ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 08, Issue, 03, pp.19420-19428, March, 2018

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

USING THE YEAST TWO-HYBRID ASSAY TO IDENTIFY LeGSTO1, A HIGHLY HOMOLOGOUS PROTEIN, TO THE MAMMALIAN GST-OMEGA FROM A TOMATO cDNA LIBRARY

***Khaled I. Sabarna**

Palestine Ahliya University, P.O. Box: 1041, Daher, Mountain, Bethlehem, West Bank, Palestine

ARTICLE INFO

Article History:

Received 10th December, 2017
Received in revised form
26th January, 2018
Accepted 23rd February, 2018
Published online 30th March, 2018

Key Words:

Yeast two-hybrid screen, *Saccharomyces Cerevisiae yeast,* peroxiredoxin protein, Protein-protein interaction, cDNA library, Oxidative Stress, mammalian GST-Omega, Plant LeTPx1, plant LeGSTO1, apoptosis.

ABSTRACT

The present study is focused on the identification of plant proteins, which interact with the newly isolated type A peroxiredoxin protein LeTPx1, using the two-hybrid assay. The leTPx1 cDNA was initially identified as an interacting protein to the glutathione S-transferase/peroxidase BI-GST/GPx (Kampranis, Damianova *et al.,* 2000). The two-hybrid screen is a powerful tool for the characterization of protein-protein interaction using yeast as a model system.To this end, we generated a LexA-LeTPx1 "bait" fusion and screened a tomato library cloned into the activation domain vector pJG4-5. A total of ninety-six interacting cDNAs were isolated, from which 26 were selected for sequencing and further characterization. The majority of the iterators have been previously implicated in stress responses. Due to the interaction observed between LeTPx1 and LeGSTO1 a highly homologous protein to the mammalian GST-Omega, in the yeast two-hybrid system and the potential physiological importance of such an interaction, we proceeded to analyze the biochemical properties of these proteins in vitro. LeGSTO1 was found to posse dehydro-ascorbatereductase activity like its human counterpart. Interestingly, LeGSTO1 was found to be able to reduce an inter-subunit disulfide present in the oxidized form of LeTPx1.The plant GSTO1 homologue was also tested for its ability to inhibit the Bax lethal phenotype in yeast. The plant homologue in contrast with its mammalian counterpart did not suppress the Bax phenotype. In parallel experiments we proceeded to characterize the antioxidant activity of the LeTPx1, the interacting glutathione S-transferases BI-GST/GPx, LeGST-T1, T2, T3, T4, T5 and the mammalian inhibitor of apoptosis Bcl-2.

Citation: Khaled I. Sabarna, 2018. "Using the Yeast Two-Hybrid Assay to Identify LeGSTO1, a Highly Homologous Protein, to the Mammalian GST-Omegafrom a Tomato cDNA Library", *International Journal of Development Research*, 8, (03), 19420-19428.

INTRODUCTION

Environmental stress and stress tolerance

Stress is usually defined as an external factor that exerts a disadvantageous influence on the plant. In most cases, stress is measured in relation to plant survival, crop yield, growth (biomass accumulation), or the primary assimilation processes (CO2 and mineral uptake). Stress is defined solely in terms of plant responses, which is the plant's fitness to cope with an unfavorable environment (Taiz, Zeiger *et al*., 2015).Under both natural and agricultural conditions, plants are frequently exposed to stress. Some environmental factors (air, temperature) can become stressful in just a few minutes, others may take days and weeks (soil water) or even months (some mineral nutrients) to become stressful. Stress plays a major role in determining how soil and climate limit the distribution of plant species (Taiz, Zeiger *et al*., 2015). Thus, understanding the physiological processes that underlie adaptation and acclimation, as well as the mechanism of stress injuries due to the environmental stresses (water deficit and drought, chilling and freezing, thermal and heat shock, salinity, heavy metal, ultra-violet (UV) light, and oxidative stress, the main topic of this study) is very crucial to agriculture and ecology.

Corresponding author: **Khaled I. Sabarna,**
Palestine Ahliya University, P.O. Box: 1041, Daher, Mountain, Bethl ehem, West Bank, Palestine.

Environmental oxidative stress and oxidative damage

In recent years it has become apparent that environmental stresses exert at least part of their effects by causing oxidative damage (McGarigal, Cushman *et al*., 2013). Consequently, the

antioxidant defense system of plants has been attracting considerable interest. Characterization of mutants and transgenic plants with altered expression of antioxidant enzymes is a potentially powerful approach to understanding the function of the antioxidant system and its role in plants against stress. Ultimately, the functional determinations of all genes that participate in stress adaptation or tolerance reaction are expected to provide an integrated understanding of the biochemical and physiological basis of stress responses in plants. Understanding specific protein-protein interactions will require the construction of protein–linkage maps using yeast two-hybrid technologies. Approaches with proteomics will be necessary to clarify the structural prediction of genome sequence information and to assess the protein modification and protein-ligands interaction that are relevant to stress tolerant phenotypes. A different and highly visible form of oxidative damage that has contributed to worldwide losses of forests and crops is ozone pollution. It binds to plasma membranes and it alters metabolism. As a result, stomatal apertures are poorly regulated, chloroplast thylakoid are damaged, rubisco is degraded, and photosynthesis is inhibited. Ozone reacts with O_2 and produces reactive oxygen species (ROS), including hydrogen peroxide H_2O_2, superoxide O_2-, singlet oxygen (1O2), and the hydroxyl radical (OH). These ROS, denature proteins, damage nucleic acids and thereby give rise to mutations.

