A novel plant glutathione S-transferase/peroxidase suppresses Bax lethality in yeast

Sotirios C. Kampranis±*, Radostina Damianova±*, Mirna Atallah±*, Garabet Toby‡, Greta Kondi†, Philip N.Tsichlis# and Antonios M. Makris±*

From ± Mediterranean Agronomic Institute of Chania, Chania 73100, Greece, ‡ Fox Chase Cancer Center, Philadelphia, PA19111 and # Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107

± These three authors contributed equally to this work

* To whom correspondence should be addressed: Mediterranean Agronomic Institute of Chania, Alsyllion agrokepion, P.O. Box 85, 73100 Chania, Greece
Tel: +30 821 81151, Fax: +30 821 81154, E-mail: antonios@maich.gr
ABSTRACT

The mammalian inducer of apoptosis Bax is lethal when expressed in yeast and plant cells. To identify potential inhibitors of Bax in plants we transformed yeast cells expressing Bax with a tomato cDNA library and we selected for cells surviving after the induction of Bax. This genetic screen allows for the identification of plant genes which inhibit either directly or indirectly the lethal phenotype of Bax. Using this method a number of cDNA clones were isolated, the more potent of which encodes a protein homologous to the class θ glutathione S-transferases. This Bax-inhibiting (BI) protein was expressed in E. coli and found to possess glutathione S-transferase (GST) and weak glutathione peroxidase (GPX) activity. Expression of Bax in yeast decreases the intracellular levels of total glutathione, causes a substantial reduction of total cellular phospholipids, diminishes the mitochondrial membrane potential and alters the intracellular redox potential. Co-expression of the BI-GST/GPX protein brought the total glutathione levels back to normal and re-established the mitochondrial membrane potential, but had no effect on the phospholipid alterations. Moreover, expression of BI-GST/GPX in yeast was found to significantly enhance resistance to H2O2 induced stress. These results indicate that oxidative stress plays an important role in Bax-induced death in yeast cells and demonstrate that the yeast-based genetic strategy described here is a powerful tool for the isolation of novel antioxidant and antiapoptotic genes.
INTRODUCTION

Apoptosis, or programmed cell death, is a crucial component of normal development and plays an important role in organogenesis, tissue homeostasis and the editing of the immune system to remove autoreactive clones. Apoptosis is a morphologically recognizable form of cell death that is implemented by a mechanism conserved through evolution from nematode to man. Homologues of the genes that implement cell death in nematodes do likewise in mammals, although in mammals the process is considerably more complex (1-4). In plants, programmed cell death is involved in the terminal differentiation of xylem vessels, and perhaps in developmental processes (5-7). Moreover, inhibition of pathogen growth in plants also involves a rapid process of programmed cell death localized at the site of invasion. The death of plant cells during this "hypersensitivity response" (HR) creates a zone of dead cells, called the "HR lesion", which limits the spread of the pathogen, playing an important role in the plant’s defense mechanism (8-10).

The process of apoptosis can be divided into three distinct components. The first component consists of the apoptotic stimulus and the signal it triggers. This signal may be delivered through surface receptors or may originate inside the cell from a secondary action of a drug, toxin or radiation. The second component consists of the detection and the transduction of the signal, while the third component includes the activation and regulation of the effectors of apoptosis (11).

A highly conserved group of genes that are involved in this third component, i.e. the regulation of the apoptotic process, is the bcl-2 family of genes. The proteins encoded by these genes interact with each other and either promote (e.g. Bax, Bcl-xS, Bak, Bid, Bik, and Hrk) or inhibit (e.g. Bcl-2, Bcl-xL, Bcl-w, Bfl-1, Brag-1, Mcl-1, and A1) apoptosis (12). A common feature of the bcl-2 family of proteins is that most
localise to the outer mitochondrial, outer nuclear, and endoplasmic reticular membranes via their hydrophobic C-terminal transmembrane domain (13). Mitochondria appear to play a central role in the induction of apoptosis, which can be controlled by a number of interrelated events. These include, a) the disruption of electron transport, oxidative phosphorylation and ATP production, b) the release of proteins that trigger the activation of the caspase family of proteases, and c) the alteration of the cellular reduction-oxidation potential which results in the release of reactive oxygen species (ROS) into the cytosol (14).

As in mammals, plant mitochondria were also shown to be the major site of ROS production (15). When produced at low levels, ROS play a double role; they act as signals that protect the plant by inducing an array of cellular protective and defense genes and they promote the oxidative cross-linking of the cell wall. The oxidative burst at the site of invading pathogen also induces systemic cellular responses in which ROS mediate a reiterative signal network required for the establishment of systemic acquired resistance to virulent pathogens (16).

The mammalian protein Bax is a proapoptotic 21-kDa protein with 21% identity to Bcl-2. The protein is thought to alter organelle function by localizing to the outer mitochondrial membrane and forming an ion channel (17). Bax can exhibit its lethal phenotype even when expressed in yeast. Despite the fact that no Bcl-2 family members exist in Saccharomyces cerevisiae, the cell death caused by Bax, or its close relative Bak, appears relevant to its function in a physiological setting. The anti-apoptotic Bcl-2 protein can rescue yeast from Bax/Bak lethality. Mutants of Bcl-2 and Bcl-xL that fail to protect mammalian cells from Bax-induced cell death are also inactive in yeast. Moreover, mutations of Bax and Bak which abolish their function in mammalian cells, do so also in yeast (18,19). Bax induces the release of cytochrome c
from mitochondria in both mammals and yeast, suggesting that its toxicity may be related to oxidative control processes and the opening of the mitochondrial permeability transition (MPT) pore (20). Bax expression is also lethal in plants. Infection of *Nicotiniana benthamiana* plants with a tobacco mosaic virus-based vector carrying the murine Bax gene caused localized tissue collapse, in a manner resembling the hypersensitivity response (21). This action of Bax was dependent on the presence of the carboxy-terminal transmembrane domain, which targets Bax to the mitochondria and, reversible on phosphorylation.

