Tomato Phospholipid Hydroperoxide Glutathione Peroxidase Inhibits Cell Death Induced by Bax and Oxidative Stresses in Yeast and Plants

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Using a conditional life or death screen in yeast, we have isolated a tomato (*Lycopersicon esculentum*) gene encoding a phospholipid hydroperoxide glutathione peroxidase (LePHGPx). The protein displayed reduced glutathione-dependent phospholipid hydroperoxide peroxidase activity, but differs from counterpart mammalian enzymes that instead contain an active seleno-Cys. LePHGPx functioned as a cytoprotector in yeast (*Saccharomyces cerevisiae*), preventing Bax, hydrogen peroxide, and heat stress induced cell death, while also delaying yeast senescence. When tobacco (*Nicotiana tabacum*) leaves were exposed to lethal levels of salt and heat stress, features associated with mammalian apoptosis were observed. Importantly, transient expression of LePHGPx protected tobacco leaves from salt and heat stress and suppressed the apoptotic-like features. As has been reported, conditional expression of Bax was lethal in tobacco, resulting in tissue collapse and membrane permeability to Evans blue. When LePHGPx was coexpressed with Bax, little cell death and no vital staining were observed. Moreover, stable expression of LePHGPx in tobacco conferred protection against the fungal phytopathogen *Botrytis cinerea*. Taken together, our data indicated that LePHGPx can protect plant tissue from a variety of stresses. Moreover, functional screens in yeast are a viable tool for the identification of plant genes that regulate cell death.

Programmed cell death (PCD) is a genetically controlled process that plays an essential role in the biology of plants and animals (Vaux and Strasser, 1996; Vaux et al., 2001; Dickman and Reed, 2003). Since the early observations of Kerr et al. (1972), which slowly gained acceptance, it has become clear that proper regulation of PCD is crucial for organisms in eliminating cells in a variety of developmental, physiological, and/or pathological contexts. Thus, altruistic cellular suicide is a property of normal physiology and homeostasis, which benefits multicellular organisms. Not only is PCD observed in widely divergent species across broad taxonomic distances, but the molecular components of the death signaling pathways show a high degree of structural and functional conservation, to the extent that cell death regulatory genes from one species (e.g. humans) can function in phylogenetically distant species (e.g. worms; Vaux et al., 1992).

In plants, PCD plays a normal physiological role in a number of developmental processes including xylogenesis, senescence, root cap growth, and responses to pathogens (Beers and McDowell, 2001). Though the biochemical mechanisms responsible for cell suicide in plants are largely unknown, a variety of reports indicate similarities to the PCD (apoptosis) that occurs in animal species. For example, PCD in plants typically requires new gene expression and thus can be suppressed by cycloheximide and similar inhibitors of protein or RNA synthesis (Havel and Durzan, 1996). The morphological characteristics of plant cells undergoing PCD also bear some striking similarities to apoptosis in animals, though the presence of a cell wall around plant cells imposes certain differences. Akin to animal cells, PCD in plants is associated with internucleosomal DNA fragmentation (DNA ladders) and the activation of proteases (Wang et al., 1996; McCabe et al., 1997; del Pozo and Lam, 1998; Navarre and Wolpert, 1999; Solomon, et al. 1999). Moreover, ectopic expression of certain animal anti-apoptosis genes in transgenic plants has been demonstrated to provide protection from crop pathogens and other insults as a result of cell death suppression (Mitsuhasha et al., 1999; Dickman et al., 2001). Conversely, expression of animal pro-apoptotic proteins, such as Bax, in plants can induce a cell death similar to the hypersensitive response program for cell suicide (Lacomme and Santa Cruz, 1999). However, to date, few endogenous plant genes have been identified that share sequence homology with the apoptosis genes of animal cells. With the completion of the Arabidopsis genome, as well as other advancing plant sequencing efforts, it has become clear that core apoptotic pathway members (e.g. Bcl-2 family, caspases) with similarity at the primary sequence level are not present in plant genomes. Thus, alternative approaches are necessary to identify candidate plant genes that regulate apoptotic-like processes. Toward this effort, we have been using baker’s
youth (Saccharomyces cerevisiae) to study regulation of heterologous gene expression with respect to PCD. Yeast has been shown to be a useful model for apoptosis research (Madeo et al., 1999; Jin and Reed, 2002). Reports from our lab and several others have provided evidence that yeast displays several of the hallmark features associated with apoptosis, including chromatin condensation, DNA fragmentation, and externalization of phosphatidylserine (Madeo et al., 1999; Chen et al., 2003). While not all of the defining features of metazoan PCD have been observed in yeast, it has become evident that PCD occurs, exhibiting at least some of these features.

Upon examination of the completed genomic sequence of yeast, with one possible exception (Madeo et al., 2002), yeast also has no apparent homologs of major metazoan apoptotic regulators (e.g. Bax/Bcl-2 family, caspases, Apaf-1/CED-4, etc.). Therefore, yeast may be considered a genetically null background system to study interactions between heterologously expressed components of apoptotic pathways (Frohlich and Madeo, 2000). Expression of various apoptotic inducers, including Bax, caspases, p53, or CED-4/Apaf-1, results in death of yeast (Tao et al., 1999; Jin and Reed, 2002). Coexpression of Bax with Bcl-2 or Bcl-xL inhibits yeast cell death, as observed in animal cells (Zha et al., 1996). In particular, the lethal phenotype observed in Bax expressing yeast has been exploited for structure/function studies, as well as gene-discovery efforts by screening for animal genes that suppress Bax-induced lethality (Xu and Reed, 1998).

