The Internal Glycine-Rich Motif and Cysteine Suppress Several Effects of the HpaG_{Xooc} Protein in Plants

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ABSTRACT

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HpaG_{Xooc}, produced by *Xanthomonas oryzae* pv. *oryzicola*, is a member of harpin group of proteins that stimulate plant growth, hypersensitive cell death (HCD), and pathogen defense. The protein contains two copies of the glycine-rich motif (GRM), a characteristic of harpins, and a cysteine, which is absent in other harpins. Genetic modification generated the protein mutants HpaG_{Xooc}MG (MG) by deleting GRMs and HpaG_{Xooc}C47T (C47T) by replacing cysteine with threonine. When applied to tobacco plants, C47T and MG were 1.2- and 1.7-fold stronger, respectively, than HpaG_{Xooc} in inducing HCD, which occurred consistently with expression of the marker genes *hin1* and *hsr203*. The proteins markedly alleviated infection of tobacco by *Tobacco mosaic virus* and *Arabidopsis* and tomato by *Pseudomonas syringae*. Treating tobacco plants with HpaG_{Xooc}, C47T,

and MG decreased the viral infection by 58, 81, and 92%, respectively. In *Arabidopsis* and tomato plants treated with HpaG_{Xooc}, C47T, or MG, *P. syringae* multiplication was inhibited; bacterial population multiplied in 5 days in these plants were ca. 160-, 1,260-, or 15,860-fold smaller than that in control plants. So pathogen defense was induced in both plants. Defense-related genes *Chia5*, *NPR1*, and *PR-1a* were expressed consistently with resistance. In response to HpaG_{Xooc}, C47T, and MG, aerial parts and roots of tomato plants increased growth by 15 and 53%, 25 and 77%, and 46 and 106%, relative to controls. The expansin gene, *EXP2*, involved in the cell expansion and plant growth was expressed coordinately with plant growth promotion. These results suggest that the presence of GRM and cysteine in HpaG_{Xooc} represses the effects of the protein in plants.

Additional keywords: hypersensitive response, *hrp* genes, systemic acquired resistance.

In many plant-pathogenic bacteria, particular hypersensitive response and pathogenicity (*hrp*) genes encode proteins of harpin group, an important category of type III effectors for *hrp* functions (2,23,29,30). The proteins contain repeats of a glycine-rich motif (GRM), as characterized earlier for HrpN_{Ea} of *Erwinia amylovora* (55) and HrpZ_{Pss} of *Pseudomonas syringae* pv. *syringae* (28). GRM is composed of six amino acid residues, sequenced as four glycines interrupted by any other amino acid at the penultimate site; the coding consensus (GRM) includes four GGC, an arbitrary code, and GGC (36,57). In bacteria of the genus *Xanthomonas*, harpin genes were identified by targeting the *hpa* (*hrp*-associated) loci (31,41,57), such as *hpaG_{Xooc}* in *X. oryzae* pv. *oryzae* (56) and *hpaG_{Xooc}* in *X. oryzae* pv. *oryzicola* (36). The *hpa* genes and Hpa proteins are peculiar in several features (37,56,57). Reported *hpa* genes are homologous with each another but not with other harpin genes. Hpa proteins are smaller than other harpins and contain two to four copies of GRM. HpaG_{Xooc} is composed of 138 amino acids, containing two GRMs that span the 72 to 77 and 107 to 112 amino acid sequences (36). Hpa proteins all contain a cysteine, which is absent in other harpins (29,30,31,41,55). The cysteine is located at the site 47 in HpaG_{Xooc} sequence (36).

Proteins of harpin group artificially applied to plants cause several beneficial effects. The proteins all elicit the hypersensitive

response (HR) (5,13,44,45,53), or hypersensitive cell death (HCD) (15,16), and systemic acquired resistance (SAR) (18,36, 43,52). HCD often develops as macroscopic HR (macro-HR) or microscopic HR (micro-HR) (3) in response to harpins (44,45). In transgenic tobacco plants expressing HpaG_{Xooc}, HCD and SAR occur independently (44). HrpN_{Ea} also stimulates abscisic acid signaling to induce drought tolerance (20). When HrpN_{Ea} (19), HpaG_{Xooc}, or HpaG_{Xooc} (36) is applied to plants, insect defense and plant growth enhancement (PGE) occur coordinately. Both processes are mediated by ethylene in response to HrpN_{Ea} (19). Thus, signaling pathways are distinct for the several effects of harpins in plants (30,34). Truncated HpaG_{Xooc} fragments can differentially activate HCD, PGE, and pathogen defense (43), suggesting that some components in the protein sequence may play important roles in activating these processes. The cysteine residue, on the other hand, may affect recognition of Hpa proteins by plants and subsequent activation of a signal transduction pathway because cysteine residues can form disulfide bonds and thus may contribute to protein-protein interactions (12,48). Moreover, GRM putatively is involved in interactions between proteins, proteins and nucleic acids as well as the cell matrix and plasma membrane (48). Such interactions may initiate a signal transduction cascade or pass exogenous signals to distinct intracellular processes (38,40). Consistently, cellular localization of harpins in transgenic plants greatly affects differential activation of HCD and SAR (44,53). Therefore, the cysteine residue and GRM may be crucial to the functions of HpaG proteins executed through distinct signal transduction pathways.

