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ORIGINAL PAPER

TAL effector-dependent *Bax* gene expression in transgenic rice confers disease resistance to *Xanthomonas oryzae* pv. *oryzae*

Yuejing Gui · Dongsheng Tian · Kar Hui Ong · Joanne Chin Yi Teo · Zhongchao Yin

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Abstract The hypersensitive response (HR) is a form of programmed cell death of plant cells occurring in the local region surrounding pathogen infection site to prevent the spread of infection by pathogens. Bax, a mammalian pro-apoptotic member of Bcl-2 family, triggers HR-like cell death when expressed in plants. However, constitutive expression of the *Bax* gene negatively affects plant growth and development. The *Xa10* gene in rice (*Oryza sativa*) is an executor resistance (*R*) gene that confers race-specific disease resistance to *Xanthomonas oryzae* pv. *oryzae* strains harboring TAL effector gene *AvrXa10*. In this study, the *Xa10* promoter was used to regulate heterologous expression of the *Bax* gene from mouse (*Mus musculus*) in *Nicotiana benthamiana* and rice. Cell death was induced in *N. benthamiana* after co-infiltration with the P_{Xa10}*:Bax:*T_{Xa10} gene and the P_{PR1}*:AvrXa10:*T_{Nos} gene. Transgenic rice plants carrying the P_{Xa10}*:Bax:*T_{Xa10} gene conferred specific disease resistance to *Xa10*-incompatible *X. oryzae* pv. *oryzae* strain PXO99^{A}(pHM1AvrXa10), but not to the *Xa10*-compatible strain PXO99^{A}(pHM1). The resistance specificity was confirmed by the AvrXa10-dependent induction of the P_{Xa10}*:Bax:*T_{Xa10} gene in transgenic rice. Our results demonstrated that the inducible expression of the *Bax* gene in transgenic rice was achieved through the control of the executor *R* gene promoter and the heterologous expression of the pro-apoptosis regulator gene in rice conferred disease resistance to *X. oryzae* pv. *oryzae*.

Keywords Bacterial blight · Bax · Hypersensitive response · Rice · *Xa10*

Abbreviations

HR	Hypersensitive response
PCD	Programmed cell death
R	Resistance
EBE	Effector binding element
BAX	Bcl-2-associated X protein

Introduction

The hypersensitive response (HR) is a form of programmed cell death (PCD) of plant cells occurring in the local region surrounding the pathogen infection site to prevent the spread of infection by pathogens.

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The HR is usually associated with plant disease resistance (*R*) gene-mediated plant immunity to pathogen infection, in which the plant *R* gene or its product perceives, either directly or indirectly, the presence of the virulence gene product from the pathogen (Balint-Kurti 2019; Zhang et al. 2015). The burst of reactive oxygen species (ROS) and the generation of nitric oxide (NO) are early events during HR (Delledonne et al. 2001). Apoptosis is a form of PCD that occurs in multicellular organisms. In animals, the onset of apoptosis is tightly regulated by the interaction between anti-apoptotic and pro-apoptotic proteins belonging to Bcl-2 family (Danial 2007; Danial and Korsmeyer 2004). The Bcl-2 family proteins contain at least one of four conserved domains known as Bcl-2 homology domains (BH1-4) (Danial 2007). Bcl-2-associated X protein (Bax) is a cardinal member of the Bcl-2 protein family (Oltval et al. 1993). Bax possesses BH1, BH2 and BH3 domains and mediates apoptosis in mammalian cells. In healthy cells, Bax is a soluble monomeric protein in cytosol. The anti-apoptotic protein Bcl-2 can bind to Bax forming heterodimers through BH3 domain to prevent Bax activation (Finucane et al. 1999; Oltval et al. 1993). Upon receipt of a death stimulus, such as DNA damage or growth factor withdrawal, Bax moves intracellularly and localizes to mitochondrial outer membrane as homodimers that may form channels to release cytochrome *c* and other pro-apoptotic factors from the mitochondria, often referred to as mitochondrial outer membrane permeabilization, leading to activation of caspases (Oltval et al. 1993; Wolter et al. 1997). Although the comparative genomic studies have failed to identify Bcl-2 family homologs in plants, heterologous expression of the murine *Bax* gene in transgenic plants can trigger plant PCD and display many similar biochemical and morphological features of apoptosis (Lacomme and Santa Cruz 1999; Yoshinaga et al. 2005). In tobacco, Bax can activate plant defense response and induce cell death that is similar to the HR in tobacco initiated by the *N* gene-mediated disease resistance to tobacco mosaic virus (TMV) (Lacomme and Santa Cruz 1999). In transgenic Arabidopsis, Bax localizes to plant mitochondria and cause the loss of mitochondrial membrane potential, which is the typical feature of early apoptosis in metazoan (Yoshinaga et al. 2005).

Bacterial blight in rice, caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most destructive bacterial diseases in rice (Nino-Liu et al. 2006). Yield loss due to bacterial blight can be as much as 50% when susceptible varieties are grown in environments favourable to the pathogens (Nino-Liu et al. 2006). During infection, *X. oryzae* pv. *oryzae* strains employ Type III secretion system (T3SS) to directly inject transcription activator-like (TAL) effectors into host cells. TAL effectors activate the expression of host susceptibility (*S*) genes, such as *SWEET* genes encoding Clade 3 sugar efflux transporters, through binding to effector binding elements (EBEs) in *S* gene promoters to foster the growth of bacterial pathogens (Chen et al. 2010; Streubel et al. 2013). To counteract *X. oryzae* pv. *oryzae* infection, rice has adapted to TAL effector activity by evolving *R* genes with matching EBEs in their promoters. The binding of cognate TAL effectors to the matching EBEs induces *R* gene expression, triggers HR and activates disease resistance to *X. oryzae* pv. *oryzae* (Gu et al. 2005; Luo et al. 2021; Tian et al. 2014; Wang et al. 2015). The rice bacterial blight *R* gene *Xa10* confers strong but narrow-spectrum disease resistance to a few Philippines *X. oryzae* pv. *oryzae* strains (Gu et al. 2008). The $EBE_{AvrXa10}$ element in the *Xa10* promoter is specifically recognized and bound by the cognate TAL effector AvrXa10 during gene activation (Tian et al. 2014). Xa10, an executor R protein localized at endoplasmic reticulum (ER) membrane, triggers Ca^{2+} depletion from the ER lumen and induces HR by a conserved mechanism involving disruption of the ER and cellular Ca^{2+} homeostasis followed by ROS generation (Tian et al. 2014). The specificity recognition between AvrXa10 and $EBE_{AvrXa10}$ in the *Xa10* promoter and the AvrXa10-dependent *Xa10* induction allowed us to use the system to test the function of executor *R* genes from other plant species in rice. Previously, the pepper *R* gene *Bs4C* under the control of the *Xa10* promoter (P_{Xa10}:*Bs4C*:T_{Xa10}) was used to generate transgenic rice (Wang et al. 2018). The expression of the P_{Xa10}:*Bs4C*:T_{Xa10} gene in transgenic rice was AvrXa10-dependent and the transgenic plants conferred specific disease resistance to the *Xa10*-incompatible *X. oryzae* pv. *oryzae* strain $PXO99^{A}$(-pHM1AvrXa10), but not to the *Xa10*-compatible strain $PXO99^{A}$(pHM1) (Wang et al. 2018). As the *Bax* gene induces PCD or HR in plants (Lacomme and Santa Cruz 1999; Yoshinaga et al. 2005), it is worth testing to see if the *Bax* gene could provide disease resistance in transgenic plants. Indeed, previous study

