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BIOTIC AND ABIOTIC STRESS

Mammalian pro-apoptotic *bax* gene enhances tobacco resistance to pathogens

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Abstract Emerging evidence suggests that plants and animals may share certain biochemical commonalities in apoptosis, or programmed cell death (PCD) pathways, though plants lack key animal apoptosis related genes. In plants, PCD has many important functions including a role in immunity and resistance to pathogen infection. In this study, a rice phenylalanine ammonia-lyase promoter is used to regulate the expression of the mouse pro-apoptotic *bax* gene in transgenic tobacco plants. Ectopic expression of the *bax* negatively affects the growth of transgenic plants. Nonetheless, results show that the *bax* transgene is induced upon infection by plant pathogens and accumulation of Bax is observed by Western blot analysis. By estimating and measuring the extent of cell death, release of active oxygen species, and accumulation defense-associated gene transcripts, it is shown that *bax* transgenic plants mount a more robust cell death response compared to control plants. The *bax* transgenic tobacco plants are also more resistant to infection by *Phytophthora parasitica* and *Ralstonia solanacearum*, but have no obvious resistance to tobacco mosaic virus. These results substantiate past studies and illustrate the powerful effects mammalian *bax* genes may have on plant development and disease resistance.

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Introduction

Apoptosis, as a form of programmed cell death (PCD), has been widely accepted as a requirement for the proper development of multi-cell organisms. The process also plays an important role in response to pathogen infection (Gilchrist 1998; Green and Reed 1998). Apoptosis is characterized by a series of distinct morphological and biochemical features, including cell shrinkage, chromatin condensation, ruffling of plasma membrane, and fragmentation of the nucleus (Kuwana and Newmeyer 2003).

Common genes that function in the induction or suppression of apoptosis have been identified in a variety of organisms (Priault et al. 2003), suggesting shared PCD-specific pathways. The mammalian Bcl-2 family is a group of conserved key regulators of cell survival and death. The Bax protein, a member of this family, is a positive regulator of cell death. Bcl-2 is proposed to induce cell death by forming channels in the outer membrance of mitochondria to release cytochrome c and subsequently trigger, by currently unknown mechanisms, complex apoptosis pathways (Manon et al. 1997; Pawlowski and Kraft 2000; Wei et al. 2001; Kuwana and Newmeyer 2003).

Plant PCD plays an important role in the process of xylogenesis, reproduction, senescence, and immunity to infection (Greenberg 1996; Kamoun et al. 1998). In incompatible plant–pathogen interactions, plants quickly recognize certain pathogens and activate defense systems, resulting in the limitation of pathogen growth at the site of infection (Lam et al. 2001). This rapid recognition and response to infection has been defined as the hypersensitive

response (HR). HR encompasses many different defense responses and often includes activation of PCD. The plant PCD induced by pathogens and elicitors may be similar to apoptosis in animals, since some of apoptosis characteristics mentioned above were present during plant PCD response to infection (Chichkova et al. 2003; Ji et al. 2005).

There is evidence to suggest that PCD in plants may share certain regulatory mechanisms with those in animals. For example, a survey of the *Arabidopsis* genome revealed a number of predicted sequences similar to animal apoptosis-related sequences. It is interesting, however, that plant homologs to genes like Bcl-2 family members that regulate animal-specific programmed cell death cannot be identified (Dickinson and Beynon 2003). Animal genes that regulate apoptosis have also been ectopically expressed in model plants to test whether the genes can induce or inhibit cell death in plant cells. Overexpression of mammalian *bcl-2*, *bcl-xl* and *ced-9* (a *Caenorhablitis elegans* homolog of *bcl-xl*) inhibited plant PCD and provided transgenic tobacco with resistance to viruses and necrotrophic fungi, and resistance to abiotic stress (Dickman et al. 2001; Mitsuhara et al. 1999; Xu et al. 2004). Furthermore, expression of anti-apoptotic baculovirus *p35* gene in tomato blocked PCD and provided broad-spectrum resistance to disease (Lincoln et al. 2002). Recently, in several plant species endogenous genes with a putative role in apoptosis have been reported, such as those encoding caspase-like proteinase (Chichkova et al. 2003; Hatsugai et al. 2004; Coffeen and Wolpert 2004), Bax-like gene (Kawai-Yamada et al. 2005), Bax inhibitor (Kawai-Yamada et al. 2001; Matsumura et al. 2003; Hückelhoven et al. 2003) and Beclin (Liu et al. 2005).

Previous reports showed that plant cell death induced by a viral vector harboring *bax* closely resembles phenotypic responses induced by tobacco mosaic virus (TMV) in tobacco plants carrying the N gene (Lacomme and Cruz 1999). Bax targets mitochondria and changes the shape of mitochondria from bacilli to round. Chloroplasts lose membrane function causing their contents to leak. These changes are followed by disruption of the vacuole (Yoshinaga et al. 2005). Ectopic expression of Bax triggered local PCD as well as increased the expression of plant defense genes, inducing those associated with systemic acquired resistance (SAR) (Lacomme and Cruz 1999).

In the present study, we tested the effectiveness of the phenylalanine ammonia-lyase promoter from rice (PALpro) (Zhu et al. 1995), to regulate bax expression. *PALpro* is a conditional promoter that is induced by stress, including wounding or challenge with TMV, or bacteria, or fungi or fungal elicitors (Zhu et al. 1995; Shadle et al. 2003). Here we generate *PALpro::bax* transgenic tobacco plants and describe their growth, defense responses, and disease response compared to control plants.

Materials and methods

Materials

Vector pTMV-*bax* used in this work for amplification of mouse *bax* cDNA was a gift from Dr. Simon Santa Cruz, Scottish Crop Research Institute. The plasmid of pGZM-7EF-PAL was donated by Prof. Guo Zejian, China Agricultural University. *Agribacterium tumefaciens* strain EHA105 and *R. solanacearum* was donated by Dr. Shao Min and Prof. Guo Jianhua Nanjing Agricultural University, respectively. *Phytophthora parasitica* virulent isolate YN-34 and TMV were from the collection of Nanjing Agricultural University.

