Identification of Specific Fragments of HpaG_{Xooc}, a Harpin from *Xanthomonas oryzae* pv. *oryzicola*, that Induce Disease Resistance and Enhance Growth in Plants

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ABSTRACT

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Harpin proteins from gram-negative plant-pathogenic bacteria can stimulate hypersensitive cell death (HCD) and pathogen defense as well as enhance growth in plants. Two of these diverse activities clearly are beneficial and may depend on particular functional regions of the proteins. Identification of beneficial and deleterious regions might facilitate the beneficial use of harpin-related proteins on crops without causing negative effects like cell death. Here, we report the identification and testing of nine functional fragments of HpaG_{Xooc}, a 137-amino-acid harpin protein from *Xanthomonas oryzae* pv. *oryzicola*, the pathogen that causes bacterial leaf streak of rice. Polymerase chain reaction-based mutagenesis generated nine proteinaceous fragments of HpaG_{Xooc}; these caused different responses following their application to *Nicotiana tabacum* (tobacco) and *Oryza sativa* (rice). Fragment HpaG₆₂₋₁₃₇, which

spans the indicated amino acid residues of the HpaG, induced more intense HCD; in contrast, HpaG₁₀₋₄₂ did not cause evident cell death in tobacco. However, both fragments stimulated stronger defense responses and enhanced more growth in rice than the full-length parent protein, HpaG_{Xooc}. Of the nine fragments, the parent protein and one deletion mutant of HpaG_{Xooc} tested, HpaG₁₀₋₄₂, stimulated higher levels of rice growth and resulted in greater levels of resistance to *X. oryzae* pv. *oryzae* and *Magnaporthe grisea*. These pathogens cause bacterial leaf blight and rice blast, respectively, the two most important diseases of rice worldwide. HpaG₁₀₋₄₂ was more active than HpaG_{Xooc} in inducing expression of several genes that regulate rice defense and growth processes and activating certain signaling pathways, which may explain the greater beneficial effects observed from treatment with that fragment. Overall, our results suggest that HpaG₁₀₋₄₂ holds promise for practical agricultural use to induce disease resistance and enhance growth of rice.

Additional keywords: hypersensitive response (HR), systemic acquired resistance (SAR).

Harpin proteins, produced by gram-negative plant-pathogenic bacteria, generally affect virulence in host plants and induce hypersensitive cell death (HCD) in nonhost plants (4,70,51). In addition to HCD, several beneficial effects also are induced following application of the proteins to plants. Resistance to various pathogens develops in a variety of plants responding to any harpins, such as HrpN_{Ea} from *Erwinia amylovora* (13,18, 19,49,68) and HrpZ_{Pss} from *Pseudomonas syringae* pv. *syringae* (61). Insect repellency occurs in HrpN_{Ea}-treated cucumber; striped cucumber beetles prefer to colonize control plants rather than HrpN_{Ea}-treated plants (77). Treating *Arabidopsis thaliana* with HrpN_{Ea} inhibits reproduction of the green peach aphid (*Myzus persicae*) feeding on the plant (14). Harpin-induced defenses often accompany enhanced plant growth (13,14,68,77). Moreover, treatment with HrpN_{Ea} increases drought tolerance of *Arabidopsis* growing under conditions of water deficit (15,74). Overall, harpins have been recognized as multifunctional elicitors in plants.

The diverse actions of harpins in plants are attributable to the activation of distinct signaling pathways. In response to harpins, HCD occurs as a consequence of the actions by reactive oxygen species (ROS) (2,10,22,47). The level of hydrogen peroxide (H₂O₂), an important ROS (2,12,35), is elevated to regulate expression of marker genes of HCD, such as *hsr203* (6,50) and *hin1* (22). When the salicylic acid-mediated systemic acquired resistance (SAR) pathway is activated (13,61), signaling by jasmonic acid is repressed (13,14); otherwise, HCD and SAR are inhibited in the presence of jasmonate (3). SAR induced by harpins (13,14,49) requires NDR1 (1,8,9) and EDS1 (41,45), which both regulate SAR (49) and race-specific resistance (46). NPR1, another SAR-regulatory protein (7,16), also is required (13,49). During concomitant induction of HCD and SAR, ethylene signal transduction is activated and the signal transducers EIN5 and EIN2 (23,66) are recruited to regulate enhanced plant growth and insect resistance, respectively (14). However, ethylene signaling does not contribute to the induction of disease resistance (13). In plants growing under conditions of water deficit, treatment with HrpN_{Ea} stimulates production of abscisic acid to induce drought tolerance; ABI2, a 2C phosphatase, plays a critical role (15). This differs from many other abscisic acid-dependent processes, such as seed development and responses to salinity, which need two 2C phosphatases, ABI1 and ABI2

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*The e-Xtra logo stands for "electronic extra" and indicates that there are three additional figures available online showing bioactivities of the cell-free proteins.

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(29,36). These studies suggest differential roles of distinct signaling pathways in plant responses to harpins.

HpaG proteins were identified as harpins in the genus *Xanthomonas* by targeting the *hrp*-associated (*hpa*) loci (32,43,75). Better-studied HpaG proteins include HpaG_{Xoo}, produced by *Xanthomonas oryzae* pv. *oryzae*, the bacterial leaf blight pathogen of rice (38,48,67); and HpaG_{Xoo} from *X. oryzae* pv. *oryzicola*, the bacterial leaf streak pathogen of rice (38,40). The HpaG harpins include two to four characteristic glycine-rich motifs (GRMs) (25, 70). HpaG_{Xoo} contains 137 amino acids, is 15.18 kD in size, and includes a GRM from amino acid 72 to 77 and another from 107 to 112 (40). All described HpaG proteins contain one cysteine residue (38,40,70), which is absent from other harpins (25,70). The cysteine residue is amino acid 47 in HpaG_{Xoo} (38,47). Despite these distinctions, HpaG proteins affect plants like other harpins, promoting plant growth (37,47) and inducing HCD (48, 67) and pathogen resistance (40,48). In HpaG_{Xoo}-transgenic rice plants, expression of the defensive protein kinase gene *OsARF1* and the expansin gene *OsEXPI* are induced coincidentally with enhanced plant growth and disease resistance (52). Clearly, there are several data indicating that, when the harpin of a rice pathogen is applied to rice, plant growth characteristics are altered and some of the alterations can be considered beneficial.

