Effects on Promoter Activity of Base Substitutions in the *cis*-Acting Regulatory Element of HrpXo Regulons in *Xanthomonas oryzae* pv. *oryzae*

Seiji Tsuge,^{1*} Shinsaku Terashima,¹ Ayako Furutani,¹ Hirokazu Ochiai,² Takashi Oku,³ Kazunori Tsuno,⁴ Hisatoshi Kaku,² and Yasuyuki Kubo¹

Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto Prefectural University, Kyoto,¹ National Institute of Agrobiological Sciences, Tsukuba,² Laboratory of Molecular Plant Pathology, School of Bioresources, Hiroshima Prefectural University, Shobara,³ and Faculty of Agriculture, Miyazaki University, Miyazaki,⁴ Japan

Received 20 October 2004/Accepted 16 December 2004

In *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight of rice, HrpXo is known to be a transcriptional regulator for the hypersensitive response and pathogenicity (*hrp*) genes. Several HrpXo regulons are preceded by a consensus sequence (TTCGC-N₁₅-TTCGC), called the plant-inducible promoter (PIP) box, which is required for expression of the gene that follows. Thus, the PIP box can be an effective marker for screening HrpXo regulons from the genome database. It is not known, however, whether mutations in the PIP box cause a complete loss of promoter activity. In this study, we introduced base substitutions at each of the consensus nucleotides in the PIP box of the *hrpC* operon in *X. oryzae* pv. *oryzae*, and the promoter activity was examined by using a β -glucuronidase (GUS) reporter gene. Although the GUS activity was generally reduced by base substitutions, several mutated PIP boxes conferred considerable promoter activity. In several cases, even imperfect PIP boxes with two base substitutions retained 20% of the promoter activity found in the nonsubstituted PIP box. We screened HrpXo regulon candidates with an imperfect PIP box obtained from the genome database of *X. oryzae* pv. *oryzae* and found that at least two genes preceded by an imperfect PIP box with two base substitutions were actually expressed in an HrpXo-dependent manner. These results indicate that a base substitution in the PIP box is quite permissible for HrpXo-dependent expression and suggest that *X. oryzae* pv. *oryzae* may possess more HrpXo regulons than expected.

Many gram-negative, plant-pathogenic bacteria possess clustered hypersensitive response and pathogenicity (*hrp*) genes. These genes encode proteins involved in the type III secretion system that delivers effector proteins from the bacteria to plant cells. These proteins are required for pathogenesis in host plants and for triggering a hypersensitive response (HR) in nonhost plants (reviewed in references 1, 6, and 41).

The expression of *hrp* genes is highly regulated. These genes are induced only in plants or certain nutrient-poor synthetic media and are not induced in nutrient-rich complex media (3, 16, 20, 30, 31, 33, 40). There are two types of *hrp* regulatory systems in plant-pathogenic bacteria (1, 14). In group I systems, which are found in *Pseudomonas syringae*, *Erwinia* sp., and *Pantoea stewartii*, a member of the ECF family of alternative sigma factors, called HrpL, functions as the regulator for other *hrp* genes (21, 42, 43). On the other hand, in group II systems, which are found in *Xanthomonas* sp. and *Ralstonia solanacearum*, either the AraC-type transcriptional activator HrpX (*Xanthomonas* sp.) or HrpB (*R. solanacearum*) regulates expression of other *hrp* genes (13, 29, 39).

In xanthomonads, the *hrp* cluster comprises six *hrp* loci, *hrpA* to *hrpF*, which encode components of a type III secretion system, and all of these genes are required for full pathogenicity (5). Two regulatory genes that control expression of the

hrp genes have been identified in the genus *Xanthomonas*. The product of *hrpG*, which belongs to the OmpR family of two-component regulatory systems, activates the expression of *hrpA* and *hrpX* (37, 38), and HrpX controls expression of other *hrp* genes (*hrpB* to *hrpF*) along with some effector proteins (4, 19, 25, 39).

Many HrpX regulons in xanthomonads are preceded by a consensus sequence motif, called the plant-inducible promoter (PIP) box (TTCGC-N₁₅-TTCGC), in the promoter regions (10, 24, 26, 39). Also, in *R. solanacearum* a similar motif (TTCG-N₁₆-TTCG; called the *hrp*_{II} box by Cunnac et al. [7]) functions as the *cis*-acting regulatory element controlling expression of HrpB regulons, such as *hrp* genes and effector proteins (7). A homodimer of HrpX or HrpB is thought to directly bind the *cis*-acting regulatory elements, although no experimental evidence for this hypothesis has been obtained (6, 7). The PIP/*hrp*_{II} box can be an effective marker for screening HrpX and HrpB regulons from the entire genomic sequence database, and several of these regulons are predicted to be involved in the pathogenicity of xanthomonads and *R. solanacearum*, respectively (8, 27; H. Ochiai, Y. Inoue, M. Takeya, A. Sasaki, and H. Kaku, unpublished data). da Silva et al. (8) found 12 and 20 candidates for HrpX regulons in *Xanthomonas campestris* pv. *campestris* and *Xanthomonas axonopodis* pv. *citri*, respectively, which did not include the genes in *hrp* clusters, and 95 candidates were found in *R. solanacearum* (7). However, genes with an imperfect PIP box, such as TTCGC-N₈-TTCGC for *hrpF*, and genes without a PIP box