Thus, functional screens using yeast present a potentially useful approach toward the identification of candidate genes that may modulate plant PCD.

The yeast Gal/Bax assay is predicated on the ability of ectopically expressed mammalian Bax to kill yeast and on the ability of cytoprotective proteins to rescue yeast from the lethal phenotype conferred by Bax (Jin and Reed, 2002). Here we describe the isolation and functional characterization of a novel tomato (Lycopersicon esculentum) gene identified by the yeast Gal/Bax screen that encodes a phospholipid hydroperoxide glutathione peroxidase (PHGPx). PHGPx have been identified in several plant species, including tomato (Depège et al., 1998; Herbette et al., 2002), and functions in the removal of phospholipid hydroperoxides, which are generated as products of lipoxygenase catalyzed oxygenation of fatty acids (Ursini et al., 1985). Functional studies show that LePHGPx inhibits not only oxidative stress induced cell death in yeast but also inhibits, salt, heat, and Bax induced PCD in tobacco (Nicotiana tabacum) plants.

RESULTS

LePHGPx Suppresses Bax Lethality in Yeast

Bax is a pro-apoptotic member of the Bcl-2 family of proteins and has been shown to induce cell death in mammals, plants, and yeast (Xu and Reed, 1998; Lacomme and Santa Cruz, 1999). To identify plant genes that inhibit Bax-induced lethality in yeast, we screened a tomato cDNA library as described in “Materials and Methods.” Transformants were selected by plating transformed cells on Gal-containing solid medium to induce Bax expression. Viable transformants presumably contain cDNAs that can suppress the Bax cytotoxicity by overexpression of a tomato cDNA. Since a single yeast transformant may contain several types of plasmids, the cDNA that is actually responsible for suppressing Bax toxicity is segregated from other irrelevant cDNAs by “passing-through-Escherichia coli” (Xu and Reed, 1998). The ability of the cDNA to neutralize Bax cytotoxicity was verified by reintroduction of the cDNA into Gal1-Bax-bearing yeast cells. Some of the cDNAs encoded proteins that somehow interfered with the expression of the Gal1 promoter as opposed to blocking the function of Bax, necessitating that each candidate clone be tested for suppression of a Gal1-lacZ gene. Those cDNAs that tested positive for suppression of Bax function but not Gal1 promoter expression were then taken forward to immunoblot analysis, where we verified that they do not interfere with Bax protein production in yeast. From $5 \times 10^6$ transformants, hundreds of colonies were obtained that grew on Gal-containing plates, which induces Bax expression and

Figure 1. The tomato LePHGPx gene product inhibits Bax-induced cell death in yeast. A, Yeast strain EGY48 carrying plasmids that encode pGilda-Bax plus Bcl-xl (positive control); pGilda-Bax plus vector pB42AD, or pGilda-Bax plus LePHGPx were spotted on plates at 5-fold dilutions. Expression of Bax was induced by Gal, whereas LePHGPx and Bcl-xl expression was constitutive. B, Yeast growth curves for the strains described in A. pGilda-Bax plus Bcl-xl (●); pGilda-Bax plus vector pB42AD (▲); pGilda-Bax plus LePHGPx (●).
normally kills the cells. Of these, 114 were picked at random and restreaked onto Gal plates, resulting in 61 colonies that displayed Bax-resistance. Plasmid DNA was recovered from these 61 yeast transformants and retransformed into yeast harboring the pGilda-Bax plasmid. Induction of Bax expression on Gal revealed that 8 of these 61 candidate Bax-suppressors conferred Bax-resistance phenotypes. Of the 8 clones thus identified, 1 encoded a predicted phospholipid hydroperoxide glutathione peroxidase (LePHGPx), which will be described in more detail below.

To show conclusively that LePHGPx rescued yeast from Bax-induced lethality, the yeast strain EGY48 containing plasmids pGilda-Bax and pB42AD-LePHGPx were grown, pelleted, washed, and resuspended as described in “Materials and Methods.” After 5 d incubation, it was apparent that yeast carrying LePHGPx was able to grow and survive on media that induce lethal Bax expression (Fig. 1A). The growth rate of EGY48 cells coexpressing Bax and LePHGPx and EGY48 expressing only the Bax or Bax and Bcl-xL was measured at regular intervals up to 48 h. As shown in Figure 1B, expression of LePHGPx enabled the Bax expressing cells to proliferate, albeit at a lower rate than the Bcl-xL expressing cells, which is a bona fide Bax inhibitor. In contrast, Bax expressing cells did not show significant growth.

The full length LePHGPx cDNA was sequenced. LePHGPx exhibited 79% identity at the amino acid level with the PHGPx from *Momordica charantia* (Li et al., 2001), 71% identity with the PHGPx from *Citrus sinensis* (Holland et al., 1993), and 69% identity with GPXle-1 from tomato (Depe`ge et al., 1998). The alignment of amino acid sequences (Fig. 2A) showed that LePHGPx shares high sequence homology with other plant PHGPx proteins, especially in the three domains (G1–G3), which are signature structural motifs of GPx proteins (Jung et al., 2002). These domains contain highly conserved amino acids, including the presumable active site Cys residue at the position occupied by SeCys in mammalian GPx proteins. The conserved Gly residue in domain G1, the Gln residue in domain G2, and a Trp-Asn-Phe motif in domain G3 are all present in the tomato PHGPx sequence. These residues are believed to form the catalytic triad of GPx proteins (Epp et al., 1983). When the phylogenetic relationship between the amino acid sequence of LePHGPx and those of other GPx isoforms is viewed as a

