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Elucidation of the *hrp* Clusters of *Xanthomonas oryzae* pv. oryzicola That Control the Hypersensitive Response in Nonhost Tobacco and Pathogenicity in Susceptible Host Rice

Li-fang Zou,[1]† Xing-ping Wang,[1]† Yong Xiang,[1]† Bing Zhang,[1] Yu-Rong Li,[1] You-lun Xiao,[1] Jin-sheng Wang,[1] Adrian R. Walmsley,[2]* and Gong-you Chen[1]*

Key Laboratory of Monitoring and Management for Plant Diseases and Insects, Ministry of Agriculture, Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China,[1] and Centre for Infectious Diseases, Wolfson Research Institute, Department of Biological and Biomedical Sciences, University of Durham, Queen's Campus, Stockton-on-Tees TS17 6BH, United Kingdom[2]

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***Xanthomonas oryzae* pv. oryzicola, the cause of bacterial leaf streak in rice, possesses clusters of *hrp* genes that determine its ability to elicit a hypersensitive response (HR) in nonhost tobacco and pathogenicity in host rice. A 27-kb region of the genome of *X. oryzae* pv. oryzicola (RS105) was identified and sequenced, revealing 10 *hrp*, 9 *hrc* (*hrp* conserved), and 8 *hpa* (*hrp*-associated) genes and 7 regulatory plant-inducible promoter boxes. While the region from *hpa2* to *hpaB* and the *hrpF* operon resembled the corresponding genes of other xanthomonads, the *hpaB-hrpF* region incorporated an *hrpE3* gene that was not present in *X. oryzae* pv. oryzae. We found that an *hrpF* mutant had lost the ability to elicit the HR in tobacco and pathogenicity in adult rice plants but still caused water-soaking symptoms in rice seedlings and that Hpa1 is an HR elicitor in nonhost tobacco whose expression is controlled by an *hrp* regulator, HrpX. Using an Hrp phenotype complementation test, we identified a small *hrp* cluster containing the *hrpG* and *hrpX* regulatory genes, which is separated from the core *hrp* cluster. In addition, we identified a gene, *prhA* (plant-regulated *hrp*), that played a key role in the Hrp phenotype of *X. oryzae* pv. oryzicola but was neither in the core *hrp* cluster nor in the *hrp* regulatory cluster. A *prhA* mutant failed to reduce the HR in tobacco and pathogenicity in rice but caused water-soaking symptoms in rice. This is the first report that *X. oryzae* pv. oryzicola possesses three separate DNA regions for HR induction in nonhost tobacco and pathogenicity in host rice, which will provide a fundamental base to understand pathogenicity determinants of *X. oryzae* pv. oryzicola compared with those of *X. oryzae* pv. oryzae.**

The interaction of many gram-negative plant-pathogenic bacteria with plants is modulated by two sets of genes: the avirulence (*avr*) or virulence (*vir*) genes determine host specificity via gene-for-gene interactions, and the *hrp* genes, which are usually clustered in a chromosomal region that spans 20 to 30 kb, are involved in induction of the hypersensitive response (HR) in resistant host and nonhost plants and pathogenicity in susceptible host plants (2, 7). HR is a rapid, local, programmed cell death that is induced upon recognition of the pathogen and concomitant with the inhibition of pathogen growth within the infected plants. Commonly, the nine *hrp* genes, which are highly conserved in plant and animal bacterial pathogens, are known as the *hrc* (*hrp* conserved) genes (6). The *hrc* genes encode the type III secretion system (TTSS) and are critical for pathogenicity and initiation of disease (2, 34). Recently, it was established that the *hrpF* gene in plant-pathogenic bacteria encodes a translocon that is a component of the TTSS (13, 31, 49). These *hrc* genes are also found in animal pathogens such as *Salmonella* and *Shigella* and are thought to be evolutionarily related to the flagellar apparatus (18, 19, 20). The *hpa* (*hrp*-associated) genes contribute to pathogenicity and to the induction of the HR in nonhost plants but are not essential for the pathogenic interactions of bacteria with plants (24, 28, 32).

Our knowledge of *hrp* genes in *Xanthomonas* arises mainly from studies of *X. campestris* species. The best characterized systems are the *hrp* gene clusters of *X. campestris* pv. vesicatoria (3, 12, 54), the causal agent of bacterial spot on pepper and tomato; *X. axonopodis* pv. glycines (31), the pathogen of bacterial pustule on soybean; *X. axonopodis* pv. citri (16), a pathogen of citrus canker; *X. campestris* pv. campestris (16), a pathogen of black rot of crucifers; and *X. oryzae* pv. oryzae (41, 49, 61), the causal agent of bacterial blight in rice. The core *hrp* cluster of xanthomonads contains six operons from *hrpA* to *hrpF*. However, little is known about the structure and function of the *hrp* cluster in *X. oryzae* pv. oryzicola.

The expression of *hrp* genes is highly regulated, being induced only in plants or certain nutrient-poor synthetic media (3, 27, 35, 46, 47, 50, 55). There are two types of *hrp* regulatory systems in plant-pathogenic bacteria (2, 23). In group I systems, which are found in *Pseudomonas syringae*, *Erwinia amylovora*, and *Pantoea stewartii*, a member of the extracytoplasmic function family of alternative sigma factors, called HrpL, functions as the regulator for the other *hrp* genes (36, 59). On the

* Corresponding author. Mailing address for Adrian R. Walmsley: Centre for Infectious Diseases, Wolfson Research Institute, Department of Biological and Biomedical Sciences, University of Durham, Queen's Campus, Stockton-on-Tees TS17 6BH, United Kingdom. Phone: 44 (0)191 334 0465. Fax: 44 (0)191 334 0468. E-mail: a.r.walmsley@dur.ac.uk. Mailing address for Gong-you Chen: Key Laboratory of Monitoring and Management for Plant Diseases and Insects, Ministry of Agriculture, Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095, China. Phone: 86 (0)25 84399092. Fax: 86 (0)25 84399002. E-mail: gyouchen@njau.edu.cn.

† These authors contributed equally to this work.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
X. oryzae pv. oryzicola		
RS105	Wild type, Rif^r	This lab
RABC	*prhA*::Cm, *prhA* mutant, Rif^r Cm^r	This study
RCX	*hrpX*::Cm, *hrpX* mutant, Rif^r Cm^r	This study
RCG	*hrpG*::Cm, *hrpG* mutant, Rif^r Cm^r	This study
RFBC	*hrpF*::Cm, *hrpF* mutant, Rif^r Cm^r	This study
M55	*hrpX* mutant generated by chemical mutagenesis	14
M1005	*hrpG* mutant generated by chemical mutagenesis	14
Escherichia coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 deoR recA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Clontech
S17-1	Tra^+ *recA* Sp^r	48
BL21(DE3)	F^- *ompT hsdB*(r_B^- m_B^-) *gal dcm* (DES)	Novagene
Bhpa1	Conjugant of BL21(DE3) with plasmid pETHpa1	This study
Plasmids		
pUFR034	Km^r, *IncW*, *Mob(p)*, Mob^+, $LacZa^+$, PK2 replicon, cosmid	17
pBlueScript II KS(−)	Phagemid, pUC derivative, Ap^r	Stratagene
pBC SK	pUC ori, Cm^r	Stratagene
pUC18	pUC ori, Ap^r	This lab
pMD-18-T	Ap^r, T-easy vector for PCR products	TaKaRa
p6	Km^r, positive *hrp* gene clone screened from *X. oryzae* pv. oryzicola genomic library	This study
pPK12.3	Km^r, a 12.3-kb fragment containing the *hrpG* and *hrpX* operons screened from *X. oryzae* pv. oryzicola genomic library	14
pA1	Km^r, positive *hrp* gene clone screened from *X. oryzae* pv. oryzicola genomic library	This study
pA1-E2	Ap^r, a 5.1-kb EcoRI fragment from pA1 in pUC18	This study
pHA1.5	Ap^r, a 1.5-kb *hrpA* of *X. oryzae* pv. *oryzae* ligated into pUC18 as a probe for screening the genomic library of *X. oryzae* pv. oryzicola	This study
pHF1.62	Ap^r, a 1.62-kb *hrpF* fragment of *X. oryzae* pv. *oryzae* ligated into pBluescript KS(−) as a probe for screening the genomic library of *X. oryzae* pv. oryzicola	This study
p6-28	Ap^r, a 9,611-bp EcoRI fragment from p6 ligated into pBluescript KS(−)	This study
p6-7	Ap^r, a 5,071-bp EcoRI fragment from p6 ligated into pBluescript KS(−)	This study
p6-8	Ap^r, a 7,808-bp EcoRI fragment from p6 ligated into pBluescript KS(−)	This study
p685	Ap^r, a 685-bp EcoRI fragment from p6 ligated into pMD-18-T vector	This study
p6-3	Ap^r, a 1,996-bp EcoRI fragment from p6 ligated into pBluescript KS(−)	This study
pBSK579	Ap^r, a 579-bp EcoRI-KpnI fragment from p6 ligated into pBluescript KS(−)	This study
p6121-2	Ap^r, a 6,381-bp KpnI fragment from p6 ligated into pBluescript KS(−)	This study
pHpa21	Ap^r, a 1,356-bp fragment containing the *hap2* and *hpa1* genes ligated into pMD-18-T	This study
p61	Km^r, a 2,163-bp *prhA* gene from pHA1.5 ligated in pUFR034	This study
phrA2.1	Ap^r, PCR product of a 2,163-bp *prhA* gene ligated into pBluescript	This study
pABC	Cm^r, a 112-bp PstI-SacII fragment from phrA2.1 ligated in pBC SK(−)	This study
pEThpa1	Ap^r, a 414-bp *hpa1* gene of *X. oryzae* pv. *oryzicola* cloned into pET21a	This study
pUhpa1	Km^r, a 614-bp *hpa1* gene plus the promoter region together with the fragment encoding a His_6 tag cloned into pUFR034	This study
pBCX147	Cm^r, a 147-bp PstI-KpnI fragment from the *hrpX* gene ligated in pBC SK(−)	This study
pBCG121	Cm^r, a 121-bp EcoRI-SacI fragment from the *hrpG* gene into pBC SK(−)	This study
pFBC	Cm^r, a 351-bp BamHI-PstI fragment from p6-3 linked into pBC SK(-)	This study
pHrpF	Km^r, a 2,409-bp *hrpF* gene by PCR linked into pUFR034	This study

