J. Microbiol. Biotechnol. (2010), **20**(1), 54–62
doi: 10.4014/jmb.0905.05027
First published online 10 October 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

HpaXm from *Xanthomonas citri* subsp. *malvacearum* is a Novel Harpin with Two Heptads for Hypersensitive Response

Miao, Wei-Guo, Cong-Feng Song, Yu Wang, and Jin-Sheng Wang*

Department of Plant Pathology, Nanjing Agricultural University, Weigang 1, Nanjing 210095, China

Received: May 16, 2009 / Revised: August 9, 2009 / Accepted: August 14, 2009

A novel harpin-like protein, HpaXm, was described from cotton leaf blight bacteria, *Xanthomonas citri* subsp. *malvacearum*. The *hpaXm* was found to be localized between *hrp2* and *hrcC*. A phylogenetic analysis of the complete amino acid sequence or solely the 13 highly conserved residues H_2N-SEKQLDQLLTQLI-COOH in the N-terminal α-helix indicates that HpaXm is evolutionarily closer to HpaGXag and HpaXac than to Hpa1Xoo and Hpa1Xoc. A synthesized peptide containing two heptads, 39-LDQLLTQ-LIMALLQ-52, from the N-terminal α-helical region of HpaXm displayed comparable activity in inducing a hypersensitive response, but two other synthesized derivatives, HpaXmΔT44C and HpaXmΔM48Q, showed reduced HR-triggering activity. The data from a GST trap test revealed that HpaXm was released into the extracellular medium, hpaXm mutant deficient for the leader peptide (1-MNSLNTQIGANSSFL-15) was unable to be secreted outside cells but still induced HR in tobacco leaves.

Keywords: *Xanthomonas citri* subsp. *malvacearum*, HpaXm, hypersensitive response, secondary structure, two heptads

Many Gram-negative phytopathogenic bacteria, along with many animal pathogens, employ type III secretion systems (TTSS or T3SS) that can inject bacterial virulence "effector" proteins into host cells [2, 5]. The T3SS pathway is encoded by *hrp* (hypersensitive response and pathogenicity) and *hrc* (HR and conserved) genes [4]. The Hrc proteins direct the secretion of T3SS substrates across the bacterial envelope; however, only a partially defined set of Hrp proteins are themselves secreted by the T3SS and direct the translocation of effectors through host cell barriers [13]. Harpins differ from true effectors, known as Avr proteins, in that they elicit the hypersensitive response (HR) from the outside rather than the inside of plant cells [33]. According to the *hrp* operon structure and the regulatory system controlling T3SS gene expression, plant bacterial *hrp* genes can be divided into two major groups [2]. The *hrp* genes of *Erwinia* spp., *Pantoea stewartii*, and *Pseudomonas syringae* belong to group I, and those of *Xanthomonas* spp. and *Ralstonia solanacearum* are in group II. Following the initial discovery of HrpN from *Erwinia amylovora* as the cell-free elicitor of HR in plants, other harpins encoded by *hrpN*, *hrpZ*, and *hrpW* were characterized from the Hrp PAI region of *Erwinia* spp. and *Pseudomonas syringae* pvs. [3, 12, 35].

Over the last few decades, the harpin-like proteins, Hpa1 and HpaG, encoded by *hrp*-associated genes, have been identified as group II *hrp* genes from *Xanthomonas oryzae* pvs. and *X. axonopodis* pvs. In addition, more effectors were found to travel through the T3SS pathway; these were designated Hop (Hrp outer protein) in *Pseudomonas syringae*, Xop (*Xanthomonas* outer protein) in *Xanthomonas* spp. [27], or Pop (*Pseudomonas* outer protein, following the previous genus designation) in *Ralstonia solanacearum* [3]. Recently, such effectors as XopA have been used in alignments and found to be more homologous to *Xanthomonas* harpins, although XopA has not yet been tested to induce HR in non-host leaves [15, 26, 34].

In fact relatively few effectors that are transported by the T3SS have been identified in plant pathogens [27]. Harpins, unlike other T3SS-secreted proteins, are characteristically glycine-rich, cysteine-lacking, and heat-stable, and possess an HR elicitor activity when infiltrated into the leaf apoplast of a non-host plant at relatively high concentrations. Although mutagenesis of the genes encoding harpins, such as *hpaG* in *X. axonopodis* pv. *glycines (Xag)* and *hrpN* in *E. amylovora (Ea)*, show that these genes function in bacterial virulence, the precise mechanisms of harpins during bacterial pathogenesis is still not fully understood [8, 15, 20, 28]. In general, harpins from different pathogenic bacteria genera are dissimilar in amino acid sequence, such as

*Corresponding author
Phone: +86-25-843-96178; Fax: +86-25-843-96178;
E-mail: wangjsh@njau.edu.cn

$harpin_{Pss}$ (*Pseudomonas syringae* pv. *syringae*) and $harpin_{Ea}$ (*Erwinia amylovora*), whereas harpins from different species in the same genera have higher identity, such as HrpN harpins from different pathovars of *Pseudomonas syringae* and harpin-like proteins from *Xanthomonas* spp. [12, 15, 21]. Currently, seven harpin-like proteins have been found to be encoded by the *Xanthomonas hpa1* homologs *hpa1*/*hpaG*; their molecular mass is approximately 15–16 kDa, compared with 35–45 kDa for the *Erwinia* and *Pseudomonas* harpins. The main difference between Hpa1 and HpaG lies in whether a cysteine residue exists in the N-terminal region [21]. Interestingly, two Hpa1 homologs, $Hpa1_{Xoo}$ and $Hpa1_{Xoc}$, from pv. *oryzae* and pv. *oryzicola* of *Xanthomonas oryzae*, were found to each have one "C" residue in the N-terminal α-helical region; this "C" was replaced by the hydrophilic "T" residue at the corresponding site of $HpaG_{Xag}$, HpaXac, and $XopA_{Xcv}$. In addition, bioinformatic analysis has indicated that harpin-like proteins encoded by *Xanthomonas hpa* genes harbor two major α-helix domains located, respectively, at the N- and C-terminal regions, and three residues, Cys "C", Thr "T", and Ala "A", were found in the N-terminal α-helix regions [15, 34]. The N-terminal coding region of the Hpa1 protein from *Xanthomonas oryzae* is essential for the induction of the hypersensitive response in tobacco. It was found that the 12 highly hydrophilic amino acids (H_2N-QGISEKQLDQLL-COOH) that partially overlap the N-terminal α-helical regions of the respective proteins are critical for the elicitation of HR in tobacco [34].