Construction of transformation vector

Mouse *bax* cDNA (GenBank Accession No.: L22472) was amplified with Bax-F (5′-TATCCCGGGATGGACGGGT CCGGGGA-3′) and Bax-R (5′-TCAGTCGACGCCCATCT TCTTCCAG-3′) primers using pTMV-*bax* as a template. PCR for obtaining the PAL promoter fragment was performed with universal primer SP6-P (5′-CATACGATTTA GGTGACACTA-3′) and specific primer PALpro-1 (5′-TATGGTACCCAGTACCAGAAGATGATGCATC-3′), using pGZM-7EF-PAL as template. The *bax* cDNA fragment and pCAMBIA1300PT (derived from pCAMBIA1300) were double digested by *Sma*I and *Sal*I, respectively, and then ligated with each other by T4 DNA ligase (Takara Biotech. Dalian), assembling the vector pCAMBIA1300PT::*bax*. Digestion of *PALpro* PCR products with *EcoR*I and *Kpn*I liberated *PALpro*. This fragment was used to replace the *CaMV35S* promoter between *EcoR*I and *Kpn*I restriction site of the binary vector pCAMBIA1300PT::*bax* to construct the final plant binary vector, pCAMBIA1300PT::*PALpro*::*bax* (Fig. 1a). DNA sequencing (Takara Biotech. Dalian) confirmed that these two fragments were subcloned into binary vector correctly.

Generation of bax transgenic plants

Agrobacterium tumefaciens strain EHA105 was used in tobacco transformations. The binary vector pCAMBIA1300PT::*PALpro*::*bax* was mobilized into EHA105 by electroporation (Instruction Manual of Gene Pulser Xcell™ Electroporation System, BIO-RAD). *Agrobacterium* transformants were selected on YEP solid media supplemented with kanamycin (100 mg/l) and identified by PCR analysis. Axenic 2-month-old W38 tobacco plants were prepared and their leaf tissues transformed and regenerated as previously described (Clark 1997; Cao et al. 1997). Plant transformants were selected on MS agar medium (Murashige and Skoog 1962) supplemented with

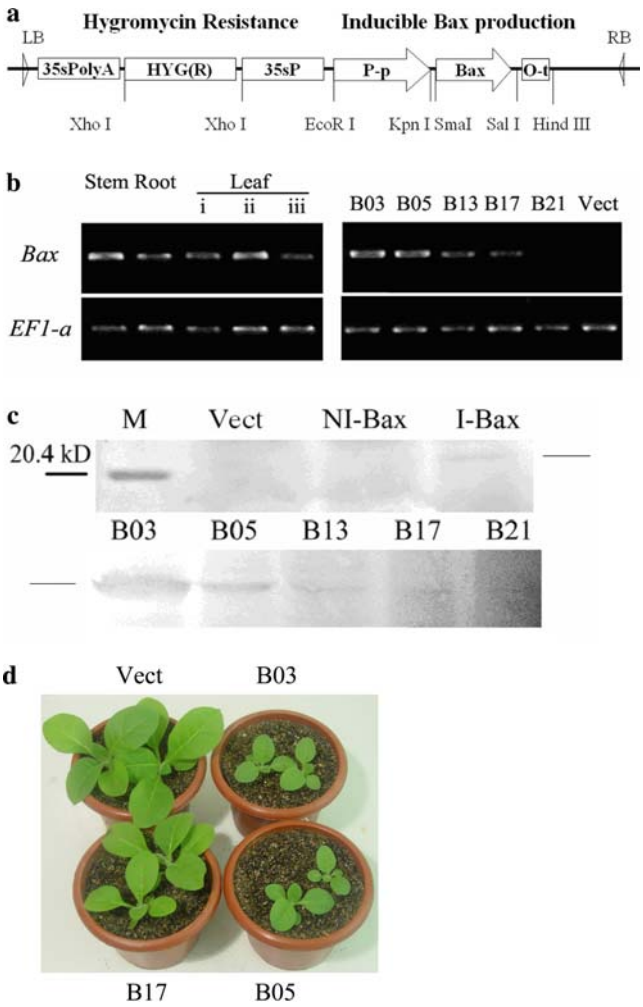

Fig. 1 **a** The expression cassette of plant binary transformation vector pCAMBIA1300PT::*PALpro*::*bax* left border (LB); cauliflower mosaic virus 35S polyA tail (35SPolyA); hygromycin B resistant marker [HYG(R)]; cauliflower mosaic virus 35S promoter (35S); *PAL* promoter (P-p); mouse *bax* cDNA (Bax); octopine synthase (ocs) terminator (O-t:); right border (RB); **b** RT-PCR analysis of *bax* expression in different tissues and lines *Left panel*: stem: Bax03 transgenic plant stem; Root: Bax03 transgenic plant root; Leaf: Bax03 plant leaf; RNA was extracted from: i: mock-inoculated leaf; ii: inoculated leaf (12 hpi); iii: leaf distal to inoculated leaf (12 hpi). *Right panel*: representative *transgenic lines* are shown here: B03, B05, infected leaves from highly resistant plants Bax03 and Bax05; B13, B17, infected leaves from intermediate resistant plants Bax13 and Bax17; B21, infected leaves from susceptible transgenic plant Bax21; leaves from plants *Vect*: transformed with empty (Vect). **c** Western blot analysis of bax accumulation in T0 and T1 transgenic tobacco. *Upper panel*: lanes are as follows: *M*, 20.4 kD protein marker; *Vect*, vector-transformed tobacco plant; NI-Bax; mock, uninfected Bax03 line; I-Bax: *P. parasitica*-infected Bax03 line. The line indicates the mouse bax protein (21.4 kD; ref). *Lower panel*: plants of Bax03, Bax05, Bax13, Bax17, Bax21 were inoculated with *P. parasitica* and the results of a Western blot analysis using anti-bax antiserum are shown. **d** Comparison of the growth of transgenic plants, shown at the 6 week-old stage. B03 and B05: Bax03 and Bax05, T1 progeny of high expression tobacco; B17, T1 progeny of low expression plants Bax17; *Vect*, empty vector transformed-plant