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Rif^r, rifampin resistance; Sp^r, spectinomycin resistance.

other hand, in group II systems, which are found in *Xanthomonas* species or pathovars and *Ralstonia solanacearum*, either the AraC-type transcriptional activator HrpX (*Xanthomonas*) or HrpB (*R. solanacearum*) regulates expression of the *hrpB*-to-*hrpF* operon along with some effector proteins (5, 22, 30, 40, 45, 54). Commonly, the HrpX regulons in xanthomonads are preceded by a consensus sequence motif, called the plant-inducible promoter (PIP) box (TTCGC-N15-TTCGC) (18, 40, 41, 51, 54). The expression of *hrpA* and *hrpX* is activated by the product of *hrpG*, which belongs to the OmpR family of two-component regulatory systems (56, 57). Interestingly, the existence of plant factors that specifically regulate *hrp* genes has been proposed. In *R. solanacearum*, the outer membrane protein PrhA (plant-regulated *hrp*) controls the plant-responsive regulatory cascade composed of PrhR, PrhI, PrhJ, HrpG, and HrpB, the final activator of *hrp* transcription units 1 to 4 and 7 (1, 11, 35). In contrast, our knowledge of the *hrp* regulation system in *X. oryzae* pv. oryzicola is rudimentary.

The harpins, i.e., HrpN of *E. amylovora* (52), HrpZ of *P. syringae* (25), HpaG of *X. axonopodis* pv. glycines (31), Hpa1 of *X. oryzae* pv. oryzae (53), and XopA of *X. campestris* pv. vesicatoria (40), have been characterized as HR elicitors in non-

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)[a]	Purpose
prhA-F1	CTGAATTCATGTCTGCGACCTTCCC	To amplify the *prhA* gene from *X. oryzae* pv. oryzicola
prhA-R1	AGGAATTCCTAGAAATCCACCTGC	
hpa1-F1	CATATGAATTCTTTGAACACACAATTC	To express the *hpa1* gene in *E. coli*
hpa1-R1	CTCGAGCTGCATCGATCCGCTGTCGTTCG	
hpa1-F2	GGATCCGATCTGTTATCGATCCTAAAAAATTTTCCAC	To express the *hpa1* gene together with a hexahistidine tag in *X. oryzae* pv. oryzicola
hpa1-R2	GGTACCTCAGTGGTGGTGGTGGTGCTGCATCGA TCCGCTGTCGTTCG	
hrpA-F1	CGGATCCACTTAACGGGCAAGAAAAAAG	To amplify the *hrpA* gene as a probe for screening for *hrp* clones from an *X. oryzae* pv. oryzicola genomic library
hrpA-R1	TGGATCCGGTACCGGGTCTGTCAAAGATTC	
hrpF-F1	GAAGGATCCGCGGTTGTTCTTCGCCATC	As a probe for screening for *hrp* clones from an *X. oryzae* pv. oryzicola genomic library
hrpF-R1	TGCAAGCTTGGAGGCACGTTCATACGAACG	
hrpF-F2	GGTACCATGTCGCTCAACATGCTTTCTAC	To express the *hrpF* gene in the *hrpF* mutant of *X. oryzae* pv. oryzicola
hrpF-R2	GGTACCTTATCTGCGACGTATCCTGACATTG	
hrpX-F	CTGGATCCATGATCCTTTCGACCTACTTTG	To amplify the *hrpX* gene from *X. oryzae* pv. oryzicola
hrpX-R	ATGGATCCTTACCGTTGCAAGGTT	
hrpG-F	GGATCCATGAACATCCCTTGCCCCCTTG	To amplify the *hrpG* gene from *X. oryzae* pv. oryzicola
hrpG-R	GGATCCTCAGCAGGCGGCTGTGCGATG	
hpa2-F	CTATTCACCAATCACACCAC	For cloning the region incorporating the *hpa2* to *hpa1* genes from *X. oryzae* pv. oryzicola
hpa1-R	TTACTGCATCGATGCGCT	

[a] Restriction sites are underlined.

host plants, but their individual contributions to pathogenicity in host plants vary greatly. The *hpa1* gene of *X. oryzae* pv. oryzae encodes a 13-kDa glycine-rich protein with a composition similar to those of harpins in xanthomonads and PopA in *R. solanacearum* (62). To date, no harpin-like proteins with elicitor activity have been reported to exist in *X. oryzae* pv. oryzicola.

X. oryzae pv. oryzicola colonizes the intercellular spaces, the apoplast, of the mesophyll to cause bacterial leaf streak in rice. This disease is of increasing importance throughout Asia, and especially in China, where many high-yield hybrids are very susceptible (15, 60). Scientifically, *X. oryzae* pv. oryzicola is considered an ideal pathogen to understand molecular mechanisms of rice-*Xanthomonas* interactions (44). The ability of the bacteria to induce the HR on nonhost plants and cause disease on host plants is controlled by *hrp* genes (14). Considering the progress in determining the rice genome (44), it is expected that knowledge of the *hrp* genes of *X. oryzae* pv. oryzicola will be key to understanding rice-bacterium interactions. However, our knowledge of the *hrp* clusters in this bacterium is still rudimentary. Consequently, we have sought to identify and clone the *hrp* clusters of *X. oryzae* pv. oryzicola, which we report upon here. In starting to characterize the individual *hrp* genes, we confirmed that the *hpa1* gene product is a TTSS-dependent HR elicitor in nonhost tobacco and that the expression of the *hpa1* gene is controlled by an *hrp* regulator gene, *hrpX*. In addition, we identified the *prhA* locus, which contributes to the hypersensitive response in nonhost tobacco and to pathogenicity in host rice.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. All of the *X. oryzae* pv. oryzicola strains used in these experiments were derivatives of the parent strain RS105. *Escherichia coli* cells were cultivated at 37°C in Luria broth (LB) or on LB agar plates. The *Xanthomonas* strains were grown at 28°C on nutrient agar (NA) (0.5% yeast extract, 1% polypeptone, and 1% NaCl) plates or in nutrient broth without agar. Antibiotics were used at the following concentrations: 100 μg/ml ampicillin, 15 μg/ml chloramphenicol, 25 μg/ml kanamycin, and 25 μg/ml spectinomycin for *E. coli* and 50 μg/ml kanamycin, 50 μg/ml spectinomycin, and 50 μg/ml rifampin for *X. oryzae* pv. oryzicola. Tetracycline was used at 10 μg/ml for *E. coli* and at 2 μg/ml for *X. oryzae* pv. oryzicola.

To obtain an *hrp*-inducing medium for *X. oryzae* pv. oryzicola, we modified XVM2 [20 mM NaC1, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM CaCl, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM fructose, 10 mM sucrose, 0.03% Casamino Acids (pH 6.7)] (46, 54) and developed a synthetic medium (termed XOM3) containing 1% sugar source, 650 μM DL-methionine, 10 mM sodium L-(+)-glutamate, 15.0 mM KH_2PO_4, 40 μM $MnSO_4$, 240 μM Fe_2-EDTA, and 5 mM $MgCl_2$, pH 6.5. Antibiotics were added to XOM3 for the growth of *Xanthomonas* bacteria as required.

Bacterial conjugation and transformation. For construction of genomic DNA (gDNA) libraries, the total genomic DNA from strain RS105 was partially digested by EcoRI, and the resulting 30- to 40-kb fragments were ligated into the vector pUFR034 (17). Subclones were first introduced into *E. coli* strain DH5α for *lacZ* selection by transformation and then into S17-1 (48) for later biparental conjugation.

As recipients, each *hrp* mutant of *X. oryzae* pv. oryzicola (10^8 CFU/ml) was harvested at 4,000 rpm for 10 min and resuspended in 200 μl of LB broth. A

portion of a single colony of the donor S17-1, harboring a corresponding cosmid with a matching *hrp* gene, was transferred onto a nylon film (2 cm^2) and then mixed with 30 μl of the recipients. The mixture on the film was incubated on NA at 28°C for 48 h and then plated on NA complemented with kanamycin and rifampin. For Hrp phenotype tests, single-colony transfers were used to purify the transconjugants after culture at 28°C for 4 to 6 days. The transformation of *X. oryzae* pv. oryzicola strains, which led to homologous recombination of the incoming DNA with marker exchange, was performed according to the method of Boucher et al. (9).

