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Genetic engineering of the *Xa10* promoter for broad-spectrum and durable resistance to *Xanthomonas oryzae* pv. *oryzae*

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Summary

Many pathovars of plant pathogenic bacteria *Xanthomonas* species inject transcription activator-like (TAL) effectors into plant host cells to promote disease susceptibility or trigger disease resistance. The rice TAL effector-dependent disease resistance gene *Xa10* confers narrow-spectrum race-specific resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of bacterial blight disease in rice. To generate broad-spectrum and durable resistance to *Xoo*, we developed a modified *Xa10* gene, designated as $Xa10^{E5}$. $Xa10^{E5}$ has an *EBE*-amended promoter containing 5 tandemly arranged *EBEs* each responding specifically to a corresponding virulent or avirulent TAL effector and a stable transgenic rice line containing $Xa10^{E5}$ was generated in the cultivar Nipponbare. The $Xa10^{E5}$ gene was specifically induced by *Xoo* strains that harbour the corresponding TAL effectors and conferred TAL effector-dependent resistance to the pathogens at all developmental stages of rice. Further disease evaluation demonstrated that the $Xa10^{E5}$ gene in either Nipponbare or 9311 genetic backgrounds provided broad-spectrum disease resistance to 27 of the 28 *Xoo* strains collected from 11 countries. The development of $Xa10^{E5}$ and transgenic rice lines provides new genetic materials for molecular breeding of rice for broad-spectrum and durable disease resistance to bacterial blight.

Introduction

Xanthomonas is a genus within the γ-proteobacteria that contains phytopathogenic species which often employ a type III secretion system (TTSS) to translocate effector proteins into plant cells where they modulate host cell functions that can benefit the infection process (Alfano and Collmer, 2004; Kay and Bonas, 2009). AvrBs3-like type III effectors, also referred to as transcription activator-like (TAL) effectors (Yang *et al.*, 2006), represent subset of type III effectors from *Xanthomonas* that activate the transcription of specific host susceptibility (*S*) genes, which, in turn, promote disease susceptibility (Antony *et al.*, 2010; Cernadas *et al.*, 2014; Hu *et al.*, 2014; Kay *et al.*, 2007; Streubel *et al.*, 2013; Sugio *et al.*, 2007; Yang *et al.*, 2006; Yu *et al.*, 2011). However, in some cases, plants take advantage of this mechanism to trigger disease resistance through TAL effector-mediated expression of *R* genes (Gu *et al.*, 2005; Romer *et al.*, 2007; Tian *et al.*, 2014). The specificity of TAL effector-mediated host gene induction is determined by the repetitive central region of each effector, which consists of near-perfect direct repeats of 33- to 35-amino acid residues. The two hypervariable amino acids at positions 12 and 13 of each repeat, also termed as repeat-variable di-residue (RVD) (Moscou and Bogdanove, 2009), preferentially associate with a different nucleotide to define the length and sequence of effector binding elements (*EBEs*) in the promoters of the targeted host genes (Boch *et al.*, 2009; Moscou and Bogdanove, 2009). Crystal structures of TAL effectors bound to DNA demonstrated that base-specific contacts are formed only by amino acid residue 13 (Deng *et al.*, 2012; Mak *et al.*, 2012).

The nature of the specific recognitions between TAL effectors and their targeted host genes makes it particularly possible to generate novel recognition specificities or to disable existing recognition specificities through engineering approaches (Boch *et al.*, 2014). For instance, passive resistance can be generated by removal of TAL effector target sites in the promoters of major host *S* genes using TAL effector-based nucleases (TALENs) (Li *et al.*, 2012) or other gene editing techniques (Feng *et al.*, 2014; Wang *et al.*, 2014b; Zhang *et al.*, 2010), whereas active resistance can be achieved by adding TAL effector target sites to promoters of TAL effector-dependent *R* genes (Hummel *et al.*, 2012; Romer *et al.*, 2009). Romer *et al.* (2009) reported that, in an *Agrobacterium*-mediated transient expression assay in *Nicotiana benthamiana*, the amended *Bs3* gene promoter containing tandemly arranged *UPT* (*UP*regulated by *T*AL effectors) boxes with distinct TAL effector specificity was capable of recognizing multiple TAL effectors. This pioneering study provides a potential approach for engineering broad spectrum and potentially durable disease resistance. Hummel *et al.* (2012) generated an *EBE*-amended *Xa27* gene that confers broad-spectrum resistance to both bacterial blight and bacterial leaf streak disease in rice. The 6 tandem *EBEs* added to the upstream of $EBE_{AvrXa27}$ in the *Xa27* promoter respond, respectively, to TAL

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effectors PthXo1, PthXo6 and Tal9a from *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99^A, the causal agent of bacterial blight disease in rice, and Tal4c, Tal2g and Tal4a from *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strain BLS256, the causal agent of bacterial leaf streak in rice (Hummel *et al.*, 2012). It should be noted that some TAL effectors perform a dual function in both virulence and avirulence (Antony *et al.*, 2010; Hopkins *et al.*, 1992; Kay *et al.*, 2007; Romer *et al.*, 2007). *R* genes that recognize these TAL effectors that otherwise contribute strong virulence are predicted to be relatively durable in the field due to high fitness cost to the pathogen of losing or modifying the TAL effectors to evade detection (Vera Cruz *et al.*, 2000). Therefore, it is more desirable for engineering durable resistance by adding virulent TAL effector binding sites to TAL effector-dependent *R* gene promoters (Hummel *et al.*, 2012).

