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Soybean Ascorbate Peroxidase Suppresses Bax-Induced Apoptosis in Yeast by Inhibiting Oxygen Radical Generation

Haejeong Moon, Dongwon Baek, Boyoung Lee, D. Theertha Prasad, Sang Yeol Lee, Moo Je Cho, Chae Oh Lim, Myung Suk Choi, Jeongdong Bahk, Myeong Ok Kim, Jong Chan Hong, and Dae-Jin Yun[1]

Division of Applied Life Science (BK21 Program) and Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Korea

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Bax, a mammalian proapoptotic member of the Bcl-2 family, can induce cell death when expressed in yeast or plant cells. To identify plant Bax inhibitors, we cotransformed a soybean cDNA library and the *Bax* gene into yeast cells and screened for expressed genes that prevented Bax-induced apoptosis. From the Bax-inhibiting genes isolated, ascorbate peroxidase (sAPX) was selected for characterization. The transcription of sAPX in plants was specifically induced by oxidative stress. Moreover, overexpression of sAPX partially suppressed the H_2O_2-sensitive phenotype of yeast cytosolic catalase T (Δ*ctt*)- and thermosensitive phenotype of cytochrome *c* peroxidase (Δ*ccp*)-deleted mutant cells. Examination of reactive oxygen species (ROS) production using the fluorescence method of dihydrorhodamine 123 oxidation revealed that expression of Bax in yeast cells generated ROS, which was greatly reduced by coexpression with sAPX. Our results collectively suggest that sAPX inhibits the generation of ROS by Bax, which in turn suppresses Bax-induced cell death in yeast.

Key Words: **ascorbate peroxidase; Bax-induced cell death; apoptosis; *Saccharomyces cerevisiae;* reactive oxygen species.**

Programmed cell death (PCD) or apoptosis, an evolutionarily conserved form of cell suicide, occurs routinely during organism development, and in response to environmental factor. This process is important for eliminating unwanted, damaged, infected or useless cells that would otherwise cause inflammation of the surrounding cells with their cytoplasmic contents (reviewed in 1). In plants, PCD is essential for normal reproductive and vegetative development, specifically, gamete development, sex determination, embryogenesis, leaf abscission, formation of tracheary element, aerenchyma formation, and hypersensitive response to environmental stress (reviewed in 2). Although very little is known about the mechanism of PCD in plants, it is suggested that the proper regulation of this process involves genetic control (2).

In animals, apoptosis is under genetic control and the signaling pathways and genes involved in apoptosis have been extensively studied (3). The Bcl-2 family of proteins are important regulators of cellular apoptosis (4). Bcl-2 proteins promote either cell survival (Bcl-SL, Bcl-2, Bcl-W, Bcl-X_L, Bfl-1 Mcl-1, A1, Brag-1, and A1) or cell death (Bax, Bak, Bcl-XS, Bid, Bik, Hrk, and Bok). Several of these regulatory proteins are located in the outer membrane of mitochondria, and play crucial roles in apoptosis by releasing apoptogenic factors such as cytochrome *c* and apoptosis-inducing factor (AIF) from intermembrane space into cytoplasm, which in turn activate caspases, hallmarks of apoptotic execution. While proapoptotic members of the Bcl-2 family induce apoptosis-associated mitochondrial release of both cytochrome *c* and AIF, the antiapoptotic members counteract this process. Ultimately, the ratio or equilibrium between pro- and antiapoptotic proteins determines the susceptibility of a particular cell to apoptosis (4).

The expression of Bax (a proapoptotic member of the Bcl-2 family) in yeast induces apoptosis, which can be suppressed by co-expression with antiapoptotic members of the same protein family, Bcl-2 or Bcl-X_L (5–7). Yeast mitochondria seems to be involved in Bax-induced cell death in the similar ways as in mammalian cells, involving release of cytochrome *c* (8) and alterations in mitochondrial membrane potential (9). The phenotype of cell death promoted by Bax in tobacco plant closely resembles hypersensitive response, a type

[1] To whom correspondence and reprint requests should be addressed. Fax: +82-55-759-9363. E-mail: djyun@nongae.gsnu.ac.kr.

