Characterization of *Xanthomonas oryzae*-Responsive *cis*-Acting Element in the Promoter of Rice Race-Specific Susceptibility Gene *Xa13*

Ting Yuan, Xianghua Li, Jinghua Xiao and **Shiping Wang**[1]

National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan 430070, China

ABSTRACT The rice *Xa13* gene, whose promoter harbors a UPT (up-regulated by transcription activator-like [TAL] effector) box, UPT_{PthXo1}, plays a pivotal role in the race-specific pathogenicity caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99. PXO99 causes rice disease by inducing *Xa13*. It is unknown, however, whether the UPT_{PthXo1} box is the only PXO99-responsive *cis*-regulating elements in the activation of *Xa13* expression. We analyzed the expression of a series of end- and site-truncated and site-mutated *Xa13* promoters in rice and the binding of PXO99 protein to the intact, partial, or site-mutated UPT_{PthXo1} boxes. In the *Xa13* promoter, UPT_{PthXo1} box is the only *Xoo*-responsive *cis*-acting element that results in PXO99-induced *Xa13* expression. The 5′-terminal second, third, and fourth nucleotides of the box are important for bacterial protein binding and gene activation; mutation of any one of these sites abolished PXO99-induced gene expression. Furthermore, the 3′-half of the UPT_{PthXo1} box is also required for protein binding and gene activation. These findings will enhance our understanding of the molecular mechanism of the interaction of rice and *Xoo* via UPT boxes and TAL effectors.

Key words: Bacterial blight; disease; *Oryza sativa*; UPT box; *Xanthomonas oryzae*.

INTRODUCTION

Bacteria blight, which is caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating diseases restricting rice production. More than 30 disease resistance (*R*) genes that mediate race-specific resistance to *Xoo* have been identified, and six of them have been characterized (Chu and Wang, 2007).

The recessive *xa13* is a new type of *R* gene that confers resistance to Philippine *Xoo* strain PXO99 (Chu et al., 2006). PXO99 infection does not influence the expression of recessive *xa13*, but it induces the expression of its dominant (susceptible) allele *Xa13*; suppressing the expression of *Xa13* can result in the same level of resistance to PXO99 as conferred by *xa13* in rice (Chu et al., 2006). Sequence analysis of a series of rice lines carrying either dominant *Xa13* or recessive *xa13* revealed a set of recessive alleles of *xa13*, whose encoding proteins are different from or identical to that encoded by dominant *Xa13*. However, all the recessive alleles had nucleotide substitutions, deletions, or insertions in a promoter region corresponding to the –86 to –69 region of the promoter of dominant *Xa13*, suggesting that promoter mutations may result in *xa13*-mediated disease resistance (Chu et al., 2006). Promoter swap analyses further confirmed that the expressional non-reaction to PXO99 infection caused by promoter mutation, not its protein composition, is the key factor for *xa13*-mediated resistance (Yuan et al., 2009). Thus, the dominant *Xa13* is a race-specific susceptibility gene. Activation of *Xa13* is required for the development of disease caused by PXO99.

A recent study has revealed that PXO99 is sensitive to copper (Yuan et al., 2010), which is an essential micronutrient of plants and an important element for a number of pesticides in agriculture. PXO99 overcomes rice defense by regulating *Xa13*,

[1] To whom correspondence should be addressed. E-mail swang@mail.hzau.edu.cn, fax 86-27-8728-7092, tel. 86-27-8728-3009.

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which incorporates with another two genes to remove copper from the xylem vessels, where *Xoo* multiplies and spreads to cause disease.

The dominant *Xa13* is also known as *Os8N3*, whose expression can be induced by a transcription activator-like (TAL) effector PthXo1 injected into rice through the type III secretion system of *Xoo* strain PXO99 (Yang et al., 2006). Effectors secreted by pathogenic bacteria play an essential role in promoting diseases in plants (Kay and Bonas, 2009). Several studies have demonstrated that pathogen TAL effectors can transcriptionally activate host genes by directly interacting with the *cis*-regulating elements, named UPT (up-regulated by TAL effectors) boxes, in the promoters of corresponding host susceptibility genes to promote diseases or in *R* genes to induce defense responses (Kay et al., 2007, 2009; Römer et al., 2007, 2009a, 2009b). This interaction is determined by the specific pairing of the repeat-variable diresidues (RVDs) of the repeat domain of a TAL effector and the nucleotides of a UPT box, with one RVD pairing to one specific nucleotide in the UPT box (Boch et al., 2009; Moscou and Bogdanove, 2009). Thus, based on the pairing codes of a given TAL effector, one can predict a putative UPT box that may interact with this TAL effector. A recent study revealed that the TAL effector PthXo1 from *Xoo* strain PXO99 directly binds to a UPT box, UPT_{PthXo1} (consisting of 25 nucleotides), in the *Xa13* promoter to mediate gene activation (Römer et al., 2010). However, it is unknown whether the UPT_{PthXo1} box is the only *cis*-regulating element that is responsible for *Xoo*-induced activation of *Xa13*.

In the present study, we analyzed the expression of truncated and mutated *Xa13* promoters in rice. We also examined the binding of PXO99 total proteins to the intact, incomplete, or site-mutated UPT_{PthXo1} box and corresponding DNA fragments from recessive *xa13* alleles. Our results suggest that, in the *Xa13* promoter, UPT_{PthXo1} box is the only *cis*-acting element that results in PXO99-induced *Xa13* expression. The important nucleotide sites of the UPT_{PthXo1} box and the neighboring sites of this box needed for efficient activation of the gene are discussed.

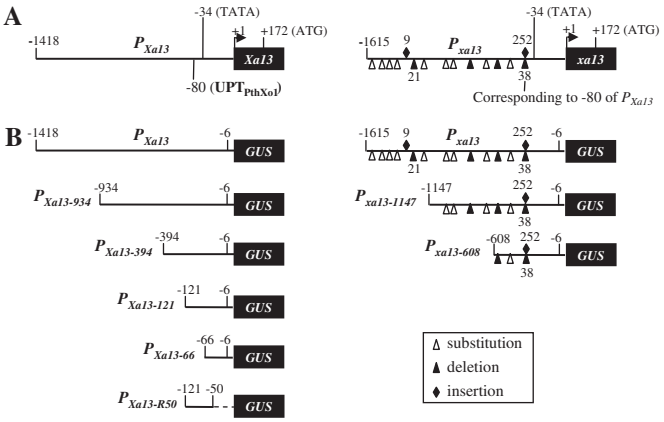

Figure 1. The Structures of Promoters P_{Xa13} and P_{xa13} from IR24 (Carrying *Xa13*) and IRBB13 (Carrying *xa13*), Respectively, and Truncated Promoters.

