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

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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 H2N-SEKQLDQLTQLI-COOH in the N-terminal α-helix indicates that HpaXm is evolutionarily closer to HpaGXag and HpaXact than to Hpa1Xoo and Hpa1Xoc. A synthesized peptide containing two heptads, 39-LDQLTQL-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-MNSLNTQIIGANSSFL-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, 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. andRalstonia 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...
harpin_psa (Pseudomonas syringae pv. syringae) and harpin_pea (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_Xcm, 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 Hpa1. XpaXc, and XopA_Xcm. 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-helices 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-N-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 hpa 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 Xianjiang, 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.

**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 hydrophobic Server of the Weizmann Institute of Science, Israel (http://bioinformatics.weizmann.ac.il/hydrophi), 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 hydrophatic 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, 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 nptI (GenBank Accession No. U76707), hsr203j (GenBank Accession No. X77136), pr-1a (GenBank Accession No. X05959), and pr-1b (GenBank Accession No. X69642). 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 purified 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-EX 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 harvested 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 Na2HPO4, 1.8 mM KH2PO4, 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-MSLNTQGIGANSFSL-15, LP)-deficient coding sequence hpaXm:L.P were inserted into pGEX-EX 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 Sciences 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 ≤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 was 86.5%, 92.5%, 60.9%, 70.7%, 69.2%, 60.2%, and 33.1% for HpaGXag (EF050509), Hpa1Xac (Xac0416), Hpa1Xoc (AY875714), Hpa1Xoxo (EF028092), Hpa1Xoo-2 (EU072048), XopAXcv (U33548), and HpaXcc (Xcc1240), respectively. By analysis performed at http://www.ncbi.nlm.nih.gov, HpaXm is rich in glycine (21.80%); this level of glycine is comparable to those of HpaGXag (21.06%), Hpa1Xoxo (24.46%), and Hpa1Xoxo (20.44%) (Fig. S1).

Two α-helical domains similar to Hpa1Xoxo were predicted in HpaXm by the HNN program; a hydrophobic h-region was found between the N- and C-terminal flanking proteins. The identical heptads, 39-β-strand resides. The latter 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-LDQLL7Q--L1MAL7Q-- S2, were found in the N-terminal α-helix region of HpaXm.
and HpaG, whereas Hpa1Xoo possesses LDQLQC--LISALLQ. Italicized letters indicate different residues in HpaXm and HpaXoo.

A leader peptide (LP) at the N-terminus of HpaXm with the sequence 1-MNSLNTQIGANSSF-15, and the cleavage sites L and Q, was identified as a putative signal peptide by signal IP. A similar sequence, 1-MNSLNTQLGANSSF-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-SEKQLDQLTQLI-COOH (Figs. 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 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
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 and BL21 (DE3)/pGEX-HpaXM∆LP. 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 indicates GST fusion protein. HR triggered by GEX-HpaXM and GEX-HpaXM∆LP. 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.

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

A. Detection of bands on SDS–PAGE with fusion proteins GST-HpaXM and GST-HpaXM∆LP from liquid culture of BL21 (DE3)/pGEX-HpaXM and BL21 (DE3)/pGEX-HpaXM∆LP. 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 indicates GST fusion protein. B. HR triggered by GEX-HpaXM and GEX-HpaXM∆LP. 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.
Two Heptads in the N-Terminal $\alpha$-Helix are Essential for Inducing HR

We previously reported that the N-terminal $\alpha$-helical regions of Hpa1Xoo are critical for the induction of HR in tobacco and found two heptads in the $\alpha$-helical regions that may be associated this activity [34]. In this study, we found two heptads, 39-LDQLTQL-MALLQ-52 (underlined letters show hydrophobic residues at $a$ and $d$ positions), in the N-terminal $\alpha$-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 HpaXm$^{\Delta T44C}$ had a lower probability of forming a coiled coil. In contrast, when the M48 residue changed to a glutamine “Q”, the derivative mutant HpaXm$^{\Delta M48Q}$ had a higher probability of forming a coiled coil. We synthesized three peptides: HpaXm-14 (LDQLTQLLLMALLQ), HpaXm-14AT6C (LDQLTQLMALLQ), and HpaXm-14AM10Q (LDQLTQLQALLQ). HpaXm, HpaXm-14, HpaXm-14AT6C, and HpaXm-14AM10Q were all diluted 10-fold (10 $\mu$M), 100-fold (1 $\mu$M), and 1,000-fold (0.1 $\mu$M). The samples (10 $\mu$l) 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 $\mu$M and even had a higher activity than the complete sequence of HpaXm (Fig. 4). The effective concentrations of HpaXm-14AT6C and HpaXm-14AM10Q for eliciting HR in tobacco leaves were 10 $\mu$M and 1 $\mu$M, 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

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<th>Treatment</th>
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<td>PBS Buffer</td>
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<td>HpaXm</td>
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Fig. 4. The ability of synthesized polypeptides to trigger HR. HpaXm was diluted 1,000-fold (0.1 $\mu$M), whereas HpaXm-14, HpaXm-14AT6C, and HpaXm-14AM10Q were diluted 10-fold (10 $\mu$M), 100-fold (1 $\mu$M), and 1,000-fold (0.1 $\mu$M). 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-14AM10Q: M was changed to Q in HpaXm-14 amino acid sequence. HpaXm-14AT6C: 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 \textit{Xcm} that is able to induce HR-mediated disease resistance on non-host plant tobacco.

Phylogenetic Relationship of \textit{Xanthomonas} Harpin Proteins

One significant feature of all described harpin proteins from the group of \textit{hrp} genes is the lack of the cysteine residue at the N-terminus; however, harpin-like proteins from \textit{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 \(\alpha\)-helix (Fig. S2). HpaXm resides in a sub-group with HpaG and HpaXac that is distinct from a subgroup containing Hpa1Xoo and Hpa1Xoc from \textit{Xanthomonas oryzae} pathovars [21]. The former contains a threonine residue “T” in the N-terminal \(\alpha\)-helix region, and the latter contains a cysteine residue “C”. We suggest that two phylogenetic clues can be deduced from \textit{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 \textit{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 \textit{Xanthomonas} harpins provides further evidence that HpaXm and HpaG reside in the same phylogenetic clue, whereas Hpa1Xoo and Hpa1Xoc from \textit{Xanthonomas 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 \textit{E. coli}. Although HpaXm homolog Hpa1 from \textit{X. oryzae} pv. oryzae and XopA from \textit{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 \textit{Xcm}? More experiments are required to verify the signal peptide at the LP site in \textit{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 \textit{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 \(\alpha\)-helix of harpins in \textit{X. oryzae} [3, 18, 34]. This study shows that a synthesized peptide containing 14 amino acids from the two heptads (\textit{LDQLLTQ-LIMALQQ}) in the N-terminal \(\alpha\)-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 (Figs. 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].

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Competing Interests

The authors declare that they have no competing interests.

Reference