hygromycin B (100 mg/l) and carbenicillin (400 mg/l). The regenerated *bax* transgenic plants were then transferred to the greenhouse to grow at 25°C with 16-h light periods after the plant was 2 cm in height. Seeds were harvested from the T0 tobacco plants and selected twice on 1/2 MS medium supplemented with hygromycin B at the concentration of 100 and 200 mg/l, respectively. Hygromycin B-resistant plant was selected and genomic DNA was isolated, then PCR was conducted by using *bax*-specific primers. Only positive T1 plants were considered as dominant homozygous or heterozygous. The T1 progeny plants were grown at 25°C with 16-h light periods in greenhouse. Transgenic tobacco plants containing binary vector alone, and wild untransformed tobacco, served as controls.

Western blot analysis of Bax protein

Two days after inoculation with *P. parasitica*, leaves from 4-month-old T0 transgenic plants were ground to powder in

liquid nitrogen. An equal volume of universal lysis buffer (Tris-HCl 10 mM, EDTA 1 mM, 1% PVP, 1% 2-mercaptoethanol, 1 mM PMSF, pH: 6.5) was added and mixed (Peng et al. 2003). The sample was mixed at 4°C for another 2 min and then centrifuged at 4°C for 15 min and the supernatant collected. A BioPhotometer (Eppendorf) was used to examine the protein concentration in the supernatant. One hundred micrograms of total protein was loaded onto a 12% SDS-PAGE gel and run in electrophoresis buffer at room temperature at 100 V for 3 h (Cao et al. 1997). Subsequently, protein in the gel was electrotransferred onto a PVDF membrane (Gelman Sciences) using a wet apparatus (BIO-RAD) at 4°C for another 3 h. The membrane was blocked in PBS (pH 6.5) containing 3% BSA for 6 h at 4°C. After washing with PBS buffer, the membrane was probed with antiserum (Santa Cruz Biotech.) raised in rabbits against Bax at 1:1,000. Alkaline phosphatase conjugated anti-rabbit IgG (Sigma) was used as the secondary antibody.

Pathogen inoculation and resistance assays

Plants including T0 and T1 progenies were inoculated with three plant pathogens 8 weeks after transplanting young plants to the soil.

P. parasitica

P. parasitica was propagated on lima bean agar plates at 25°C. Two different inoculation methods were used to assess the resistance of transgenic plants to *P. parasitica,* (1) Leaf inoculation: a minimum of two leaves per plant from each of the independently transformed plants were inoculated by placing 5-mm-diameter agar plugs containing actively growing hyphal tips from 3-day-old colonies of *P. parasitica* on leaves. Agar plugs were covered by wet cotton balls in order to maintain high humidity for 24 h. The inoculated tobacco plants were incubated at 25°C with 16-h light periods in the greenhouse and disease development was recorded every 12 h after cotton balls were removed. (2) Stem inoculation: 20 plants per line from T1 progeny of Bax03, Bax05 and Bax17 transgenic plants were inoculated by placing 5-mm-diameter agar plugs of *P. parasitica* on plant hypocotyls with wet cotton balls to maintain high humidity.

R. solanacearum

T1 progeny from transgenic plants were tested for their resistance to the bacterial plant pathogen *R. solanacearum* by leaf infiltration and steam inoculation. For leaf infiltration, a bacterial suspension of 1.0×10^8 CFU/ml in a 50 µl volume was infiltrated into tobacco leaves by use of a disposable 1 ml syringe. For stem inoculation, plants were inoculated by stabbing a 200 µl disposable pipette tip containing 100 µl of 1.0×10^7 CFU/ml into the third leaf axil down from each plant's apex, and then inoculated plants were placed in a 25°C greenhouse. Pipette tips were removed after all the bacterial suspension entered the stem (Peng et al. 2003; Dannon and Wydra 2004).

TMV

A minimum of five plants per line from T1 progeny of Bax03 and Bax05 were inoculated with TMV (1 µg/ml of solution) by gently rubbing leaves using a finger in the presence of abrasive diatomaceous earth (Zhang et al. 2004).

Assessment of host cell response to infection by *P. parasitica*

Trypan blue staining and cell death computation

Approximately 1.5-cm squares of infected leaves 60 hpi were soaked in Farmers mixture for 30 s and then 0.4% trypan blue stain (GibcoBRL) was infiltrated overnight into the leaf intercellular spaces with the aid of a vacuum pump. Infiltrated leaves were boiled in 95% ethanol until tissues were cleared (Peng et al. 2003). The 5-mm-diameter agar plugs inoculated areas were examined using a compound microscope and images were photographed. Diseased and control parts (1 mm × 1 mm) were randomly selected and images depicting equal leaf areas were collected. Photos were then imported into Morphologic Image Analysis System (Nanjing University, China) for cell death analysis. The similar color detection tool provided in this soft was used to calculate the stained and unstained area, taking the area ratio output as cell death ratio.

Ion leakage detection

A minimum of four leaves per plant from T1 progeny of Bax plants were inoculated with *P. parasitica* by placing 5-mm-diameter pathogen agar plugs as described previously. Then 20-mm-diameter plant discs were excised with a circle model, taking the inoculation site as circle center. These discs were washed with 20 ml deionized water at 12 h intervals after removal of cotton balls. The electrical conductivity of the washing solution was determined with Syperscan 510 (EUTEOH) as shown previously (Mitsuhara et al. 1999).

Oxygen burst detection

Oxygen burst was observed based on H_2O_2 accumulation after staining tobacco leaves with diaminobenzidine (DAB). *P. parasitica* inoculation was performed as

previously described and 4 cm × 4 cm squares of excised leaves were soaked in DAB aqueous solution (pH 3.5) at 1 mg/ml and maintained at 25°C for 8 h. Leaf sections were cleared by boiling in 95% ethanol (Alvarez et al. 1998).

