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Constitutive heterologous expression of *avrXa27* in rice containing the *R* gene *Xa27* confers enhanced resistance to compatible *Xanthomonas oryzae* strains

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SUMMARY

The vascular pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and nonvascular pathogen *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) cause bacterial blight (BB) and bacterial leaf streak (BLS) diseases of rice, respectively. We have previously identified the avirulence gene *avrXa27* from *Xoo* PXO99^A, which specifically induces the expression of the rice resistance gene *Xa27*, ultimately leading to resistance against BB disease in rice. In this study, we have generated a transgenic rice line (L24) that expresses *avrXa27* constitutively under the control of the *PR1* promoter, and have examined its role in the host–pathogen interaction. L24 is not more susceptible to BB, indicating that *avrXa27* does not contribute to virulence. AvrXa27 retains avirulence activity in L24 and, after crossing with a line containing *Xa27*, progeny display phenotypic changes including inhibition of tillering, delay in flowering, stiff leaves, early leaf senescence and activation of pathogenesis-related (*PR*) genes. On challenge with a variety of compatible strains of *Xoo* and *Xoc* strain L8, lines with both *avrXa27* and *Xa27* also show enhanced resistance to bacterial infection. The induction of *Xa27* and subsequent inhibition of *Xoc* growth in *Xa27* plants are observed on inoculation with *Xoc* L8 harbouring *avrXa27*. Our results indicate that the heterologous expression of *avrXa27* in rice containing *Xa27* triggers *R* gene-specific resistance and, at the same time, confers enhanced resistance to compatible strains of *Xoo* and *Xoc*. The expression of AvrXa27 and related proteins in plants has the potential to generate broad resistance in plants.

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) cause bacterial blight (BB) and bacterial leaf streak (BLS) diseases of rice, respectively (Nino-Liu *et al.*, 2006). Despite their close relationship, *Xoo* and *Xoc* cause two distinct diseases. *Xoo* is a vascular pathogen that enters rice leaf typically through hydathodes at the leaf margin, multiplies in the intercellular spaces of the underlying epitheme, and moves to the xylem vessels to cause systemic infection (Noda and Kaku, 1999; Ou, 1985). *Xoc*, by contrast, is a nonvascular pathogen that penetrates the leaf mainly through the stomata, multiplies in the substomatal cavity, and colonizes the intercellular spaces of the parenchyma (Ou, 1985). Host genetic resistance is the most effective way to control BB in rice, and nearly 30 *R* genes or loci with race-specific resistance to *Xoo* have been identified (Nino-Liu *et al.*, 2006). Genetic resistance for BLS in rice is limited to quantitative resistance (Gnanamanickam *et al.*, 1999; Sheng *et al.*, 2005; Tang *et al.*, 2000). More recently, solutions for BLS resistance involving genetic engineering have been proposed (Zhao *et al.*, 2005).

Many plant pathogenic bacteria, including *Xoo* and *Xoc*, rely on the type III secretion system to inject virulence effectors into the host cell to cause infection (He *et al.*, 2004; Zhu *et al.*, 2000). Although most of the type III effectors have a virulence function during pathogenesis, some may betray the pathogen to plant defences during host–pathogen interaction. In the latter case, the type III effectors are called avirulence (Avr) proteins (Kjemtrup *et al.*, 2000). Avr proteins elicit a hypersensitive response in the resistant hosts that express a corresponding *R* gene. In the absence of *avr* or *R* genes, or both, no recognition takes place and disease occurs (Flor, 1971). Recently, we have isolated a pair of *avr* and *R* genes, *avrXa27* and *Xa27*, from *Xoo* PXO99^A and rice line IRBB27, respectively (Gu *et al.*, 2005). The *avrXa27* gene encodes a type III effector that is similar to members of the AvrBs3/PthA family, whereas the *Xa27* gene encodes a novel R protein. Members of the AvrBs3/PthA family of type III effectors are found in many strains of *Xanthomonas* (Bonas *et al.*, 1989; De Feyter and Gabriel, 1991; Hopkins *et al.*, 1992; Swarup *et al.*, 1991) and at least one strain of *Ralstonia solanacearum* (Salanoubat *et al.*, 2002). AvrBs3/PthA family members appear to function as gene-specific transcription factors in the host (Kay *et al.*, 2007). The member proteins are remarkably similar, except

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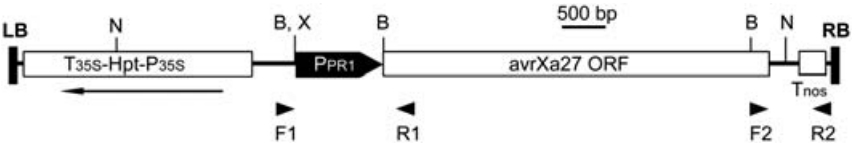

Fig. 1 Schematic map of the T-DNA region of pCPR1avrXa27. The arrow indicates the direction of transcription of the hygromycin phosphotransferase gene. Polymerase chain reaction (PCR) primers F1, R1, F2 and R2 are shown by the arrowheads. avrXa27 ORF, coding region of a*vrXa27*; B, *Bam*HI; Hpt, hygromycin phosphotransferase gene; LB, left border; N, *Nde*I; P_{35S}, promoter of cauliflower mosaic virus (CaMV) 35S gene; P_{PR1}, promoter of rice *PR1* gene; RB, right border; T_{35S}, terminator of CaMV 35S gene; Tnos, terminator of nopaline synthase (nos) gene; X, *Xba*I.

for a difference in the number and apparent nature of near-identical, 34-amino-acid, direct repeats in the central portion of the protein known as the repetitive region. These direct repeat sequences show a major variation at positions 12 and 13. The order and type of central repeats are involved in the governing of resistance specificity of a particular gene family (Bonas *et al.*, 1989; Gu *et al.*, 2005; Herbers *et al.*, 1992; Yang *et al.*, 1994). Members of the AvrBs3/PthA family also contain conserved C-terminal nuclear localization signal motifs and a functional eukaryotic transcriptional activation domain at the extreme C-terminus (Gu *et al.*, 2005; Szurek *et al.*, 2001; Van den Ackerveken *et al.*, 1996; Yang and Gabriel, 1995; Zhu *et al.*, 1998). Except for AvrBs4 (Schornack *et al.*, 2004), most of the members of the AvrBs3/PthA family require the nuclear localization signal motifs and activation domain for their virulence and/or avirulence functions (Gu *et al.*, 2005; Szurek *et al.*, 2001; Van den Ackerveken *et al.*, 1996; Zhu *et al.*, 1998).