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of PCD in plants induced by tobacco mosaic virus, signify that expression of Bax in plants is also lethal (10). Earlier mutational analyses demonstrate that deletion of the carboxyl-terminal transmembrane domain of Bax necessary for mitochondrial localization abolishes protein lethality, thereby indicating that targeting of Bax to mitochondria is crucial for cell death in plants (10).

Observations on Bax-induced cell death in animals, yeasts and plants suggest that some elements of this mechanism may be conserved among various organisms. We employed yeast, a powerful genetic tool, to identify the molecular determinants of Bax-induced apoptosis in plants. Soybean cDNA library was cotransformed with the *Bax* gene into yeast cells, and overexpressed genes that could suppressed Bax-induced cell death were isolated. From the repertoire of the Bax-inhibiting proteins obtained, we characterized a soybean ascorbate peroxidase (sAPX) in detail. Here we show that ROS accumulated in yeast cells during Bax-promoted cell death, which was inhibited by the overexpression of sAPX. Previous studies suggest that ROS accumulation is a prerequisite for ensuing cell death (11–13). Our experiments indicate that the yeast genetic system is a powerful tool in the isolation of plant cell death suppressors and confirm the above hypothesis.

MATERIALS AND METHODS

Media, strains, and plasmids. Standard procedures were used for yeast culture and growth media preparation (14). Yeast transformation was performed using the lithium acetate method of Elble (15). *Saccharomyces cerevisiae* strains W303-1a (*MAT*a *ura3-1 leu2-3, 112 his3-11,15 ade2-1 trp1-1 can1-110*), ccp1::HIS3 (isogenic to W303-1a with *cpp::HIS3*), Ga74-1A (*MAT*a *leu2 his3 trp1 ade8 cta1-2 ura3::CTT1-lacZ*), GA74T-1A (isogenic to Ga74-1A with *ctt::URA3*) (16, 17) and the construction of the pGilda-Bax plasmid (18) are described elsewhere.

Screening strategy. The pADGal4-2.1 vector (Stratagene) containing the soybean (*Glycin max* L. cv. Williams) cDNA library was transformed into W303-1a strain harboring pGilda-Bax. The transformed cells (2×10^5) were plated onto galactose-based synthetic minimal medium (SD) [0.67% yeast nitrogen base, 2% galactose, (10×) amino acids dropout solution] deficient in leucine and histidine. Selected growing colonies were further evaluated for their ability to suppress Bax lethality, by reintroducing isolated library plasmids into W303-1a cells containing pGilda-Bax. DNA sequencing was performed with an automated sequencing apparatus (Applied Biosystems).

Western blot analysis. Yeast cells harboring either pGilda-Bax/pADGal4-2.1 or pGilda-Bax/pADGal4-2.1-sAPX were grown to an OD_{600} of 1.0 to 2.0 in SD-glucose medium. To induce Bax protein, cells were collected by centrifugation, transferred to fresh SD-galactose medium, and incubated for 12 h at 30°C. Cells were collected by centrifugation and proteins extracted, as described previously (14). Protein samples (20 μg) were loaded on SDS–polyacrylamide gels (10%), and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Amersham). Blots were subsequently probed with anti-Bax polyclonal antibody (Santa Cruz Biotechnology) and secondary anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). Signals were detected with the enhanced chemiluminescence system (Amersham).

Electron microscopy. Exponentially growing W303-1a cells (containing either pGilda-Bax/pADGal4-2.1 or pGilda-Bax/pADGal4-2.1-sAPX) in SD-glucose medium was washed in water, and grown in SD-galactose medium for a further 12 h at 30°C. After washing once in water, cells were fixed with 0.1 M phosphate buffer (pH 7.2), 3% glutaraldehyde for 12 h at 4°C. Cells were postfixed with osmium tetroxide, and dehydrated to obtain stationary phase cells. Next, cells were rinsed with 100% ethanol followed by 100% acetone, and infiltrated with 50% acetone/50% epon for 30 min and 100% epon for 20 h. Thin sections were applied to a grid and imaged with an electron microscope.