IR24 and IRBB13 are near-isogenic rice lines.

(A) The predicted basal regulatory element TATA in P_{Xa13} and P_{xa13}. The transcription initiation site is indicated as +1. The nucleotide substitution, deletion, or insertion in P_{xa13} compared with P_{Xa13} were based on a previous report (Chu et al., 2006); the figure with a sign of deletion or insertion indicates the numbers of nucleotides inserted or deleted and the sign without a figure represents single-nucleotide deletion or insertion. ATG, translation start codon.

(B) The full-length and truncated promoters.

RESULTS

Identification of the Pathogen-Responsive Region of the *Xa13* Promoter

Sequence analysis suggested that the putative TATA boxes, which provide the binding sites for RNA polymerase, were at –34 for the promoters of both the dominant *Xa13* gene (P_{Xa13}) and recessive *xa13* gene (P_{xa13}) (Figure 1A). Based on the putative locations of TATA boxes, previous identification of the promoter regions of *Xa13* and *xa13* genes (Yuan et al., 2009), and the location of UPT box for PthXo1 effector binding (Römer et al., 2010), the DNA fragment located at the –1418 to –6 region of *Xa13* and the corresponding DNA fragment located at the –1615 to –6 region of *xa13* were fused with the reporter gene β-glucuronidase (*GUS*) to detect the pathogen-responsive regions; two truncated promoters $P_{Xa13-934}$ and $P_{Xa13-394}$ for P_{Xa13} and $P_{xa13-1147}$ and $P_{xa13-608}$ for P_{xa13} were also fused with *GUS* (Figure 1B). These constructs were transferred separately into rice, and each construct generated approximately 20 independent positive transgenic plants. Each transgenic plant was divided into two parts by separating the tillers at the tillering stage: one part for inoculation with *Xoo* strain PXO99 and another part for mock-inoculation at the booting (panicle development) stage.

Because *Xa13* expression was markedly induced at 1–5 d after PXO99 infection (Chu et al., 2006; Yuan et al., 2009, 2010), the expression level of marker gene in the transgenic plants was examine from 8 h to 5 d after infection. The expression of GUS in the transgenic plants carrying P_{Xa13}*:GUS* was strongly induced at 2 d and increased continually until 5 d after PXO99 infection compared with mock-inoculated control plants (Figure 2). Neither PXO99 infection nor mock-inoculation influenced GUS expression in the transgenic plants carrying P_{xa13}*:GUS* (Figure 2). The GUS level in the plants carrying P_{Xa13}*:GUS* at 5 d after PXO99 infection was 23-fold higher than that in the same plants at 5 d after mock-inoculation. Plants carrying $P_{Xa13-934}$ and those carrying $P_{Xa13-394}$ showed a similar induced expression pattern of GUS to plants carrying P_{Xa13}*:GUS* after PXO99 infection (Figure 2). However, plants carrying $P_{Xa13-934}$ and those carrying $P_{xa13-1147}$ showed a significantly higher level ($P < 0.01$) of GUS than the plants carrying P_{Xa13}*:GUS* or P_{xa13}*:GUS* at 8 h after infection or even without

pathogen infection, respectively. Compared with mock-inoculation, PXO99 infection induced approximately two and three-fold increases of GUS levels in plants carrying $P_{Xa13\text{-}934}$ at 8 h, 2, and 5 d after infection, respectively. PXO99 infection did not markedly influence GUS expression in plants carrying $P_{xa13\text{-}1147}$ compared with the same plants after mock-inoculation. Plants carrying $P_{Xa13\text{-}394}$ and those carrying $P_{xa13\text{-}608}$ showed similar levels of GUS to the plants carrying P_{Xa13}*:GUS* or P_{xa13}*:GUS* when without pathogen infection. PXO99 infection showed approximately nine- and 10-fold increases in GUS levels in plants carrying $P_{Xa13\text{-}394}$ as compared with the same plants after mock-inoculation at 2 and 5 d after infection, respectively (Figure 2). PXO99 infection did not markedly influence GUS levels in the plants carrying $P_{xa13\text{-}608}$ compared to the same plants after mock-inoculation. These results suggest the following possibilities. First, the –1418 to –935 region of P_{Xa13} and the corresponding –1615 to –1148 region of P_{xa13} may contain PXO99-independent *cis*-regulating element(s) that suppress gene expression. Second, the –934 to –395 region of P_{Xa13} and the corresponding –1147 to –609 region of P_{xa13} may contain PXO99-independent *cis*-regulating element(s) that stimulate gene expression. Last, only the –394 to –6 region of P_{Xa13} may contain PXO99-responsive element(s) that induce gene expression.

The –394 to –6 region of P_{Xa13} harbors a PXO99-responsive element, the UPT_{PthXo1} box, located at –80 to –56 (Figure 1A; Römer et al., 2010). To determine whether this region may contain other PXO99-responsive elements in addition to the UPT box, two additional 5′-end truncated promoters, $P_{Xa13\text{-}121}$ and $P_{Xa13\text{-}66}$, and one 3′-end truncated promoter, $P_{Xa13\text{-}R50}$, of P_{Xa13} were fused with GUS (Figure 1B). The three constructs as well as the construct carrying P_{Xa13}*:GUS* were transiently expressed in rice calli. The expression of GUS in the calli carrying $P_{Xa13\text{-}121}$*:GUS* was strongly induced by PXO99-inoculation compared with mock-inoculated control calli, as was the case in the calli carrying P_{Xa13}*:GUS* (Figure 3). PXO99-inoculation did not obviously influence GUS expression in the calli carrying $P_{Xa13\text{-}66}$*:GUS* or $P_{Xa13\text{-}R50}$*:GUS* compared with mock-inoculated calli. Because the $P_{Xa13\text{-}66}$*:GUS* construct only harbored incomplete UPT_{PthXo1} box, these results suggest that the –121 to –65 region of P_{Xa13} may contain another PXO99-responsive element that induces gene expression or the –121 to –6 region may contain only one PXO99-responsive element, the UPT_{PthXo1} box. However, PXO99 infection did not obviously influenced GUS expression in the calli carrying $P_{Xa13\text{-}R50}$*:GUS*, which harbored the –121 to –50 region; this may be due to the lack of the basal transcriptional element, the TATA box. This assumption is