Harpins can function as effector proteins to trigger hypersensitive programmed cell death (PCD), establishment of systemic acquired resistance (SAR), and various beneficial effects for plants such as increasing yield and improving quality [8, 29, 35]. Meanwhile, diverse defense responses and related gene expression involved in signaling pathways are enhanced in the harpin-treated plants [1, 28, 32].

In this study, HpaXm, a new type of harpin-like protein, was first identified from cotton leaf blight bacteria, *X. citri* subsp. *malvacearum* (*Xcm*, a synonym of *Xanthomonas campestris* pv. *malvacearum* [Smith 1901] Dye 1978, *Xcm*) [30] by PCR, using primers designed from the $hpa1_{Xoo}$ sequence. The HpaXm protein was characterized and positioned in the *hrp* region. HpaXm, like other harpins, can elicit HR and induce SAR in tobacco leaves. A synthesized peptide containing two heptads from the N-terminal α-helical region of HpaXm is adjustable with the change of a single amino acid for inducing HR. In addition, secondary structure prediction of HpaXm revealed a signal peptide-like sequence that is only present at the N-terminus of HpaXm and HpaG homologs in *X. axonopodis* pathovars. A mutant deficient for the leader peptide was unable to be secreted outside cells but induced HR.

MATERIALS AND METHODS

Bacteria and Plant

Two strains, ISO and V1 of *X. citri* subsp. *malvacearum* (*Xcm*), were provided by Dr. P. Thaxton (Department of Soil and Crop Science, Texas A&M University, U.S.A.). XCM143 was isolated from Xinjiang, China (Table S1). The *Xcm* strains were grown at 28°C on NA-agar medium, and a pathogenicity assay was performed with the infiltration method previously described [34]. *Escherichia coli* DH5α was grown at 37°C in LB medium. The medium was supplemented with a final concentration of 100 μg/ml ampicillin and 100 μg/ml kanamycin. *E. coli* strain DH5α was used as the host for gene manipulation. *E. coli* strain BL21 (DE3) was used to express the recombinant fusion protein GST–HpaXm. *E. coli* strains were purchased from Pharmacia (Uppsala, Sweden). Tobacco (*Nicotiana tabacum* L. cv. NC89) seeds were kindly provided by the Tobacco Laboratory of Shandong Agricultural University (Taian, China).

Prediction of Secondary Structure and Signal Peptide of HpaXm

The secondary structure, including α-helical domains, was predicted by the HNN Secondary Structure Prediction Method (http://npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl). A coiled-coil conformation analysis of several Hpa1 homologs was performed using the COILS2 [24] program (http://www.ch. embnet. org/software/COILS_form.html), as in our previous research [34]. The signal peptide (SP) was predicted by signal IP (SignalP 3.0 server; http://www.cbs.dtu.dk/services/SignalP/). The Hydropathic Server of the Weizmann Institute of Science, Israel (http://bioinformatics.weizmann.ac.il/hydroph), TMHMM version 2.0 programs (http://www.cbs.dtu.dk/services/TMHMM), and PRED-TMBB Prediction of TransMembrane Beta-Barrel Proteins (http://bioinformatics.biol.uoa.gr/PRED-TMBB/input.jsp.) were used to predict the hydropathic profile and transmembrane helices, respectively [17, 31].

PCR

PCR was used to clone the entire DNA sequence of *hpaXm* from *Xcm* [34]. The primers were designed based on the $hpa1_{Xoo}$ sequence (GenBank Accession No. EF028092) from *Xoo* (Table S2). *Xcm* genomic DNA was extracted using a AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, U.S.A.). A PCR reaction was performed as follows: pre-denaturation at 95°C for 5 min, followed by 35 cycles of amplification (95°C for 45 s, 56°C for 45 s, 72°C for 45 s) and then a final extension of 7 min at 72°C. The PCR product was purified using a Gel Extraction Kit (TaKaRa, Dalian, China), ligated into pMD18-T vector (TaKaRa, Dalian, China), transformed into the *E. coli* strain DH5α, and sequenced. Based on the sequencing results, specific primers were designed for *hpaXm*, and amplification was repeated using the same cycle parameters as above.

The TAIL–PCR method (thermal asymmetric interlaced PCR) of recovering the DNA fragments flanking the known *hpaXm* sequence (GenBank accession No. DQ643828) was performed as described previously [22, 37]. Three nested sequence-specific primers (Br1, Br2, Br3, and Bf1, Bf2, Bf3) with shorter arbitrary degenerated (AD) primers were used; their sequences are listed in Table S2. Each aliquot (1 μl) of *Xcm* genomic DNA was added to the TAIL–PCR mixture. The MicroAmp reaction tubes contained 50 μl of the primary TAIL–PCR mixture (Table S3), according to the method described by Liu and Huang [22]. The thermal cycling conditions are summarized in Table S4. Primary, secondary, and tertiary

amplifications were programmed and produced in the proper order (Table S4). Final PCR products were cloned and sequenced. The Blastn and Blastx programs of the NCBI (http://www.ncbi.nlm.nih.gov) were used to obtain information on the functions of the left and right segments flanking *hpaXm*. Specific primers were designed for *hpa2* and *hrcC* of *X. citri* subsp. *malvacearum* according to *hpa2* (GenBank Accession No. AY875714) and *hrcC* (GenBank Accession No. AY875714) of *Xoo* and the fragments in *X. citri* subsp. *malvacearum*.

Quantitative PCR (qRT–PCR) was conducted to measure the relative transcriptional expression levels of a plant defense-related gene in the fully expanded leaves of tobacco (*Nicotiana tabacum* L. cv. NC89) treated with purified HpaXm protein using a TaKaRa AMV RT–PCR kit and EvaGreen dye (Biotium Ltd.) on an ABI 7000 Sequence Detection System (Applied Bio-Systems, Foster City, CA, U.S.A.) according to the manufacturer's instructions. The complete laminas of plant leaves were harvested individually at 0, 1, and 3 h post spraying and frozen in liquid nitrogen. The RNA was extracted with an RNAiso for Plant Tissue [TaKaRa Biotechnology (Dalian) Co., Ltd, China]. Two-step qRT–PCR was performed on an ABI PRISM 7000 (ABI, Foster City, CA, U.S.A.) according to the procedures recommended for the SYBR Premix Ex *Taq* (Perfect Real Time) kit (TaKaRa Biotechnology). The specific primers listed in Table S2 were designed to measure the expression of *npr1* (GenBank Accession No. U76707), *hsr203J* (GenBank Accession No. X77136), *pr-1a* (GenBank Accession No. X05959), and *pr-1b* (GenBank Accession No. X66942). To normalize expression levels, *ef-1α* (GenBank Accession No. AJ223969) was used as an internal control, which proved to be a stable reference gene [29]. All PCR reactions were performed in duplicate, and a biological replicate was also included. The resulting data were normalized to *ef-1α* using the CT method [32].