Northern blot analysis. Total RNA was purified from 2-week-old soybean seedlings treated with H_2O_2 (100 μM), methyl viologen (10 μM), $FeCl_3$ (100 mM) and UV (260 nm) for 6 h. Samples (10 μg/lane) were electrophoresed on a 1.2% formaldehyde agarose gel and transferred to an Immobilon-Ny^+ transfer membrane (Millipore). Membranes were UV cross-linked and hybridized to gene-specific probes in Church buffer (1% BSA, 1 mM EDTA, 0.25 M $NaHPO_4$ (pH 7.2), 7% SDS at 65°C. Following incubation with gene-specific probes, membranes were washed with 2× SSC for 10 min, 1× SSC for 10 min, 0.5× SSC for 10 min, and rewashed with 0.1× SSC/0.1% SDS at 65°C for 10 min.

H_2O_2 sensitivity test. For spot assays, cells were grown at 23°C to an OD_{600} of 0.3. After treatment with 5 mM H_2O_2, the temperature was shifted to 37°C for 15 min. H_2O_2 sensitivity was measured by spotting aliquots (4 μl) of 10-fold serial dilutions from an exponentially growing culture of the strain (at OD_{600} of 0.4) on YPD plates. Plates were incubated at 30°C and examined for surviving cells after 2 days.

Thermosensitivity assay. Lethal thermosensitivity assays were performed as described earlier (16). Briefly, strains were grown to mid-logarithmic phase in YPD medium and concentrated to 2×10^8 cells per milliliter. Aliquots (100 μl) were pipetted into Eppendorf microfuge tubes at each time-point and placed on ice. Samples were subjected to non-permissive temperature at 50°C, removed at each time point and immediately incubated on ice. Aliquots were diluted accordingly and plated onto solid YPD. Colonies were counted after 2 days of incubation at 30°C. Percentage viability was calculated in relation to non-heat-treated control cells.

Flow cytometry and confocal microscopy studies. Wild-type W303-1a cells and W303-1a cells containing either pGilda-Bax/pADGal 4-2.1 or pGilda-Bax/pADGal4-2.1-sAPX were grown to OD_{600} of 0.5 in SD-glucose medium without leucine and histidine. After washing in water, cells were cultured in SD-galactose medium for 12 h at 30°C. Cells were rewashed in water and resuspended in 50 mM Tris–HCl, pH 7.5. To measure ROS, 2×10^5 cells were incubated for 2 h at room temperature with 50 μM dihydrorhodamine 123 or 50 μM 2,7-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes) and analyzed microscopically, as described previously (13). Briefly, dihydrorhodamine 123 and DCFH-DA fluorescence was excited at 450–490 nm, and emission was monitored at 515–565 nm. Fluorescence intensities of rhodamine 123 were analyzed by recording Fl-1 fluorescence with a flow cytometer (Becton–Dickinson). Experiments were repeated three times, with approximately about 20,000 cells per assay. Data were collected with a FACscan fluorescence activated cell scanner, using the data acquisition program QCELL Quest (Becton–Dickinson).

RESULTS

Cloning of sAPX, a Suppressor of Bax-Induced Cell Death in Yeast

Bax is a proapoptotic members of the Bcl-2 family of proteins that regulates cell death in animals (reviewed

in 4). Recently, it has been demonstrated that animal Bax also induces apoptosis in yeast and plant cells (6–10). It is strongly suggested that Bax-induced cell death may be a conserved process amongst animals, yeasts and plants (12, 13). To study Bax-induced-apoptosis in plants, we screened for expression of plant genes inhibiting cell death promoted by the regulatory protein in yeast.

A soybean cDNA library was co-transformed with *Bax* into a W303-1a strain, and transformants were selected on glucose containing medium. For conditional expression of *Bax* in yeast, the gene was placed under the control of a GAL1 promoter that allowed specific expression of the protein when cells were grown in galactose-containing medium instead of dextrose as carbon source. Approximately, 2×10^5 independent transformants were collected. After extensive washing in water, transformants were plated onto a galactose-containing medium and plasmids from selected surviving clones were isolated and analyzed by restriction mapping. Based on their restriction patterns, plasmid inserts corresponding to five different loci were designated *PBI1*, *PBI2*, *PBI3*, *PBI4*, and *PBI5* (for plant Bax inhibitor). Since *PBI1* conferred the highest tolerance to Bax-induced cell death (data not shown), this gene was selected for further study. Proteins encoded by *PBI2*, *PBI3*, *PBI4*, and *PBI5* are not structurally related to *PBI1*, and will be described elsewhere.

