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Bax-induced cell death of *Arabidopsis* is meditated through reactive oxygen-dependent and -independent processes

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Abstract

An *Arabidopsis* protoplast system was developed for dissecting plant cell death in individual cells. Bax, a mammalian pro-apoptotic member of the Bcl-2 family, induces apoptotic-like cell death in *Arabidopsis*. Bax accumulation in *Arabidopsis* mesophyll protoplasts expressing murine *Bax* cDNA from a glucocorticoid-inducible promoter results in cytological characteristics of apoptosis, namely DNA fragmentation, increased vacuolation, and loss of plasma membrane integrity. *In vivo* targeting analysis monitored using jellyfish green fluorescent protein (GFP) reporter indicated full-length Bax was localized to the mitochondria, as it does in animal cells. Deletion of the carboxyl-terminal transmembrane domain of Bax completely abolished targeting to mitochondria. Bax expression was followed by reactive oxygen species (ROS) accumulation. Treatment of protoplasts with the antioxidant *N*-acetyl-L-cysteine (NAC) during induction of Bax expression strongly suppressed Bax-mediated ROS production and the cell death phenotype. However, some population of the ROS depleted cells still induced cell death, indicating that there is a process that Bax-mediated plant cell death is independent of ROS accumulation. Accordingly, suppression of Bax-mediated plant cell death also takes place in two different processes. Over-expression of a key redox-regulator, *Arabidopsis* nucleoside diphosphate kinase 2 (AtNDPK2) down-regulated ROS accumulation and suppressed Bax-mediated cell death and transient expression of *Arabidopsis* Bax inhibitor-1 (AtBI-1) substantially suppressed Bax-induced cell death without altering cellular ROS level. Taken together, our results collectively suggest that the Bax-mediated cell death and its suppression in plants is mediated by ROS-dependent and -independent processes.

Abbreviations: AtBI-1, *Arabidopsis* Bax inhibitor-1; AtNDPK2, *Arabidopsis* nucleoside diphosphate kinase; DAPI, 4′,6-diamino-2-phenylindole dihydrochloride; DEX, dexamethasone; GFP, green fluorescent protein; NAC, *N*-acetyl-L-cysteine; PCD, programmed cell death; RFP, red fluorescent protein; ROS, reactive oxygen species; TM, terminal transmembrane

Introduction

Programmed cell death (PCD) is a highly conserved cellular suicide process in organisms and is important for development and eliminating damaged or infected cells in response to environmental factors and disease (Steller, 1995). Animal apoptosis, a morphologically and biochemically defined type of PCD, have been extensively studied and its signalling pathways are relatively well known (Willis *et al*., 2003). Among the important regulators of apoptosis in metazoans are the Bcl-2 family of proteins (Gross *et al*., 1999; Scorrano and Korsmeyer, 2003). Some Bcl-2 proteins promote cell survival (Bcl-SL, Bcl-2, Bcl-W, Bcl-X_L, Bfl-1 Mcl-1, Brag-1 and A1) while others promote cell death (Bax, Bak, Bcl-XS, Bid, Bik, Hrk, and Bok). Several of the pro-apoptotic family members, including Bax, are mitochondrial outer membrane proteins. Bax mediates apoptosis by facilitating the release of cytochrome *c* and apoptosis-inducing factor from the intermembrane space to the cytoplasm where it activates PCD. Anti-apoptotic members of the Bcl-2 family, on the other hand, inhibit mitochondrial release of both cytochrome *c* and apoptosis-inducing factor. Thus, survival or death is controlled by the equilibrium between pro- and anti-apoptotic proteins (Gross *et al*., 1999; Scorrano and Korsmeyer, 2003).

Informatics has failed to identify Bax, Bcl-2, and Bcl-XL sequence homologs in the *Arabidopsis* and yeast (*Saccharomyces cerevisiae*) genomes, although evidence implicates the existence of functional orthologs. PCD in yeast cells is induced by the localization of mammalian Bax to the outer mitochondrial membrane. Bax-induced PCD in yeast cells can be suppressed by co-expression of anti-apoptotic Bcl-2 family protein (e.g. Bcl-2 or Bcl-X_L; Hanada *et al*., 1995; Greenhalf *et al*., 1996; Jurgensmeier *et al*., 1997). Even though details of Bax-mediated PCD in yeast are still unclear, reactive oxygen species (ROS) are effectors of Bax-induced PCD in yeast, as it is in mammalian cells (Madeo *et al*., 1999; Punj and Chakrabarty, 2003).

In plants, PCD is essential for normal reproductive and vegetative development. Specifically, it is essential for gamete development, sex determination, embryogenesis, leaf abscission, tracheary element and aerenchyma formation, and the hypersensitive response to abiotic and biotic environmental stress (reviewed in Dangl *et al*., 2000). Many plant pathogens suppress PCD during infection, which facilitates propagation (Abramovitch *et al*., 2003) and environmental stress induces PCD in plants (Huh *et al*., 2002). Transgenic modification of pathways regulating PCD is therefore a potential strategy to develop multiple stress tolerances in plants (Mitsuhara *et al*., 1999; Qiao *et al*., 2002). Very little is known about the molecular mechanism underlying PCD of plant cells (Dangl *et al*., 2000). However, there is evidence that the basic regulatory mechanisms underlying the activation of PCD in animal and plant systems are conserved (Kawai *et al*., 1999; Lacomme and Cruz, 1999; Mitsuhara *et al*., 1999; Solomon *et al*., 1999; Lam *et al*., 2001; Elbaz *et al*., 2002). In particular, substantial morphological and biochemical evidence has suggested the similarity between the plant hypersensitive response (that is, plant PCD associated with plant disease resistance) and animal Bax-induced cell death in plants (Lacomme and Cruz, 1999; Abramovitch *et al*., 2003).

Modulation of PCD in plants by expression of animal Bax has led to identification of cell survival genes (Kawai-Yamada *et al*., 2001; Moon *et al*., 2002, 2003; Hückelhoven *et al*., 2003; Matsumura *et al*., 2003). Among them, plant Bax Inhibitor-1 (BI-1)s that are homologs of human Bax inhibitor that suppresses Bax-mediated cell death in yeast were most extensively studied (Xu and Reed, 1998). Plant BI-1s function as suppressors of cell death in plants induced by either Bax (Kawai-Yamada *et al*., 2001) or fungal elicitor (Matsumura *et al*., 2003). Furthermore, it is demonstrated that barley BI-1 is a suppressor protein of *mlo*-mediated penetration resistance to *Blumeria graminis* (Hückelhoven *et al*., 2003).