We sought to identify the roles of GRM and cysteine in the effects of HpaG proteins applied to plants. By modifying HpaG_{Xooc}, we produced HpaG_{Xooc}MG (MG), which no longer contains GRM, and HpaG_{Xooc}C47T (C47T), in which the original

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47-site cysteine has been replaced with threonine. HpaG_{X_{hoo}} is less active than C47T and MG in stimulating HCD, PGE, and pathogen defense, indicating that GRM and cysteine act to suppress the effects of the full-length protein.

MATERIALS AND METHODS

Plasmids and microbes. The vectors pET30a(+) and pMD18-T were from EMD Bioscience, Inc. (Darmstadt, Germany) and Takara Biotech. Co., Ltd. (Dalian, China). Both vectors contain *KpnI* restriction bases and pET30a(+) additionally contains an *NdeI* site used in this study. Selective markers are kanamycin resistance in pET30a(+) and both *lacZ* and ampicillin resistance in pMD18-T. The 77 promoter in pET30a(+) is inducible by isopropyl-β-D-thiogalactoside (IPTG). Aqueous solution of *Tobacco mosaic virus* (TMV) was stored under 4°C. Bacteria used were *X. oryzae* pv. *oryzicola* strain RS105, *P. syringae* pv. tomato DC3000, and *Escherichia coli* BL21 and DH5α harboring pET30a(+) and pMD18-T, respectively. They were lyophilized, stored at -80°C, and multiplied in nutrient broth (25,33). The medium was supplemented with kanamycin (20 μg/ml) or ampicillin (100 μg/ml) to grow *E. coli* cells.

Nucleic acid and plasmid operation. DNA and cDNA were manipulated by standard methods (50). They were fractionated by electrophoresis in agarose gel and visualized by staining with ethidium bromide (EB), using the DL2000 marker for sizing. Plasmids were isolated from bacteria using the 3S Plasmid Mini-prep Kit (Shanghai Shenergy Biocolor Biosci. & Tech. Co., Shanghai, China). Polymerase chain reaction (PCR) protocols were optimized to amplify DNA or cDNA for 25 cycles (44). PCR products and vectors were digested with *KpnI* and *NdeI*, respectively. After fraction in gel, desired fragments were purified with the Agarose Gel DNA Purification Kit (Takara Biotech. Co., Ltd., Dalian, China). The *KpnI/NdeI* fragments from PCR products and pMD18-T were ligated with the T4 DNA ligase. Recombinant vectors were transferred into DH5α by electroporation. Cultured DH5α cells were used to sequence the cloned PCR products. Sequences were confirmed by the Blast Search programs (provided online by the National Center for Biotechnology Information [NCBI]). Subsequently, the recombinant pMD18-T vectors containing correct gene sequences were isolated from DH5α and digested with *NdeI* and *KpnI*. Desired fragments were ligated with the *NdeI/KpnI* fragment excised from pET30a(+) and purified similarly as for pMD18-T. Recombinant pET30a(+) vectors containing desired gene inserts were subject to transformation to express the genes. Reagents were from Takara Biotech. and were used according to the vender's instructions.

Modification of *hpaG_{X_{hoo}}*. The gene sequence was checked to find elements that might facilitate the modification (Fig. 1A). PCR and enzymatic protocols were performed to produce *hpaG_{X_{hoo}}*, *hpaG_{X_{hoo}}*C47T (C47T), and *hpaG_{X_{hoo}}*MG (MG). Specific primers (Table 1) were synthesized according to the database in GenBank (accession no. AY875714). Full-length *hpaG_{X_{hoo}}* was cloned subsequent to amplifying RS105 genomic DNA with the P-*hpaG* primers. To obtain C47T, site-directed mutagenesis was done by PCR with the P-C47T primers, pMD18-T::*hpaG_{X_{hoo}}*, and the Takara MutanBEST Kit. Operation was as per the manufacturer's manual. MG was formed after assembling the up-, middle-, and down-stream regions interrupted by the two GRMs (Fig. 1A). A PCR product amplified with the P-MG-Up 1 primers was used as template for the second round of PCR accomplished with the P-MG-Up 2 primers. Resulting product was used to generate the upstream region. The PCR product amplified with the P-MG-Middle primers was used directly to generate the middle region. To obtain the downstream region, the PCR product of full-length *hpaG_{X_{hoo}}* was used. The three products were treated with *MspI*, which acts at bases originally present in *hpaG_{X_{hoo}}* and introduced into related PCR products by downstream primers