Special regions of harpin proteins are responsible for eliciting different plant phenotypes. Deletion of GRMs and the sole cysteine residue increases the effects of HpaG_{Xoo} on plant growth, HCD, and pathogen defense (40). Tobacco HCD induced by HpaG of *X. axonopodis* pv. *glycines* is attributable to a 17-amino-acid region of the protein (31). HrpN_{Ea} fragments acting to elicit HCD and several beneficial effects also were characterized (34,69). Functional regions of HpaG_{Xoo} induce plant growth, HCD, and defen-

sive responses consistently with activating specific promoters in transgenic tobacco (48). We hypothesized that individual portions of HpaG_{Xoo} are responsible for the several phenotypes elicited by the full-length protein. We reasoned that, if this hypothesis is supported, the specific portions of HpaG needed to induce beneficial effects might be used in plants without sustaining HCD, a phenotype that increases photosynthesis and respiration without contributing to plant growth and productivity (21).

We created specific fragments of HpaG_{Xoo} and evaluated each for effects in plants (Fig. 1). Polymerase chain reaction (PCR)-based mutagenesis applied to the *hpaG_{Xoo}* gene created nine fragments. Recombinant proteins were purified and bioassayed in the lab and in small field plot experiments. HpaG₁₋₉₄, HpaG₆₂₋₁₃₇, and HpaG₁₀₋₄₂, which cover HpaG_{Xoo} regions composed of amino acids as indicated by the subscript numbers, were found more effective than HpaG_{Xoo} in stimulating plant growth and inducing HCD in tobacco and disease resistance in rice. HpaG₁₀₋₄₂ was most active in eliciting resistance to rice bacterial leaf blight and blast, the most devastating diseases of rice worldwide (58,76). Here, we describe identification of the fragments, their characteristics, and their activities in plants. The effects of some of the same fragments applied to extensive rice plantings are reported in the companion article.

MATERIALS AND METHODS

Plasmids, microbes, and plants. Materials used in molecular operations throughout this report were all from EMD Biosciences, Inc. (Darmstadt, Germany) unless otherwise noted. The vectors pET30a(+) and pMD18-T Simple (TaKaRa Biotechnology Co., Ltd., Dalian, China) pET30a(+) contain *EcoRI* and *HindIII* restriction sites, which were both added to pMD18-T by the primers. Following insertion of a gene of interest, the selective markers available are kanamycin resistance (*Kan^r*) in pET30a(+) and both *lacZ* and ampicillin resistance (*Amp^r*) in pMD18-T Simple (Fig. 2A). The T7 promoter present in pET30a(+) is inducible by isopropyl-β-D-thiogalactoside (IPTG). Strain PX099 of *X. oryzae* pv. *oryzae*, labeled with a gene encoding a green fluorescent protein (56), was used to inoculate the susceptible rice (*Oryza sativa*) cv. R109. Strain RS105 of *X. oryzae* pv. *oryzicola* was used as a source of HpaG_{Xoo}. Modifications of *hpaG_{Xoo}* were done in *Escherichia coli* strains BL21 and DH5α harboring pET30a(+) and pMD18-T Simple, respectively. Bacteria were lyophilized, stored at -80°C, and cultured in NB medium (33). For growth of *E. coli*, the media were supplemented with kanamycin at 20 µg/ml or ampicillin at 100 µg/ml. The virulent strain G1 of the rice blast pathogen (*Magnaporthe grisea*) was provided by our colleague Y.-J. Zhou; it was cultured as described (17,42).

Plants of the *Arabidopsis* ecotype Col-0 were grown as previously described (13) and used when 30 days old. Tobacco (*Nicotiana tabacum*) cv. Xanthi was grown at 25°C in controlled-environment chambers for 30 days before use. Depending on the specific experiment, plants of the *indica* rice cv. R109 were grown at 25 to 30°C in petri dishes, controlled-environment chambers, or in the field, in plots protected from insect infestation by netting, on the campus of Nanjing Agricultural University. The field plots for protein bioassay contained three replicates of 5 m² for each treatment.

Nucleic acid manipulation. Standard methods (56) were used. Unless otherwise stated, reagents and kits were obtained from TaKaRa Biotechnology Co. Ltd. PCR protocols were optimized to amplify DNA or cDNA for 25 cycles (40,47). DNA and cDNA were fractionated by electrophoresis in agarose gel and visualized by staining with ethidium bromide, using DL2000 as a size marker. PCR products were purified with the DNA Fragment Purification Kit and digested with *EcoRI* and *HindIII*. Resulting fragments were subjected to electrophoresis and purified with the

Fig. 1. Scheme for generation of fragments of HpaG_{Xoo} and evaluation of their activity in plants. Fragment derivatives of *hpaG_{Xoo}* were generated by conventional polymerase chain reactions (PCRs). Nine cDNA fragments were created and used to produce nine derivatives of the HpaG_{Xoo} protein. Bioactivities of the proteins were assayed on a lab scale with tobacco and rice seedlings, as depicted. Three derivatives with differential effects on plants were identified and confirmed by experiments with rice growing in small field plots. Only fragment HpaG₁₀₋₄₂ provided high levels of disease resistance and enhanced growth of rice without inducing noticeable hypersensitive cell death (HCD). HpaG₁₀₋₄₂ was tested in comparison with HpaG_{Xoo} under grower field conditions; the results are reported in a companion article.

Agarose Gel DNA Purification Kit. Purified *Eco*RI and *Hind*III fragments were cloned into pMD18-T Simple vector. Recombinant vectors were transferred to DH5 α by electroporation. Cultured DH5 α cells were used to sequence the cloned PCR products. Subsequently, recombinant pMD18-T Simple vectors containing correct gene sequences were isolated from DH5 α and digested with *Eco*RI and *Hind*III. Desired fragments were ligated with the larger fragment from similar digestion of pET30a(+) isolated from BL21 cells using the 3S Plasmid Miniprep Kit. Recombinant pET30a(+) vector was transformed into BL21; positive clones were sequenced. Sequences were confirmed with Blast Search programs.