* Corresponding author. Mailing address: Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto Prefectural University, Kyoto 606-8522, Japan. Phone: 81-75-703-5614. Fax: 81-75-703-5614. E-mail: s_tsuge@love.kpu.ac.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference(s) or source
<i>X. oryzae</i> pv. <i>oryzae</i> strains		
T7174R	Spontaneous rifampicin-resistant mutant derived from T7174	9, 36
74ΔHrpXo	<i>hrpXo</i> mutant	32
<i>E. coli</i> DH5αMCR	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsd RMS-mcrBC</i>) <i>recA</i> φ80 <i>dlacZ</i> ΔM15	Gibco-BRL
Plasmids		
pBluescript II SK(+)	Plasmid vector, Am ^r	Stratagene
pUC118	Plasmid vector, Am ^r	Takara
pHM1	Broad-host-range cosmid vector, pSa <i>ori</i> , Sp ^r	17
pBSGUS	<i>gus</i> gene cloned in pBluescript II KS(+)	33
pUCGUS	<i>gus</i> gene cloned in pUC118	This study
pHMPiPGUS2	Promoterless <i>gus</i> gene preceded by 5' end of <i>hrcU</i> and promoter region cloned in pHM1	33
pHMPiPGUS7G10G	PIP box in pHMPiPGUS2 replaced with TTCGC-N ₁₅ -TGCGG	This study
pHMPiPGUS9T10G	PIP box in pHMPiPGUS2 replaced with TTCGC-N ₁₅ -TTCTG	This study
pBSPIPORF1	<i>orf1</i> with imperfect PIP box cloned in pBluescript II SK(+)	This study
pHMPiPORF1	<i>orf1</i> with imperfect PIP box cloned in pHM1	This study
pHMPiPORF1M1	<i>orf1</i> with base-substituted imperfect PIP box TTAGC-N ₁₅ -TGCGG cloned in pHM1	This study
pHMPiPORF1M2	<i>orf1</i> with base-substituted imperfect PIP box TTCGC-N ₁₅ -TGAGG cloned in pHM1	This study
pBSPIPORF2	<i>orf2</i> with imperfect PIP box cloned in pBluescript II SK(+)	This study
pHMPiPORF2	<i>orf2</i> with imperfect PIP box cloned in pHM1	This study
pHMPiPORF2M1	<i>orf2</i> with base-substituted imperfect PIP box TTAGC-N ₁₅ -TTCTG cloned in pHM1	This study
pHMPiPORF1M2	<i>orf2</i> with base-substituted imperfect PIP box TTCGC-N ₁₅ -TTATG cloned in pHM1	This study

have been found to be expressed in an HrpX-dependent manner (23; Terashima, unpublished data)

Xanthomonas oryzae pv. *oryzae* is the causal agent of bacterial leaf blight of rice (34). Like other xanthomonads, *X. oryzae* pv. *oryzae* possesses an *hrp* gene cluster, which is essential for pathogenicity on susceptible rice cultivars and induction of an HR on resistant rice cultivars and nonhost plants and is controlled by HrpG and HrpXo (*X. oryzae* pv. *oryzae* HrpX) (19, 26, 25, 33; Ochiai et al., unpublished; Tsuge, unpublished data). In addition, some reports have shown that effector proteins are secreted via the type III secretion system in this bacterium (11, 12, 44).

Recently, the whole genome sequence of *X. oryzae* pv. *oryzae* has been determined (Ochiai et al., unpublished). Prior to screening for HrpXo regulons preceded by a PIP box in the genome database, it is important to examine whether a sequence in a PIP box is absolutely required for expression of the following gene or whether some base substitutions in the sequence are permissible for a functional level of the activity.

Here, we constructed plasmids harboring an *hrcU*-β-glucuronidase (GUS) gene fusion (*hrcU::gus*) preceded by a base-substituted PIP box and showed that some base substitutions in the consensus sequence of the PIP box (two TTCGC sequences) do not result in a complete loss of promoter activity. Two genes preceded by the imperfect PIP box (TTCGC-N₁₅-TTCTG or TTCGC-N₁₅-TGCGG) are actually expressed in an HrpXo-dependent manner in *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. A spontaneous rifampin-resistant mutant derived from *X. oryzae* pv. *oryzae* strain T7174, T7174R, has the same pathogenicity as the parental strain and was used as the wild type in this study (9, 36). Mutant 74ΔHrpXo is an *hrpXo*-deficient mutant derived from T7174R (32). Strains of *X. oryzae* pv. *oryzae* were cultured at 28°C on the nutrient-rich non-*hrp*-inducing medium NBY (35) or on nutrient-poor *hrp*-inducing medium XOM2 (33). *Escherichia coli* DH5αMCR was grown at 37°C in Luria-Bertani medium or LM agar (28). Media were supplemented with antibiotics at the following concentrations:

rifampin, 20 μg/ml; ampicillin, 50 μg/ml; cycloheximide, 50 μg/ml; kanamycin, 25 μg/ml for *X. oryzae* pv. *oryzae* and 50 μg/ml for *E. coli*; and spectinomycin, 25 μg/ml for *X. oryzae* pv. *oryzae* and 50 μg/ml for *E. coli*.

Plasmid pHMPiPGUS2 contains a promoterless β-glucuronidase gene (*gus*) fused to the 5' coding region of *X. oryzae* pv. *oryzae* *hrcU* (the first gene of the *hrpC* operon) and the preceding promoter region (positions -188 to 89; position 1 is the A of the *hrcU* start codon) in the broad-host-range and low-copy-number vector pHM1 (17, 33).