In this study, we report the development of a unicellular system for the molecular dissection of Bax-mediated cell death and its suppression mechanisms in plants using *Arabidopsis* protoplasts. We demonstrate that Bax-mediated PCD in *Arabidopsis* protoplasts is meditated through reactive oxygen-dependent and -independent processes. In addition, *Arabidopsis* BI-1 (AtBI-1) functions as a negative regulator of cell death by either participating in the downstream of oxidative burst or alternative processes lacking the involvement of ROS.

Materials and method

Plant materials and growth conditions

Wild-type, *Bax* and *Bax*ΔTM transgenic (T_3) *Arabidopsis thaliana* plants (ecotype Columbia) used in this study were described in Kawai-Yamada *et al*. (2001). Plants were grown either in sterile culture on Murashige and Skoog medium (Murashige and Skoog, 1962) at 22 °C or in soil in a greenhouse with 16-h-light/8-h-dark photoperiod.

Protoplast preparation

Leaf tissues (5 g) of 3- to 4-week-old *Arabidopsis* plants were cut into small squares (5–10 mm^2) with a new razor blade and incubated with 30 ml of enzyme solution containing 0.25% Macerozyme R-10 (Yakult Honsha Co., Ltd., Tokyo, Japan), 1.0% cellulase R-10 (Yakult Honsha Co., Ltd., Tokyo, Japan), 500 mM mannitol, 1 mM $CaCl_2$, and 5 mM Mes–KOH (pH 5.6) at 22 °C for 9 h with gentle agitation (50–75 rpm). After incubation, the protoplast suspension was filtered through 100 μm mesh and protoplasts were overlaid on top of 20 ml of 21% sucrose, and centrifuged for at 720 rpm for 10 min. The intact protoplasts at the interface were transferred to a new falcon tube containing 30 ml of W5 solution containing 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes–KOH (pH 5.6). The protoplasts were pelleted again by centrifugation at 500 rpm for 5 min and resuspended in 30 ml of W5 solution. The protoplasts were then incubated on ice for 5 h for stabilization.

Western blot analysis

Total proteins were extracted from *Arabidopis* protoplasts in 30 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA, 1 × plant protease inhibitor mixture (Sigma, St. Louis, MO, USA) and separated on a 12% gel by SDS-PAGE. Then, they were blotted onto a poly vinylidene difluoride membrane (Immobilon, Millipore, Billerica, MA, USA) by using a semidry electroblotter. Anti-Bax antibody (#06-499, Upstate Biotechnology, Waltham, MA, USA) was used for detection by using a standard protocol (Moon *et al*., 2002).

Plasmid construction, transient gene expression and in vivo *targeting of proteins*

The cDNAs encoding Bax and BaxΔTM, a deletion of the C-terminal 21 amino acids of Bax, were isolated from pGilda-BAX by PCR (Moon *et al*., 2002). The primers were 5′-TCTAGAATGGACG GGTCCGGGGAGCA-3′ (Bax-5′), 5′-AGATC TGCCCATCTTCTTCCAGATGG-3′ (Bax-3′) and 5′-AGATCTGGTCCAACCACCCTGGTC TT-3′ (BaxΔTM-3′). To construct *AtBI-1:GFP* and *AtNDPK2:GFP*, *AtBI-1* and *AtNDPK2* were amplified from an *Arabidopsis* cDNA library by PCR using primers designed from the nucleotide sequence information deposited in the EST database. The primers were 5′-TCTAGAATGGATGC GTTCTCTTCCTT-3′ (AtBI-1-5′) and 5′- GGAT CCGTTTCTCCTTTTCTTCTTCT-3′ (AtBI-1-3′) for AtBI-1, and 5′-GCTCTAGAATGGTGGGA GCGACTGTA-3′ (AtNDPK2-5′) and 5′-GGATC CGCTCCCTTAGCCATGTAGCTA-3′ (AtNDP K2-3′) for AtNDPK2. All PCR products were confirmed by nucleotide sequencing and were inserted into *Xba*I and *Bam*HI sites of plasmid 326-sGFP (kindly provided by Inhwan Hwang, POSTEC, Korea) to create chimeric GFP-fusion constructs under the control of the 35S promoter. Plasmid 326-sGFP is a pUC-based vector containing CaMV35S-sGFP-NOS3′ for protoplast expression (S65T; Niwa *et al*., 1999).

For expression in protoplasts, all plasmid constructs were purified using Qiagen (Qiagen Inc., Valencia, CA, USA) columns according to the manufacturer's protocol. The plasmids were introduced into *Arabidopsis* protoplasts that had been prepared from leaf tissues by polyethylene glycol-mediated transformation (Jin *et al*., 2001; Kim *et al*., 2001). Expression of the fusion constructs was monitored at various time points after transformation and images were captured with a cooled CCD camera and a Zeiss Axioplan fluorescence microscope (Carl Zeiss Co., Jena, Germany). The filter sets used were XF116 (exciter, 474AF20; dichroic, 500DRLP; and emitter, 605DF50) and XF137 (exciter, 540AF30; dichroic, 500DRLP; and emitter, 585ALP; Omega, Inc., Brattlebora, VT) for GFP and RFP, respectively. Data were then processed using Adobe Photoshop software (Adobe System, Mountain view, CA) and presented in pseudocolor format.

Measurement of cell death

The percentage of dead cells was quantitatively determined by 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) staining (Molecular Probes, Eugene, OR, USA). An aliquot of protoplast cells ($\sim 2 \times 10^5$ ml^{-1}) was treated with aqueous DAPI (0.25 μg ml^{-1}) for 5 min. The UV absorbance (excitation, 364 nm; emission, 454 nm) of the supernatant was measured after centrifugation. DAPI-stained cells were counted microscopically using more than 100 cells for each experiment.

DNA fragmentation analysis

Protoplasts of Bax and BaxΔTM transgenic plants were treated with dexamethasone (DEX) (5 μM). Genomic DNA was isolated from DEX-treated protoplasts after 12, 24, 36, and 48 h treatment and from untreated protoplasts (control) after 48 h using the DNeasy Plant Kit (Qiagen Inc., Valencia, CA, USA). For each sample, 5 μg DNA was loaded per lane and electrophoresed in 1.5% agarose gel. ^{32}P-labeled total genomic DNA from Bax transgenic plants was used as probe. DNA blot hybridization and membrane washing were performed as described (Moon *et al.*, 2002).