Hpa1 protein expression and purification in *E. coli*. The *hpa1* gene (accession no. AY875714) was amplified by PCR from genomic DNA of the *X. oryzae* pv. oryzicola strain RS105 with the primers *hpa1*-F1 and *hpa1*-R1 (Table 2) and ligated into pGEM-T Easy vector (Promega). After restriction enzyme digestion of the vector with NdeI and XhoI, the resulting *hpa1* fragment was ligated into pET21a (Novagen), generating a construct (pEThpa1) to express the *hpa1* gene with a C-terminal hexahistidine tag. The construct was transformed into *E. coli* strain BL21(DE3) (Invitrogen). Bhpa1 cells were grown in an orbital shaker at 37°C and 220 rpm until the culture reached an absorbance, at 600 nm, of 0.5 to 0.6 and then were induced by the addition of 1 mM isopropyl-β-*D*-thiogalactopyranoside at 37 °C and 200 rpm for 3 h. The cells were harvested by centrifugation at 7,000 × *g* for 8 min at 4°C, resuspended in buffer A (20 mM Tris [pH 7.4], 300 mM NaCl, 20% glycerol, 5 U/ml DNase I, one 100-ml protease inhibitor mixture tablet), and disrupted by three passages through a Constant System cell disrupter (15 kpsi, model Z-plus 1.1 kW; Constant Systems). Disrupted cells were subjected to ultracentrifugation at 220,000 × *g* for 90 min at 4°C. The supernatant fraction was purified by affinity chromatography using a 1-ml HiTrap chelating column (Amersham Bioscience) immobilized with Ni^{2+} equilibrated with buffer C (20 mM Tris [pH 7.8], 100 mM NaCl, 10% glycerol). The column was washed with 50 mM imidazole added to the buffer C, and the extracted harpin was eluted with 500 mM imidazole in buffer B.

Detection of Hpa1 in the supernatant of the *Xanthomonas* cell culture. The promoter and coding regions of the *hpa1* gene were amplified by PCR with the primers *hpa1*-F2 and *hpa1*-R2, which incorporated the sequence encoding the His tag (Table 2). The resulting PCR product was cloned into the BamHI/KpnI sites of pUFR034, giving pUhpa1. The cosmid was transconjugated into the *hrpX* mutant RCX and the wild-type RS105, as described elsewhere in this paper, producing conjugants of RCX and RS105 with the pUhpa1 plasmid. The conjugants were grown at 28°C for 16 h in 500 ml of XOM3 medium. After two successive centrifugations (8,000 × *g*, 15 min), the culture supernatant was concentrated 20-fold by ultrafiltration with an Amicon Centricon 10 miniconcentrator (10,000-M_r cutoff) (37). This concentrated supernatant preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Meanwhile, harvested cells were resuspended in 0.05× the original volume of buffer A. *Xanthomonas* cells were disrupted and Hpa1 protein purified as described above.

Electrophoresis of proteins by 12.5% SDS-PAGE was performed as described by Laemmli (33). Before electrophoresis, the purified protein was treated with 0.5 M Tris(hydroxypropyl)phosphine solution (Novagen) in order to dissociate any dimers. After electrophoresis, proteins were either stained using the Stain Plus kit (Bio-Rad) or transferred onto a nitrocellulose membrane. Immunoblotting experiments were performed with mouse anti-His_6 monoclonal antibody (Roche Molecular Biochemical), which allows the detection of His_6-tagged recombinant proteins, according to the manufacturer's protocol.

Hrp phenotype tests in planta. Hypersensitive response and pathogenicity assays were performed as described by Hopkins et al. (26). *Xanthomonas* bacteria grown in NA broth with appropriate antibiotics to 10^8 CFU/ml were infiltrated into tobacco leaves (*Nicotiana tobacum* cultivar NC89) by using needleless syringes and inoculated into leaves of adult rice plants (IR24, susceptible to the pathogen) by using leaf needling for lesion length measurement or needleless syringes for detection of water soaking in rice seedlings. Plant responses were scored at 24 h (for HR), 3 days (for water soaking), and 14 days (for pathogenicity) after inoculation. Scores are the means of those for three leaves. All plants were grown in growth chambers at 28°C with a 12-h photoperiod. Experiments were repeated at least three times.

RT-PCR assay. The expression of the *hpa1* gene of *X. oryzae* pv. oryzicola was assayed by reverse transcription-PCR (RT-PCR) with the primers *hpa1*-F1 and *hpa1*-R1 (Table 2). First, the xanthomonad bacteria were preincubated in NA medium for 16 h, suspended at an optical density of 600 nm of 2.0 in sterilized water, and washed twice. Then, 40 μl of this bacterial suspension was inoculated into 1 ml of the modified XOM3. As a template, total RNA from the bacteria was prepared using the RNeasy plant minikit (QIAGEN). Reverse transcription and PCR for the *hpa1* gene with the primers were performed using ReverTra Ace (TaKaRa, China) according to the manufacturer's directions.

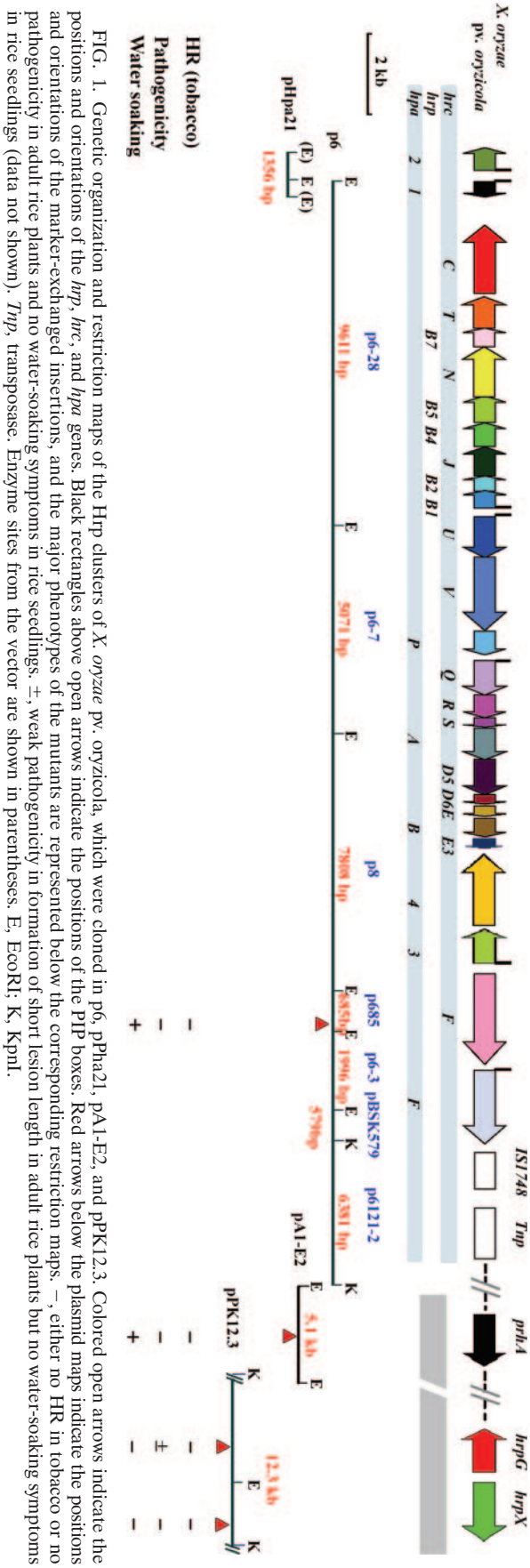

FIG. 1. Genetic organization and restriction maps of the Hrp clusters of *X. oryzae* pv. oryzicola, which were cloned in p6, pHa21, pA1-E2, and pPK12.3. Colored open arrows indicate the positions and orientations of the *hrp*, *hrc*, and *hpa* genes. Black rectangles above open arrows indicate the positions of the PIP boxes. Red arrows below the plasmid maps indicate the positions and orientations of the marker-exchanged insertions, and the major phenotypes of the mutants are represented below the corresponding restriction maps. −, either no HR in tobacco or no pathogenicity in adult rice plants and no water-soaking symptoms in rice seedlings. ±, weak pathogenicity in formation of short lesion length in adult rice plants but no water-soaking symptoms in rice seedlings (data not shown). *Tnp*, transposase. Enzyme sites from the vector are shown in parentheses. E, EcoRI; K, KpnI.