Individual *Xoo* strains harbour 11–19 TAL effectors (White *et al.*, 2009). The TAL effector PthXo1 from *Xoo* strain PXO99^A targets the disease *S* gene *Os8N3*, which is a member of the nodulin 3 family of proteins and shown to be a member of a family of sugar transporters in rice and other plants (Chen *et al.*, 2010; Yang *et al.*, 2006). Members of the nodulin 3 gene family have been renamed as *SWEETs*, and *Os8N3* is commonly referred to as *OsSWEET11* (Chen *et al.*, 2010). Recessive alleles of *OsSWEET11* exist that are unresponsive to PthXo1 and comprise the *R* gene locus known as *xa13* (Chu *et al.*, 2006). Plants with *xa13* are resistant to the *Xoo* strains that rely solely on the PthXo1 as the essential effector for virulence (Yang *et al.*, 2006). The *xa13*-mediated resistance to rice bacterial blight can be defeated by induction of any one of five members of the clade III of SWEET proteins (Streubel *et al.*, 2013). *Os11N3,* now *OsSWEET14*, is targeted by *Xoo* strains harbouring TAL effectors AvrXa7, PthXo3, TalC and Tal5 (Antony *et al.*, 2010; Streubel *et al.*, 2013; Yu *et al.*, 2011). PthXo6 and PthXo7 are two other TAL effectors from PXO99^A and target, respectively, to two transcription factor genes, *OsTFX1* and *OsTFIIAγ1*, in rice (Sugio *et al.*, 2007). *OsTFX1* encodes a bZIP transcription factor, whereas the gene product of *OsTFIIAγ1* is the small subunit of the transcription factor IIA (Sugio *et al.*, 2007). The induction of *OsTFIIAγ1* located on chromosome 1 by PthXo7 may reflect the adaptation of PXO99^A to the resistance mediated by *xa5* (Iyer and McCouch, 2004), an allele of *OsTFIIAγ5* encoding a second form of the TFIIA small subunit on chromosome 5 of rice (Sugio *et al.*, 2007). AvrXa27 and *Xa27* are a cognate pair of TAL effector and host resistance (*R*) gene from *Xoo* and rice, respectively, that requires full TAL effector function to trigger resistance (Gu *et al.*, 2005). A 16- to 18-bp DNA *cis*-element, termed as $UPT_{AvrXa27}$ (*UP*regulated by *T*AL effector AvrXa27) (Romer *et al.*, 2009) or $EBE_{AvrXa27}$ (Tian *et al.*, 2014), was identified in the promoter of *Xa27* to be specifically recognized by AvrXa27. *Xa10* is another TAL effector-dependent *R* gene that confers narrow-spectrum race-specific resistance to a few Philippine races of *Xoo* (Gu *et al.*, 2008; Yoshimura *et al.*, 1995). The cognate avirulence (*Avr*) gene *avrXa10* from the *Xoo* strain PXO86 encodes a TAL effector containing 15.5 repeats that targets to $EBE_{AvrXa10}$ in *Xa10* promoter (Hopkins *et al.*, 1992; Tian *et al.*, 2014). The *Xa10* gene product XA10 is an ER membrane protein, which triggers calcium depletion in the ER and induces host cell death during the host–pathogen interaction (Tian *et al.*, 2014). Here, we report the genetic engineering of *Xa10* promoter for broad-spectrum and durable resistance to *Xoo* by adding five *EBEs* to the promoter of *Xa10*, which include binding sites to virulent TAL effectors PthXo1, PthXo6 and PthXo7 and avirulent TAL effectors AvrXa10 and AvrXa27.

Results

Production of transgenic $Xa10^{E5}$ rice plants

A 142-bp DNA fragment containing five tandem *EBEs*, including EBE_{PthXo7}, EBE_{PthXo1}, $EBE_{AvrXa10}$, EBE_{PthXo6} and $EBE_{AvrXa27}$, that are respondent to the corresponding TAL effectors PthXo7, PthXo1, AvrXa10, PthXo6 and AvrXa27 was used to replace $EBE_{AvrXa10}$ and its adjacent flanking sequences in the native promoter of *Xa10* (Figure 1; Table 1). The *EBE*-amended *Xa10* gene, designated as $Xa10^{E5}$, was reconstructed based on the 4671-bp *Avr*II-*Sac*I genomic clone of the *Xa10* gene. Similar to the *Xa10* gene, the $Xa10^{E5}$ gene consists of a 2456-bp promoter, a 381-bp open reading frame, a 378-bp terminator and a 1456-bp downstream region. Theoretically, $Xa10^{E5}$ would show recognition or resistance specificity to the *Xoo* strains that harbour the five corresponding TAL effectors.

$Xa10^{E5}$ was used to produce transgenic rice plants through *Agrobacterium*-mediated transformation of the japonica cultivar Nipponbare. Eight independent transgenic T_0 plants were regenerated from 1896 independent hygromycin-resistance calli. Disease evaluation of the T_0 plants with the *Xoo* strain PXO99^A (pHM1avrXa10) indicated that two T_0 plants, T_0-2 and T_0-5, conferred resistance to the *Xoo* strain. T_0-5 had stress-related phenotypes, including lesion mimics, stiff leaves and growth retardation. The stress-related phenotypes of T_0-5 progeny in the T_2 or T_3 generations became more severe in homozygous transgenic plants. The progeny of T_0-5 was eventually abandoned in the further study. T_0-2 showed normal morphological phenotype, growth duration and yield compared to that of Nipponbare.

Genetic and molecular characterization of T_0-2

Southern blot analysis using the hygromycin phosphotransferase (*hpt*) gene probe identified at least 6 copies of T-DNA in the genome of T_0-2 (data not shown). These T-DNAs were separated in the T_1 and subsequent generations (Figure 2a and b). However, only one copy of the T-DNAs, which showed the hybridized band of the *hpt* gene at about 5.0 kb in Southern blot analysis, co-segregated with the resistant phenotype (Figure 2a and b). Five T_2 plants (T_2-27, T_2-36, T_3-39 and T_2-40 and T_2-43) that only carried the T-DNA with functional $Xa10^{E5}$ gene(s) were obtained from T_1-80 (Figure 2b). Among which, T_2-36 showed no segregation of the *hpt* gene in the T_3 generation (data not shown). T_2-36 had two hybridization bands of the $Xa10^{E5}$ gene with the size at about 5.0 and 7.4 kb, respectively (Figure 2c). The two bands co-segregated with each other as well as with the resistance phenotype in the T_3 generation (Figure 2c). However, neither of the two bands matched the expected size of the *BamH*I-*Xba*I fragment of the $Xa10^{E5}$ gene at 2866 bp (Figures 1 and 2c). The structure of $Xa10^{E5}$ was further determined by PCR amplification and DNA sequencing of a contiguous 3649-bp fragment recovered from T_2-36. The 3649-bp fragment comprises an intact 2456-bp $Xa10^{E5}$ promoter, a 381-bp open reading frame of *Xa10*, an intact 378-bp *Xa10* terminator and a 434-bp region downstream of the *Xa10* promoter. The remaining 1022-bp fragment at the downstream of the 434-bp region in the 4671-bp *Avr*II-*Sac*I clone of $Xa10^{E5}$ could not be amplified from T_2-36 due to truncated T-DNA insertion. In summary, the genetic and molecular analyses demonstrated that T_2-36 has two truncated T-DNAs, which were inserted to the same locus or closely related locations in Nipponbare genome. Each truncated T-DNA carries a $Xa10^{E5}$ gene, but only one of them contains the