![Figure 2. Amino acid alignment and phylogenetic tree of LePHGPx with other GPx polypeptides from plants and humans. A, The deduced amino acid sequences for comparison are from tomato (LePHGPx), tomato (GPxle-1), *Momordica charantia* (McGPx), Arabidopsis (AtGPx1 and AGPpx2), spinach (SoGPx), cotton (GhGPx), *Citrus sinensis* (CsGPx), *Brassica napus* (BnPx), *Pisum sativum* (PsGPx). B, A phylogenetic tree was generated from the sequences in A. The sequences were aligned and compared to construct the tree using (1) mammalian GPx, (2) nonspecific targeted GPx, and (3) plastid targeted GPX.

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dendrogram, it is apparent that LePHGPx belongs to the same branch of the phylogenetic tree as other PHGPx proteins and that the PHGPx group, as a whole, is distinct from other GPx isoforms (Fig. 2B).

LePHGPx Overexpression Protects against H₂O₂ and Heat Stress in Yeast

LePHGPx-expressing yeast cells were treated with 3 mM or 9 mM H₂O₂ for 6 h and washed extensively prior to subsequent transfer into yeast peptone dextrose (YPD) plates. During the 6 h treatment, H₂O₂ concentrations in the medium were still greater than 50% from the starting levels of wild type and LePHGPx expressing yeast cells as determined by the Guiaicol assay (Tiedmann, 1997; data not shown). The number of growing colonies was counted after 48 h of incubation at 30°C, and the percentage of surviving cells was calculated. Expression of the LePHGPx significantly protected EGY48 cells from death induced by 3 mM H₂O₂ (Fig. 3A). Of the cells with LePHGPx, 38% remained viable compared to only 8% of the yeast cells containing the vector alone. CED-9 containing yeast served as a positive control (Chen et al., 2003). As the concentration of H₂O₂ was increased to 9 mM, almost all of the yeast cells (wild type and transformed) were killed.

For heat stress assays, yeast harboring LePHGPx were pretreated at 37°C for 30 min and then heat shocked at 50°C for 30 min. Under these conditions, in wild-type cells the viability was 16%, but the LePHGPx expressing cells had a 52% level of viability (Fig. 3B).

LePHGPx Delays Senescence in Yeast

Senescence occurs in all organisms, and a number of studies suggest that reactive oxygen species participate in senescence (Lee et al., 1999; Serrano and Blasco, 2001). This process is genetically programmed and thus may be apoptotic-like. The median life span of most laboratory strains of yeast is about 3 d (Jazwinski, 1993). To evaluate the senescence profile of selected yeast strains, cells were grown from lag phase to log phase to stationary phase and the viability of control yeast strains and transformants was compared. The doubling times of all yeast strains were nearly equivalent (4.7 ± 0.3 h). Aliquots of cells were removed at specified intervals up to 48 h and the A₆₀₀ was measured. Senescence was determined by monitoring cell viability after 72 h continuing to 288 h and was measured by plating serial dilutions of the yeast cultures onto YPD plates. The cell density was approximately equivalent between samples at the start of the senescence evaluation. LePHGPx expression significantly delayed the progression of yeast into senescence, relative to the wild-type control and the negative control CED-9 expressing yeast (Fig. 4 and Chen et al., 2003).

LePHGPx Displays Reduced Glutathione-Dependent GPx Activity

In order to determine whether the LePHGPx gene encodes a functional enzyme and to evaluate its activity, the enzyme's glutathione-dependent GPx activity was determined.

LePHGPx protects yeast from H₂O₂ and heat stress induced cell death. A, H₂O₂: Yeast strains containing CED-9 (dark stipples); pB42AD vector alone (hatching) or LePHGPx (light stipples) were treated with 3 mM or 9 mM H₂O₂ as described in "Materials and Methods." Surviving colonies were counted and compared to untreated wild type control (not shown). B, Heat stress: Yeast cells containing the same constructs as in A were either preheated (37°C) and heat shocked (50°C) or just heat shocked. Percent viability was calculated as in A. Data are presented as the average of three experiments with the s.e.

Figure 3. LePHGPx protects yeast from H₂O₂ and heat stress induced cell death. A, H₂O₂: Yeast strains containing CED-9 (dark stipples); pB42AD vector alone (hatching) or LePHGPx (light stipples) were treated with 3 mM or 9 mM H₂O₂ as described in “Materials and Methods.” Surviving colonies were counted and compared to untreated wild type control (not shown). B, Heat stress: Yeast cells containing the same constructs as in A were either preheated (37°C) and heat shocked (50°C) or just heat shocked. Percent viability was calculated as in A. Data are presented as the average of three experiments with the s.e.

Figure 4. LePHGPx delays senescence in yeast. Yeast strains as described in Figure 3 were grown to stationary phase, and viability was determined by colony counts of the control yeast strain (vector alone, ▲) compared to transformants containing CED-9 (●) or LePHGPx (○). Data are presented as the average of three experiments with the s.e.
substrate specificity, recombinant enzymes were prepared in E. coli BL21 cells. Purified LePHGPx showed a single band of about 19 kD in denaturing SDS-PAGE gels (data not shown). LePHGPx displayed GPx activities and catalyzed reduced glutathione-dependent reduction of H₂O₂, cumene hydroperoxide, t-butyl hydroperoxide (200 μM each), and phospholipid hydroperoxide (PL-OOH; approximately 800 μM; Table I). Specific activities with PL-OOH were low when compared to other substrates but in the same order of magnitude as PHGPx activities from citrus, tobacco, and sunflower (Beeor-Tzahar et al., 1995; Herbette et al., 2002). No activity was observed with extracts from E. coli transformed with the empty vector and affinity purified on glutathione S-transferase (GST)-coupled agarose.