Sequence determination of *PBI1* and comparison to GenBank/EMBL nucleic acid databases revealed a 98% identity at the amino acid level with an ascorbate peroxidase from soybean, and the novel protein was accordingly re-designated as *sAPX*. To verify that sAPX suppresses Bax-induced cell death in yeast, colony formation was observed on a galactose-based medium (Fig. 1A). Colonies formed by cells harboring both pGilda-Bax and pADGal4-2.1-sAPX on glucose-based medium were detected with approximately the same efficiency as control transformants containing plasmid pGilda-Bax with an empty vector. However, while transformants containing Bax with an empty vector on galactose medium induced complete inhibition of colony formation, those containing Bax with sAPX restored cell growth. Immunoblot analyses were performed to examine whether sAPX affects the expression levels of Bax protein (Fig. 1B). No Bax protein was detected in glucose-based medium. Upon transfer of cells from glucose-based medium (in which the GAL1 promoter is repressed) to galactose-based medium, Bax protein accumulated to easily detectable levels within 12 h in the cells containing plasmid pGilda-Bax without or with sAPX. These findings suggest that sAPX is one of the specific proteins in plants that suppress Bax-induced cell death in yeast.

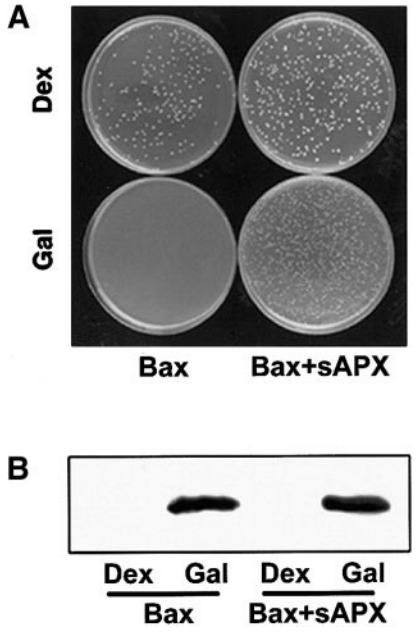

FIG. 1. sAPX protein suppresses Bax-induced cell death in yeast. (A) sAPX-promoted resistance to Bax lethality. W303-1a cells harboring either pGilda-Bax/pADGal4-2.1 (left) or pGilda-Bax/pADGal4-2.1-sAPX (right) were spread onto SD-glucose (Dex)- or SD-galactose (Gal)-containing plates. Photographs were taken after culturing at 30°C for 2 days. (B) Western blot analyses. W303-1a transformants used in (A) containing pGilda-Bax/pADGal4-2.1 (Bax) or pGilda-Bax/pADGal4-2.1-sAPX (Bax + sAPX) were grown in glucose-containing medium (Dex), subsequently transferred to galactose-containing medium (Gal) and cultured for 12 h. Total protein extracts (20 μg/lane) were subjected to SDS–PAGE and immunoblot analyses were performed, using anti-mouse Bax antiserum.

sAPX Suppresses Bax-Induced Apoptotic Marker in Yeast

The hallmarks of metazoan apoptosis observed in yeast cells undergoing PCD include phosphatidyl serine externalization, membrane blebbing, increased vacuolation, DNA fragmentation, nuclear fragmentation, and apoptotic body formation (13, 19). We employed electron microscopy (EM) to further examine the function of sAPX as a Bax suppressor (Fig. 2). Cells containing Bax alone developed a variety of morphological abnormalities that presumably reflect a continuum of severity. Numerous cells showed enlargement of cytoplasmic vacuoles, dissolution of nucleus, and evidence of mitochondrial vacuole swelling (Fig. 2A). Furthermore, some cells demonstrated nearly complete autophagy (absence of the nucleus and most cytosolic organelles), containing only the remnants of ruptured mitochondria (Fig. 2B). In contrast, EM analyses of yeast cells containing both Bax and sAPX displayed morphology typical for normal *S. cerevisiae*, with a central vacuole, several mitochondria, and homogeneous nuclear chromatin (Figs. 2C and 2D).