Xa27-dependent resistance is always associated with the specific induction of *Xa27* by incompatible pathogens harbouring *avrXa27*. We have shown previously that ectopic expression of *Xa27* under the control of the rice *PR1* promoter provides enhanced resistance to otherwise compatible *Xoo* strains. In this study, we carried out functional studies of *avrXa27* in transgenic rice to investigate whether its product has a similar avirulence activity to that shown in *Xoo*, and whether the induction of *Xa27* by AvrXa27 in transgenic rice can confer enhanced resistance to compatible strains of *Xoo* and the nonvascular pathogen *Xoc*.

RESULTS

Generation of transgenic plants expressing *avrXa27*

Considering that the constitutive expression of *avrXa27* in rice might cause an unfavourable effect on rice cells, we chose the inducible promoter from the rice pathogenesis-related (*PR*) gene *PR1* to drive *avrXa27* expression in transgenic rice (Fig. 1). Thirty-four independent transgenic T_0 plants were obtained by *Agrobacterium*-mediated transformation. However, only four lines (L1, L14, L24 and L25) contained an intact *avrXa27* coding region on the basis of Southern blot hybridization analysis

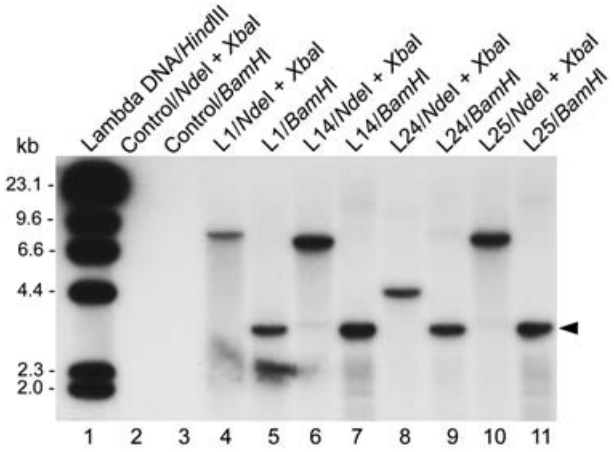

Fig. 2 Southern blot analysis of *avrXa27* in transgenic plants. The control transgenic plant (Control) was derived from the transformation of Nipponbare with pC1305.1. The Southern blot filter was probed with a ^{32}P-labelled 3234-bp *Bam*HI fragment from *avrXa27*. The arrowhead indicates the position of the *Bam*HI fragment of *avrXa27*.

(Fig. 2). The remaining transgenic plants contained only the truncated *avrXa27* coding region and were excluded from the study (data not shown). Using the 3234-bp *Bam*HI fragment of *avrXa27* as a probe, the 4.5-kb *Nde*I–*Xba*I band corresponding to the *PR1* promoter and *avrXa27* coding region was detected in L24 by Southern blot analysis (Fig. 2, lane 8). However, it was not detected in L1, L14 and L25. The *Nde*I–*Xba*I bands detected in L1, L14 and L25 were larger than 4.5 kb (Fig. 2, lanes 4, 6 and 10). At the same time, the 3.2-kb *Bam*HI band covering most of the *avrXa27* coding region was detected in all four lines (Fig. 2, lanes 5, 7, 9 and 11). We further verified the presence of the *PR1* promoter, 5′ and 3′ coding regions of *avrXa27* and the terminator of the nopaline synthase gene (Tnos) in all four lines by polymerase chain reaction (PCR) amplification, followed by DNA sequencing (data not shown). The results from PCR and Southern blot analysis indicated that L24 carried the intact P_{PR1}-*avrXa27-Tnos* expression cassette, whereas L1, L14 and L25 contained an expression cassette with a modification probably caused by DNA mutations and rearrangements in the *PR1*

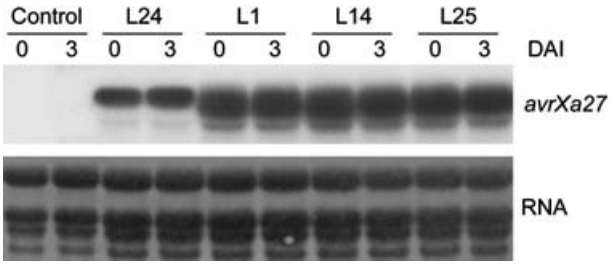

Fig. 3 Expression of *avrXa27* in transgenic plants. Total RNA was isolated from noninoculated plants (0) and plants at 3 days after inoculation (DAI) (3) with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) PXO99^{A}. Approximately 30 μg of total RNA was loaded on to each lane for Northern blot analysis. The control transgenic plant (Control) was derived from the transformation of Nipponbare with empty vector pC1305.1. L24, L1, L14 and L25 are independent transgenic *avrXa27* lines. The Northern blot filter was probed with a ^{32}P-labelled 3234-bp *BamH*I fragment from *avrXa27*.

promoter region. Southern blot analysis with the hygromycin phosphotransferase (*hpt*) gene as a probe also showed that each of the four lines carried a single T-DNA insertion (data not shown). The morphological phenotypes of the four transgenic *avrXa27* lines were similar to that of nontransgenic Nipponbare or control transgenic plants that were generated by transformation of Nipponbare with empty vector pC1305.1. The four transgenic *avrXa27* lines were selfed, and homozygous progeny were chosen for further studies.

Expression analysis of *avrXa27* in transgenic rice

Transcripts corresponding to P_{PR1}-*avrXa27-Tnos* were detected in all four lines using the 3234-bp *BamH*I fragment of *avrXa27* as a probe (Fig. 3). However, expression of the gene in L1, L14, L24 and L25 did not show significant variations in induction on inoculation with *Xoo* PXO99^{A} (Fig. 3). The wild-type mRNA molecules of P_{PR1}-*avrXa27-Tnos* produced in L24 were approximately 4 kb in Northern blot analysis (Fig. 3). In contrast with the sharp band detected in L24, less intense and diffuse bands were observed in L1, L14 and L25. The reasons for the differences in expression are unknown, and we continue only with the analysis of L24.