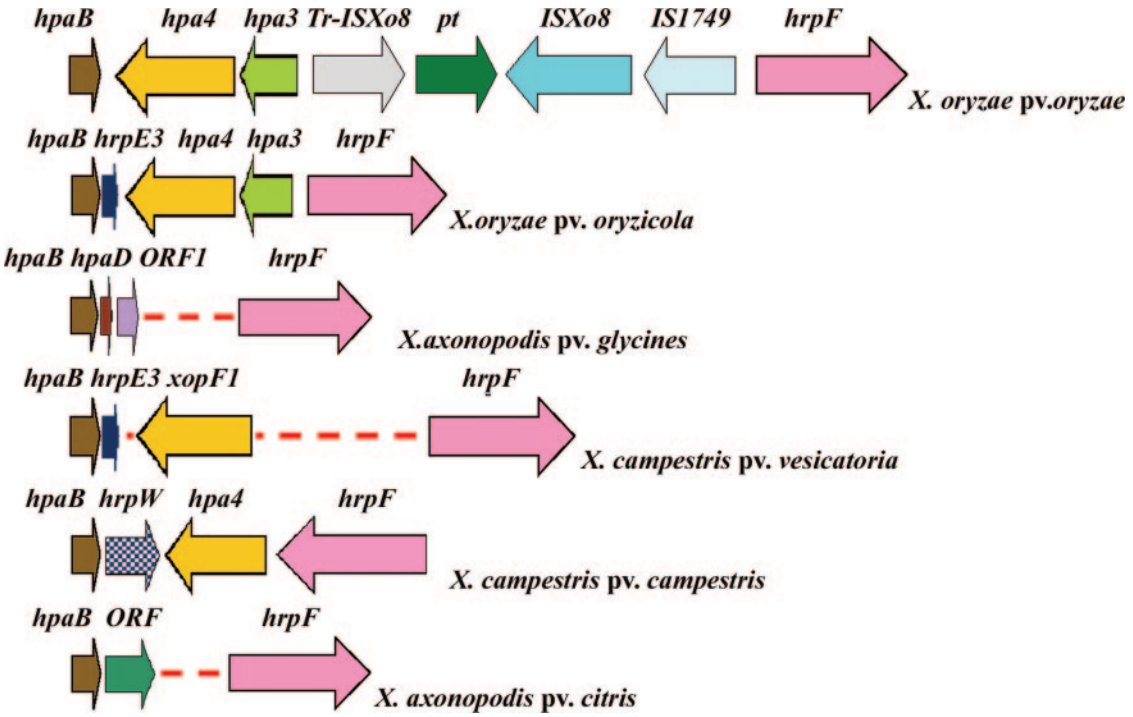

FIG. 2. Comparisons of the *hpaB-hrpF* interspace region in the core *hrp* clusters of six *Xanthomonas* species or pathovars. Colored arrows display the orientations and genetic organizations of genes in the *hpaB-hrpF* region. The gene size and organization are based on the sequence from the GenBank database, with accession numbers AB115081 for *X. oryzae* pv. oryzae, AY875714 for *X. oryzae* pv. oryzicola, AF499777 for *X. axonopodis* pv. glycines, AF056246 for *X. campestris* pv. vesicatoria, AE008922 for *X. campestris* pv. campestris, and AE008923 for *X. axonopodis* pv. citri. *pt*, putative transposase.

Generation of *hrpG*, *hrpX*, *prhA*, and *hrpF* mutants of *X. oryzae* pv. oryzicola. The *hrpG*, *hrpX*, *prhA*, and *hrpF* mutants of *X. oryzae* pv. oryzicola were constructed using the methodology described by Mongkolsuk et al. (38, 39). To create a polar insertion of *hrpX*, the pBCX147 plasmid was constructed in the following manner. A 1,430-bp fragment of the *hrpX* gene was amplified (with primers described in Table 2) using gDNA of strain RS105 as a template. The product was ligated into the vector pMD-18-T and digested with PstI and KpnI. The excised 147-bp fragment was inserted into the PstI and KpnI sites of pBC SK(−), giving pBCX147. Using the same strategy, a 121-bp fragment from the *hrpG* gene (primers are described in Table 2) was cloned into the EcoRI and SacI sites of pBC SK(−), a 112-bp fragment from the *prhA* gene (primers are described in Table 2) was cloned into the PstI and SacII sites of pBC SK(−), and a 351-bp fragment from p6-3 was ligated into the BamHI and PstI sites of pBC SK(−), giving pBCG121, pABC, and pFBC, respectively. Subsequently, the reconstructed plasmids were electroporated into strain RS105, using the methodology described by Mongkolsuk et al. (39), and single transformants were selected on chloramphenicol-NA plates after a 4-day incubation. Mutants at a single crossing with marker exchange were identified by Southern blotting with corresponding probes (unpublished data), and these strains were termed RABC for the *prhA* mutant, RCX for the *hrpX* mutant, RCG for the *hrpG* mutant, and RFBC for the *hrpF* mutant, respectively.

Nucleotide sequencing and data analysis. The inserted DNA in p6 digested with single EcoRI or KpnI enzymes was mapped physically first and then subcloned into pBluescript II SK(−) prior to sequencing. Universal and reverse primers were used for the primary reactions, and synthesized primers were then used to sequence both strands completely. DNA sequencing was performed using ABI PRISM dideoxy terminator kits and analyzed on an ABI model 373A automated sequencer in TaKaRa (Dalian, China). The sequence data were analyzed with the BLAST program at the National Center for Biotechnology Information, MEGALIGN software (DNASTAR), and Vector NTI software (Invitrogen). Since there are no regions containing the *hpa2* gene and the *hpa1* promoter in p6, we PCR amplified a 1,356-bp fragment containing the *hpa2* and *hpa1* genes with the primers *hpa2*-F and *hpa1*-R (Table 2), using *X. oryzae* pv. oryzicola gDNA as the template. The fragment was ligated into pMD-18-T vector to give pHpa21. Using primers outside the fragment, with *X. oryzae* pv. oryzicola gDNA as the template, we were able to confirm that the amplified DNA was native to *X. oryzae* pv. oryzicola (data not shown).

Nucleotide sequence accession numbers. The core *hrp* cluster, the cluster of the *hrpG* and *hrpX* genes, and the *prhA* gene have been assigned GenBank accession numbers AY875714, AY272885, and AY129230, respectively.

RESULTS

DNA sequence and putative ORFs of the core *hrp* region. Based on the sequence of the *hrp* cluster of *X. oryzae* pv. oryzae (accession no. AB115081), we PCR amplified a 1.5-kb *hrpA* operon with the primers *hrpA*-F1 and *hrpA*-R1 (Table 2) and a 1.62-kb *hrpF* gene fragment with the primers *hrpF*-F1 and *hrpF*-R1 (Table 2) from the gDNA of *X. oryzae* pv. oryzae and used these as probes for screening a genomic library for the *hrp* cluster of *X. oryzae* pv. oryzicola. In situ hybridization with the probes used for screening the genomic library established that p6 was an *hrp*-positive clone (data not shown). Furthermore, a Southern hybridization with the two probes and the p6 clone, digested with EcoRI, indicated that the clone contained the *hrp* region from the *hrpA* to the *hrpF* operons (Fig. 1). Subsequent subcloning and sequencing demonstrated that clone p6 contained a 26,243-bp *hrp* cluster of *X. oryzae* pv. oryzicola, comprising 26 open reading frames (ORFs) that included the region from the partial *hpa1* gene to the *hpaF* gene (Fig. 1 and 2; Table 3). There were two genes for a transposase and an IS*1748* sited at the right border of *hpaF* (Fig. 1). In contrast to the case for *X. campestris* pv. vesicatoria (12), *X. axonopodis* pv. glycines (31), and *X. axonopodis* pv. citri (16), there are no tRNAs

TABLE 3. Nucleotide sequence homology between clustered *hrp* genes of *Xanthomonas oryzae* pv. oryzicola and the *hrp* genes of *X. oryzae* pv. oryzae

Gene	Size (bp) in:		% Identical nucleotides compared with *X. oryzae* pv. oryzae	Gap (bp)	PIP box[a]	
	X. oryzae pv. oryzicola	*X. oryzae* pv. oryzae			Sequence	Position
hpa2	564	474	98	90	I	147
hpa1	414	420	86	12	P	160
hrcC (*hrpA*)	1,824	1,818	97	6		
hrcT (*hrpB8*)	831	831	98	0		
hrpB7	510	510	97	0		
hrcN (*hrpB6*)	1,329	1,329	98	0		
hrpB5	702	702	98	0		
hrpB4	630	630	97	0		
hrcJ (*hrpB3*)	765	762	94	3		
hrpB2	393	393	96	0		
hrpB1	456	456	98	0	P	121
hrcU (*hrpC1*)	1,074	1,080	97	6	P	96
hrcV (*hrpC2*)	1,938	1,938	99	0		
hpaP (*hrpC3*)	618	618	97			
hrcQ (*hrpD1*)	915	915	97		P	273
hrcR (*hrpD2*)	645	645	98			
hrcS (*hrpD3*)	261	261	98			
hpaA (*hrpD4*)	828	828	96			
hrpD5	939	939	96			
hrpD6	243	243	97			
hrpE	282	282	97			
hpaB	471	471	97			
hrpE3	260					
hpa4	1,932	1,986	95	56		
hpa3	447	447	96	0	II	121
hrpF	2,409	2,409	98	0		
hpaF	2,409	2,040	97	9	III	239
IS*1748*	1,062					
Putative transposase gene	1,059	1,059	97	0		

[a] A PIP box or similar sequence upstream of the putative translational start site of the gene. I, imperfect PIP box TTCGC-N15-TTCGT; P, PIP box TTCGC-N15-TTCGC; II, imperfect PIP box TTCGT-N15-TTCGC; III, imperfect PIP box TTCGC-N9-TTCGC.

bordering after the *hpaF* gene (data not shown). The *hpa2* and *hpa1* genes were directly adjacent to the left of the *hrpA* (Fig. 1). Therefore, the length of the core *hrp* cluster from *hpa2* to *hpaF* is about 27 kb.