Expression of HpaXm and Trapping of the Putative Signal Peptide in HpaXm

HpaXm protein was prepared based on the procedure previously described [20]. The PCR-amplified products of *hpaXm* were obtained with high-fidelity *Taq* (TaKaRa) using pMD18-T as a template, digested with BamH1 and Sac1, cloned into the pGEX-EF vector [19], and sequenced. The recombinant plasmid pGEX-HpaXm was introduced into *E. coli* BL21 (DE3). The transformed *E. coli* strain was grown in liquid LB medium up to an optical density of 0.8 at 600 nm. The overproduction of glutathione S-transferase (GST)–HpaXm was induced by the addition of 0.05 mM isopropyl-β-D-thiogalactoside (IPTG). The harvested bacterial cells were subjected to sonication in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.3). After centrifugation at 3,500 ×*g* for 5 min, the GST–HpaXm fusion protein was purified directly from bacterial lysate using GenScript High-Affinity GST Resin (GenScript Corporation, U.S.A.). HpaXm was cut from the GST–HpaXm fusion protein and eluted according to the instruction of the Thrombin Cleavage Capture Kit (Novagen, Madison, WI, U.S.A.).

To trap the putative signal peptide (SP), the full-length *hpaXm* and the leader peptide (1-MNSLNTQIGANSSFL-15, LP)-deficient coding sequence *hpaXmΔLP* were inserted into pGEX-EF containing the GST tag to express intact HpaXm and a LP-deficient mutant in BL21 (DE3) cells. After inducing with IPTG (0.05 mM) at 1, 2, and 3 h, the cell-free supernatants were gathered by the Positive GST–Protein Electrophoresis Detection Kit (Genmed Scientifics Inc. U.S.A.) to identify the GST–protein by 0.1% SDS and 10% PAGE. The sonicated cells of the two strains were treated the same as a positive control.

HpaXm-Treated Plant Reaction Assay

The hypersensitive response and induced resistance assays were performed on tobacco plants (*Nicotiana tabacum*) by the methods previously described [30, 34]. HR assay was performed by infiltrating the protein solution at 1 to 10 μM into leaf apoplasts, and the induced resistance assay by foliar spraying with harpin before TMV infection as per a previous method [34]. A visible HR was observed at 16 h postinoculation, and induced resistance was assessed by the reduction in lesion number on untreated leaves comparing with treated leaves. The experiment with 30 plants per treatment was repeated three times. The number of lesions induced by TMV infection was recorded 3 days after the inoculations. The experiment with 10 plants per treatment was repeated three times, and the data were analyzed with a Student's *t*-test. One-way analysis of variance (ANOVA) and multiple comparisons (Tukey's, $p \leq 0.01$) were used to evaluate the resistance induced by the proteins.

Peptide Synthesis

Peptides were synthesized by the GenScript Corporation (Nanjing, China). The purities of the peptides were 86.7%, 77.4%, and 84.6% for HpaXm-14, HpaXm-14ΔT6C, and HpaXm-14ΔM10Q, respectively. All stock solutions were 100 μM.

RESULTS

Primary, Secondary, and Tertiary Structures of HpaXm

The DNA sequence of three *hpaXm* clones was 402 bp in length, which shared 100% identity and contained a 60.70% GC content (GenBank Accession No. DQ643828). The putative protein (HpaXm) contained 133 amino acids, and the molecular mass was estimated to be 13.3 kDa. HpaXm is a glycine-rich, cysteine-lacking protein. The percent identity between the amino acid sequences of HpaXm and the seven homologs from the *Xanthomonas* strains are 86.5%, 92.5%, 60.9%, 70.7%, 69.2%, 60.2%, and 33.1% for HpaGXag (EF050509), Hpa1Xac (Xac0416), Hpa1Xoc (AY875714), Hpa1Xoo (EF028092), Hpa1Xoo-2 (EU072048), XopAXcv (U33548), and HpaXcc (Xcc1240), respectively. By analysis performed at http://www.ebi.ac.uk, HpaXm is rich in glycine (21.80%); this level of glycine is comparable to those of HpaGXag (21.06%), Hpa1Xoo (24.46%), and Hpa1Xoc (20.44%) (Fig. S1).

Two α-helical domains similar to Hpa1Xoo were predicted in HpaXm by the HNN program; a hydrophobic h-region was found between the N- and C-terminal flanking regions. The former contains the 1st to 18th residues, in which a β-strand resides. The latter contains the 101st to 133rd residues. H-regions were also found in other harpin-like proteins. The identical heptads, 39-LDQLL*T*Q--LI*M*ALLQ-52, were found in the N-terminal α-helix region of HpaXm

and HpaG, whereas Hpa1Xoo possesses **LDQLL***C***Q--LI**S**ALLQ**. Italicized letters indicate different residues in HpaXm and HpaXoo.

A leader peptide (LP) at the N-terminus of HpaXm with the sequence 1-MNSLNTQIGANSSFL-15, and the cleavage sites L and Q, was identified as a putative signal peptide by signal IP. A similar sequence, 1-MNSLNTQ**L**GANSSF**F**-15 (bolds showing residues different from HpaXm), was found in HpaXac and HpaG (Fig. S1).

Phylogenetic analysis of the entire amino acid sequence or of the 13 highly conserved amino acids H2N-SEKQLDQLLTQLI-COOH (Fig. S2A and S2B) in the N-terminal α-helix region reveals that the eight proteins can be divided into three evolutionary branches: HpaGXag/HpaXac/HpaXm, Hpa1Xoo/Hpa1Xoc, and HpaXcc/Xop1Xcv.