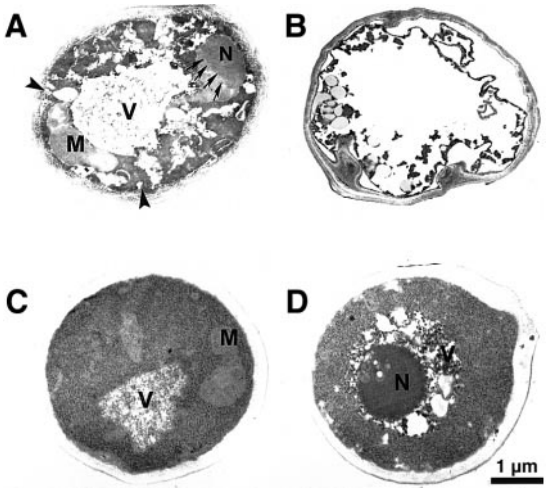

FIG. 2. Overexpression of sAPX suppresses Bax-induced apoptotic markers in yeast. W303-1a cells harboring either pGilda-Bax/pADGal4-2.1 (A and B) or pGilda-Bax/pADGal4-2.1-sAPX (C) were cultured in SD-galactose medium for 12 h. Cell morphology was analyzed by electron microscopy. (D) Control wild-type W303-1a cells. Arrows indicate regions of nucleus with condensed and marginalized chromatin. Arrowheads mark blebbing of the plasma membrane. N, nucleus; V, vacuole; M, mitochondria (scale bar is 1 μm).

sAPX Functions as an Antioxidant in Yeast Cell

To identify the physiological role of sAPX in plants, RNA expression levels were analyzed under various stress conditions. Total RNA was isolated from stress-treated soybean seedlings and Northern blot analysis was performed using *sAPX* cDNA as a probe (Fig. 3). Transcription of *sAPX* was induced individually with H_2O_2, MV, $FeCl_3$, or UV treatment. Our results indicate that the biological function of sAPX in plant is related to oxidative stress.

To further determine the antioxidant function of sAPX, we overexpressed the protein in yeast cells and performed H_2O_2 sensitivity and thermosensitivity assays. Two different yeast strains were used, specifically, the *ctt1* mutant (Δ*ctt*) strain, which lacks catalase T, and the *ccp* mutant (Δ*ccp*) strain, lacking

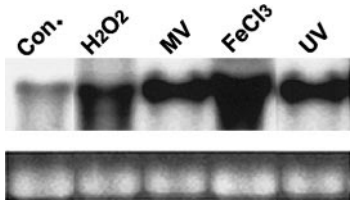

FIG. 3. *sAPX* transcript accumulates during oxidative stress. Total RNA was prepared from 2-week-old soybean seedlings treated for 6 h with 100 μM H_2O_2, 10 μM methyl viologen (MV), 100 mM $FeCl_3$, and UV. A fixed amount of total RNA (10 μg) was loaded onto each lane. Equal loading for each lane was confirmed by prestaining gels with ethidium bromide (lower). RNA blots were probed with ^{32}P-labeled sAPX-cDNA.