Modification of HpaG_{Xooc} and characterization of derivatives. The *hpaG_{Xooc}* gene and its derivatives were produced by PCR with *X. oryzae* pv. *oryzicola* genomic DNA and specific primers (Table 1). PCR products were cloned, sequenced, and confirmed as described. Each correct sequence was inserted into the pET30a(+) vector between *Eco*RI and *Hind*III sites to reserve the upstream histidine tag (His-tag) close to T7 promoter; the downstream His-tag was excluded by adding proper stop codons in the primers (Fig. 2A; Table 1). After transformation and sequencing, recombinant BL21 cells were incubated to produce protein preparations, termed cell-free preparations, by methods described previously (13,40). BL21 cells containing only the empty pET30a(+) vector were grown similarly to produce an inactive protein preparation, termed empty vector preparation (EVP), which was used as a control. Cell-free preparations were dissolved in imidazole-binding buffer (pH 7.4) and phenylmethylsulfonyl fluoride (PMSF) at 100 μ g/ml. Concentrations of the buffer differed depending on the proteins and were determined during process of protein purification by nickel chromatography with the HisTrap HP Kit (Amersham Biosciences Corp., Piscataway, NJ). The purification protocol was started by saturating the resin with 10 ml of imidazole-binding buffer at the same concentrations as used to dissolve cell-free preparations. Proteins loaded in the resin were eluted with 10 mM phosphate buffer containing imidazole at the concentrations determined for each protein. Molecular masses were predicted with the DNASTar program and verified by tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (T-SDS-PAGE) of the proteins (57).

Protein bioassays. Two rounds of bioassays were done. In the first, cell-free preparations were tested to select HpaG_{Xooc} fragments with differential effects on plants. Selected fragments were purified and subjected to a second round of bioassays after the His-tag was excised by treatment with the Novagen Enterokinase Cleavage Capture Kit (EMD Biosciences Inc.). HrpN_{Ea} was prepared (4,13) and used as a positive control in some assays. Purified proteins in aqueous solutions, suspected proteins in cell-free preparations, and total proteins in EVP were quantified as previously described (13,40). Proteins were used at 12 μ g/ml together with 0.03% of the surfactant Silwet-77 (Momentum Performance Materials Inc., China Branch, Beijing).

HCD in the forms of macro-HR and micro-HR was observed by eye and with the aid of a microscope following straining leaves with trypan blue (49); levels of cell death were determined based on Evan's blue uptake by dead cells (3). To observe macro-HR, the fifth youngest fully expanded leaves on 30-day-old tobacco plants were infiltrated with aqueous solutions of the proteins tested; the infiltrated areas were examined for macro-HR 24 h after infiltration. To test micro-HR and cell death levels, plants were sprayed with solutions of the proteins. Micro-HR was monitored at 12 h posttreatment (hpt) by microscopic observation of the treated leaves after staining with trypan blue, a protocol that can visualize only a few dead cells, which are not easily detectable by eye (49). Levels of cell death that occurred in leaf tissues were estimated at 12 hpt by Evan's blue uptake assay (3). In the assay, leaf discs were incubated for 20 min with 0.05% Evan's blue and washed extensively with highly purified water to

remove unbound dye. Dye bound to dead cells was solubilized by incubating for 45 min at 50°C in a 50% (vol/vol) aqueous solution of methanol, containing 1% (wt/vol) SDS. The absorbance of the solubilized dye was determined by spectrophotometry at 600 nm.

Proteins were tested for their ability to promote growth and induce disease resistance in rice. To observe root growth, seed were disinfested, soaked separately in filter-sterilized solutions of the proteins, and then incubated on filter paper previously wetted with MS medium (56) and placed in 9-cm-diameter petri dishes. The lengths of roots were measured after incubating for 6 days. Seedlings were transplanted into field plots to observe subsequent growth. For testing resistance to leaf blight and blast diseases on

Fig. 2. Vectors and constructs used to generate fragment derivatives of *hpaG_{Xooc}* and their characteristics. **A**, Regions of the sequencing and expression vectors showing critical sites and genes. The *hpaG_{Xooc}* gene and its derivative fragments were cloned individually in pMD18-T Simple. Confirmed cDNA sequences were cloned in pET30a(+) for the second sequencing and transformation of *Escherichia coli* cells. Two stop codons had been added to the 3' terminus of each insert in the recombinant pET30a(+) vector, to reserve the histidine tag (His-tag) close to T7 promoter and exclude the downstream His-tag (shaded). **B**, Electrophoresis of fragments of *hpaG_{Xooc}* cloned in pMD18-T Simple and digested with *Eco*RI and *Hind*III. EVP denotes empty vector preparation. The truncated *hpaG_{Xooc}* fragments were named after the spans of nucleotides indicated by subscript numbers. **C**, Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis of cell-free preparations of the proteins produced by recombinant *E. coli* cells. The protein fragments were named after the spans of amino acids indicated by subscript numbers. Each protein preparation (5 μ l) was loaded in the gel; estimated amounts (μ g) were loaded are indicated. The experiments depicted in B and C were done three times with similar results; data from one experiment is shown.

plants growing in the field plots, rice seed without treatment were sown. At 40 days after sowing, the seedlings were sprayed with the protein solutions. Five days later, separate groups of plants were inoculated with strain PXO99 of *X. oryzae* pv. *oryzae* and isolate G1 of *M. grisea*. PXO99 cells were inoculated into three expanded leaves by cutting leaf tips (1 cm) with scissors dipped in a 5×10^7 CFU/ml of bacterial suspension (39). Inoculated plants were incubated as described previously (13). Bacterial populations in leaf tissues were determined 2 days after inoculation. Bacteria-containing plant homogenates were suspended in phosphate buffer (0.2 mM, pH 7.0), diluted, and plated on NB agar medium (56). Resulting bacterial colonies were observed with a fluorescent binocular microscope. Leaf blight severity was scored 20 days after inoculation. To induce leaf blast, 15-day-old plants were inoculated by spraying the top two leaves with conidial suspensions of G1. Inoculated plants were placed in a glass box and incubated in the dark at 25°C and 100% relative humidity. Blast symptoms were observed 10 days after inoculation. Induced resistance was evaluated based on symptom severity of plants treated with the individual proteins relative to the severity of plants that had been treated with EVP.