Detection of ROS and flow cytometric analysis

To measure ROS, an aliquot of protoplast suspension ($\sim 2 \times 10^5$ ml^{-1}) was incubated with 20 μM dihydrorhodamine123 (Molecular Probes, Eugene, Oregon, USA) for 15 min and were visualized under a Zeiss Axioplan fluorescence microscope using XF33/E (exciter, 535DF35; dichroic, 570DRLP; emitter, 605DF50 ; Omega, Inc., Brattlebora, VT, USA). The fluorescence intensity levels were measured in a flow cytometer with excitation and emission settings of 535DF35 nm and 605DF50 nm, respectively. Data were collected with a FACscan fluorescence activated cell scanner (Becton-Dickinson, San Jose, CA, USA) using the QCELL Quest data acquisition program (Becton-Dickinson, San Jose, CA, USA).

Results

Expression of Bax in Arabidopsis *protoplasts induces cell death*

Protoplasts were isolated from *Arabidopsis* wild-type plants or transgenic plants transformed to express either *Bax* or *Bax*ΔTM (deletion of the 21 amino acid carboxyl-terminal transmembrane (TM) domain of Bax) under the control of the glucocorticoid promoter (Kawai-Yamada *et al.*, 2001) that is induced by DEX solution (Figure 1A). Cell viability was assessed by staining with DAPI, a nucleic acid stain. DAPI is excluded from living protoplast cells but enters cells within seconds after loss of membrane integrity fluoresces brightly in nuclei within seconds after cell death (Bethke *et al.*, 1999). Treatment of protoplasts from wild type seedlings with 20 μM or higher concentration of DEX did not influence protoplast viability over 48 h (Figure 1B, C). However, DEX induced Bax expression (Figure 1A) and reduced protoplast viability in *Bax* transformed plants (Figure 1B, C). Mitochondrial membrane targeting is essential for the cytotoxic activity of Bax in yeast and tobacco cells (Lacomme and Cruz, 1999; Harris *et al.*, 2000). Additionally, it is known that the carboxyl-TM domain of Bax is necessary for Bax-induced cell death in both tobacco and *Arabidopsis* (Lacomme and Cruz, 1999; Kawai-Yamada *et al.*, 2001). DEX treatment of *Arabidopsis* protoplasts transformed with *Bax*ΔTM, which lacks the domain necessary for attachment to the mitochondrial outer membrane, did not induce cell death (Figure 1B, C). The protein level of Bax and BaxΔTM in the transgenic lines following induction with DEX was similar (Figure 1A). Bax level was positively correlated with the severity of Bax-mediated cell death of protoplasts (data not shown).

Expression of Bax in Arabidopsis *protoplasts induces cytological aberrations that are hallmarks of apoptosis*

Clonigenic survival (Figure 1C) as well as the appearance of morphological changes (Figure 2) were examined at discrete time intervals after treatment of *Bax*-transformed *Arabidopsis* protoplasts with DEX. DEX induced cell death was preceded by the abnormal cytology (Figure 2A), and accumulation of ROS (Figure 5).

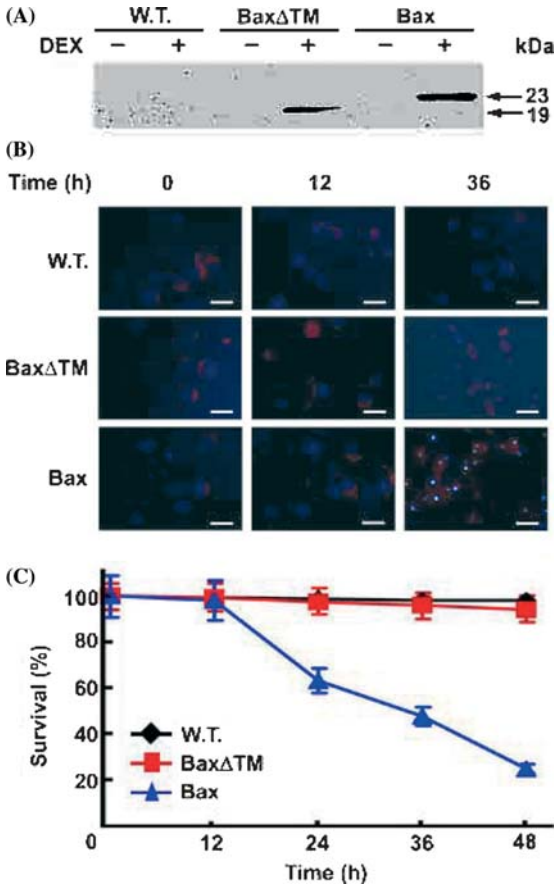

Figure 1. Bax-induces cell death in *Arabidopsis* protoplasts. Protoplasts from three week-old-seedlings of wild-type (W.T.), transgenic Bax (Bax) and transgenic BaxΔTM plants (BaxΔTM) were incubated on medium containing 5 μM DEX for various time periods. (A) Protoplasts were treated with DEX for 0 (−) or 24 h (+). Shown are immunoblots of total protein extracts (20 μg per lane) that were subjected to SDS-PAGE and analyzed using anti-mouse Bax antiserum (Moon *et al.*, 2002). (B,C) Loss of protoplast viability. The protoplasts were stained with DAPI after the indicated intervals of DEX treatment and observed with epifluorescence microscopy. Photographs (B) show that the nuclei of inviable protoplasts are stained blue. Bar indicates 100 μm. The percentage of inviable cells in the population (C) are means ± mean deviation of nine independent experiments (n = 300–500).