The complete DNA sequence of the core *hrp* cluster of *X. oryzae* pv. oryzicola revealed that there were 10 *hrp* genes, 9 *hrc* genes, and 8 *hpa* genes (Fig. 1; Table 3). For most genes, there was 86% to 99% identity in the nucleotide sequences for each ORF between the two pathovars of *X. oryzae* (Table 3). However, significant differences were found in the sequences of four genes: the ORF for the *hpa2* genes was 90 bp larger at the 5′ end than that of *X. oryzae* pv. oryzae, the *hpa1* gene had only 86% similarity with that of *X. oryzae* pv. oryzae, the *hpa4* gene lacked a 63-bp fragment that existed in *hpa4* of *X. oryzae* pv. oryzae, and the 5′ end of *hpaF* was 96 bp longer than that in *X. oryzae* pv. oryzae (accession numbers AY536514 and AB115081) (Table 3). In total, there were seven predicted PIP boxes in the *hrp* cluster. Four genes (*hpa1*, *hrpB1*, *hrcU*, and *hrcQ*) had perfect PIP boxes (TTCGC-N15-TTCGC), while three genes (*hpa2*, *hpa3*, and *hpaF*) had imperfect PIP boxes (*hpa2*, TTCGC-N15-TTCGT; *hpa3*, TTCGT-N15-TTCGC; *hpaF*, TTCGC-N9-TTCGC) in their putative promoter regions (Fig. 1; Table 3). The imperfect PIP box in the *hpaF* locus was found only in *X. oryzae* pv. oryzicola among *Xanthomonas* species or pathovars, including *X. oryzae* pv. oryzae. Additionally, Hpa1 of *X. oryzae* pv. oryzicola had 23.1% identity to PopA1 of *R. solanacearum* and 42% to 72.1% identity to Hpa1 proteins of other *Xanthomonas* species or pathovars (see Fig. 6), indicating that the *hpa1* locus is variable in xanthomonads.

The interspace region from *hpaB* to *hrpF* is variable in the highly conserved *hrp* cluster of xanthomonads. We used a combination of BLAST, PSI-BLAST, and FASTA programs to search for homology in the deduced amino acid sequence of each Hrp protein of *X. oryzae* pv. oryzicola and to predict its site of localization in the bacterial or plant cell (data not shown). The 20 genes from *hrcC* to *hpaB*, which were the main genes for the TTSS, of the core region were present in all six *Xanthomonas* species tested and were highly conserved with the corresponding genes from *X. oryzae* pv. oryzae (Table 3). In contrast to the corresponding region in *X. oryzae* pv. oryzae, we could not find an insertion sequence or a transposase in the interspace region between *hpaB* and *hrpF*, which encodes the *hrpE3*, *hpa4*, and *hpa3* genes (Fig. 2). The *hrpE3* gene, which encodes an 86-amino-acid protein, is highly homologous to *hrpE3* of *X. campestris* pv. vesicatoria. The *hpa4* gene, which encodes a 643-amino-acid protein that is predicted to be soluble and located in the cytoplasm, was homologous to *hpa4* in *X. oryzae* pv. oryzae (accession no. AB115081) (49), which is also located in the interspace region between *hpaB* and *hrpF* of the *hrp* gene cluster (Fig. 2). A homology search revealed a 254-residue protein with homology to the protein XopF1 in *X. axonopodis* pv. citri (AE011919-5) (16) (Fig. 2). The *hpa3* gene,

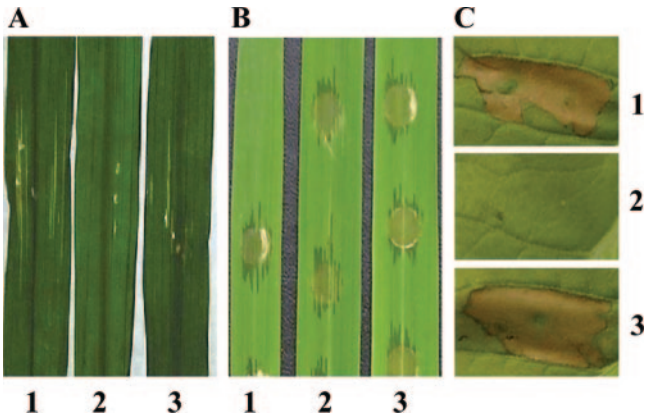

FIG. 3. Phenotypic analysis of a *prhA* mutant derived from *X. oryzae* pv. oryzicola. The mutation in the *prhA* gene resulted in the loss of the hypersensitive response in tobacco (Nc89) (C) and pathogenicity in adult rice (A), but the ability to cause water-soaking symptoms in rice seedlings (3 weeks old, IR24) was retained (B). 1, wild-type strain RS105 with an empty vector pUFR034; 2, *prhA* mutant RFBC produced by marker exchange via a single crossover event when the reconstructed plasmid pFBC was electroporated into the wild-type strain RS105; 3, the conjugate of the *prhA* mutant complemented with plasmid pA1 harboring the *prhA* gene of *X. oryzae* pv. oryzicola.

which is predicted to encode a 148-residue lipoprotein that is anchored to the inner and/or outer membrane, is similar to *hpa3* of *X. oryzae* pv. oryzae but differs by the presence of an imperfect PIP box (TTCGT-N15-TTCGC) that is not present in the promoter region in *X. oryzae* pv. oryzae (49). However, the *hrpW* homolog from *P. syringae* pathovars and *Erwinia amylovora* were present only in the interspace region of *X. campestris* pv. campestris and not in *X. oryzae* pv. oryzicola (Fig. 2). In contrast to the case for other xanthomonads, there is an IS*1748* and a gene for a putative transposase following the *hpaF* gene (Fig. 1) in *X. oryzae* pv. oryzicola. There are no tRNAs after the *hpaF* gene in *X. oryzae* pv. oryzicola, in contrast to those found in *X. campestris* pv. vesicatoria, *X. axonopodis* pv. glycines, and *X. axonopodis* pv. citri (data not shown).

Hrp regulatory genes, *hrpG*, *hrpX*, and *prhA*, are outside of the core *hrp* cluster. To isolate the Hrp regulatory genes of *X. oryzae* pv. oryzicola, we transferred individual clones of the RS105 genomic library into the *hrp* mutants M55 and M1005, which were previously confirmed as *hrpX* and *hrpG* mutants (14), and isolated a cosmid clone (pPK12.3) with the function of restoring the *hrp* mutants to HR induction in tobacco and pathogenicity in rice. A restriction enzyme digestion analysis established that pPK12.3 had an insert of approximately 12.3 kb (Fig. 1), while a Southern blot confirmed that the inserted fragment was colinear with the RS105 genome (data not shown). The complete DNA sequence of the 12.3-kb insert in pPK12.3 was determined and found to contain two clustered *hrp* regulatory genes, *hrpG* and *hrpX*, which were highly conserved with those in other xanthomonads (data not shown). However, the sequences on either side of the *hrpG* and *hrpX* locus had no homology to *hrp* genes in other xanthomonads, indicating that the *hrpG* and *hrpX* genes are clustered but located outside the core *hrp* cluster, elsewhere in the chromosome (Fig. 1).

We were interested in identifying a gene encoding a putative siderophore receptor similar to PrhA in *Ralstonia solanacearum* (35), which acts as a sensor that detects the plant cell wall, triggering the transcriptional activation of bacterial virulence genes (1). Using the *phrA* gene in a BLAST search, we identified a homolog that encodes a putative siderophore receptor in *X. oryzae* pv. oryzae (accession number AF325732). Based upon this sequence, we designed primers (Table 2) and PCR amplified the gene from *X. oryzae* pv. oryzae, which was used to construct a *prhA* mutant of *X. oryzae* pv. oryzicola that was conjugated with each clone of the RS105 genomic library (unpublished data) in order to identify the adjacent genes and to determine whether they are related to any genes involved in Hrp phenotypes, HR induction in nonhost plants, and pathogenicity in host plants. The *prhA* mutant lost HR induction in tobacco and pathogenicity in rice (Fig. 3). The subsequent determination of the phenotypes of the conjugants in tobacco and rice led to the isolation of a cosmid clone (pA1) that restored the ability of the mutant to cause HR induction in tobacco and pathogenicity in rice (Fig. 3). A subclone, pA1-E2, which harbored a 5.1-kb DNA fragment was rescued as the smallest DNA fragment with the ability to restore the Hrp phenotype to the mutant (Table 1; Fig. 1). The complete sequence of this fragment showed that it contained a gene, which we term *prhA*, encoding a putative siderophore receptor which had 29% identity to PrhA of *R. solanacearum* (35). The sequences adjacent to the *prhA* locus had no homology with *hrp* genes in other xanthomonads (data not shown).

Phenotypes of the *hrpF*, *hrpG*, *hrpX*, and *prhA* mutants. Most of the *hrp*, *hrc*, and *hpa* genes of *Xanthomonas oryzae* have been mutated in an attempt to understand their roles in the hypersensitive response in nonhosts and pathogenicity in rice (41, 49, 62). To elucidate the *hrp* regulatory cassette for the core *hrp* cluster of *X. oryzae* pv. oryzicola, we constructed *hrpF*, *prhA*, *hrpG*, and *hrpX* mutants by using the homologous recombination and marker exchange methodology described by Rabibhadana et al. (42). Each mutant was confirmed by Southern hybridization (data not shown) and assayed for HR induction in tobacco and pathogenicity in rice. Aside from the fact that the *hrpF*, *hrpX*, and *prhA* mutants had lost their ability to induce the HR in tobacco and pathogenicity in adult rice, interestingly, the *hrpF* and *prhA* mutants, when infiltrated into leaves of rice seedlings (21 days old, cultivar IR24), retained the ability to trigger water soaking comparable to that caused by the wild-type strain RS105 (Fig. 3, 4, and 5). In contrast, the *hrpG* mutant had completely lost its ability to cause water soaking in IR24 seedling leaves and HR induction in tobacco but retained weak pathogenicity in the form of short lesion length when inoculated into leaves of adult rice plants by the leaf-needling method (Fig. 4). All of the mutant strains were complemented in HR induction and pathogenicity with the clones that incorporated the corresponding genes: p6 complemented the *hrpF* mutant (Fig. 1 and 5), pA1-E2 complemented the *prhA* mutant (Fig. 1), and pPK12.3 complemented the *hrpG* and *hrpX* mutants (Fig. 1). Since the three clones p6, pA1-E2, and pPK12.3 were nonoverlapping clones, derived from the RS105 genomic library, this confirms that the Hrp phenotype is controlled by three separate DNA regions: a core *hrp* cluster, an *hrpG* and *hrpX* cluster, and a *prhA* locus. The identification of genes in addition to *prhA* in pA1-E2 is ongoing.

