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

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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-TTCG), 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 hrc 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 Xanthomonas sp. and Pantoaea Stewartii, a member of the ECF family of alternative sigma factors, called HrpL, functions as the regulator for hrpF operon in Xanthomonas campestris pv. citri, respectively, which did not include the genes 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-TTCG), in the promoter regions (10, 24, 26, 39). Also, in R. solanacearum a similar motif (TTCG-N16-TTCG; called the hrpD 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 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 TGGC-N15-TTCG for hrpF, and genes without a PIP box...
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* (9, 36). Mutant *HrpXo* is an *hrp*-dependent manner in *X. oryzae*.

Recently, the whole genome sequence of *X. 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 gene (GUS) fused to the 5′ coding region of *X. oryzae* pv. *oryzae* hrcU (the first gene of the *hrc* 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 pPHM1 (17, 33). Base substitutions in the PIP box of the *hrcU* promoter region. Base substitutions in the PIP box of the *hrcU* promoter region were generated by a recombinant PCR method (15). An approximately 300-bp EcoRI-BamHI fragment containing the PIP box of *hrcU* was excised from pHMPlPGUS2 and cloned into blueScriptII vector pHM1. The PCR product was digested with EcoRI and BamHI, and the corresponding region in pHMPIPGUS2 was purified by agarose gel electrophoresis and annealed with each primer. The PCR product as a template, a second PCR was performed with the T3 and T7 primers; for base substitutions in the PIP box of the *hrcU* promoter region.

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. oryzae</em> pv. <em>oryzae</em> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T7174R</td>
<td>Spontaneous rifampicin-resistant mutant derived from T7174</td>
<td>9, 36</td>
</tr>
<tr>
<td>744HrpXo</td>
<td>hrcXo mutant</td>
<td>32</td>
</tr>
<tr>
<td>E. coli DH5αMCR</td>
<td>F′ mcrA Δ(mrr-hsd RMS-mcrBC) recA Δ808lacZΔM15</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>Plasmids</td>
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<td></td>
</tr>
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<td>Plasmid vector, Am′</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUC118</td>
<td>Plasmid vector, Am′</td>
<td>Takara</td>
</tr>
<tr>
<td>pHM1</td>
<td>Broad-host-range cosmid vector, pSa</td>
<td>17</td>
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<tr>
<td>pUCGUS</td>
<td>gus gene cloned in pBluescript II KS(+)</td>
<td>33</td>
</tr>
<tr>
<td>pHMPIPGUS9T10G</td>
<td>PIP box in pHMPIPGUS2 replaced with TTCGC-N15-TTCTG</td>
<td>This study</td>
</tr>
<tr>
<td>pHMPIPGUS2</td>
<td>Promoterless gus gene preceded by 5′ end of hrcU and promoter region cloned in pHM1</td>
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<td>pHMPIPORF1M2</td>
<td>orf2 with base-substituted imperfect PIP box cloned in pHM1</td>
<td>This study</td>
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**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. A spontaneous rifampicin-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 744HrpXo is an *hrp*-deficient mutant derived from T7174R (32). Strains of *X. oryzae* pv. *oryzae* were cultured at 28°C on the nutrient-rich non-β-glucuronidase gene (*gus*) gene fusion (*hrcU::gus*) preceded by a base-substituted imperfect PIP box cloned in pHM1 (Terashima, unpublished data).

**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 pBGUS (33) was cloned between BamHI and SalI sites of pUC18 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 pPSPIPORF1, was digested with EcoRI and HindIII and cloned into the broad-host-range vector pHM1 to generate pHMPI-
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 Smal and KpnI to obtain pBSPIPORF2. A NotI-HindIII fragment containing the orf2 promoter region and the orf2-gus fusion was inserted into pHM1, generating pHMPIP- PORF2. 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 (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$_{max}$ 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 74A HrpXo, 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 (TTGC sequences) in pHMPIP GUS2 and transformed T7174R with each of the plasmids. All transformers 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% of wild-type activity was observed when the last nucleotide of each TTGC 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 TTGC-N$_{15}$-TTCGC, as well as TTGC-N$_{15}$-TTCGGC and TTGC-N$_{15}$-ATCGG, but little activity was found in the case of TTGC-N$_{15}$-T(A/C)CGG, although the same nucleotides were substituted (base substitutions are underlined).

Generally, the effects of a base substitution in the first TTGC 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 74A HrpXo (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 TTGC-N$_{15}$-TGGGG (19.6% activity compared to the perfect PIP box), TTGC-N$_{15}$-TGGGT (19.3%), TTGC-N$_{15}$-TTCGG (21.2%), TTGC-N$_{15}$-TTCCTT (22.3%), TTGG-N$_{15}$-TTCGG (22.1%), and TTGG-N$_{15}$-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 74A HrpXo (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
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-TGCTG, TTCGC-N15-TTCGG, 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 74/H9004HrpXo 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 74/H9004HrpXo, 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 pHMPISGUS7G10G, 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 TTCTG into the imperfect PIP boxes. T7174R transformed with each of the resulting plasmids, pHMPIPORF1M1 and pHMPIPORF2M2, 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 pHMPIPGUS2 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).
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 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-N₁₅-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 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 σ⁷⁰ 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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative GUS activity (%)</th>
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<tbody>
<tr>
<td>T7174R(pHMPiPGUSG710G)</td>
<td>19.6 ± 6.0</td>
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<tr>
<td>T7174R(pHMPiPORF1)</td>
<td>49.5 ± 10.4</td>
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<tr>
<td>74ΔHrpXo(pHMPiPORF1)</td>
<td>1.9 ± 1.5</td>
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<tr>
<td>T7174R(pHMPiPGUS910G)</td>
<td>21.2 ± 5.9</td>
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<tr>
<td>T7174R(pHMPiPORF2)</td>
<td>226.8 ± 38.8</td>
</tr>
<tr>
<td>74ΔHrpXo(pHMPiPORF2)</td>
<td>3.7 ± 0.3</td>
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</tbody>
</table>

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

**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-TTCGG), pHMPIPORF1M2 (TTAGC-N₁₅-TGAAG), pHMPIPORF2M1 (TTAGC-N₁₅-TTCTG), and pHMPIPORF2M2 (TTAGC-N₁₅-TTATG). Transformants with each plasmid were inoculated in XOM2, and the GUS activity was measured. The values are activities relative to the wild-type PIP box and are averages and standard deviations for five independent experiments.
nucleotides. In particular, the activity in the transformant harboring orf2-gus was higher than that observed with hrcU:gus with a perfect PIP box. The results suggest that at least one factor 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 TTTGC 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-indepen-}

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