Fig. 1. Annotations of *hpaG_{X_{hoo}}* and HpaG_{X_{hoo}} sequences for modification of the gene and protein. **A**, Sequence annotations. Capital and small letters indicate the nucleotide and amino acid sequences, respectively, numbered in black (nucleotides) and blue (amino acids) on left for locations. Numbers on top indicate locations of the vertically enlarged nucleotides in horizontal lines. Colors in the sequences highlight factors critical to the study. As per nucleotides 1 to 18 in red and 403 to 414 in purple, P-*hpaG* primers (Table 1) were synthesized to amplify the bacterial DNA, producing full-length *hpaG_{X_{hoo}}*. Full length also was used as template to create C47T in polymerase chain reaction (PCR) with the P-C47T primers, and was used in the enzymatic reaction to generate MG (Table 1). Using the upstream P-*hpaG* primer with the downstream primers of P-MG-Up 1 and P-MG-Up 2 (Table 1) made on nucleotides 100 to 213 and 233 to 241 (orange), respectively, could generate the second product by two rounds of PCR. The upstream P-*hpaG* primer and the downstream P-MG-Middle primer (made as per nucleotides 306 to 318 in azure) were used together to create the third product, which could have a nucleotide shift of the original guanosine319 replacing the guanosine337 without changing the code. Digesting the three PCR products with *MspI*, which acts at the bases labeled in green, would generate several fragments. Three of them were assembled to form MG by excluding the GRM consensus, which encodes glycine (g)-rich motif (GRM) in the protein. Shown in blue are two copies of both GRM (214 to 231 and 319 to 336 nucleotides) and GRM (72 to 77 and 107 to 112 amino acids). The 139 to 141 nucleotide TGC coding for the 47-sited cysteine (c), in pink, was subjected to a substitution with AGC that encodes threonine (t). **B**, A diagram of the gene and protein versions. Bars in the left vertical panel depict *hpaG_{X_{hoo}}* (*hpaG*), *hpaG_{X_{hoo}}*C47T (C47T), and *hpaG_{X_{hoo}}*MG (MG); bars in the right panel indicate the proteins. Shown on the left to bars are numbers of nucleotides (Nnt) and numbers of amino acids plus predicted molecular masses (Naa/kD). The top numbers in parentheses indicate locations of the molecular modifications. The codes and amino acids as targets of the modification are shown in pink; blue areas indicate the motifs.

(Fig. 1A; Table 1). After digested products were fractioned in agarose gel, desired fragments were selected and ligated to make *MG*. Sequences of the three genes were confirmed and their encoded proteins were predicted, both using the Blast Search programs. Molecular masses of the proteins were estimated by the DNASTar EditSeq program (DNASTAR, Inc., Madison, WI; available online from DNASTAR website).

Protein production and analysis. Each of the recombinant pET30a(+) vectors containing correct genes was transferred into BL21 cells by electroporation. Transformed cells were incubated on a shaker at 200 rpm for 18 to 20 h to optical density at 620 nm (OD_{620}) ≥ 2.0 of bacterial density, determined under a light pathway with a 1-cm length. IPTG (1 mM) was added to the medium 3 h prior to harvesting bacteria. BL21 cells containing only the empty pET30a(+) vector were grown similarly to produce inactive protein preparation (pET) used as a control. Proteins were isolated from BL21 (5). Bacterial cells were precipitated by centrifuging at $7,000 \times g$ for 15 min, suspended in water, and incubated with Ready-Lyse (1 μ l/ml) (Epicenter Biotech., Rockford, IL) and phenylmethylsulfonyl fluoride (PMSF, 100 μ g/ml, prepared in dimethyl formamide) on ice for 10 min. Bacterial cells were sonicated in an ice bath, using a 1.5-cm probe driven by the Cell Disruptor (model 305, Branson Sonic Power Co., Indianapolis). The resulting bacterial lysate was mixed with a third addition of PMSF and centrifuged at $10,000 \times g$ for 20 min. Supernatant fractions were heated in a boiling water bath for 10 min, allowed to cool, and centrifuged ($12,000 \times g$, 20 min). To further remove unwanted sundries, the supernatant fractions were chilled overnight under -20°C , thawed, and centrifuged ($10,000 \times g$, 20 min) to obtain new supernatant fractions, which were regarded as protein preparations. Their bioactivities were tested based on the induction of an HR in tobacco leaves (5,18). Proteins were applied to dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7); HrpN_{Ea} (44 kDa), supplied in the product Messenger (Eden Bioscience Corp., Bothell, WA), and bovine serum albumin (66 kDa) were used to verify protein migration in the gel. Protein preparations were purified by fractionation in ammonium sulfate and anion HiPre Q XL exchange chromatography (AKTA Explore 100, Amersham Pharmacia Biotech Ltd., Piscataway, NJ) as described (44). Purified proteins were analyzed by SDS-PAGE to determine molecular masses.