Location of *hpaXm* in the *hrp* Cluster of *Xcm*

HpaXm with Hpa1 and HpaG has proven to have a high degree of homology. In order to determine HpaXm encoded by a *hrp* gene, the *hpaXm* flanking sequences should also be identified by the TAIL–PCR method with the designed primers (Table S2). Two amplified products were obtained, and the sequences were blasted at http://www.ncbi.nlm. nih.gov. The right fragment adjacent to *hpaXm* contained a homolog of the *hrp* conserved gene *hrcCXoo* (GenBank Accession No. AY875714) and a 704-bp intergenic region between *hrcCXm* and *hpaXm*. The left fragment of *hpaXm* was a homolog of *hpa2Xoo*, named *hpa2Xm* (GenBank Accession No. AY875714). Complete sequences of *hpa2Xm* and *hrcCXm* were obtained from *Xcm* by PCR amplification with the specific primers (Table S2). The *hpa2Xm* (GenBank Accession No. FJ769158) and the *hrcCXm* (GenBank Accession No. FJ769159) products were 707 bp and 1,826 bp in size, respectively, and shared 89% and 93% identity to *hpa2Xoo* and *hrcCXoo*, respectively. Thus, *hpaXm* is clearly localized between *hpa2Xm* and *hrcCXm* in *Xcm*, like *hpa1* homolog from other *Xanthomonas* species.

HR and Induced Resistance by HpaXm

Unlike the negative control GST protein, the expressed fusion protein GST–HpaXm was infiltrated into the intercellular space of fully expanded tobacco leaves, resulting in HR production. Likewise, both unboiled and boiled (10 min) purified HpaXm produced HR similar to other harpins (Fig. 2)

Resistance against TMV was induced in tobacco plants treated with purified HpaXm, as were plants treated with Hpa1Xoo. After 3 days, only a few lesions were found, unlike the negative control plants treated with PBS (Fig. 1).

To further understand the induced resistance, transcriptional levels of several HR- and SAR-associated genes, including *hsr203J*, *npr1*, *pr-1a*, and *pr-1b*, were detected by qRT–PCR. In general, an increased level of four defense-related

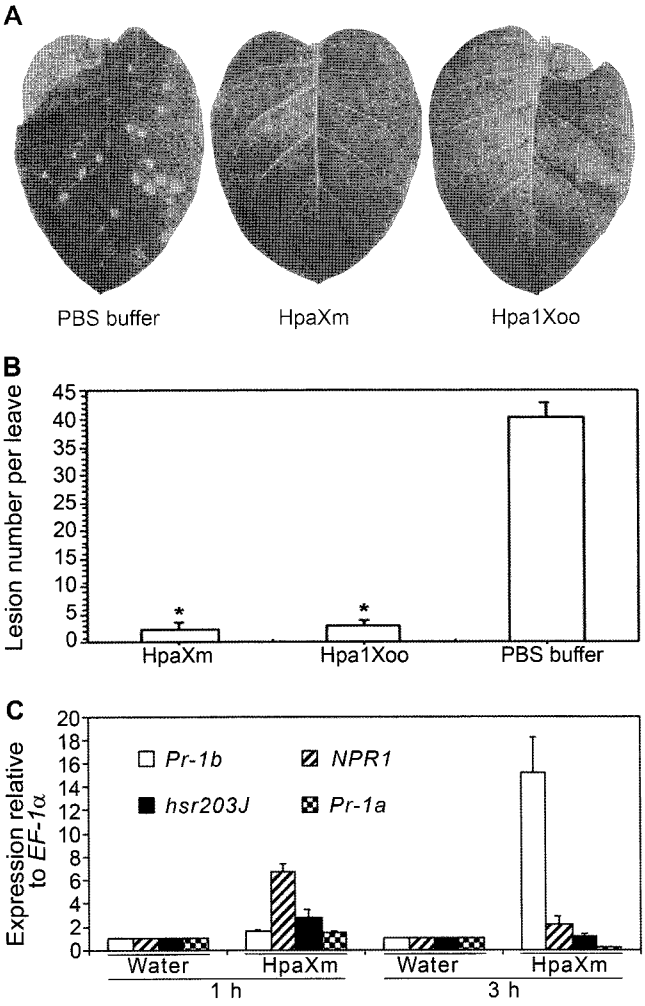

Fig. 1. The Enhanced resistance of tobacco to TMV by HpaXm and Hpa1Xoo.

A. Photographs of tobacco leaves inoculated with TMV 3 days after pretreatments with HpaXm and Hpa1Xoo at concentrations of 10 μM. PBS buffer was used as a control. **B.** Lesion number of tobacco leaves treated by the proteins at a concentration of 10 μM. PBS buffer was used as a control. TMV was inoculated 16 h later, and necrotic lesions were observed after 3 days. Data marked with asterisks are significantly different as assessed by one-way ANOVA with post hoc multiple comparisons Tukey test ($p \leq 0.01$). The experiment was repeated three times with similar results. **C.** The relative expression of the defense genes in tobacco leaves at different times (1 h, 3 h) after exposure to HpaXm. The complete laminas of plant leaves were harvested individually at 0, 1, and 3 h post spraying and frozen in liquid nitrogen. cDNA was reverse-transcribed from total RNA and subjected to quantitative reverse-transcriptase polymerase chain reaction. Expression levels of *npr1*, *hsr203J*, *pr-1a*, and *pr-1b* were quantified using a standard curve of pooled cDNA samples. Relative expression was obtained by normalizing expression to that of *ef-1α*. Water was used as the control. Error bars indicate standard error of the mean (n=3).

genes was detected in the leaves treated with HpaXm at 1 h and 3 h intervals, compared with the treatment with PBS buffer; however, the expression levels were variable at different time intervals and for different defense genes. For example, the expression of *pr-1b* at 3 h was higher