Activity of AvrXa27 in transgenic rice

AvrXa27 in *Xoo* specifically induces the expression of the *Xa27* gene in rice (Gu *et al*., 2005). To determine whether AvrXa27 in transgenic rice has a similar activity, the expression of *Xa27* in F_1 plants derived from crosses between IRBB27 (female) and L24 (IRBB27 × L24) or IRBB27 (female) and control transgenic plants (IRBB27 × Control) was examined (Fig. 4). Northern blot analysis indicated that *Xa27* was induced in F_1 plants from the IRBB27 × L24 cross, but not in those from the IRBB27 × Control

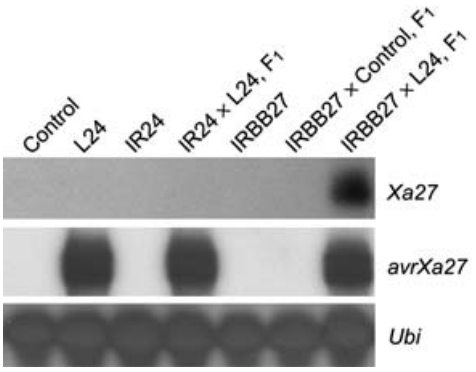

Fig. 4 Induction of *Xa27* but not *xa27* in F_1 plants by AvrXa27 in transgenic rice. About 5 μg of mRNA was loaded on to each lane for Northern blot analysis. Control, control transgenic plant derived from the transformation of Nipponbare with empty vector pC1305.1; L24, line 24 of transgenic rice carrying *avrXa27*; IR24, susceptible rice variety with the *xa27/xa27* genotype; 'IR24 × L24, F_1', F_1 plants of IR24 × L24; IRBB27, near-isogenic line of *Xa27* in IR24 background; 'IRBB27 × Control, F_1', F_1 plants of IRBB27 × Control; 'IRBB27 × L24, F_1', F_1 plants of IRBB27 × L24.

cross (Fig. 4). IR24 carries susceptible alleles of the *Xa27* gene (*xa27*) that are not induced by *Xoo* strains harbouring *avrXa27* (Gu *et al*., 2005), and Northern blot analysis indicated that *xa27* was not induced in the F_1 plants of IR24 × L24 (Fig. 4). These results indicate that P_{PR1}-*avrXa27-Tnos* in transgenic rice functions in the specific induction of *Xa27* expression.

The expression of *avrXa27* in rice also afforded the opportunity to determine whether AvrXa27 has any effect on the susceptibility of the host to bacterial infection in the absence and presence of the cognate *R* gene *Xa27*. Previously, no virulence activity has been attributed to AvrXa27 in *Xoo* (Gu *et al*., 2005). However, the expression of *avrXa27* in the plant may provide a more sensitive assay for virulence effects on host physiology. The inoculation of the susceptible rice lines Nipponbare and IR24 (*xa27/xa27*) with *Xoo* strains PXO99^{A}ME1(pHM1) and PXO99^{A}ME1(pHM1avrXa27) indicated that, as observed previously, lesions on Nipponbare or IR24 caused by PXO99^{A}ME1(pHM1avrXa27) were comparable with or even shorter than those caused by PXO99^{A}ME1(pHM1), and that AvrXa27 contributes no (or undetectable) virulence activity in *Xoo* (Fig. 5). L24 was inoculated with four strains including PXO99^{A}, AXO1947, K202 and ZHE173. The disease lesions on L24 were either comparable with or slightly shorter than those on the control transgenic or Nipponbare plants (Table 1). The results failed to detect any effect on host susceptibility of expression of P_{PR1}-*avrXa27-Tnos* in the plant.

P_{PR1}-*avrXa27-Tnos*-dependent expression of *Xa27* is associated with pleiotropic phenotypes

Although F_1 and backcross progeny of IRBB27 × L24 (BC_1F_1–BC_4F_1) did not show local lesions, other phenotypes were observed in

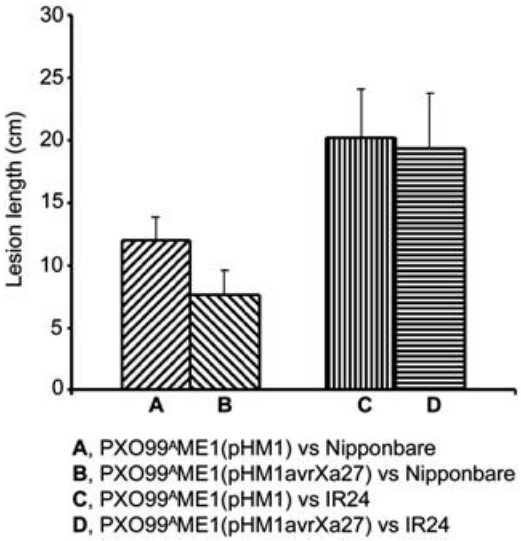

Fig. 5 Virulence activity of AvrXa27 in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) on susceptible rice. The data represent the mean and standard deviation of the lesion length from three independent experiments.

comparison with BC_1F_1 plants of IR24 × L24. The BC_1F_1 plants of IRBB27 × L24 produced fewer tillers, which was more obvious when BC_1F_1 plants were homozygous at the *Xa27* locus. The BC_1F_1 plants of IR24 × L24 (*xa27/xa27, avrXa27/–*), IRBB27 × L24 (*Xa27/xa27, avrXa27/–*) and IRBB27 × L24 (*Xa27/Xa27, avrXa27/–*) developed 10–14, 7–9 and 4–6 tillers, respectively (Fig. 6A,B). F_1 plants (*Xa27/xa27, avrXa27/–*) of IRBB27 × L24 showed a delay in flowering when compared with F_1 plants (*xa27/xa27, avrXa27/–*) of IR24 × L24. The lifespan of the F_1 plants of IRBB27 × L24 was 2 weeks longer than that of the F_1 plants of IR24 × L24. The delay in flowering and increase in lifespan became more obvious in the subsequent backcross progeny of IRBB27 × L24. In the BC_4F_1 generation, the lifespan of BC_4F_1 plants (*Xa27/xa27, avrXa27/–*) of IRBB27 × L24 was about 4 weeks longer than that of BC_4F_1 plants of IR24 × L24 (Fig. 6C). The backcross progeny of IRBB27 × L24 also developed stiff leaves. Cell wall thickening occurred at the vascular elements of the stiff leaves, but was not detected in the leaves of the backcross progeny of IR24 × L24 (Fig. 6D,E). The stiff leaves of the backcross progeny of IRBB27 × L24 also showed early leaf senescence (Fig. 6F). Interestingly, the phenotypes of stiff leaves and early leaf senescence were not as prominent in the F_1 plants of IRBB27 × L24 as in the backcrossed progeny. To determine whether the phenotypes could be associated with defence-related gene expression, the expression of *PR* genes *PR1* and *PBZ1* was measured. Northern blot analysis revealed that both genes were expressed in the backcross progeny of IRBB27 × L24 (Fig. 7).