demonstrated that transgenic tobacco carrying the mouse *Bax* gene exhibited increased resistance to *Phytophthora parasitica* and *Ralstonia solanacearum* (Dong et al. 2008). In this study, a synthetic gene based on the *Bax* gene of house mouse (*Mus musculus*) was put under the control of *Xa10* promoter to form P_{Xa10}:*Bax*:T_{Xa10} gene. The P_{Xa10}:*Bax*:T_{Xa10} gene was used for the transient expression in *N. benthamiana* and the generation of transgenic rice plants for evaluation of disease resistance to rice bacterial blight. The results demonstrated that the heterologous transient expression of the *Bax* gene in *N. benthamiana* induced cell death and the AvrXa10-dependent gene expression of the P_{Xa10}:*Bax*:T_{Xa10} gene in transgenic rice plants conferred specific disease resistance to *X. oryzae* pv. *oryzae* strains harbouring the cognate *AvrXa10* gene.

Materials and methods

Plants and growth conditions

Oryza sativa ssp. *japonica* cv. Nipponbare, which is susceptible to many *X. oryzae* pv. *oryzae* strains, was used to produce transgenic plants. Xa10NB is a near-isogenic line of the *Xa10* gene in Nipponbare genetic background. Rice plants were grown in greenhouse at 30 °C under natural sunlight for 12.5 h and 25 °C at night for 11.5 h with relative humidity at 80–85%. The *N. benthamiana* plants were grown in growth room at 25 °C with relative humidity at 60% and under 16 h light with density at 200 μmol m^{-2} s^{-1} and 8 h in darkness.

Genes and constructs

The binary construct pCPR1AvrXa10 contains the TAL effector gene *AvrXa10* from *X. oryzae* pv. *oryzae* strain PXO86 under the promoter of rice *PR1* gene (P_{PR1}:*AvrXa10*:T_{Nos}) (Tian et al. 2014). The mouse (*Mus musculus*) *Bax* gene, which encodes apoptosis regulator Bax (Accession no.: NP_031553), was synthesized by GenScript® (Piscataway, NJ 08,854, USA) after codon optimization for gene expression in rice. The coding region of the synthetic *Bax* gene was fused with the *Xa10* promoter through PCR amplification. The 1280-bp *Bam*HI-*Apa*I fragment of the fusion gene was used to replace the corresponding *Bam*HI-*Apa*I fragment of the *Xa10* gene in the binary construct pCSA4671 (Tian et al. 2014) to generate binary construct pC4671Bax that contains the P_{Xa10}:*Bax*:T_{Xa10} gene.

Rice transformation

Agrobacterium-mediated transformation of Nipponbare was conducted according to the method described previously (Hiei et al. 1994; Zeng et al. 2015). The regenerated hygromycin-resistant plants were transferred to plantlet medium (1/2 MS_0 medium with 50 mg/L hygromycin) for shoot and root elongation. Regenerated plants were transplanted to the soil in pots and grown in greenhouse.

Southern blot analysis

About 2–3 μg of rice genomic DNA was digested with appropriate restriction enzymes. The digested DNA samples were separated on 0.8% agarose gel and blotted to Hybond-N^+ nylon membrane (Amersham Biosciences, Piscataway, NJ 08,855-1327, USA). DNA probe labelling and DNA hybridization were conducted using the DIG DNA labelling and detection kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany) in accordance with the manufacturer's instructions. The DNA probe for detecting the copy number of T-DNA in transgenic rice plants was the *Hpt* probe derived from the coding region of the *Hpt* gene. The DNA probe for the detection of the P_{Xa10}:*Bax*:T_{Xa10} fusion gene was the *PXa10* probe derived from the *Xa10* promoter. The oligo primers for the *Hpt* probe were Hpt-F(5′AAAAAGCCTGAACTCACCGCG3′) and Hpt910-1(5′TACTTCTACACAGCCATCGGT3′). The oligo primers for the *PXa10* probe were PXa10-F (5′AGCTTACGAAGGTTGAGAGC3′) and PXa10-R (5′GAGGAGTGAACGTGATTGCG3′).

qRT-PCR

Total RNA extraction and qRT-PCR were carried out according to the method described previously (Wang et al. 2018). The internal control genes for qRT-PCR analysis were the *Actin 1* gene of *N. benthamiana* (AY594294) (*NbActin-1*) and the elongation factor 1α gene of rice (Os03g0178000) (*OsEf1α*), respectively. The qRT-PCR experiments were conducted in

triplicate and the data were presented as means ± SD. The oligo primers for the *Bax* gene were Bax-F4(5'TTAGGGTTGCAGCCGATATG3') and Bax-R4(5′CCTGATGAGCTCAGGAACTTTAG3′). The oligo primers for the *Xa10* gene were Xa10-F (5′GGCATCATCTTCTCCGGCG3′) and Xa10-R (5′GCAGCTATACGGGCATAAG3′). The oligo primers for the *NbActin-1* gene were NbAct1-F (5′TCCTGATGGGCAAGTGATTAC3′) and NbAct1-R (5′TTGTATGTGGTCTCGTGGATTC3′). The oligo primers for the *OsEf1α* gene were OsEf1a-F2 (5′GCACGCTCTTCTTGCTTTC3′) and OsEf1a-R2 (5′AGGGAATCTTGTCAGGGTTG3′).