It is likely that Bax-inhibiting plant proteins may prevent apoptosis in plant cells. To test this possibility, we utilized the lethal phenotype of Bax in yeast in an attempt to characterize plant molecules linked to the apoptotic process. We devised a yeast genetic screen that would enable us to isolate plant genes which inhibit its lethality either by interacting directly with it or by indirectly alleviating its toxic effects. Here we present the characterisation of the most potent amongst the Bax inhibiting proteins we isolated, a novel GST/GPX. Our aim is to understand the function of this protein with respect to the lethal Bax phenotype and to reveal components of the plant machinery that may be relevant to the process of apoptosis.
MATERIALS AND METHODS

Plasmid constructs. A LexA-Bax construct was generated by PCR amplification of mouse Bax using the primers 5’-GGT G↓EcoRI AA TTC ATG GAC GGG TCC GGG GAG CAG-3’ (which introduces an EcoRI restriction site before the N-terminus of Bax, MDGSGEQ) and 5’-CTT C↓XhoI TC GAG TCA GCC CAT CTT CTT CCA GAT-3’ (which incorporates a XhoI restriction site after the stop codon at the C-terminus of Bax, IWKKMG). The amplified product was subcloned into the EcoRI and XhoI sites of pGILDA vector (Clontech, USA), giving rise to a construct (pGILDA/Bax) that expresses a LexA fusion with Bax under the control of GAL1 promoter. A hemaglutinin (HA) fusion construct of Bcl-2 (pJG4-6/Bcl-2) was generated by subcloning the open reading frame (ORF) of this gene from the pJG4-5/Bcl-2 construct into the EcoRI and XhoI sites of the pJG4-6 yeast expression vector, which expresses proteins as fusions to a single hemaglutinin tag under the control of GAL1 promoter. A HA fusion to the library clone 2.37 (pJG4-6/2.37) was generated by subcloning the library clone 2.37 into the pJG4-6 vector (22-24).

Yeast genetic screen. A tomato cDNA library cloned into the pJG4-5 vector expresses library clones as fusion to a nuclear localization sequence, a portable transcriptional activation domain (the acid blob B42AD), and a hemaglutinin epitope (25). Bait and library plasmids were transformed into the yeast strain EGY48 (ura3 trpl his3 6LexA-operator-LEU2), in which the upstream regulatory elements of the chromosomal LEU2 gene have been replaced by six copies of the LexA operator. Approximately 0.5 million transformed cells were plated in gal-raff/CM-his, trp and gal-raff/CM-his, trp, leu media. Growing colonies were further evaluated for their ability to inhibit Bax by replating on selection plates. The isolated library plasmids were
reintroduced into LexA-Bax carrying cells and examined for their ability to grow in the
presence of Bax.

The full length GST clone was isolated from a ripening UC82-B tomato cDNA
library (Stratagene) which was screened using the 2.37 clone as a probe. Three
independent clones containing the full length sequence were isolated and sequenced.

*Growth curve.* EGY48 cells containing the plasmids coding for LexA-Bax, or
LexA-Bax and the GST/GPX protein were grown in glu/CM-his, and EGY48 cells
containing the plasmids coding for LexA-Bax and Bcl-2 were grown in glu/CM-his,
trp, until the optical density (OD\textsubscript{600}) reached 0.5. At this point, the cells were pelleted
and resuspended to OD\textsubscript{600} = 0.05 in a medium containing 2% galactose and 1% raffinose (gal-raff) as the carbon source, instead of glucose, to induce expression of the
fusion proteins from the GAL1 promoter (cells containing the plasmids coding for
LexA-Bax, or LexA-Bax and the GST/GPX protein were grown in gal-raff/CM-his,
and EGY48 cells containing the plasmids coding for LexA-Bax and Bcl-2 were grown
in gal-raff/CM-his, trp. Aliquots of cells were removed at regular intervals up to 48 h
and the OD\textsubscript{600} was measured.

*Measurement of sensitivity to \(H_2O_2\).* Fresh overnight cultures of EGY48 yeast
cells expressing the 2.37 cDNA in gal-raff/CM-trp medium, as well as the other
isolated library clones, were washed with sterile \(H_2O\) and diluted to OD\textsubscript{600} = 0.5. 100
\(\mu\)L aliquots were used to inoculate 5 mL of gal-raff/CM-trp liquid medium containing
5 mM \(H_2O_2\). The cultures were incubated at 30°C with shaking. 25 \(\mu\)L aliquots
(~5x10\(^4\) cells) were removed, plated on gal/CN-trp, and the surviving colonies were
count.

*Expression of the cDNA clone 2.37 in bacteria.* The full length open reading
frame of the newly isolated 2.37 cDNA clone was PCR amplified using the primers 5'-
ATG GCT AAC GAT GAA GTG ATT CTG-3' and 5'-TTC AAT TCC AAA CTT TTG CCT TAG-3'. The PCR product was cloned into the pBad TOPO TA vector according to the manufacturer's instructions (Invitrogen, CA, USA). Expression of protein was induced by supplementing logarithmically growing bacterial cells with 0.2% L-arabinose.

**Purification of the His-tagged BI-GST/GPX protein.** 50 mL L-broth medium supplemented with 80 µg/mL ampicillin, was inoculated with 1 mL of fresh overnight culture of bacterial cells harboring the pBad/BI-GST/GPX construct. Cells were grown at 37°C with vigorous shaking to OD$_{600}$~0.7. L-arabinose was added and the culture was grown as described above for 5 h. The induced culture was centrifuged to remove the medium and the cells were resuspended in 5 mL lysis solution (10 mM Tris (pH 7.6), 0.5 M NaCl, 0.1% NP-40, 10% glycerol, 4 mM DTT). The cells were broken by sonication and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected and incubated with Ni$^{2+}$-agarose resin. Imidazole was added to a final concentration of 1 mM, and the mixture was further incubated for 3 h at 4°C. At the end of the incubation the beads were washed several times with wash solution (20 mM Tris (pH 7.6), 1 M KCl, 20% glycerol, 4 mM DTT and 100 mM imidazole) by repeated centrifugations at low speed. The His-tag fused GST protein was eluted by incubating in 200 µL of elution solution (20 mM Tris (pH 7.6), 0.3 M KCl, 20% glycerol, 4 mM DTT and 100 mM imidazole) for 1 h at room temperature.

**Binding of the BI-GST/GPX to glutathione.** Cells were grown and expression of the protein was induced as described above. The induced culture was centrifuged and the cells were resuspended in Phosphate-Buffered Saline (PBS) (10 mM potassium phosphate buffer (pH 7.4), 2.7 mM KCl, 120 mM NaCl). The cells were broken by sonication, and centrifuged at 12,000 rpm for 2 min at 4°C. The supernatant was
collected and incubated with 200 µL of a suspension of GSH-agarose in PBS. The mixture was incubated for 1 h at 4ºC and the beads were subsequently washed several times with PBS supplemented with 0.1% NP-40. The bound protein was eluted by adding 300 µL of PBS containing 15 mM GSH. The bound protein was analyzed by SDS-PAGE.

