laine et al. 1996). The *chp-7*^{S232T} derivative was made by polymerase chain reaction (PCR) using KOD Hot Start DNA polymerase (Novagen) using pRLSTC7 (Syverson 2011) as template, with primers C7_S232T_F and C7_S232T_R, followed by self-ligation with T4 DNA ligase (New England Biolabs), resulting in pRLSTC7ST. The *EcoRI* fragment containing *chp-7*^{S232T} was ligated into pHN216, resulting in pHNC7ST. C-terminal FLAG epitope tags were inserted into pHNC7 and pHNC7ST by PCR with primers C7_OLpHN_F, C7_FLAG_R, and FLAG_OLpHN_R, followed by ligation using the Gibson assembly cloning kit (New England Biolabs) into pHN216 cut open with *EcoRI*, resulting in pHNC7-F and pHNC7ST-F. Both plasmids were transformed into competent cells of CmsC7X20 by electroporation at 1.5 kV in cuvettes with a 1-mm gap width as previously described (Nissinen et al. 2009). Colonies were selected on YGM medium supplemented with chloramphenicol at $10\text{ }\mu\text{g/ml}$ and kanamycin at $25\text{ }\mu\text{g/ml}$.

The DNA sequence of *chp-7* was commercially codon-optimized for expression in *E. coli*. The optimized sequence *chp-7E* was cloned into expression vector pJExpress401 (DNA2.0), resulting in pJEC7H. The plasmid was transformed into *E. coli* BL21 (DE3) pLysS (Invitrogen), using the heat-shock method, according to the manufacturer's protocol. Colonies were selected on LB medium supplemented with chloramphenicol and kanamycin.

The entry clones for transient expression experiments were created by TOPO TA cloning of the *chp-7* or *chp-7*^{S232T} coding sequences, omitting the native signal peptide region and using the primers Chp7v35_F and C7+279R, into pCR8/GW/TOPO vector (Invitrogen), resulting in pCR8_C7 and pCR8_C7ST, respectively. All the *chp-7* constructs used for transient expression in plants contain the second intron *PIV2* from the potato *ST-LS1* gene (Vancanneyt et al. 1990), which was cloned from pAD1339 (Ke et al. 2001) using primers OLC7KpnI_PIV2_F and OLC7KpnI_PIV2_R. The resulting fragment contains sequences overlapping ones flanking the unique *KpnI* site in the wild-type *chp-7* sequence. The *PIV2* fragment was ligated using the Gibson assembly cloning kit (New England Biolabs) into pCR8_C7 and pCR8_C7ST cut open with *KpnI*. The *chp-7* construct for transient expression and targeting to the apoplast was then made by ligating the DNA sequence of the signal peptide of *N. benthamiana* *PR1a*, cloned using the primers PR1a_sp_F and OLC7v35_PR1a_sp_R, to the N-terminus of the *chp-7v35* sequence, cloned using the primers Chp7+35F and C7+279R, resulting in pCR8_spC7 and pCR8_spC7ST. The entry clones were then recombined into binary vector pMDC32, containing a C-terminal HPB epitope (Qi and Katagiri 2009), through LR reactions (Invitrogen), resulting in pMDC-C7HPB, pMDC-spC7HPB, and pMDC-spC7STHPB.

All the constructs derived from PCR products were verified by sequencing. The sequences of the PCR primers are listed in the Supplementary Table S1.

Cloning of *pat-1* homologs

from *C. michiganensis* subsp. *michiganensis* Cm15-2.0sm.

To create a linearized plasmid containing the *chp-7* native promoter pGemC7pro, pRLSTC7 (Syverson 2011) was amplified by PCR, using the primers Pro_c7_R and Term_c7_F. *pat-1*, *phpA*, and *phpB* were cloned as PCR fragments using KOD Hot Start DNA polymerase (Novagen, CA) with the primers OLC7pro_pat1_F and OLC7term_pat1_R, OLC7pro_phpA_F and OLC7term_phpA_R, and OLC7pro_phpB_F and OLC7term_phpB_R, respectively, using DNA from Cm15-2.0sm as the template. pGemC7pro_pat1, pGemC7pro_phpA and pGemC7pro_phpB were subsequently constructed by ligating the pGemC7pro vector with the PCR fragment of *pat-1*, *phpA* and *phpB*, respectively, using the Gibson assembly cloning kit (New England Biolabs, MA). The three plasmids were then digested with *EcoRI* and the fragments were ligated with pHN216 cut open with *EcoRI*, resulting in pHNC7pro_pat1, pHNC7pro_phpA, and pHNC7pro_phpB.

chpG was cloned as a PCR fragment using KOD Hot Start DNA polymerase (Novagen) with the primers OLPR1a_chpGn38_F and OLC7_chpG_R, using DNA from Cm15-2.0sm as the template and ligated with the fragment PCR-amplified from pCR8_spC7 with the primers PR1a_R and pCR8attL2_F, resulting in pCR8_spG. This entry clone was then recombined into pEARLEYGATE100 (Earley et al. 2006) using the LR reaction (Invitrogen) to generate pEG100_spG. pEG100_spC was cloned following the same procedure with the starting primers OLPR1a_chpCt33_F and OLC7_chpC_R.