P_{PR1}-*avrXa27-Tnos*-dependent expression of *Xa27* confers enhanced resistance to compatible *Xoo* strains

To determine whether the constitutive induction of *Xa27* by AvrXa27 in transgenic rice confers enhanced resistance to additional compatible strains of *Xoo*, F_1 plants of IRBB27 × L24 were inoculated with three strains AXO1947, K202 and ZHE173. The F_1 plants of IRBB27 × L24 showed enhanced resistance to these *Xoo* strains, whereas the parental line IRBB27 and the control F_1 plants of IRBB27 × Control were susceptible to compatible strains (Table 1). F_1 plants of IR24 × L24 were susceptible to all *Xoo* strains tested (Table 1). Compared with the complete resistance of IRBB27 to $PXO99^A$, the control F_1 plants of IRBB27 × Control showed moderate resistance to $PXO99^A$ (Table 1). The partial resistance displayed by these control F_1 plants to $PXO99^A$ may have resulted from a dosage effect and/or influence of the genetic background from Nipponbare.

Table 1 Disease evaluation of *Xa27* rice plants carrying *avrXa27* for resistance to different *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains.

	Lesion length (cm) and disease score*			
Line or plant	$PXO99^A$	AXO1947	K202	ZHE173
Nipponbare	20.5 ± 4.1 (S)	18.9 ± 4.2 (S)	16.5 ± 4.9 (S)	15.6 ± 4.0 (S)
Control†	19.6 ± 4.2 (S)	19.5 ± 2.8 (S)	16.4 ± 2.3 (S)	18.8 ± 3.6 (S)
L24	20.2 ± 2.4 (S)	16.9 ± 2.5 (S)	16.5 ± 3.1 (S)	14.0 ± 3.2 (S)
IR24	29.0 ± 3.6 (S)	27.5 ± 3.1 (S)	19.9 ± 3.5 (S)	24.5 ± 4.3 (S)
IRBB27	0.3 ± 0.2 (R)	28.6 ± 5.0 (S)	23.3 ± 2.3 (S)	27.3 ± 3.5 (S)
IR24 × L24, F_1	35.9 ± 6.6 (S)	38.1 ± 4.8 (S)	21.4 ± 2.9 (S)	25.4 ± 4.7 (S)
IRBB27 × Control, F_1	5.3 ± 6.3 (MR)	20.7 ± 3.2 (S)	20.4 ± 4.7 (S)	18.9 ± 9.6 (S)
IRBB27 × L24, F_1	0.4 ± 0.4 (R)	0.3 ± 0.2 (R)	0.1 ± 0.1 (R)	0.2 ± 0.2 (R)

*The average lesion length and standard deviation were calculated on the basis of two independent experiments. For each strain, at least 60 leaves from eight individual plants were inoculated. For the resistance score, see 'Experimental procedures'. MR, moderately resistant; R, resistant; S, susceptible.
†Control transgenic plant produced by transformation of Nipponbare with empty vector pC1305.1.

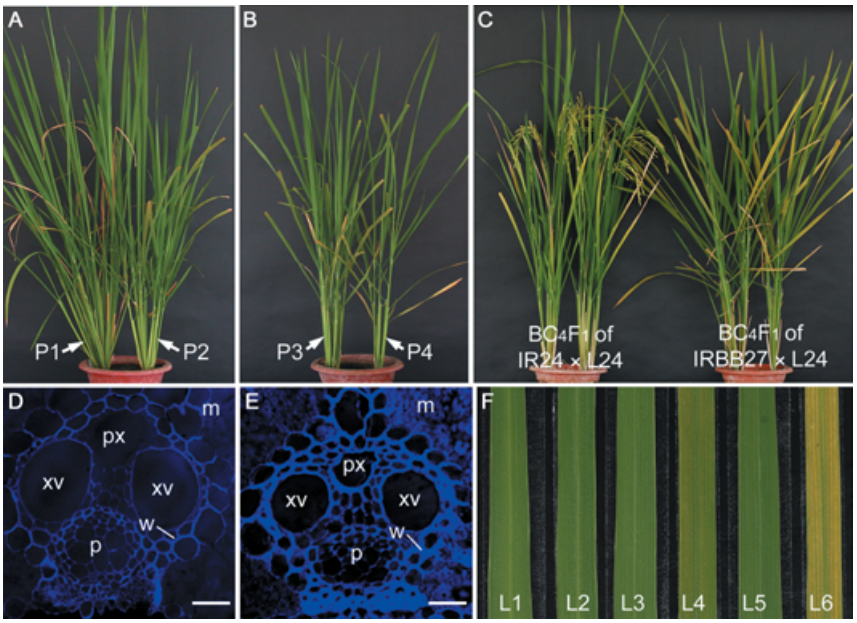

Fig. 6 Constitutive induction of *Xa27* by AvrXa27 in transgenic rice leads to pleiotropic phenotypes. (A, B) Morphological phenotype of BC_1F_1 plants of IR24 × L24 (P1) and IRBB27 × L24 (P2–P4) at 56 days after sowing. The plants were inoculated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain AXO1947 and the photographs were taken at 14 days after inoculation (DAI). Genotypes of the plants: P1, *xa27/xa27, avrXa27/–*; P2 and P3, *Xa27/xa27, avrXa27/–*; P4, *Xa27/Xa27, avrXa27/–*. (C) Morphological phenotype of BC_4F_1 plants of IR24 × L24 and IRBB27 × L24 at 95 days after sowing. (D) Cross-section of a leaf vascular bundle from a 77-day-old BC_4F_1 plant of IR24 × L24. Bar, 20 μm. (E) Cross-section of a leaf vascular bundle from a 77-day-old BC_4F_1 plant of IRBB27 × L24. Bar, 20 μm. (F) Leaves from 77-day-old BC_4F_1 plants of IR24 × L24 (L1, L3 and L5) and IRBB27 × L24 (L2, L4 and L6). Leaves L1, L3 and L5 are the first (flag leaf), second and third leaves from a typical BC_4F_1 plant of IR24 × L24, and leaves L2, L4 and L6 are the corresponding leaves from a typical BC_4F_1 plant of IRBB27 × L24. Abbreviations in (D) and (E): m, mesophyll cells; p, phloem; px, protoxylem lacuna; w, cell wall; xv, xylem vessel.