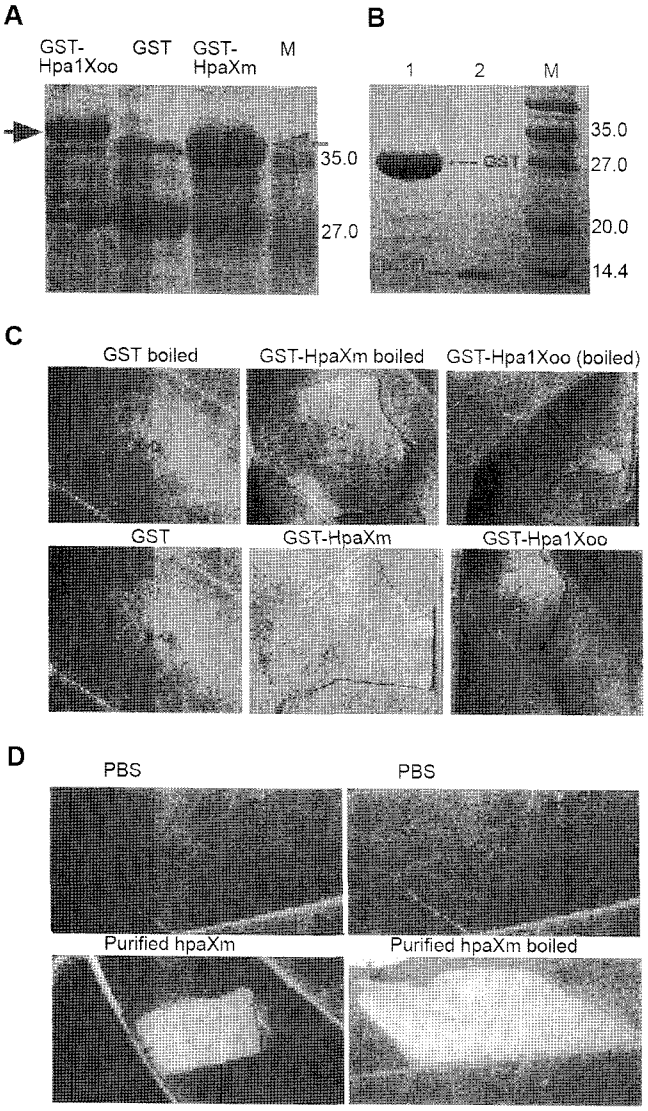

Fig. 2. SDS–PAGE analysis and HR elicited by expressed GST–HpaXm and purified HpaXm.

A. SDS–PAGE analysis of the expressed fusion proteins GST–HpaXm and GST–Hpa1Xoo. The arrow indicates the expression of the GST fusion protein in *E. coli*. **B.** SDS–PAGE analysis of purified HpaXm. Lane 1, the purified glutathione S-transferase (GST) from *E. coli* BL21 (DE3)/pGEX-EF; 2, purified HpaXm. The arrow indicates the expression of GST or purified HpaXm. **C.** HR elicited by the expressed fusion proteins GST–HpaXm and GST–Hpa1Xoo. GST from *E. coli* BL21 (DE3)/pGEX-EF was used as a negative control. **D.** HR triggered by purified HpaXm. PBS buffer was used as a negative control. M: protein marker for "**A**" and "**B**". The experiment was repeated twice with the same result.

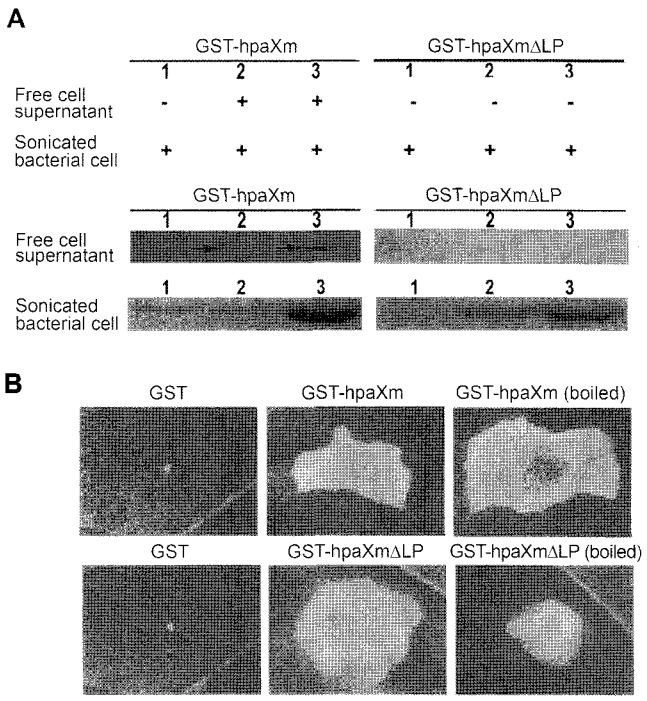

Fig. 3. Test of signal peptide function in secretion of BL21 (DE3)/pGEX-HpaXm and BL21 (DE3)/pGEX-HpaXmΔLP and in HR triggering activity.

A. Detection of bands on SDS–PAGE with fusion proteins GST–HpaXm and GST–HpaXmΔLP from liquid culture of BL21 (DE3)/pGEX-HpaXm and BL21 (DE3)/pGEX-HpaXmΔLP. Crude protein from the sonicated bacterial cells: 1, 2, 3 represent the bacterial cells cultured for 1, 2, and 3 h before harvest. Cell free supernatants: 1, 2, and 3 represent the protein extracted from the liquid cultures after the bacteria culture for 1, 2, and 3 h, respectively. M: protein marker. The red arrow indictes GST fusion protein. **B.** HR triggered by GEX-HpaXm and GEX-HpaXmΔLP. GST: The purified glutathione S-transferase (GST) from *E. coli* BL21 (DE3)/pGEX-EF was used as a control. The experiment was repeated twice, with the same result.

than at 1 h post-treatment, and the expressions of *hsr203J, npr1,* and *pr-1a* at 1 h were lower than at 3 h post-treatment (Fig. 1).

Leader Peptide-Deficient Mutant of HpaXm is not Secreted

To investigate whether the leader peptide is a putative signal peptide, and whether it has a critical role in the secretion of HpaXm, wild-type HpaXm containing the LP segment and a LP-deficient mutant in *E. coli* BL21 (DE3) were expressed. HpaXm was recovered only from the cell-free liquid medium of BL21 (DE3)/pGEX-HpaXm at 2 h and 3 h post-inoculation. The cell-free supernatant was concentrated 5-fold, and the expressed fusion protein GST–HpaXm was detected with a peak at OD_{600} of 0.475 A at 2 h or 0.717 A at 3 h post-cultivation by a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany). Positive bands were discovered on SDS–PAGE from the concentrated samples with a GST–protein electrophoresis detection kit (Fig. 3A). The mutated protein HpaXmΔLP expressed in BL21 (DE3)/pGEX-HpaXmΔLP could not be detected at the same time. As expected, bands from sonicated cell solutions were observed for fusion proteins expressed in BL21 (DE3)/pGEX-HpaXm and BL21 (DE3)/pGEX-HpaXmΔLP at 1 h or 2 h post-inoculation. When infiltrated into tobacco leaves, purified HpaXmΔLP was able to induce HR, similar to wild-type HpaXm (Fig. 3B).