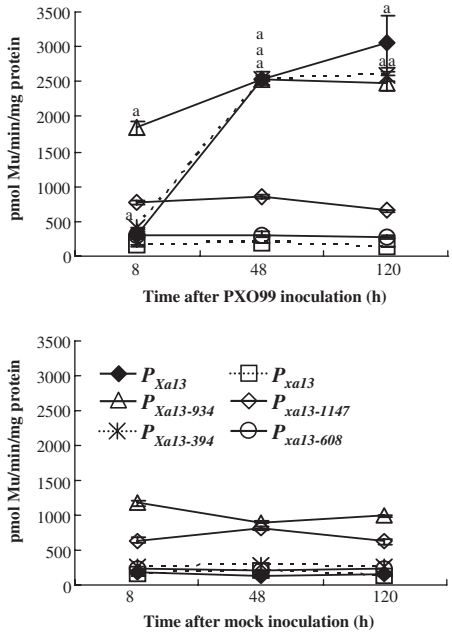

Figure 2. The Expression of *GUS* Driven by Native (P_{Xa13} and P_{xa13}) or Truncated ($P_{Xa13\text{-}934}$, $P_{Xa13\text{-}394}$, $P_{xa13\text{-}1147}$, and $P_{xa13\text{-}608}$) Promoters after PXO99-Inoculation or Mock-Inoculation at the Booting Stage.

GUS activity was determined by measuring the amount of 4-methylumbelliferone (Mu) produced under the catalysis of GUS in 1 mg total protein per minute. Sixteen to 22 T_0 transgenic plants carrying each construct were used for analyses. For each time point examined, leaf fragments were collected from all the plants carrying the same construct and mixed to prepare a sample. The GUS activity at each time point was the average of three measurements ± standard deviation. The 'a' indicates that a significant difference ($P < 0.01$) was detected between pathogen-inoculated and mock-inoculated plants in the same time point.

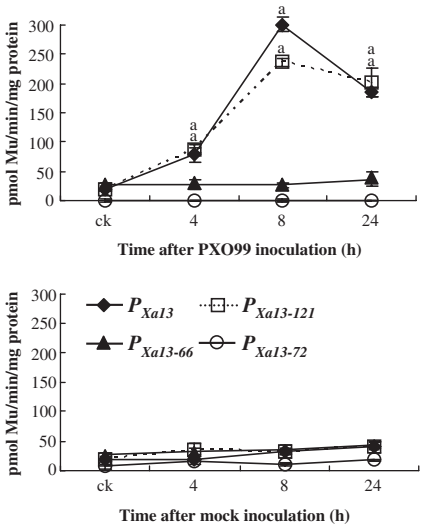

Figure 3. Transient Expression of *GUS* Driven by Native (P_{Xa13}) and Truncated ($P_{Xa13\text{-}121}$, $P_{Xa13\text{-}66}$, and $P_{Xa13\text{-}R50}$) Promoters in Rice Calli.

GUS activity was determined by measuring the amount of 4-methylumbelliferone (Mu) produced under the catalysis of GUS in 1 mg total protein per minute. Each sample was from 4.5 ml calli; all the calli were mixed to prepare a sample. The GUS activity of each sample was the average of three measurements ± standard deviation. ck, without pathogen- or mock-inoculation. The 'a' indicates that a significant difference ($P < 0.01$) was detected between pathogen-inoculated and mock-inoculated plants in the same time point. This experiment was biologically repeated twice and similar results were obtained.

supported by evidence that the GUS levels in the calli carrying $P_{Xa13\text{-}66}$*:GUS* were constantly approximately three-fold higher than those in the calli carrying $P_{Xa13\text{-}R50}$*:GUS* with either PXO99- or mock-inoculation.

More *Xoo* Proteins Bind to the Promoter Fragment of *Xa13* than to the Promoter Fragment of *xa13*

Although our findings indicated that the –121 to –66 region may harbor a PXO99-responsive element, a previous study showed that a region corresponding to the –86 to –69 region of P_{Xa13} might be responsible for PXO99-induced expression of dominant *Xa13*, based on the comparison of the promoter sequences of seven rice lines carrying dominant *Xa13* and 11 rice lines carrying recessive *xa13* or its recessive alleles (Chu et al., 2006). To determine whether the protein(s) of PXO99 could directly bind to the putative PXO99-responsive element of P_{Xa13}, 14- to 15-nt DNA probes from the –86 to –69 region of P_{Xa13}, which harbored the 5′-half of the UPT_{PthXo1} box, and the corresponding regions of some promoters of recessive *xa13* and its recessive alleles were designed based on sequence-specific analysis and used for protein binding analyses (Figure 4A).

Because the TAL effector PthXo1 of PXO99 transcriptionally activates *Xa13* (Yang et al., 2006), we expected that PXO99 proteins would bind more intensively to the probe from P_{Xa13} than the probes from P_{xa13}, if there was protein binding. Unexpectedly, an electrophoretic mobility shift assay (EMSA) showed that PXO99 proteins bound more intensely to the four probes, R15-Tepa 1, R15-IRBB13, R14-AUS274, and R15-AC19-1–1, from the promoters of recessive *xa13* and its recessive alleles than to probe D15-IR24 from P_{Xa13} (Figure 4B). The protein-binding of D15-IR24 and R15-Tepa 1 was reduced or abolished by the competition of unlabelled probes, indicating specificity of the binding. To determine which nucleotide influenced the binding of PXO99 proteins, three site-mutated short DNA probes (D15M1, D15M2, and D15M3) were used for analysis of protein binding (Figure 4A). By comparing the protein binding intensity of D15M1, D15M2, and D15M3 with that of D15-IR24, substitution of C with A or A with G in probes D15M2 and D15M3, respectively, appeared to be important for protein binding (Figure 4B).