**Dimerisation of the BI-GST/GPX.** The full length open reading frame of 2.37 cDNA was PCR amplified using the primers 5'-CAG GAA TTC ATG GCT AAC GAT GAA GTG ATT-3' and 5'-TCC CTC GAG TTA TTC AAT TCC AAA CTT TTG-3'. The PCR product was digested with *Eco*RI and *Xho*I, purified and cloned into the pEG202 and pJG4-5 interaction trap vectors. The constructs pEG202/BI-GST/GPX and pJG4-5/BI-GST/GPX were introduced into the yeast reporter strain EGY48 by lithium acetate transformation (24). EGY48 cells expressing the LexA-(BI-GST/GPX) fusion with the B42AD-(BI-GST/GPX) or the LexA-(BI-GST/GPX) fusion with the B42AD alone were replica plated on glu/CM-his, trp, leu and gal-raff/CM-his, trp, leu and incubated at 30ºC for 24 h.

**Determination of total glutathione, glutathione disulfide and lipid analysis in yeast cells.** EGY48 cells containing LexA-Bax, LexA-Bax and HA-(BI-GST/GPX), HA-(BI-GST/GPX), HA-Bcl-2 or no plasmids were grown to OD$_{600}$=0.5 using glucose as the carbon source. Cells were subsequently washed and resuspended in galactose-containing medium in which they were cultured with shaking for an additional 8 h period.

Total glutathione and glutathione disulfide were measured in equal amount of cellular material according to the method described by Griffith (26). The cells were pelleted, resuspended in 5 mL 1% oxalic acid, and broken by sonication. After the samples were centrifuged, 200 µL of the supernatant was removed. To the samples
analysed for [GSSG], 4 µL of 2-vinyl pyridine was added. 45 µM final concentration of triethanolamine was then added to all the samples. 1 mL samples containing 125 mM Na-phosphate (pH 7.5), 6.3 mM EDTA, 0.21 mM NADPH, 0.1 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB, Sigma), and 200 µL of the samples were equilibrated at 30°C for 5 min. 0.5 Units of glutathione reductase was added and the absorbance at 412 nm was monitored. The concentration of glutathione was calculated by comparing the rate of the reaction to a glutathione standard curve.

Lipid extraction was performed using the method described by Komagata (27). 0.5 g of lyophilized cells were incubated with 5 mL chloroform/methanol (2:1 v/v) overnight at room temperature in a screw-capped glass tube. The mixture was subsequently filtered through Whatman paper No 1 and the supernatant was transferred to a new tube. The pellet was rinsed with 2 mL of chloroform/methanol (2:1 v/v) and the supernatants were pooled. To partition the two solvents, one fourth volume of 1% KCl solution was added. The lipophilic layer was removed and filtered through Na₂SO₄ powder to absorb the remaining water in the extract. The solvent was removed by a stream of N₂. Subsequently, 0.4 mL of a 1.5% solution of sodium methylate was added, and the samples were incubated at 80°C for 90 min. 0.4 mL of heptane was added, the samples were vortexed, and the phases were allowed to separate. To concentrate the samples, the upper phase was transferred to high recovery vials (Hewlett Packard, USA), the solvent was evaporated, and the residual was resuspended in 50 µL of heptane.

Quantitative lipid analysis was performed using a gas chromatograph (Hewlett Packard 5890 series II) equipped with flame ionization detector. A cross-linked fused silica capillary column HP-FFAP, 50 m long, 0.2 mm id., 0.33 µm film thickness, was used under the following conditions: injector temperature, 250°C; oven temperature,
220ºC; detector temperature, 280ºC; attenuation, -2; column head pressure, 30 psi; column flow, 0.4 m/min. Split vent flow was 15 mL/mm. GC-MS was performed with a coupled system HPGC 5890II-VG TRIO 2000 mass spectrometer equipped with the MAS LYNX software. Chromatographic conditions were similar to those described above. Mass spectra were taken at 70 eV and the scanning speed was $1 \text{ s}^{-1}$ from 40 to 230 m/z. Identification of peaks was carried out by comparison with authentic samples and spectra from the Wiley electronic library.

**Measurement of GST and GPX activity.** For the determination of GST and GPX activity, induced and uninduced bacterial cells containing the vector pGILDA/BI-GST were broken by sonication. GST activity of the cell extract was measured by monitoring the absorbance at 340 nm of a 1 mL reaction containing a range of sample volumes in 50 mM Tris-HCl (pH 6.5), 0.1 mM EDTA, 1 mM GSH, and 5 mM 1-chloro-2,4-dinitrobenzene (CDNB) at 25ºC. 1 unit of GST activity is defined as the amount that transforms 1 µmol of CDNB per min at 25ºC. The total protein concentration of the bacterial extracts was measured using the Bio-Rad protein assay reagent and the activity of the sample was expressed as units of GST per mg of total protein. Bovine liver GST (Sigma) was used as a positive control. GPX activity was measured by monitoring the absorbance at 340 nm of a 1 mL reaction containing a range of sample volumes in 50 mM Tris (pH 7.5), 0.1 mM EDTA, 1 mM GSH, 0.2 mM NADPH, 50 µg/mL cumene hydroperoxide, and 1 unit of glutathione reductase at 25ºC. 1 unit of GPX is defined as the amount of enzyme that transforms 1 µmol of NADPH to NADP per min at 25ºC. As above the GPX activity was measured as units of GPX per mg of total protein. Bovine erythrocyte GPX (Sigma) was used as a positive control.
**Flow cytometric studies.** The full length ORF of the putative GST/GPX was cloned into the pJG4-6 yeast vector, which directs the expression of proteins fused with to a hemaglutinin epitope tag from a GAL1 promoter. EGY48 yeast cells harboring the constructs pGILDA/Bax, pGILDA/Bax and pJG4-6/Bcl-2, pGILDA/Bax and pJG4-6/GST, and pJG4-6/GST were grown overnight in glu/CM media lacking the amino acids necessary for plasmid maintenance. The following day cells were washed and resuspended to final OD$_{600}$ ≅ 0.1 in galactose based selective medium to induce protein expression. The cultures were induced for 6 h at 30ºC with vigorous shaking. Following this, the cells were resuspended in 500 µL of 50 mM Tris-HCl (pH 7.5) and sonicated briefly to disrupt any aggregates. After sonication the cells were treated with 50 µL RNAsel A (10 mg/mL) and incubated for 1 h at 37ºC. After the addition of 5 µL proteinase K (20 mg/mL) the sample were incubated for 1 h at 50ºC. The pelleted cells were washed in 300 µL distilled H$_2$O, centrifuged, and subsequently resuspended in 500 µL of 50 mM Tris-HCl (pH 7.5). Intracellular ROS production was measured by staining with the dye 2′,7′-dichlorodihydrofluorescein diacetate (DCF; D-399, Molecular Probes), at a final concentration of 50 µM for 15 min. Mitochondrial membrane potential was measured by staining cells with 3,3′dihexylocarbocyanine iodide (DiOC$_6$(3); D-273, Molecular Probes) at a final concentration of 40 nM for 15 min. To measure the DNA content, cells subsequent to proteinase K treatment were resuspended on 300 µL H$_2$O and they were fixed by the addition of 700 µL 100% ethanol and incubation for 1 h at room temperature. Fixed cells were stained with 5 µL of propidium iodide (2.5 mg/mL) and incubated for 1 h at room temperature. Data were collected with a FACScan fluorescence activated cell scanner using the data acquisition program CELLQuest (Becton Dickinson, CA). 20,000 cells were measured for the DCF and DiOC$_6$(3) staining and 10,000 cells for the propidium iodide staining.
RESULTS