Agroinfiltration of *N. benthamiana*

A. tumefaciens strain GV3101 harbouring binary constructs were cultured in 5 ml of LB liquid medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH7.0) with appropriate antibiotics at 28 °C until the density of bacteria reached an optical density of 0.8 at OD600, and then sub-cultured in 100 ml of LB liquid medium at 28 °C until the density of bacteria reached 0.8 at OD600. The bacteria were collected and re-suspended in 10 mM $MgCl_2$ to the density of 0.6 ~ 0.8 at OD600. 2-(N-morpholino) ethanesulfonic acid (MES) and Acetosyringone (AS) were then added to the bacterial suspensions at the final concentration of 10 mM and 200 μM, respectively. Bacterial suspensions were infiltrated into leaves of *N. benthamiana* as described previously (Kay et al. 2007). The infiltrated plants were grown under 16 h light and 8 h darkness at 25 °C. The phenotype was checked at 24–48 h after infiltration (HAI). The experiment was conducted with three biological repeats for each gene and construct.

Trypan blue staining

Trypan blue staining was conducted according to the method described previously (Tian et al. 2014). The trypan blue staining solution was prepared by diluting trypan blue stock solution (10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water and 0.02 g trypan) (Sigma-Aldrich, St. Louis, Missouri, USA) with 96% ethanol (1:2 v/v). Plant tissues were boiled in trypan blue staining solution for 1 min and left in the solution at room temperature for 1 d. The stained leaves were de-stained in chloral hydrate solution (25 g in 10 ml of water) for 3 d and then equilibrated with 70% glycerol for photography.

Bacterial blight inoculation

The *X. oryzae* pv. *oryzae* strains were cultured in the PSA plate (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, 16 g/L bacto-agar, and pH 7.0) at 28 °C for 2 d. Bacterial cells were collected and re-suspended in sterile water to the density of 0.5 at OD600. Both infiltration and leaf-clipping methods were used for bacterial blight inoculation. For bacterial inoculation with infiltration method, leaves of four-week-old plants were infiltrated with bacterial inoculum using a needleless syringe. Leaf tissues were collected for gene induction analysis at 48 h after infiltration. For leaf-clipping method, fully expanded leaves on 6-week old rice plants were inoculated according to the procedures described previously (Kauffman 1973). The lesion length (L.L.) was measured at 14 days after inoculation.

Immunoblot analysis

The *N. benthamiana* leaf tissues were harvested at 24 h after agroinfiltration and frozen in liquid nitrogen. Total protein was extracted by grinding frozen leaf tissues in homogenization buffer containing 100 mM Tris–HCl (pH 8.0), 10 mM MgCl2, 18% sucrose (w/v), 2% SDS, 40 mM 2-mercaptoethanol, and plant protein protease inhibitor cocktail (Sigma-Aldrich). The samples were centrifuged at 12,000 g for 10 min at 4 °C to remove cellular debris. About 10 μl supernatant was mixed with 4 × Laemmli buffer and boiled for 5 min. Proteins were separated on 12% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane. Immunoblotting was performed following the standard protocol. Bax protein was detected by using anti-Bax monoclonal primary antibody (Abcam) and anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Abcam). Signals were detected by Clarity™ Western ECL Substrate (Bio-Rad). Proteins separated on a duplicate SDS-PAGE gel were stained with Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad), photographed, and used as the protein loading control.

Statistical analysis

Student's *t*-test was performed to evaluate statistical significance, and P value < 0.01 was considered statistically significant. All statistical analyses were performed using SPSS Statistics package.

Results

AvrXa10-dependent heterologous expression of the P_{Xa10}:*Bax*:T_{Xa10} gene in *N. benthamiana* induced cell death

The heterologous expression of the *Bax* gene in *N. benthamiana* was previously found to induce cell death and disease resistance (Dong et al. 2008; Lacomme and Santa Cruz 1999). In this study, a synthetic mouse *Bax* gene was used to replace the coding sequence of the *Xa10* gene to generate P_{Xa10}:*Bax*:T_{Xa10} gene. To determine the specific inducible expression of the P_{Xa10}:*Bax*:T_{Xa10} gene by AvrXa10 and the function of the synthetic *Bax* gene, the P_{Xa10}:*Bax*:T_{Xa10} gene was transiently expressed in *N. benthamiana* leaves through agroinfiltration with the P_{PR1}:*AvrXa10*:T_{Nos} gene. Cell death was observed in the infiltrated area at 1–2 days after co-infiltration with the P_{Xa10}:*Bax*:T_{Xa10} gene and the P_{PR1}:*AvrXa10*:T_{Nos} gene (Fig. 1a). No cell death was observed after infiltration with the P_{Xa10}:*Bax*:T_{Xa10} gene, the P_{PR1}:*AvrXa10*:T_{Nos} gene or control buffer alone (Fig. 1a). Cell death was also observed in infiltrated *N. benthamiana* leaves at 24 h after co-infiltration with the *Xa10* gene and the P_{PR1}:*AvrXa10*:T_{Nos} gene, but not in the control experiment after infiltration with the *Xa10* gene alone (Fig. 1a). Dead cells at the infiltrated *N. benthamiana* leaves could be detected by trypan blue staining at 24 h after co-infiltration of the P_{PR1}:*AvrXa10*:T_{Nos} gene with either the P_{Xa10}:*Bax*:T_{Xa10} gene or the *Xa10* gene (Fig. 1b). No or only background trypan blue staining was detected in the infiltrated *N. benthamiana* leaves after infiltration with the P_{Xa10}:*Bax*:T_{Xa10} gene or the *Xa10* gene alone (Fig. 1b). The qRT-PCR analysis demonstrated that AvrXa10-dependent transient expression of the *Bax* gene in *N. benthamiana* was only detected in leaves co-infiltrated with the P_{Xa10}:*Bax*:T_{Xa10} gene and the P_{PR1}:*AvrXa10*:T_{Nos} gene, but not in the control experiments infiltrated with the P_{Xa10}:*Bax*:T_{Xa10} gene alone or buffer solution (Fig. 1c). Immunoblot analysis with anti-Bax antibody also confirmed that the Bax protein was only detected in *N. benthamiana* leaves co-infiltrated with the P_{Xa10}:*Bax*:T_{Xa10} gene and the P_{PR1}:*AvrXa10*:T_{Nos} gene (Fig. 1d). These results demonstrated that the P_{Xa10}:*Bax*:T_{Xa10} gene was tightly regulated and specifically induced by TAL effector AvrXa10 and the heterologous expression of the proapoptotic protein Bax in *N. benthamiana* triggered cell death.