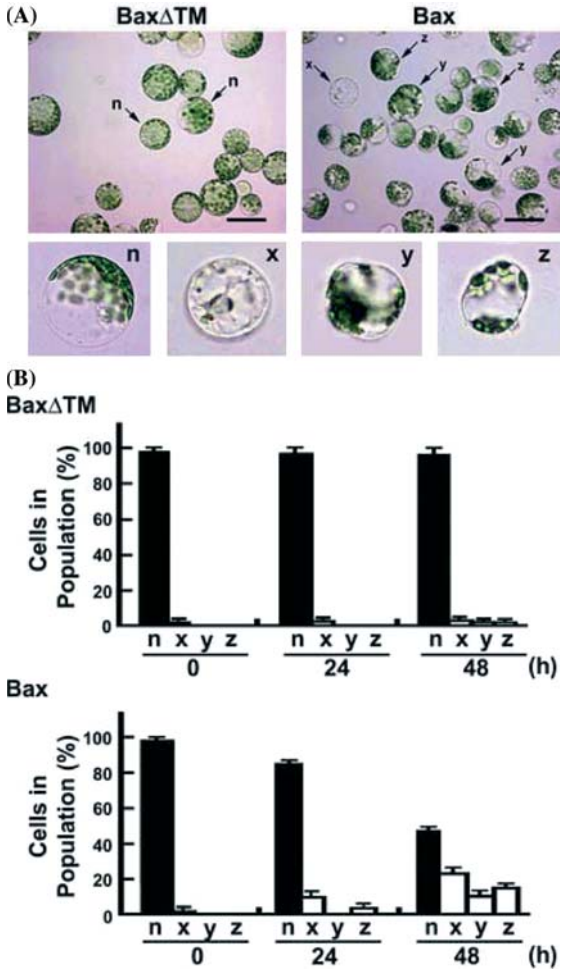

Figure 2. Morphological changes associated with Bax expression are progressive. Protoplast cell ($\sim 2 \times 10^5$ ml^{-1}) from Bax (Bax) and BaxΔTM (BaxΔTM) transgenic plants were treated with DEX (5 μM). Cell morphology was observed by microscopy at intervals after DEX treatment. (A) Shown are the types of morphology observed after 12 h DEX treatment. Symbols: n, normal cells; x, cells with disappearing cytosol (vacuolation); y, cells with cytosol shrinkage, and disorganization; z, cells with greater cyosolic shrinkage and cytosol density lower than normal. Bar, 100 μm. (B) Morphological changes associated with different degrees of Bax expression were quantified by counting protoplasts after DEX treatment for the indicated time intervals. The Figure shows the percentage of protoplasts in the morphological categories indicated above (n, x, y, and z). The data are means ± mean deviation of seven independent experiments (n = 300–500).

Non-laddered DNA degradation, detected by agarose gel electrophoresis, was significant in Bax cells after 24, 36 and 48 h of DEX treatment (Figure 3).

The cytological aberrations were classified into three groups (Figure 2A); vacuolation of cells with lower cytosol density than normal (category x), swelling, disorientation, and disorganization of the cell which may be due to loss of turgor pressure (category y), shrinkage in the protoplast size, enlargement of the vacuoles, condensation of the organelles to the plasma membrane, increased density (category z). All these morphological

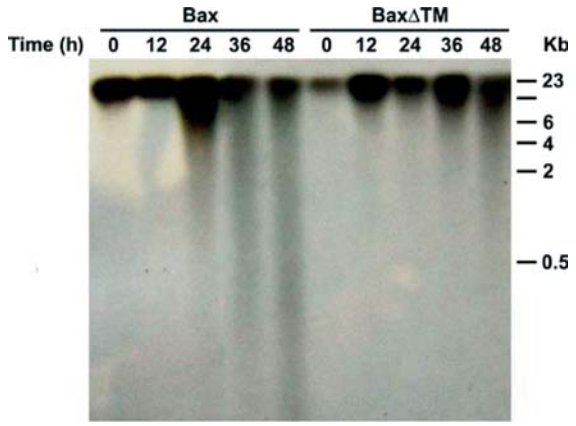

Figure 3. DNA laddering is associated with Bax-induced cell death. Protoplasts of Bax (Bax) and BaxΔTM (BaxΔTM) transgenic plants were treated with 5 μM DEX as described (Figure 1). Internucleosomal DNA fragmentation was detected by Southern blotting of total genomic DNA extracted from these protoplasts, harvested after the indicated period of DEX treatment, using genomic DNA from Bax transgenic plants as probe.

characteristics are considered to be hallmarks of cells undergoing apoptosis (Filonova *et al*., 2000; Houot *et al*., 2001; Fath *et al*., 2002). The fraction of inviable cells and cells with abnormal morphology in the population increased proportionately with the duration of DEX treatment (Figures 1 and 2B). A very low percentage (less than 2%) of structure-less ghosts and cellular debris that are typical for necrotic cell death were also observed after 24 h DEX treatment (data not shown). However, even after 36 or 48 h treatment, the majority of cells belonged to categories x, y and z (Figure 2B and data not shown). Thus, PCD-like death appears to be the major form of cell death induced by Bax, although necrotic cell death can also occur. The triggering of apoptosis and necrosis in the same cell type by a given signal has been reported and has been ascribed to factors such as the ATP level in the cell and activation of pro-apoptotic proteins (Green and Reed, 1998). The cytological changes associated with cell death occurred in only 2% of cells expressing BaxΔTM (Figure 2 and data not shown).

Bax is localized to the mitochondria of Arabidopsis

The targeting of Bax to mitochondria in *Arabidopsis* protoplasts and the necessity of the TM domain for targeting and cell death (Lacomme and Cruz, 1999; Kawai-Yamada *et al*., 2001) were confirmed using green fluorescent protein (GFP) and red fluorescent protein (RFP) imaging (Davis and Vierstra, 1998; Heikal *et al*., 2000). Chimeric *Bax:GFP* or *BaxΔTM:GFP* cDNAs (Figure 4A), was introduced into protoplasts isolated from wild type *Arabidopsis* seedlings together with a *F_1-ATPase-gamma:RFP* construct encoding a mitochondrial marker protein (Jin *et al*., 2003). The subcellular distribution of the Bax:GFP signal mainly overlapped (over 95%) with those of the F_1-ATPase-gamma:RFP red fluorescent signal indicating co-localization on the mitochondria membrane whereas BaxΔTM:GFP signal was uniformly distributed in the cytosol (Figure 4B).