Screening for plant proteins that inhibit the Bax phenotype in yeast. Bax is a proapoptotic member of the Bcl-2 family of proteins and has been shown to induce cell death in mammalian, plant and yeast cells. To screen for plant proteins that inhibit Bax-induced apoptosis, we utilized the yeast two hybrid system. The system was set up to detect a) proteins that inhibit directly the lethal phenotype of Bax via dimerisation with the Bax protein, and b) proteins that indirectly alleviate the toxic effects of Bax.

A tomato library cloned into the yeast interaction trap vector pJG4-5 (25) was introduced into the EGY48 yeast two-hybrid reporter strain harboring the pGILDA/Bax construct. EGY48 cells are unable to grow in the absence of leucine in the medium, unless there is physical interaction of the LexA-DNA binding domain-bait fusion protein with the transcription activation domain-library fusion protein, which directs the complex to the LexA operator sites upstream of the LEU2 gene and activates transcription. Approximately $5 \times 10^5$ independent transformed colonies, grown in glucose containing medium, were collected. To identify plant proteins that dimerise with Bax and block its activity, the LexA-Bax and the library B42AD fusion proteins were induced by switching the cells into galactose-containing medium for 1 h and by subsequently plating them on gal-raff/CM-his, trp, leu semi-solid medium. This would select for proteins that not only inhibit Bax lethality but also physically interact with the LexA-Bax protein in the two hybrid system, thus enabling them to survive in the absence of leucine. To identify plant proteins that only indirectly (without physical interaction) inhibit Bax toxicity, following their exposure to galactose-containing media, the cells were plated on gal-raff/CM-his, trp medium. No colonies grew in the medium lacking leucine, which indicated the absence of direct Bax-interactors in this pool of transformants. However, screening for indirect
inhibitors of Bax toxicity (in leucine-containing media) yielded six independent colonies. Transfer of these cDNA clones into fresh pGILDA/Bax cells reproduced the phenotype, indicating that this was specifically induced by the proteins encoded by the transfected library clones.

The library plasmids were isolated and the cDNAs sequenced. Of the six isolated clones four could be involved in oxidative metabolism. Clone 2.37 exhibits 88% identity at the amino acid level with a glutathione S-transferase (GST) from *N. tabacum* (28). GSTs catalyze the nucleophilic attack of the sulfur atom of glutathione (GSH, γ-glutamylcysteinylglycine) to the electronic center of a wide variety of substrates (29). Glutathione is the chief intracellular non-protein thiol compound that mediates redox cycling between ascorbate and NADPH and functions as a cellular storage pool of reduced thiol. During oxidative stress, rapid changes are observed in the cellular glutathione content (30). Library clone 7.40 exhibits 68% aminoacid identity to a cytochrome P450 protein from *Helianthus tuberosus* while library clone 8.44 is 75% identical with the gene encoding a tobacco cytochrome P450-like protein (31). Both these heme-containing enzymes are involved in the oxidative metabolism of diverse endogenous and exogenous lipophilic substrates. Finally, library clone 7.52 exhibits 67% amino acid identity to the senescence induced protein SRG1, a member of the Fe(II) ascorbate oxidase superfamily (32).

The two remaining clones include: Clone 2.46, which exhibits 68% amino acid identity with the Endochitinase cytoplasmic precursor of *Arabidopsis thaliana* (Z25683, Genbank accession number) and 40% identity with a second characterized chitinase from *Arabidopsis thaliana*; and library clone 6.19, which has 96% amino acid identity to the mitochondrial serine hydroxymethyl transferase protein of *Solanum*.
*tuberosum* (33), a folate requiring enzyme that channels the amino acid serine into the pathway of nucleotide biosynthesis.

Expression of selected library clones confers resistance to apoptosis induced by reactive oxygen species (ROS). These findings suggest that Bax toxicity may be due, at least in part, to the generation of oxidative stress. Results from other studies also suggest that ROS are important regulators of apoptosis (30). Pro-oxidants and redox cycling agents, such as H$_2$O$_2$ (34), diamide (35), etoposide (36,37) can induce apoptosis. To assess the ability of the proteins encoded by the newly isolated cDNAs to function as antioxidants, we evaluated their role in resistance to oxidative stress-induced cell death (hydrogen peroxide, H$_2$O$_2$).

EGY48 cells expressing the library B42AD fusion proteins were exposed to 5 mM H$_2$O$_2$ for 6 and 18 h. The choice of concentration and length of exposure to H$_2$O$_2$ was established from survival curves for the EGY48 strain and represented the minimal concentration inducing cell death in more than 90% of the cells (Figure 1). Cells were subsequently transferred into plates containing galactose as carbon source and the number of growing colonies were counted after 72 h of incubation at 30ºC. All experiments were repeated in triplicate and the percentage of surviving cells compared to timepoint 0 was calculated. Expression of the B42AD-2.37 fusion enhanced significantly the viability of EGY48 cells exposed to H$_2$O$_2$ for 6 h (Fig. 2). 70% of cells remained viable compared to only 8% of the wild type cells. Cellular viability decreased rapidly after prolonged exposure to H$_2$O$_2$ reaching zero after exposure for 30 h. Expression of the B42AD-7.40 cytochrome P450 homologue and the B42AD-7.52 (FeII) ascorbate oxidase homologue also enhanced resistance to H$_2$O$_2$ albeit their effect was less pronounced than that of clone 2.37.
The endochitinase AD-2.46 clone and the serine hydroxymethyl transferase B42AD-6.19 clone did not enhance resistance to H$_2$O$_2$ suggesting that their mechanism of action on the Bax phenotype does not relate to oxidative stress.