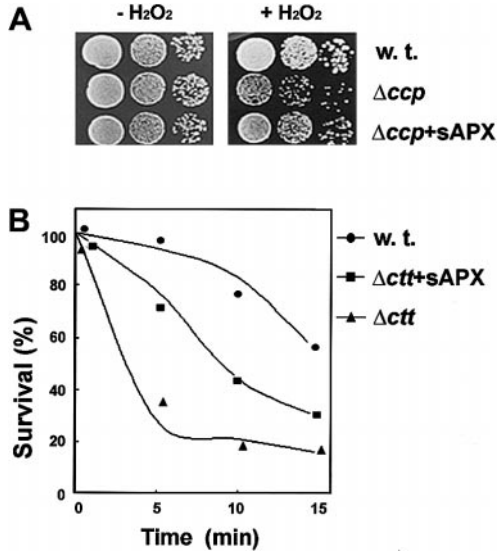

FIG. 4. sAPX suppresses H_2O_2 sensitivity of Δ*ccp* and thermosensitivity of Δ*ctt* mutant cells. (A) Spot assay. Exponentially growing cells of strain W303-1a (upper), and isogenic Δ*ccp* mutant strain transformed with either pADGal4-2.1 (middle) or pADGal4-2.1-sAPX (lower) were treated with 5 mM H_2O_2 for 15 min. H_2O_2 sensitivities were measured by spotting aliquots (4 μl) of 10-fold serially diluted cultures (growing until OD_{600} of 0.3) on YPD plates. Plates were photographed after 2 days of incubation at 30°C. (B) Lethal thermal survival assay. Ga74-1A (wild-type), and isogenic Δ*ctt* mutant strains transformed with either pADGal4-2.1 or pADGal4-2.1-sAPX were exposed to 50°C for indicated times and plated onto solid YPD medium. The number of viable cells was determined after incubating at 30°C for 2 days and normalized to the value of cells not subjected to heat treatment.

cytochrome peroxidase c. Cell viability of Δ*ccp* mutant strains decreased rapidly after exposure to H_2O_2. However, overexpression of sAPX in mutant cells enhanced resistance to H_2O_2-induced stress (Fig. 4A). Similar results were observed with a thermosensitivity assay on Δ*ccp* mutant cells (Fig. 4B). As predicted, *ctt1* mutant was extremely sensitive to heat stress, compared to wild-type control cells. Again, overexpression of sAPX greatly suppressed the heat sensitivity of mutant cells. The data collectively imply that sAPX functions as an antioxidant in the yeast *S. cerevisiae.*

sAPX Can Block Hyperproduction of Intracellular ROS by Bax

Increasing evidence suggests that ROS are effectors of PCD in animals and plants (12) and play a role in stress adaptation in prokaryotes (20). In yeast, depletion of free radical generation during hypoxia prevented PCD induced by the mutant $cdc48^{S565G}$ allele or overexpression of Bax (13). We analyzed the production of ROS during Bax-induced cell death in yeast, using dihydrorhodamine 123 and DCFH-DA. Upon oxidation by ROS, nonfluorescent dihydrorhodamine 123 becomes a fluorescent chromopore, rhodamine 123 (21),

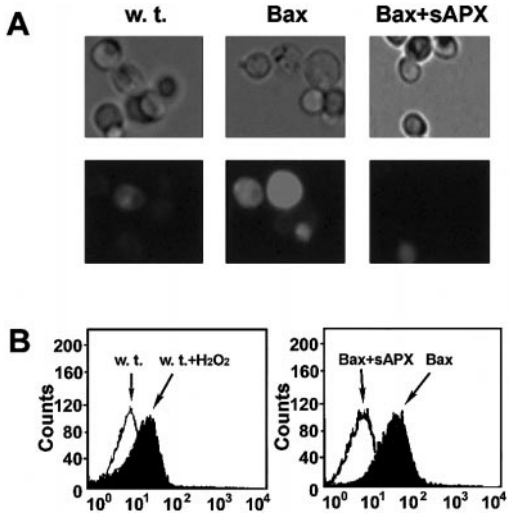

FIG. 5. Overexpression of sAPX suppresses generation of ROS by Bax. (A) Microscopy analyses. Wild-type W303-1a cells and W303-1a cells harboring either pGilda-Bax/pADGal4-2.1 or pGilda-Bax/pADGal4-2.1-sAPX constructs were grown in galactose medium for 12 h. Following this, cells were incubated with 50 μM dihydrorhodamine 123 for 2 h and subjected to microscopy. Fluorescence data after incubation with dihydrorhodamine 123 (lower) and the corresponding phase-contrast display (upper) are depicted. (B) Flow cytometric analyses. W303-1a transformants containing pGilda-Bax/pADGal4-2.1 (Bax) or pGilda-Bax/pADGal4-2.1-sAPX (Bax + sAPX) were grown in galactose medium for 12 h. Cells were further incubated with 50 μM dihydrorhodamine 123 for 2 h for flow cytometric analyses (right). Wild-type W303-1a cultures grown in media without or with 1 mM H_2O_2 for 15 min were employed as control (left).

and DCFH-DA is deacylated and oxidized to fluorescent compound dichlorofluorescein (22).