Generation of transgenic rice plants carrying P_{Xa10}:*Bax*:T_{Xa10} gene for disease resistance to *X. oryzae* pv. *oryzae*

The P_{Xa10}:*Bax*:T_{Xa10} gene was then used to produce transgenic rice in the Nipponbare background via *Agrobacterium*-mediated rice transformation. In total, 107 transgenic T0 plants were generated. After bacterial blight inoculation with *X. oryzae* pv. *oryzae* strain PXO99^{A}(pHM1AvrXa10), 4 bacterial blight resistant T0 plants (T0-24, T0-27, T0-68 and T0-74) were selected for further disease evaluation in the T1 and T2 generations. Fourteen bacterial blight and hygromycin resistant T2 plants derived from the 4 bacterial blight resistant T0 plants were subjected to Southern blot analysis. The copy number of T-DNA inserts in the T2 plants was detected by the *Hpt* probe and the P_{Xa10}:*Bax*:T_{Xa10} gene was detected by the *PXa10* probe, respectively. Southern blot analysis with the *Hpt* probe revealed that 3 to 6 copies of T-DNA inserts were detected in one T2 plant derived from T0-24 (24-26-2), 5 T2 plants derived from T0-27 (27-47-70, 27-47-88, 27-54-101, 27-54-109 and 27-54-112) and 1 T2 plant derived from T0-68 (68-78-170) (Fig. 2). An expected 4887-bp *Eco*RI-*Pst*I band harbouring the P_{Xa10}:*Bax*:T_{Xa10} gene was also detected by the *PXa10* probe in the 7 T2 plants (Fig. 2). The *Hpt* probe detected 4 copies of co-segregated T-DNA inserts in 7 T2 plants derived from T0-74 (74-80-177, 74-82-179, 74-82-184, 74-84-194, 74-85-195, 74-85-207 and 74-89-214) (Fig. 2). The *PXa10* probe also detected 4 *Eco*RI-*Pst*I bands in the 7 T2 plants; however, none of them had molecular size at 4887 bp (Fig. 2). The results indicated that the 7 T2 plants derived from T0-74 should carry 4 copies of truncated T-DNAs inserted at the same location in rice genome due to illegitimate T-DNA integration. As the 7 T2 plants still conferred disease resistance to PXO99^{A}(pHM1AvrXa10), it was assumed

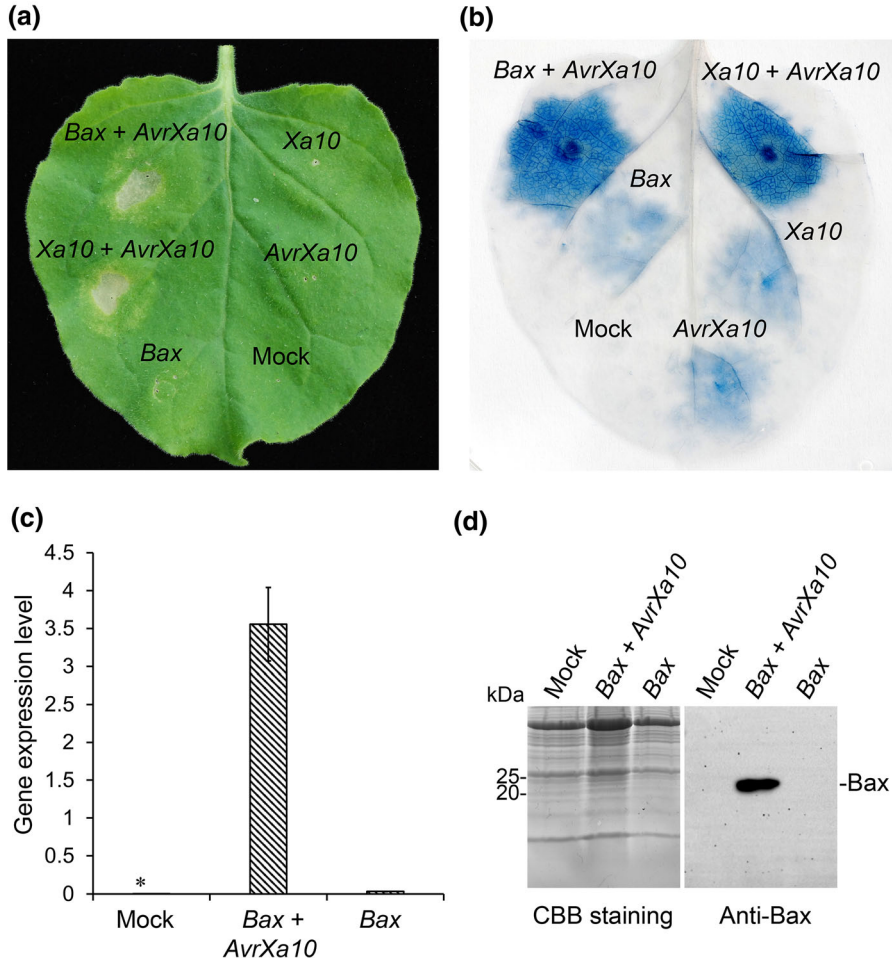

Fig. 1 AvrXa10-dependent transient *Bax* expression triggered cell death in *N. benthamiana*. **a** Phenotype of *N. benthamiana* leaf at 3 days after infiltration with *A. tumefacien* strain GV3101 harbouring different binary constructs. **b** Trypan blue staining of leaf tissues of *N. benthamiana* at 24 h after infiltration with *A. tumefacien* strain GV3101 harbouring different binary constructs. **c** Transient expression of the *Bax* gene in *N. benthamiana* at 24 h after infiltration detected by qRT-PCR. Asterisk (*) stands for no amplification signal in qRT-PCR analysis. *AvrXa10*, infiltrated with the P_{PR1}*:AvrXa10:*T_{Nos} gene; *Bax*, infiltrated with the P_{Xa10}*:Bax:*T_{Xa10} gene; *Xa10*, infiltrated with the *Xa10* gene; *Bax* + *AvrXa10*, co-infiltrated with the P_{Xa10}*:Bax:*T_{Xa10} gene and the P_{PR1}*:AvrXa10:*T_{Nos} gene. *Xa10* + *AvrXa10*, co-infiltrated with the *Xa10* gene and the P_{PR1}*:AvrXa10:*T_{Nos} gene. Mock, infiltration with control buffer. **d** Detection of Bax proteins transiently expressed in *N. benthamiana* by immunoblot analysis. Anti-Bax, immunoblot detected by anti-Bax antibody; CBB staining, proteins separated on a duplicate SDS-PAGE gel stained with Coomassie Brilliant Blue and used as the protein loading control. kDa, kilodalton

that at least one of the T-DNA harbours functional P_{Xa10}*:Bax:*T_{Xa10} gene.