Concentrations of HpaG_{Xooc}, MG, and C47T in protein solutions before fractionation in ammonium sulfate were determined by a combination of methods. Total proteins were quantified with the BCA Protein Assay Regent Kit (Epicenter Biotech.). HpaG_{Xooc}, MG, or C47T, in contrast to total proteins, was portioned after SDS-PAGE by a scanner attached to the Gel Doc XRS 170-8071 system (Bio-Rad Laboratories, Hercules, CA). Concentrations of the proteins were estimated based on their proportion in total protein. The estimate was confirmed by determining purified proteins with the kit.

Plant response studies. *Arabidopsis thaliana* (ecotype Col-0) plants were grown as described (18). Tobacco (*Nicotiana tabacum*)

cv. Xanthi (nc) and tomato (*Lycopersicon esculentum*) cv. Xiafen were grown at 25°C in environment-controlled chambers. Except when specified, 40-day-old tobacco seedlings and 20-day-old tomato and *Arabidopsis* seedlings were treated with aqueous protein solutions (10 μ g/ml) in the presence of the surfactant Silwet-77 (0.03%).

To determine HCD, the fourth and fifth leaves of tobacco plants were infiltrated separately with protein solutions. Macro-HR was observed at 16 to 24 h posttreatment (hpt). Micro-HR was monitored at 12 hpt by trypan blue-staining visualization protocol (45,54). Levels of HCD were estimated at 12 hpt based on Evan's blue up-take by dead cells in leaves (16). Leaf disks were incubated for 20 min with 0.05% Evan's blue and extensively washed to remove unbound dye. Dye bound to dead cells was solubilized by incubating 45 min at 50°C in a solution of 50% methanol and 1% SDS. OD_{600} of the solubilized dye was determined by spectrophotometry and noted as OD/pET, OD/H, and OD/M for pET, HpaG_{Xooc}, and C47T or MG, respectively. HCD caused by C47T or MG compared with HpaG_{Xooc} was quantified using the following formula: relative level of HCD = $100\% \times (\text{OD/M} \cdot \text{OD/pET}) / (\text{OD/H} \cdot \text{OD/pET})$.

Infection of tobacco plants by TMV and plants of tomato and *Arabidopsis* by *P. syringae* pv. tomato DC3000, respectively, was investigated by established methods (17,18). The fourth and fifth leaves of tobacco, leaves at the second and third shoots of tomato or lower leaves of *Arabidopsis* were sprayed separately with protein solutions. At 5 days posttreatment (dpt), the sixth leaves of tobacco were inoculated with TMV (1 μ g/ml); tomato leaves at the fourth shoots were inoculated with a DC3000 suspension containing 5×10^6 CFU/ml and 0.03% Silwet-77. Size of lesions caused by TMV on tobacco leaves was measured at 4 days after inoculation. The bacterial population in tomato and *Arabidopsis* leaves during 1 to 5 days after inoculation was monitored.

To investigate effects of the proteins on plant growth, tomato seeds were soaked for 6 h in a solution of each protein tested. Soaked seeds were sown in a mixture of sand and potting soil in 60-mm pots, and incubated at 25°C . Length of roots and fresh weight of the aerial parts of plants were monitored.

Plant gene expression was studied by reverse transcriptional PCR (RT-PCR) as described (11,19,44), using the *EF1 α* gene as a standard because of its constitutive expression in prokaryotes (6,24). RNA was isolated from the untreated top three leaves of *Arabidopsis*, sixth leaves of tobacco, and untreated expanding leaves on the fourth shoots of tomato. The fourth and fifth leaves of the tobacco plants and leaves below the fourth shoots of the tomato plants had been sprayed with solutions of the proteins tested. For *Arabidopsis*, lower leaves had been sprayed when tops were protected by an overlay with facial tissue and plastic wrapper. Primers were the same as in a previous study (44). The first-strand cDNA was synthesized with 2 μ g of RNA that had been treated with RNase-free DNase. An equal volume of cDNA was amplified 25 cycles. Fidelity of the protocol in producing desired genes was determined by sequencing RT-PCR products (19,44) and the Blast Search analysis. RT-PCR products were

TABLE 1. Polymerase chain reaction (PCR) primers used to produce three versions of *hpaG_{Xooc}* and product description