To the counter these effects, a complex system of scavenging activated oxygen exists in plant cells with complementary and interdependent strategies. Scavengers detoxify activated oxygen directly or serve to recycle other protective components back to their reduced state(Bewley,1997). The enzymes that catalyze the synthesis, degradation and recycling of these antioxidants are essential to viability. Consequently they are highly conserved among plants, and exist in multiple forms in different subcellular compartments and different tissues to allow precise regulation. The most well-known antioxidant defenses include several small molecules such as Vitamin E, and C, carotenoids and thiols, and several enzymes including superoxide dismutase (SOD), catalase and the glutathione system [glutathione peroxidase (GPx), glutathione reductases and glutathione, and recently Peroxiredoxins. It is important to mention that, the concept of effect-cause between the environmental oxidative stress and living organisms and particularly plants emerged with the discovery of the superoxide dismutase in 1938 by Mann and Keilis. This enzyme converts superoxide radicals to hydrogen peroxide, which then converted to water by peroxides: O2 +. O2 +2H+ → H2O2 + O2. In anoxia-tolerant rhizomes of Iris Pseudacorus, SOD activity increases 13-fold during 28 days of anoxia(Taiz, Zeiger *et al.,* 2015). Since SOD is presented in all aerobic organisms and most (if not all) subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in defense against environmental oxidative stress (Bowler, Montagu *et al.,* 1992).

Mitochondria as a site of activated oxygen production

There are many sites for production of activated oxygen in eukaryotic cells such as the chloroplast, endoplasmic reticulum, plasma membranes, and micro-bodies and mitochondria(Winston, Harvey *et al*., 1983). Most of the oxygen is consumed by the cytochrome oxidase enzyme in the mitochondrial electron transport system, and involves the sequential transfer of four electrons to oxygen. Nevertheless, plant mitochondria performed an additional oxygen reduction at the alternative oxidase, distinguished from cytochrome oxidase by its resistance to cyanide, but neither of these sites produce significant quantities of (Neiman, Barcos *et al.,* 1981). However isolated mitochondria produce H_2O_2 and O_{-2} in the presence of (Loschen, Azzi *et al.,* 1974). The various Fe-S proteins and NADH dehydrogenases have also been implicated as possible sites of superoxide and hydrogen peroxide formation (Turrens, Freeman *et al.,* 1982) as shown in Figure 1. It is well established that oxygen-utilizing cells have evolved defense mechanisms to protect to protect against the damage caused by oxidative stress called the oxidative stress response (ORS. Despite the importance of (ORS) in maintaining homeostasis, little is known about how this response is regulated. One family of the recently characterized proteins with antioxidant activity is the Peroxiredoxins. This group will be mostly the focus of the current work presented in this study.

Peroxiredoxins

Peroxiredoxin (Prx) is a family of distinct antioxidant enzymes, with diverse biological activities. The first peroxidoxin to be reported, thioredoxin peroxidase (TPx), was detected in yeast Saccharomyces cerevisiae, (TSA1) where its expression is induced specifically by exposure to oxidative stress. (Jamieson, 1992). Peroxiredoxins do not possess activities associated with the well-known antioxidant enzymes They have no amino acid sequence similarities with other enzymatic antioxidants like catalases, peroxidases superoxide dismutases(Turrens, Freeman *et al.,* 1982). They do not contain tightly bound metal and their activity is due to their ability to decompose alkyl hydroperoxides and H2O2. (Turrens, Freeman *et al.,* 1982). Thus, it prevents formation of dangerous hydroxyl radical. A highly conserved redox- active cysteine (Cys47) is involved in catalytic mechanism of these enzymes.

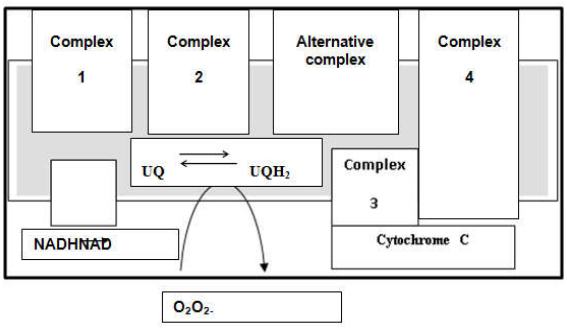

Figure 1. Schematic representation of the electron transport system in the mitochondrial membrane showing a possible site of superoxide production by reduced ubiquinone

The (Prx) family can be divided into two main groups, one with proteins containing two conserved cysteine residues (Cys47 and Cys170) and another with only one conserved Cys47 only (Turrens, Freeman *et al.,* 1982). Furthermore, the 1-cys and the 2-cys groups are differ in the conserved region surrounding Cys47 The similarity within the two groups of peroxidoxins extends over their entire sequence lengths and ranges between 20% and 99% (Turrens, Freeman *et al.,* 1982). The fact that these are so well conserved and their protein products are so abundant suggests a fundamental and essential oxidative homeostatic function for peroxidoxins in living organisms, and a gene duplication event, leading to these two forms of Prx, which took place prior to the evolution of eukaryotes (Perez, Neto *et al.,* 1998).