Studies of plant signaling events. Plant H_2O_2 burst and expression of defense-related and growth-related genes were studied. The two youngest fully unfolded leaves of treated tobacco plants were assayed at 12 hpt for accumulation of H_2O_2 , which was visualized by staining leaves with diaminobenzidine (DAB) (3,47). Reverse-transcriptional PCR (RT-PCR) was conducted to determine expression of *hsr203* and *hin1* (23,50) in tobacco, *NPRI* and *PR1* in *Arabidopsis*, and *OsARF1* and *OsEXPI* in rice. Solutions of the evaluated proteins were applied by spraying the tops of 30-day-old *Arabidopsis* and tobacco plants growing in a controlled-environment chamber. Twenty-day-old rice seedlings growing in a field plot were treated similarly, except they were treated also with the fungicide Tricycloazole at 4 mg/ml (20% wettable powder; Guoying Kunshan Biochemical Co., Ltd., Naging, China). At

2 days post treatment (dpt), RNA was isolated from the youngest two fully expanded leaves as described (13,52). RT-PCR was done using RT-PCR Beads (Amersham Pharmacia Biotechnology Inc.), as per provided protocols. The previously designed primers for tobacco *hsr203* and *hin1* (47), rice *OsARF1* and *OsEXPI* (52), and *Arabidopsis NPRI* and *PR1* (49) were synthesized for use in this study. The *EF1 α* gene, which is constitutively expressed in eukaryotes (5,20), was used as a standard, and the primers designed previously for the plant species were synthesized (47,49, 52). Genes were amplified for 25 cycles by the RT-PCR protocol to estimate levels of expression (47,49); products were cloned, sequenced, and confirmed as described (49). After electrophoresis and staining with ethidium bromide, relative levels of gene expression were estimated from band intensities aided by a gel documentation system (Molecular Imager Gel Doc XR System and Quantity One 1-D analysis software; Hercules, CA).

Data presentation. All experiments were repeated at least three times with similar results. Numbers of replicates used and plants tested are stated in the figure legends.

RESULTS

HpaG_{Xooc} truncation. To identify functional domains within the HpaG_{Xooc} protein, we first mutated the *hpaG_{Xooc}* sequence by conventional PCR (Fig. 2A and B) using restriction endonucleases with single sites within the gene sequence (Table 1). Putative truncated gene fragments were confirmed by sequencing from recombinant pMD18-T Simple constructions (Fig. 2A, top panel). The fragments (Table 1) were cloned into pET30a(+), which allowed for fusion with His-tag-encoding nucleotides (Fig. 2A, bottom panel) to facilitate subsequent protein purification (63). Nine fragments were created, which were designated based on their nucleotide spans (Table 2; Figs. 2B and 3). The fragments of *hpaG_{Xooc}* were sequenced from recombinant pET30a(+) units and the derived sequences were compared

TABLE 1. Polymerase chain reaction primers used to produce *hpaG_{Xooc}* and its derivatives

Primers ^a	Product
5' <u>CCC</u> ATATGATGAACCTCTTTGAACAC, 5' <u>CCC</u> ATATGCTATTACGTCATCGA	<i>hpaG_{Xooc}</i>
5' <u>GGA</u> ATTCATGAACCTCTTTGAACACAC, 5' <u>CC</u> ATATGCTATTAACTTCTGCGCTGGAGAA	<i>hpaG₁₋₃₁₅</i>
5' <u>GCG</u> GGAATTCATGAACCTCTTTGAACACA, 5' <u>ATT</u> CATATGCTATTACAGCATCTGGG	<i>hpaG₁₋₂₈₂</i>
5' <u>GCG</u> AATTCATGAACCTCTTTGAAC, 5' <u>CC</u> ATATGCTATTACTCAGCATT	<i>hpaG₁₋₁₈₃</i>
5' <u>GCG</u> GGAATTCATGAACCTCTTTGAACACA, 5' <u>CC</u> ATATGCTATTAGCACAGCAACTGGTCCA	<i>hpaG₁₋₁₄₁</i>
5' <u>GAA</u> ATTCATGGCGTCCAACCTCCAGGTT, 5' <u>CC</u> ATATGCTATTACTCAGCATT	<i>hpaG₁₉₋₁₈₃</i>
5' <u>CGG</u> TGAATTCGAAGGTAAGGGTCAGCAG, 5' <u>CGC</u> CATATGCTATTACTGCATTGATGCA	<i>hpaG₁₈₄₋₄₁₄</i>
5' <u>GCG</u> AATTCGAAGGTAAGGGTGC, 5' <u>ATT</u> CATATGCTATTACAGCATCTGG	<i>hpaG₂₈₋₁₂₆</i>
5' <u>CG</u> GAAATTCATATCGTCGGAGAGA, 5' <u>CGC</u> ATATGCTATTACTGCATTGATG	<i>hpaG₂₈₃₋₄₁₄</i>
5' <u>GGA</u> ATTCGAAGGTAAGGGTCAGCA, 5' <u>CG</u> ATATGCTATTACAGCATCTGGGTGAGTG	<i>hpaG₂₅₀₋₂₈₂</i>

^a Primers were synthesized as per the sequence of *hpaG_{Xooc}* accessioned in GenBank (AY875714). To exclude the downstream histidine tag in pET30a(+), two stop codes (CTA and TTA in italics) were added to the downstream primers. Bases underlined are restriction sites of *Eco*RI and *Hind*III in the upstream and downstream primers, respectively. Protection bases were added to the 5' termini.

TABLE 2. Characteristics of the *hpaG* gene and HpaG protein and derivatives^a

Gene name	No. of nucleotides	Protein	No. of amino acids	Protein size (kD)	
				- His-tag	+ His-tag
<i>hpaG</i>	414	HpaG	137	15.18	21.50
<i>hpaG₁₋₃₁₅</i>	315	HpaG ₁₋₁₀₅	105	11.55	17.87
<i>hpaG₁₋₂₈₂</i>	282	HpaG ₁₋₉₄	94	10.34	16.66
<i>hpaG₁₋₁₈₃</i>	183	HpaG ₁₋₆₁	61	6.71	13.03
<i>hpaG₁₋₁₄₁</i>	141	HpaG ₁₋₄₇	47	5.17	11.49
<i>hpaG₁₉₋₁₈₃</i>	165	HpaG ₇₋₆₁	55	5.50	11.82
<i>hpaG₁₈₄₋₄₁₄</i>	231	HpaG ₆₂₋₁₃₇	77	8.58	14.90
<i>hpaG₂₈₋₁₂₆</i>	99	HpaG ₁₀₋₄₂	33	3.63	9.95
<i>hpaG₂₈₃₋₄₁₄</i>	132	HpaG ₉₅₋₁₃₇	44	4.84	11.16
<i>hpaG₂₅₀₋₂₈₂</i>	33	HpaG ₈₄₋₉₄	11	0.40	6.72
<i>hpaGΔGRM</i>	378	HpaGΔGRM	126	14.20	20.52

^a His-tag = histidine tag; HpaGΔGRM was produced previously (40).

with those reported for HpaG_{Xooc} (GenBank accession no. AY875714) using the National Center for Biotechnology Information Blast search tool. Cell-free preparations of the proteins, produced by recombinant *E. coli* cells, were analyzed by T-SDS-PAGE in comparison with EVP. The putative proteins resolved by T-SDS-PAGE had mobility (Fig. 2C) consistent with their predicted sizes (Table 2).