DNA and RNA manipulation

Plant genomic DNA was extracted as previously described (Sambrook and Russell 2001). Plant tissues form roots, leaves, stems were ground in liquid nitrogen, and total RNA was isolated by Trizol reagent (Invitrogen) according to the manufacturer's instructions. PCR and Southern blot were conducted for detection of *bax* inserts into tobacco genomes, and transcript of *bax* and defense-related genes were studied by reverse transcriptional RT-PCR. In DNA-PCR identification, PCR was conducted with a 58°C annealing temperature for 28 cycles using 1 ng genomic DNA as template and the *bax* primers, producing intact *bax* fragment (579 bp). For Southern blot hybridization, intact *bax* cDNA, produced by PCR from pTMV-*bax*, was used as a probe in DNA blot analysis. A sum of 10 μg of genomic DNA was digested with *Sma*I, *Sal*I and *BamH*I respectively prior to electrophoresis and the following procedures were as described by Sambrook and Russell (2001). For RT-PCR analysis, total RNA (2 μg) was treated with RNase-free DNase and used as template to synthesize the first-strand cDNA with the aid of a reverse transcription kit (Promega). The *EF1-α* gene was used as an internal marker to be amplified and then the relative quantity of RNA was adjusted. An equal volume of cDNA was amplified for 28 cycles using specific primers (*hin1*, *Bax*, *PR1-a* and *PR1-c*) and electrophoresis was conducted to analysis the expression of defense-related genes (Gopalan et al. 1996; Ji et al. 2005). The NCBI accession number of genes monitored in RT-PCR and their primers sequences are provided *PR-1a* (D90196): 5′-ATGCCCATAACAGCTCG-3′/5′-GAGG ATCATAGTTGCAAGAG-3′, *PR-1c* (X05454):,5′-AT GCCCATAACAGCTCG-3′/5′-GGATCATAGTTGCAAG AGAC-3′. *EF1-α* (AF120093): 5′-AGACCACCAAGTAC TACTGCAC-3′/5′-CCACCAATCTTGTACACATCC-3′. *hin1* (Y07563): 5′-GGTTTGATAGTACAAATTTGG-3′/ 5′-TCCATCGAGATGTGAAGATGA-3′.

Results

Generation of transgenic plants and analysis of gene expression

A total of 37 independent transgenic plants including 15 pCAMBIA1300- transformed control plants and 22 *bax* transgenic plants were generated for this study using *Agrobacterium* mediated plant transformation. Shown in Fig. 1a is the transformation construct. PCR and Southern blot analysis was used to verify *bax* transgenic plants (data not shown). After triggering *bax* expression by inoculation with *P. parasitica*, RT-PCR (Fig. 1b) and Western blot analysis (Fig. 1c) were used to examine the expression level in transgenic plants. After T0 progeny plants were grown up on MS agar media supplemented with hygromycin B, PCR and Southern blot analyses were performed to select 22 *bax* transformants, with the criteria of single or two-copy transgene integrations. The T1 progeny of transgenic plants were then generated and at least 60 plants from each independent line were obtained. The expression of *bax* was measured by RT-PCR and Western blot analysis. Firstly, the transcript levels of *bax* in different plant tissues were examined by RT-PCR. As shown in Fig. 1b, *bax* is transcribed in leaves, stems and roots. Results from *P. parasitica* leaf inoculation assay also demonstrated that infected leaves, which are responsive to invasion, accumulate a higher level of *bax* transcript than both mock-inoculated leaves or leaves distal to the infected leaves. These data suggested that *bax* transcript is induced upon pathogen challenge. We also found that *bax* transcripts accumulate at different levels in transgenic plants. Some transgenic plants (B03 and B05) accumulate comparatively high levels of *bax*, while some (B013 and B17) show a relatively low levels. However, some transgenic plants, such as B21, did not transcribe *bax*. These results were confirmed by Western blot analysis (Fig. 1c). Furthermore, *bax* transgenic plants were one week delayed in growth compared to pCAMBIA1300 vector-transformed plants in both T0 and T1 progeny (Fig. 1d).

Bax transgenic plants exhibit resistance to *P. parasitica*

To rule out the possibility that differences in growth may have an effect on plant resistance, we tested the resistance of *bax* transgenic plants one week earlier than control plants in T0 progeny and T1 *bax* transgenic plants one week earlier than controls in order to keep all the tested plants at the same growth stage. Two different inoculation methods, using leaves or stems, were employed to test the susceptibility of *bax* transgenic plants to infection by *P. parasitica*. Using either method of inoculation, control tobacco plants were highly susceptible to *P. parasitica*. At 6 days post-inoculation (dpi) T0 progeny leaves were classified as high resistance, intermediate resistance and susceptible, with necrotic lesions of 0.4 cm ± 0.2 cm, 2.5 cm ± 0.8 cm, 7.1 cm ± 1.1 cm in diameter, respectively. As shown in Figs. 2a, b, pathogen lesion diameter increased at the same rate during the early stages of infection in control and transgenic plants. However, 4–5 dpi, lesion diameter on Bax03, Bax05, Bax09, Bax14 and Bax19 plants stabilized and did not grow much larger,

compared to the other plants. Furthermore, the infection lesions on Bax13, Bax17 and 11 other plants (Bax02, Bax06, Bax07, Bax11, Bax12, Bax15, Bax17, Bax20, Bax23, Bax25 and Bax29) that expressed intermediate resistance were reduced or delayed in development. Ten days after inoculation, disease progression ceased in highly resistant transgenic tobacco whereas control plants collapsed and were overcome by infection at this time. To confirm the results, T1 progeny tobacco plants were inoculated with *P. parasitica*, using inoculation methods as above. In stem inoculation tests of T1 progeny tobacco, necrotic lesions (at 4 dpi) of 1 cm ± 0.8 cm, 6.5 cm ± 1.5 cm, 11.1 cm ± 3.1 cm diameter were classified as high resistance, intermediate resistance and susceptible, respectively. It was difficult to differentiate high resistance versus intermediate resistance with the stem inoculation test. As shown in Table 1, T1 progeny of highly resistant transgenic tobacco lines were generally classified as highly resistance, with the exceptions of B03 and B05 plants.