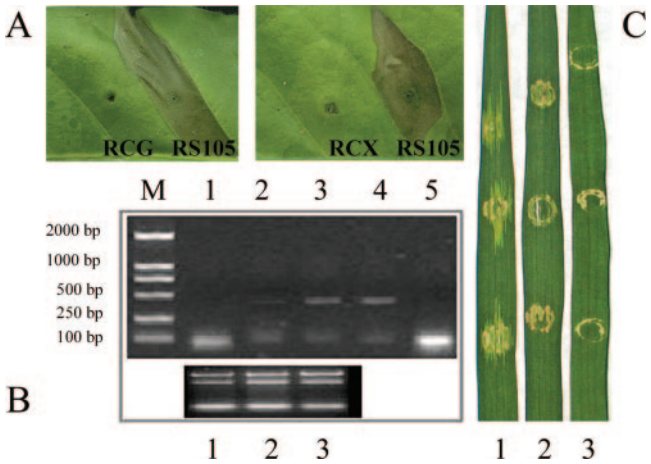

FIG. 4. The *hrpX* gene of *X. oryzae* pv. oryzicola controls expression of the *hpa1* gene in XOM3 medium. *Xanthomonas* strains were incubated in XOM3, and the phenotypes of the *hrp* regulatory gene mutants, *hrpX* and *hrpG*, were tested in tobacco leaves (cultivar Nc89) (A) and in rice (IR24) (C), respectively. The induction of *hpa1* expression in XOM3 medium was identified by RT-PCR (B), which was performed using total RNA as the template. The *hrpX* mutant RCX (1), the *hrpG* mutant RCG (2), and the wild-type strain RS105 (3) were grown at 28°C in XOM3 (pH 6.5). Total RNA was extracted from each bacterium after incubation for 16 h. Specific primers that amplified a 414-bp DNA fragment corresponding to the *hpa1* gene were used for RT-PCR. PCR products (B, top) and rRNA (B, bottom) were separated by agarose gel electrophoresis and stained with ethidium bromide. A positive PCR control using RS105 gDNA as the template (4) and a negative PCR control with no RT (5) are also indicated.

Hpa1 is a TTSS-dependent HR elicitor. Hpa1 is a harpin-like protein encoded by the *hpa1* gene of *X. oryzae* pv. oryzae (61) and is considered to be an Hrp type III-secreted elicitor (31). The sequence of the putative Hpa1 protein from *X. oryzae* pv. oryzicola had quite high similarities with Hpa1 of *X. oryzae* pv. oryzae (71.3% to 72.1% identity), Hpa1 of *X. axonopodis* pv. citri (67.4% identity), HpaG of *X. axonopodis* pv. glycines (64.4% identity), XopA of *X. campestris* pv. vesicatoria (53.0% identity), Hpa1 of *X. campestris* pv. campestris (42.0% identity), and PopA1 of *R. solanacearum* (23.1% identity) (Fig. 6). In contrast to XopA (8% glycine) of *X. campestris* pv. vesicatoria and Hpa1 (13% glycine) of *X. campestris* pv. campestris, Hpa1 (21% glycine) of *X. oryzae* pv. oryzicola, HpaG (21% glycine) of *X. axonopodis* pv. glycines, Hpa1 (22% glycine) of *X. axonopodis* pv. citri, and Hpa1 (26% glycine) of *X. oryzae* pv. oryzae had relatively high glycine contents (Fig. 6). Another significant difference was that both Hpa1 of *X. oryzae* pv. oryzicola and Hpa1 of *X. oryzae* pv. oryzae had a cysteine residue that is not present in Hpa1 or HpaG from other xanthomonad species (Fig. 6). Indeed, a feature of the harpins found in *P. syringae* pathovars, *Erwinia* species, *R. solanacearum*, and other xanthomonads is that they do not possess cysteine residues. A phylogenetic analysis of the relationship of Hpa1 with others of xathomonads and *R. solanacearum* demonstrated that Hpa1of *X. oryzae* pv. oryzicola is grouped with Hpa1 of *X. oryzae* pv. oryzae (Fig. 6).

To explore how the expression of the *hpa1* gene is modulated in *X. oryzae* pv. oryzicola, an *hrp*-inducing medium, XOM3, was developed based on XOM2, which was used for *X. oryzae* pv. oryzae (50). Total RNAs were extracted from the *Xanthomonas* strain RS105 and the *hrpG* and *hrpX* mutants growing in NA and XOM3 media, respectively, and used as templates for RT-PCR assays of the expression of the *hpa1* gene. This analysis indicated that *hpa1* expression is induced by the nutrient-poor medium XOM3 (Fig. 4) but not by a nutrient-rich medium, NA (data not shown), and that the expression of *hpa1* in *X. oryzae* pv. oryzicola is under the control of the *hrpX* gene, because the *hpa1* gene was not expressed when the *hrpX* gene was mutated (Fig. 4). The expression level of the *hpa1* gene in the wild type was higher than that in the *hrpG* mutant of *X. oryzae* pv. oryzicola (Fig. 4), suggesting that the *hrpG* gene participated in regulating the expression of *hpa1* when the bacterium was grown in the *hrp*-inducing medium for 16 h.

In order to investigate whether Hpa1 of *X. oryzae* pv. oryzicola had HR elicitor activity in the nonhost tobacco, Hpa1 that was expressed and purified from *E. coli* (Fig. 7A) was injected (at, e.g., 1 μg/ml) into tobacco leaves, where it was found to elicit the HR (Fig. 7B). Interestingly, the purified Hpa1 existed both as a monomer and a dimer (Fig. 7A), possibly due to cross-linking of the cysteine residue in Hpa1 (Fig. 6A). An immunoblotting analysis was performed to determine if Hpa1 secretion was via the type III secretion system; Hpa1 was not detected in the disrupted cells of the *hrpX* mutant harboring the pUhpa1 plasmid that carries the *hpa1* gene, whereas it was detected both in the disrupted cells and in the culture supernatant of the wild-type strain (Fig. 8), thus indicating that Hpa1 is a TTSS effector that triggers the HR in nonhost tobacco.

DISCUSSION

Here we report on the isolation of a core cluster of *hrp* genes, a clustered pair of *hrp* regulatory genes (*hrpX* and *hrpG*), and a *prhA* gene from the Chinese strain RS105 of *X. oryzae* pv. oryzicola. Mutations in the isolated *hrp* genes, *hrp* regulatory genes, and a *prhA* gene of *X. oryzae* pv. oryzicola resulted in a loss of pleiotropic phenotypes for both the ability to induce the HR in nonhost tobacco and pathogenicity in host rice. When the nucleotide sequence for the core *hrp* cluster from *X. oryzae* pv. oryzicola was compared with those from *X. oryzae* pv. oryzae (41, 49), *X. campestris* pv. campestris and *X. axonopodis* pv. citri (17), *X. campestris* pv. vesicatoria (8, 28, 40), and *X. axonopodis* pv. glycines (31), we found that, in common with other xanthomonads, the *hrc* genes encoding the TTSS apparatus were completely conserved. A comparison of the Hrp pathogenicity islands of six *Xanthomonas* species demonstrated variabilities in the *hpaB-hrpF* interspace region (Fig. 2). Only the *hrpE3*, *hpa4*, and *hpa3* genes were present in the *hpaB-hrpF* region of *X. oryzae* pv. oryzicola, which differed greatly from the regions of the other five *Xanthomonas* species. The *hpa4* and *hpa3* genes appear to be linearly placed within an operon that is preceded by a PIP box (TTCGT-N15-TTCGC) located 121 bp upstream of the putative operon (Table 3). This indicates that the interspace region is part of the core *hrp* cluster of *X. oryzae* pv. oryzicola and that the expression of the *hpa4* and *hpa3* genes is regulated by HrpX. However, direct evidence for the involvement of *hrpE3*, *hpa4*, and *hpa3* in the pathogenicity of *X. oryzae* pv. oryzicola is required.

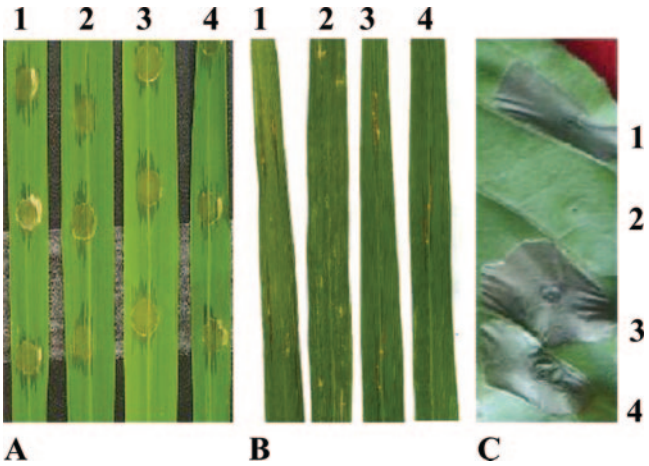

FIG. 5. Phenotypic analysis of the *hrpF* mutant derived from *X. oryzae* pv. oryzicola. The *hrpF* mutant had lost pathogenicity in adult rice (IR24) when inoculated into leaves by using leaf needling for lesion length measurements (B) and had lost the hypersensitive response in tobacco (Nc89) when infiltrated into leaves with needleless syringes (C), but it retained to the ability to cause water-soaking symptoms in rice seedlings (A). A. Water-soaking symptoms caused by the *hrpF* mutant. The third leaf of 14-day-old IR24 seedlings was infiltrated using needleless syringes individually with the wild-type strain RS105 harboring pUFR034 (empty plasmid) (1), RFBC (*hrpF* mutant) (2), RFBC harboring p6 (the core *hrp* cluster) (3), and RFBC harboring pHrpF (the *hrpF* gene) (4). The water-soaking symptoms after 3 days of infiltration are shown. B. Measurements of lesion length caused by *hrpF* mutants. The third leaf of IR24 adult plants was inoculated with corresponding bacteria as described above, using leaf needling. The lesion lengths after 14 days are shown. C. Hypersensitive response in tobacco induced by *hrpF* mutants. The leaves were infiltrated using needleless syringes with the bacteria as described above, and the reaction was recorded within 24 h. Three replicates were conducted for identification of the phenotypes.