Primer name	Primer sequences ^a	Product description
P-hpaG	5'-CCC <u>ATATG</u> TGAACCTCTTGAACAC-3' 3'-AGCTACGTCATTATCC <u>ATGGCC</u> -5'	Full-length <i>hpaG_{Xooc}</i> , also used to generate the downstream region of the <i>hpaG_{Xooc}MG</i> version
P-C47T	5'-ACCAAGTTGCTGAGCCAGCTC-3' 3'-CCATAGAGCCTTTTCGTTGACC-5'	The <i>hpaG_{Xooc}C47T</i> version created by mutagenesis for the base in bold
P-MG-Up 1	5'-GGGGG <u>CATATG</u> TGAATTCTTTGAACA-3' 3'-AGTCGTCCACCGCTCTTATTAGTC-5'	Product resulted from two rounds of PCR, used as substance to generate the upstream region of <i>hpaG_{Xooc}MG</i>
P-MG-Up 2	5'-GGGGG <u>CATATG</u> TGAATTCTTTGAACA-3' 3'-CGCTCTTATTAGTCGTC <u>CGCC</u> -5'	
P-MG-Middle	5'-GGGGG <u>CATATG</u> TGAACCTCTTGAACACA-3' 3'-GAGGTCCGCGCTTAC <u>GGCC</u> -5'	Product directly used as substance to generate the middle region of <i>hpaG_{Xooc}MG</i>

^a The bases underlined are restriction sites specific for *Nde*I (CA↓ATG), *Kpn*I (GGTAC↓C), and *Msp*I (C↓CGG). Protection bases are included at 5' termini.

fractionated in agarose gel, stained with EB, and quantified by the gel documentation system.

Data analysis. Each assay was done three times; three replicates were involved each time; 45 *Arabidopsis* plants, 15 tobacco plants, and 20 tomato plants were tested in each replicate. Quantitative data were subject to *t* and analysis of variance (ANOVA) tests using the SAS/STAT tools (available online from the SAS Institute, Cary, NC) to determine significant differences between treatments.

RESULTS

Generation of HpaG_{Xooc}, HpaG_{Xooc}C47T, and HpaG_{Xooc}MG.

Checking of *hrpG_{Xooc}* and *HpaG_{Xooc}* sequences (Fig. 1A) established the success of the PCR and enzymatic protocols in modifying the gene and protein. Full-length *hpaG_{Xooc}* (Fig. 1B) was obtained from the bacterial DNA amplified by PCR with the P-*hpaG* primers (Table 1). The *hpaG_{Xooc}C47T* (C47T) version with the Cysteine47 residue mutated to threonine was generated by a site-directed mutagenesis PCR protocol using the P-C47T primers (Table 1) and *hpaG_{Xooc}* as template (Fig. 1B). The *hpaG_{Xooc}MG* (MG) mutant, lacking the two GRM regions (Fig. 1B), was created by reassembling three PCR-amplified fragments that flank the GRM regions (Fig. 1A). The three constructs were confirmed by DNA sequencing and the sequence of the MG construct has been deposited in GenBank (accession no. QD273269). Predicted proteins also showed the anticipated characteristics (Fig. 1B). C47T contained 138 amino acids and a threonine, instead of cysteine, at the site 47. There were no GRMs in MG, which contained 126 amino acids. Molecular masses of HpaG_{Xooc}, C47T, and MG were predicted to be 15.3, 15.3, and 14.2 kDa, respectively. The protein preparations migrated in SDS-PAGE as estimated by computer analysis and verified by the behaviors of HrpN_{Ea} and BSA in the gel (Fig. 2A). Similar analysis of purified proteins confirmed that they had molecular masses as predicted (Fig. 2B).

Effects on HCD responses. In macro-HR assays done with bacterial suspensions, *E. coli* strains that contained full-length *hpaG_{Xooc}*, C47T, and MG were active. In contrast, the strains containing the empty vector pET30a(+) did not cause the HR. This result was confirmed by similar tests with preparations of the proteins. As shown in Figure 3A, C47T and MG were similar to HpaG_{Xooc} in eliciting tobacco macro-HR observed at 24 h after infiltrating leaves with solutions of the proteins. No HR trace was seen in the control panel infiltrated with pET, which was made from the bacterial cells harboring only the pET30a(+) vector and contained neither HpaG_{Xooc} nor its derivatives. Based on determining Evan's blue up-take by dead leaf cells (Fig. 3B), HCD levels varied markedly between treatments. Relative to the control, HCD elicited by HpaG_{Xooc}, C47T, or MG was significant (*t* and ANOVA tests, *P* = 0.05). C47T and MG induced cell death 1.2- and 1.7-fold higher, respectively, than that induced by HpaG_{Xooc}.

Micro-HR may occur in plant leaves sprayed with harpins when macro-HR does not appear and both resistance and HCD are stimulated (45). We monitored micro-HR by observing leaves stained with trypan blue (Fig. 3C). Micro-HR occurred 12 hpt with each of the three proteins but was not evident in the control. When HpaG_{Xooc} was applied, micro-HR occurred in some epidermal areas. Micro-HR was distributed more extensively over the epidermis of leaves treated with C47T or MG. MG caused the optimal effect.