P_{PR1}-*avrXa27-Tnos*-dependent expression of *Xa27* confers enhanced resistance to rice nonvascular pathogen *Xoc*

To determine whether the constitutive expression of *Xa27* induced by *avrXa27* in rice provides enhanced resistance to the nonvascular pathogen *Xoc*, BC_3F_1 plants of IRBB27 × L24 were inoculated with *Xoc* strain L8. Control BC_3F_1 plants of IRBB27 × Control and those of IR24 × L24 were susceptible to *Xoc* L8 (Fig. 8A, leaves 1 and 2). Large BLS lesions developed towards the leaf bases and leaf tips of BC_3F_1 plants at 10 days after inoculation (DAI), and numerous yellow beads of *Xoc* exudate were observed at the lesion area. BC_3F_1 plants of IRBB27 × L24, by contrast, were resistant to *Xoc* L8 (Fig. 8A, leaf 3). The development of BLS lesions on these BC_3F_1 plants was slower than that on control BC_3F_1 plants. However, unlike *Xa27*-mediated resistance to *Xoo*, the resistance of BC_3F_1 plants of IRBB27 × L24 to *Xoc* L8 was incomplete. Yellow beads of *Xoc* were observed at the margins of the lesions on BC_3F_1 plants of IRBB27 × L24 at 10 DAI (Fig. 8A, leaf 3), and, although 63- to 7825-fold lower than the *Xoc* populations in control BC_3F_1 plants, *Xoc* populations in the inoculated leaves of BC_3F_1 plants of IRBB27 × L24 continued to grow at 10 DAI (Fig. 8B).

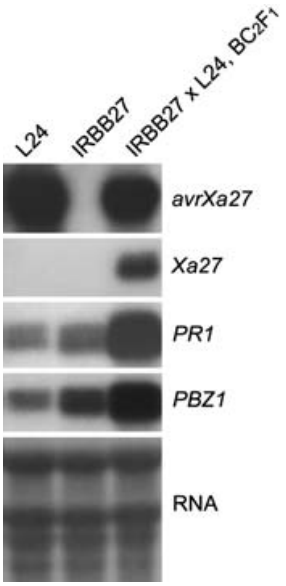

Fig. 7 Expression of *avrXa27*, *Xa27*, *PR1* and *PBZ1* in BC_2F_1 plants of IRBB27 × L24 and their parental lines. Approximately 30 μg of total RNA was loaded on to each lane for Northern blot analysis.