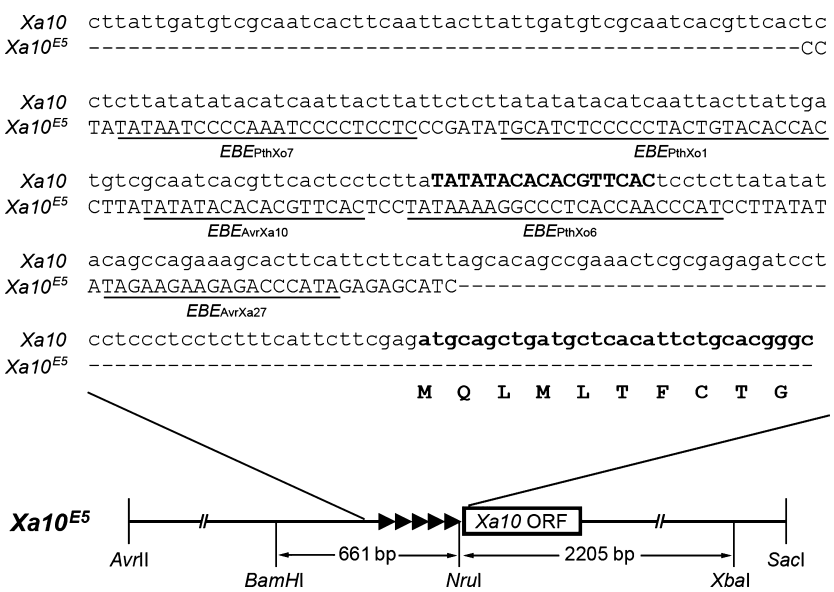


Figure 1 Schematic map of *Xa10*^{E5} and the nucleotide sequences of *Xa10* and *Xa10*^{E5} at the promoter regions. The nucleotide sequences of *Xa10* promoter and partial open reading frame (bold) are shown in lower-case letters except that *EBE*$_{AvrXa10}$ is displayed in bold upper-case letters. The engineered region in the *Xa10*^{E5} promoter is shown in capital letters with *EBE*$_{PthXo7}$, *EBE*$_{PthXo1}$, *EBE*$_{AvrXa10}$, *EBE*$_{PthXo6}$ and *EBE*$_{AvrXa27}$ underlined. Other nucleotide sequences in the *Xa10*^{E5} promoter and coding region that are identical to that of *Xa10* are shown with '—'. The restriction sites of *Avr*II, *BamH*I, *Nru*I, *Xba*I and SacI, and the size of DNA restriction fragments in *Xa10*^{E5} are indicated. Map was not drawn to scale. *Xa10* ORF, open reading frame of the *Xa10* gene.

Table 1 TAL effectors and the corresponding *EBE*s used in this study

TAL effector	*EBE*[†]	References
AvrXa10	TATATACACACGTTCAC	(Hopkins *et al.*, 1992; Tian *et al.*, 2014)
AvrXa27	TAGAAGAAGAGACCCATA	(Gu *et al.*, 2005; Romer *et al.*, 2009; Tian *et al.*, 2014)
PthXo1	TGCATCTCCCCCTACTGTACACCAC	(Boch *et al.*, 2009; Yang *et al.*, 2006)
PthXo6	TATAAAAGGCCCTCACCAACCCAT	(Boch *et al.*, 2009; Sugio *et al.*, 2007)
PthXo7	TATAATCCCCAAATCCCCTCCTC	(Boch *et al.*, 2009; Sugio *et al.*, 2007)

[†]*EBE*$_{PthXo1}$, *EBE*$_{PthXo6}$ and *EBE*$_{PthXo7}$ were identified based on the comparison between the effector binding sequences predicted by Boch *et al.* (2009) and the nucleotide sequences of the targeted gene promoters.

hpt gene. At least one of the two *Xa10*^{E5} genes, which is intact and only has DNA deletion at the downstream of the *Xa10* terminator, is fully functional. T_2-36 and progeny were designated as Xa10^{E5}NB for further studies.

Xa10^{E5} confers race-specific resistance to *Xoo* strain 1947 harbouring the corresponding TAL effectors at all developmental stages

Our initial disease evaluation experiments showed that the African *Xoo* strain 1947 (1947) caused susceptible phenotype on both Xa10^{E5}NB and Nipponbare plants (data not shown). The result indicated that 1947 does not carry any one of the 5 TAL effectors that can target the corresponding *EBE*s in the *Xa10*^{E5} promoter. Cosmid constructs that contain either one of the five TAL effector genes or empty vector pHM1 were introduced into 1947, respectively. The resulted *Xoo* strains were used to inoculate Nipponbare and Xa10^{E5}NB plants. The Xa10^{E5}NB plants conferred high resistance to 1947(pHM1avrXa10), 1947 (pHM1avrXa27), 1947(pHM1pthXo1), 1947(pHM1pthXo6) and 1947(pHM1pthXo7), whereas Nipponbare was susceptible to all these *Xoo* strains (Figure 3). In control experiments, both Xa10^{E5}NB and Nipponbare were susceptible to 1947(pHM1) (Figure 3). We previously found that *Xa10* provides resistance to PXO99^{A}(pHM1avrXa10) at all developmental stages of rice (Gu *et al.*, 2008). Disease evaluation showed that the Xa10^{E5}NB plants provided high resistance to the 5 *Xoo* strains harbouring the corresponding TAL effectors at the developmental stages from the seedling stage at 2 weeks after sowing to the booting stage at 6 weeks after sowing (Figure 4). The results demonstrated that, similar to the *Xa10* gene, *Xa10*^{E5} provides resistance to *Xoo* at all developmental stages.

Xa10^{E5} was specifically induced by *Xoo* strain 1947 harbouring the corresponding TAL effectors

The induction of *Xa10* in Xa10NB and *Xa10*^{E5} in Xa10^{E5}NB by individual TAL effectors was detected by qRT–PCR. In control experiments, no *Xa10* or *Xa10*^{E5} transcripts were detected in Xa10NB or Xa10^{E5}NB plants after mock inoculation or inoculation with 1947(pHM1), indicating that there was no TAL effector-independent *Xa10* or *Xa10*^{E5} expression in the Xa10NB or Xa10^{E5}NB plants (Figure 5). *Xa10* in Xa10NB was only induced by 1947(pHM1avrXa10), while *Xa10*^{E5} in Xa10^{E5}NB plants was induced by 1947 harbouring either one of the 5 corresponding TAL effectors (Figure 5). The levels of *Xa10*^{E5} transcripts in Xa10^{E5}NB plants at 24 h after inoculation (HAI) with 1947 (pHM1pthXo1) (1.11-fold), 1947(pHM1pthXo6) (1.29-fold) or 1947(pHM1avrXa27) (1.33-fold) were slightly higher than that of *Xa10* transcripts in the Xa10NB plants at 24 HAI with 1947 (pHM1avrXa10), while the levels of *Xa10*^{E5} transcripts were lower in the Xa10^{E5}NB plants inoculated with 1947(pHM1pthXo7) (0.37-fold) and 1947(pHM1avrXa10) (0.41-fold), possibly due to the less efficiency of the interaction between the two TAL effectors and its cognate *EBE*s in the *Xa10*^{E5} promoter for gene activation than that of the other three pairs (Figure 5). Nevertheless, the *Xa10*^{E5} transcripts induced by the TAL effectors in the latter two interactions were still sufficient to provide complete resistance to 1947(pHM1pthXo7) or 1947(pHM1avrXa10) (Figures 3 and 4b,f). The results demonstrated that the 5 *EBE*s in the *Xa10*^{E5} promoter respond specifically and efficiently to the corresponding 5 TAL effectors.