Base substitutions in the PIP box of the *hrpC* promoter region. Base substitutions in the PIP box of the *hrpC* promoter region were generated by a recombinant PCR method (15). An approximately 300-bp EcoRI-BamHI fragment containing the PIP box of *hrpC* was excised from pHMPiPGUS2 and cloned into pBluescript II SK(+) (Stratagene, La Jolla, Calif.). By using the plasmid as a template, PCRs were performed with KOD-Plus polymerase (TOYOBO, Osaka, Japan) used according to the manufacturer's instructions and the following two sets of primers: (i) T3 primer and the sense primer corresponding to the PIP box with a base substitution (for base substitutions in the first TTCGC sequence, 5'-CAAATCATTCGGCTTCGCCAGGCCATCC-3'; for base substitutions in the second TTCGC sequence, 5'-CAGGCCATCCACACATTCGCCTGGAAAACG-3'; underlined nucleotides were base substituted) and (ii) T7 primer and the antisense primer corresponding to the reverse of the sense primer (for base substitutions in the first TTCGC sequence, 5'-GGATGGCCTGGCAAGCCGGAATGATTTG-3'; for base substitutions in the second TTCGC sequence, 5'-CGTTTTCCAGGCGAATGTGTGGATGGCCTG-3'). Then two amplified fragments were purified by agarose gel electrophoresis and annealed with each other; this was followed by a DNA extension reaction with KOD-Plus. By using the product as a template, a second PCR was performed with the T3 and T7 primers. The PCR product with a base substitution in the PIP box was digested with EcoRI and BamHI, and the corresponding region in pHMPiPGUS2 was substituted.

Assay of GUS activity. GUS activity was assayed as described previously (18, 33).

Reverse transcription PCR (RT-PCR). The total RNA of bacteria cultured in XOM2 for 1 day was extracted with an RNeasy kit (QIAGEN, Valencia, Calif.). cDNA synthesis and PCR were performed with RiverTra-Ace reverse transcriptase (TOYOBO) and KOD Dash polymerase (TOYOBO), respectively, used according to the manufacturer's instructions.

Plasmid construction. A BamHI-EcoRI fragment containing a *gus* gene from pBSGUS (33) was cloned between BamHI and SalI sites of pUC118 to generate pUCGUS. An approximately 800-bp BglII (filled-in)-EcoRI fragment containing 400 bp of the 5' coding region of *orf1* and 400 bp of the promoter region were ligated with pUCGUS treated with BamHI, T4 DNA polymerase, and EcoRI. The plasmid obtained, designated pBSPIPORF1, was digested with EcoRI and HindIII and cloned into the broad-host-range vector pHM1 to generate pHMPi-

FIG. 1. Relative promoter activities of imperfect PIP boxes with base substitutions in the first (A) and second (B) TTCGC sequences. Each nucleotide of the consensus sequences of the PIP box (TTCGC sequences) in pHMIPGUS2 was base substituted. Transformants with each of the plasmids were incubated in XOM2 for 15 h, and the GUS activity was measured. The values are activities relative to the activity with the wild-type PIP box and are averages and standard deviations for five independent experiments.

PORF1. An approximately 600-bp NotI-EcoRI fragment containing 400 bp of the 5' coding region of *orf2* and 200 bp of the promoter region were cloned into pBluescript II SK(+), and a 600-bp EcoRI (filled-in)-KpnI fragment was excised from the plasmid and ligated with pBSGUS treated with SmaI and KpnI to obtain pBSPIPORF2. A NotI-HindIII fragment containing the *orf2* promoter region and the *orf2::gus* fusion was inserted into pHM1, generating pHMIPORF2. For base substitutions in the PIP box upstream of *orf1* and *orf2*, recombinant PCR were performed with pBSPIPORF1 and pBSPIPORF2, respectively, and this was followed by cloning of the regions containing the promoter and the *gus* gene fusion into pHM1. The plasmids were introduced into *X. oryzae* pv. *oryzae* by electroporation as described previously (32).

Isolation of an *orf1* mutant and an *orf2* mutant of *X. oryzae* pv. *oryzae* T7174R. From the genomic library of T7174R, plasmids harboring an approximately 8.5-kb fragment containing *orf1* and an 8.2-kb fragment containing *orf2* were selected, and transpositions with each of the plasmids and transposon EZ::TN <KAN-2> (Epicentre, Madison, Wis.) were performed according to the manufacturer's instructions. Clones with a transposon at position 57 of *orf1* (position 1 was A of the initiation codon) and at position 157 of *orf2* were screened by restriction analysis followed by sequence analysis. By using the plasmids, marker exchange mutagenesis of *X. oryzae* pv. *oryzae* T7174R was performed as described previously (32).

Pathogenicity test. Bacteria grown on NBY agar for 2 days at 28°C were suspended in distilled water to a concentration equivalent to an A_{600} of 0.3. Pathogenicity tests with rice plants (*Oryza sativa* cv. IR24) were performed by the clipping method (22). Lesion length was measured 2 weeks after inoculation. The ability to induce an HR in tomato (*Lycopersicon esculentum* cv. Momotaro) was tested by infiltration of bacterial suspensions into the leaf parenchyma.

RESULTS

Effects of a base substitution in the PIP box on promoter activity. The plasmid harboring the promoter regions of the *hrpC* operon followed by an *hrcU::gus* gene fusion was introduced into *X. oryzae* pv. *oryzae* wild-type strain T7174R and 74ΔHrpXo, which is deficient in the *hrp* regulatory gene *hrpXo*. Each transformant was incubated in the *hrp*-inducing medium XOM2 for 15 h, and then GUS activity was measured, revealing the *hrpXo*-dependent expression of HrcU::GUS, as described previously (33) (data not shown). We introduced a base substitution into each of the nucleotides in the consensus sequences (TTCGC sequences) in pHMIPGUS2 and transformed T7174R with each of the plasmids. All transformants had reduced GUS activity after 15 h of incubation in XOM2 compared with the strain with the plasmid containing the perfect PIP box (Fig. 1). The effects of a base substitution on

promoter activity depended not only on the location of the substituted nucleotide but also on the base used after substitution, and several base-substituted PIP boxes retained considerable promoter activity. In particular, 50 to 70% the wild-type activity was observed when the last nucleotide of each TTCGC was changed to G or T. Interestingly, the base substitution resulting in TTCGA for each TTCGC resulted in less promoter activity. Twenty-five to 35% activity was found in the case of TTCGC-N₁₅-TGCGC, as well as TTCGC-N₁₅-TTCTC and TTCGC-N₁₅-ATCGC, but little activity was found in the case of TTCGC-N₁₅-T(A/C)CGC, although the same nucleotides were substituted (base substitutions are underlined). Generally, the effects of a base substitution in the first TTCGC sequence on the promoter activity were greater than the effects of a base substitution in the second sequence. Little GUS activity was observed when the transformants were incubated in non-*hrp*-inducing medium NBY or when each plasmid was introduced into 74ΔHrpXo (data not shown).