Bioactivities of cell-free proteins. The activities of the nine new fragments of HpaG_{Xooc}, and the previously generated HpaGΔGRM, were tested in plants relative to HpaG_{Xooc} and EVP (40). Within 24 h of infiltration into leaf intercellular spaces of tobacco, all the protein fragments, except HpaG₁₀₋₄₂, induced HR; EVP did not. Staining of excised leaves with Evan's blue 24 h after spraying plant tops with solutions of the protein fragments revealed markedly different levels of induced cell death as a function of the different fragments; similar observations were made at 12 hpt (Fig. 4A). Cell death levels induced by HpaG₁₋₉₄, HpaG₉₅₋₁₃₇, HpaG₈₄₋₉₄, and HpaG_{Xooc} were similar and ≈1.6 times greater than the response to HpaG₁₋₁₀₅, HpaG₁₋₆₁, HpaG₁₋₄₇, HpaG₇₋₆₂, or HpaGΔGRM and twice as great as that induced by HpaG₆₂₋₁₃₇ compared with HpaG_{Xooc}. In contrast, HpaG₁₀₋₄₂ was benign relative to the effect of its parent protein or any of the other fragments in inducing HCD in tobacco leaves.

The cell-free preparations of proteins also were evaluated for their effects on rice bacterial leaf blight. Rice plants were sprayed with the protein or fragment solutions and inoculated with the pathogen *X. oryzae* pv. *oryzae* 5 dpt; leaf blight symptoms developed within 20 days after inoculation to different extents. Based on the lengths of blight lesions, all the HpaG-derived proteins markedly reduced the severity of the disease relative to EVP (Fig. 4B). The 10 variants of HpaG_{Xooc} all had greater effects than the parent protein (Fig. 4B). Of the 10, HpaG₁₋₆₁ and HpaG₇₋₆₁ were

much more active than the others, but HpaG₁₀₋₄₂ clearly was most effective. In contrast, treatment with HpaG₆₂₋₁₃₇ resulted in the least disease resistance.

To confirm the conspicuous difference, rice plants treated with HpaG₆₂₋₁₃₇, HpaG₁₀₋₄₂, and HpaG_{Xooc} were inoculated with *X.*

Fig. 3. The *hpaGXooc* gene and several fragments cloned by polymerase chain reaction and tested for biological activity in plants. Bold bars indicate the spans of DNA included.

Fig. 4. Biological activities of cell-free preparations of HpaG_{Xooc}, nine fragments derived from HpaG_{Xooc}, HpaGΔGRM (ΔGRM), a derivative that lacks the glycine-rich motif, and empty vector preparation (EVP), which contains inactive proteins. **A to C,** Activities of the same proteins in three assays are depicted vertically and their names are indicated under histogram **C**. **A,** Tobacco hypersensitive cell death levels. Aqueous solutions of the protein preparations were sprayed onto 30-day-old tobacco plants. At 12 h post treatment, the fifth youngest fully expanded leaves (five leaves from each treatment) were excised and treated with Evan's blue to estimate relative levels of cell death. **B,** Rice bacterial leaf blight severities. Forty-day-old rice plants growing in field plots were sprayed to runoff with protein solutions; at 5 days posttreatment, the plants were inoculated with *Xanthomonas* pv. *oryzae*. The lengths of leaf blight lesions (five plants from each treatment) were scored 20 days after inoculation. **C,** Length of rice roots. Seed (50 per treatment) were soaked in solutions of proteins for 6 h, drained, and incubated on filter papers marinated in MS medium. Root length was scored 6 days later. The three assays represented in histograms **A, B,** and **C** were done three times with similar results. The mean results of one experiment are shown ± standard deviations.

oryzae pv. *oryzae*, which had been labeled with a green fluorescent protein, and populations in planta were assayed subsequently. Monitoring of bacterial colonies by plating on appropriate media and fluorescent microscopy of inoculated leaves

Fig. 5. Purification by nickel chromatography of histidine (His)-tagged proteins of HpaG_{X00C} and selected fragments derived from HpaG_{X00C}. **A**, Tricine sodium dodecyl sulfate (T-SDS) electrophoretic analyses of the proteins. Cell-free preparations of the proteins were bound to nickel-polystyrene beads, which were eluted with the indicated concentrations of imidazole. Electrophoresis of the eluents indicated purity of HpaG_{X00C} and selected fragments. Arrows on the four gel panels indicate those fractions that contained mainly the desired proteins and were collected and pooled for bioassays. The gel for HpaG_{X00C} included markers (M) with the most brilliant ladder indicated for size and cell-free preparation (CFP) of the protein. **B**, T-SDS polyacrylamide gel electrophoresis comparison of His-tagged proteins following purification by nickel chromatography. The pooled samples collected from A were loaded to electrophorese $\approx 5 \mu\text{g}$ per sample.

indicated that bacterial populations recovered from leaves were consistent with leaf blight severity. Pathogen populations were 20-fold greater in plants treated with EVP than those that had been treated with HpaG₁₀₋₄₂. The populations recovered from plants that had been treated with HpaG₆₂₋₁₃₇ and HpaG_{X00C} were similar and only $\approx 0.001\%$ of those recovered from plants that had been treated with EVP.