Two Heptads in the N-Terminal α-Helix are Essential for Inducing HR

We previously reported that the N-terminal α-helical regions of Hpa1Xoo are critical for the induction of HR in tobacco and found two heptads in the α-helical regions that may be associated this activity [34]. In this study, we found two heptads, 39-**L**DQ**L**LTQ--**L**IM**A**LLQ-52 (underlined letters show hydrophobic residues at *a* and *d* positions), in the N-terminal α-helical region of HpaXm, and computer simulation was used to predict its ability to form coiled coils (Fig. S3). The T44 residue changed to a cysteine, and the derivative mutant HpaXmΔT44C had a lower probability of forming a coiled coil. In contrast, when the M48 residue changed to a glutamine "Q", the derivative mutant HpaXmM48Q had a higher probability of forming a coiled coil. We synthesized three peptides: HpaXm-14 (**L**DQ**L**LTQ-**L**IM**A**LLQ), HpaXm-14ΔT6C (**L**DQ**L**L**C**Q-**L**IM**A**LLQ), and HpaXm-14ΔM10Q (**L**DQ**L**LTQ-**L**I**Q**A**L**LQ). HpaXm, HpaXm-14, HpaXm-14ΔT6C, and HpaXm-14ΔM10Q were all diluted 10-fold (10 μM), 100-fold (1 μM), and 1,000-fold (0.1 μM). The samples (10 μl) were infiltrated into tobacco leaves for HR induction. The synthesized peptides and wild-type HpaXm had comparable activity in inducing HR at 10-fold dilutions. Surprisingly, the oligopeptide HpaXm-14 containing only 14 amino acids was able to induce HR on tobacco leaves at a concentration of 0.1 μM and even had a higher activity than the complete sequence of HpaXm (Fig. 4). The effective concentrations of HpaXm-14ΔT6C and HpaXm-14ΔM10Q for eliciting HR in tobacco leaves were 10 μM and 1 μM, respectively.

DISCUSSION

Harpin is well known for their elicitor activity and capability of inducing HR and systemic acquire resistance, by pretreatment of leaf spraying or infiltration [15, 34, 35]. HR induction is often, but not always, involved in incompatible plant–pathogen interactions. Harpins induce

Treatment	Dilution fold	HR
PBS Buffer	-	-
HpaXm	Wild type	+
	1000x	-
	Boiled(WT)	+
HpaXm-14	10x	+
	100x	+
	1000x	+
	Boiled(10x)	+
HpaXm-14ΔM10Q	10x	+
	100x	+
	1000x	-
	Boiled(10x)	+
HpaXm-14ΔT6C	10x	+
	100x	-
	1000x	-
	Boiled(10x)	+

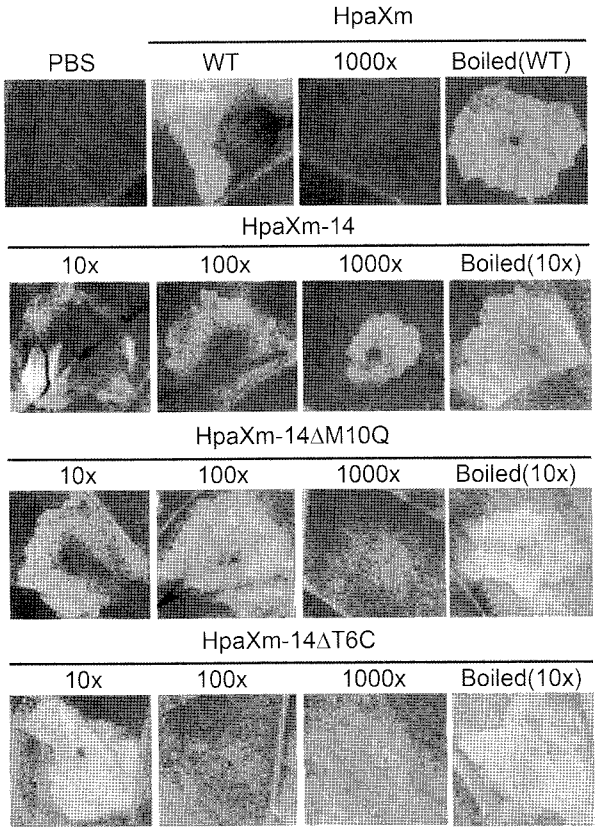

Fig. 4. The ability of synthesized polypeptides to trigger HR.
HpaXm was diluted 1,000-fold (0.1 μM), whereas HpaXm-14, HpaXm-14ΔT6C, and HpaXm-14ΔM10Q were diluted 10-fold (10 μM), 100-fold (1 μM), and 1,000-fold (0.1 μM). The 10-fold diluted samples were boiled for 10 min before inoculation. All the samples were infiltrated into tobacco leaves for HR induction. PBS buffer: phosphate-buffered saline buffer; HpaXm: purified HpaXm; HpaXm-14: 14 amino acids (39–52) in HpaXm protein sequence. HpaXm-14ΔM10Q: M was changed to Q in HpaXm-14 amino acid sequence. HpaXm-14ΔT6C: T was changed to C in HpaXm-14 amino acid sequence. WT: wild type. The experiment was repeated three times, with the same result.

systemic acquired resistance (SAR) and associated defense responses in many plants that may or may not be accompanied by HR. Accumulating evidence suggests that harpins induce plant resistance against pathogen infection and coordinate the expression of defense-related genes, but in some cases without involving visible HR [29, 34]. This study provided evidences that HpaXm is a harpin protein from *Xcm* that is able to induce HR-mediated disease resistance on non-host plant tobacco.