Endogenous LePHGPx RNA Expression in Tomato Following Stress

Tomato plants were exposed to abiotic and biotic stresses, and RNA was extracted at selected time points and hybridized to radioactively labeled LePHGPx. Since PHGPx is part of a gene family in tomato (Depe`ge et al., 1998) and Arabidopsis (Rodríguez Milla et al., 2003) with relatively high levels of sequence similarity (Fig. 2A), LePHGPx specific probes were designed from 3' untranslated regions of the gene, as described in “Materials and Methods.” In all treatments, control leaves showed low, basal levels of LePHGPx expression. However, upon exposure to heat, cold, or salt, a considerable increase in transcript levels was observed (Fig. 5, A–C). In a similar manner, abscisic acid (ABA) treatment also induced expression of LePHGPx (Fig. 5D). When tomato leaves were inoculated with two necrotrophic compatible fungal pathogens, Sclerotinia sclerotiorum and Alternaria alternata, again, the induction of LePHGPx expression was evident (Fig. 5E). It should be noted that when leaves were wounded, an increase in this transcript also occurred (not shown). Taken together, biotic and abiotic stresses induce transcriptional activity of LePHGPx.

LePHGPx Transient Expression Prevents Bax-Induced Cell Death in Tobacco

We next determined whether LePHGPx could protect plant cells from Bax-induced cell death as it does in yeast. Bax expression was conditionally regulated by the dexamethasone-inducible promoter. Bax infiltrated tissue showed clear indications of cell death as indicated by tissue collapse, loss of chlorophyll, and membrane permeability to the vital stain, Evans blue (Fig. 6A). Similarly treated tobacco coexpressing LePHGPx + Bax showed either no cell death or very small sectors of cell death in the infiltrated areas as indicated by Evans blue staining (compare Fig. 6, A and B). Control plant tissue expressing vector only or mock infiltrations did not show any indications of cell death, nor did such tissue stain with Evans blue.
indicating that the cell membranes were intact. These results show that LePHGPx functions to protect tobacco cells from the cell death pathway induced by Bax.

**LePHGPx Transient Expression Protects Plant Cells against Abiotic Stress**

Since it has been shown that LePHGPx protein levels increase during salt stress (Beeor-Tzahar et al., 1995; Gueta-Dahan et al., 1997), we were interested in determining whether this increase in expression was possibly related to an increased resistance to oxidative stress conditions as observed with yeast. Leaf discs transiently expressing LePHGPx were exposed to 350 mM salt concentrations. After 48 h treatment, empty vector-infiltrated tobacco leaf discs showed a significant yellowing indicative of loss of chlorophyll, while the LePHGPx expressing leaf tissue appeared green with only slight losses of chlorophyll (Fig. 7A and data not shown). DNA extracted from 3 independent transient assays, after 48 h salt treatment, showed DNA laddering in the vector and mock infiltrated samples, while the DNA from LePHGPx expressing leaves was intact (Fig. 7B). In addition, wild type, but not LePHGPx expressing cells, stained positively for the transferase-mediated dUTP nick end labeling (TUNEL) reaction (Fig. 7C), indicating DNA fragmentation had occurred with morphologies reminiscent of animal apoptotic bodies (Fig. 7C, arrowhead). These results indicate that tobacco leaves exposed to lethal doses of salt exhibit features that resemble apoptotic-like cell death and that expression of LePHGPx confers protection against salt injury and prevents DNA fragmentation.

Excessive heat exposure is also known to cause oxidative stress (Samali et al., 1999). We therefore were interested in determining whether LePHGPx expression could protect plant tissue from lethal exposure to heat. Empty vector infiltrated leaf samples subjected to heat stress showed a progression from yellowing of leaves to browning with leaf tissue showing dead cells over more than one-third of the leaf area. Comparatively, LePHGPx infiltrated leaves showed minimal heat damage as indicated by browning being restricted to the leaf margins (Fig. 8).

**LePHGPx Stable Expression Protects Plant Cells against Biotic Stress**

Transgenic tobacco plants were generated harboring single copy number, kanamycin-resistant LePHGPx. A minimum of 5 leaves from 3 independent transformed lines of 5-week-old transgenic tobacco harboring LePHGPx or empty vector were inoculated with the broad host range necrotrophic fungus, *Botrytis cinerea*. Five-millimeter-diameter agar plugs containing actively growing hyphal tips from 3-d-old colonies of *B. cinerea* were placed on detached leaves. When transgenic tobacco harboring LePHGPx was inoculated with *B. cinerea*, leaves were highly resistant to infection and showed only tiny necrotic spots around the agar plugs (Fig. 9). In contrast, leaves transformed with vector alone were highly susceptible and the damaged cells extended far from the agar plugs (Fig. 9). Similar results were obtained with *S. sclerotiorum*, also a broad, host range necrotroph (data not shown).
Thus, expression of LePHGPx confers protection from both abiotic and biotic stresses.