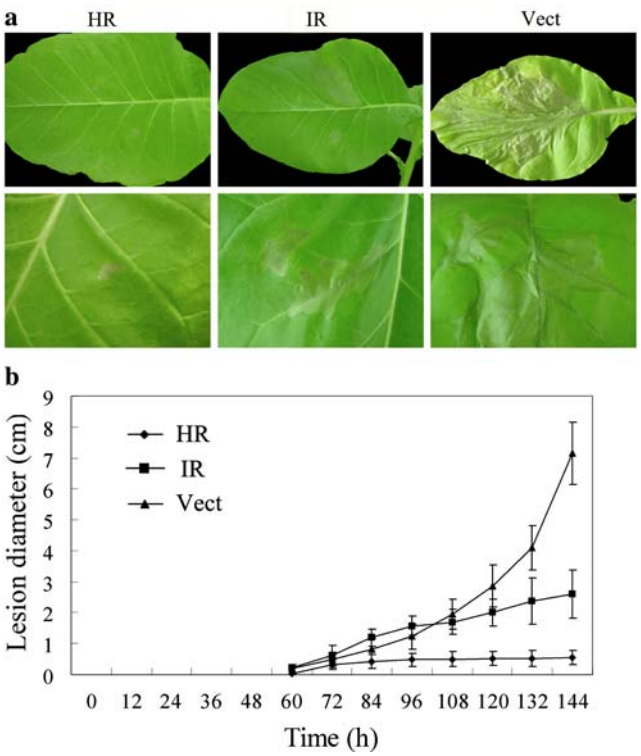

Fig. 2 Analysis of resistance to the oomycete pathogen *P. parasitica* in *bax* transgenic plants, and in control vector transformed plants. *HR*, highly resistant plant; *IR*, intermediate resistant plant; *Vect*, vector transformed alone plant. Pictures are representative of 12 leaves from a single experiment, and the experiment was done three times. **a** Disease severity in Bax transgenic plants and control plants 6 dpi. Bax03 and Bax05 is representative of highly resistant plants, and displayed minimal disease symptoms. The spread of the disease was somewhat limited somewhat in intermediate resistant plants Bax13 and Bax17. Conversely, plants transformed with binary vector alone were sensitive to pathogen attack. **b** Disease lesion diameters on the above transgenic plants were measured at 60 hpi and at 12 h intervals after that time

Basic resistance mechanism of Bax transgenic plants to pathogens

Combined with previous RT-PCR, western blot analysis and inoculation results, we found that the resistance level of *bax* transgenic plants to *P. parasitica* was positively correlated with the accumulated level of Bax protein. In trypan blue staining, there was a clear boundary between healthy and diseased tissues in the highly resistant *bax* transgenic lines, as shown in Fig. 3a. In these plants, the diseased parts stained intensely blue, suggesting extensive cell death in the vicinity of *P. parasitica* infection. In contrast, no obvious cell death appeared in control, untransformed plants. As shown in Fig. 3b, quantitative analysis of cell trypan blue staining indicated that 21% of highly resistant *bax* transgenic tobacco leaf cells died about tenfold higher than that of cell death in control plants.

Ion leakage was used as an additional indicator of plant cell death (Fig. 3c). The amount of ion leakage detected after 24 hpi was much greater in *bax* transgenic plants compared to controls. In control tobacco plants, maximum ion leakage appeared at 24 hpi, whereas ion leakage in *bax* transgenic tobacco plants reached a maximum at 36 hpi.

Staining with DAB, as an indicator for H_2O_2 generation, resulted in more intense reactions in highly resistant *bax* transgenic tobacco compared to controls at 1 and 3 h time points, as shown in Fig. 4a. This result suggests that a greater H_2O_2 burst occurs in highly resistant plants.

Results from gene expression analysis of the *bax* transgene and three other endogenous tobacco genes are shown in Fig. 4b. The *bax* transgene displayed weak constitutive expression but was up-regulated after *P. parasitica* inoculation. The endogenous *PR1-a* was only detected upon pathogen challenge, although this expression differed between control plants and *bax* trangenic plants; the *PR1-a* gene was more rapidly induced in the *bax* transgenic plants. The expression of *PR1-c* in *bax* transgenic plants was also different from that in control tobacco plants. This gene displayed higher constitutive levels of expression and more rapid induction in response to infection in the *bax* transgenic plants than it did in the control plants.

Plant resistance to *R. solanacearum*

Pro-apoptotic gene *bax* provided transgenic plants with resistance to *R. solanacearum*, an important causal agent of bacterial wilt disease of a variety of plants. Its colonization of stems results in browning of the xylem, foliar epinasty

Table 1 Disease evaluation after stem inoculation of wild-type and three *bax* transgenic tobacco lines with *P. parasitica* and *R. solanacearum*

Cultivar/line (**a**)	*P. parasitica* (**b**)			*R. solanacearum*(**c**)	
	Susceptible	High resistance	Intermediate resistance	Susceptible	Resistance
Vect	18	0	2	15	0
B03	2	15	3	3	12
B05	1	14	4	2	13
B17	1	10	9	6	9

Values indicate number of plants in each category. Because of the delayed effects of transiently expressed *bax* on plant growth and development, all the plants are tested at the same developmental stage. **a** Transgenic plants inoculated and evaluated in this table were 3-month-old T1 progeny of Line Bax03, Bax05 and Bax17. Vect: empty vector transformed tobacco. **b** Twenty plants of each line were challenged with *P. parasitica* and evaluated 5 dpi. **c** Fifteen plants of each line were challenged with *R. solanacearum* and evaluated 7 dpi