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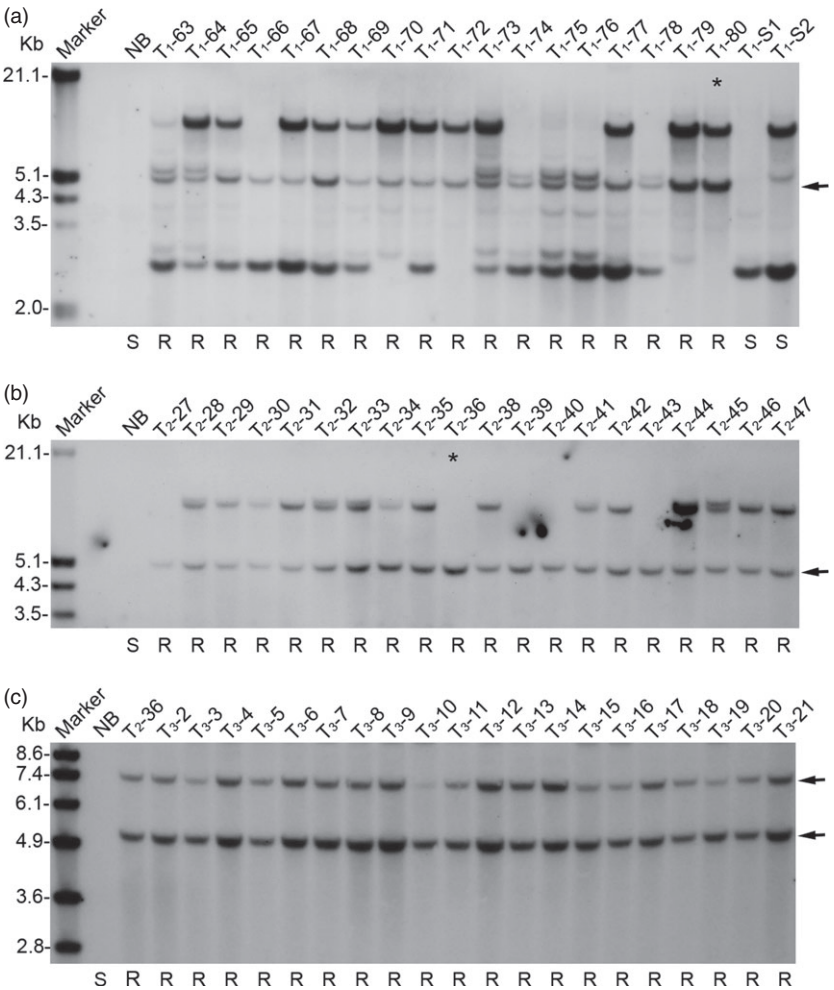


Figure 2 Southern blot analysis of transgenic plants. (a) Southern blot analysis of T_1 progeny of T_0-2 using *hpt* probe. (b) Southern blot analysis of T_2 progeny of T_1-80 using *hpt* probe. (c) Southern blot analysis of T_2-36 and its T_3 progeny using *Xa10*^{E5} probe. Southern blot analyses were carried out using about 2 μg of genomic DNA digested with restriction enzymes *BamH*I and *Xba*I and separated in 0.8% agarose gel. The phenotypes of plants were shown below the images of Southern blot analysis. Arrows indicate the DNA hybridization bands that co-segregated with the disease resistance phenotype.

Xa10^{E5} confers broad-spectrum resistance to multiple *Xoo* strains

To test if *Xa10*^{E5} also functions in the genetic background of indica rice and, more practically, to engage *Xa10*^{E5} in a rice breeding program in developing new parental lines of Chinese hybrid rice, we introduced *Xa10*^{E5} in Xa10^{E5}NB into cultivar 9311, an elite paternal line of Chinese two-line hybrid rice, through crossing and backcrossing using 9311 as the recurrent female lines. The BC_6F_2 plants that did not show segregation of the *Xa10*^{E5} gene in the BC_6F_3 generation was selected and designated as Xa10^{E5}9311. The Xa10^{E5}9311 plants showed similar morphological phenotype, growth duration and yield to that of 9311 (Figure 6). To detect the resistance spectrum of *Xa10*^{E5} in indica and japonica backgrounds, Xa10^{E5}9311, Xa10^{E5}NB and the control lines, including IR24, IRBB10A, Nipponbare and 9311 were challenged with 28 *Xoo* strains, which were collected from 11 countries. IR24 was susceptible to 27 of the 28 strains tested and only showed moderate resistance to Aust-2031 (Table 2). Similar to that of IR24, Nipponbare was susceptible to 26 of the 28 strains tested and only showed moderate resistance to GD1358 and CIAT1185 (Table 2). Cultivar 9311 conferred complete or moderate resistance to 8 strains [Aust-2031, Aust-R3, HB17, ZHE173, T7174, K202, PXO112(R5) and Thai R2] but was susceptible to the remaining 20 *Xoo* strains (Table 2). The *Xa10* line IRBB10A displayed complete resistance to PXO86(R2) and PXO112(R5) and moderate resistance to Aust-2031, but were susceptible to other 25 *Xoo* strains (Table 2). Both Xa10^{E5}NB and Xa10^{E5}9311 showed high resistance to 27 *Xoo* strains and were only susceptible to 1947 (Table 2). The inoculated Xa10^{E5}NB and Xa10^{E5}9311 plants only displayed HR phenotype with very short disease lesion (~0.1 cm) at the infection sites (Table 2; Figure 3).

Discussion

Xa10 and *Xa27* have been demonstrated to act as TAL effector-dependent *R* genes (Gu *et al.*, 2005; Tian *et al.*, 2014). We chose *Xa10* rather than *Xa27* for promoter engineering for broad-spectrum and durable resistance to *Xoo* due to the following reasons: (i) *Xa10* induces stronger HR with faster cell death and shorter lesions than *Xa27* in incompatible interactions (Gu *et al.*, 2004, 2008; Tian *et al.*, 2014); (ii) XA10 functions broadly than XA27. XA10 induces cell death by disruption of ER and Ca^{2+} homoeostasis and functions broadly across plant and animal kingdoms (Tian *et al.*, 2014), whereas XA27 localizes to apoplast and may be involved in apoplast- and cell wall-associated defence and its function in other plant species remains to be detected (Gu *et al.*, 2005; Tian *et al.*, 2014; Wu *et al.*, 2008); (iii) *Xa10* confers full disease resistance to *Xoo* at all developmental stages of rice (Gu *et al.*, 2008), whereas *Xa27* only provides complete disease resistance to the pathogen at adult stage or at 5 weeks after germination (Gu *et al.*, 2004); (iv) *Xa10*-mediated disease resistance to *Xoo* is not affected by rice genetic background (Gu *et al.*, 2008; Tian *et al.*, 2014), while the near-isogenic line of

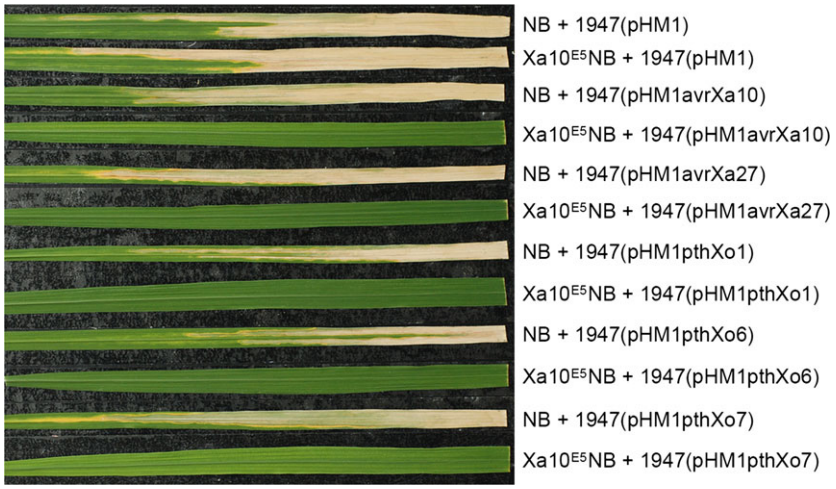


Figure 3 Bacterial blight phenotype of Nipponbare and Xa10^{E5}NB plants after inoculation with *Xoo* strains. Six-week-old Nipponbare and Xa10^{E5}NB plants were inoculated with *Xoo* strains 1947(pHM1), 1947(pHM1avrXa10), 1947 (pHM1avrXa27), 1947(pHM1pthXo1), 1947 (pHM1pthXo6) or 1947(pHM1pthXo7). Image was taken at 2 weeks after inoculation. NB, Nipponbare; Xa10^{E5}NB, homozygous *Xa10^{E5}* plants derived from T_2-36 plants.