Effects of two base substitutions in the PIP box on the promoter activity. We constructed plasmids containing imperfect PIP boxes with two base substitutions, each of which did not result in a complete loss in promoter activity, and transformed T7174R with the plasmids. The GUS activities of transformants with two base substitutions was much lower than the GUS activities of transformants with only one of the substitutions (Fig. 2). However, several substituted PIP boxes, such as TTCGC-N₁₅-TGCGG (19.6% activity compared to the perfect PIP box), TTCGC-N₁₅-TGCGT (19.3%), TTCGC-N₁₅-TTCTG (21.2%), TTCGC-N₁₅-TTCTT (22.3%), TTCGC-N₁₅-TTCGG (22.1%), and TTCGC-N₁₅-TTCGT (34.2%), exhibited considerable promoter activity. Little GUS activity was detected when the transformants were incubated in NBY or when each plasmid was introduced into 74ΔHrpXo (data not shown). The results suggest that even genes preceded by an imperfect PIP box with certain one- or two-base substitutions might be expressed in an HrpXo-dependent manner.

HrpXo-dependent expression of ORFs preceded by a base-substituted PIP box. Our results showed that some base substitutions in the PIP box of the *X. oryzae* pv. *oryzae* *hrpC*

FIG. 2. Relative promoter activities of imperfect PIP boxes with two base substitutions. Two nucleotides (indicated by X at the top and by underlining at the bottom) of the consensus sequences of the PIP box (TTCGC sequences) in pHMPIPUS2 were base substituted. Transformants with each of the plasmids were incubated in XOM2 for 15 h, and the GUS activity was measured. The values are activities relative to the activity with the wild-type PIP box. For comparison, the activity of the imperfect PIP box with each of two base substitutions is indicated. The data are averages and standard deviations for five independent experiments.

operon did not necessarily result in loss of promoter activity. We also screened open reading frames (ORFs) preceded by imperfect PIP boxes with one or two base substitutions, which were predicted to have more than ca. 20% of the promoter activity of the perfect PIP box according to the data shown in Fig. 1 and 2, from the genome database of *X. oryzae* pv. *oryzae*. More than 250 ORFs that had an imperfect PIP box in a region extending from 50 to 500 bp upstream of the start codon of each ORF were found. We selected 12 ORFs with TTCGC-N₁₅-ATCGG, TTCGC-N₁₅-ATCGT, TTCGC-N₁₅-TGCGG, TTCGC-N₁₅-TGCGT, TTCGC-N₁₅-TTCTG or TTCGC-N₁₅-TTCTT (two ORFs for each imperfect PIP box) and examined the HrpXo-dependent expression of these ORFs. Strain T7174R and the HrpXo mutant 74ΔHrpXo were incubated in XOM2, and total RNA was extracted; this was followed by RT-PCR analysis with a set of primers specific for each ORF. Although HrpXo-dependent expression was not confirmed for most of the variants (some of the ORFs were expressed even in 74ΔHrpXo, and in other cases no specific fragment was amplified even in T7174R), at least two ORFs preceded by TTCGC-N₁₅-TGCGG and TTCGC-N₁₅-TTCTG (*orf1* and *orf2*, respectively) were found to be expressed in the wild type but not in the HrpXo mutant (Fig. 3). These ORFs corresponded to XOO0804 and XOO3803, respectively, in the genomic database of *X. oryzae* pv. *oryzae* (Ochiai et al., unpublished). A search with a BLAST program (2) revealed that XOO0804 and XOO3803 are homologous to XAC1124 (88%) and XAC0601 (91%) in *X. axonopodis* pv. *citri* (8), but they have no homology with known protein genes.

To confirm HrpXo-dependent expression of *orf1* and *orf2* preceded by TTCGC-N₁₅-TGCGG and TTCGC-N₁₅-TTCTG, respectively, we constructed plasmids harboring a promoter-less *gus* gene fused with the N-terminal coding region of each ORF preceded by the promoter region containing an imperfect PIP box. Plasmid pHMPIPORF1, harboring *orf1::gus*, was introduced into T7174R and 74ΔHrpXo. T7174R(pHMPIPORF1) transformants incubated in XOM2 for 15 h had very

high GUS activity that was higher than that of T7174R transformed with pHMPIPUS7G10G, which contained an *hrcU::gus* gene fusion preceded by the original promoter region, except that the PIP box was replaced with TTCGC-N₁₅-TGCGG (Table 2). On the other hand, 74ΔHrpXo(pHMPIPORF1) had little GUS activity. T7174R(pHMPIPORF1) incubated in the non-*hrp*-inducing medium NBY also had little GUS activity (data not shown). We introduced another base substitution in TTCGC and TGCGG into the imperfect PIP boxes. T7174R transformed with each of the resulting plasmids, pHMPIPORF1M1 and pHMPIPORF1M2, containing TTAGC-N₁₅-TGCGG and TTCGC-N₁₅-TGAGG, respectively, showed dramatic decreases in GUS activity (Fig. 4).