A recent study reported that the UPT_{PthXo1} box of P_{Xa13} consists of 25 nucleotides (Figure 4A; Römer et al., 2010). Thus, we designed a new set of long DNA probes that harbored the full-length UPT_{PthXo1} box from *Xa13* or the corresponding regions from *xa13* or its recessive alleles (Figure 4A). Among these long probes, R28-IRBB13, R28-Tepa 1, R28-AUS274, and R28-AC19-1–1 from the promoters of recessive *xa13* (IRBB13) and its recessive alleles (Tepa 1, AUS274, and AC19-1–1), respectively, harbored DNA fragments that had 1-, 3-, 10-, or 16-nucleotide differences from the UPT_{PthXo1} box. EMSA showed that PXO99 proteins bound intensively to probe D28-IR24 harboring the UPT_{PthXo1} box, but only very weakly to probes D28-IRBB13, D28-Tepa 1, D28-AUS274, and D28-AC19-1–1 (Figure 4C). Substitution of the third C nucleotide of the UPT_{PthXo1} box with A (probe D28M2) or the fourth A nucleotide with G (D28M3) markedly reduced the binding (Figure 4C). However, substitution of the sixth C of the UPT_{PthXo1} box with A (probe D28M1) did not influence the binding affinity of PXO99 proteins. The protein-binding signals of the probes were abolished by the competition of unlabelled probes, indicating specificity of the binding (Figure 4C). These results suggest that an intact UPT_{PthXo1} box is essential for bacterial protein binding. Furthermore, at least the second, third, and fourth nucleotides of the box are important for bacterial protein binding, whereas the sixth nucleotide of this box may not be important for protein binding.

Mutation of UPT_{PthXo1} Box Influences PXO99-Induced Transcriptional Activity

To ascertain whether the differential binding activities of the various probes to bacterial proteins affected transcriptional regulation of the promoter, we constructed four site-mutated promoters ($P_{Xa13\Delta 79T}$, $P_{Xa13\Delta 78A}$, $P_{Xa13\Delta 77G}$, and $P_{Xa13\Delta 75A}$) of P_{Xa13} harboring the UPT_{PthXo1} box, in which the second, third, fourth, and sixth nucleotides were mutated, respectively (Figure 5A). These mutated promoters were fused with *GUS* and transferred separately into rice. Each construct generated approximately 20 independent positive transgenic plants. Each plant was divided into two parts by separating the tillers at the tillering stage for inoculation with *Xoo* strain PXO99 and mock-inoculation, respectively. Transgenic plants carrying different promoter constructs showed the similar levels of GUS expression when mock-inoculated. The PXO99-induced expression of GUS in the transgenic plants carrying $P_{Xa13\Delta 79T}$, $P_{Xa13\Delta 78A}$, or $P_{Xa13\Delta 77G}$ was completely or partially suppressed compared to the expression of GUS in the plants carrying P_{Xa13}*:GUS* (Figure 5B). However, plants carrying $P_{Xa13\Delta 75A}$ showed a similar level of PXO99-induced GUS expression to the plants carrying P_{Xa13}*:GUS*. The mutated UPT_{PthXo1} boxes in the promoters $P_{Xa13\Delta 79T}$, $P_{Xa13\Delta 78A}$, and $P_{Xa13\Delta 77G}$ corresponded to DNA probes R28-Tepa1, D28M2, and D28M3, respectively, which showed only weak binding of PXO99 proteins (Figure 4C). The mutated UPT_{PthXo1} box in $P_{Xa13\Delta 75A}$ was consistent with probe D28M1, which showed strong binding to PXO99 proteins (Figure 4C). These results suggest that the suppression or loss of PXO99-induced transcriptional activation in the transgenic plants carrying $P_{Xa13\Delta 79T}$, $P_{Xa13\Delta 78A}$, or $P_{Xa13\Delta 77G}$ may have resulted from the unavailability or low affinity of binding bacterial protein to the promoters due to the mutation of the key sites of the UPT_{PthXo1} box. These results also suggest that the UPT_{PthXo1} box is the only PXO99-responsive element in the promoter of *Xa13*.

We also constructed seven site-truncated promoters. Three to 11 nucleotides either in front of the UPT_{PthXo1} box ($P_{Xa13\text{-}F3}$) or at 3′-terminal of the UPT_{PthXo1} box ($P_{Xa13\text{-}E3}$, $P_{Xa13\text{-}E5}$, $P_{Xa13\text{-}E7}$, $P_{Xa13\text{-}E9}$, $P_{Xa13\text{-}E11}$, and $P_{Xa13\text{-}E13}$) were deleted from P_{Xa13} promoter (Figure 6A). These site-truncated constructs and the construct carrying P_{Xa13}*:GUS* were transiently expressed in separate rice calli. The expression of GUS in the calli carrying

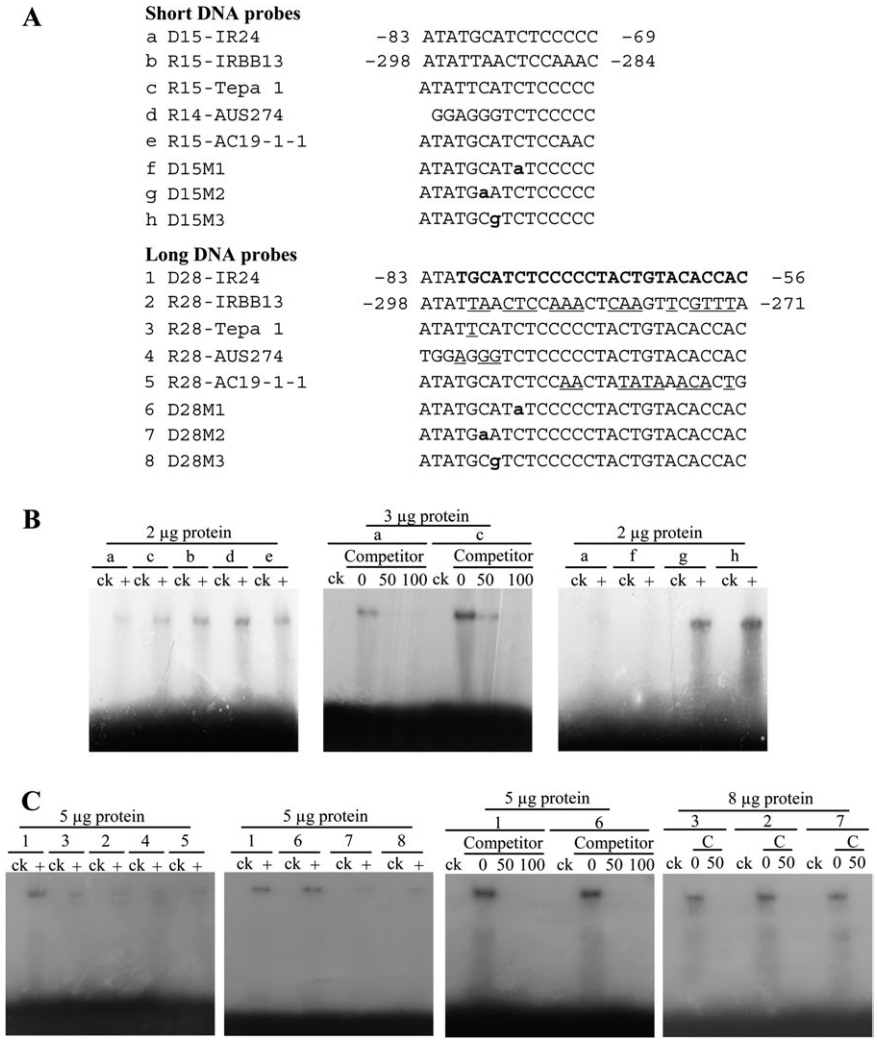

Figure 4. The Binding Ability of the Promoter Probes of Dominant *Xa13* and Recessive *xa13* to the Total Proteins of *Xoo* Strain PXO99 Analyzed by EMSA.