DISCUSSION

Under aerobic conditions, cells are constantly exposed to the possibility of oxidative damage mediated by reactive oxygen species. Cells possess a range of nonenzymatic and enzymatic defense systems to counter oxidative stress including glutathione, thioredoxin, ascorbate, superoxide dismutase, and peroxidases such as catalases, glutathione peroxidases, and ascorbate peroxidases (Herbette et al., 2002; Moon et al., 2002). In mammals there are at least five isoforms of glutathione peroxidases (GPx), including a phospholipid hydroperoxide GPx (PHGPx or GPX4). PHGPx is a monomeric enzyme, associated with both soluble and membrane fractions, that reduces lipid hydroperoxides (Jung et al., 2002). Phospholipid hydroperoxides are key intermediates in the lipid peroxidation chain reaction, one of the major types of oxidative damage in cells, associated with membrane perturbation, inactivation of membrane proteins, and cell lysis. Lipid peroxidation has also been linked to pathological conditions such as ischemic injury, apoptosis, atherosclerosis, and carcinogenesis (Nomura et al., 2000). Thus, PHGPx is an important cellular enzyme capable of halting membrane lipid peroxidation and oxidative damage in animal cells.

In this report we describe the identification and characterization of a tomato LePHGPx, using a conditional Gal/Bax screen in yeast. Expression of the proapoptotic mammalian Bax gene arrests growth and eventually causes cell death in yeast. This cell death appears to be physiologically relevant since Bax expression induces cytochrome c release from yeast mitochondria and the death phenotype can be rescued by Bcl-2 or Bcl-xl. We and others (Xu and Reed, 1998; Kawai et al., 1999; Chen et al., 2003) have exploited the experimental advantages of yeast as a heterologous system for screening and identification of candidate genes that functionally regulate apoptosis. While plant genes have been identified that inhibit Bax induced...
lethality in yeast, this report extends this observation to plants, thus indicating that function in yeast can translate to function in plants (also see Kawai-Yamada et al., 2001). Importantly, data is presented that indicates that the LePHGPx functions as a cytoprotective protein under various conditions of lethal stress, suggesting that LePHGPx may serve in an analogous manner to animal antiapoptotic genes. Although these results are all based on overexpression of LePHGPx, the northern blots show that the LePHGPx transcript is also up-regulated under diverse lethal stress conditions, suggesting that endogenous LePHGPx is involved in oxidative stress responses. Moreover, heat, cold, salt, and ABA have been shown to induce expression of the citrus PHGPx (Avsian-Kretchmer et al., 1999). A number of genes are similarly induced by these (and other) abiotic stresses, although whether there is a direct relationship for ABA regulating these processes is not clear, as there is evidence for both ABA-dependent and -independent modulation of stress response genes. The induction of LePHGPx by Sclerotinia and Alternaria is of interest, but in the experiments described above, disease ensued. To address whether regulated expression of LePHGPx can confer disease tolerance and/or resistance, transgenic tobacco plants constitutively expressing LePHGPx were generated. Results clearly indicated that transgenic expression was sufficient to provide cell survival when challenged by the necrotrophic fungus, *B. cinerea*. This fungus is also known to generate oxidative stress during infection (Kuzniak and Sklodowska, 2004).

Our interest in LePHGPx stems from the fact that not only does it inhibit Bax-induced cell death in yeast and that in animals it is involved in ameliorating oxidative stress, but also because PHGPx functions as an anti-apoptotic agent in animals. Moreover, Bax-induced cell death in yeast occurs at least in part by generation of toxic levels of reactive oxygen species (Chen et al., 2003). Involvement of PHGPx in mammalian signal transduction pathways is suggested by studies of the mitochondrial PHGPx, which functions as an anti-apoptotic agent in mitochondrial death signaling (Nomura et al., 1999, 2000). In plants the role(s) of PHGPx is not well defined. Expression of a PHGPx activity has been shown to increase during exposure to NaCl in citrus (Beeor-Tzahar et al., 1995; Gueta-Dahan, et al., 1997), although the functional implications for these observations are presently unknown. A Chinese cabbage cDNA with sequence similarity to PHGPx has been biochemically characterized and shown to have thioredoxin-dependent peroxidase activity and was suggested to be chloroplast encoded (Jung et al., 2002). The first functional description of a plant protein with PHGPx activity occurred when a tobacco GST/PHGPx was overexpressed. Transgenic seedlings exhibited enhanced growth rates over wild type when exposed to chilling or salt stress (Roxas et al., 1997, 2000). In addition, two PHGPx-like proteins distinct from LePHGPx have been described from tomato and sunflower and were shown to have bifunctional enzyme activities: PHGPx and thioredoxin peroxidase activities (Depêge et al., 1998; Herbette et al., 2002). Again, the functional role(s) for these plant enzymes is not clear. It should be mentioned that Predotar (http://Genoplante-info.infobiogen.fr/predotar/ predotar.html) and ChloroP 1.1 (Emanuelsson et al., 1999) ruled out the presence of a predicted chloroplast transit peptide indicating a cytoplasmic subcellular location for LePHGPx.

A distinct difference between plant and animal GPx family members is the presence of an active site Cys in plants as compared to animal proteins that contain selenocysteine (SeCys; Stadtman, 1996). The
importance of the SeCys in the PHGPx catalytic activity has been demonstrated for the animal proteins (Gladyshev et al., 1996). Replacement of the active site Cys by SeCys in a PHGPx from citrus resulted in an increased lipid peroxidase activity, supporting the catalytic role of this residue also in plant PHGPxs (Hazebrouck et al., 2000). The low in vitro activity observed with the recombinant LePHGPx in this study is comparable to that observed with other plant PHGPxs (Beeor-Tzahar et al., 1995; Herbette et al., 2002). The nature of the catalytic activity of the tomato PHGPx is also supported by the high conservation of the GPx motifs in the primary sequence (Fig. 2A). However, the peroxidase activity with PL-OOH as a substrate, and reduced glutathione as the electron donor, is orders of magnitude lower than that of the animal proteins. It is possible that the low level of PL-OOH peroxidase activity of LePHGPx is sufficient to support the protective effect from oxidative lipid damage.