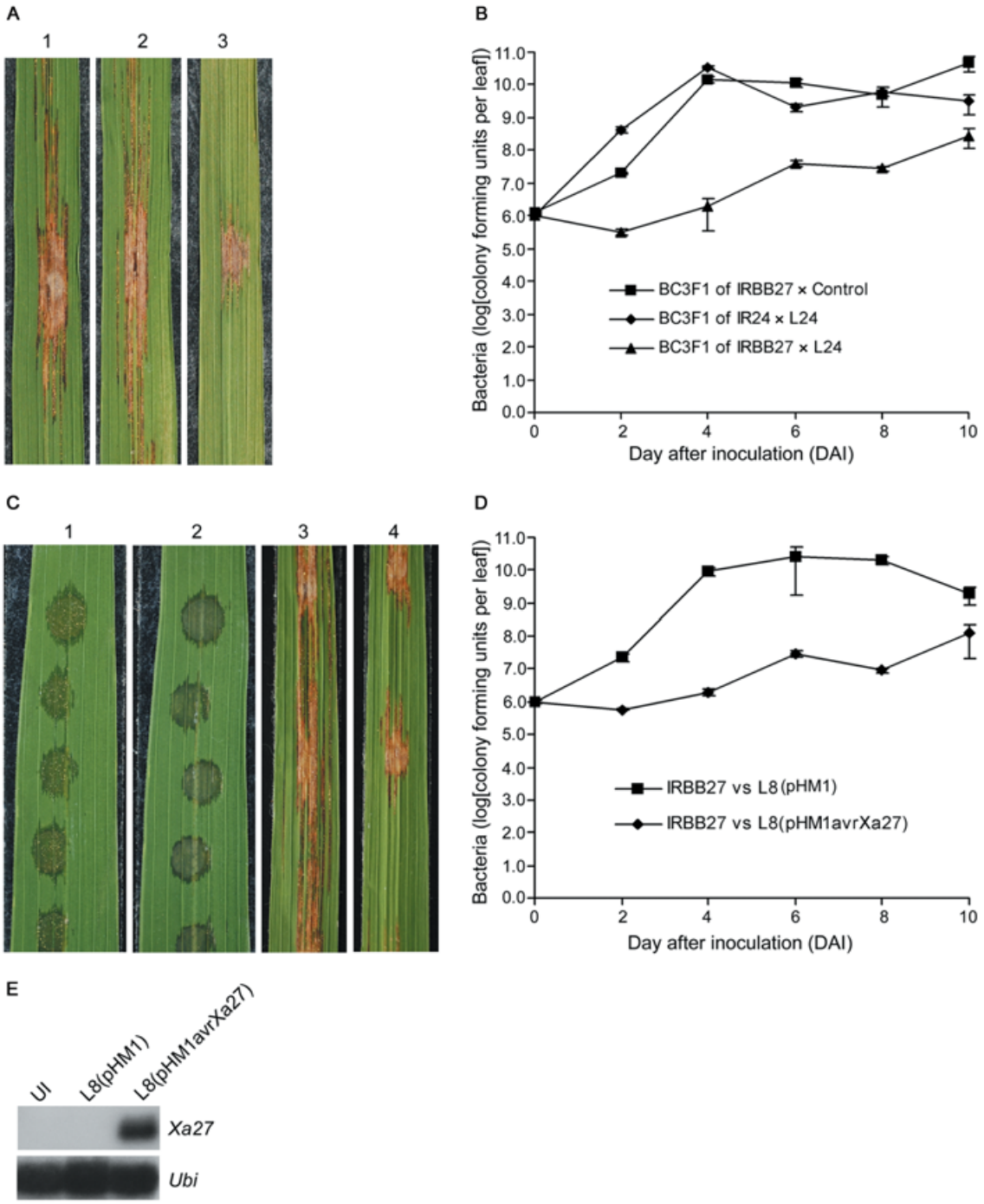

Fig. 8 Expression of the *Xa27* gene confers enhanced resistance to *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) L8. (A) Phenotype of bacterial leaf streak on leaves of BC_3F_1 plants of IRBB27 × Control (leaf 1), IR24 × L24 (leaf 2) and IRBB27 × L24 (leaf 3) at 10 days after inoculation (DAI) with *Xoc* L8. Control, control transgenic plant derived from the transformation of Nipponbare with empty vector pC1305.1. (B) Bacterial population of *Xoc* L8 in leaves of BC_3F_1 plants of IRBB27 × Control, IR24 × L24 and IRBB27 × L24 over 10 days by syringe infiltration. (C) Phenotype of bacterial leaf streak on leaves of IRBB27 plants at 3 DAI (leaves 1 and 2) and 10 DAI (leaves 3 and 4) with *Xoc* strains L8(pHM1) (leaves 1 and 3) and L8(pHM1avrXa27) (leaves 2 and 4). (D) Bacterial population of *Xoc* strains L8(pHM1) and L8(pHM1avrXa27) in leaves of IRBB27 plants over 10 days by syringe infiltration. (E) Induction of *Xa27* in leaves of IRBB27 plants by inoculation with *Xoc* strains. Lane 1, noninoculated IRBB27 plants; lane 2, IRBB27 plants infiltrated with *Xoc* L8(pHM1) at 3 DAI; lane 3, IRBB27 plants infiltrated with *Xoc* L8(pHM1avrXa27) at 3 DAI.

To further verify that the enhanced resistance of BC_3F_1 plants of IRBB27 × L24 to *Xoc* resulted from *Xa27* expression, IRBB27 plants were inoculated with *Xoc* strains L8(pHM1) and L8(pHM1avrXa27). Three days after syringe infiltration of the bacterium, leaf tissues at the infection sites in both interactions were water-soaked in appearance (Fig. 8C, leaves 1 and 2). Although few or no beads of *Xoc* were found at the infection sites on IRBB27 plants inoculated with *Xoc* L8(pHM1avrXa27) (Fig. 8C, leaf 2), many tiny yellow beads of *Xoc* were observed on IRBB27 plants inoculated with *Xoc* L8(pHM1) (Fig. 8C, leaf 1). The development of BLS lesions on IRBB27 plants inoculated with L8(pHM1avrXa27) was slower than that on IRBB27 plants inoculated with L8(pHM1) (Fig. 8C, leaves 3 and 4). *Xoc* populations in IRBB27 plants inoculated with L8(pHM1avrXa27) were 16- to 4583-fold lower than those in IRBB27 plants inoculated with L8(pHM1) (Fig. 8D). Northern blot analysis indicated that *Xa27* in IRBB27 was induced by AvrXa27 in L8(pHM1avrXa27), whereas no induction was detected when L8(pHM1) was used for inoculation (Fig. 8E).

DISCUSSION

Type III effectors can have a bifunctional role in virulence and host recognition (Alfano and Collmer, 2004). The virulence function of several type III effectors, when expressed in plants, results in cell death or necrosis and suppresses host defences in plants lacking the cognate *R* genes (Alfano and Collmer 2004; Hauck *et al.*, 2003). The gene product of *avrXa27* in PXO99^A has been shown to elicit resistance in plants containing *Xa27* (Gu *et al.*, 2005). PXO99^{A}ME1, an *avrXa27* knockout mutant in the PXO99^A background, is compatible with both IRBB27 and IR24 (Gu *et al.*, 2005). We noted that the disease lesions on IR24 caused by PXO99^{A}ME1 were slightly shorter than those caused by PXO99^A, suggesting that AvrXa27 may contribute to virulence activity (Z. Yin, unpublished data). However, recent studies have shown that an *avrXa27* paralogue called *pthXo6* is mutated together with *avrXa27* in PXO99^{A}ME1, and that *pthXo6* is responsible for the induction of the host susceptibility gene *OsTFX1*, encoding a bZIP transcription factor (Sugio *et al.*, 2007). In this study, we did not detect any virulence activity of AvrXa27 in either *Xoo* strain PXO99^{A}ME1(pHM1avrXa27) or rice. Furthermore, the BB lesion length on Nipponbare plants inoculated with PXO99^{A}ME1(pHM1avrXa27) was even shorter than that on plants challenged by PXO99^{A}ME1(pHM1). The shorter BB lesions indicate the presence of the avirulence activity of AvrXa27. However, the corresponding *R* gene that confers partial resistance to PXO99^{A}ME1(pHM1avrXa27) is yet to be identified. PXO99^A harbours 19 members of the AvrBs3/PthA family, of which *pthXo1* and *pthXo6* are known to contribute major virulence activity in rice (Salzberg *et al.*, 2008; Sugio *et al.*, 2007; Yang and White, 2004; Yang *et al.*, 2006). Evidence indicates that others may provide additional virulence activity (Bai *et al.*, 2000). The results obtained in this study indicate that AvrXa27 contributes little or no virulence activity.