The binding assays were biologically repeated twice, with similar results ((B) and (C)).

(A) Probe sequences. Rice line IR24 carries dominant *Xa13*. Rice lines IRBB13, Tepa 1, AUS274, and AC19-1–1 carry different alleles of recessive *xa13*; another five rice lines, Chinsurah Boro2 (11484), Chinsurah Boro2 (11760), Chinsurah Boro2 (50930), Long Grain (64950), and BJ1 that carry different *xa13* alleles from that of Tepa 1, have the same DNA sequence as Tepa 1 in the region corresponding to the –86 to –69 region of P_{Xa13} (Chu et al., 2006). Probe D28-IR24 harbors the UPT_{PthXo1} box of P_{Xa13} (bold italic letters). Compared with D28-IR24, the substitution sites of the probes from the promoters of *xa13* and its recessive alleles are underlined. Mutation sites are shown in lower-case letters.

(B) The binding ability of PXO99 total proteins to different short (14- or 15-nt) probes. The labeled probes plus 50- or 100-fold unlabelled competitor probes or without plus competitor (0) were used for the binding assays. ck, without PXO99 proteins; +, with PXO99 proteins.

(C) The binding ability of PXO99 total proteins to different long (28-nt) probes. The labeled probes plus 50-fold unlabelled competitor (C) probes or without plus competitor (0) were used for the binding assays.

only P_{Xa13}*:GUS* or $P_{Xa13\text{-}F3}$*:GUS* but not other constructs was significantly induced ($P < 0.01$) by PXO99-inoculation compared to mock-inoculation (Figure 6B). However, GUS level in the calli carrying $P_{Xa13\text{-}F3}$*:GUS* was significantly lower than that in the calli carrying P_{Xa13}*:GUS* at 4–24 h after infection (Figure 6B). These results suggest that both the flanking sequence and the 3′-terminal part of UPT_{PthXo1} box may be important for *Xoo*-induced expression.

DISCUSSION

Xoo-induced *Xa13* expression is critical for *Xa13*-facilited susceptibility (Yuan et al., 2009, 2010). The PthXo1 effector of *Xoo* strain PXO99 transcriptionally activates *Xa13* by binding to a *cis*-acting element, the UPT_{PthXo1} box, in the gene's promoter (Yang et al., 2006; Römer et al., 2010). We analyzed a series of end- and site-truncated and site-mutated promoters, and our findings suggest that the UPT_{PthXo1} box is the only PXO99-responsive element in the *Xa13* promoter. Thus, interaction of PthXo1 effector and UPT_{PthXo1} box results in the susceptibility of rice to PXO99. The promoters of recessive *R* gene *xa13* and its recessive alleles carry mutated UPT_{PthXo1} boxes, which is the key reason for *xa13*-mediated resistance.

Although the present study used the total proteins of PXO99 instead of PthXo1 for DNA-protein binding analyses, the proteins bound to the DNA probe harboring the UPT_{PthXo1} box are likely mainly the PthXo1 effector, as supported by the following evidence. First, EMSA showed that PXO99 proteins bound intensively to the DNA probe harboring the full-length UPT_{PthXo1} box but weakly to the probes harboring mutated UPT_{PthXo1} boxes from the promoters of recessive *xa13* and its recessive alleles (Figure 4C). These results agree with a recent report that His:PthXo1 fusion protein binds strongly to the DNA fragment harboring the UPT_{PthXo1} box but to a lesser extent to the corresponding promoter region of recessive *xa13* (Römer et al., 2010). Second, replacement of the second G nucleotide of the UPT_{PthXo1} box with T in a natural mutation (the recessive allele of *xa13* from rice variety Tepa 1) markedly

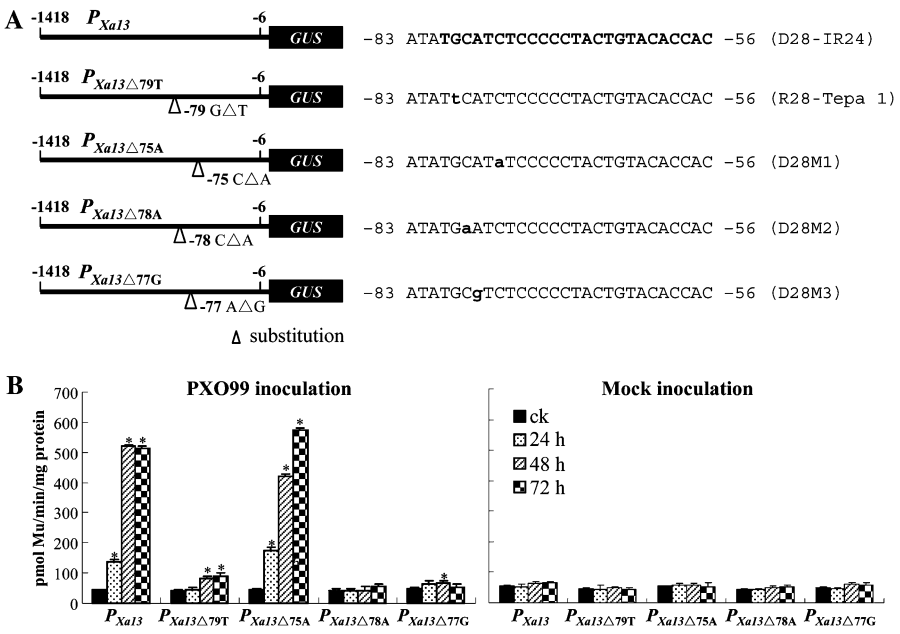

Figure 5. Expression of *GUS* Driven by Native (P_{Xa13}) or Mutated ($P_{Xa13\Delta 79T}$, $P_{Xa13\Delta 75A}$, $P_{Xa13\Delta 78A}$, and $P_{Xa13\Delta 77G}$) Promoters after PXO99 Infection or Mock-Inoculation at the Booting Stage.