To determine whether HpaG₁₀₋₄₂ and HpaG₆₂₋₁₃₇ affect another rice disease as they affect bacterial leaf blight, the effect on leaf blast was determined. Plants were treated with the HpaG-derived materials and inoculated with spores of *M. grisea* 5 days later. Subsequent severities of leaf blast varied with treatments. Blast symptoms (tissue necrosis) appeared on leaves that had been treated with EVP but were quite limited on leaves that had been treated with HpaG_{X00C}. Leaves treated with HpaG₆₂₋₁₃₇ had some signs of infection but necrosis was not evident. No evidence of infection was seen on leaves treated with HpaG₁₀₋₄₂. Thus, treatment of rice plants with HpaG₁₀₋₄₂ reduced disease caused by both a bacterial and a fungal pathogen.

Next, we studied the growth of rice roots on filter papers in petri dishes after soaking seed in solutions of cell-free preparations of the HpaG-derived materials. Compared with EVP, all the HpaG-derived materials tested induced better growth of roots tested at 6 dpt. HpaG₁₋₁₀₅, HpaG₁₋₆₂, HpaG₁₋₄₇, HpaG₆₂₋₁₃₇, and HpaG₈₄₋₉₄ were less active in stimulating root growth compared with HpaG_{X00C}. In contrast, HpaG_{AGRM}, HpaG₁₋₉₄, HpaG₇₋₆₁, and HpaG₁₀₋₄₂ increased root length by 15, 55, 20, and 48%, respectively. Clearly, HpaG₁₋₉₄ and HpaG₁₀₋₄₂ stimulated greater growth of rice roots.

Characterization of purified proteins. The basic characteristics of the nine HpaG_{X00C}-derived proteins and fragments and the parent protein, including predicted and estimated molecular masses following T-SDS-PAGE analyses after purification, were determined (Table 2). During protein purification, we optimized the concentration of imidazole needed in the binding buffer and the composition of the elution buffer for each protein (57). Test conditions were optimized for the four proteins used in further studies and monitored by SDS-PAGE (Fig. 5). The indicated fractions were collected and pooled (Fig. 5A) and the mixtures were electrophoresed again (Fig. 5B) to estimate molecular masses of the purified proteins, which were consistent with predictions (Fig. 5B; Table 2). These protocols resulted in high purification of HpaG₁₋₉₄, HpaG₆₂₋₁₃₇, and HpaG₁₀₋₄₂ proteins, which differed sharply in molecular mass (Fig. 5B). Fragment HpaG₁₀₋₄₂ contains 33 amino acids and has a molecular mass of 3.63 kD, which is $\approx 24\%$ of the molecular mass of the parent protein HpaG_{X00C} (Table 2).

Effects of selected purified HpaG_{X00C}-derived fragments in plants. The effects of the four nickel-purified proteins were evaluated in plants in manners similar to those used for evaluation of the cell-free preparations from which they were derived. HR was tested by infiltrating leaf intercellular spaces of tobacco plants separately with solutions of the proteins or HrpN_{Ea} from *Erwinia amylovora* (70). Water and EVP did not cause evident HR in 24 to 120 hpt. At 24 hpt, however, HR was induced by HpaG₁₋₉₄, HpaG₆₂₋₁₃₇, and HpaG_{X00C}. HpaG₁₀₋₄₂ was not active in eliciting HR. When protein solutions were sprayed onto plants, microscopic examination of leaves stained with trypan blue showed that cell death was induced by 12 hpt. The extent of staining or dye uptake varied with the protein; however, EVP and water resulted in no cell death (Fig. 6B). Defining death level induced by HpaG_{X00C} as 1.0, HpaG₁₋₉₄ induced a level close to 1.0, while HpaG_{AGRM} and HpaG₆₂₋₁₃₇ rated 1.6 and 2.4, respectively. In contrast, HpaG₁₀₋₄₂ rated 0.1 (Fig. 6C).

Growth of roots following soaking rice seed in solutions of the five proteins was enhanced compared with the EVP control (Fig. 6D, top panel). HpaG₆₂₋₁₃₇ and HpaG_{X00C} had similar effects, whereas HpaG₁₀₋₄₂ was greater and HpaG₁₋₉₄ had the greatest

effect. After transplanting into pots, seedling growth was rather similar to root growth as a function of the protein used for treatment of the seed (Fig. 6D, bottom panel).

At 20 days after inoculation, leaf blight severity was reduced markedly in plants treated with purified HpaG_{Xooc} and its derivatives relative to EVP, in which blight lesions had expanded vertically on leaves (Fig. 6E, top panel with leaves). Lesion lengths on plants treated with HpaG₆₂₋₁₃₇ and HpaG_{Xooc}, HpaG₁₋₉₄, Δ GRM, and HpaG₁₀₋₄₂ were reduced relative to lengths in responses to EVP and water (Fig. 6E, bottom panel). The purified preparation of HpaG₁₀₋₄₂ was the most active of those tested.

Activation of relevant signaling events. Fragments HpaG₆₂₋₁₃₇ and HpaG₁₀₋₄₂ differed widely in their inductive effects on HCD versus growth and pathogen resistance, respectively (Fig. 6). Thus, we tested the two fragments and HpaG_{Xooc} for activation of plant signaling associated with HCD, plant defense, and growth. Tobacco plants were assayed for ROS burst revealed as H₂O₂ accumulation in leaves by staining with DAB. At 6 hpt, the effects of spraying each solution of protein was evaluated (Fig. 7A). Substantial H₂O₂ accumulated in leaf cells following the application of HpaG_{Xooc} and more accumulated in leaves treated with HpaG₆₂₋₁₃₇ versus HpaG₁₀₋₄₂ (data not shown) and EVP, which accumulated little or none.

The expression of *hsr203* and *hin1* in leaves was analyzed by RT-PCR using the constitutively expressed gene *EF1 α* (5,20) as a standard (Fig. 7B). In leaves treated with HpaG₆₂₋₁₃₇, *hsr203* and *hin1* both were induced to accumulate transcripts beginning 6 hpt; expression of the genes in leaves generally increased to 48 hpt. At 48 hpt, many more transcripts of both genes were detected in leaves treated with HpaG₆₂₋₁₃₇ than with HpaG₁₀₋₄₂ (data not shown) and HpaG_{Xooc}, consistent with the ROS burst results.