Peroxiredoxin's sub-cellular Localization

The Sub-cellular localization has been studied for few Peroxiredoxins. Among the 2-Cys proteins, the Thiol Specific Antioxidant (TSA) is found in the cytoplasm (Chase, Soltis *et al.,* 1993) However, a bovine Prx was found to have an N-terminal extension localizing it to mitochondria (Chase, Soltis *et al.,* 1993)while the barley 2-Cys Prx, is localized to the chloroplast. (Baier and Dietz 1997). As for the 2-Cys proteins, the sub cellular localization of 1-Cys Prxs does not seem to be identical in all organisms. Investigations of 1-Cys human Prxs indicate a cytosolic localization(Kang, Nakamura *et al.,* 1998). The yeast protein has an N-terminal extension of about 45 residues compared to other 1-Cys Prx, and preliminary evidence indicates mitochondrial localization (North, Gu *et al.,* 1999).

Peroxiredoxin Types and Biochemical Properties

Peroxiredoxins catalyze the reduction of either hydrogen peroxide or various alkyl hydroperoxides to water and the corresponding alcohol in the presence of a hydrogen donor (Thioredoxin), which in turn is converted to oxidized form. Peroxiredoxins associated with catalases or other peroxidases are believed to participate in signal transduction by regulating the intracellular concentration of H2O2, which in turn controls gene transcription and cell signaling through phosphorylation cascades(Pan, Li *et al.,* 1997). Unlike all the peroxidases, Prx isomers can be differentiated by the position of the Cys(Mukherjee, Soe *et al.,* 1999) and by lacking prosthetic groups containing a metal ion, normally needed for the catalytic reaction to occur. Peroxiredoxins overcome this problem by using the conserved N terminal catalytic acid and regenerated via a proton donor. Site-directed mutagenesis has clearly identified this Cys residue (Cys47) in yeast Saccharomyces cerevisiae(Chae, Chung *et al.,* 1994). A new classification of peroxiredoxin family was proposed by (Elias, Blot *et al.,* 2001)suggesting that, based on amino acid comparisons, Prx can be divided into three main groups: the so-called 2-Cys Prx (referred to as type A), the 1-Cys (type B), and a new isotype that will call type C.

MATERIALS AND METHODS

Materials

DNA (Stratagene)

- DNA, strains, plasmids, primers,
- DNA, strains, plasmids, primers, Material, Media, and Solutions
- A cDNA library from lycopersiconesculenum cloned into the pJG4-5 vector was kindly donated by Dr. G. Martin

Bacterial Strains

Escherihia coli strains

- XL1-blue (STRATAGENE) chemically competent cells were prepared according to the protocol describe by Sambrook *et al* Saccharomyces cerevisiae strains:
- EGY48 Matα ura3 trp1 his3 3LexA operator. This strain is a derivative of strain U457 in which homologous recombination was used to replace the sequences upstream of the chromosomal LEU2 gene operators for LexA derived from the colE1 gene.

Yeast strain *Saccharomyces cerevisiae yeast*

Plasmids

- pGilda, HIS3, AmpR. Plasmid expressing LexA fusion proteins under the control of the GAL1 inducible promoter. The bait protein is fused to the carboxy terminal domain of LexA.
- pJG4-5, TRP1, AmpR. Plasmid for making nuclear localization sequence-activation domain-hemaglutinin epitope tag fusion to a specific protein or a cDNAlibrary. Expression is driven from the galactose-inducible promoter. The cDNA tomato library was subcloned into this plasmid.
- pEG202, HIS3, AmpR. Plasmid expressing LexA fusion proteins under the control of the GAL1 inducible promoter. The bait protein is fused to the carboxyl terminal domain of LexA.
- PYES plasmid.

Sequencing Primers

- pJG4-5 forward: 5′-CTG AGT GGA GCC TCC-3′
- pJG4-5-reverse: 5′-CTG GCA AGG TAG ACA AGC CG-3′

Media and solutions

Routine Bacterial and yeast media and solutions according to the standard procedures and protocols of cloning and Biotechnology

Methods

The following protocols were implemented according to the standard procedures and protocols of cloning and Biotechnology

- The yeast system and Transformation for the identification of proteins Interaction
- Growing and preparation of the yeast cells for Transformation
- Lithium acetate Transformation
- Transforming EGY48, pEG202/TPx with cDNA Tomato Library
- Yeast Two-Hybrid Screen
- Collection of Primary Transformants
- Library screening
- Preparation of master plate
- Preparation of replica plates
- Isolating of Library Plasmid from Yeast cells
- Introduction of the Isolated Plasmid DNA into E.Coli
- DNA Transformation using frozen competent cells
- Identification of insert size (Digestion)
- Agarose gel electrophoresis
- Induction of yeast protein for Western Blots (Results are not shown)
- Freezing of transformation competent cells
- QIAprep Spin Miniprep Kit Protocol