LePHGPx was identified by its ability to protect yeast cells during forced expression of Bax. Expression of the pro-apoptotic Bax protein is known to cause depletion of glutathione levels in yeast (Kampranis et al., 2000). This observation has also been noted in response to a number of apoptotic inducers in mammalian systems including infectious disease, FAS, and TNF-α (Cai and Jones, 1998). Thus the ability of LePHGPx to protect yeast in the Gal/Bax screen prompted a detailed examination to determine whether or not this enzyme can function as a cytoprotectant in yeast under more physiological stresses and, more importantly, whether these observations could be extended to plants. When yeast was treated with 3 mM H₂O₂, a concentration which kills yeast in an apoptotic-like manner (Chen et al., 2003), LePHGPx expression considerably prevented cell death from occurring (Fig. 3A). Moreover, TUNEL positively reacting wild-type yeast were observed following H₂O₂ administration, which were absent in the LePHGPx expressing protected yeast strains (data not shown). We also observed a similar situation during heat stress. A common feature of Bax, H₂O₂ and heat treatments is the generation oxidative stress. Interestingly, LePHGPx also delayed senescence of aging yeast cells grown for extended incubation periods.

A crucial question in using heterologous functional screens, such as the one described, is whether candidate plant genes identified in yeast have any meaningful or analogous role in plants. In other words, is the use of yeast screens valid? Significantly, we show a strong correlation in plants to what was observed in yeast, when LePHGPx is expressed under conditions of oxidative stress. We used the pSfinx vector system (Takken et al., 2000) for transient expression of LePHGPx, which combines the advantages of Agrobacterium binary vectors and plant viral vectors. A potato (Solanum tuberosum) virus X (PVX) component with LePHGPx was agro-infiltrated into leaves. The PVX component ensures abundant expression of the gene of interest as well as movement between cells. Since increases in PHGPx expression has been associated with high salt concentrations, we wanted to see if LePHGPx expression would protect plant tissue from salt stress. Indeed this was shown to be the case, thereby establishing that LePHGPx functions as a cell protectant against salt. Moreover, salt treatment of wild type or mock inoculated plant tissue kills tissue with features associated with mammalian PCD, namely, fragmented DNA resulting in a characteristic ladder. LePHGPx expression also prevented the fragmentation of DNA and maintained membrane integrity. Similar observations occurred during Bax transient expression and coexpression of Bax and LePHGPx. Taken together these data show that yeast screens are a viable tool for the identification of plant genes that regulate cell death. In addition, stable expression of LePHGPx in tobacco plants conferred protection against necrotrophic fungi. Thus, LePHGPx overexpression can protect plants against biotic and abiotic stresses and thus shares properties associated with mammalian anti-apoptotic genes.

MATERIALS AND METHODS

Yeast Gal/BAX Assay

pGilda-Bax (from John Reed, Burnham Institute, La Jolla, CA; Zha et al., 1996) was transformed into yeast strain EGY48, and the transformed cells were plated on SD/glu/-his/-media. Growing colonies were further evaluated for their ability to kill cells on SD/gal/raff/-his/-media. A tomato cDNA library, constructed from tobacco mosaic virus infected tomato VF36 leaves, was cloned into yeast expression vector pB42AD and transformed into Bax containing yeast cells. The transformed cells were plated on SD/glu/-his/-trp media. Growing cells were collected, washed, and plated on SD/gal/-raff/-his/-trp media. After 5 d incubation at 30°C, the colonies rescued from Bax lethality were streaked onto SD/gal/raff/-his/-trp plates again to confirm the growth phenotype. Tomato cDNAs that conferred resistance to Bax-induced death were plasmid-rescued and the resulting plasmid DNA was retransformed into EGY48 yeast containing pGilda-Bax, and the cells were grown on SD/gal/raff/-his/-trp plates to confirm ability to rescue from Bax-induced lethality. To evaluate yeast cell viability, EGY48 cells containing the plasmids pGilda-Bax and pB42AD were grown in SD/glu/-his/-trp overnight. The cells were pelleted, washed, and resuspended in water. For the plate assay, the yeast cultures were serial 5-fold diluted, and 5 μL of each dilution was dropped onto SD/glu/-his/-Trp or SD/gal/raff/-his/-trp plates and incubated at 30°C for 5 and photographed. For liquid assays, the cells were resuspended to A₀₅₀ = 0.05 in SD medium containing 2% Gal and 1% raffinose (gal-raff) as the carbon source, instead of Glc, to induce expression of the fusion proteins from the GAL1 promoter. Aliquots of cells were removed at regular intervals up to 48 h and the A₀₅₀ was measured.