Expression of the *Arabidopsis* SAR pathway genes *NPR1* and *PR1* (16,55,59) was tested in response to treatment with HpaG₁₀₋₄₂ and HpaG_{Xooc}. RT-PCR analyses, conducted using *EF1 α* as a standard, indicated that expression of both *NPR1* and *PR1* increased with time from 6 hpt following treatment with HpaG₁₀₋₄₂. The expression of *PR1*, especially, seemed dependent on induction because few transcripts were detected immediately after treatment. The fragment HpaG₁₀₋₄₂ was markedly more effective than HpaG_{Xooc} in inducing expression of *NPR1* and *PR1* at 48 hpt.

We next determined expression of the defense-regulatory protein kinase gene *OsARF1* and the growth-promoting expansin gene *OsEXPI* as a function of treatment with HpaG_{Xooc} and HpaG₁₀₋₄₂. Both genes were expressed markedly in rice plants treated with HpaG_{Xooc} or HpaG₁₀₋₄₂. HpaG₁₀₋₄₂ induced levels of expression that were 80 and 65% greater for *OsARF1* and *OsEXPI*, respectively, than HpaG_{Xooc}. This result indicates that the action by HpaG₁₀₋₄₂ is consistent in stimulating defense and growth regulatory genes with the effects on disease resistance and growth in rice. Interestingly, Tricycloazole, a fungicide often used for control of rice blast control (37), did not activate expression of *OsARF1* or *OsEXPI*.

DISCUSSION

Harpins, proteins produced by many gram-negative plant-pathogenic bacteria, induce multiple biological effects following application to plants. Some effects, such as induced resistance to pathogens and pests and stimulation of plant growth (28), are considered beneficial whereas others, including HCD and the HR, are considered deleterious. Several fragments of HpaG (31,48,67) and HrpN_{Ea} (34,69) have been shown to be responsible for inducing plant growth and defenses (13–15,61). In addition, studies of plant signaling pathways activated by treatment with HrpN_{Ea}- and HpaG_{Xooc}-derived proteins have indicated differential responses as a function of particular portions of the molecules. However, the protein fragments responsible have not been well characterized and mechanistic linkage between the phenotypic functions and

Fig. 6. Biological activities of HpaG_{Xooc} and selected fragment derivatives after purification by nickel chromatography. **A**, Macroscopic views of the hypersensitive response (HR). Leaves of 30-day-old tobacco plants were infiltrated with solutions containing the indicated proteins. Photos were taken at 24 h posttreatment (hpt) and represent 15 leaves tested in three experiments. **B**, Binocular microscope views of micro-HR in tobacco leaves. Leaves of 30-day-old tobacco plants were sprayed with solutions containing the indicated proteins or water. Leaves were excised at 12 hpt and stained with trypan blue, which stains dead cells blue. Photos represent 12 leaves tested in three experiments for each treatment. Bar = 0.5 cm. **C**, Relative hypersensitive cell death (HCD) levels determined at 12 hpt by an Evan's blue uptake assay of leaves from **B**. Histograms represent mean $\pm$ standard deviation (SD) bars of the replicate results. **D**, Growth of rice roots and seedlings observed at 6 and 15 days, respectively, after soaking seed in solutions of the indicated proteins. Assays were done three times with similar results; 15 seeds were treated with empty vector preparation (EVP) or an individual protein in each assay. **E**, Rice bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae*. Forty-day-old rice plants were sprayed with solutions of the indicated compounds and inoculated 5 days later by cutting the top 1 cm of leaves with pathogen-contaminated scissors. Symptoms were observed 20 days after inoculation. Length of lesions is presented as mean $\pm$ SD from three replicates, each involving 15 plants.

Fig. 7. Effects of HpaGXooc and selected fragment derivatives on expression of selected plant genes involved in signaling. **A**, H₂O₂ accumulation in tobacco. Plants were sprayed with solutions of the indicated proteins. At 6 h post-treatment, the fifth youngest fully expanded leaves were harvested and stained with diaminobenzidine to visualize H₂O₂ accumulation (black spots under black and white background). Photos are representative of the 12 leaves tested in each treatment. EVP = empty vector preparation. **B**, Expression of hypersensitive cell death marker genes in tobacco. Both *hsr203* and *hin1* were studied together with the constitutively expressed *EF1a* gene by reverse-transcriptional polymerase chain reaction (RT-PCR) conducted with RNA isolated from leaves equivalent to those tested in **A**. Experiments were done three times with similar results. **C**, Expression of *NPR1* and *PR1* genes in *Arabidopsis*. Thirty-day-old plants were sprayed with each protein solution. RNA was isolated from the two youngest fully expanded leaves at the indicated times and used for RT-PCR analysis conducted as in **B**. Experiments were done three times, each with 15 plants; results were similar. **D**, Expression of *OsARF1* and *OsEXP1* in rice plants. Rice seedlings (20 days old) were sprayed with solutions containing EVP, HpaGXooc, HpaG₁₀₋₄₂, or the fungicide Tricycloazole. Gene expression was tested at 2 days post treatment as in **B**. Expression levels were quantified as arbitrary units (au). Data represent means $\pm$ standard deviation of results from three experiments; 15 plants were tested in each experiment with three replicates.

Fig. 8. Summary of harpin-affected signaling processes and effects of representative fragments of HpaGXooc in plants. **A**, Harpin-affected pathways in tobacco and *Arabidopsis*. Bold arrows and bars indicate codes for the pathways that are activated and inhibited; the H-shaped symbol and the two-way arrows denote antagonistic and synergistic interactions, respectively. Processes in P1 through P5 are induced when plants grow normally. P1 to P3 each involve transduction of the indicated signal. P4 is performed when the Raf-like protein kinase CTR1 is active in the absence of an ethylene signal to inhibit ethylene-dependent processes (23,24,59). Inversely, when ethylene is elevated to inactivate CTR1, P5 is activated through ethylene sensing by the receptor ETR1. P6 operates under conditions of drought stress. Definitions of signaling regulations are as follows: ABF, abscisic acid-binding factor (30); ABH, abscisic acid-hypersensitive (27); COI1, coronatine-insensitive1 that regulates jasmonate signal transduction (72); ERF1, ethylene responsive factor 1 (44); JAR1, jasmonic acid-resistant1 (60); TTG1, transparent testa glabra1 that regulates development of leaf trichomes (64). Effectors that are characteristic of P1 to P6 are shown in the penultimate line. In the bottom line, phenotypes are indicated. **B**, Schematic maps and relative effects of the three fragment derivatives of HpaGXooc versus full-length HpaGXooc. The maps are drawn with amino acid sites indexed to the right of the schematic representation of the genes; hatched portions in bars indicate glycine-rich motifs. The protein fragments were rated in their proportional effects, compared with HpaGXooc, in inducing plant hypersensitive cell death (HCD) and disease resistance, and enhancing growth.

plant response mechanisms have not been defined. In this study, we aimed to determine whether particular domains or portions of the harpin protein HpaG_{Xooc} produced by *X. oryzae* pv. *oryzicola* are responsible for all or some of the distinct effects that HpaG_{Xooc} induces in plants. The data presented indicate that distinct regions of HpaG_{Xooc} induce beneficial effects, while other regions induce deleterious effects; clones responsible for the distinct effects were produced. Consistent with these results, we found that certain subclones of *hpaG_{Xooc}* encode proteins that activate distinct plant signaling pathways (Fig. 8A).