- Sequencing of Library Plasmids
- Growth Curve
- Measurement of Sensitivity to Prooxidants
- Determination of Resistance to Pro Oxidants of Yeast Cells

REAULTS AND DISCUSSION

Screening for plant proteins that interact with the LeTPx1 protein in yeast

In the present study, we have initially attempted to identify plant proteins that interact with the LeTPx1 peroxiredoxin clone. The LeTPx1 cDNA was initially identified as an interacting protein to the recently published antioxidant glutathione S-transferase/peroxidase protein BI-GST/GPx. Thus, the possibility of the existence of cross-talking antioxidant complexes prompted us to try to further identifies the interacting partners of the LeTPx1 protein. The two-hybrid in yeast is a powerful genetic screening strategy, utilizing yeast, which is used an in vivo assay for detecting protein-protein interaction (Fields and Song 1989). The term "two-hybrid" derives from the two classes of chimeric proteins used in a screen. The first referred to, as "Bait" is a fusion of the protein of interest, in this case the LeTPx1 a DNA-binding domain (LexA). The second referred to, as "Prey" is a fusion of cDNA library to a transcriptional activation domain (B42 "acid blob").Both proteins are expressed from multi-copy (2µ) plasmids; the LexA-LeTPx1 fusion is expressed from the pEG202 plasmid containing the HIS3 marker, under the control of the constitutive yeast ADH1 promoter. The prey library plasmid, pJG4-5, expresses protein fused to the B42 activation domain, the SV40 nuclear localization signal, and an epitope tag derived from hemaglutimin, all driven by the yeast GAL1 promoter which is active only in yeast grown on galactose (Golemis, 1997).

The two plasmids are introduced in a yeast reporter strain that carries upstream LexA binding sites (operators) of the LEU2 gene integrated into the genome. Physical interaction between the "Bait" proteins with a B42AD-library clone brings into proximity the DNA binding domain (LexA) to the B42AD transcriptional activation domain turning on transcription of the LEU2 genes. Cells plated on media that lack leucine upon interaction are able to grow. The generation of a construct expressing the LeTPx1 protein as a fusion to the bacterial DNA binding domain LexA is the commencement of the two-hybrid screen. The full length LeTPxcDNA that was previously identified in the BI-GST/GPx two-hybrid screen was excised from the pJG4-5 library plasmid using EcoRI and XhoI restriction enzymes and ligated to the pEG202 vector, which will fuse to the LeTPx1 to the C- terminals of the LexA. We subsequently proceed to determine whether cells would express the right size fusion protein. To this aim fresh cells harboring the construct were broken using glass beads. Samples from the extracted protein (40µgs) were resolved on SDS-PAGE and transferred on PVDF membrane by western blot. The presence of the fusion protein was detected using antibodies that recognize the LexA part of the fusion (see Figure 2). As seem on figure 1 the protein size is near to 35kDa, which is close to the calculated size of the fusion protein (22kDa LexA + 17kDa LeTPx = 39kDa).The cells were tested for bait "self-activation" by plating them on Glu/ CM-trip, leu medium.

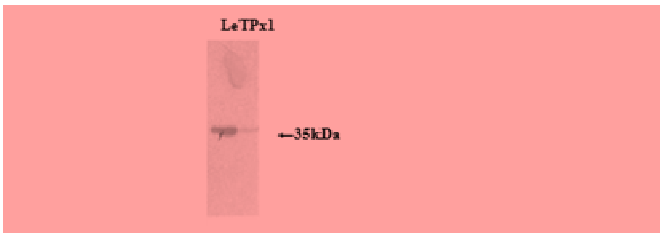

Figure 2. Lex A-Le TPx1 fusion protein is expressed in the expected size

The absence of growth (data not shown) indicated that LexA – LeTPx1 did not non-specifically activate transcription of the LEU2 gene. A tomato library cloned into the yeast two-hybrid vector pJG4-5 (Zhou *et al*., 1995) was introduced into the EGY48 cells harboring the pEG202/LeTPx construct. Approximately 5x105 independent transformed colonies, grown in glucose containing medium, were collected. To identify plant library fusion proteins that bind to LeTPx, the cells were switched for 1 hour into galactose containing medium to induce expression and they were subsequently plated on gal-raff /CM-his, trip, leu. Individual colonies growing on the selective plates were picked and transferred on a glu/ CM-his, trip master plate (See Figure 3).

Figure 3. Master plate of yeast cells carrying the LexA-LeTPx1 and the pJG4-5 tomato library on glu/CM-his, trp plates

The initial specificity test involves replanting from the master plate on glu/CM-his, trp, leu and gal-raff/CM-his, trpleu plates (see Figure 3).Since the library proteins are expressed under the control of GAL1 promoter, growth due to the physical interaction should be manifested only on galactose media.The vast majority of isolated exhibited specific growth on galactose but were unable to grow in glucose-based media indicating specific interactions (See Figure 4).