P_{PR1}-*avrXa27-Tnos*-dependent expression of *Xa27* conferred enhanced resistance to compatible *Xoo* strains. Similar results were obtained in a previous study, in which ectopic expression of *Xa27* led to a broad-spectrum resistance to both compatible and incompatible strains (Gu *et al.*, 2005), indicating that the *Xa27* gene product provides nonspecific resistance to *Xoo*. The Xa27 protein is different from other characterized R proteins that function specifically in perception and/or signalling of a particular pathogen infection. It is possible that the *Xa27* gene evolved from a nonspecific resistance or defence gene, whose product confers nonspecific resistance to *Xoo*. This gene may have acquired a mimic promoter that perceives the presence of AvrXa27 and, consequently, confers specific resistance to incompatible *Xoo* strains that harbour *avrXa27*.

The constitutive expression of *Xa27* induced by AvrXa27 in BC_1F_1 rice plants or subsequent backcross progeny of IRBB27 × L24 resulted in the constitutive activation of the defence response. Although a typical hypersensitive response was not observed in these plants, pleiotropic phenotypes were developed throughout the developmental stages, and may be a side-effect of the constitutive expression of *Xa27*. The pleiotropic phenotypes were not as prominent in the F_1 plants of IRBB27 × L24 as in the backcrossed progeny, and may have been masked by the strong vegetative plant growth as a result of heterosis of the F_1 plants derived from the inter-subspecies cross between indica rice IRBB27 and japonica rice L24. The pleiotropic phenotypes, such as cell wall thickening, especially in the vascular elements, were also found in *Xa27* ectopic lines that constitutively expressed the *Xa27* gene (Gu *et al.*, 2005). Wild-type IRBB27 plants have no pleiotropic phenotype as the induction of *Xa27* by AvrXa27 in incompatible *Xoo* strains occurs only in the immediate vicinity of infected tissues. The pleiotropic phenotypes hinder application of the *R* gene for enhanced resistance to compatible *Xoo* strains lacking *avrXa27*. Enhanced resistance may be achievable by either controlling the expression of *Xa27,* as reported previously (Gu *et al.*, 2005), or by regulating the *in planta* expression of *avrXa27* in rice, as shown here. In either case, an inducible promoter that can be activated by either incompatible or compatible *Xoo* strains is required. The promoter should have strong but not leaky expression, because constitutive expression of *Xa27* causes unfavourable pleiotropic phenotypes.

The nonvascular bacterial pathogen *Xoc* is closely related to *Xoo* (Nino-Liu *et al.*, 2006). Like *Xoo*, *Xoc* produces multiple members of the AvrBs3/PthA family, but no function has yet been ascribed to these members (Yang and White, 2004). *Xoc* can deliver type III effector AvrXa10 expressed from a plasmid into rice cells as efficiently as can *Xoo* (Makino *et al.*, 2006). Given that *Xoo* and *Xoc* have many similarities, we investigated

whether the constitutive induction of *Xa27* by AvrXa27 in transgenic rice was able to provide enhanced resistance to *Xoc*. We found that the constitutive expression of *Xa27* specifically induced by AvrXa27 in transgenic rice conferred enhanced resistance to *Xoc* L8. This enhanced resistance to *Xoc* was further verified through the expression of *Xa27* specifically induced by AvrXa27 in *Xoc*. Although a single strain was tested, the results indicate that the strategy could provide broad resistance against xanthomonads. We also tested the disease resistance of the backcross progeny of IRBB27 × L24 and *Xa27* over-expressing lines to other rice pathogens, such as *Magnaporthe grisea* isolate PO6-6, the causal agent of rice fungal blast, and *Erwinia chrysanthemi* pv. *zeae* EC1, a vascular bacterial pathogen causing rice bacterial foot rot disease. However, we did not find any *Xa27*-mediated enhanced resistance to these fungal or bacterial pathogens (data not shown). Nevertheless, the finding of *Xa27*-mediated enhanced resistance to *Xoc* augments host resistance by controlling BLS in rice, which presently lacks an *R* gene for resistance. In another successful example of host resistance, transgenic rice carrying *Rxo1* from maize showed resistance to *Xoc* (Zhao *et al.*, 2005).

EXPERIMENTAL PROCEDURES

Construction of binary vector carrying *avrXa27*

The binary construct pCPR1avrXa27, which contains *avrXa27* under the control of the *PR1* promoter, was prepared using the CAMBIA vector pC1305.1, available shot-gun subclones of *avrXa27*, cosmid 99-avrXa27-20 harbouring the wild-type of *avrXa27* (Gu *et al.*, 2005), and pZWavrXa27 containing a modified *avrXa27* gene with a FLAG epitope tag in its C-terminal region (Gu *et al.*, 2005; Zhu *et al.*, 1998). Briefly, a 257-bp *BamH*I-*Pst*I fragment of the 3′ region of *avrXa27*, including a 69-bp 3′ noncoding region, was isolated from shot-gun clone V5G05 and cloned into vector pGBKT7 to generate pGavrXa27Ter. The 3234-bp *BamH*I fragment of *avrXa27* was isolated from cosmid 99-avrXa27–20 and cloned into the *BamH*I site of pGavrXa27Ter to generate pGavrXa27B1Ter. A 379-bp fragment containing the 5′ coding region of *avrXa27* was amplified from shot-gun clone V3B01 with primers A-F (5′-GGGGTACCATGGATCCCATTCGT-TCGCGC-3′) and A-R (5′-ACGCGTCGACAGTGACAGCGACACG-CACGGTG-3′). The PCR product was cloned into pGEM®-T Easy vector (Promega) to generate pTA27. A 3410-bp *Pst*I fragment, including the 3327-bp coding region of *avrXa27*, was isolated from pGavrXa27B1Ter and cloned into the *Pst*I site of pTA27 to generate pTavrXa27. The 3234-bp *BamH*I fragment of *avrXa27* in pTavrXa27 was replaced with the *BamH*I fragment of *avrXa27* from pZWavrXa27 (Gu *et al.*, 2005) to create pTZWavrXa27. The *Sac*I-*Ase*I fragment from pTZWavrXa27 was isolated, blunted and cloned downstream of the rice *PR1* promoter in pC1305.1 to generate pCPR1avrXa27. pCPR1avrXa27 was introduced to *Agrobacterium tumefaciens* AGL1 by electroporation (Sambrook *et al.*, 1989).

Xoo and *Xoc* strains

The *Xoo* and *Xoc* strains used in this study are listed in Table 2. *Xoc* strain L8 has been verified previously to be an *Xoc* strain by serological test (Zeng *et al.*, 1995). The cosmid vector pHM1 or its derivative pHM1avrXa27 was introduced into *Xoo* or *Xoc* strains by electroporation (Sambrook *et al.*, 1989).