Previous studies have shown that mutations in either *hpa4* or *hpa3* of *X. oryzae* pv. oryzae did not affect the virulence (49) and that the product of *xopF1*, an homolog of *hpa4*, is a TTSS effector of *X. campestris* pv. vesicatoria (43). The variability in the *hpaB-hrpF* region revealed in this report suggests that, in comparison to the *hrp* clusters of other xanthomonads, the core *hrp* cluster of *X. oryzae* pv. oryzicola is novel.

The core *hrp* cluster of gram-negative plant pathogenic bacteria forms a TTSS apparatus to deliver virulence effectors into the host cytosol that interfere with and alter host processes. The proposed function of HrpF as a translocon and as a secreted protein places the protein at the plant-bacterium interface (13, 49). Interestingly, mutation of the *hrpF* locus of *X. oryzae* pv. oryzicola resulted in the loss of pathogenicity in rice and the ability to induce HR in nonhost tobacco. This was consistent with previous reports that mutations in *hrpF* of *X. campestris* pv. vesicatoria or *X. axonopodis* pv. glycines resulted in strains that were nonpathogenic in host plants and unable to elicit race-specific HRs (13, 31). This contrasts with the behavior of the *hrpF* mutant of *X. oryzae* pv. oryzae, which retained its pathogenicity but displayed both a reduced ability to grow within rice and a reduced ability to cause lesions (49). However, in common with the *hrpF* mutant of *X. oryzae* pv. oryzae, our investigation revealed that the *hrpF* mutant of *X. oryzae* pv. oryzicola still caused water-soaking symptoms in susceptible rice seedlings (Fig. 5). Importantly, the mutation in the *hrpF* gene of *X. oryzae* pv. oryzae had no effect on HR induction in rice when there was an *avr* gene in the pathogen that correspondingly matched an *R* gene in rice (49). The production of water-soaking symptoms in susceptible rice seedlings is one of the functions of *avrBs3* family members in *X. oryzae* pv. oryzae (58). In this study, we found that mutation of the *hrpX* gene led to a complete loss of pathogenicity, not only as measured by lesion length in adult rice plants but also in a lack of ability to cause water-soaking symptoms in rice seedlings (Fig. 1 and 4). However, the *hrpG* mutant retained weak pathogenicity in lesion length only at the inoculation site and had completely lost its ability to cause water soaking in rice seedlings (Fig. 4). Recently, it was reported that there are diverse members of the *avrBs3* family in *X. oryzae* pv. oryzicola (15, 62). Considering the fact that the expression of *hrpA* and *hrpX* is activated by HrpG and the activation of *hrpB-hrpF* operons by HrpX established in other xanthomonads (51, 54, 56, 57), we postulate that AvrBs3 family members, the critical TTSS effectors, are not delivered through the HrpF translocon of *X. oryzae* pv. oryzicola into rice cells, but further evidence is needed to support this postulate. Importantly, the *prhA* mutant of *X. oryzae* pv. oryzicola retained the ability to cause water-soaking symptom in rice seedlings, indicating that the *prhA* gene had no effect on the roles of *avrBs3* family members in *X. oryzae* pv. oryzicola. Therefore, modulating the expression of *avrBs3* family genes in xanthomonads at the *hrpF* and *prhA* loci should elucidate the process of secretion when *X. oryzae* pv. oryzicola interacts with rice.

Our knowledge and understanding of the *hrp* regulatory cascade in *X. oryzae* pv. oryzicola are still rudimentary. Previous studies revealed that the *hrp* genes in xanthomonads, which are induced in plants, are not expressed when bacteria are grown in rich media but are strongly expressed in media that mimic the plant apoplastic medium (35, 47, 48, 50, 55). In this report, we developed an XOM2-based *hrp*-inducing medium, XOM3, that is suitable for *X. oryzae* pv. oryzicola (50). The *hpa1* gene, as an indicator detected by RT-PCR, was expressed strongly in the wild-type strain and constitutively in the *hrpG* mutant but was not expressed in the *hrpX* mutant when the bacteria were grown on XOM3 (Fig. 4). The His-tagged Hap1 was detected by immunoblotting in the supernatant and disrupted cells of the wild-type strain that had been transformed with the pUhpa1 plasmid and grown in XOM3, while it was not detected in disrupted cells of the *hrpX* mutant (Fig. 8). However, a *prhA* mutant of *X. oryzae* pv. oryzicola, which had lost the ability to trigger HR in tobacco and pathogenicity in adult rice but retained the ability to cause water-soaking symptoms in rice seedlings, had no effect on *hpa1* expression in XOM3 (data not shown). The phenotypes in tobacco and rice caused by the *prhA* mutant were not comparable to those caused by the *prhA* mutant of *R. solanacearum*. In *R. solanacearum*, PrhA perceived plant cell wall-derived signals during the bacterium-plant interaction and activated *hrp* gene expression through a six-gene regulatory cascade (e.g., *prhA*, *prhR*, *prhI*, *prhJ*, *hrpG* and *hrpB*) (1, 10). Recently, Genin et al. (21) reported that the repression of *hrp* genes in nutrient-rich medium was relieved in a *phcA* mutant of *R. solanacearum* and that the Prh plant-responsive pathway and an unidentified minimal medium pathway connected *hrp* gene regulation to the global virulence