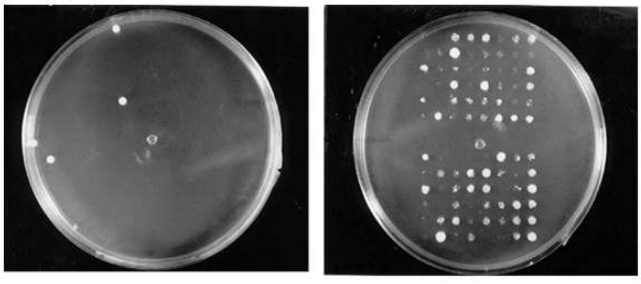

Figure (4)

LexA /TPx + pJG4-5 Tomato Library on Glu/CM-his, trp, leu Plates

LexA/TPx + pJG4-5 Tomato Library on Gal-Raff/CM-trp, his, leu Plates

Selected colonies were also streaked on plates to assess the extent of growth differences between the two media (see Figure 5)

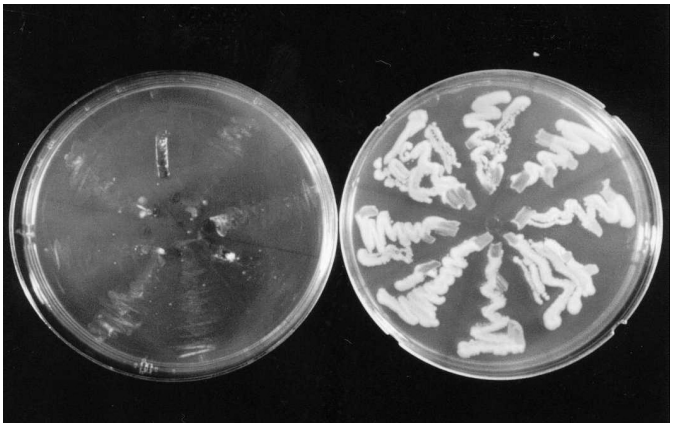

Figure 5. LexA/TPx1 + pJG4-5 Tomato Library on Gal-Raff/CM-trp, his, leu Plates (Positive growth), and Glu/CM-his, trp, leu plates (Negative growth)

Plasmid Extraction from Yeast and Bacterial Transformation

Positive colonies that passed the initial specificity test were grown in liquid cultures in glu/CM-trp, which selects only for the presence of the library plasmid. Plasmid DNA was isolated and digested with EcoRI and XhoI restriction enzymes, which cleave at the beginning and the end of the cDNA insert respectively. The digested products were run on an agarose gel to assess the size of the inserts (see Figure 6). Plasmid DNA was extracted and transformed into chemically competent XL1-blue Bacterial cells.

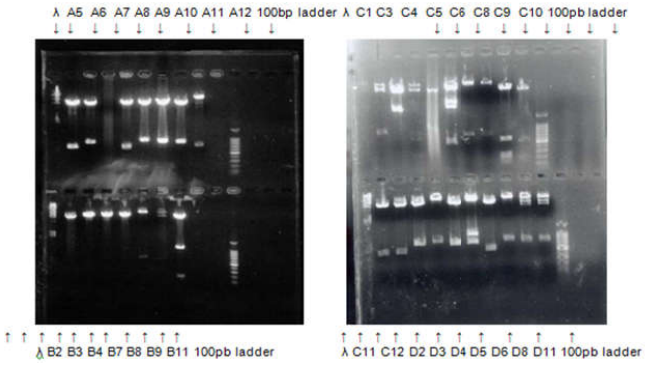

Figure 6. Plasmid DNAof LeTPx1 positive inter-actors

The estimated were mostly variable which indicated the presence of multiple interacting proteins.

General Scheme of Plating for Transformed Yeast Cells

8 large plates of (~ 1million cfus) EGY48cells carrying pEG202/LeTPx1 +pJG4 /Tomato Library on Glu/CM-his, trp 500 ng of a set of selected library plasmids were mixed with 3.2 pM of sequencing primers and were submitted to the Thomas Jefferson University Sequencing Facility by Dr. A. Makris, courtesy of Prof. Philip N. Tsichlis. The sequenced information from the end sequencing was compared to Gene Bank, and the EST database at TIGR (www.tigr.org). From the set of 26 sequenced (see Table 1) isolates the following grouping of proteins with related functions can be inferred A) Antioxidant proteins. Those are 1) LeTPx1 with two independent isolates 2) LeTPx2, a full length highly homologous Peroxiredoxin with a unique N-terminal extension 3) LeGST-T2 4) a partial cDNA that is homologous to the type III Glutathione S-transferase from Glycine max, 5) novel type III (Tau) glutathione S-transferase which shares significant homology in the conserved catalytic residues to the