Transformant T7174R(pHMPIPORF2) also had high GUS activity only when it was incubated in XOM2 (Table 2). Surprisingly, the activity was higher than even that of T7174R transformed with pHMPIPUS2 harboring an *hrcU::gus* gene fusion controlled by the perfect PIP box. Similar to expression of *orf1*, 74ΔHrpXo(pHMPIPORF2) and T7174R transformed with plasmids (pHMPIPORF2M1 and pHMPIPORF2M2) that harbor an additional base-substituted PIP box (TTAGC-N₁₅-TTCTG and TTCGC-N₁₅-TTATG, respectively) had less GUS activity than T7174R(pHMPIPORF2) (Table 2 and Fig. 4).

FIG. 3. RT-PCR analysis of transcriptional regulation of *orf1* (upper gel) and *orf2* (lower gel) by HrpXo. The wild type (lanes 2 and 4) or 74ΔHrpXo (lanes 3 and 5) was incubated in XOM2, and RT-PCR (lanes 2 and 3) or PCR (lanes 4 and 5) was performed by using total RNA as a template. The PCR product obtained by using genomic DNA of T7174R (100 ng) as a template was used as a size marker (lane 1). The arrows indicate the locations of specific amplified fragments. The other bands are nonspecific.

TABLE 2. Relative GUS activities of transformants with plasmids harboring an *orf1::gus* or *orf2::gus* gene fusion

Strain	Relative GUS activity (%) ^a
T7174R(pHMPiPGUS7G10G).....	19.6 ± 6.0
T7174R(pHMPiPORF1).....	49.5 ± 10.4
74ΔHrpXo(pHMPiPORF1).....	1.9 ± 1.5
T7174R(pHMPiPGUS9T10G).....	21.2 ± 5.9
T7174R(pHMPiPORF2).....	226.8 ± 38.8
74ΔHrpXo(pHMPiPORF2).....	3.7 ± 0.3

^a The values are GUS activities expressed relative to the GUS activity of T7174R(pHMPiPGUS2) and are averages ± standard deviations for five independent experiments.

Virulence and HR-inducing ability of *orf1* and *orf2* mutants.

We generated mutants that had a kanamycin resistance gene inserted into *orf1* and *orf2* by marker exchange mutagenesis. Rice cultivar IR24, which is susceptible to parental strain T7174R, was inoculated with the mutants. The lesions formed by the mutants were similar to those formed by the parental strain (data not shown). Also, there were no difference in induction of an HR on tomato between the mutants and the wild type.

DISCUSSION

Several previous reports showed that the *hrp* HrpX regulatory proteins in xanthomonads and the corresponding protein of *R. solanacearum*, HrpB, regulate not only *hrp* genes but also pathogenicity-related genes other than *hrp* (6, 7). These proteins directly or indirectly interact with the *cis*-acting regulatory element called the plant-inducible promoter box (Cunnac et al. [7] designated the motif of HrpB regulons of *R. solanacearum* the *hrp_{II}* box). Recently, the whole genome sequences of several plant-pathogenic bacteria, including *X. oryzae* pv. *oryzae*, *X. campestris* pv. *campestris*, *X. axonopodis*

pv. *citri*, and *R. solanacearum*, have been determined (8, 27; Ochiai et al., unpublished). Thus, screening candidates for HrpX/HrpB regulons is now possible. For screening for regulon candidates, it is important to know whether the sequence of the PIP box is absolutely necessary or if the gene can still be expressed after mutations in the PIP box.

In this study, we investigated the relationships between base substitutions in the consensus sequences (TTCGC sequences) and the promoter activities and showed that considerable activities were observed for several imperfect PIP boxes with one or two base substitutions. The conclusions of our study are as follows. (i) Generally, base substitutions in the TTCGC sequences resulted in highly reduced promoter activities, although in some cases considerable activity remained. (ii) The last nucleotide of each TTCGC can be changed to G or T with retention of more than 50% of the wild-type promoter activity. These results correspond to those of other researchers who found a TTCG-N₁₆-TTCG PIP box. It is very interesting, however, that substitution of A for the last nucleotide (C) led to less promoter activity. Cunnac et al. (7) also reported decreased promoter activity after substitution with A in these nucleotides in the *hrp_{II}* box of *hrpY* of *R. solanacearum*. (iii) Generally, base substitutions in the first TTCGC may affect promoter activity more than base substitutions in the second TTCGC. (iv) Two base substitutions in the TTCGC sequences may multiply the effect of the individual substitutions, but some imperfect PIP boxes with two base substitutions may function.

We proved that expression of at least two genes (*orf1* and *orf2*) preceded by an imperfect PIP box with two base substitutions is actually HrpXo dependent by RT-PCR and by using constructs harboring *gus* gene fusions with *orf1* and *orf2*, respectively. An additional base substitution in each of the imperfect PIP boxes resulted in loss of the promoter activities, revealing that the boxes actually function as the *cis*-acting regulatory element.

Cunnac et al. (7) compared the promoter regions of HrpB regulons in *R. solanacearum* and found conservation of the distance between the PIP box and the -10 box, which resembled the -10 binding element of the RNA polymerase σ^{70} factor. We compared the flanking regions of the imperfect PIP boxes of *orf1* and *orf2* with those of *hpa1*, *hrpB*, *hrpC*, and *hrpD* in *X. oryzae* pv. *oryzae* and those of the *popABC* and *hrpY* operons in *R. solanacearum* and found that the distance between the PIP box and the -10 box-like sequence was well conserved not only upstream of *hpa1*, *hrpB*, *hrpC*, and *hrpD* but also upstream of *orf1* and *orf2* (Fig. 5). The findings suggest that in addition to the presence of the PIP box, the distance between the PIP box and the -10 box is important in *X. oryzae* pv. *oryzae* and that transcription of HrpX/HrpB regulons is likely to be regulated similarly, although the transcription start site of *orf1* and *orf2* could not be determined in this study.