(A) The structures of mutated promoters and the sites of mutations. The UPT_{PthXo1} box of P_{Xa13} is shown with bold italic letters. The figure with a sign of substitution indicates the site of nucleotides substitution.

(B) Expression of GUS in transgenic plants. GUS activity was determined by measuring the amount of 4-methylumbelliferone (Mu) produced under the catalysis of GUS in 1 mg total protein per minute. Twenty to 36 T_0 transgenic plants carrying each construct were used for analyses. For each time point examined, leaf fragments were collected from all the plants carrying the same construct and mixed for preparing sample. The GUS activity at each time point was the average of three measurements ± standard deviation. ck, without pathogen- or mock-inoculation. The asterisk (*) indicates that a significant difference ($P < 0.01$) was detected between PXO99-inoculated plants and non-inoculated control (ck) plants carrying the same promoter.

reduced protein binding (Figure 4C). Likewise, substitution of the second nucleotide of the UPT_{PthXo1} box with A, C, or T significantly reduced or almost complete negated PthXo1-mediated promoter activation (Römer et al., 2010). Finally, the same mutations in the UPT_{PthXo1} box that reduced PXO99 protein binding abolished PXO99-induced *Xa13* expression (Figure 5), which was similar to the knockout of PthXo1 in PXO99 (Yang et al., 2006).

Recently, several UPT boxes have been predicted by using the TAL effector code, the RVDs that are the hypervariable residues 12 and 13 in each repeat unit of the repeat domain of TAL effectors (Boch et al., 2009; Moscou and Bogdanove, 2009). Although about half of the identified RVDs of TAL effectors have degenerated pairing nucleotides based on the identified and predicted UPT boxes, with some RVDs codes varying between two to four nucleotides (Boch et al., 2009; Moscou and Bogdanove, 2009), this degeneracy appears to be influenced by the position of a RVD in TAL effectors or/and by the position of a nucleotide in a UPT box. For example, the RVD NN appears to recognize four types of nucleotide in UPT boxes (Boch et al., 2009; Moscou and Bogdanove, 2009). However, matching the NN-type RVD of PthXo1 to the 5′-end second G nucleotide but not the A, C, or T nucleotide of the UPT_{PthXo1} box is crucial for the interaction of the PthXo1 and UPT_{PthXo1} box (Römer et al., 2010). Our present results also showed that substitution of the second G to T in a natural mutation markedly reduced PXO99 protein binding and abolished PXO99-induced gene expression. Furthermore, the 5′-end third and forth nucleotides of the UPT_{PthXo1} box are also important for PXO99 protein binding and gene transcriptional activation. Both the RVDs pairing to the third and sixth nucleotides are HD-type (Römer et al., 2010), which is suggested to have degenerated pairing nucleotides in the UPT boxes (Boch et al., 2009; Moscou and Bogdanove, 2009). Interestingly, substitution of the third C to A in the UPT_{PthXo1} box abolished PXO99-induced gene expression and substitution of the sixth C to A did not influence gene activation (Figure 5). Similar results were observed in another study. The RVDs of TAL effector AvrBs3 pairing the 14th and 15th nucleotides of the UPA_{AvrBs3} box (also a UPT box) in the promoter of pepper *R* gene *Bs3* are HD-type, but substitution of the 15th nucleotide C to A in the UPA_{Bs3} box abolished the *Bs3*-mediated hypersensitive response, while substitution of the 14th nucleotide C to A did not influence *Bs3* function (Römer et al., 2009b). These results suggest that the three-dimensional positions of some RVDs may influence the interaction of a TAL effector and UPT box.

One RVD pairs with one nucleotide in a UPT box in the host–bacterium interaction; thus, the number of RVDs in a TAL

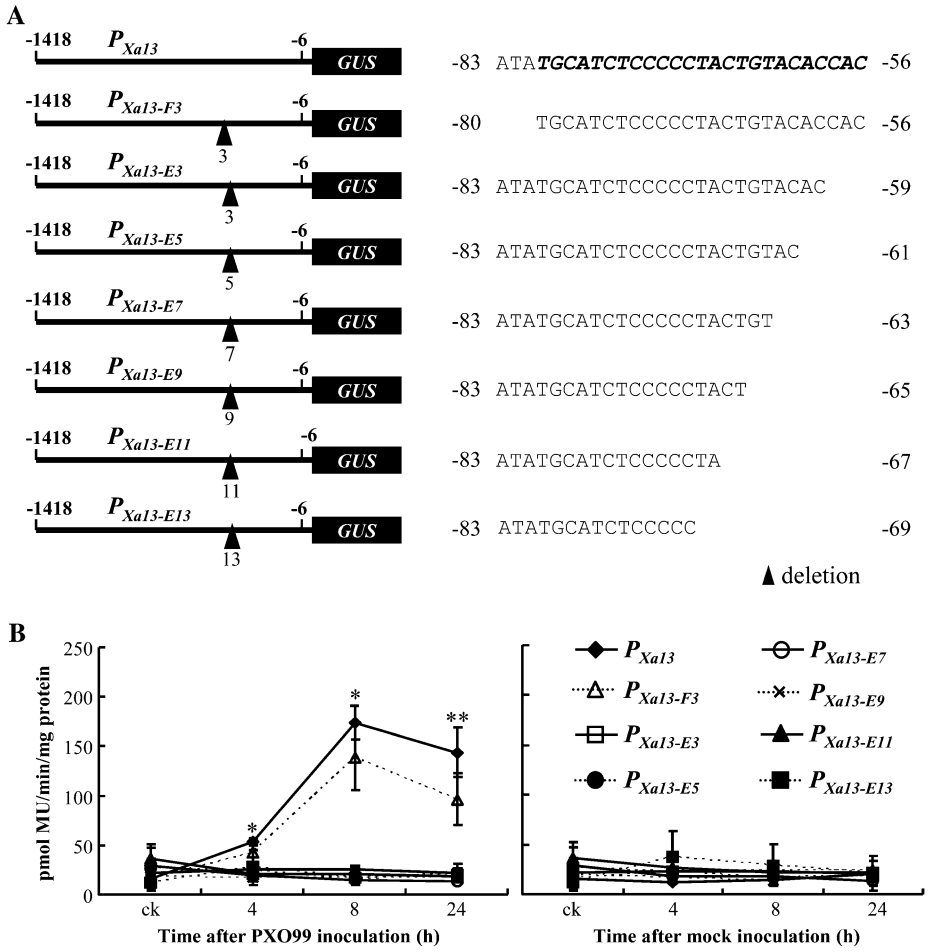

Figure 6. Transient Expression of *GUS* Driven by Native (P_{Xa13}) and Site-Truncated ($P_{Xa13\text{-}F3}$, $P_{Xa13\text{-}E3}$, $P_{Xa13\text{-}E5}$, $P_{Xa13\text{-}E7}$, $P_{Xa13\text{-}E9}$, $P_{Xa13\text{-}E11}$, and $P_{Xa13\text{-}E13}$) Promoters in Rice Calli.