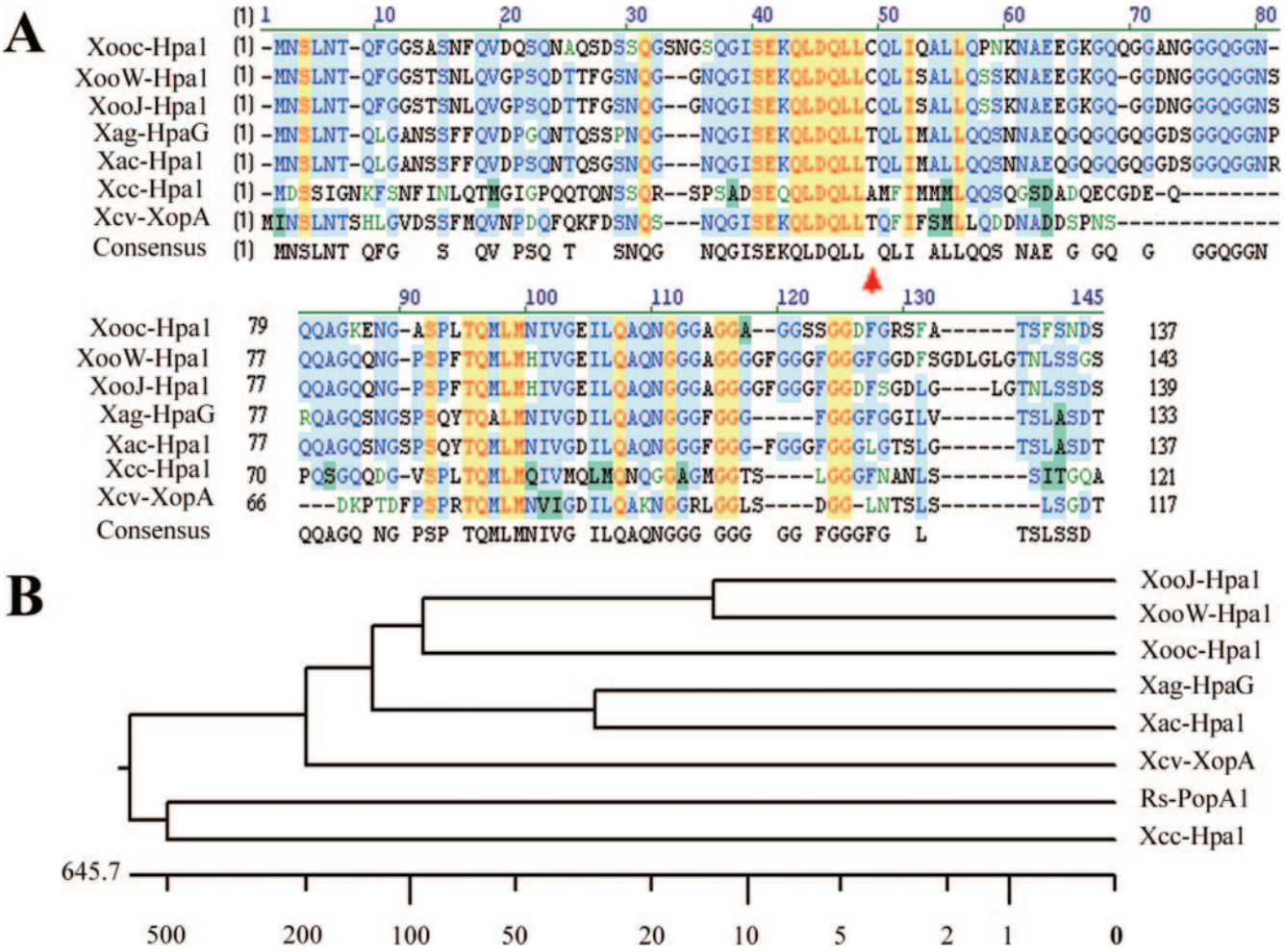

FIG. 6. Comparison of Hpa1 proteins among xanthomonads and *Ralstonia solanacearum*. A neighbor-joining bootstrap tree is derived from the amino acid sequences of all the Hpa1 proteins in plant-pathogenic bacteria by using the Vector NTI align program. The protein names are indicated as the abbreviated names of the corresponding plant-pathogenic bacteria. The abbreviations are as follows, with accession numbers in GenBank or Gene ID in parentheses: Xooc, *X. oryzae* pv. oryzicola (AY875714); XooJ, in strain MAFF301237 of *X. oryzae* pv. oryzae (NC006834); XooW, in strain PXO99^{A} of *X. oryzae* pv. oryzae (AB115081); Xag, *X. axonopodis* pv. glycines (AF4997777); Xac, *X. axonopodis* pv. citri (Xac0416); Xcv, *X. campestris* pv. vesicatoria (U33548); Rs, *Ralstonia solanacearum* (AB026629); and Xcc, *X. campestris* pv. campestris (Xcc1240). A. Sequence alignment of xanthomonad Hpa1 proteins. The red arrow indicates the position of the cysteine residue in Hpa1 of *X. oryzae*. B. Phylogenetic relationship of Hpa1 protein among xathomonads and *R. solanacearum*.

regulator PhcA at the branching point of HrpG. Therefore, further genetic evidence is required in order to elucidate the *hrp* regulatory network in xanthomonads, especially in *X. oryzae* pv. oryzicola.

The expression of some TTSS effector genes that have a PIP box with the consensus nucleotide sequence TTCGC-N15-TTCGC in their promoter region is HrpX dependent (7, 18, 51). In analogy to *hpa1* in *X. oryzae* pv. oryzae, we found that there was a PIP box in the promoter region of the *hpa1* gene of *X. oryzae* pv. oryzicola. The *hpa1* gene was not expressed in the *hrpX* mutant when the bacterium was grown in the *hrp*-inducing medium XOM3 but was expressed in the *hrpG* mutant at a much lower level. Moreover, immunoblotting failed to detect the Hpa1 protein in the *hrpX* mutant of *X. oryzae* pv. oryzicola, consistent with the proposal that Hpa1 is a TTSS effector and that the transcriptional expression of the *hpa1* gene was directly controlled by HrpX.

Harpins constitute a family of secreted effector proteins which are translocated via the type III pathway in plant-pathogenic bacteria, triggering disease resistance-associated responses, such as hypersensitive cell death, and thus activating the plant's surveillance system (20, 32). Genes encoding such proteins have been identified in *E. amylovora* and *Erwinia chrysanthemi* (*hrpN*) (52); *P. syringae* pv. syringae, tomato, and glycinea (*hrpZ*) (25); *R. solanacearum* (*popA*) (4); *X. axonopodis* pv. glycines (*hpaG*) (31); *X. campestris* pv. vesicatoria (*xopA*) (40); and *X. oryzae* pv. oryzae (*hpa1*) (53, 61). Prior to the present study, no harpin-like proteins had been isolated in *X. oryzae* pv. oryzicola. Zhao et al. (60) demonstrated that *avrRxo1* of *X. oryzae* pv. oryzicola induced an HR on maize with *Rxo1* but not on maize without *Rxo1*. In our study, the putative translation product of the *hpa1* gene of *X. oryzae* pv. oryzicola was similar to the harpins mentioned above: it is a heat-stable, glycine-rich protein. The purified product of the

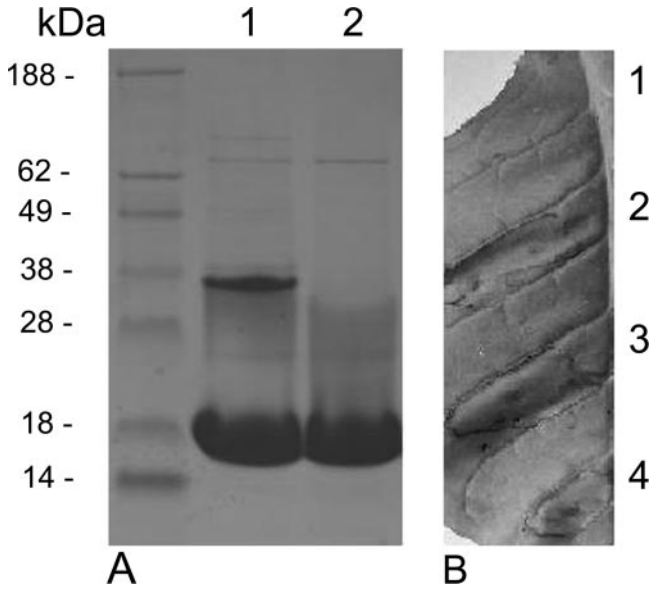

FIG. 7. Hypersensitive reaction of the purified Hpa1 protein of *X. oryzae* pv. oryzicola. A. A 12.5% SDS-polyacrylamide gel showing Hpa1 that was expressed and purified from *E. coli* (BL21). Two bands are apparent in lane 1, while only one band is apparent in lane 2 after treatment of the protein with 0.5 M Tris(hydroxypropyl)phosphine (Novagen), suggesting that the higher-M_r band is a dimer due to cysteine cross-linking. B. Comparison of Hpa1 activity with that of well-known harpin HrpN. 1, BL21 harboring empty vector pET21a; 2, RS105 (10^8 CFU/ml); 3, Hpa1 (1 μg/ml); 4, HrpN (5 μg/ml) (kindly provided by Z. Wei, Eden Biotech). The tobacco leaves were photographed 24 h after injection using needleless syringes. Three replicates of the assay were conducted. The first lane in panel A is the protein marker.

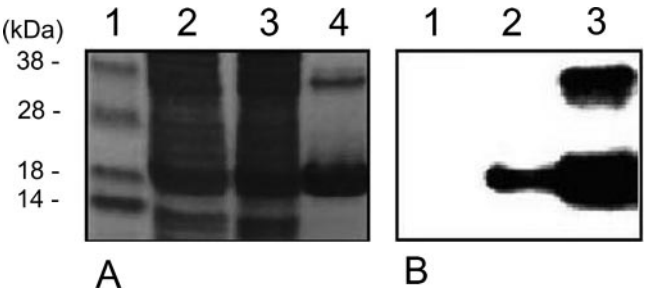

FIG. 8. In vitro secretion analysis of *X. oryzae* pv. oryzicola expressing Hpa1. Purified proteins from disrupted cells and the culture supernatant were analyzed by 12.5% SDS-PAGE (A) and immunoblotted with the monoclonal antihexahistidine antibody (B). The plasmid pUhpa1 was used to express the His-tagged Hpa1 in bacteria that were cultured in XOM3 for 16 h. Lanes 1, disrupted cells of the *hrpX* mutant RCX harboring pUhpa1; lanes 2, disrupted cells of the wild-type RS105 harboring pUhpa1; lanes 3, supernatant of the wild-type RS105 with plasmid pUhpa1. The Hpa1 monomer and dimer are shown as the lower and upper bands in lanes 3. The first lane in panel A is the protein marker.

hpa1 gene, which elicited an HR in tobacco at 1.0 μM, is the first HR-eliciting protein identified in *X. oryzae* pv. oryzicola.

Among the Hpa1 homologs of xanthomonads, only Hpa1 from *X. oryzae* pv. oryzicola, Hpa1 from *X. oryzae* pv. oryzae (53), and HpaG from *X. axonopodis* pv. glycines (31) have demonstrated harpin-like elicitor activity. Although all of the homologs exhibited high levels of identity at the amino acid level, two interesting differences were found in the amino acid alignment of Hpa1, HpaG, and XopA. There were two regions containing more glycine residues in Hpa1 of *X. oryzae* pv. oryzicola (Fig. 6A). HpaG lacked the residues GFGGG that corresponded to positions 114 to 117 in Hpa1 of *X. oryzae* pv. oryzicola. XopA lacked two glycine-rich regions that were present in Hpa1, suggesting that this region is critical for Hpa1 homologs to act as elicitors on nonhost plants. Currently, we are investigating which amino acid residues in Hpa1 are critical for conferring HR activity. Another notable difference is that only Hpa1 of *X. oryzae* pv. oryzicola and *X. oryzae* pv. oryzae possess a cysteine residue. The purified Hpa1 of *X. oryzae* pv. oryzicola, expressed both in *X. oryzae* pv. oryzicola and in *E. coli*, displayed two bands when run on SDS-polyacrylamide gels, which we have putatively assigned as the monomer and dimer. We postulated that these oligomers are due to cross-linking of the monomer due to disulfide bond formation, but we do not know if the dimer has any functional significance. In order to gain further insight into the functional role of Hpa1, we are using biophysical methods to determine if it undergoes structural changes upon exposure to artificial membranes, and we have succeeded in its crystallization. Such studies may lead us to determine if the harpin-induced HR is mediated by direct insertion of harpin into plant membranes or by a specific interaction with a proteinaceous or nonproteinaceous receptor.

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