It seems that some *cis*-acting elements other than the PIP box and the -10 box remain to be clarified. Cunnac et al. (7) also showed the importance of the sequences and the distance between TTCG sequences for the activity of *R. solanacearum* *hrpY*, although no consensus motif was found. It is very interesting that the promoter activities of the imperfect PIP boxes of *orf1* and *orf2*, as demonstrated by GUS activity, were found to be quite high, although they had base substitutions at two

FIG. 4. Effects of base substitutions in the imperfect PIP boxes preceding *orf1* (A) and *orf2* (B) on promoter activity. Each imperfect PIP box preceding *orf1* and *orf2* in pHMPiPORF1 and pHMPiPORF2, respectively, was further base substituted to obtain pHMPiPORF1M1 (TTAGC-N₁₅-TGCGG), pHMPiPORF1M2 (TTCGC-N₁₅-TGAGG), pHMPiPORF2M1 (TTAGC-N₁₅-TTCTG), and pHMPiPORF2M2 (TTCGC-N₁₅-TTATG). Transformants with each plasmid were incubated in XOM2, and the GUS activity was measured. The values are activities relative to the activity with the wild-type PIP box and are averages and standard deviations for five independent experiments.

orf1	TTTCGGTTTAAACGCGACCGGT	TTGGGGGCCAGTACGT	CAGACCAGCCGCCCGCA	--GGTAAATTCGGGGCAT
orf2	TTTCGGCAAATCGCACATCGA	TTTCGCTGTGAGCGGTATG	CAGGTTCTGGCTGC	--CTTACATTTTCAGCTC
hpa1	TTTCGGCGGTACAAGCGCAAT	TTTCGCAAAATTTCTGGCGAT	GATGGGCTTCCGCT	-TCTACTGTTTGTATCGG
hrpB	TTTCGGCAGCGACTTCCGAT	TTTCGCTTCCGGAAGGATCT	GACCGCGTGGCGCG	-CGTAGCTTCCCCCGCA
hrpC	TTTCGGCAGGCCATCCACACA	TTTCGCTTGGAAAACGAAT	CGCTCGACCCAGCTC	-CCACAAATCGGGGGCG
hrpD	TTTCGGCACACCGCTATCGC	TTTCGCTTCAATACCAGT	TCGCCAGACGCGCGCA	-GGTACTTTGGTGTGGA
hrpY	TTTCGTACGCTTGACACAAGG	TTTCGGGGCAGCGCGCAT	GTGTGCAACGGACTTATCC	CATCAT-AAAACCA
popABC	TTTCGGGTGCATGACACGAT	TTTCGGCATGGCAGGGGCCC	CGACGCCAACACTCTCT	TAAGGTTCAGCGA
Consensus	TTTCGC	TTTCGC		YANNRT

FIG. 5. Sequence alignment for the promoter regions of *orf1*, *orf2*, *hrpB*, *hrpC*, and *hrpD* of *X. oryzae* pv. *oryzae* and *hrpY* and *popABC* of *R. solanacearum*. The open boxes indicate the PIP box and the -10 box. The underlined final nucleotides of *hrpY* and *popABC* are the transcription start determined by Cunnac et al. (7).

nucleotides. In particular, the activity in the transformant harboring *orf2::gus*, which was preceded by TTTCGC-N₁₅-TTCTG, was higher than even the activity observed with *hrcU::gus* with a perfect PIP box. The results suggest that an unknown factor(s) might be involved in efficient expression of the following gene and that even genes with imperfect PIP boxes could be efficiently expressed by addition of other factors.

In this study, we investigated effects of base substitutions in the TTTCGC sequences of the *hrpC* promoter regions on the promoter activity. Different results might be obtained for other genes with a PIP box. However, our results revealed that not only genes with a perfect PIP box but also genes with a base-substituted imperfect PIP box could be expressed in an HrpXo-dependent manner. The results suggest that more HrpXo regulons than expected might be scattered in the genome of *X. oryzae* pv. *oryzae* and that several of them are involved in pathogenicity of the bacterium. We found more than 250 HrpXo regulon candidates with perfect or imperfect PIP boxes that were thought to have more than 20% of the promoter activity of the perfect PIP box, according to the data obtained after base substitution in the *hrpC* PIP box. Many of them may be pseudogenes or may be expressed in an HrpXo-independent manner because RT-PCR analysis showed that only 2 of the 12 candidate genes which we tested (*orf1* and *orf2*) were expressed in an HrpXo-dependent manner. For other candidate genes, an amplified signal was found even in the HrpXo mutant, or no signal was found even in the wild-type strain, which might have been due to technical problems.

Some of the HrpX regulons have been found to not have a PIP box (23; Terashima, unpublished). It is likely that expression of these genes is regulated by a protein(s) that is preceded by a perfect or imperfect PIP box and that is expressed in an HrpX-dependent manner, although we found no ORF homologous to a known regulatory gene in the HrpXo regulon candidates.

ACKNOWLEDGMENT

This work was supported by Grant-in-Aid for Scientific Research B 13460024 from the Ministry of Education, Science, Sports and Culture, Japan.