(A) The structures of site-truncated promoters and the sites of deletion. The UPT_{PthXo1} box of P_{Xa13} is shown with bold italic letters. The figure with a sign of deletion indicates the numbers of nucleotides deleted.

(B) Expression of GUS driven by different promoters in rice calli. GUS activity was determined by measuring the amount of 4-methylumbelliferone (Mu) produced under the catalysis of GUS in 1 mg total protein per minute. Each sample was from 4.5 ml calli; all the calli were mixed to prepare a sample. The GUS activity of each sample was the average of three measurements ± standard deviation. One (*) or two (**) asterisks indicate that a significant difference between plants carrying P_{Xa13}:*GUS* and $P_{Xa13\text{-}F3}$:*GUS* in the same time point was detected at $P < 0.05$ or $P < 0.01$, respectively. ck, without pathogen- or mock-inoculation. This experiment was repeated twice biologically and similar results were obtained.

effector determines the size of the corresponding UPT box (Boch et al., 2009; Moscou and Bogdanove, 2009). However, the length of a functional UPT box is not always as long as that predicted using the TAL effector code. The nucleotides in the 3′-end of some UPT boxes appear to not be required for gene activation. The AvrXa27 effector contains 17 RVDs (Moscou and Bogdanove, 2009). Omission of the last three nucleotides of the predicted $UPT_{AvrXa27}$ box can still trigger hypersensitive response by AvrXa27 (Römer et al., 2009a). The AvrBs3Δrep16 effector contains 14 RVDs (Römer et al., 2010). Omission of the last two nucleotides of the predicted $UPA_{AvrBs3\Delta rep16}$ box (also a UPT box) can also trigger hypersensitive response by AvrBs3Δrep16 (Römer et al., 2009b). A recent study reported that at least 6.5 RVDs were required to recognize the target DNA box and to activate gene expression, and 10.5 or more RVDs could efficiently activate gene expression (Boch et al., 2009). Our present results showed that the 12 5′-terminal nucleotides of the UPT_{PthXo1} box could not provide correct pathogen protein binding (Figure 4). Even deletion of the three nucleotides at the 3′-end of the UPT_{PthXo1} box abolished PXO99-induced gene expression (Figure 6). Likewise, an incomplete $UPA_{AvrBs3\Delta rep16}$ box (lacking the first nucleotide) had a lower affinity for the AvrBs3Δrep16 effector than the complete UPA_{AvrBs3} box (Römer et al., 2007). However, the complete $UPA_{AvrBs3\Delta rep16}$ box showed a higher affinity for AvrBs3Δrep16 than the complete UPA_{AvrBs3} box (Römer et al., 2009b). These results suggest that in some cases, an incomplete UPT box may result in non-specific protein binding.

The 5′-terminal T of UPT boxes has been reported to be crucial to transcriptional activation by TAL effectors (Boch et al., 2009;

Römer et al., 2009b, 2010). Interestingly, the flanking sequence of the UPT_{PthXo1} box appears to influence PXO99-induced gene expression. The deletion of the three nucleotides flanking the 5′-end of the UPT_{PthXo1} significantly reduced the expression level of the gene activated by PXO99 (Figure 6). The neighboring nucleotides of at least some plant *cis*-acting elements also contribute to high-affinity binding of regulating proteins (Ciolkowski et al., 2008). It remains to be demonstrated whether it is a common feature that neighboring nucleotides of UPT boxes contribute to binding affinity of TAL effectors. In conclusion, the molecular mechanism of the specific interactions of TAL effectors and UPT boxes still needs to be refined. Investigating the binding of TAL effectors to UPT boxes at the three-dimensional level may help to clarify these issues.

METHODS

Constructing End-Truncated Promoters

Truncated promoters of dominant *Xa13* gene (P_{Xa13}) and recessive *xa13* gene (P_{xa13}) were obtained by PCR amplification from the 5′-end using the intermediate vector containing P_{Xa13} and P_{xa13} (Yuan et al., 2009), respectively, as a template. The 5′-end primers of Xa13P-F (5′-GGATCCGATGTTGAGCTTTAGGATTAGCGGGTT-3′), 5′-deletion-1147 (5′-GGATCCTCCCTTTCTTCAAGTTACCTCTCTC-3′), and 5′-deletion-608 (5′-GGATCCGCATCATTGTCCATGGTTGT-3′), which were specific to both P_{Xa13} and P_{xa13} and contained the *Bam*HI digestion site (underlined), as well as primers XP-90F (5′-GGATCCGAAATATCAAGCACAAG-3′) and XP-60F (5′-GGATCCCTGTACACCACCAAAAG-3′), which were specific to P_{Xa13}, were used in combination with the 3′-end primer X13-promR (5′-GGCAAGCTTGGCCTTGGCCATGGCTCAGT-3′), which was specific to both P_{Xa13} and P_{xa13} and contained the *Hin*dIII digestion site (underlined). The 5′-end primer XP-90F was also used in combination with the 3′-end primer XP-60R (5′-AAGCTTCTTTTGGTGGTGTACAG-3′), which was specific to P_{Xa13} and contained the *Hin*dIII digestion site (underlined). The PCR products were ligated to vector pGEM-T (Promega Corporation, Madison, WI, USA) for sequencing, then the truncated promoter fragments were digested with *Bam*HI and *Hin*dIII and ligated to vector pCAMBIA1381 to form truncated promoters fused with reporter gene *GUS*.