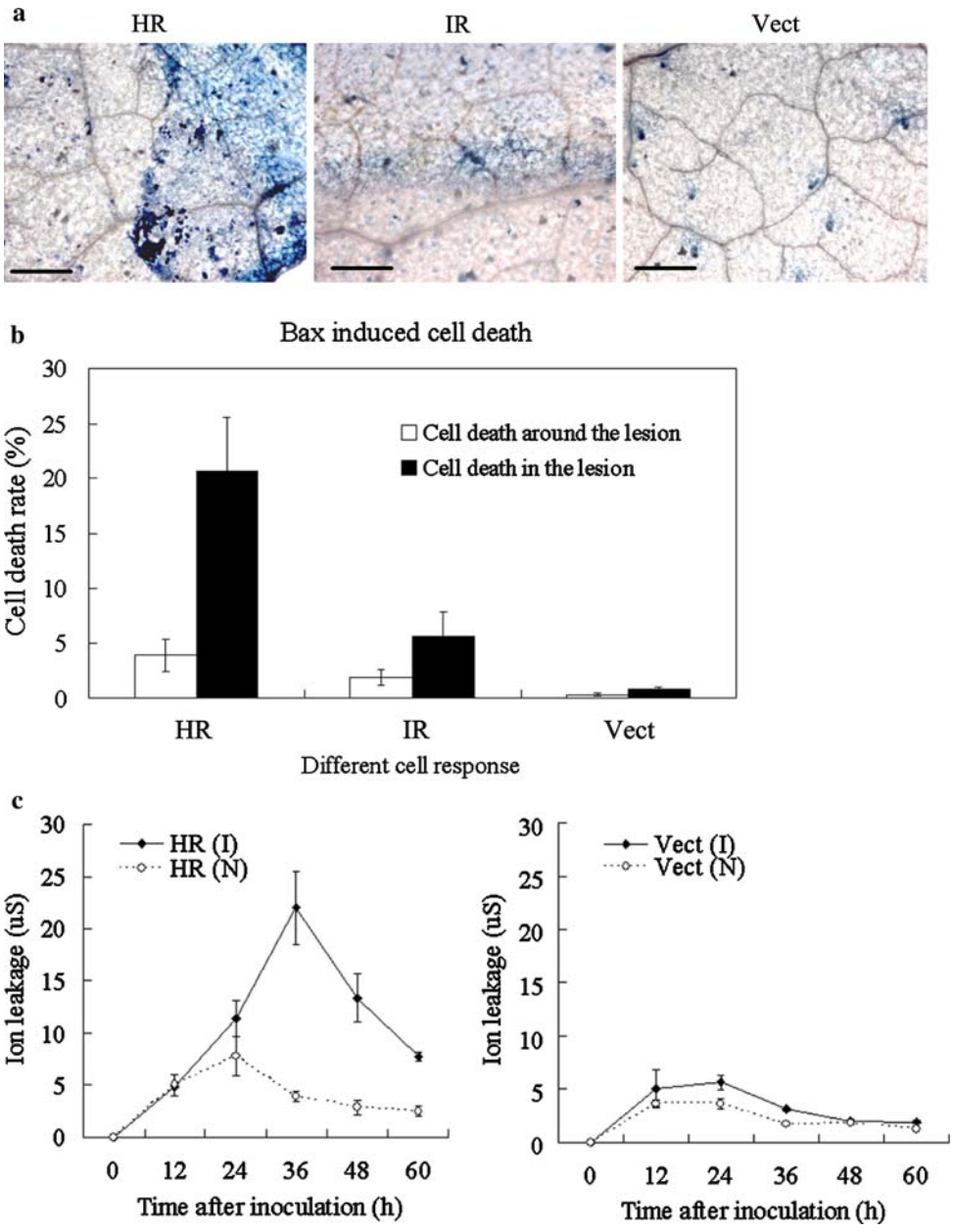

Fig. 3 Cells of leaves of *bax* expressing and wild type tobacco were analyzed after plants were inoculated with *P. parasitica*. *HR*, high resistance; *IR*, intermediate resistance; *Vect*, empty vector-transformant. **a** Inoculated leaves 60 hpi were strained with trypan blue stain and observed under a microscope. *Scale bar* 0.3 mm. **b** Results of cell death measurements. Cell death in the lesion: cell death was measured inside of the 5-mm-diameter agar plugs inoculated areas. Cell death around the lesion: cell death was calculated outside of the 5-mm-diameter agar plugs inoculated areas. **c** Ion leakage detection was performed by measuring electrical conductivity of leaf wash. The leaves were inoculated with *P. parasitica* and sampled at 12, 24, 36, 48 and 60 h. HR(I): inoculated Bax plants; HR(N), mock-inoculated Bax plants Vect(I): inoculated vector-transformed plants; Vect(N): mock-inoculated Vector transformed plants

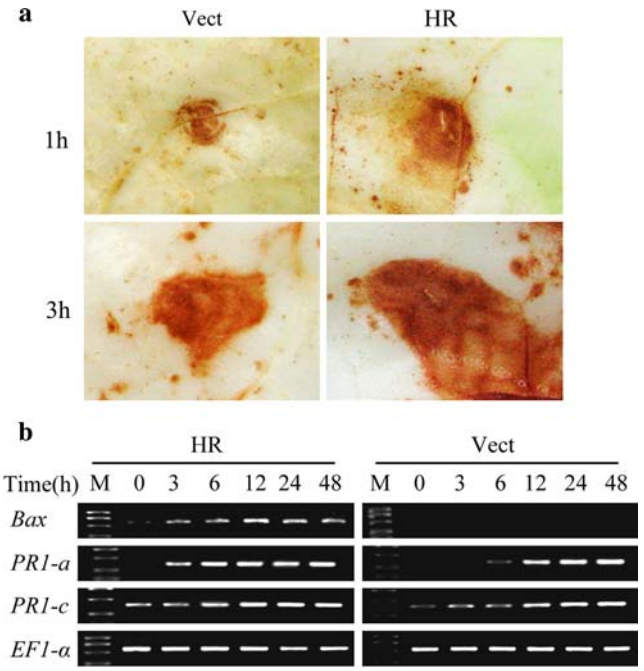

Fig. 4 Kinetics of plant oxidative burst response and PR gene expression. Bax05 is taken as a representative of five high resistant transgenic plants. *HR*, high resistant plant (Bax05); *Vect*, vector transformed alone plant. **a** Leaves from control plants and highly resistant plants were stained with DAB at 1 and 3 h after inoculation, respectively. **b** RNA extracted from leaves of control and *P. parasitica*-inoculated Bax transgenic plants at 0, 3, 6, 12, 24, 48 hpi and amplified with specific primers (*bax*, *PR1-a* and *PR1-c*) by reverse transcription-polymerase chain reaction, using *EF1-α* as an internal standard