REFERENCES

- Alfano, J. R., and A. Collmer. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. *J. Bacteriol.* **179**:5655–5662.
- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Arlat, M., C. Gough, C. E. Barber, C. Boucher, and M. Daniels. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. *Mol. Plant-Microbe Interact.* **4**:593–601.
- Astua-Monge, G., G. V. Minsavage, R. E. Stall, M. J. Davis, U. Bonas, and J. B. Jones. 2000. Resistance of tomato and pepper to T3 strains of *Xanthomonas campestris* pv. *vesicatoria* is specified by a plant-inducible avirulence gene. *Mol. Plant-Microbe Interact.* **13**:911–921.
- Bonas, U., R. Schulte, S. Fenselau, G. V. Minsavage, B. J. Staskawicz, and R. E. Stall. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. *Mol. Plant-Microbe Interact.* **4**:81–88.
- Büttner, D., and U. Bonas. 2002. Getting across—bacterial type III effector proteins on their way to the plant cell. *EMBO J.* **21**:5313–5322.
- Cunnac, S., C. Boucher, and S. Genin. 2004. Characterization of the *cis*-acting regulatory element controlling HrpB-mediated activation of the type III secretion system and effector genes in *Ralstonia solanacearum*. *J. Bacteriol.* **186**:2309–2318.
- da Silva, A. C. R., J. A. Ferro, F. C. Reinach, C. S. Farah, L. R. Furlan, R. B. Quaggio, C. B. Monteiro-Vitorello, M. A. Van Sluys, N. F. Almeida, Jr., L. M. C. Alves, A. M. do Amaral, M. C. Bertolini, L. E. A. Camargo, G. Camarotte, F. Cannavan, J. Cardozo, F. Chamberg, L. P. Ciapina, R. M. B. Cicarelli, L. L. Coutinho, J. R. Cursino-Santos, H. El-Dorri, J. B. Faria, A. J. S. Ferreira, R. C. C. Ferreira, M. I. T. Ferro, E. F. Formighieri, M. C. Franco, C. C. Greggio, A. Gruber, A. M. Katsuyama, L. T. Kishi, R. P. Leite, Jr., E. G. M. Lemos, M. V. F. Lemos, E. C. Locali, M. A. Machado, A. M. B. N. Madeira, N. M. Martinez-Rossi, E. C. Martins, J. Meidanis, C. F. M. Menck, C. Y. Miyaki, D. H. Moon, L. M. Moreira, M. T. M. Novo, V. K. Okura, M. C. Oliveira, V. R. Oliveira, H. A. Pereira, Jr., A. Rossi, J. A. D. Sena, C. Silva, R. F. de Souza, L. A. F. Spinola, M. A. Takita, R. E. Tamura, E. C. Teixeira, R. I. D. Tezza, M. Trindade dos Santos, D. Truffi, S. M. Tsai, F. F. White, J. C. Setubal, and J. P. Kitajima. 2002. Comparison of the genomes of two *Xanthomonas* pathogens with differing host specificities. *Nature* **417**:459–463.
- Ezuka, A., and O. Horino. 1974. Classification of rice varieties and *Xanthomonas oryzae* strains on the basis of their differential interactions. *Bull. Tokai Kinki Natl. Agric. Exp. Stn.* **27**:1–19.
- Fenselau, S., and U. Bonas. 1995. Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa and Fli secretion systems. *Mol. Plant-Microbe Interact.* **8**:845–854.
- Furutani, A., S. Tsuge, T. Oku, K. Tsuno, Y. Inoue, H. Ochiai, H. Kaku, H., and Y. Kubo. 2003. HpaI secretion via type III secretion system in *Xanthomonas oryzae* pv. *oryzae*. *J. Gen. Plant Pathol.* **69**:271–278.
- Furutani, A., S. Tsuge, K. Ohnishi, Y. Hikichi, T. Oku, K. Tsuno, Y. Inoue, H. Ochiai, H. Kaku, and Y. Kubo. 2004. Evidence for HrpXo-dependent expression of type II secretory proteins in *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* **186**:1374–1380.
- Genin, S., C. L. Gough, C. Zischek, and C. A. Boucher. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. *Mol. Microbiol.* **6**:3065–3076.
- Gophna, U., E. Z. Ron, and D. Graur. 2003. Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. *Gene* **312**:151–163.
- Higuchi, R. 1989. Using PCR to engineer DNA, p. 61–70. In H. A. Erlich (ed.), *PCR technology: principles and applications for DNA amplification*. M. Stock Press, Inc., New York, N.Y.
- Huang, H., S. W. Hutcheson, and A. Collmer. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and *TnphoA* tagging of genes encoding export of membrane spanning Hrp proteins. *Mol. Plant-Microbe Interact.* **4**:469–476.
- Innes, R. W., M. A. Hirose, and P. L. Kuempel. 1988. Induction of nitrogen-fixing nodules on clover requires only 32 kilobase pairs of DNA from the *Rhizobium trifolii* symbiosis plasmid. *J. Bacteriol.* **170**:3793–3802.
- Jefferson, R. A., T. A. Kavanagh, and M. W. Bevan. 1987. GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**:3901–3907.
- Kamdar, H. V., S. Kamoun, and C. I. Kado. 1993. Restoration of pathogenicity of avirulent *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* patho-