**Characterization of clone 2.37.** Of the proteins identified, the most potent inhibitor of ROS (Figure 1) and Bax toxicity (data not shown) was the protein encoded by clone 2.37. We therefore proceeded with the biochemical and biological characterisation of this protein with the aim to improve our understanding of the function of Bax. Independent colonies containing both constructs were transferred from a glucose master plate into a galactose plate (Figure 2 A, B). Most of the transferred colonies (46/56, ~82%) grew well in the presence of Bax (data not shown). The expression of LexA-Bax was confirmed by western blotting using anti-LexA antibodies (not shown). Cells harboring both constructs were also grown in liquid cultures and the growth rate of EGY48 cells co-expressing the LexA-Bax and B42AD-2.37 was compared to the growth rate of cells expressing only the LexA-Bax or LexA-Bax and Bcl-2. As shown in Figure 2C, expression of cDNA clone 2.37 enables the LexA-Bax expressing cells to proliferate and the culture to reach saturation after 48 h, albeit at a lower rate than wild-type cells. In contrast LexA-Bax expressing cells never reached saturation even after 72 h of incubation (data not shown). Cells expressing the protein encoded by clone 2.37 proliferated faster than cells expressing Bcl-2. However, by comparison with the total lack of growth of Bax expressing cells in semi-solid media, the effect of Bax in liquid cultures was less pronounced. This could be explained by the extent of exposure to air and the degree of respiration, since cells spread thinly on semi-solid medium would be more exposed to air. Alternatively, there are indications of a nonfunctional pump system in the Bax expressing cells that could also explain the disparity of the phenotype. The full effect of Bax expression was
manifested 7-8 h after the induction of LexA-Bax expression. Cells in liquid cultures stopped dividing when Bax was expressed, but maintained their viability and resumed growth when transferred to glucose based medium (data not shown).

To isolate full length cDNA clones, a tomato cDNA library was screened using the 2.37 insert as a probe. Three independent clones were isolated and sequenced and all of them contained the full length ORF. This encodes a 220 amino acid long protein, that is 88% identical with the auxin-induced glutathione S-transferase from *N. tabacum*, a member of the θ class of GSTs (28,38,39). Clone 2.37 was shown to be an in-frame fusion between B42AD and the truncated Bax-inhibiting (BI) GST protein starting at amino acid 40.

*The protein encoded by clone 2.37 is a GST/GPX enzyme.* The GST activity of the full length cDNA was established by initially showing that the bacterially expressed protein can bind to glutathione. A carboxy-terminal fusion of the protein to a V5 epitope and a His-tag was expressed inducibly in *E. coli*. Duplicate lysates from induced bacterial extracts were used to purify the protein using Ni²⁺-agarose beads, which bind the protein through the His-tag, and GSH-agarose beads which should specifically bind a GST protein. As shown in Figure 3A the induced protein can be purified using GSH agarose, confirming its ability to bind glutathione.

Glutathione S-transferases exhibit large variation in their substrate specificity. However, most of the enzymes are active against 1-chloro-2,4-dinitrobenzene (CDNB) (40). To determine whether the protein encoded by the full length clone possesses GST activity, we examined its ability to conjugate CDNB with GSH. Conjugation increases absorbance at 340 nm. Using this assay we observed that induced bacterial extracts expressing the protein encoded by the full length clone exhibit >30 times
higher GST activity than uninduced ones (Table 1). Taken together, these results confirm that the Bax-inhibiting protein encoded by the cDNA clone 2.37 is a GST.

Many GSTs are multifunctional in that they have been shown to also possess glutathione peroxidase (GPX) activity. Glutathione peroxidase catalyzes the GSH-dependent reduction/inactivation of H$_2$O$_2$ forming glutathione disulfide (GSSG) and increasing GSH synthesis by feedback induction (29). The closest homologue to our protein, a GST from *N. tabacum*, was shown to possess both GST and GPX activity, and when it was overexpressed in tobacco plants it was shown to enhance acclimation to chilling and salt stress (28). To determine whether our protein possesses GPX activity, we assessed the ability of bacterial cell extracts expressing this protein to oxidize GSH using cumene peroxide as a substrate. Induced cell extracts expressing the full length clone were found to exhibit 4-times higher GPX activity than uninduced ones (Table 1).

The novel BI-GST/GPX protein encoded by clone 2.37 forms dimers. Glutathione S-transferases are generally active as dimers, mostly homodimers, although they have also been found as heterodimers with other GST molecules (29). To assess the homodimerisation potential of our protein, we cloned the full length ORF in frame with LexA and B42AD (22). Expression of the LexA-GST alone or with B42AD does not activate transcription of the LEU2 gene and cells cannot grow in the absence of leucine (Figure 3B-1). When LexA-GST is co-transformed with B42AD-GST in the reporter strain, the GST fusion proteins interact, bringing into proximity LexA and the B42 domain, inducing transcription of the LEU2 gene, thus conferring a leu+ phenotype (Figure 3B).

Expression of Bax in mammalian cells induces apoptosis by generating ROS (41) and by decreasing the mitochondrial membrane potential (42). To assess the
effect of Bax expression in *S. cerevisiae* and the mechanism by which the BI-GST/GPX protein alleviates the Bax phenotype, we proceeded to characterize the intracellular glutathione levels, the fatty acid profile, the redox potential, the mitochondrial membrane potential, and the DNA content of yeast cells expressing Bax with or without, the rescuing BI-GST/GPX protein.

*Effects of Bax, Bcl-2, and the novel BI-GST/GPX on the levels of intracellular GSH and GSSG.* To assess the role of intracellular glutathione levels in the Bax phenotype, EGY48 cells expressing LexA-Bax, LexA-Bax and BI-GST-GPX, BI-GST-GPX alone, and LexA-Bax and Bcl-2, or Bcl-2 alone, were processed and the intracellular levels of total glutathione (GSH and GSSG) and glutathione disulfide (GSSG) were measured. Cells expressing the LexA-Bax protein showed a 40% reduction in the levels of total glutathione and a marginal increase in its oxidized form (GSSG). Co-expression of the BI-GST/GPX protein reverted total glutathione to wild type levels. GSSG was increased to levels almost three times higher than normal, leading to an increase in the ratio of GSSG to GSH. Expression of only the BI-GST/GPX clone in EGY48 cells results in a similar higher ratio of GSSG to GSH which indicates that the shift is due to the peroxidase activity of the GST/GPX enzyme (Figure 4). Co-expression of Bcl-2 in LexA-Bax carrying cells also reverted GSH to wild type levels but did not alter the ratio GSH/GSSG.