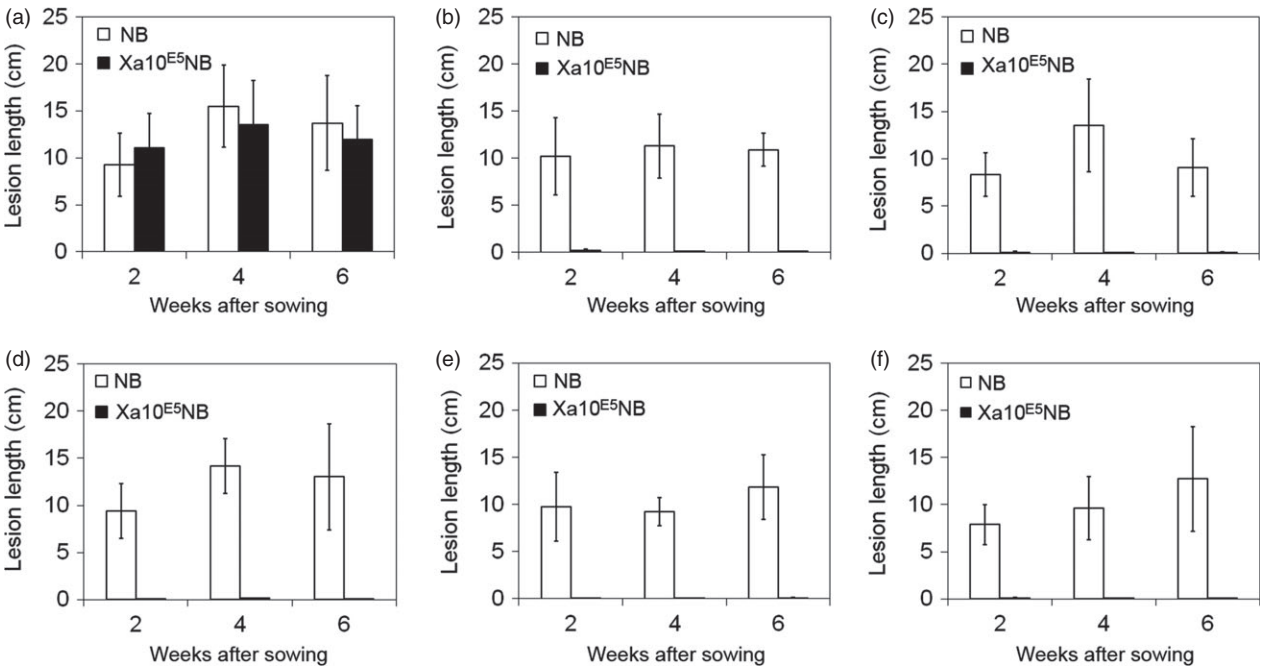


Figure 4 Resistance of *Xa10^{E5}* to *Xoo* strains at different developmental stages of rice. Nipponbare and Xa10^{E5}NB plants at the developmental stages from 2 to 6 weeks were inoculated with *Xoo* strains 1947(pHM1) (a), 1947(pHM1avrXa10) (b), 1947(pHM1avrXa27) (c), 1947(pHM1pthXo1) (d), 1947 (pHM1pthXo6) (e) or 1947(pHM1pthXo7) (f), and disease phenotype was scored at 2 weeks after inoculation. The lesion lengths of bacterial blight are the average values of 6–12 inoculated leaves with standard deviations.

Xa27 in Nipponbare genetic background is susceptible to *Xoo* strain PXO99^{A}, which harbours AvrXa27 (K. Gu and Z. Yin, unpublished data); (v) The *Xa27* native promoters show high levels of AvrXa27-independent gene expression in transgenic plants, which is different from the strictly controlled AvrXa27-dependent *Xa27* expression in wild-type IRBB27 plants (Gu *et al.*, 2005; Wu *et al.*, 2008). These *Xa27* transgenes or its derivatives might still be respondent to AvrXa27; however, the AvrXa27-independent *Xa27* expression usually results in stress phenotype and yield loss on transgenic plants, which also made it difficult to do disease evaluation. The reasons for AvrXa27-independent *Xa27* expression in transgenic plants are complicated and remain to be addressed. The *Xa10* gene under its native promoters in transgenic plants might also show AvrXa10-independent gene expression. However, *Xa10* would cause lethal phenotype on rice if it is expressed constitutively (Tian *et al.*, 2014). This *Xa10*-induced cell death can be used as negative selection during plant transformation as the transformed cells showing AvrXa10-independent *Xa10* expression would be eliminated. Therefore, the chance to obtain transgenic plants with normal morphological phenotype and AvrXa10-dependent *Xa10* expression would be higher. Indeed, only two resistant transgenic T_0 plants were obtained in this study, one of them showed strictly controlled TAL effector-dependent *Xa10^{E5}* expression. Our results demonstrated again that *Xa10* is a strong cell death inducer, belonging to so-called 'executor' *R* gene (Bogdanove *et al.*, 2010), whereas *Xa27* is more like a TAL effector-dependent 'defence' gene, which may have been evolved from a downstream defence gene involving in