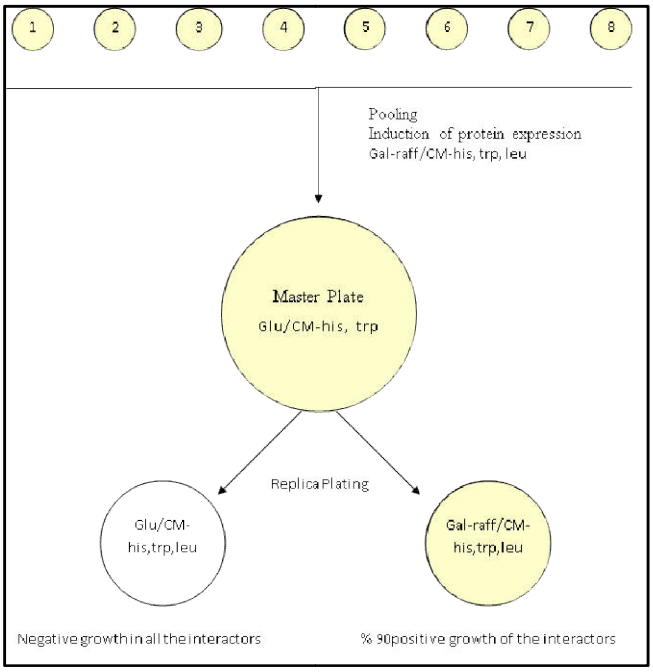

Figure 7. General Scheme of Plating for Transformed Yeast Cells

mammalian GSTO1 of the omega class. 6) Cytoplasmic Superoxide dismutase (SOD2). 7) Ascorbate Peroxidase. 8) A protein homologous to yeast Glyoxalase. 9) Heat shock protein 70. This group represents the majority of protein interactors to the LeTPx1 protein (11/26). The fact that LeTPx1 a known homodimer can pull out from the two-hybrid screen its own self, highly homologous LeTPx2 and two type III (Tau) Glutathione S-transferases is a good indicator of the screen specificity.It should be noted that LeTPx1 was initially isolated as an inteactor to BI-GST/GPx, a type III (Tau) Glutathione S-transferase(Kampranis, Damianova *et al.,* 2000)together with 5 novel typeIII (Tau) GSTs (LeGST-T1, T2, T3, T4, T5). It was subsequently tested for interaction with all the isolated LeGST and was shown to be capable of interaction with all LeGST Proteins in the two-hybrid system.

Figure 8. LexA-TPx1 interacts with B42AD fusions of BI-GST/GPx (1), LeGST-T1 (2), LeGST-T2 (3), LeGST-T3 (4), LeGST-T4 (5), and LeGST-T5 (6)

Table 1. The sequencedLeTPx1Interactors

I D	LeTPx1 Interactor	Gene	Accession number
1	A10	SOD	Q43779
2	A9	LeGST-T2	AY007559
3	B11	HSP70 homologue	P27322, S14950
4	B2	Homology to protein from Deinococcusradiodurans	D75542
5	B4	GST(Omega) homologue Mammalian GST-omega homologue	U80819
6	B7	GST(Omega) homologue Mammalian GST-omega homologue	U80819
7	B9	Unknown protein, homology to glyoxalase from fusion yeast	NP_180769.1
8	C1	LeTPx1, Peroxiredoxin	
9	C10	Putative Calcium binding protein	NP_198235.1,T0717
10	C11	Hypothetical protein	NP_189390.1
11	C12	Hypothetical protein	NP_189390.1
12	C6	Calmodulin homologue	X65016
13	D2	Hypothetical protein	NP_1890913.1
14	D6	Chlorophyll a/b binding protein type III	S04125
15	D7	Homologues to 60S acidic ribosomal protein P2	X78213
16	E10	Unknown protein 019904	
17	G11	GTX1 homologue, partial	P32111
18	G12	Import in alpha subunit, tomato	O22478
19	G2	Hypothetical	TC1876
20	G3	Unknown protein	ACO10657_3
21	G4	Importin, alpha	AF369706
22	G5	Hypothetical, same as TpxintG2	TC1876 EST
23	G6	Adenine phosphoribosyltransferase	L19637
28	H10	Ascorbate peroxidase	Y16773
24	H4	Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH)	S38570
25	H5	Homologous to glucosyltransferase	X85138
26	H6	Probable glycosyltransferase	T07404 EST

Work subsequently performed by Kilili *et al* (manuscript in preparation), which proceeded to characterize the dimerization domain of BI-GST/GPx, showed that LeTPx1 specifically binds to the conserved N-terminal region of BI-GST (a a 30-107) most probably as a hetrodimer. The dimerization domain is conserved among all types III (tau) plant GSTs. The nature of the interaction between the type III (Tau) GSTs and the peroxiredoxin is currently the subject of the investigation in the laboratory of Natural Products and Biotechnology. To mention briefly what is currently known about the function of the interacting GSTs, the first gene characterized BI-GST/GPx was found to possess glutathione S-transferase activity (GST) and weak glutathione peroxidase activity. Expression of the mammalian apoptotic protein Bax conferred a lethal phenotype in yeast cells. Expression of BI-GST/GPx inhibited Bax lethality. Some of the manifestations of the Bax phenotype in yeast involve dramatic decreases in the levels of intracellular glutathione, a substantial reduction of the total cellular phospholipids, collapse of the mitochondrial membrane potential and alterations in the intracellular redox potential. Co-expression of the BI-GST/GPx protein brought the total glutathione levels back to normal and reestablished the mitochondrial membrane potential but had no effect on the phospholipid alterations. Moreover, the BI-GST/GPx protein was found to enhance resistance to H2O2 induced stress (Kampranis, Damianova *et al.,* 2000). Subsequent work on the characterization of the 5 novel type III (Tau) GSTs isolated, part of which is also presented in the current thesis showed that coexpression of the LeGSTs with the proapoptoticBax protein partially restored cell viability and growth by effecting the mitochondrial functions. When examined for their antioxidant capacity against H2O2, t-BOOH and CHP, the proteins exerted a differential protective effect in yeast cells, unique for each protein. Additionally each LeGST protein exhibited selectivity in protein interactions against the 10 stress related BI-GST/GPx interacting proteins(Kilili, Atanassova *et al.,* 2004).