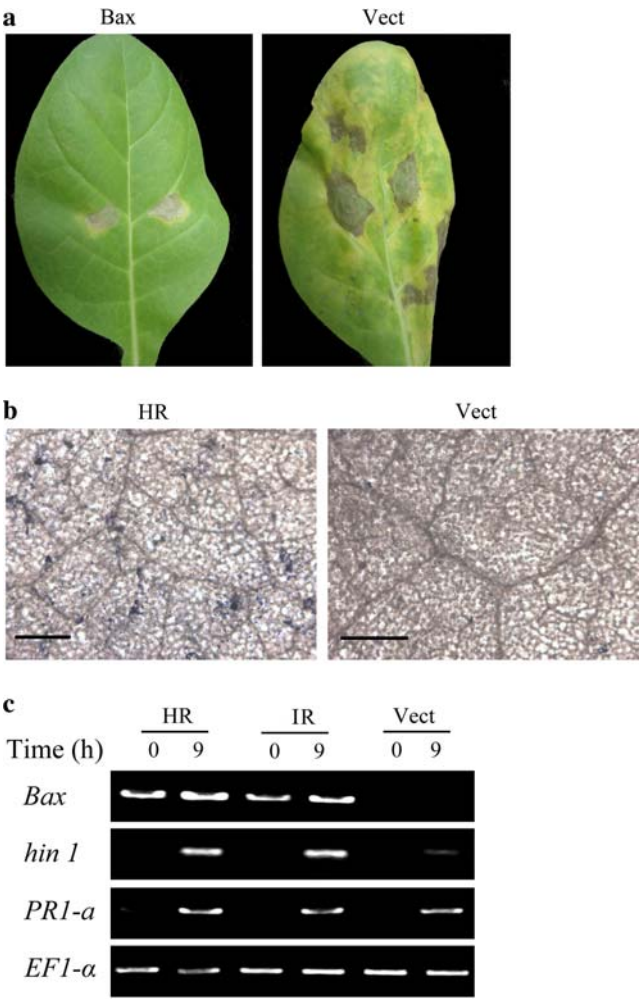

Fig. 5 Representative *bax* transgenic plants were inoculated with *R. solanacearum* or TMV. **a** Bax transgenic and control tobacco plants were infiltrated with bacteria suspension of 1.0×10^8 CFU/ml. Photos were taken 10 days after infiltration. *Bax*, Bax 03 transformed plant; *Vect*, empty vector transformed alone plant. **b** TMV inoculated tobacco leaves were stained with trypan blue and observed under microscope at 5 dpi. *Scale bar* 0.3 mm. HR: Bax 03 transformed plant. **c** Expression of *PR* gene and cell death gene at 0 and 9 h time points, in T1 progeny of *bax* transgenic plants that were inoculated with TMV. HR (high resistant plant line Bax03) and IR (intermediate resistant plant line Bax17) were compared plant transformed with empty vector only

and lethal wilt. The disease assay of T1 progeny showed that *bax* transgenic plants were resistant to *R. solanacearum*. After leaf infiltration inoculation, control plants were nearly collapsed and bacteria spread freely, whereas the leaves of *bax* transgenic plants formed necrotic flecks and disease expansion was delayed (Fig. 5a). Stem inoculation of T1 plants confirmed that expression of *bax* improved tobacco resistance (Table 1). Controls wilted 3–4 days after stem inoculation but this response was delayed to 7–9 days in transgenic tobacco.

TMV assay

The tobacco used in this experiment is an n-type tobacco, therefore TMV should not trigger N gene mediated cell death. There were no obvious phenotypic differences between *bax* transgenic and control plants following virus challenge. Therefore, histological and molecular methods were used to explore whether transgenic plants differed in their response to the virus. Compared to vector-transformed tobacco, tissues of transgenic plants stained more intensely with trypan blue after viral challenge (Fig. 5b). Furthermore, the harpin-induced 1 gene (*hin1*) (Gopalan et al. 1996) expression was greater in transgenic plants 9 h after inoculation, suggesting a greater extent of cell death in the transgenic plants (Fig. 5c). However, no difference in *PR1a* expression was noted between transgenic plants and controls. Likewise, DAB staining showed no obvious difference after TMV challenge (data not shown). Since these results show no significant difference, Bax may not improve plant resistance to TMV.

Discussion

The infection of plants by pathogenic microbes is a complex process that involves many biotic and abiotic factors. From the perspective of cell death, the equilibrium and control of host PCD may play a critical role during infection. This study shows that ectopic expression of a mammalian *bax* pro-apoptosis gene may protect plants against pathogen infection.

Previous studies have focused on anti-apoptotic genes that facilitate cell survival, protecting plant cells from biotic or abiotic damages. For example, the genes *bcl-2*, *bcl-xl*, c*ed-9* and *p35* have all been introduced and expressed in plants. The resulting transgenic plants displayed an improved resistance to toxins such as AAL-toxin and paraquat, and to physical challenge such as UV-B irradiation and cold treatment (Mitsuhara et al. 1999; Xu et al. 2004). These genes additionally promoted cell survival and improved resistance to necrotrophic pathogens such as *Sclerotinia sclerotiorum*, *Cercospora nicotianae*, and *Botrytis cinerea* (Dickman et al. 2001; Xu et al. 2004). Necrotrophs commonly use degradative enzymes and toxins to overwhelm and kill host cells, and subsequently feed upon the dead cells. There is mounting evidence that necrotrophs may even trigger host PCD to accelerate their own growth. The HR, a form of plant PCD, may facilitate plant infection by necrotrophic pathogens (Govrin and Levine 2000). Thus, inhibition of host PCD by expression of anti-apoptotic genes may enhance resistance to necrotrophs. Conversely, resistance to biotrophs and hemibiotrophs may be compromised since these pathogens feed on living cells, at least during the early stages of infection. This was demonstrated by overexpression of *BI-1* from barley. Upon challenge with the biotrophic fungus *Blumeria graminis*, pathogen penetration efficiency was greater in the *BI-1* transgenic plants (Hückelhoven et al. 2003).