Supplementing the growth medium with GSH did not revert the Bax-induced growth inhibition of EGY48 cells. However, cells co-expressing Bax and the GST/GPX protein grew slightly better when supplemented with 2 mM GSH (data not shown). Addition of GSSG in the medium had no effect in the growth of cells expressing Bax or co-expressing Bax with Bcl-2 and Bax with the GST/GPX (data not shown).
Effects of Bax and the BI-GST/GPX protein on the fatty acid profile of yeast cells. Recently Asoh et al. showed alterations in the fatty acid profile including accumulation of monounsaturated fatty acids in Bax-expressing E. coli (43). The effect of LexA-Bax expression on lipid composition was analyzed by methanol/chloroform (2:1 v/v) extraction of wild-type EGY48 cells, EGY48 cells expressing LexA-Bax alone, and EGY48 cells expressing LexA-Bax and the BI-GST/GPX protein 7 h after induction of these proteins (Figure 5). As expected the major fatty acids of S. cerevisiae EGY48 cells are hexadecanoic (palmitic), hexadec-9-enoic (palmitoleic), octadecanoic (stearic), octadec-9-enoic (oleic), but also octadec-9,12-dienoic (linoleic) acids (44). The fatty acid profiles at time zero were identical between all samples. Seven hours after induction of protein expression, a relatively proportional quantitative decrease in all fatty acids is observed in cells expressing the LexA-Bax fusion, suggesting an inhibition in fatty acid biosynthesis due to the expression of Bax (Figure 5). Co-expression of the GST/GPX protein did not revert these changes, even though it enhanced the viability of the cells.

Effects of Bax, the GST/GPX protein and Bcl-2 on the generation of ROS and the mitochondrial membrane potential $\Delta \Psi_m$. The nature of the genes isolated as Bax inhibitors, the effect of the expression of Bax and some of its inhibitory genes on the glutathione levels, and recent data from other laboratories (14), implicate oxidative stress in the manifestation of the Bax phenotype. To assess the effect of Bax expression on the generation of ROS, DCFH-DA was utilized as a substrate for the detection of $H_2O_2$ and other hydroperoxides (45). The non polar compound DCFH-DA is hydrolyzed to DCFH when incorporated into the hydrophobic regions of the cell. In the presence of ROS, DCFH gives rise to DCF, which is membrane impermeable, highly fluorescent and can be detected by FACS analysis. EGY48 cells
expressing LexA-Bax, LexA-Bax and BI-GST/GPX, LexA-Bax and Bcl-2 and BI-GST/GPX alone, were treated and stained with DCFH-DA at seven hours after induction. Contrary to expectations, expression of Bax did not increase ROS. Expression of LexA-Bax caused an initial small transient increase in ROS, which upon prolonged expression stabilized to levels slightly lower than those in control cells (Figure 6). However, co-expression of Bcl-2 led to a substantial increase in the amount of ROS. A plausible explanation for this effect could be that the Bax protein, targets the mitochondrial outer membrane and as a result it causes a significant inhibition of the respiratory machinery in yeast, thus ceasing the production of ROS. Expression of Bcl-2 or BI-GST/GPX in these cells antagonizes the action of Bax and enhances respiration. This in turn generates large amounts of ROS which can leak into the cytosol. The antioxidant capacity of Bcl-2 does not completely compensate for this, whereas expression of the BI-GST/GPX effectively neutralizes ROS. In a mammalian system where Bcl-2 family members are continuously present, overexpression of Bax leads to an increase in ROS generation, for as long as, the respiratory system remains functional.

To assess the status of the mitochondrial function upon Bax expression and the effects of the BI-GST/GPX, we proceeded to stain replicate samples of cells with DiOC₆(3). The fluorescent dye DiOC₆(3) localizes to the mitochondria as a consequence of the mitochondrial membrane potential ΔΨₘ. Dissipation of ΔΨₘ can be the result of either the disruption of the integrity of the mitochondrial outer membrane or the opening of the MPT pore. Overexpression of Bax in mammalian systems has been associated with the opening of MPT and loss of ΔΨₘ (42). Expression of Bax in yeast cells also caused a collapse of ΔΨₘ (Figure 6). This effect was largely reversed by co-expression of the BI-GST/GPX or Bcl-2 (Figure 6). The collapse of ΔΨₘ, upon
LexA-Bax expression reasonably fits with the concomitant decrease in ROS production, as assessed by DCF staining. \( \Delta \Psi_m \) restored by BI-GST/GPX and Bcl-2 did not return to wild type levels. Taken together with the DCF fluorescence data, this could explain the increased ROS, when Bcl-2 was expressed.

**Effects of Bax, Bcl-2 and BI-GST on the cell cycle.** To assess the effect of expression of Bax and the Bax-inhibitory genes on the cell cycle, ethanol fixed cells were stained with propidium iodide and analysed by fluorimetry. The DNA content profile of Bax expressing cells, obtained by this analysis, was similar to the profile of normal cells. This suggests that expression of Bax froze the cells at the stage of the cell cycle they were while replicating (Figure 7). In contrast, cells co-expressing Bax and BI-GST/GPX or Bax and Bcl-2 kept dividing, albeit cell division was prolonged. These cells accumulated in the G1 phase of the cell cycle suggesting that cells expressing these proteins exhibit a partial G1 to S block.
DISCUSSION

Expression of Bax in yeast cells leads to growth inhibition and eventually cell death (46-48). This phenotype appears relevant to its function in a physiological setting. Previous reports showed that Bax induces the release of cytochrome c from yeast mitochondria, and its activity can be suppressed when dimerised by Bcl-2 or Bcl-xL. Our data elaborate on this by showing that expression of Bax in yeast causes a collapse of the mitochondrial membrane potential, ΔΨm, a significant decrease of total glutathione levels and a decrease in fatty acids.