- vars by reciprocal complementation with the *hrpXo* and *hrpXc* genes and identification of HrpX function by sequence analyses. *J. Bacteriol.* **175**:2017–2025.
20. Marena, M., B. Brito, D. Callard, S. Genin, P. Barberis, C. Boucher, and M. Arlat. 1998. PrhA controls a novel regulatory pathway required for the specific induction of *Ralstonia solanacearum* *hrp* genes in the presence of plant cells. *Mol. Microbiol.* **27**:437–453.
 21. Merighi, M., D. R. Majerczak, E. H. Stover, and D. L. Coplin. 2003. The HrpX/HrpY two-component system activates *hrpS* expression, the first step in the regulatory cascade controlling the Hrp regulon in *Pantoea stewartii* subsp. *stewartii*. *Mol. Plant-Microbe Interact.* **16**:238–248.
 22. Morinaka, T. H., H. Kaku, M. Hori, and T. Kumura. 1978. Applying conditions of the clipping inoculation technique for evaluation of resistance of rice to *Xanthomonas oryzae*. *Bull. Chugoku Agric. Exp. Stn. Ser. E* **13**:1–16.
 23. Noël, L., F. Thieme, D. Ninnstiel, and U. Bonas. 2001. cDNA-AFLP analysis unravels a genome-wide *hrpG*-regulon in the plant pathogen *Xanthomonas campestris* pv. *vesicatoria*. *Mol. Microbiol.* **41**:1271–1281.
 24. Noël, L., F. Thieme, D. Nennstiel, and U. Bonas. 2002. Two novel type III system-secreted proteins of *Xanthomonas campestris* pv. *vesicatoria* are encoded within the *hrp* pathogenicity island. *J. Bacteriol.* **184**:1340–1348.
 25. Oku, T., A. M. Alvarez, and C. I. Kado. 1995. Conservation of the hypersensitivity-pathogenicity regulatory gene *hrpX* of *Xanthomonas campestris* and *X. oryzae*. *DNA Seq.* **5**:245–249.
 26. Oku, T., K. Tanaka, M. Iwamoto, Y. Inoue, H. Ochiai, H. Kaku, S. Tsuge, and K. Tsuno. 2004. Structural conservation of *hrp* gene cluster in *Xanthomonas oryzae* pv. *oryzae*. *J. Gen. Plant Pathol.* **70**:159–167.
 27. Salanoubat, M., S. Genin, F. Artiguenave, J. Gouzy, S. Mangenot, M. Arlat, A. Billault, P. Brottier, J. C. Camus, L. Cattolico, M. Chandler, N. Choisne, C. Claudel-Renard, S. Cunnac, N. Demange, C. Gaspin, M. Lavie, A. Moisan, C. Robert, C. W. Saurin, T. Schiex, P. Sigui, P. Thebault, M. Whalen, P. Wincker, M. Levy, J. Weissenbach, and C. A. Boucher. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* **415**:497–502.
 28. Sambrook, J., F. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 29. Schell, M. A. 2000. Regulation of virulence and pathogenicity genes in *Ralstonia solanacearum* by a complex network. *Annu. Rev. Phytopathol.* **38**:263–292.
 30. Schulte, R., and U. Bonas. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria* *hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J. Bacteriol.* **174**:815–823.
 31. Schulte, R., and U. Bonas. 1992. A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acid. *Plant Cell* **1992**:79–86.
 32. Tsuge, S., A. Furutani, R. Fukunaka, Y. Kubo, and O. Horino. 2001. Growth complementation of *hrpXo* mutants of *Xanthomonas oryzae* pv. *oryzae* by virulent strains in rice cultivars resistant and susceptible to the parental strain. *J. Gen. Plant Pathol.* **67**:51–57.
 33. Tsuge, T., A. Furutani, R. Fukunaka, T. Oku, K. Tsuno, H. Ochiai, Y. Inoue, H. Kaku, and Y. Kubo. 2002. Expression of *Xanthomonas oryzae* pv. *oryzae* *hrp* genes in XOM2, a novel synthetic medium. *J. Gen. Plant Pathol.* **68**:363–371.
 34. Vauterin, L., B. Hoste, K. Kersters, and Swing, J. 1995. Reclassification of *Xanthomonas*. *Int. J. Syst. Bacteriol.* **45**:472–489.
 35. Vidaver, A. K. 1967. Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: effect of the carbon source. *Appl. Microbiol.* **15**:1523–1524.
 36. Watabe, M., M. Yamaguchi, I. Furusawa, and O. Horino. 1993. Virulence, and bacterial multiplication and movement in rice leaves of *Xanthomonas campestris* pv. *oryzae* mutants impaired in productivity of extracellular polysaccharide. *Ann. Phytopathol. Soc. Jpn.* **59**:544–550.
 37. Wengelnik, K., G. Van den Ackerveken, and U. Bonas. 1996. HrpG, a key *hrp* regulatory protein of *Xanthomonas campestris* pv. *vesicatoria* is homologous to two-component response regulators. *Mol. Plant-Microbe Interact.* **9**:704–712.
 38. Wengelnik, K., O. Rossier, and U. Bonas. 1999. Mutations in the regulatory gene *hrpG* of *Xanthomonas campestris* pv. *vesicatoria* result in constitutive expression of all *hrp* genes. *J. Bacteriol.* **181**:6828–6831.
 39. Wengelnik, K., and U. Bonas. 1996. HrpXv, an AraC-type regulator, activates expression of five of the six loci in the *hrp* cluster of *Xanthomonas campestris* pv. *vesicatoria*. *J. Bacteriol.* **178**:3462–3469.
 40. Wengelnik, K., C. Marie, M. Russel, and U. Bonas. 1996. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. *J. Bacteriol.* **178**:1061–1069.
 41. Willis, D. K., J. J. Rich, and E. M. Hrabak. 1991. *hrp* genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* **4**:132–138.
 42. Xiao, Y., S. Heu, J. Yi, Y. Lu, and S. W. Hutcheson. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hmrA* genes. *J. Bacteriol.* **176**:1025–1036.
 43. Xiao, Y., and S. W. Hutcheson. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J. Bacteriol.* **176**:3089–3091.
 44. Zhu, W., M. M. Magbanua, and F. F. White. 2000. Identification of two novel *hrp*-associated genes in the *hrp* gene cluster of *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* **182**:1844–1853.