TPx intB4 and TPx intB7 homologues of Mammalian GSTO1

BI-GST/GPx and all the isolated LeGSTs belong to the larger ancient theta (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 theta class is representative of a progenitor GST involved in protecting cellular component from oxidative damage (Pemble and Taylor, 1992). The mouse theta class GST p28, which shares a 28% identity with BI-GST/GPx and the LeGSTs, has recently been implicated in the development of resistance to radiation-induced apoptosis in lymphoma cells (Kodym, Calkins *et al.,* 1999) Subsequently to the publication of the p28 mouse protein, the human homologue GST-omega (GSTO1) was identified, biochemically characterized and its crystal structure elucidated (Board, 2000). GSTO1 exhibits higher similarity to the type III (Tau) class of plant GSTs, than to other human proteins. 1A unique library clone representing a new plant GST named TPXintB4, sharing even greater homology to GSTO1 than all other proteins, was identified in the present work as an interactor to another antioxidant protein, a type II peroxiredoxin (LeTPx1). In comparison to all other theta class (Type III) GSTs, the plant GST Omega protein shares in common a unique N-terminal extension and a cysteine residue at the active center, rather than the serine found in all the rest of the homologous plant GSTs (Whitbread, Masoumi *et al.,* 2005). Searching the database of tomato EST clones for proteins similar to the plat GST-Omega, revealed three partial clones that code for GST Omega-like proteins. The two of these three partial clones possess a Cys residue in their active-site (the sequence around the active-site of the third clone is not available), which confidently classifies them in the Omega class. A pile-up of the two human GST-Omega enzymes and the four Omega-like tomato proteins is shown in Figure 9. Based on the crystal structure of the human counterpart (Board *et al.,* 2000), it can be seen that most of the residues involved