In our genetic screen, we identified six plant cDNA clones which were capable of indirectly inhibiting the Bax phenotype in yeast. Four out of six, rescuing library clones, were found to be homologues to known antioxidant proteins, suggesting a central role for oxidative stress in the Bax phenotype. The most potent inhibitor of Bax lethality was further characterised and was shown to be a glutathione S-transferase, which also possesses weak peroxidase activity. GSTs are ubiquitous enzymes in aerobic organisms and catalyse the conjugation of glutathione to a wide variety of substrates. The peroxidase activity of the BI-GST/GPX converts H2O2 to H2O by oxidizing reduced glutathione. The BI-GST/GPX is a member of the θ class of GSTs. The θ class is evolutionarily the most ancient group of GSTs and is found in vertebrates, insects, plants and in Methylobacterium. It has been suggested that the θ class is representative of a progenitor GST involved in protecting cellular components from oxidative damage (39). A mouse GST of the θ class with 28% homology to BI-GST/GPX was recently cloned as a protein implicated in the development of resistance to radiation-induced apoptosis in lymphoma cells (49). The θ class GST from the Australian sheep blowfly, Lucilia cuprina (Lucilia GST; EC 2.5.1.18) has been crystallized and its structure has been elucidated. The highly conserved residues Glu64
and Ser\textsuperscript{65} which appear to interact with the γ-glutamyl residue of GSH in the crystal structure of the Lucilia enzyme, are homologous to Glu\textsuperscript{67} and Ser\textsuperscript{68} of the BI-GST protein (Figure 8). Histidine 50, one of the two histidines (at positions 38 and 50) of the Lucilia enzyme which appear to interact with the glycine of the glutathione molecule, is found to be homologous to His\textsuperscript{52} of BI-GST. Also, Ile\textsuperscript{52} of the Lucilia enzyme, which appears to hydrogen bond to the cysteinyl moiety, corresponds to Ile\textsuperscript{55} of BI-GST. Ser\textsuperscript{9} of the Lucilia enzyme has been shown by site-directed mutagenesis to be involved in the activation of the thiol group of GSH (50). This amino acid is homologous to Ser\textsuperscript{14} of BI-GST. However, the protein encoded by clone 2.37, which lacks the first 40 aminoacids, is an active transferase.

Yeast cells expressing Bax exhibit a depletion of intracellular glutathione levels. This effect has also been observed in response to many inducers of apoptosis in mammalian cells, including Tumor Necrosis Factor α, FAS, chemotherapeutic agents, viral infections and glutamate (41). Glutathione levels are known to play a regulatory role in key enzymes involved in the apoptotic process, such as a neutral sphingomyelinase which controls the synthesis of ceramide and several apoptotic proteases (51). Expression of the BI-GST/GPX protein restored total levels of glutathione in the Bax expressing yeast cells.

The activity of Bax in the mitochondria is thought to involve the opening of the MPT pore, a multiprotein complex located at the contact sites between the inner and outer mitochondrial membranes. This complex is composed of cytosolic proteins, such as hexokinase; outer membrane proteins, such as the benzodiazepin receptor and the Voltage Dependent Anion Channel; an intermembrane protein creatine kinase; an inner membrane protein, the Adenine Nucleotide Translocator and at least one matrix protein, cyclophilin D (52-54). Under normal circumstances, the inner mitochondrial
membrane is nearly impermeable. This feature is required for maintaining the $\text{H}^+$ gradient across the inner mitochondrial membrane, or the inner transmembrane potential, $\Delta \Psi_m$. The MPT involves the opening of a large channel ($\sim 2.9 \text{ nm}$, $\sim 1.3 \text{ nS}$), allowing the diffusion of solutes with a Mw of $<1500 \text{ Da}$ causing the dissipation of $\Delta \Psi_m$ (55,56). This is associated with the release of $\text{Ca}^{2+}$ and glutathione from the mitochondrial matrix, the uncoupling of oxidative phosphorylation with cessation of ATP synthesis, inhibition of mitochondrial protein synthesis, inhibition of protein import into the mitochondria and hyperproduction of ROS by the uncoupled respiratory chain. These changes occur almost universally during the apoptotic process (42).

This work permits a better understanding of the function of Bax which could not be achieved by studying the role of Bax in cells from higher eukaryotes. In yeast cells the mitochondrial membrane damage proceeds unopposed because of the lack of Bcl-2. As a result, mitochondrial function is inhibited and the increased ROS production ceases. This allows the cells to survive although, because of the decreased energy production, they proliferate slowly. In higher eukaryotic cells, which express Bcl-2, the effect of ROS on the mitochondrial membrane potential is partially blocked (This may be either because Bcl-2 downregulates the increased production of ROS bringing it into levels that can be tolerated, or because Bcl-2 may have a direct effect). As a result, Bax continues to stimulate the production of ROS, which leads to cell death by apoptosis.

The BI-GST/GPX protein was able to restore the $\Delta \Psi_m$ in Bax expressing yeast cells. Although the nature of the effect of the protein on the restoration of $\Delta \Psi_m$ is not yet known, it has recently been shown that another antioxidant enzyme thioredoxin peroxidase specifically prevented the occurrence of MPT by neutralizing
extramitochondrial \( \text{H}_2\text{O}_2 \) \(^{(57)}\). The BI-GST/GPX could either exert its effect by removing ROS from the cytosol or directly affecting the MPT pore. It should be noted that upon inclusion of 1 mM \( \text{H}_2\text{O}_2 \) in the growth medium, despite of the fact that DCF fluorescence is significantly increased, the mitochondrial membrane potential became hyperpolarized and not collapsed, suggesting that the mechanism of BI-GST/GPX may be other than neutralizing ROS.

This work shows that the BI-GST/GPX, when co-expressed in yeast cells with Bax, restores mitochondrial function. Considering that mitochondria are ultimately involved in apoptosis, the isolated protein presents an attractive candidate as a regulator of apoptosis. Future work will address the involvement of mitochondria in plant apoptosis and will characterize the BI-GST/GPX protein and its effects on organellar function under various types of apoptotic stimuli. Oxidative stress has been shown to occur in response to many types of environmental stress, caused by chilling, ozone, salt and draught. Along these lines it is interesting that the closest relative of the BI-GST/GPX, isolated from \( N. \text{tabacum} \), was shown to enhance resistance to chilling and salt stress. A potential link between mitochondrial function and resistance to certain types of environmental stress is of great interest because BI-GST/GPX, as well as other antioxidant genes isolated from the yeast genetic screen, can be important in engineering plants resistant to environmental stress.
ABBREVIATIONS

GST, glutathione S-transferase; GPX, glutathione peroxidase; ORF, open reading frame; MPT, mitochondrial permeability transition; B42AD, activation domain B42; ROS, reactive oxygen species; HR, hypersensitivity response; OD, optical density; gal-raff/CM, galactose and raffinose supplemented complete medium; glu/CM, glucose supplemented complete medium; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, γ-glutamylcystinylglycine; FACS, fluorescence associated cell sorting

BI-GST/GPX GenBank accession number: AF193439
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Table 1.