Yeast Stress Treatment Assays

Early log phase yeast cultures (A₀₅₀ = 0.5) were diluted to a density of A₀₅₀ = 0.05 with SD/gal/raff/-his or SD/gal/raff/-his/-trp and treated in one of the following ways. For chemical treatment, H₂O₂ was added at different concentrations and incubated at 30°C with vigorous shaking for 6 h. For heat stress, yeast cells were incubated at 37°C for 30 min with vigorous shaking then transferred to a water bath at 30°C for 5 to 30 min and incubated at 30°C with vigorous shaking for 6 h. Following these treatments, viability was determined by plate counting of colony forming units. Ten microliters of cells were sampled, diluted, and spread onto YPD medium with 2% agar then incubated at 30°C for 48 h. The number of colonies forming units from treated cells was compared to the colonies forming units of untreated cells. All experiments were repeated at least in triplicate.
Yeast Growth and Senescence Assay

Doubling times were determined by taking early log phase yeast cultures (A600 = 0.5) in SD media containing 2% Gal, 1% raffinose then diluting and incubating at A600 of 0.05 with shaking in the same medium. Aliquots of cells were removed at specified intervals up to 48 h, and the A600 was measured. Senescence was determined by monitoring cell viability after 72 h to 288 h and was measured by plating serial dilutions of the yeast cultures onto YPD plates. Colonies forming units were counted after 2 d incubation at 30°C. The cell density was approximately equivalent between samples at the start of the senescence evaluation.

Plant Transient Expression Assays

The pSfinx vector system (Takken et al., 2000) was used for plant transient assays. The LePHGPx open reading frame was cloned into the CaMV-35S sites of the PVX-based vector pSfinx (obtained from Dr. Mathew Joosten, Wageningen University, The Netherlands). Expression was driven by the duplicated PVX coat protein promoter. This construct was electroporated into Agrobacterium tumefaciens MG101 electrocompetent cells containing the helper plasmid pC5-Arep (Jones et al., 1992) and transformed cells were selected on kanamycin (100 mg/L) and ampicillin (5 mg/L) containing media. A single colony of A. tumefaciens MG101 harboring the LePHGPx construct was inoculated into 5 mL YEP medium with antibiotics and grown overnight at 30°C. Cultures were pelleted and resuspended in induction medium (K2HPO4, 10.5 g/L; KH2PO4, 4.5 g/L; (NH4)2SO4, 1 g/L; sodium citrate, 2H2O, 0.5 g/L; MgSO4·1H2O; 1 mM; Gic, 0.2%; Glyceral, 0.5%; MES, 10 mM). Prior to use, 50 mM acetoxyringsone in dimethyl formamide was added to the induction medium. Bacteria were grown in induction medium for 6 to 8 h at 30°C, after which cells were pelleted and resuspended in infiltration medium (0.5 × Murashige and Skoog basal salts/L; 10 mM MES, pH 5.6) at an A600 of 0.8. Acetoxyringsone (150 μg/mL in dimethyl formamide) was added to infiltration medium just prior to use. Nicotiana tabacum (cv Glurk) plants at the 6-leaf stage were watered prior to infiltration and kept in a growth chamber at 23°C. Infiltration was carried out on the underside of healthy young leaves using a needleless tuberculin syringe. Control cultures containing vector only were also infiltrated and a mock infiltration using just infiltration medium was also carried out. Plants were covered and kept in a dark chamber with high humidity. The following day, plants were returned to the growth chamber and kept at 25°C for 5 d.

Cell Death Assays

To evaluate DNA fragmentation (laddering), leaf tissues, following salt stress, were frozen and ground in liquid nitrogen. DNA was extracted using standard protocols (White and Kaper, 1989). Fifteen micrograms of DNA was loaded onto a 2% agarose gel and electrophoresed at 3 V/cm overnight. Gels were photographed and processed for southern blotting according to standard protocols (Sambrook et al., 1989). Membranes were blocked with 5×-TBS-1% BSA (Sigma) 1×TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature. The membranes were probed with 32P-labeled tobacco genomic DNA. To evaluate DNA fragmentation via terminal deoxynucleotidytransferase-mediated dUTP nick end labeling staining, plant tissue were fixed with 3.7% fresh formaldehyde, digested with lyticase (5 units/L for 30 min at 37°C), then electrostatically bound to a glass slide (Fisherbrand Superfrost Plus, Chicago). Plant tissues were also stained with propidium iodide, a fluorescent DNA stain, which shows that TUNEL labeling occurs specifically with fragmented DNA. The slides were rinsed with phosphate-buffered saline (PBS), incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice, rinsed twice with PBS, incubated with 10 μL TUNEL reaction mixture (200 units/mL terminal deoxynucleotidytransferase, 1 mM FITC-labeled dUTP, 25 mM Tris/HCl, 100 mM sodium cacodylate, 5 mM cobalt chloride; in situ cell death detection kit, Fluorescin, Roche, Indianapolis) for 60 min at 37°C, and rinsed 3 times with PBS. The slides were then incubated with 1 μg/mL diaminophenylindole in PBS for 10 min at room temperature, rinsed twice with PBS, and analyzed under a fluorescence microscope (Zeiss Axioskop, Jena, Germany) coupled to an imaging system (AxioCam HR, Thornwood, NY).

Salt and/or Heat-Stress Assay

Cultures containing the pSfinx vector alone or pS-LePHGPx were prepared as described above. Infiltration of bacteria was done at A600 = 0.6. Agro-infiltration was carried out on the underside of tobacco (cv Glurk) leaves using a 1-mL tuberculin syringe and up to 4 to 5 leaves were completely infiltrated per sample. Plants were kept overnight in the dark after which they were returned to light. Five days after infiltration, 1-cm-diameter discs were excised from infiltrated leaves using a cork-borer and floated on 350 mM NaCl solution, followed by incubation at room temperature under constant illumination. After 48 h, discs were photographed, evaluated for chlorophyll content, and collected for DNA extraction. For heat stress, infiltrated leaves were excised from plant and placed in petri dishes containing moist filter paper. Heat stress was administered at 53°C for 20 min, after which the leaf samples were returned to room temperature and placed under constant illumination and high humidity. Leaves were photographed 48 h after application of stress.