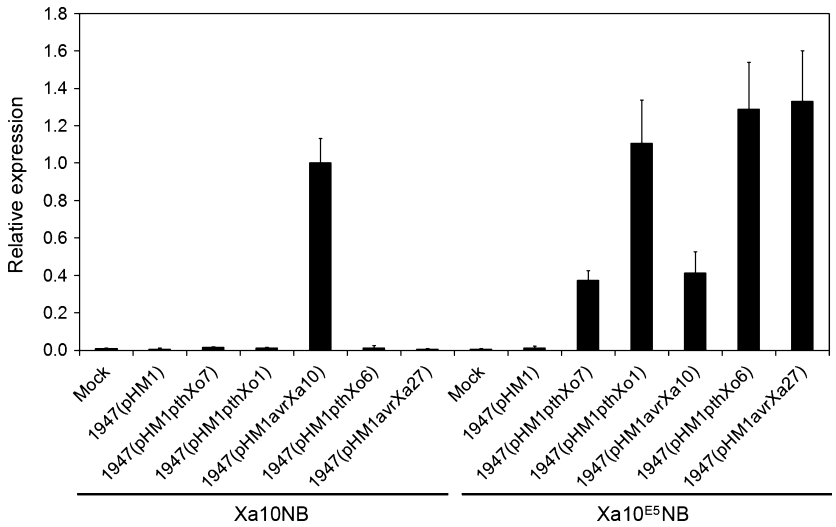


Figure 5 Expression of *Xa10* in Xa10NB and *Xa10^{E5}* in Xa10^{E5}NB after inoculation with *Xoo* strains. The rice plants were infiltrated with water (Mock) or *Xoo* strains 1947(pHM1), 1947 (pHM1pthXo7), 1947(pHM1pthXo1), 1947 (pHM1avrXa10), 1947(pHM1pthXo6) or 1947 (pHM1avrXa27). *Xa10* transcripts in Xa10NB plants and *Xa10^{E5}* transcripts in Xa10^{E5}NB plants at 24 h after inoculation (HAI) with *Xoo* strains were detected by qRT–PCR. The expression level of *Xa10* in Xa10NB at 24 HAI with 1947 (pHM1avrXa10) was set as '1'. The expression of rice ubiquitin gene 1 (*Ubi1*) was used as the internal control.



Figure 6 Morphological phenotype of 9311 and Xa10^{E5}9311 plants at mature stage.

apoplast- and cell wall-associated defence after acquiring an *EBE*$_{AvrXa27}$ in its promoter. As *Xa10* is more associated with HR initiation and functions broadly in different organisms, the *R* gene has a potential of application in heterologous plant species for generating broad-spectrum resistance not only to *Xanthomonas* species but also to other plant pathogens.

AvrXa10 targets to *EBE*$_{AvrXa10}$ in the *Xa10* promoter to activate the *R* gene (Tian *et al.*, 2014). However, AvrXa10 may only be present in a few Philippine *Xoo* races (Hopkins *et al.*, 1992). On the contrary, AvrXa27 seems to be more broadly present across the geographically diverse strains of *Xoo* as the cognate *Xa27* gene conferred complete or moderate resistance to 30 of 35 *Xoo* strains collected from 11 countries (Gu *et al.*, 2004). However, neither AvrXa10 nor AvrXa27 has been detected to contribute to virulence (Tian and Yin, 2009). To engineer a *Xa10* promoter that responds to both virulent and avirulent TAL effectors, we added 5 *EBEs* to the *Xa10* promoter. The *EBE*-amended *Xa10* promoter responds to two avirulent TAL effectors, AvrXa10 and AvrXa27, and three virulent TAL effectors, PthXo1, PthXo6 and PthXo7. In addition to providing resistance to 24 *Xa10*- or *Xa27*-incompatible *Xoo* strains, the *Xa10^{E5}* conferred strong resistance to three *Xa10*- and *Xa27*-compatible *Xoo* strains, ZHE173, K202 and Thai R2 (Table 2) (Gu *et al.*, 2004). These 3 *Xoo* strains may harbour at least one of the three virulent TAL effectors, PthXo1, PthXo6 or PthXo7, which remains to be determined. Disease evaluation also demonstrated that the *Xa10^{E5}* plants are still susceptible to African *Xoo* strain 1947 (Table 2), suggesting that 1947 does not harbour any one of the five TAL effectors. Therefore, a special design for *EBEs* in the *Xa10* promoter that can respond to TAL effectors from 1947 is needed to combat against this super virulent *Xoo* strain from Africa. The absence of TAL effectors from 1947 that can activate *Xa10^{E5}* makes the *Xoo* strain suitable for detection of specific activation of the *EBE*-amended *Xa10* by individual TAL effectors used in this study. The *Xoo* strain 1947 that harbours the corresponding TAL effectors specifically activated *Xa10^{E5}* expression. Although the levels of *Xa10^{E5}* transcripts induced by different TAL effectors varied significantly, the TAL effector-activated *Xa10^{E5}* expression efficiently provided disease resistance to incompatible *Xoo* strains, including the 27 *Xoo* strains collected from 11 countries. Hummel *et al.* (2012) previously described that the *EBE*-amended *Xa27* with specificity for distinct virulent TAL effectors from *Xoo* strain PXO99^{A} and *Xoc* strain BLS256 confers resistance to a broader spectrum of *Xoo* strains and to all tested *Xoc* strains. Considering XA10 functions broadly in different organisms (Tian *et al.*, 2014), more *EBEs* with different TAL effector specificity can be employed in the future research to amend *Xa10* promoter and to generate broad-spectrum and durable *R* genes against *Xoc* infection of rice and possibly other species of *Xanthomonas* that infect other crops (Hummel *et al.*, 2012). In terms of *EBEs* for promoter engineering for resistance to *Xoo*, *EBE*$_{AvrXa7}$ and *EBE*$_{PthXo3}$ are two good candidates because AvrXa7 and PthXo3 are two important TAL effectors present in most *Xoo* strains that do not carry PthXo1 (Antony *et al.*, 2010). Similarly, *EBE*$_{AvrXa23}$ should also be particularly useful as AvrXa23 is widely distributed among *Xoo* strains (Wang *et al.*, 2014a).

The transgenic *Xa10^{E5}* line obtained in this study showed strictly controlled TAL effector-dependent *Xa10^{E5}* expression and normal morphological phenotype with no yield penalty. The *Xa10^{E5}* line provides initial material for breeding of the engineered *R* gene into other elite rice varieties for broad-spectrum and durable resistance to bacterial blight. Both

Table 2 Disease evaluation of *Xa10*^{E5} for resistance to multiple *Xoo* strains