The *hin1* and *hsr203* genes are molecular markers of HCD (46,47) and are activated by applying harpins to plants (43, 44). RT-PCR protocol was conducted to study whether the HCD induction in treated leaves was coincident with the gene expression induced in the untreated sixth leaves of tobacco (Fig. 3D). When determined at 72 hpt, both genes were expressed evidently

in leaves treated with HpaG_{Xooc}, C47T, or MG compared with pET. The use of the constitutively expressed *EF1α* gene in the assay indicated uniform loading of RNA and amplification of the genes. Based on ANOVA test (*P* = 0.05), however, C47T and MG were significantly more effective than HpaG_{Xooc}. Thus, HCD and its marker genes coordinately responded to the proteins.

Effects on pathogen defense. In response to an elicitor, induced resistance nullifies infection of plants by pathogens (17, 18). On tobacco leaves, sizes of lesions caused by TMV greatly varied with treatments, as observed at 4 days after inoculation (Fig. 4A). Differences in lesion size were significant between treatments with pET and with HpaG_{Xooc}, C47T, or MG (*t* test, *P* = 0.05). Relative to the control, the application of HpaG_{Xooc}, C47T, or MG decreased the different proportion of lesion size to leaf size (Fig. 4B). The optimal reduction in lesion size was provided by MG. C47T was less active than MG and more active than HpaG_{Xooc} in the effect. Therefore, resistance developed to inhibit

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteins. **A**, Gel patterns of the protein preparations evaluated in comparison with known proteins. The inactive protein preparation pET and preparations of HpaG_{Xooc}, C47T, and MG were analyzed before purification. A product containing the 44-kD HrpN_{Ea} and a 66-kD bovine serum albumin (BSA) were used at 3 and 10 µg, respectively, to verify protein migration in gel. **B**, Gel patterns of the purified proteins. The preparations were purified by chemical fractionation and chromatography. Resulting aqueous solutions of purified proteins were concentrated to 0.3 µg/µl (300 µg/ml); 10 µl of each solution was loaded in gel. In **A** and **B**, the same makers were used; gel was stained with Coomassie brilliant G-250.

TMV infection in the plants responding to HpaG_{X_{00C}}, C47T, and MG; MG induced the greatest level of resistance.

We then assayed for induced resistance against *P. syringae* pv. tomato DC3000 in *Arabidopsis* and tomato plants following treatments with HpaG_{X_{00C}}, C47T, and MG compared with the control. When observed at 7 days after inoculation by spraying plants with the bacterial suspension, symptoms appeared on leaves as chlorosis and necrosis, which varied with treatments (Fig. 4C; data not shown). Symptoms spread from leaf margin to an extensive area in control plants. In contrast, symptom was less severe on leaves of plants treated with HpaG_{X_{00C}}, C47T, and MG. Consistently, bacterial growth in leaves was reduced differently by the application of the different proteins (Fig. 4D). At each time point, bacterial number in plants treated with HpaG_{X_{00C}}, C47T, or MG was less than that in control plants. During 1 to 5 days, bacterial numbers increased ca. 16,000-fold in control plants and ca. 1,000- or ca. 200-fold in plants treated with HpaG_{X_{00C}} or C47T, respectively. The increase in bacterial growth was only ca. 100-fold in plants treated with MG during the same period. Thus, induced resistance in the plants inhibited bacterial multiplication and reduced severity of symptoms. MG was most active in the effect.

The *NPR1* gene regulates SAR signal transduction (9,21) and is required by harpins to activate SAR (18,45) and expression of SAR genes, like *PR-1a* and *Chia 5* (12,22,49). Analysis by RT-PCR, using *EF1α* as a standard, indicated that expression of

NPR1, *PR-1a*, and *Chia 5* were markedly induced or enhanced in untreated leaves of *Arabidopsis*, tobacco, and tomato plants treated with HpaG_{X_{00C}}, C47T, or MG (Fig. 4E; data not shown). Expression of *NPR1* and *Chia 5* was constitutive at low levels but was enhanced, evidently by the proteins, whereas that of *PR-1a* was induced, not constitutive. The three genes were expressed to the greatest levels in MG treatment, relative to other treatments. Clearly, the genes behaved consistently with pathogen resistance in response to the proteins.