Plant Bax and Fungal Assays

The open reading frames of mouse Bax and LePHGPx were cloned into the Xhol, SpeI sites of the demethasamine inducible vector pTA7002 (McNellis et al., 1998). These constructs were introduced into the A. tumefaciens strain C58C1 via electroporation, and transformants were selected on media containing kanamycin (50 μg/mL), rifampicin (50 μg/mL), and gentamicin (50 μg/mL). Cultures were prepared as described for the plant transient assays. After 6 to 8 h of growth in induction medium, cells were pelleted and resuspended in induction medium (0.5 × MS-B, 10 mM MES, pH 5.6; and 150 μg/mL acetoxyringsone prior to use). A600 of cultures was adjusted to 0.6 and cultures (4-6 leaf stage tobacco sectors were infiltrated with cultures harboring either Bax, LePHGPX, Bax+LePHGPX (1:1), or pTA7002. Mock infiltrations were carried out using infiltration buffer only. After infiltration, plants were kept in the dark overnight, and 24 h later infiltrated sectors were sprayed with 20 μM demethasamine (with 0.005% Silwet L-77). Leaf tissue was examined at 36 h postinduction, stained with Evans blue (10 μg/mL in PBS) overnight, and cleared in 10% aceticelcohol.

To generate transgenic tobacco plants, separate binary vectors were constructed that contained LePHGPx under the control of the cauliflower mosaic virus 35S promoter and the Agrobacterium nopaline synthase terminator. Plant transformation and transgenic evaluations were done as described (Dickman et al., 2001). From three independent transformation events, a minimum of 5 leaves from 5-week-old transgenic tobacco harboring LePHGPX or empty vector were inoculated by placing 5-mm-diameter agar plugs containing actively growing hyphal tips from 3- to 4-d-old colonies of Botrytis cinerea grown on potato (Solanum tuberosum) dextrose agar. All experiments were repeated at least three times.

Northern Analysis

Three-week-old wild-type Rutgers tomato plants grown at 25°C with 16 h light periods in greenhouse were used for all RNA expression experiments. For ABA treatments, tomato plants were sprayed with 250 μM of cis-trans-ABA (Sigma, St. Louis) until run off. The ABA solution was prepared from a 1 mM stock solution containing 1% (v/v) ethanol. Control plants were treated with the same solution minus ABA. For salt stress, plants were placed in a solution containing 300 mM NaCl for 24 to 48 h after which RNA was extracted. Control plants were treated similarly with water alone.

Plants were exposed to 55°C for 20 min for heat stress or 4°C for 3 h for cold treatment; plants were then returned to room temperature. RNA was extracted from samples collected before the stress was imposed (control), time 0 (immediately after stress), and 6 and 12 h following stress treatment. To evaluate LePHGPx expression following pathogen challenge, Sclerotinia sclerotiorum and Alternaria alternata were inoculated on the tomato leaves by placing 5-mm-diameter agar plugs containing actively growing hyphal tips from 3- and 4-d-old colonies, respectively. Inoculated plants were placed in a growth chamber at 25°C with 16 h light periods and 100% relative humidity. Leaves treated with agar plugs only were used as a control. RNA was extracted as described by Reuber and Ausubel (1996) and northern analysis was done as described by Sambrook et al. (1989). To generate an LePHGPx gene-specific probe, a unique fragment of 200 bp was identified by sequence alignment of plant PHGPx genes. This fragment corresponds to the 5′ untranslated region and was obtained by PCR using primers 5′-tagacactcagcggtaa-3′ and 5′-tactatgccacattattac-3′.

Enzyme Assays

LePHGPX cDNA was subcloned into the EcoRI and XhoI sites of the GST fusion protein expression vector pGEX-4T-1 (Pharmacia Biotech, Piscataway,
Expression of GST fusion proteins was carried out as described (Choi et al., 2000). The LePHGPx protein was cleaved by thrombin and purified according to the manufacturer’s manual. LePHGPx activity was determined by using PL-OH, t-butyldihydroperoxide, cumene hydroperoxide, and \( \text{H}_2\text{O}_2 \) as the substrates. Enzyme activity was determined as described by Maiorino et al. (1990) by detecting the oxidation of glutathione in a coupled assay using glutathione reductase (Sigma) and saturating concentrations of NADPH. The oxidation of NADPH was recorded at 340 nm in an SLM-Amino DW2000 spectrophotometer in split beam mode at 37°C. The activity was calculated using an extinction coefficient of 6.220 \( \mu \text{mol}^{-1} \text{cm}^{-1} \). Nonspecific NADPH oxidation activity was recorded in the absence of substrates and taken into account in the activity calculation. PL-OH was synthesized according to Maiorino et al. (1990) using soybean phosphatidyl choline (Avanti PolarLipids, Alabaster, AL) and soybean lipoxynogenase (Sigma; Type IV). PL-OH oxidation concentrations were determined as described by El-Saadani et al. (1989). The GenBank accession number for LePHGPx is AY301280.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY301280.

ACKNOWLEDGMENTS

We thank Young-ki Park for technical assistance.

Received December 23, 2003; returned for revision March 30, 2004; accepted March 30, 2004.

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