Rice transformation and characterization of transgenic plants

Agrobacterium-mediated transformation of rice cultivar Nipponbare was carried out according to the procedures described previously (Yin and Wang, 2000). Control transgenic plants were derived from Nipponbare transformed with empty vector pC1305.1. The

Table 2 *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strains used in this study.

Strain	Description*	Reference
PXO99^{A}	*Xoo*, wild-type, *Xa27* incompatible	Gu *et al.* (2004, 2005)
AXO1947	*Xoo*, wild-type, *Xa27* compatible	Gu *et al.* (2005)
K202	*Xoo*, wild-type, *Xa27* compatible	Gu *et al.* (2004, 2005)
ZHE173	*Xoo*, wild-type, *Xa27* compatible	Gu *et al.* (2004, 2005)
PXO99^{A}ME1	*Xoo*, *avrXa27* knockout mutant	Gu *et al.* (2005)
PXO99^{A}ME1(pHM1)	*Xoo*, PXO99^{A}ME1 harbouring pHM1	This study
PXO99^{A}ME1(pHM1avrXa27)	*Xoo*, PXO99^{A}ME1 harbouring pHM1avrXa27	Gu *et al.* (2005)
L8	*Xoc*, wild-type	Zeng *et al.* (1995)
L8(pHM1)	*Xoc*, L8 harbouring pHM1	This study
L8(pHM1avrXa27)	*Xoc*, L8 harbouring pHM1avrXa27	This study

*pHM1, cosmid vector pHM1 (Hopkins *et al.*, 1992); pHM1avrXa27, pHM1 carrying *avrXa27*. pHM1avrXa27 was also designated as pZWavrXa27 (Gu *et al.*, 2005).

avrXa27 gene in transgenic plants was detected by Southern blot hybridization (Sambrook *et al*., 1989). In brief, 2–5 μg of DNA isolated from transgenic plants was digested with either restriction enzymes *Nde*I and *Xba*I, or *BamH*I, and blotted onto a nylon filter (Hybond-N$^+$, Amersham). The DNA filters were probed with a ^{32}P-labelled 3234-bp *BamH*I fragment from *avrXa27*. The copy number of T-DNA in the transgenic plants was detected by hybridizing the DNA filters with a probe from the *hpt* gene. The *PR1* promoter, 5′ and 3′ regions of *avrXa27* and Tnos in transgenic plants were isolated by PCR and verified by DNA sequencing. The DNA primers used to amplify the *PR1* promoter and 5′ region of *avrXa27* were F1 (5′-CCATGATTACGAATTC-GAGCTCGG-3′) and R1 (5′-AAGAAGCGACGGATCGAACTGAC-3′). Similarly, those used to amplify the 3′ region of *avrXa27* and Tnos were F2 (5′-CGAAGAGGAGCTCGCATGGTTGAT-3′) and R2 (5′-ACGCTCTTTTCTCTTAGGTTTAC-3′). The amplified PCR products were cloned into the pGEM®-T Easy vector (Promega) and verified by DNA sequencing.

Rice lines and genetic crossing

The rice lines used in this study were Nipponbare, a variety of japonica subspecies that does not carry the *Xa27* gene, IR24, a variety of indica subspecies that carries the susceptible allele of the *Xa27* gene (*xa27*), and IRBB27, a near-isogenic line of *Xa27* in a IR24 background (Gu *et al*., 2004). IRBB27 was used as the recurrent female to cross and backcross with transgenic *avrXa27* line L24. Similar crossings were carried out between IR24 (recurrent female) and L24 or IRBB27 (recurrent female) and the control transgenic plants, and at least four backcrosses were performed for each combination.

Northern blot analysis

Northern blot analysis was carried out according to the standard procedures described in Sambrook *et al*. (1989). Total RNA was isolated from rice leaves using the RNeasy Plant Mini Kit (Qiagen). mRNA was purified with an mRNA Midi Kit (Qiagen). Approximately 30 μg of total RNA or 5 μg of mRNA were used for each lane in Northern blot analysis. The RNA loading was assessed by staining RNA blots with methylene blue or hybridizing RNA blots with the rice ubiquitin gene 2 (*Ubi*). The probe for *avrXa27* was the 3234-bp *BamH*I fragment from the gene (Gu *et al*., 2005). The probe for the *Xa27* gene was the full-length cDNA of the gene (Gu *et al*., 2005). Probes were labelled with ^{32}P-dCTP (Amersham).

Bacterial inoculations and disease scoring

Xoo and *Xoc* strains were grown on PSA medium (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, 16 g/L bacto-agar, pH 7.0) with appropriate antibiotics for 2–3 days. The bacterial cells of *Xoo* and *Xoc* were suspended in sterile water at an optical density at 600 nm (OD_{600}) of 0.5. BB inoculation was carried out on the two youngest fully expanded leaves on each tiller of 6-week-old rice plants using the leaf-clipping method (Kauffman *et al*., 1973). The disease symptoms were scored at 14 DAI according to the criteria described previously (Gu *et al*., 2004). For BLS inoculation, fully expanded leaves of 6-week-old rice plants were infiltrated with *Xoc* suspension using a needleless syringe (Schaad *et al*., 1996). The *Xoc* population in inoculated plants was determined using the method reported by Makino *et al*. (2006) with slight modifications. In brief, infiltrated areas of rice leaves were removed and ground in 5 mL of sterile water. Serial dilutions were made and spread on to PSA agar plates with appropriate antibiotics. The plates were incubated at 28 °C until single colonies could be counted. The number of colony-forming units (CFU) per leaf was counted, and the standard deviation was calculated using colony counts from replicate experiments.

Histological analysis

Cell wall thickening at vascular elements was measured according to the method described previously (Gu *et al*., 2005). Leaf segments, 3 mm in length, were fixed in 50 mM $NaPO_4$ buffer (pH 7.4) containing 2.5% glutaraldehyde. After dehydration with ethanol, samples were embedded using a Leica Historesin Embedding Kit. The 3-μm unstained sections were examined for autofluorescence from phenolic compounds using a Zeiss LSM 510 confocal microscope (excitation, 405 nm; emission, 420–480 nm).

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