There have been fewer studies on pro-apoptotic genes such as *bax*, with regard to their effect on disease progression in transgenic plants. Generation and recovery of the transgenic plants themselves is an obstacle, because the introduction and expression of cell death-promoting genes presents obvious challenges and necessitates that the transgene be under tight regulatory control. Anti-apoptotic transgenes in maize can circumvent the cell death response normally triggered by exposure to *Agrobacterium*, thereby increasing transformation frequencies (Hansen 2000), Conversely, the introduction of pro-apoptotic gene into plants decrease transformation frequencies (unpublished data from our lab). Furthermore, overexpression of *Bcl-xl* and *Ced-9* may seriously impair normal plant growth and development (Xu et al. 2004). Two different strategies have been successfully employed to overcome these difficulties including transient expression using plant viral vectors (Lacomme and Cruz 1999), and dexamethasone-inducible expression systems (Kawai-Yamada et al. 2001). However, nither approach is a practical methodology for agricultural applications, because both virus release or dexamethasone spray in crop field and triggering strong plant cell death may cause ecological and economical consideration. Therefore we employed a stress-inducible promoter, *PALpro,* to control *bax* expression in transgenic plants and to study its effect on plant disease resistance.

We found that expression of the pro-apoptotic *bax* gene negatively affected plant growth and reproduction. We observed that *bax* transgenic plants grew more slowly, with about 1 week delay, compared to control plant (Fig. 1d). The number of flowers, the fruit size, and the number of seeds harvested from *bax* transgenic plants were comparatively smaller than those from controls. We attribute these effects to low-level constitutive expression of the *bax* transgene conferred by the *PALpro* promoter as shown in Fig. 1b. Similar developmental effects have been observed for transgenic plants expressing *bcl-2* and *bcl-xl* (Xu et al. 2004), illustrating that both anti-apoptotic and pro-apoptotic genes may impact plant development and reproduction.

After inoculation with the hemi-biotrophic pathogen *P. parasitica, bax* trangenic plants displayed greater resistance to the pathogen and more dramatic responses with regard to cell death, ion leakage, ROS generation, and defense gene activation. Many of these effects have been previously observed in other systems (Priault et al. 2003; Madeo et al. 1999; Kawai-Yamada et al. 2004; Lacomme and Cruz 1999; Yoshioka et al. 2003). In this paper, plant resistance related genes *PR1*a and *PR1c* have also been shown to be earlier and more intensely induced upon pathogen challenge in *bax* transgenic plants. The timing and intensity of induced defense responses are believed to be crucial to plant immunity, especially against biotrophic pathogens (Peng et al. 2003; Glazebrook 2005; Torres and Dangl 2005, Gechev and Hille 2005).

It was somewhat surprising that phenotypic responses of *bax* trangenic plants to TMV did not differ from control plants. However, RT-PCR analysis and histological tests suggested that cell death may have been greater in the *bax* transgenic plants after TMV challenge. We checked the difference between *P. parasitica* and TMV in their ability to induce the PAL promoter using RT-PCR. (data not shown) The result showed no transcriptional level difference, suggesting that the *PALpro* promoter is as equally responsive to TMV infection as to *P. parasitica* infection in the transgenic tobacco plants. Therefore, we assumed that TMV may employ more effective counter-strategies than *P. parasitica,* to inhibit host cell death and ensure its propagation.

Although it is difficult to define *R. solanacearum* as a biotroph or as necrotroph, our results show that *bax* expression lessened the severity of this disease. These results suggest that the pathogen may rely, in part, on biotrophic

growth. Moreover, these data, coupled with those of Lacomme and Cruz (1999) indicate that *bax* expression increases the transcription of *PR* genes, which may contribute to plant resistance to bacterial infection. This interpretation is in accord with a previous report that *PR* gene expression and salicylic acid (SA) pathways are involved in tobacco resistance to *R. solanacearum* (Kiba et al. 2003).

Another consideration is the expression level and location of *bax* in the transgenic plants. Although *bax* transgenic plants exhibit enhanced disease resistance, the plants generally remain susceptible to pathogen attack. Western analysis demonsrated low levels of bax, suggesting that the *PALpro* promoter may not be ideal for *bax* regulation. A dicot-specific, pathogen-inducible promoter may serve to better regulate *bax* enhanced plant resistance while reducing the detrimental effects on plant growth and development. Transgenic line Bax17 showed an interesting phenotype. *P. parasitica*- challenged T0 leaves, resulted in intermediate resistance, whereas, the stem-inoculated T1 plants yielded higher resistance (Table 1). We compared the leaf to stem inoculation in T1 plants and consistently found that stems were more resistant to *P. parasitica* challenge compared to leaves. Other intermediate transgenic plants demonstrated the same phenotype. The previous studies have shown that *PAL* is transcribed higher in stems than in leaves, which could account for a quicker and more robust cell death response in steam (Zhu et al. 1995).

In conclusion, expression in tobacco plants of a mammalian pro-apoptotic *bax* gene controlled by the rice *PAL pro* promoter enhanced plant disease resistance but resulted in negative effects on plant growth and development. Overall, the work substantiates and enlarges upon past research describing the use of ectopically expressed pro-apoptotic genes in transgenic plants. The development of appropriate promoters to control transgene expression could help to mitigate negative effects of this technology and improve the prospects for practical applications.

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