ROS are effectors of Bax-induced cell death in plant

Mitochondria are one of the major sites of ROS generation in plants, and ROS are implicated as

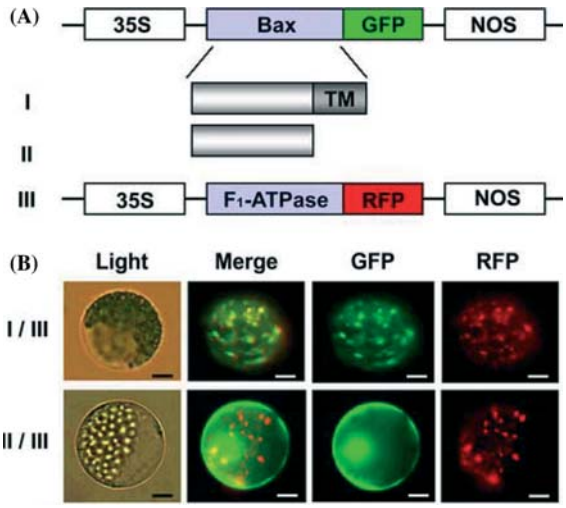

Figure 4. Localization of Bax protein in the mitochondria is dependent on the C-terminal transmembrane domain. (A) Schematic representation of constructs used for protoplast transformation. Symbols are: 35S, cauliflower mosaic virus 35S promoter; Bax, full length murine Bax cDNA; TM, the 21 carboxyl-terminal amino acids of Bax; GFP, green fluorescent protein; NOS, nopaline synthase gene poly A signal; RFP, red fluorescent protein; F_1-ATPase, F_1-ATPase-gamma cDNA; I, *Bax:GFP* construct; II, *BaxΔTM:GFP* construct; III, *F_1-ATPase-gamma:RFP* construct. (B) Subcellular localization of Bax. Protoplasts prepared from *Arabidopsis* seedlings were co-transformed with two constructs *Bax:GFP* plus *F_1-ATPase-gamma:RFP* (I/III) or *BaxΔTM:GFP* plus *F_1-ATPase-gamma:RFP* (II/III). The transformed protoplasts were examined under fluorescent microscope at 12–24 h after transformation. Green and red images are GFP and RFP signals, respectively. Bar indicates 20 μm.

effectors of PCD in animals (Jabs, 1999). Since Bax localized to mitochondria in *Arabidopsis* protoplasts (Figure 4), the role of ROS, if any, in Bax-induced cell death was examined. ROS production was monitored using dihydrorhodamine123, which upon oxidation by ROS, becomes the fluorescent chromophore, rhodamine123 (Schulz *et al*., 1996).

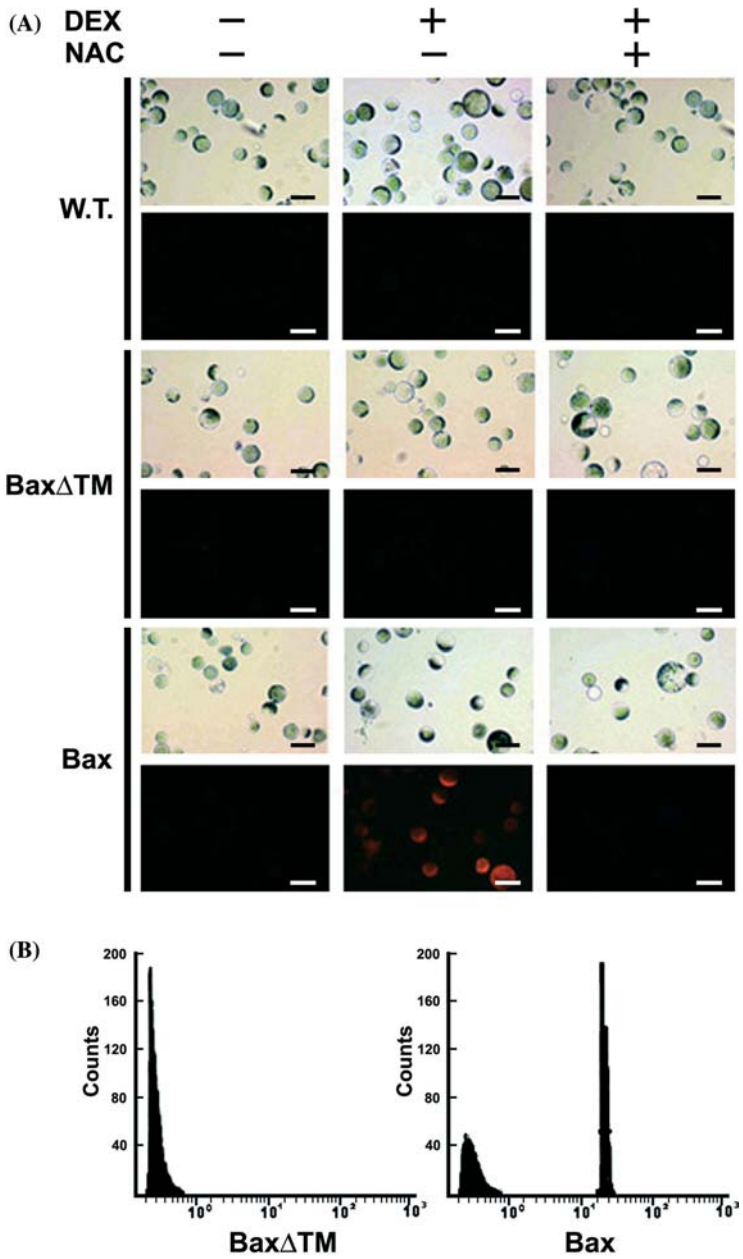

Figure 5. Bax expression is associated with ROS accumulation. Proptoplasts from three week-old-seedlings of wild-type (W.T.), Bax (Bax) and BaxΔTM (BaxΔTM) transgenic plants were treated with DEX (5 μM). ROS accumulation was monitored at the end of DEX treatment by incubating protoplasts with 20 μM dihydrorhodamine123 for 15 min and subjecting to microscopy (A) and flow cytometric analysis (B). (A) Fluorescence data (lower panel) after 0 h (−) or 12 h (+) DEX treatment in the absence (−) or presence (+) of the thiol reductant *N*-acetyl-L-cysteine (NAC, 1 mM) and the corresponding phase-contrast display (upper panel) are depicted. Bar indicates 100 μm. (B) Flow cytometric analysis was performed after 24 h DEX treatment.

As shown in Figure 5A, protoplasts expressing Bax exhibited a strong fluorescence when incubated with dihydrorhodamine123 indicating ROS accumulation, whereas, wild-type protoplasts or protoplasts expressing BaxΔTM exhibited no significant fluorescence. Virtually all cells remain viable up to 12 h after DEX treatment (Figure 1B), indicating that the generation of ROS by Bax precedes loss of viability and is not a symptom of cell death. The number of cells in the population that accumulated ROS was quantified by flow cytometric analysis and found to be significant in protoplasts expressing Bax but negligible in protoplasts expressing BaxΔTM (Figure 5B).