Effects on plant growth. In plants that have been tested for PGE by harpins, tomato is best to evaluate the phenotype. Soaking tomato seeds in a solution of HrpN_{Ea} promotes growth of roots and aerial parts of the plants (19). Thus, PGE by the three proteins was evaluated with tomato. After soaking seeds in a solution of HpaG_{X_{00C}}, C47T, or MG, plants grew better than those from the pET treatment, as observed at 15 dpt (Fig. 5A). When compared with HpaG_{X_{00C}}, nevertheless, C47T and MG both provided greater PGE based on fresh weight per plant; the best effect was observed with the MG treatment. Root growth also was stimulated by treatment with HpaG_{X_{00C}}, C47T, or MG (Fig. 5B). Within 15 dpt, aerial parts and roots of the plants increased growth by 15 and 53%, 25 and 77%, and 46 and 106%, respectively, in response to HpaG_{X_{00C}}, C47T, and MG, relative to controls (*t* test, *P* = 0.05). Clearly, MG provided the greatest PGE. In an assay for dose effects monitored at 10 dpt, plant growth varied subsequent to

Fig. 3. Tobacco hypersensitive cell death (HCD) responses. **A**, A representative leaf showing macro-hypersensitive response (HR). Leaves were infiltrated with a solution of the proteins and photographed at 24 h posttreatment (hpt). **B**, Relative levels. Leaves equivalent to that in **A** were treated with Evan's blue at 12 hpt. The dye that stained dead cells was determined to evaluate relative levels of cell death while defining the level provided by HpaG_{X_{00C}} as 1. Histograms represent means $\pm$ standard deviation of results from three replicates. **C**, Views of trypan blue-stained leaves. Leaves were equivalent to those in **A** and **B**. Epidermal cells with micro-HR stained blue. Scale bar is 10 μ m. **D**, Expression of the HCD-marker genes *hsr203* and *hin1* compared with *EF1α* used as a standard. Reverse transcription-polymerase chain reaction was done with RNA isolated at 24 hpt from untreated leaves of treated plants. Experiments were done three times with similar results.

soaking seeds with MG solutions of different concentrations compared with pET (Fig. 5C). When a range of 0 to 30 $\mu\text{g/ml}$ was tested in increments of 0.25 $\mu\text{g/ml}$, 5 to 10 $\mu\text{g/ml}$ was most effective although doses higher than 10 $\mu\text{g/ml}$ still supported PGE.

To determine if the proteins stimulate growth signaling events, the tomato expansin gene *EXP2* was studied because it contributes to PGE as function of harpins under regulation by ethylene signaling (19). Figure 5D shows results of RT-PCR assays verified by use of *EF1 α* . *EXP2* was not expressed in control plants,

but it was expressed at a similar time course in untreated leaves of plants treated with HpaG_{X00C}, MG, or C47T. High levels of *EXP2* expression were detected at 48 and 72 hpt. Compared with HpaG_{X00C}, C47T and MG both induced higher levels of the expression, as determined at 72 hpt; MG was better than C47T (Fig. 5E). These results suggest that the expansin gene expression is consistent with PGE in response to the proteins.

DISCUSSION

Bacterial harpin proteins belong to an important group of type III effectors that elicit multiple plant responses when secreted by

Fig. 4. Tobacco and *Arabidopsis* responses to *Tobacco mosaic virus* (TMV) and *Pseudomonas syringae* following treatment with proteins evaluated. **A and B**, TMV infection of tobacco. The fourth and fifth leaves of plants were sprayed separately with solutions of the proteins. The sixth leaves were inoculated with TMV at 5 days posttreatment (dpt) and investigated 4 days later. Means in sizes of lesions caused by the virus on leaves (LS) are shown in red on photos. The proportion of lesion size to leaf size was determined as mean $\pm$ standard deviation (SD) of three replicates. **C and D**, Bacterial infection of *Arabidopsis*. Plants were sprayed with the proteins when top two leaves were protected from spraying. Treated plants were inoculated at 5 dpt. Seven days later, untreated and inoculated leaves were photographed. Bacterial population was monitored for untreated but inoculated upper leaves of treated and inoculated plants. Curves represent mean $\pm$ SD of results from three replicates. **E**, Expression of defense-related genes in tobacco plants. Gene expression was analyzed similar to that in Figure 4. Experiments were done three times with similar results.

Fig. 5. Effects of the proteins on tomato growth and expression of the expansin gene *EXP2*. **A and B**, Growth of aerial parts and roots. Seeds were soaked for 6 h in a solution (10 $\mu\text{g/ml}$) of the proteins tested, sown in pots, and incubated in a chamber. The numbers on photos and the histograms refer to averages of fresh weight per plant and root length, both determined at 15 days posttreatment (dpt) for three replicates. **C**, Dose effects of MG versus pET. Assay was similar to that in **A**. Seedlings were observed at 10 dpt. Means of fresh weight per plant are indicated. **D**, Pattern and level of the *EXP2* gene expression. Plants were grown from untreated seeds. Leaves below the fourth shoot were sprayed with solutions of the proteins indicated on left. RNA was isolated from expanding leaves at the fourth shoots. A reverse transcription-polymerase chain reaction protocol was conducted for *EXP2* (top four gel panels), involving *EF1 α* as a standard, for which partial results are shown (bottom two panels). Relative levels of *EXP2* expression at 72 h posttreatment were quantified. Histograms represent means $\pm$ standard deviation of the results from three replicates.

pathogens during infection (2,23,31,41,51), and that cause beneficial effects when applied to plants (13,18–20,34–36,43–45,52). We focus on the second aspect to characterize functional and structural features of the proteins. As reported here, two mutant versions of HpaG_{Xooc} were more effective than the wild-type protein in inducing plant defense and growth. This finding has potential relevance in applied agriculture and is implicated in mechanisms that underlie bacterial pathogenicity.