Transgenic T3 plants with bacterial blight resistance were obtained from T2 plants 24-26-2, 27-54-101, 68-78-170 and 74-82-179. They were designated as transgenic lines L24, L27, L68 and L74, respectively. The L24 plants had similar plant height and panicle length to Nipponbare, whereas the L27, L68 and L74 plants displayed slightly shorter plant height and panicles than the non-transgenic control (Fig. 3a, b and c). In addition, the L24 plants (84 ± 4%) and Nipponbare (84 ± 3%) showed similar seed setting rates, whereas the L27, L68, L74 plants had slightly lower seed setting rates at 77 ± 6%, 67 ± 4% and 66 ± 8%, respectively.

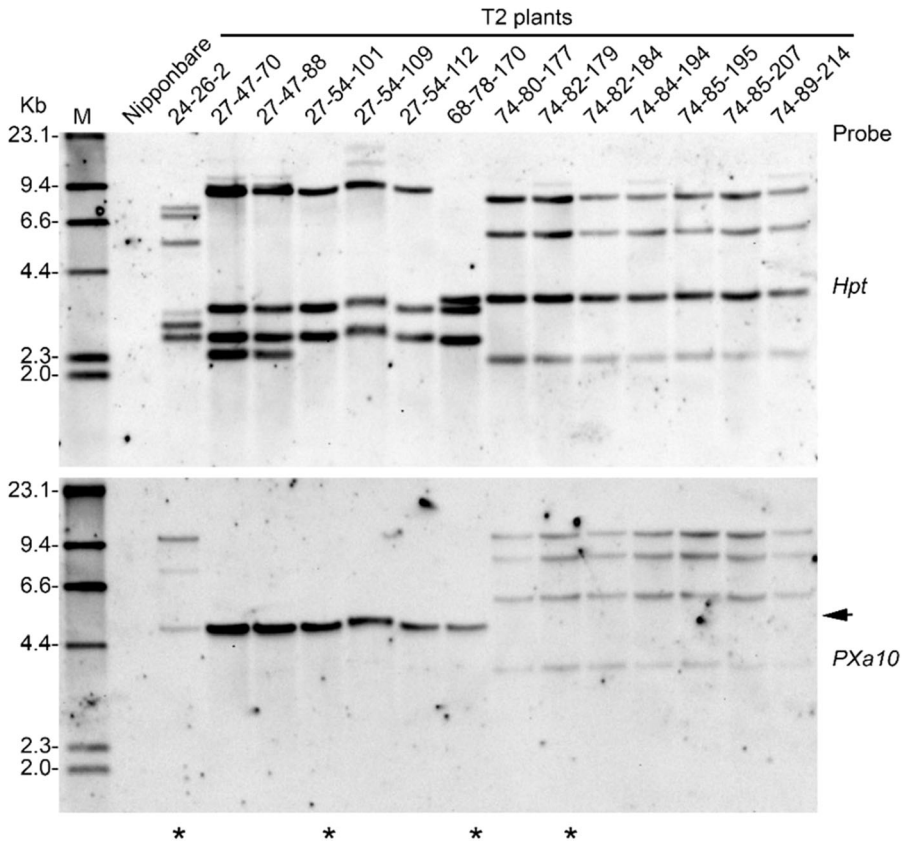

Fig. 2 Southern blot analysis of transgenic T2 plants. Rice genomic DNAs were double digested with restriction enzymes *Eco*RI and *Pst*I. Southern blots were hybridized with the *Hpt* probe (upper panel) and the *PXa10* probe (lower panel), respectively. Arrow indicates the position of the expected 4887-bp bands of the P_{Xa10}:*Bax*:T_{Xa10} gene. T2 plants denoted with Asterisks were selected for further disease evaluation and gene expression in the T3 generation. Arrow at the lower panel indicates the position for the expected *Eco*RI-*Pst*I band at 4887 bp harbouring the P_{Xa10}:*Bax*:T_{Xa10} gene. M, molecular marker; 24-26-2, T2 plant derive from T0 plant T0-24; 27-47-70 to 27-54-112, T2 plants derived from T0 plant T0-27; 68-78-170, T2 plant derive from T0 plant T0-68; 74-80-177 to 74-89-214, T2 plants derived from T0 plant T0-74

The P_{Xa10}:*Bax*:T_{Xa10} gene in transgenic rice conferred AvrXa10-dependent specific disease resistance to *X. oryzae* pv. *oryzae*

The disease resistance specificity of L24, L27, L68 and L74 was investigated by inoculation with *X. oryzae* pv. *oryzae* strains, PXO99^{A}(pHM1AvrXa10) and PXO99^{A}(pHM1), respectively. Nipponbare and Xa10NB were served as the negative and positive controls, respectively. Like Xa10NB, T3 plants derived from the 4 P_{Xa10}:*Bax*:T_{Xa10} transgenic lines conferred disease resistance to *Xa10*-incompatible strain PXO99^{A}(pHM1AvrXa10), but were still susceptible to *Xa10*-compatible strain PXO99^{A}(pHM1) (Fig. 4a and b). Nipponbare was susceptible to both PXO99^{A}(pHM1AvrXa10) and PXO99^{A}(pHM1) (Fig. 4a and b). The results demonstrated that, like the *Xa10* gene, the P_{Xa10}:*Bax*:T_{Xa10} gene has similar AvrXa10-dependent resistance specificity to *X. oryzae* pv. *oryzae* strains harbouring *AvrXa10*. It was noted that the lesion length of bacterial blight on the transgenic P_{Xa10}:*Bax*:T_{Xa10} plants was slightly longer than that on the Xa10NB plants after they were inoculated with PXO99^{A}(pHM1AvrXa10) (Fig. 4a and b). The result indicated that the P_{Xa10}:*Bax*:T_{Xa10} gene was slightly weaker than the *Xa10* gene in conferring disease resistance to the *Xa10*-incompatible strain. In addition, all of the transgenic P_{Xa10}:*Bax*:T_{Xa10} plants had shorter lesion length than Xa10NB plants when they were inoculated with PXO99^{A}(pHM1) (Fig. 4a and b). The result suggested that the P_{Xa10}:*Bax*:T_{Xa10} plants showed low levels of