Phylogenetic Relationship of *Xanthomonas* Harpin Proteins

One significant feature of all described harpin proteins from the group of *hrp* genes is the lack of the cysteine residue at the N-terminus; however, harpin-like proteins from *Xanthomonas* spp. can be divided into two subgroups based on a clustering analysis of the complete amino acid sequence or the conserved amino acids in the N-terminal α-helix (Fig. S2). HpaXm resides in a sub-group with HpaG and HpaXac that is distinct from a subgroup containing Hpa1Xoo and Hpa1Xoc from *Xanthomonas oryzae* pathovars [21]. The former contains a threonine residue "T" in the N-terminal α-helix region, and the latter contains a cysteine residue "C". We suggest that two phylogenetic clues can be deduced from *Xanthomonas* harpins: "C" and "T" type harpins. Unlike the horizontal gene transfer of T3SS proteins [9], the harpin-like proteins have evolved vertically into different branches containing structural and functional differentiations by single codon mutagenesis. The codon preference in HpaXm is close to HpaG and different from Hpa1Xoo. Like other harpin-like proteins from *Xanthomonas* pv., HpaXm has a preference for codons with a higher G and C content; only CGG, encoding Arg, was found in HpaXm and HpaG. With regard to synonymous codon usage, HpaXm and HpaG possess 15 common codons with the same usage number, and Hpa1Xoo and Hpa1Xoc have 19 common codons with the same usage number. HpaXm and Hpa1Xoo only have seven common codons with the same usage number. Similarly, there are 10 common codons with the same usage number between Hpa1Xoc and HpaG (data not shown). Thus, the codon preference of *Xanthomonas* harpins provides further evidence that HpaXm and HpaG reside in the same phylogenetic clue, whereas Hpa1Xoo and Hpa1Xoc from *Xanthomonas oryzae* pathovars belong in another.

N-Terminal Region of HpaXm May Contain a Secretion and Translocation Signal

Harpins, like avirulence (Avr) proteins of plant pathogenic bacteria, are thought to be secreted through the Hrp T3SS, which directly or indirectly interacts with corresponding plant resistance proteins (R) [2, 11]. However, harpins have yet to be shown to have any role in effector delivery, and no evidence indicates whether or not harpins have signal peptides [16, 35]. How harpins are secreted out of bacterial cells and why harpins localize to the plant apoplast and interact with the plant have not been well understood. This study predicted that a signal peptide-like sequence located at the N-terminus of HpaXm functions for probably *sec*-dependent secretion, and demonstrated the critical role in HpaXm secretion in the early growth phase of *E. coli*. Although HpaXm homolog Hpa1 from *X. oryzae* pv. *oryzae* and XopA from *X. axonopodis* (*campestris*) pv. *vesicatoria* are shown to be secreted *via* TTSS instead of the *sec*-dependent pathway [26]. HpaXm is predicted to own a putative signal sequence rather than hpa1Xoo and XopA not (data not shown) by signal IP prediction [7]. Throughout evolution, numerous bacterial pathogens have acquired several specialized protein secretion pathways [36]. Beside the TTSS pathway, is HpaXm also actually secreted *sec*-dependently in *Xcm*? More experiments are required to verify the signal peptide at the LP site in *Xcm*. The SP is an amino-terminal extension of the secretary protein that is necessary for the correct targeting to the translocation pathway. The function and structure of SP is conserved in all domains of life; typically, they have an average length of 20 amino acid residues and a tripartite structure (*i.e.*, a positively charged amino-terminus, a hydrophobic core, and a polar carboxyl-terminal region) [25]. The twin-arginine sequence motif (Z-R-R-x-Φ-Φ) was not found at the N-terminal region of HpaXm [6, 36]. Harpins are delivered across the bacterial envelope into the surrounding environment, where they can interact with harpin-interacting protein on the plant cell membrane [28].

Two Heptads are Adjustable with the Change of a Single Amino Acid for Inducing HR

In contrast to *Pseudomonas* strains, which have active domains for HR elicitation in the C-terminal region of their harpins, the key functional region for HR elicitation resides in the N-terminal α-helix of harpins in *X. oryzae* [3, 18, 34]. This study shows that a synthesized peptide containing 14 amino acids from the two heptads (LDQLLTQ--LIMALLQ) in the N-terminal α-helical region have comparable activity for eliciting HR in tobacco leaves. This peptide contains two heptads with hydrophobic amino acid residues at positions *a* and *d*; it has the potential to form a coiled-coil conformation. If the hydrophilic threonine "T" at the *f* position changes to a cysteine "C", or the hydrophobic methionine "M" at the *c* position changes to a glutamine "Q", the probability of forming a coiled coil will be reduced significantly and the constructive ability of inducing HR on tobacco leaves will also be reduced, especially for HpaXm-14ΔT6C. Thus, we supposed that the C residue, as in the mild conditions of the amino acid oxidation, may be unfavorable for coiled-coil formation and interferes with protein–protein interactions (Fig. 1 and S3). Accordingly, the coiled-coil conformation is further proved

to be critical for the harpin to induce HR on non-host plants to initiate pathogenesis [34].

Acknowledgments

This research was supported by grants from the National Key Basic Research Plan of China (No. 2006CB101902), National Key Science Plan (No. 2004BA901A36), the Communal Program Specially for Agricultural Research (No. nyhyzx07-052), and the National High Technology Research and Development Program of China (863 Program) (No. 2006AA02Z180).