Rice Transformation

The promoter constructs were transferred into rice variety Mudanjiang 8 (*Oryza sativa* L. ssp. *japonica*) by *Agrobacterium*-mediated transformation (Lin and Zhang, 2005). A pair of PCR primers, GusF (5′-CCAGGCAGTTTTAACGATCAGTTCGC-3′) and GusR (5′-GAGTGAAGATCCCTTTCTTGTTACC), designed according to the sequence of *GUS* was used for detecting positive transgenic plants.

Pathogen Inoculation

Rice plants were inoculated with *Xoo* strain PXO99 by the leaf-clipping method at the booting (panicle development) stage (Sun et al., 2004). Mock-inoculated plants were treated under the same conditions except that the *Xoo* suspension was replaced with water.

Analysis of GUS Activity

Leaf fragments about 1 cm long right next to the inoculation sites were used for analysis of GUS expression. Quantitative analyses of GUS activity were conducted as described previously (Cai et al., 2007). Total protein concentration in the supernatant was quantified with the Bradford assay (Bradford, 1976). GUS protein in the supernatant was determined fluorometrically with an INFINITE 200 (Tecan Austria Gmbh, Ltd, Grodig, Austria).

Agrobacterium-Mediated Transient Expression

The calli of rice variety Zhonghua 11 (*O. sativa* ssp. *japonica*) were prepared as described previously (Lin and Zhang, 2005). *Agrobacteria* containing different transformation construct were co-cultured with the calli for over 16 h in a MS medium. The calli were then washed 10 times using autoclaved water and dried in a laminar flow cabinet. The calli were treated with *Xoo* strain PXO99 at 10^9 cfu ml^{-1} or mock-treated with autoclaved water for a certain period of time.

Site-Directed Mutation and Truncation

The GeneTailor Site-Directed Mutagenesis System (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for site-directed mutation of *Xa13* promoter as described previously (Cai et al., 2007). The pUC19 plasmid that contained the native promoter (P_{Xa13}) used as a PCR template was methylated before use. The mutagenic primer pair, in which the regulatory element was mutated, was used to amplify the promoter-containing target mutation. Primer pairs Xa13PM1F (5′-AAAGCAAAGGTTAGATATTCATCTCCCCCT-3′)/Xa13PM1R (5′-ATATCTAACCTTTGCTTTTTTTTTTC-3′), Xa13PM2F (5′-CAAAGGTTAGATATGCATATCCCCCTACTG-3′)/Xa13PM2R (5′-ATGCATATCTAACCTTTGCTTTTTTTTTTC-3′), Xa13PM4F (5′-AAGCAAAGGTTAGATATGAATCTCCCCCT-3′)/Xa13PM4R (5′-CATATCTAACCTTTGCTTTTTTTTTC-3′), and Xa13PM5F (5′-AGCAAAGGTTAGATATGCGTCTCCCCCTAC-3′)/Xa13PM5R (5′-GCATATCTAACCTTTGCTTTTTTTTTC-3′) were used for site-directed mutation of P_{Xa13}, in which the mutation points were underlined. To help select the mutated construct, the PCR product was transferred into *Escherichia coli* strain DH5a-T1, in which the methylated plasmid could not replicate.

The site-directed truncation of *Xa13* promoter was performed by nested PCR amplification using the intermediate vector containing P_{Xa13} (Yuan et al., 2009) as a template. First, the forward primer BXa13(IR24)M17F (5′-TAGATATGCATCTCCCCCTACAAAAGTGGAG-3′), which contained a deletion in the target site, was used in combination with the reverse primer X13-promR to amplify the downstream of the deletion site. Simultaneously, the reverse primer BXa13(IR24)M17R (5′-GGGGGAGATGCATATCTAACCTTTGCTTTT-3′), which was

overlapped with BXa13(IR24)M17F and also contained the same deletion as in BXa13(IR24)M17F, was paired with the forward primer Xa13P-F to amplify the upstream of the deletion site. The amplification products from the two PCR reactions were purified and mixed and used as the template for the second round of PCR using primers Xa13P-F and X13-promR. Another six pairs of primers, BXa13(IR24)M15F (5′-TAGATATGCATCTCCCCCCAAAAGTGGAGGG-3′)/BXa13(IR24)M15R (5′-GGGAGATGCATATCTAACCTTTGCTTTTTT-3′), BXa13(IR24)M19F (5′-GATATGCATCTCCCCCTACTCAAAAGTGGAG-3′)/BXa13(IR24)M19R (5′-TAGGGGGAGATGCATATCTAACCTTTGCTT-3′), BXa13(IR24)M21F (5′-TATGCATCTCCCCCTACTGTCAAAAGTGGAG-3′)/BXa13(IR24)M21R (5′-AGTAGGGGGAGATGCATATCTAACCTTTGC-3′), BXa13(IR24)M23F (5′-TGCATCTCCCCCTACTGTACCAAAAGTGGAG-3′)/BXa13(IR24)M23R (5′-ACAGTAGGGGGAGATGCATATCTAACCTTT-3′), BXa13(IR24)M25F (5′-CATCTCCCCCTACTGTACACCAAAAGTGGAG-3′)/BXa13(IR24)M25R (5′-GTACAGTAGGGGGAGATGCATATCTAACCT-3′), and BXa13(IR24)M-3F (5′-GAAAAAAAAAAGCAAAGGTTAGTGCATCTCCCC-3′)/BXa13(IR24)M-3R (5′-AACCTTTGCTTTTTTTTTTCTTGTGCTTGA-3′), were also used to construct other site-directed truncated promoters in the same way as described above.

Electrophoretic Mobility Shift Assay

To isolate the total proteins of *Xoo* strain PXO99, the fresh bacteria were ground with liquid nitrogen and suspended in buffer (10 mM Tris-HCl at pH 8.0, 1 mM DTT, 200 μM phenylmethanesulfonyl fluoride) and homogenized completely. The mixture was centrifuged at 4°C at 1000 *g* to collect the supernatant. The protein content in the nuclear extract and in the total proteins of *Xoo* strain PXO99 was quantified using the Bradford assay. EMSA was applied as described previously (Qiu et al., 2007).

Promoter Sequence Analysis

The TATA boxes of promoters P_{Xa13} and P_{xa13} were predicted using the computer programs TSSP provided at the Softberry website (www.softberry.com) and PROSCAN (http://bimas.dcrt.nih.gov/molbio/proscan).

Statistical Analysis

The significant differences between control and treatment of the samples were analyzed by the pair-wise *t*-test installed in the Microsoft Office Excel program.

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