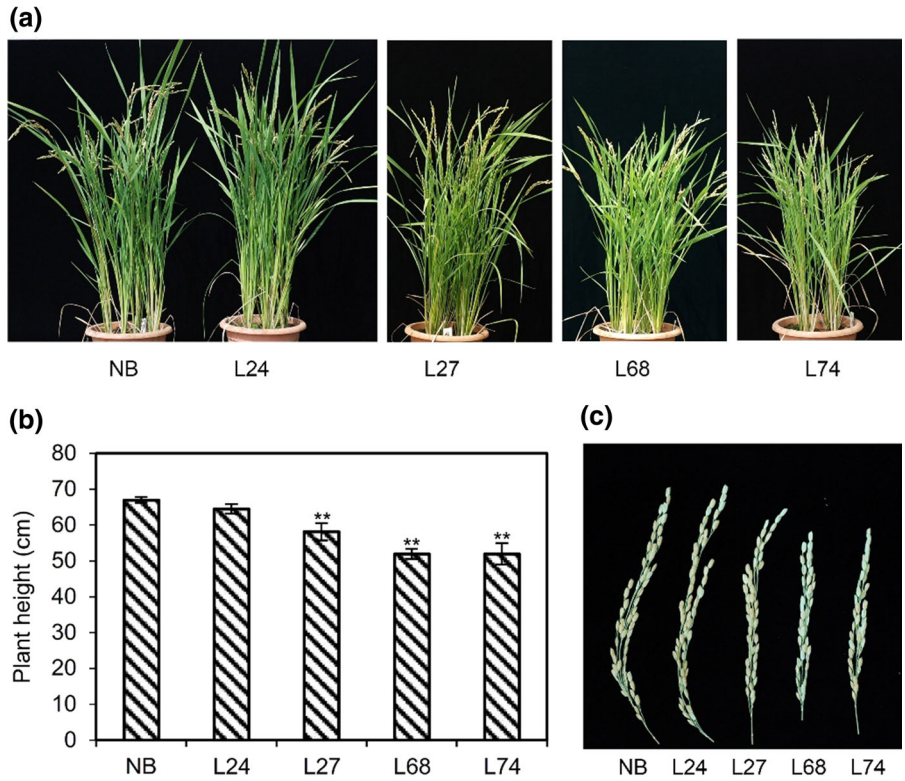

Fig. 3 Morphology phenotype of Nipponbare and transgenic plants of P_{Xa10}:*Bax*:T_{Xa10} at mature stage. **a** Phenotype of whole plants of Nipponbare and transgenic plants. **b** Plant height of Nipponbare and transgenic plants. The data are presented as means ± SD (n = 5). The asterisks (**) represent a significant difference from wild type ($P < 0.01$; *t* test). **c** Panicles of Nipponbare and transgenic plants. NB, Nipponbare; L24, L27, L68 and L74, T3 plants of transgenic lines L24, L27, L68 and L74, respectively

enhanced disease resistance to *Xa10*-compatible strain.

Our previous study reported that TAL effector AvrXa10 specifically binds to $EBE_{AvrXa10}$ in the *Xa10* promoter and induces *Xa10* expression (Tian et al. 2014). The qRT-PCR analysis demonstrated that, similar to *Xa10* gene in Xa10NB, the P_{Xa10}:*Bax*:T_{Xa10} genes in transgenic plants were specifically induced by PXO99^{A}(pHM1AvrXa10), but not by PXO99^{A}(-pHM1) (Fig. 5). Compared with no or trace level of background expression of the *Xa10* gene in uninoculated Xa10NB plants, a low level of leaking expression of the P_{Xa10}:*Bax*:T_{Xa10} gene was detected in uninoculated transgenic plants (Fig. 5). It remained unchanged when the transgenic plants were inoculated with PXO99^{A}(pHM1) (Fig. 5). This low level of leaking expression of the P_{Xa10}:*Bax*:T_{Xa10} gene could be the major reason that resulted in slightly enhanced disease resistance to PXO99^{A}(pHM1) (Fig. 4a and b). Nevertheless, the results demonstrated that the P_{Xa10}:*Bax*:T_{Xa10} gene in transgenic plants was specifically induced by AvrXa10 from *X. oryzae* pv. *oryzae* and the heterologous expression of pro-apoptosis regulator Bax from mouse conferred disease resistance to bacterial blight in rice.

Discussion

The executor genes in rice are a small group of *R* genes with promoter traps for TAL effectors and confer immunity to the *X. oryzae* pv. *oryzae* strains carrying these TAL effectors (Gu et al. 2005; Luo et al. 2021; Tian et al. 2014; Wang et al. 2015). Unlike traditional R proteins that mainly trigger HR in their native plant species, some rice executor R proteins, such as Xa7, Xa10 and Xa23, can cause PCD in dicotyledonous plant *N. benthamiana* (Luo et al. 2021; Tian et al.