Directed mutation in HpaG_{Xooc} created the HpaG_{Xooc}C47T (C47T) and HpaG_{Xooc}MG (MG) versions, which lacks the glycine-rich motif (GRM) and replaces the cysteine residue with threonine, respectively. Both versions were active in the bioassay by an established method (5,45). Tobacco plants concomitantly responded to the mutant proteins in developing HCD and expressing the genes regarded as HCD markers (15,46,47). Both events can be induced by HpaG_{Xooc} (36) and several other harpins (44, 45,53). In *Arabidopsis*, tobacco, and tomato, C47T and MG coincidentally elicited resistance to the pathogens and the expression of *NPR1* and PR genes involved in the SAR pathway (21,49). Earlier studies have demonstrated that the pathway regulates pathogen defense in response to HpaG_{Xooc} (36,56) and other harpins (18,44,52). When the proteins were applied to seeds and seedlings of tomato, subsequent growth of roots and aerial parts of the plant was promoted; expression of the expansin gene *EXP2* was induced as well. The cell expansion and plant growth are attributed to induced accumulation of expansin transcripts and proteins under mediation by ethylene (10,37). In plants treated with HrpN_{Ea}, ethylene signaling is responsible to the expression of expansin genes and the PGE phenotype (19). These results suggest that C47T and MG act similarly as do intact molecules of harpins (18,44,45,52) in stimulating HCD, defense, and growth pathways.

Proteins of harpin group have a great potential for use in agriculture because they cause several beneficial effects, including resistance to pathogens (18,43–45,52) and insects (19), promotion of plant growth (19), and drought tolerance (20). Some of the effects have been found to be useful in a variety of crops. Increasing the effects was an important purpose of modifying the proteins. Both C47T and MG were more effective than HpaG_{Xooc} in stimulating pathogen defense and plant growth. Although C47T was similar to HpaG_{Xooc} in eliciting HCD, C47T induced greater levels of defense and growth. Unlike C47T, MG was much more active than HpaG_{Xooc} in eliciting the three phenotypes. These results could promise more effective agricultural use of the mutant proteins in contrast to the intact molecule. In laboratory assays, HrpN_{Ea}, HpaG_{Xooc}, or HpaG_{Xooc} of 0.341 μ M in aqueous solutions can provide optimal effects in inducing defenses against pathogens (45), insects (19), and drought stress (20). The molar concentration is equal to, in practical scale, 15 g kg⁻¹ HrpN_{Ea}, 5.3 g kg⁻¹ HpaG_{Xooc}, and 5.2 g kg⁻¹ HpaG_{Xooc}. In this study, 5 μ g/ml (5 g kg⁻¹) MG conferred the best promotion of the plant growth. Thus, molecular modification of harpins could increase economic efficiency in agricultural application.

It is intriguing that the presence of GRM and the cysteine residue in HpaG_{Xooc} experimentally represses the several effects of the protein. Recently, avirulence proteins, the other important group of type III effectors (2,5,27,29,51), have been shown to facilitate pathogenicity by suppressing plant defense signaling (4,8,14,26,32). Our results indicate that harpins may act similarly by applying an intramolecular regulation mechanism to the different functions. Several regions in HpaG_{Xooc} (43) and HpaG_{Xooc} (H. Dong, unpublished data), when isolated from the proteins, are more active than the intact molecules to differentially elicit HCD, PGE, and pathogen defense. Thus, the intact proteins impair the observed full effects of the functional regions. Inversely, GRM and cysteine in HpaG_{Xooc} repress the effects of the full-length molecule, indicating that GRM and cysteine antagonize the regions in the effects. However, harpins have the potential to activate distinct signaling pathways (18,27,44) but often prioritize

one pathway over others instead of executing all the pathways simultaneously (19,34,35). Partial reasons for the phenomenon seem to be the challenges being exerted to plants (18–20). When harpins are applied to plants, the opposite modes of the supposed intramolecular regulation may act to balance the pathways. Similar modes also may affect pathogenicity when harpins get access to plants after secreted by bacteria (39,51). Whereas harpins induce plant defense via the SAR pathway and indirectly through PGE (18,20), repressing both processes is critical to successful infection (1,2,42). GRM present in all the harpins and cysteine in Hpa proteins may facilitate pathogenicity by inhibiting the proteins to activate plant defense and growth signaling pathways. The hypothesis remains to be examined.

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