All the clones from Cm15-2.0sm were verified by sequencing. The sequences of all the clones are identical to the sequences in the reference genome *C. michiganensis* subsp. *michiganensis* NCPPB 382.

Plant assays.

Tobacco plants were grown to 10- or 11-leaf stages in a Conviron growth chamber at 22°C and 75% relative humidity with a 16-h photoperiod under $100\text{ }\mu\text{M m}^{-2}\text{ s}^{-1}$ fluorescent illumination. For complementation assays, the seventh and eighth leaves were infiltrated, using needleless syringes, with *C. michiganensis* strains suspended in potassium phosphate buffer ($3.3\text{ mM K}_2\text{HPO}_4$, $6.7\text{ mM K}_2\text{HPO}_4$, pH 6.5) to an optical density at 600 nm (OD_{600}) = 0.4. The infiltrated leaves were examined for the HR phenotype at 48 hpi, unless otherwise noted. For transient expression assays, *A. tumefaciens* GV3101 strains were suspended to a final OD_{600} = 0.2 in MES buffer (10 mM MES , 10 mM MgSO_4 , pH 5.6) containing $150\text{ }\mu\text{M}$ acetosyringone and were incubated at 27°C for 90 min, before infiltrating into tobacco leaves. The infiltrated leaves were examined for the HR phenotype at 48 hpi.

Expression and purification of Chp-7 from *E. coli*.

E. coli BL21 (DE3) pLysS carrying pJEC7H was grown in 20 ml of LB broth supplemented with chloramphenicol and kanamycin. Cultures were grown with shaking at 250 rpm at 37°C until early log phase (OD_{600} = 0.2), at which point IPTG was added to a final concentration of $500\text{ }\mu\text{M}$ to induce *chp-7* expression. Cultures were then transferred to 25°C with continued shaking at 250 rpm for 3 h. After incubation, the *E. coli* cells were suspended in lysis buffer ($50\text{ mM NaH}_2\text{PO}_4$, 300 mM NaCl , 0.5% (vol/vol) NP-40, pH 7.6). Tandem histidine-tagged proteins from the cell lysates were pulled down by cobalt-based TALON metal affinity resin chromatography (Clontech). Bound proteins were eluted with imidazole buffer ($50\text{ mM KH}_2\text{PO}_4$, 300 mM KCl , 200 mM imidazole , pH 7.8). The resulting eluate was desalted in exchange to potassium phosphate-KCl buffer ($3.3\text{ mM K}_2\text{HPO}_4$, $6.7\text{ mM KH}_2\text{PO}_4$, 50 mM KCl , pH 6.5), using Quick Spin Protein Columns (Roche).

Cya assay.

The DNA sequence of *cya* was cloned from pDD62 (Casper-Lindley et al. 2002) as a fragment using the primers Cya_OLC7_F and Cya_OLpGEM_R. The fragment contains a 5' overlapping sequence in-frame to the 3' end of *chp-7* and was ligated with linearized pRLSTC7 (Syverson 2011), using the Gibson assembly cloning kit (New England Biolabs). The *chp-7-cya* fusion construct as a plasmid was then digested with *EcoRI* restriction enzyme and was ligated into *EcoRI* sites of pHN216, resulting in pHNC7Cya, and was transformed into competent cells of CmsC7X20. Inoculum was prepared as described above and was infiltrated into *N. benthamiana* leaves. Leaf samples were collected at 0, 10, and 24 hpi and were processed to extract cAMP, as described (Casper-Lindley et al. 2002). The cAMP concentration was measured with a cAMP enzyme-linked immunosorbent assay kit (ENZO Life Sciences), following the manufacturer's protocol. Statistical analysis was performed using the lme4 package (Bates et al. 2014) in the R environment.

Immunoblots.

Protein samples in 1× sodium dodecyl sulfate (SDS) loading buffer were boiled for 5 min, were separated on 10% SDS-polyacrylamide gels, and were blotted to polyvinylidene difluoride membranes according to the manufacturer's instructions (Bio-Rad). Membranes were incubated with 5% (wt/vol) nonfat milk dissolved in TBST buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1% [vol/vol] Tween-20, pH 7.6), before incubating with anti-FLAG (clone M2) (Sigma), anti-HA (clone 3F10) (Roche), or antibiotin antibody (clone D5A7) (Cell Signaling), corresponding to the epitope in samples. After washing the membrane with TBST buffer, the secondary antibody antirat immunoglobulin G (IgG) horseradish peroxidase (HRP) conjugate (Cell Signaling) or antimouse IgG HRP conjugate (Promega) was incubated with the membrane. All the antibodies used were diluted according to their manufacturers' recommendations. The blots were developed using the Pierce ECL Western blotting substrate (Pierce Biotechnology).

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