We noted that more than 35% cells exhibited fluorescence when yeast cells harboring pGilda-Bax were incubated with dihydrorhodamine 123 (Fig. 5A, middle). However, most of the corresponding wild-type cells, and cells expressing both Bax and sAPX exhibited no fluorescence, appearing dark against faint background fluorescence (Fig. 5A, left and right). The production of ROS by Bax in yeast cells was further confirmed by flow cytometry analyses with dihydrorhodamine 123. Yeast cells harboring pGilda-Bax accumulated a large number of oxygen radicals, which was significantly inhibited by co-expression with sAPX (Fig. 5B). Similar results were obtained on ROS generation by DCFH-DA (data not shown). Our results suggest that the antioxidant capacity of sAPX blocks the hyperproduction of intracellular ROS promoted by Bax.

DISCUSSION

All organisms produce ROS, such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH^-$) as by-products of normal metabolic processes (11, 23). Higher levels of ROS in cells induce toxic damage to lipids, carbohydrates, proteins and DNA. Although ROS are conventionally viewed as toxic by-products of cellular metabolism, increasing evidence indicates that low doses of ROS play a key role in PCD in both animals and plants (12). In animals, antioxidants that scavenge peroxide, thiol-reductants like *N*-acetylcysteine, and overexpression of manganese superoxide (MnSOD) block or delay apoptosis (22). PCD in animals is additionally blocked by an endogenously produced protein, Bcl-2, that employs an antioxidant mechanism (24). Analogous to PCD in animals, ROS participate in plant cell death processes. During a hypersensitive response that results from the activation of an intrinsic PCD pathway by plant-pathogen interactions, ROS are utilized as second messengers in the execution of cell death (12). In accordance with this theory, transgenic plants with lowered antioxidative capacity showed higher rates of PCD following pathogen infection than wild-type plants. *S. cerevisiae* cells also undergo cell death in response to H_2O_2 treatment and glutathione depletion (13). PCD induced by the CDC48 mutation also exhibits accumulation of ROS (13). It would appear that ROS is a common element of PCD in animals, plants and yeasts as part of a basic, evolutionarily conserved mechanism.

Although Bcl-2 like proapoptotic members have not been identified in yeast and plants to date, the expression of animal Bax in these organisms similarly induces apoptosis. The mechanism of action of Bax in yeast cells is dependent on the localization of the protein to the outer mitochondrial membrane, which may result in the impairment of oxidative phosphorylation, electron transport, ATP production and mitochondrial transmembrane potential, and subsequent ROS production (reviewed in 25, 26). However, the details of this molecular mechanism remain to be elucidated. Release of ROS from mitochondria to cytosol alters the cellular redox potential, which may be an important determinant of cell death in yeast cells. Consistent with this theory, we observed that (i) the plant antioxidant enzyme, sAPX, suppressed Bax-induced cell death in yeast; (ii) oxygen radicals accumulated during Bax expression in yeast cells, which was greatly reduced on coexpression of the protein with sAPX.

In plants, ascorbate peroxidase (APX) is an important H_2O_2-detoxifying enzyme, and expression of APX is rapidly induced in response to stress that promotes the ROS accumulation. In this report, we demonstrate that a plant antioxidant gene functions as a suppressor of Bax-induced PCD in yeast cells. It is essential to clarify the mechanisms through which cells adapt by expressing a cascade of genes that regulate cellular ROS levels. Catalases and peroxidases appear to modulate ROS levels directly, while the redox state of cysteine residues involved in the structure and function of numerous enzymes receptors and transcription factors, protect from ROS mediated oxidation by S-thiolation, a

process that can be reversed by glutaredoxin, thioredoxin, glutathione and protein disulfide isomerases *in vivo* (27–29).

In summary, we propose that yeast *S. cerevisiae* is a powerful and ideal eukaryotic model system to identify plant genes involved in antioxidant and antiapoptotic function and should facilitate clarification of the PCD mechanism conserved within various organisms.

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