Reference

1. Adam, A. L., S. Pike, M. E. Hoyos, J. M. Stone, J. C. Walker, and A. Novacky. 1997. Rapid and transient activation of a myelin basic protein kinase in tobacco leaves treated with harpin from *Erwinia amylovora*. *Plant Physiol.* **115:** 853–861.
2. Alfano, J. R. and A. Collmer. 2004. Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* **42:** 385–414.
3. Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pernollet, and C. A. Boucher. 1994. PopA1, a protein which induces a hypersensitivity-like response on specific *Petunia* genotypes, is secreted *via* the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J.* **13:** 543–553.
4. Bogdanove, A. J., S. V. Beer, U. Bonas, C. A. Boucher, A. Collmer, D. L. Coplin, *et al.* 1996. Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol. Microbiol.* **20:** 681–683.
5. Cornelis, G. R. and F. Van Gijsegem. 2000. Assembly and function of type III secretory systems. *Annu. Rev. Microbiol.* **54:** 735–774.
6. Dilks, K., R. W. Rose, E. Hartmann, and M. Pohlschroder. 2003. Prokaryotic utilization of the twin-arginine translocation pathway: A genomic survey. *J. Bacteriol.* **185:** 1478–1483.
7. Emanuelsson, O., S. Brunak, G. von Heijne, and H. Nielsen. 2007. Locating proteins in the cell using TargetP, SignalP, and related tools. *Nat. Protoc.* **2:** 953–971.
8. Galan, J. E. and H. Wolf-Watz. 2006. Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444:** 567–573.
9. Gophna, U., E. Z. Ron, and D. Graur. 2003. Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. *Gene* **312:** 151–163.
10. Guermeur, Y. 1997. Combinaison de classifieurs statistiques, Application a la prediction de structure secondaire des proteines, PhD thesis, Université de Paris 06.
11. Gurlebeck, D., F. Thieme, and U. Bonas. 2006. Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interaction with the host plant. *J. Plant Physiol.* **163:** 233–255.
12. He, S. Y., H. C. Huang, and A. Collmer. 1993. *Pseudomonas syringae* pv. *syringae* harpinPss: A protein that is secreted *via* the Hrp pathway and elicits the hypersensitive response in plants. *Cell* **73:** 1255–1266.
13. Kay, S., S. Hahn, E. Marois, G. Hause, and U. Bonas. 2007. A bacterial effector acts as a plant transcription factor and induces a cell size regulator. *Science* **318:** 648–651.
14. Kim, J. F. and S. V. Beer. 1998. HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. *J. Bacteriol.* **180:** 5203–5210.
15. Kim, J. G., B. K. Park, C. H. Yoo, E. Jeon, J. Oh, and I. Hwang. 2003. Characterization of the *Xanthomonas axonopodis* pv. *glycines* Hrp pathogenicity island. *J. Bacteriol.* **185:** 3155–3166.
16. Kim, J. G., E. Jeon, J. Oh, J. S. Moon, and I. Hwang. 2004. Mutational analysis of *Xanthomonas* harpin HpaG identifies a key functional region that elicits the hypersensitive response in nonhost plants. *J. Bacteriol.* **186:** 6239–6247.
17. Krogh, A., B. Larsson, G. von Heijne, and E. L. Sonnhammer. 2001. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. *J. Mol. Biol.* **305:** 567–580.
18. Lee, J., D. F. Klessig, and T. Nurnberger. 2001. A harpin binding site in tobacco plasma membranes mediates activation of the pathogenesis-related gene HIN1 independent of extracellular calcium but dependent on mitogen-activated protein kinase activity. *Plant Cell* **13:** 1079–1093.
19. Levy, R., M. Wiedmann, and G. Kreibich. 2001. *In vitro* binding of ribosomes to the beta subunit of the Sec61p protein translocation complex. *J. Biol. Chem.* **276:** 2340–2346.
20. Li, C. M., M. Haapalainen, J. Lee, T. Nurnberger, M. Romantschuk, and S. Taira. 2005. Harpin of *Pseudomonas syringae* pv. *phaseolicola* harbors a protein binding site. *Mol. Plant Microbe Interact.* **18:** 60–66.
21. Li, P., X. Lu, M. Shao, J. Long, and J. Wang. 2004. Genetic diversity of harpins from *Xanthomonas oryzae* and their activity to induce hypersensitive response and disease resistance in tobacco. *Sci. China C Life Sci.* **47:** 461–469.
22. Liu, Y. G. and N. Huang. 1998. Efficient amplification of insert end sequences from bacterial artificial chromosome clones by thermal asymmetric interlaced PCR Pl. *Plant Mol. Biol. Rep.* **16:** 175–181.
23. Livak, K. J. and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* **25:** 402–408.
24. Lupas, A., M. Van Dyke, and J. Stock. 1991. Predicting coiled coils from protein sequences. *Science* **252:** 1162–1164.
25. Natale, P., T. Bruser, and A. J. Driessen. 2008. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane – distinct translocases and mechanisms. *Biochim. Biophys. Acta* **1778:** 1735–1756.
26. Noel, L., F. Thieme, D. Nennstiel, and U. Bonas. 2002. Two novel type III-secreted proteins of *Xanthomonas campestris* pv. *vesicatoria* are encoded within the *hrp* pathogenicity island. *J. Bacteriol.* **184:** 1340–1348.
27. Noel, L., F. Thieme, J. Gabler, D. Buttner, and U. Bonas. 2003. XopC and XopJ, two novel type III effector proteins from *Xanthomonas campestris* pv. *vesicatoria*. *J. Bacteriol.* **185:** 7092–7102.
28. Oh, C. S. and S. V. Beer. 2007. AtHIPM, an ortholog of the apple HrpN-interacting protein, is a negative regulator of plant growth and mediates the growth-enhancing effect of HrpN in *Arabidopsis*. *Plant Physiol.* **145:** 426–436.

29. Peng, J. L., Z. L. Bao, H. Y. Ren, J. S. Wang, and H. S. Dong. 2004. Expression of harpin(xoo) in transgenic tobacco induces pathogen defense in the absence of hypersensitive cell death. *Phytopathology* **94:** 1048–1055.
30. Schaad, N. W., E. Postnikova, G. Lacy, A. Sechler, I. Agarkova, P. E. Stromberg, V. K. Stromberg, and A. K. Vidaver. 2006. Emended classification of xanthomonad pathogens on citrus. *Syst. Appl. Microbiol.* **29:** 690–695.
31. Spyropoulos, I. C., T. D. Liakopoulos, P. G. Bagos, and S. J. Hamodrakas. 2004. TMRPres2D: High quality visual representation of transmembrane protein models. *Bioinformatics* **20:** 3258–3260.
32. Strobel, N. E., C. Ji, S. Gopalan, J. A. Kuc, and S. Y. He. 1996. Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 HrpZPss protein. *Plant J.* **9:** 431–439.
33. Tampakaki, A. P. and N. J. Panopoulos. 2000. Elicitation of hypersensitive cell death by extracellularly targeted HrpZPsph produced in planta. *Mol. Plant Microbe Interact.* **13:** 1366–1374.
34. Wang, X. Y., C. F. Song, W. G. Miao, Z. L. Ji, X. Wang, Y. Zhang, 2008. Mutations in the N-terminal coding region of the harpin protein Hpa1 from *Xanthomonas oryzae* cause loss of hypersensitive reaction induction in tobacco. *Appl. Microbiol. Biotechnol.* **81:** 359–369.
35. Wei, Z. M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S. Y. He, A. Collmer, and S. V. Beer. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* **257:** 85–88.
36. Wooldridge, K. 2009. *Bacterial Secreted Proteins: Secretory Mechanisms and Role in Pathogenesis*. Caister Academic Press, Norfolk
37. Ying, G., W. Wu, and C. Z. He. 2002. Cloning of *Xanthomonas campestris* pv. *campestris* pathogenicity-related gene sequences by TAIL–PCR. *Sheng Wu Gong Cheng Xue Bao* **18:** 182–186.