Bax-induced cell death in plant is meditated through reactive oxygen-dependent and -independent processes

N-acetyl-L-cysteine (NAC) is an antioxidant known to increase cellular pools of free radical scavengers (Joo *et al.*, 2001). Bax-induced ROS generation and toxicity were suppressed in the presence of NAC (Figures 5A and 6A), as expected if ROS are effectors of Bax-induced cell death in *Arabidopsis*. However, depletion of ROS in the cell with NAC, a strong antioxidant, could not prevent Bax-mediated cell death completely (Figure 6A). Importantly, most of the unsuppressed cells by NAC showed death cell phenotype without ROS accumulation (Figure 6B), indicating that the existence of alternative cell death process that is independent of ROS. Thus these results collectively indicate that although ROS is a common element and key event of PCD in animals, plants and yeasts as part of a basic, evolutionarily conserved mechanism (Madeo *et al.*, 1999; Moon *et al.*, 2003; Punj and Chakrabarty, 2003), cell death of plants can involve a ROS independent process also.

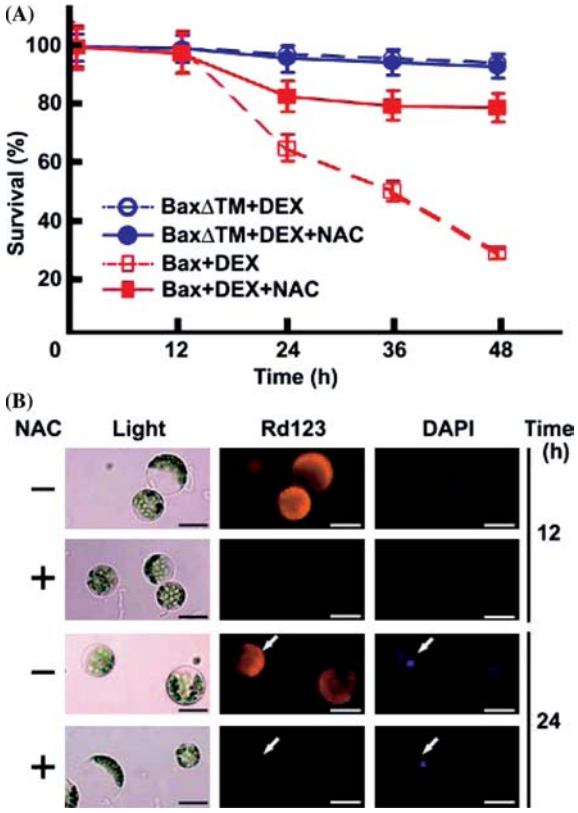

Figure 6. Bax-induced cell death of *Arabidopsis* is meditated through reactive oxygen-dependent and -independent processes. (A) Bax-induced cell death is inhibited by NAC. Protoplasts from three week-old-seedlings of Bax (Bax) and BaxΔTM (BaxΔTM) transgenic plants were treated with DEX (5 μM) in the absence or presence of the NAC for the periods indicated and stained with DAPI (described in Figure 1). Percentage of viable cells in population was determined by counting (n = 250–300). The data are the average ± SE of seven independent experiments. (B) Cell death in plant is mediated by ROS independent process. Protoplast from three week-old-seedlings of Bax transgenic plants were treated with DEX (5 μM) in the absence (−) or presence (+) of the NAC (1 mM) for the periods indicated and stained with DAPI (described in Figure 1). Shown are representative of three independent experiments. Bar indicates 100 μm.

Arabidopsis *Bax inhibitor-1 (AtBI-1) suppresses Bax-induced cell death independent of ROS*

AtBI-1 suppresses Bax induced cell death in *Arabidopsis* plants (Kawai-Yamada *et al.*, 2001). AtBI-1 has seven transmembrane domains and thought to be localized in the endoplasmic reticulum membrane (Xu and Reed, 1998; Kawai-Yamada *et al.*, 2001). *AtBI-I:GFP*, a chimeric gene construct that expresses a functional AtBI-1 protein (Kawai-Yamada *et al.*, 2001), was used to investigate the possibility that AtBI-1 can suppress Bax toxicity in *Arabidopsis* protoplasts also. When protoplasts from wild type *Arabidopsis* seedlings were co-transformed with the *AtBI-I:GFP* construct and a chimeric construct containing chaperone binding protein fused to RFP (*BiP:RFP*) as marker for the endoplasmic reticulum (Kim *et al.*, 2001), the subcellular distribution of green and red fluorescent signals were found to closely overlap each other (Figure 7A), indicating that AtBI-1 is targeted to the ER. The data also show that similar

BiP:reporter gene fusion constructs can be used as a control for experiments involving *AtBI-1:GFP*.

Twelve hours after transformation of *BiP:GFP* and *AtBI-1:GFP* into protoplasts derived from leaf tissues of *Bax* transgenic plants, 60% of the protoplasts expressed both of the proteins (data not shown). AtBI-1:GFP protected cells against Bax-induced cell death (Figure 7B) indicating that AtBI-1 is an efficient suppressor of Bax toxicity in the protoplasts as well in cells in plants (Kawai-Yamada *et al*., 2001).

Since ROS accumulation was a key mediator of Bax-induced cell death in *Arabidopsis* protoplasts (Figure 5), the role of AtBI-1 to attenuate ROS accumulation was investigated. ROS production was examined 24 h after DEX (5 μM) treatment (Figure 8). In these experiments, *Arabidopsis* NDP kinase was expressed (*AtNDPK2:GFP*) as a control. AtNDPK2 is a protein that suppresses Bax-mediated cell death in yeast by attenuating oxygen radical generation (Moon *et al*., 2002) and is involved in cellular redox regulation in plants (Fukamatsu *et al*., 2003; Moon *et al*., 2003). Twenty four hours after DEX (5 μM) treatment, ROS were detected in protoplasts of transgenic Bax protoplasts whether or not they had been further transformed with *BiP:GFP* or *AtBI-1:GFP* (Figure 8). However, transgenic *Bax* protoplasts transformed with *AtNDPK2:GFP* failed to accumulate ROS (Figure 8). Suppression of cell death by ectopic expression of AtBI:GFP was 17.5% greater than suppression of cell death by ectopic expression of AtNDPK2:GFP (cell survival was measured 24 h after DEX treatment). These results suggest that, although ROS are effectors of Bax-induced cell death in plants (Figures 5 and 6) protection afforded by AtBI-1 in Bax-induced cell death is independent of the oxidative burst.