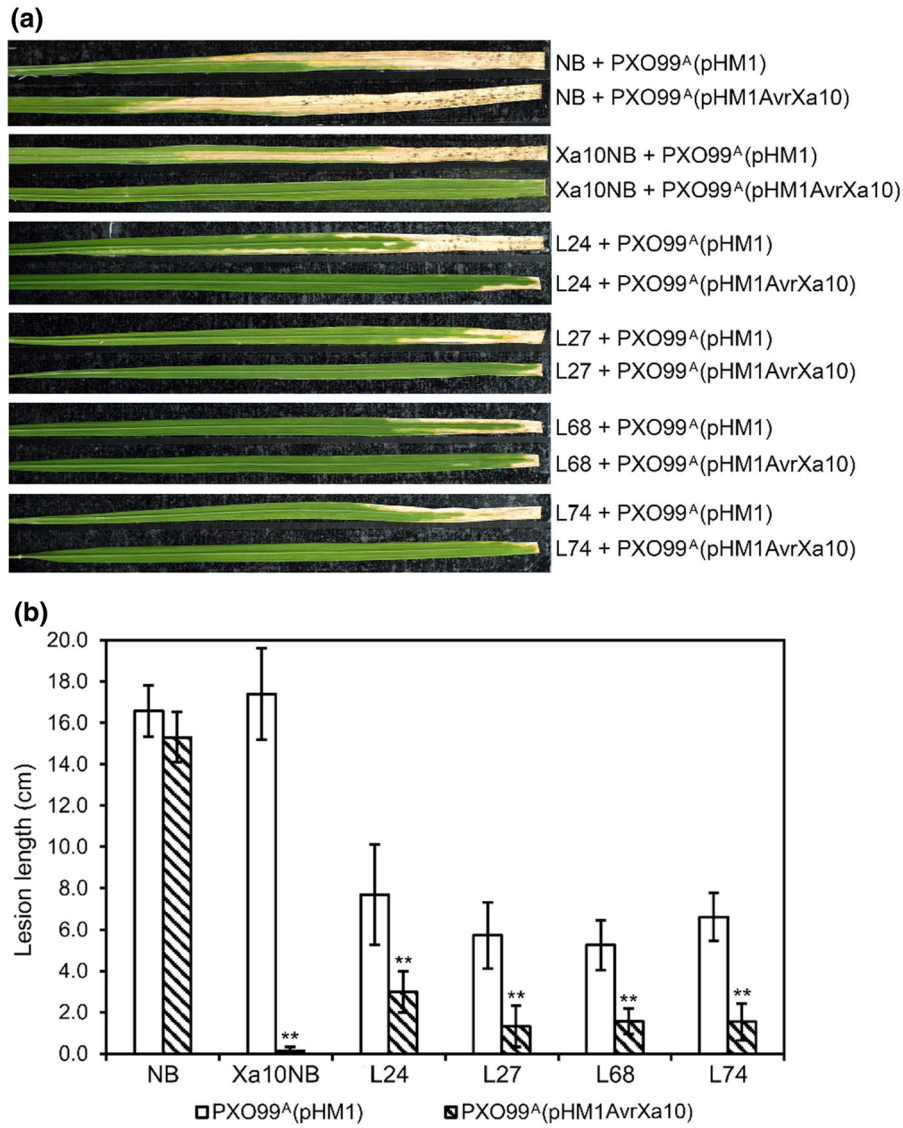

Fig. 4 Disease evaluation of transgenic plants of P_{Xa10}:-*Bax*:T_{Xa10} for disease resistance to bacterial blight. **a** Bacterial blight phenotype of transgenic and control plants. Images of inoculated leaves were taken at two weeks after inoculation with *X. oryzae* pv. *oryzae* strains harbouring pHM1AvrXa10 or empty vector pHM1. **b** Lesion length of bacterial blight on the inoculated rice leaves. Bacterial blight lesions were scored at two weeks after inoculation with *X. oryzae* pv. *oryzae* strains. NB, Nipponbare; Xa10NB, near-isogenic line of the *Xa10* gene in Nipponbare genetic background. L24, L27, L68 and L74, T3 plants derived from T0-24, T0-27, T0-68 and T0-74, respectively. The data are represented as means ± SD ($n \geq 5$). The asterisks (**) indicate a significant difference from inoculation with *X. oryzae* pv. *oryzae* strains harbouring empty vector pHM1 ($P < 0.01$; *t* test)

2014; Wang et al. 2015). Xa10 can even induce apoptosis in HeLa cells (Tian et al. 2014). Previous reports demonstrated that the mammalian Bax proteins induced HR-like PCD in tobacco and Arabidopsis (Lacomme and Santa Cruz 1999; Yoshinaga et al. 2005). It seems that both Bax and Xa10 have a common feature in inducing PCD broadly in plant and animal cells, though they have a significant difference in their protein structures and may function through different mechanisms. A previous study also demonstrated that transgenic tobacco plants carrying the mouse *Bax* gene exhibited increased resistance to

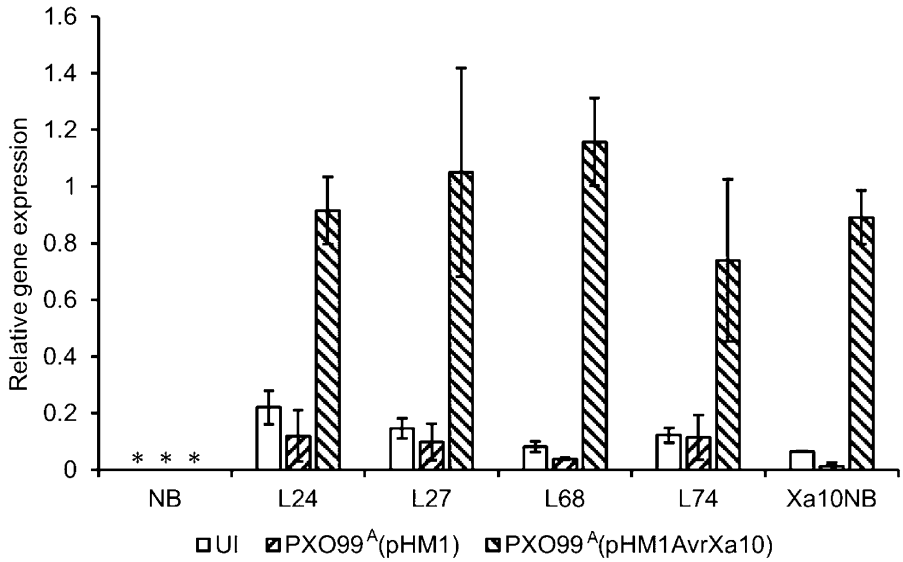

Fig. 5 *Bax* gene expression in transgenic plants at 48 h after inoculation with *X. oryzae* pv. *oryzae* strains. Gene expression was detected by qRT-PCR. The expression of rice elongation factor 1α gene (Ef1α) (Os03g0178000) was used as the internal control. The qRT-PCR experiments were performed in triplicate and the data are presented as means ± SD. Asterisk (*) stands for no amplification signal in qRT-PCR analysis. The expression of *Xa10* in Xa10NB was used as induction control. L24, L27, L68 and L74, T3 plants of transgenic lines L24, L27, L68 and L74, respectively; UI, uninoculated control; Xa10NB, near-isogenic line of the *Xa10* gene in Nipponbare genetic background