		Lesion length and resistance score*					
Strain	Origin	IR24	IRBB10A†	NB	Xa10^{E5}NB‡	9311	Xa10^{E5}9311§
1947	Africa	27.0 ± 6.3 (S)	28.6 ± 4.8 (S)	15.8 ± 3.8 (S)	10.1 ± 0.9 (S)	6.2 ± 2.2 (MS)	3.4 ± 2.2 (MR)
Aust-2031	Australia	5.5 ± 1.4 (MR)	4.4 ± 1.6 (MR)	9.4 ± 1.8 (S)	0.2 ± 0.1 (R)	2.4 ± 0.6 (R)	0.1 ± 0.0 (R)
Aust-R3	Australia	8.6 ± 3.4 (MS)	6.8 ± 2.0 (MS)	10.9 ± 4.2 (S)	0.1 ± 0.0 (R)	5.3 ± 5.4 (MR)	0.1 ± 0.0 (R)
GD1358	China	19.0 ± 7.3 (S)	21.1 ± 3.6 (S)	4.0 ± 1.6 (MR)	0.1 ± 0.0 (R)	15.1 ± 2.1 (S)	0.1 ± 0.0 (R)
HB17	China	29.8 ± 10.3 (S)	24.7 ± 4.0 (S)	21.2 ± 2.0 (S)	0.1 ± 0.0 (R)	4.3 ± 1.4 (MR)	0.1 ± 0.0 (R)
HB21	China	19.6 ± 2.6 (S)	15.2 ± 3.5 (S)	20.8 ± 1.7 (S)	0.1 ± 0.0 (R)	10.5 ± 2.8 (S)	0.1 ± 0.0 (R)
HLJ72	China	13.0 ± 4.4 (S)	9.8 ± 3.0 (S)	12.1 ± 1.3 (S)	0.1 ± 0.0 (R)	9.4 ± 1.7 (S)	0.1 ± 0.0 (R)
JS49-6	China	27.9 ± 3.9 (S)	21.6 ± 4.0 (S)	18.3 ± 2.6 (S)	0.1 ± 0.0 (R)	8.4 ± 2.6 (MS)	0.1 ± 0.0 (R)
LN57	China	27.6 ± 3.6 (S)	25.4 ± 3.5 (S)	20.1 ± 4.8 (S)	0.1 ± 0.0 (R)	8.3 ± 2.0 (MS)	0.1 ± 0.0 (R)
NX42	China	29.4 ± 4.1 (S)	23.2 ± 4.8 (S)	20.6 ± 2.2 (S)	0.1 ± 0.0 (R)	6.7 ± 1.5 (MS)	0.1 ± 0.0 (R)
ZHE173	China	24.2 ± 5.0 (S)	23.0 ± 4.5 (S)	14.5 ± 2.8 (S)	0.1 ± 0.0 (R)	5.0 ± 2.5 (MR)	0.1 ± 0.0 (R)
CIAT1185	Columbia	18.1 ± 4.8 (S)	13.6 ± 3.5 (S)	4.6 ± 2.1 (MR)	0.1 ± 0.0 (R)	11.4 ± 3.2 (S)	0.1 ± 0.0 (R)
A3842	India	22.4 ± 3.8 (S)	21.5 ± 3.9 (S)	16.6 ± 3.4 (S)	0.1 ± 0.0 (R)	14.1 ± 2.5 (S)	0.1 ± 0.0 (R)
A3857	India	19.1 ± 2.7 (S)	18.3 ± 4.6 (S)	19.0 ± 1.9 (S)	0.1 ± 0.0 (R)	28.1 ± 2.7 (S)	0.1 ± 0.0 (R)
IXO56	Indonesia	24.1 ± 5.2 (S)	23.9 ± 3.6 (S)	13.3 ± 2.5 (S)	0.1 ± 0.0 (R)	19.4 ± 8.4 (S)	0.1 ± 0.0 (R)
H75373	Japan	25.7 ± 5.7 (S)	24.4 ± 4.3 (S)	17.9 ± 2.0 (S)	0.1 ± 0.0 (R)	7.7 ± 2.7 (MS)	0.1 ± 0.0 (R)
T7174	Japan	23.3 ± 3.6 (S)	19.1 ± 3.0 (S)	19.7 ± 2.1 (S)	0.2 ± 0.1 (R)	5.0 ± 2.5 (MR)	0.1 ± 0.0 (R)
JW89011	Korea	28.6 ± 5.2 (S)	26.6 ± 5.1 (S)	16.7 ± 2.3 (S)	0.1 ± 0.0 (R)	8.9 ± 1.7 (MS)	0.1 ± 0.0 (R)
K202	Korea	25.6 ± 3.9 (S)	26.3 ± 4.3 (S)	12.3 ± 2.3 (S)	0.1 ± 0.0 (R)	2.1 ± 0.7 (R)	0.1 ± 0.0 (R)
NXO260	Nepal	22.4 ± 3.4 (S)	22.4 ± 4.5 (S)	16.6 ± 2.0 (S)	0.1 ± 0.0 (R)	21.5 ± 4.6 (S)	0.1 ± 0.0 (R)
PXO86(R2)	Philippines	28.1 ± 4.2 (S)	0.2 ± 0.1 (R)	12.7 ± 2.1 (S)	0.1 ± 0.0 (R)	20.2 ± 3.1 (S)	0.1 ± 0.1 (R)
PXO79(R3)	Philippines	22.1 ± 3.9 (S)	20.2 ± 4.1 (S)	6.7 ± 2.3 (MS)	0.1 ± 0.0 (R)	14.8 ± 3.5 (S)	0.1 ± 0.0 (R)
PXO71(R4)	Philippines	23.1 ± 3.3 (S)	23.2 ± 4.7 (S)	15.9 ± 1.8 (S)	0.1 ± 0.0 (R)	7.4 ± 1.7 (MS)	0.1 ± 0.0 (R)
PXO112(R5)	Philippines	13.4 ± 2.5 (S)	0.1 ± 0.0 (R)	11.8 ± 3.1 (S)	0.1 ± 0.0 (R)	2.3 ± 1.6 (R)	0.1 ± 0.0 (R)
PXO113(R4)	Philippines	18.5 ± 2.1 (S)	18.6 ± 3.7 (S)	11.0 ± 3.6 (S)	0.1 ± 0.0 (R)	7.7 ± 2.0 (MS)	0.1 ± 0.0 (R)
PXO99(R6)	Philippines	23.4 ± 3.3 (S)	23.2 ± 2.9 (S)	14.2 ± 3.1 (S)	0.1 ± 0.0 (R)	21.3 ± 3.5 (S)	0.1 ± 0.0 (R)
Thai R2	Thailand	25.4 ± 3.6 (S)	26.4 ± 3.9 (S)	18.6 ± 4.4 (S)	0.1 ± 0.0 (R)	1.1 ± 0.6 (R)	0.1 ± 0.0 (R)
Thai R7	Thailand	10.0 ± 6.5 (S)	6.9 ± 2.7 (MS)	9.9 ± 2.3 (S)	0.1 ± 0.0 (R)	6.3 ± 0.8 (MS)	0.1 ± 0.0 (R)

*Six-week-old rice plants were inoculated with *Xoo* strains. Lesion length and disease phenotype of the inoculated plants were scored at 2 weeks after inoculation. For disease scoring: R, resistant, lesion length ≤3.0 cm; MR, moderately resistant, 3.0 cm <lesion length ≤6.0 cm; MS, moderately susceptible, 6.0 cm <lesion length ≤9.0 cm; S, susceptible, lesion length >9.0 cm.

†IRBB10A, near-isogenic line of *Xa10* in IR24 genetic background (Gu *et al.*, 2008).

‡Xa10^{E5}NB, homozygous transgenic *Xa10*^{E5} plants in Nipponbare genetic background derived from T_2-36.

§Xa10^{E5}9311, homozygous transgenic *Xa10*^{E5} plants (BC_6F_3) in 9311 genetic background derived from the cross between 9311 and Xa10^{E5}NB followed by backcrossing with 9311 (recurrent female) for 6 generations and self-pollination for 2 generations.

Xa10^{E5}NB and Xa10^{E5}9311 display similar broad-spectrum resistance to *Xoo* strains, which demonstrates that, such as *Xa10*, *Xa10*^{E5} functions in both japonica and indica genetic backgrounds. The *Xa10*^{E5} gene is stable in transgenic rice, and it consistently provided high levels of disease resistance to bacterial blight in many generations of disease evaluation (9 generations in Nipponbare and 9311 backgrounds, respectively). *Xa10*^{E5} is assumed to provide durable resistance to *Xoo* strains that rely on the 5 TAL effectors, especially PthXo1, PthXo6 and PthXo7, for virulence as loss or modification of the TAL effectors in a pathogen to evade detection by host plants will result in decrease or loss of fitness of the pathogen (Vera Cruz *et al.*, 2000).