Enzymatic activity of the protein encoded by the library clone 2.37

<table>
<thead>
<tr>
<th>Enzymatic Activity</th>
<th>Units/mg of total protein</th>
<th>Uninduced</th>
<th>Induced</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST activity</td>
<td></td>
<td>6.3x10^{-5}</td>
<td>2.1x10^{-3}</td>
</tr>
<tr>
<td>GPX activity</td>
<td></td>
<td>0.7x10^{-5}</td>
<td>2.8x10^{-5}</td>
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FIGURE LEGENDS

Fig. 1. Plant inhibitors of Bax lethality inhibit H$_2$O$_2$-induced cell death. EGY48 cells expressing the library fusion proteins isolated from the genetic screen in yeast for Bax inhibitors were grown in gal-raff/CM-trp and evaluated for their ability to maintain cellular viability when subjected to stress treatment using 5 mM H$_2$O$_2$ for 6 and 18 h. Library clones 2.37 (GST homologue), 7.40 (cytochrome P450 homologue), and 7.52 (ascorbate oxidase homologue) significantly enhance viability in the presence of H$_2$O$_2$. Control EGY48 cells were grown in gal-raff/CM.

Fig. 2. The plant GST homologue inhibits Bax-induced lethality in yeast. A, EGY48 yeast cells harboring a LexA-Bax construct under the control of GAL1 promoter, were transferred to glu/CM-his plates (left) and to gal-raff/CM-his plates (right). Expression of LexA-Bax inhibits cell growth. B, EGY48 cells harboring the LexA-Bax with B42AD (left) and the LexA-Bax with B42AD-2.37 constructs (right) were transferred to gal-raff/CM-his, trp plates. Coexpression of the candidate GST fusion protein alleviates Bax induced growth inhibition. C, cells expressing LexA-Bax, LexA-Bax and HA-2.37, and LexA-Bax and HA-Bcl-2 were grown in liquid cultures containing gal-raff/CM-his for LexA-Bax and gal-raff/CM-his, trp for the other two. The inhibition of cell proliferation due to Bax expression is less pronounced in liquid medium. However, the GST co-expressing cells exhibit rapid proliferation and quickly reach saturation; their rate of proliferation exceeds that of cells co-expressing LexA-Bax and HA-Bcl-2.
**Fig. 3.** A, The plant GST homologue binds to glutathione. Bacterial cells harboring a GST fusion construct to a V5 epitope and a His-tag to the carboxy terminus were grown in LB broth and induced to express the protein by the addition of a final concentration of 0.2% L-arabinose. (1) Sonicated uninduced bacterial lysates, (2) Sonicated lysates induced for GST expression using L-Arabinose, (3) Purification of the GST-V5-His-tag protein using Ni\(^{2+}\)-agarose column, (4) Purification of GST-V5-HIS using a GSH-agarose column. B, The plant GST/GPX homodimerises in the yeast two-hybrid system. LexA-GST interacts with the B42AD fusion of GST in the yeast interaction trap system when plated on gal-raff/CM-his, trp(1). However, LexA-GST fails to interact with the activation domain B42AD alone (2). The observed homodimerisation interaction occurred only in the presence of galactose in the medium, which induces the expression of B42AD-GST.

**Fig. 4.** Determination of total glutathione and glutathione disulfide. EGY48 cells were induced to express LexA-Bax, LexA-Bax and BI-GST/GPX, LexA-Bax and Bcl-2, BI-GST/GPX alone, and Bcl-2 alone for 8 h in gal-raff/CM-his, gal-raff/CM-his, trp, gal-raff/CM-his, trp, gal-raff/CM-trp and gal-raff/CM-trp medium respectively. The cells were subsequently freeze dried and equal amounts were used to enzymatically determine GSH and GSSG levels. There is a significant reduction in total glutathione (GSH + GSSG) in the LexA-Bax-expressing cells. Coexpression of the GST/GPX or Bcl-2 reverses the levels of total glutathione to normal. However, the ratio of the GSH/GSSG is altered in the GST/GPX-expressing cells due to the glutathione peroxidase activity of this protein.
Fig. 5. Expression of Bax causes alterations in fatty acid composition. Wild-type EGY48 yeast cells (A), EGY48 cells harboring the LexA-Bax construct (B), and EGY48 cells harboring the LexA-Bax and the B42AD-GST construct (C), were incubated for 7 h in gal-raff/CM, gal-raff/CM-his, and gal-raff/CM-his, trp medium respectively. The cells were washed, freeze-dried, and equal amounts of dried material were used for the extraction of fatty acid methyl esters. The extracts were subjected to gas chromatography and the identity of the peaks was confirmed by GC-coupled mass spectrometry. Expression of Bax caused a quantitative decrease in the amounts of fatty acids, which was not reversible by GST coexpression.

Fig. 6. Changes in the intracellular ROS and mitochondrial membrane potential ($\Delta \Psi_m$). EGY48 cells harboring the LexA-Bax, LexA-Bax and Bcl-2, LexA-Bax and GST/GPX, and GST/GPX constructs were induced in gal-raff/CM-his, gal-raff/CM-his, trp, gal-raff/CM-his, trp, and gal-raff/CM-trp medium respectively for 7 h. Cells growing in medium with or without 1 mM H$_2$O$_2$ were used as controls. A, the cells were stained with the oxidation-sensitive probe DCFH-DA at 30°C for 30 min and subjected to flow cytometry (left). Duplicate cells were stained with DiOC$_6$(3) which localizes to the mitochondria as a consequence of $\Delta \Psi_m$ (right).

Fig. 7. Cell cycle distribution of cells expressing Bax, Bcl-2, BI-GST/GPX, and Bax with Bcl-2 or Bax with BI-GST/GPX. Cells were fixed and stained with propidium iodide to determine the nuclear DNA content. The two peaks shown in the control cells represent cells accumulated in the G1 phase (left) and cells accumulated in the G2 phase (right).
Fig. 8. Sequence alignment of the plant GST homologue with related proteins from mouse, yeast and *Lucilia*. The peptide sequence of the novel GST/GPX was compared with the sequences of related proteins from mouse, yeast and *Lucilia*. Black rectangles indicate residues of identity between the four sequences while gray boxes show amino acids that are similar in at least two of the sequences.
REFERENCES


Kampranis et al., Figure 2
Propidium Iodide Staining

Bax

Counts

0 200 400 600 800 1000

PI/FL2

Bax + Bcl-2

Counts

0 200 400 600 800 1000

PI/FL2

BI-GST/GPX

Counts

0 200 400 600 800 1000

PI/FL2

Bax + BI-GST/GPX

Counts

0 20 40 60 80 100

0 200 400 600 800 1000

PI/FL2

Control cells

Counts

0 20 40 60 80 100

0 200 400 600 800 1000

PI/FL2

Kampranis et al., Figure 7
A novel plant glutathione S-transferase/peroxidase suppresses Bax lethality in yeast
Sotirios C. Kampranis, Radostina Damianova, Mirna Atallah, Garabet Toby, Greta Kondi, Philip N. Tsichlis and Antonios M. Makris

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