Nine subclones of the *hpaG_{Xooc}* gene, fused with the His-tag sequence to facilitate subsequent purification of the encoded protein fragments, were produced by PCR-based procedures. Partially purified protein preparations were screened in tobacco for induction of HCD and in rice for resistance to both bacterial and fungal pathogens and effects on root growth. HpaG₆₂₋₁₃₇ showed greater effects than HpaG_{Xooc} in eliciting HCD, whereas HpaG₁₋₉₄ induced greater growth of rice than HpaG_{Xooc}. Remarkably, the small protein HpaG₁₀₋₄₂ was 7.5 times more effective in inducing disease resistance in rice and 1.5 times more effective in stimulating growth of rice than similar amounts of HpaG_{Xooc} (Fig. 8B, lower panels). Also, HpaG₁₀₋₄₂ did not induce evident HCD (Fig. 8B, lower panels). Fragment HpaG₁₀₋₄₂ contains 33 amino acids and has a molecular mass of 3.63 kD, which constitutes ≈24% of the molecular mass of the parent protein HpaG_{Xooc} (Table 2). The plant assay results are consistent with earlier findings that HCD is separated in performance from disease resistance, and these phenotypes are induced by different domains of harpin (8,49).

The nine protein fragments were further purified and their molecular masses were estimated through T-SDS-PAGE using proteins of known molecular masses as standards. The predicted and estimated molecular masses agreed closely (Table 2). Based on the differential effects of crude protein preparations in plants, highly purified preparations of fragments HpaG₁₀₋₄₂ and HpaG₆₂₋₁₃₇ were chosen for further study in comparison with the parent protein HpaG_{Xooc} and HpaGΔGRM, a deletion mutant of HpaG_{Xooc}, which lacks a glycine-rich region and cysteine (40).

When applied topically, highly purified HpaG₁₀₋₄₂ clearly lacked ability to induce HR and micro-HR relative to the other tested fragments and the parent protein HpaG_{Xooc}. Furthermore, and consistent with the earlier results with cell-free supernatant preparations, rice plants treated with solutions of purified HpaG₁₀₋₄₂ exhibited greater root and top growth and greater degrees of resistance to the bacterial leaf blight pathogen than plants treated with the other tested fragments and the parent protein (Fig. 6).

Consistent with our phenotypic results, fragments HpaG₆₂₋₁₃₇ and HpaG₁₀₋₄₂ were more effective than HpaG_{Xooc} in activating relevant signaling events. ROS burst (8,35) is critical to the activation of HCD marker genes, such as *hsr203* and *hin1* (22,50), during development of HCD in tobacco plants treated with HpaG_{Xooc} (47). Similarly, the signal was elevated and the marker genes were expressed at greater extents by treating tobacco plants with HpaG₆₂₋₁₃₇ than HpaG_{Xooc}. In contrast, HpaG₁₀₋₄₂ did not significantly affect expression of *hsr203* and *hin1* (data not shown). On the other hand, expression of genes encoding pathogenesis-related (PR) proteins, regulated by the transcription factor NPR1 in SAR signal transduction (11,16,53-55), was strongly induced in *Arabidopsis*. HpaG₁₀₋₄₂ also was more effective than HpaG_{Xooc} in inducing expression of *PR1* in *Arabidopsis*.

In rice plants, the defensive protein kinase gene *OsARF1* (52, 65) and the growth-promoting expansin gene *OsEXPI* (26,52) were both strongly induced by treatment of plants with HpaG₁₀₋₄₂. Transcripts of these genes accumulated at greater levels than they did in HpaG_{Xooc}-treated plants. These results establish a mechanistic connection between the dual beneficial functions of HpaG₁₀₋₄₂ and signaling pathways that regulate plant defense and growth.

In addition to HpaG₁₀₋₄₂, the deletion mutant HpaGΔGRM was more active than HpaG_{Xooc} in inducing beneficial effects in plants,

including rice (40). However, one GRM is present in HpaG₁₀₋₄₂ and HpaG₁₋₉₄ and two are present in HpaG₆₂₋₁₃₇ (Fig. 8B, top panel). Thus, the role of GRMs in plant interaction is unclear. Modifying harpin proteins is assumed to change the dimensional structures and, thereby, affect the functions of the protein in plants (31,73). Although identifying structure–function relationships requires much more prodigious work, rational identification and testing of several fragments of HpaG_{Xooc} may well stimulate further studies and perhaps lead to even more effective use of harpin-derived materials for use in agriculture.

Treatment of plants with harpin is an attractive practical possibility (62) based on the several beneficial effects of harpins and especially the fact that the protein is biodegradable in the environment. However, application of harpin sometimes injures healthy plants (8,21) through the development of the HCD, and the micro-HR develops extensively in leaves that are sprayed with harpin solutions (49). Therefore, if plants are to be treated with harpins to induce disease resistance and to promote greater growth, use of a harpin or harpin derivative that lacks the ability to induce HCD would be highly desirable. Of the fragments tested in our studies, HpaG₁₀₋₄₂ appears to be less injurious to plants than other tested fragments. On the other hand, HpaG₁₀₋₄₂ appears to induce more beneficial effects in treated rice. Thus, treatment of plants with HpaG₁₀₋₄₂ on a large scale may result in real beneficial results of effective control of diseases and increased crop productivity. The results of applying the HpaG-derived products to rice on a moderate scale are reported in the companion article.

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