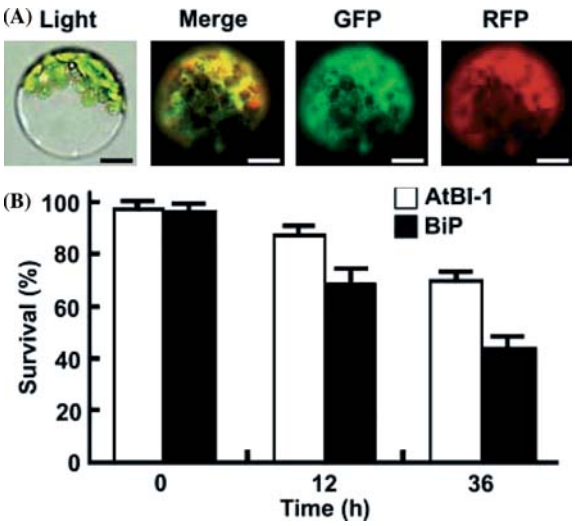

Figure 7. Bax-induced cell death is suppressed by AtBI-1. (A) Localization of AtBI-1 in *Arabidopsis* protoplasts. Protoplasts isolated from wild type *Arabidopsis* seedlings were co-transformed with the reporter gene fusion constructs, *AtBI-1:GFP* or *BiP:RFP*. Co-localization of the encoded proteins in the ER was observed by examining the protoplasts under fluorescent microscope 12–24 h after transformation. Shown from left to right are the phase contrast (Light), red and green fluorescence images merged (Merge), green (GFP) and red (Red) fluorescent images of one protoplast, respectively. Bar indicates 20 μm. (B) Suppression of Bax-induced cell death by AtBI-1. Protoplasts were isolated from Bax transformed *Arabidopsis* seedlings transformed with the reporter gene fusion constructs, *AtBI-1:GFP* or *BiP:GFP*. Twelve hours after transformation, protoplasts were treated with DEX (5 μM) for the indicated times and stained with DAPI (as in Figure 1). Percentage of viable cells in population was determined by counting (n = 250–300). The data are the average ± SE of four independent experiments.

Discussion

In this paper, we establish an *Arabidopsis* protoplast system for functional dissection of Bax-in-

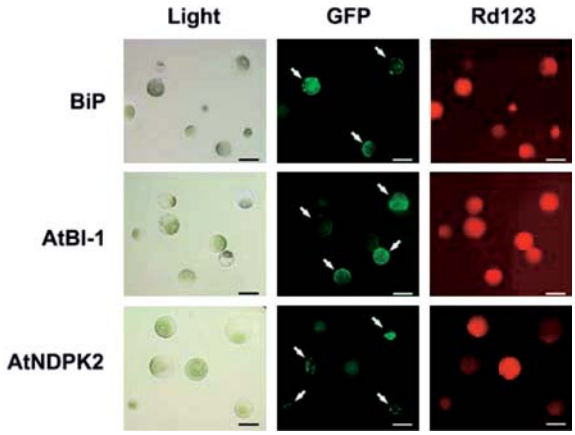

Figure 8. AtBI-1 does not suppress Bax-induced accumulation of ROS. Protoplasts isolated from three week-old-seedlings of Bax transgenic plants were transformed with *BiP:GFP* (BiP), *AtBI-1:GFP* (AtBI-1), and *AtNDPK2:GFP* (AtNDPK2) reporter gene fusion constructs. Twelve hours after transformation, the protoplasts were treated with 5 μM DEX for 24 h. They were then incubated with 20 μM dihydrorhodamine123 for 15 min and observed by microscopy. The phase-contrast (Light), green fluorescence (GFP) and red fluorescence (Rd123) images of one aliquot of protoplasts of each transformant are depicted. Arrows point to GFP-expressing cells. Bar indicates 100 μm.

duced cell death in plants. By expressing murine *Bax* cDNA from a tightly regulated glucocorticoid-inducible promoter, the timing and extent of cell death could be controlled by application of DEX, a synthetic glucocorticoid. The advantage of the protoplast system over a whole-plant system is that Bax-induced morphological changes and cell death can be quantified on a cellular basis (Figures 1, 2, and 6). Furthermore, this system facilitates efficient and timely targeting of pharmacological agents and molecular genetic reagents for indepth dissection of events leading to cell death (Figures 4–8).

Bax-induced cell death in plants is mediated by ROS generation

ROS participate in plant cell death processes in animals, yeast and plants (Jabs, 1999; Madeo *et al*., 1999, 2002). Toxicity of ROS, that are by-products of normal metabolic processes in cells, is due to damage inflicted on to cellular lipids, carbohydrates, proteins and DNA (Byczkowski and Gessner, 1998; Kannan and Jain, 2000). Low levels of ROS, however, play a signaling role in PCD in animals, yeasts and plant cells (Jabs, 1999). Thus, improved ROS scavenging by exogenous application of antioxidants and thiol-reductants such as NAC, or endogenously by over-expression of ROS scavenging enzymes or antioxidant Bax-family proteins such as Bcl-2, blocks PCD in yeast and animal cells (Hockenbery *et al*., 1993; Jacobson and Raff, 1995). During a hypersensitive response that is the most well-studied PCD phenomenon in plants, ROS are utilized as second messengers in the execution of cell death (Jabs, 1999; Dangl *et al*., 2000). In accordance with these results, Bax-induced cell death in *Arabidopsis* protoplasts was also found to be mediated by ROS generation. Therefore, it would appear that ROS is a common element and key event of PCD in animals, plants and yeasts as part of a basic, evolutionarily conserved mechanism.