oomycete pathogen *Phytophthora parasitica* and bacterial pathogen *Ralstonia solanacearum* (Dong et al. 2008). In this study, the synthetic *Bax* gene was put under the control of the *Xa10* promoter and the expression of the P_{Xa10}:*Bax*:T_{Xa10} gene was tightly regulated and specifically induced by AvrXa10. The AvrXa10-dependent transient expression of the P_{Xa10}:*Bax*:T_{Xa10} gene in *N. benthamiana* induced cell death (Fig. 1a and b). The result was consistent with the finding in the previous report (Lacomme and Santa Cruz 1999). Further disease evaluation with transgenic rice plants containing the P_{Xa10}:*Bax*:T_{Xa10} gene demonstrated that the *Bax* gene conferred disease resistance to *Xa10*-incompatible *X. oryzae* pv. *oryzae* strain harbouring the *AvrXa10* gene (Fig. 4). It should be noted that both *R. solanacearum* and *X. oryzae* pv. *oryzae* are soil-borne gram-negative bacteria that infect and multiply in plant vascular systems, though they belong to distinct classes of pathogenic bacteria with different plant hosts. *X. oryzae* pv. *oryzae* enters the rice leaf and gain access to the xylem typically through hydathodes at leaf margins or wounds on plants (Nino-Liu et al. 2006). Within the xylem, *X. oryzae* pv. *oryzae* presumably interacts with xylem parenchyma cells and injects the TAL effectors into host cells to activate the expression of host *S* genes, such as the *SWEET* genes encoding Clade 3 sugar efflux transporters (Chen et al. 2010). The executor R protein-induced HR and its associated immune responses in the xylem parenchyma cells targeted by *X. oryzae* pv. *oryzae* prevent bacteria from gaining access to nutrition and restricts pathogens from propagating and spreading within the vascular system. In this study, the Bax protein might induce HR-like PCD in the xylem parenchyma cells of the transgenic rice plants after inoculation with *Xa10*-incompatible *X. oryzae* pv. *oryzae* strain. Also, a similar immune response to the executor R protein-mediated disease resistance might be activated as the consequence of PCD in pathogen-infected cells. The results also demonstrated the importance of HR or PCD in disease resistance to *X. oryzae* pv. *oryzae* − Once the HR or PCD in the cells targeted by bacteria is initiated, the disease resistance to the pathogen would be activated. The disease lesions of bacterial blight on the P_{Xa10}:-*Bax*:T_{Xa10} plants were slightly longer than that on the Xa10NB plants when they were inoculated with *Xa10*-incompatible strain PXO99^{A}(pHM1AvrXa10) (Fig. 4a and b). One of the possible reasons could be that, compared with the Xa10 protein, the Bax protein

might be less toxic in inducing cell death in rice cells and thereby reducing the efficiency in conferring disease resistance to the bacterial pathogen. Further studies may include the similarities and differences between Xa10 and Bax in the initiation of PCD and its downstream signalling in plant cells. Finally, the TAL effector-dependent inducible gene expression system developed in this study could be used to test the function of R protein homologs/orthologs, mammalian proapoptotic proteins or toxic proteins in cell death induction in plants and genetically engineer novel disease resistance in plants (Wang et al. 2018).

Due to the toxicity of executor R proteins on cell death induction, the expression of executor *R* genes is tightly regulated in plants. They are only induced upon infection by incompatible pathogens. Usually, the background expression of executor *R* genes is hardly detected by northern analysis with mRNA or qRT-PCR (Gu et al. 2005; Tian et al. 2014). However, the leaking expression of the executor *R* gene could be detected in some transgenic plants when the genomic clones of the *R* genes containing the different lengths of native promoters were used for genetic complementation study (Gu et al. 2005). Rice plants with constitutively leaking expression of executor *R* genes usually cause stress morphological phenotypes with few tillers, stiff leaves, lesion mimics and growth retardation (Gu et al. 2005; Tian et al. 2014; Tian and Yin 2009). The reasons for leaking expression of executor *R* genes in transgenic plants were not known. One of the possible reasons could have resulted from chromosomal position effect on transgene expression as they were randomly inserted in the plant genome. The frequency of obtaining transgenic plants harbouring executor *R* genes with leaking expression may be related to the degree of the toxicity of executor R proteins. The Xa10 protein should have a high degree of toxicity as the frequency of obtaining transgenic plants with *Xa10* transgenes with leaking expression was low, possibly due to the negative selection by the strong toxicity of the Xa10 protein (Tian et al. 2014; Zeng et al. 2015). The high degree of toxicity of the Xa10 protein enabled us to genetically engineer the *Xa10* gene for broad-spectrum resistance to different *X. oryzae* pv. *oryzae* strains by adding multiple EBEs to the *Xa10* promoter (Zeng et al. 2015). In this study, no leaking expression of the P_{Xa10}:*Bax*:T_{Xa10} gene or the Bax protein was detected in *N. benthamian* leaves infiltrated with the *Bax* gene alone (Fig. 1c and d). The expression of the *Bax* gene and its product were only induced in *N. benthamian* leaves that were co-infiltrated with the P_{Xa10}:*Bax*:T_{Xa10} and P_{PR1}:*AvrXa10*:T_{Nos} genes (Fig. 1c and d). The results indicated that the expression of the P_{Xa10}:*Bax*:T_{Xa10} gene in *N. benthamiana* was tightly regulated and specifically induced by AvrXa10 under transient expression condition. However, the transgenic plants obtained in this study still showed low levels of leaking expression of the P_{Xa10}:*Bax*:T_{Xa10} gene (Fig. 5). This might be resulted from the weak toxicity of the Bax protein in cell death induction, which led to low efficiency in negative selection by eliminating transformed cells with leaking expression of the P_{Xa10}:*Bax*:T_{Xa10} gene. The low leaking expression of the P_{Xa10}:*Bax*:T_{Xa10} gene enabled the transgenic plants to confer slightly enhanced disease resistance to the *Xa10*-compatible strain PXO99^{A}(pHM1) (Fig. 4). However, the leaking expression of the *Bax* gene also caused a slight stress phenotype on transgenic plants (Fig. 3). Previous studies also reported that the constitutive expression of the *Bax* gene in transgenic plants negatively affected plant growth and development (Dong et al. 2008; Kawai-Yamada et al. 2001). Although the T-DNA insertion in the rice genome and the chromosomal position effect on transgene expression in transgenic rice plants might be out of our control or unavoidable, the leaking expression of the P_{Xa10}:*Bax*:T_{Xa10} gene did not overwrite the specific interaction between the EBE_{AvrXa10} in the *Xa10* promoter and AvrXa10. The transgenic plants still retained AvrXa10-dependent induction of the P_{Xa10}:*Bax*:T_{Xa10} gene and strain-specific disease resistance to PXO99^{A}(-pHM1AvrXa10) (Figs. 4 and 5).

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Author's contribution THE authors thank RM for editing the manuscript. DT and ZY designed the experiments. YG, DT, JT and KHO conducted the experiments. YG, DT, JT and ZY wrote the article. All authors read and approved the article for publication.

Declarations

Conflict of interest The authors state that they have no conflict of interest.

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