Experimental procedures

Plants and growth conditions

IRBB10A is a near-isogenic line (NIL) of *Xa10* in the IR24 genetic background (Gu *et al.*, 2008). Nipponbare is a japonica cultivar, which is susceptible to most of the *Xoo* strains (Table 2; Gu *et al.*, 2005). Xa10NB is another near-isogenic line of Xa10 in the Nipponbare genetic background (Tian *et al.*, 2014). Rice cultivar 9311 is a paternal line that is used to produce two-line hybrid rice. Rice plants, including transgenic plants, were grown in the greenhouse at 32 °C for 12.5 h (light) and 25 °C for 11.5 h (dark).

TAL effector genes and *Xanthomonas oryzae* pv. *oryzae* strains

TAL effector genes, *avrXa10*, *avrXa27*, *pthXo1*, *pthXo6* and *pthXo7*, have been reported previously (Gu *et al.*, 2005; Hopkins *et al.*, 1992; Sugio *et al.*, 2007; Yang *et al.*, 2006). TAL effector genes were cloned into vector pBluescript and placed under lacZ promoter. The intermediate constructs were then fused with cosmid vector pHM1 (Accession no. EF059993) at the *Hind* III site. Cosmid constructs containing the TAL effector genes were introduced into *Xoo* strains by electroporation. Twenty-eight field *Xoo* strains used in this study were collected from 11 countries (Gu *et al.*, 2004, 2008).

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Binary construct

Binary construct pCXA10E5 that contains the *Xa10*^{E5} gene (Figure 1) was made based on pCSA4671, which contains 4671-bp *Avr*II-*Sac*I genomic clone of *Xa10* in binary vector pC1300 (Tian *et al.*, 2014). The 661-bp *BamH*I-*Nru*I fragment in the native promoter of *Xa10* gene was replaced with a 661-bp synthetic *BamH*I-*Nru*I DNA fragment containing *EBE*$_{PthXo7}$, *EBE*$_{PthXo1}$, *EBE*$_{AvrXa10}$, *EBE*$_{PthXo6}$ and *EBE*$_{AvrXa27}$. pCXA10E5 was introduced into *Agrobacterium tumeficiens* strain AGL1 by electroporation.

Rice transformation

Agrobacterium-mediated transformation of Nipponbare was carried out using the method described previously (Hiei *et al.*, 1994) with slight modification. Briefly, vigorously growing embryogenic calli derived from the scutellum of mature embryos were co-cultured with *A. tumefaciens* strain AGL1(pCXA10E5) for 2 days. After co-culture, the rice calli were washed and then cultured on the selection medium (NB_0 medium containing 2 mg/L 2, 4-D, 250 mg/L cefotaxime and 50 mg/L hygromycin) at 26 °C in the dark. After 4-week selection, the hygromycin-resistant calli were excised from co-cultivated callus and transferred to regeneration medium (NB_0 medium containing 2.0 mg/L KT, 0.02 mg/L NAA and 50 mg/L hygromycin). The regenerated hygromycin-resistant plantlets were transferred to plantlet medium ($1/2MS_0$ medium with 50 mg/L hygromycin) for shoot and root elongation. Regenerated plantlets were subsequently transplanted to the soil in pots and grown in a greenhouse.

Bacterial blight inoculation and disease scoring

Bacterial inoculation was carried out using the leaf-clipping method (Kauffman *et al.*, 1973). Briefly, *Xoo* strains were grown on PSA medium (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, 16 g/L bacto-agar, and pH 7.0) for 2 days at 28 °C. The bacterial cells were collected and resuspended in sterile water at an optical density of 0.5 at OD600. The bacterial cell suspension was applied to the two youngest, but fully expanded, leaves of each tiller by clipping about 5 cm from the tips of the leaves using a pair of scissors dipped in the inoculum. The inoculated plants were grown in the greenhouse at 32 °C for 12.5 h (light) and 25 °C for 11.5 h (dark). Lesion length was measured 2 weeks after inoculation. The disease symptom was scored as described previously (Gu *et al.*, 2004).

Southern blot analysis

Rice genomic DNA was isolated from leaf tissues according to the procedures described previously (Dellaporta *et al.*, 1983). About 2 μg of DNA was digested with the appropriate restriction enzymes, separated on 0.8% agarose gel and blotted to Hybond™-N$^+$ nylon membrane (Amersham Biosciences, Piscataway, NJ). DNA hybridization was conducted according to standard procedures (Sambrook *et al.*, 1989). DNA probes were labelled with DIG system (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The DNA probe for the *hpt* gene was amplified by PCR using DNA primers Hpt-F (5′AAAAAGCCTGAACTCACCGCG3′) and Hpt-R (5′TACTTCTACACAGCCATCGGT3′). The DNA probe for *Xa10*^{E5} was amplified from the *Xa10* gene by PCR using DNA primers Xa10-F (5′CACGGGCCCCCTCCTGTTTGC3′) and Xa10-R (5′CCTCGTCGTCTTCACCAATGCAG3′), which covers 341 bp of the 381-bp open reading frame of *Xa10*.

PCR analysis and oligo primers

Quantitative reverse transcription–PCR (qRT–PCR) was carried out in accordance with the procedures described previously using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA) (Tian *et al.*, 2014). Total RNA was extracted from rice leaves at 24 h after inoculation (HAI) with *Xoo* strains. The total RNA samples were first treated with DNase I, and the first-strand cDNAs were synthesized using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. A standard reaction mixture (15 μL) contained 2 μL cDNA template, 1× SsoFast EvaGreen supermix (Bio-Rad) and 500 nM forward and reverse primers. The PCR reaction was conducted at an initial denaturing step of 98 °C for 3 min, followed by 40 cycles of 98 °C for 5 s, 55 °C for 5 s. PCR product specificity was confirmed by melting curve analysis and agarose gel electrophoresis to ensure that the PCRs were free of primer dimers. The rice ubiquitin gene 1 (*Ubi1*) was used as an internal reference to normalize the relative amount of total RNA for all samples. The qRT–PCR experiments were repeated for three times. The specific primers for *Xa10* were 10RTF2 (5′GGCATCATCTTCTCCGGCG3′) and 10RTR2 (5′GCAGCTATACGGGCATAAG3′). The specific primers for *Ubi1* were RBQ3 (5′CCAGTAAGTCCTCAGCCATG3′) and RBQ4 (5′TTTCAGACACCATCAAACCAG3′). The DNA oligo primers for PCR amplification of the *Xa10*^{E5} gene from transgenic T_2-36 were Xa10^{E5}-F1 (5′CTAGGTTTATTGGCTGAGCAATG3′) and Xa10^{E5}-R2 (5′TCCTCGCTAAATTAAAGTGATG3′).

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Conflict of interest

Temasek Life Sciences Laboratory has filed an international patent on the *Xa10*^{E5} gene.

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