Function of AtBI-1 and ROS independent cell death process

The results presented herein that over-expression of AtBI-1 mediated suppression of Bax induced cell death is independent from ROS accumulation indicate that AtBI-1 functions as a negative regulator of cell death independent from ROS accumulation, downstream of ROS, or both (Figure 9). How does AtBI-1 suppress Bax-mediated cell death in plants? Since the ER performs several essential functions implicated in cell death regulation such as calcium homeostasis and vesicle transport, it is possible to speculate that AtBI-1, an integral membrane protein mainly localized in the ER (Figure 7), could possibly function as regulator preventing Ca^{2+} efflux from ER with functional ortholog of Bcl-2 or solely in plant as hypothesized in animal (Xu and Reed, 1998; Kawai-Yamada *et al*., 2001; Demaurex and Distelhorst, 2003). According to animal apoptosis model, Ca^{2+} released into cytoplasm can induce mitochondrial permeability transition (PT) pore opening, which induces release of apoptotic activators to cytoplasm and consequently activate caspase-mediated PCD independent of ROS accumulation (Green and Reed, 1998; Xu and Reed, 1998; Demaurex and Distelhorst, 2003; Scorrano and Korsmeyer, 2003). Thus, we hypothesize that decrease of the basal efflux of Ca^{2+} from ER by over-expression of AtBl-1 might close PT pore and prevent apoptotic activators from releasing to cytoplasm, resulting in suppression of ROS-independent PCD process. This speculation is supported with the results that strong antioxidant could not completely suppress Bax-mediated cell death in *Arabidopsis* protoplast cells and the cells that are not suppressed with antioxidant clearly showed cell death without ROS accumulation. These results also suggest that

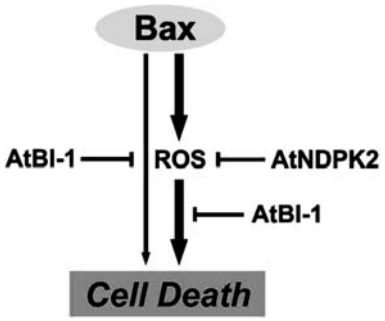

Figure 9. Model for Bax-induced cell death processes in plant. Bax-induced cell death in plant is mediated through either ROS-dependent or -independent processes. AtNDPK2, a cellular redox regulator, suppresses the cell death by inhibiting ROS generation. In addition, AtBI-1 suppresses the cell death by either participated in the downstream of oxidative burst or alternative processes lacking the involvement of ROS.

Bax-mediated cell death in plants mainly results from oxidative stress as well as result from a process independent of ROS (Figure 9).

Alternatively, AtBl-1 could function as downstream effecter of Bax-mediated ROS generation by regulating membrane recycling and vesicle trafficking to intracellular membranes. Oxidative stress damages membranes by lipid peroxidation and protein oxidation. Therefore, membrane recycling is important for tolerance to oxidative stress and it is postulated that failure to repair the membranes during oxidative stress will result in the loss of membrane integrity, ion leakage, and ultimately, cell death. In accordance with this theory, a vesicle-associated membrane protein of *Arabidopsis* (AtVAMP), which is involved in membrane recycling, suppressed Bax toxicity in yeast and plant downstream of the oxidative burst (Levine *et al.* 2001; our unpublished results). However, we cannot rule out another possibility that AtBl-1 functions as a negative regulator in the both ROS-dependent and independent processes (Figure 9).

Mitochondrial targeting is essential for the cytotoxic activity of Bax

Mitochondria are highly susceptible to oxidative damage. Because of their importance in cellular energetics and their sensitivity to cellular changes, loss of mitochondrial membrane integrity is an early event in the apoptotic pathway (Salvioli *et al.*, 2001). The importance of the mitochondria to apoptosis mediated by the Bcl-2 family proteins has been well documented in animal models (Gross *et al.*, 1999). However, the connection of mitochondria to plant PCD, particularly in the hypersensitive response, is not well understood (Jones, 2000). Lacomme and Cruz (1999) showed that TMV-mediated Bax expression in tobacco plants results in protein phosphatase-dependent cell death. This hypersensitive response-like cell death closely resembles the *N* gene-mediated hypersensitive response and requires localization of Bax to mitochondria. The inducible expression of Bax in protoplasts from transgenic *Arabidopsis* plants also activated PCD and the localization of Bax to plant mitochondria was required for this activity, confirming the importance of the mitochondrial-Bax connection in plants. Cell death induced by Bax expression was mediated by the accumulation of ROS following mitochondrial targeting of Bax. This is consistent with the hypothesis that localization of Bax to the yeast outer mitochondrial membrane impairs oxidative phosphorylation, electron transport, ATP production and mitochondrial transmembrane potential, and subsequent ROS production (Gross *et al.*, 1999; Jones, 2000; Kroemer and Reed, 2000). Even though the details of this molecular mechanism remain to be clarified, Bax-mediated ROS accumulation in cytosol can alter the cytoplasmic redox homeostasis, which may be an important determinant of plant cell death.

The evolutionally conserved cell death mechanism by Bax

Does the Bax-mediated pathway for plant cell death share signalling components with pathogen-mediated hypersensitive response cell death? Several lines of evidence indicate overlap between elements of Bax-mediated cell death and the pathogen-mediated hypersensitive response in plants. For example, plant Bl-1 is rapidly expressed during pathogen challenge (Sanchez *et al.*, 2000), and suppresses fungal elicitor- and fungal-mediated cell death plant (Hûckelhoven *et al.*, 2003; Matsumura *et al.*, 2003). Peptide inhibitors of caspases abolish HR cell death induced by pathogen (del Pozo and Lam, 1998). Tobacco transgenic plants expressing the pro-survival cell death regulator Bcl-x_L and Ced-9 can delay HR cell death (Mitsuhara *et al.*, 1999). Bax can activate HR-like cell death (Lacomme and Cruz, 1999). AvrPtoB, an *Pseudomonas* type III effector, which can induce plant disease susceptibility by inhibition of host PCD, suppressed Bax-induced cell death in yeast (Abramovitch *et al.*, 2003). These results suggest that regulatory mechanisms in plant and animal cell death maybe similar. Therefore, a direct comparison of Bax-mediated and hypersensitive response-mediated cell death at the cellular level would provide valuable information concerning plant PCD.

In summary, we propose that the protoplast system is powerful tool to study a specific cell death program in plant induced by Bax. Using the transgenic *Arabidopsis* line expressing Bax from the inducible glucocorticoid promoter, we have isolated plant genes responsible for suppressing Bax-induced cell death in plants (our unpublished

results). Further studies on plant homologs of well known animal cell death-related genes, such as those of the metacaspase gene family (Uren *et al*., 2000) and those encoding mitochondrial permeability transition components, such as the adenine nucleotide translocator or the voltage dependent anion channel (Lam *et al*., 2001; Belzacq *et al*., 2002; Tsujimoto and Shimizu, 2002), will shed light on the molecular mechanism underlying not only Bax-mediated cell death in plants but also plant-specific PCD.

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