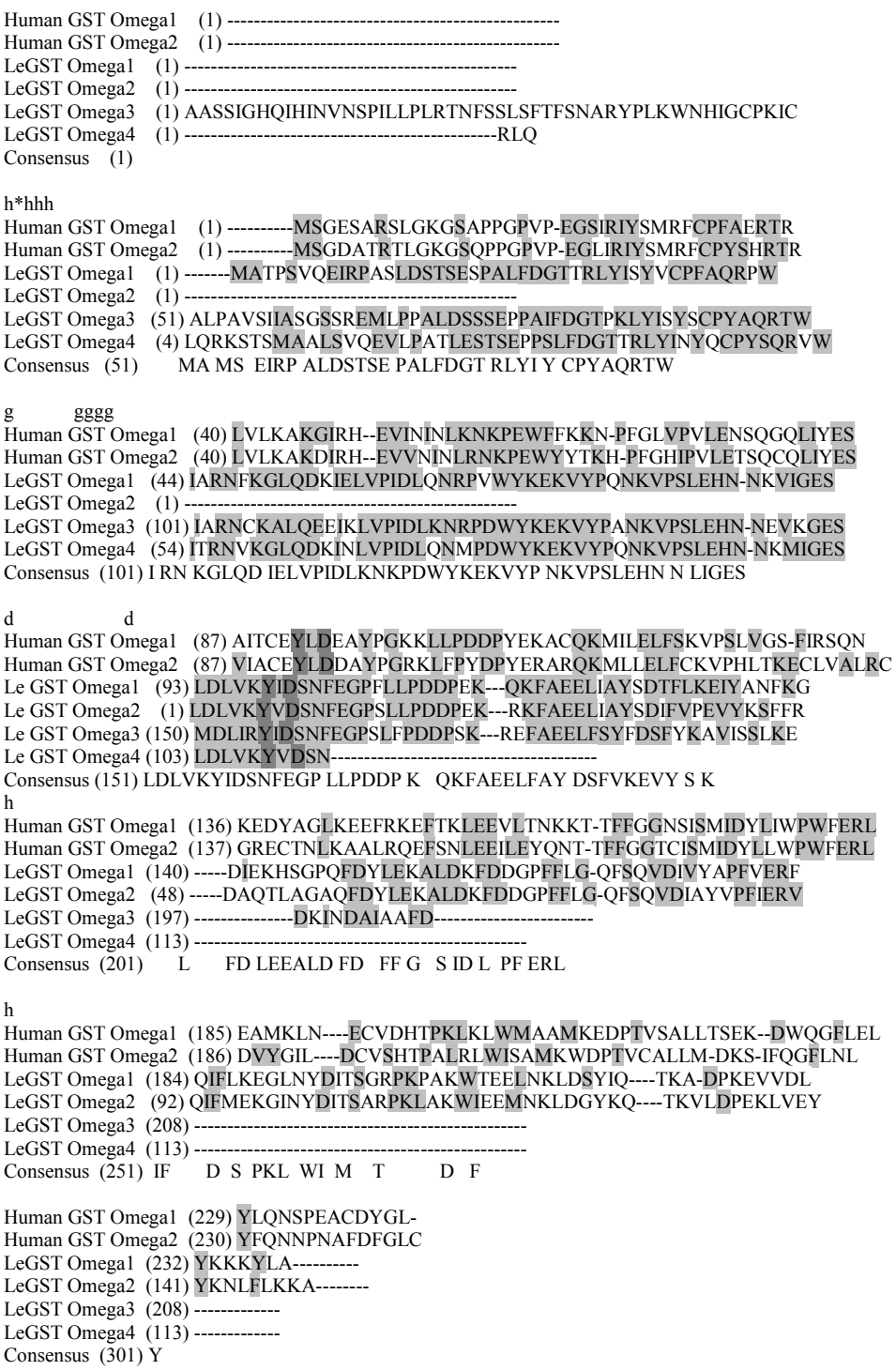

Figure 9. Sequence alignment: Sequence alignment of human GSTO1 with its plant homologue and three other plant omega-like GSTs. The peptide sequence of human GST-Omega was compared to the sequence of the recently isolated closest plant homologous GST (TPXintB4 or LeGST-Omega1) and three partial clones identified from a plant EST database. The active site Cys is indicated by (*), (g) denotes residues that participate in glutathione binding, (h) denotes residues that line the substrate binding pocket, and (d) amino acids that participate in the interaction with the other monomer. Interestingly, GST-omega has been implicated in ion channel modulation, a novel and unrecognized role for a GST

in glutathione binding and substrate binding are conserved or conservatively substituted in this group of proteins. It is of interest to note that the relatively polar nature of the H-pocket is conserved in the tomato proteins, which in conjunction with the evidence that tomato GSTO1 interacts with LeTPxII-1, supports the notion that the binding partner for the GSTs of the Omega class could be a large and not entirely hydrophobic molecule (Whitbread, Masoumi *et al.,* 2005). Two residues that may play a significant role in determining the nature of the interacting partner for these proteins are those corresponding to Phe-34 and Ala-35 of the human GSTO1. These residues, which line the H-site, are conserved between the human enzyme and tomato GST-Omega1. However, in human GSTO2 and the other tomato enzymes there is a marked substitution to the polar Tyr and Ser amino acids, respectively, which is expected to make the H-site even more hydrophilic. Substitutions in this region may be responsible for determining the nature of the interacting ligand. Based on this observation, it seems that tomato GSTO1 is functionally closer to human GSTO1, while tomato GSTO 2, 3, and 4 are more likely to

have a substrate specificity more similar to human GSTO2. Tomato GST-Omega3 is characterized by a longer (by 57 aa) N-terminal extension whose role is not yet clear (see Figure9). Work presented by Dulhunty *et al* indicates that GSTO1-1 is able to either inhibit or potentate RyR calcium channels, which provide an additional mechanism by which Redox State might be conveyed to the channel. Cytoplasmic (Ca+2) increases during oxidative stress, partly through oxidative activation of RyR(Dulhunty, Gage *et al.,* 2001).

Conclusion

The aim of our study was to identify plant proteins that interact with LeTPx1 peroxiredoxin clone, in the effort to understand the intracellular functions of these proteins. By performing functional screen for LeTPx1 interacting protein. 96 interacting cDNAs were isolated, from which 26 of them were sequenced. From the set of 26 sequenced isolates the following grouping of proteins with related functions could be inferred A) Antioxidant proteins. Those are 1) LeTPx1 with two independent isolates 2) LeTPx2, a full length highly homologous Peroxiredoxin with a unique N-terminal extension which likely codes for a chloroplastic sequence signal. 3) LeGST-T2 4) a partial cDNA that is homologous to the type III Glutathione S-transferase from Glycine max, GTX1 5) two novel type III (Tau) glutathione S-transferases which shares significant homology in the conserved catalytic residues to the mammalian GSTO1 of the omega class. 6) Cytoplasmic Superoxide dismutase (SOD2). 7) Ascorbate Peroxidase. 8) A protein homologous to yeast Glyoxalase. 9) Heat shock protein 70. B) Two glucosytransferase homologous proteins [H5, H6]. C) Two chloroplastic proteins [D6, H4]. D) Four hypothetical proteins [C11, C12, D2, G2, G5]. E) Three of the isolated interactors did not show any identity with any known protein.
These groups represent the majority of protein interactors to the LeTPx1 protein. We concentrated our efforts in the characterization of the newly isolated and characterized LeTPx1 Peroxiredoxin, and GST-omega homologue Mammalian GST-omega homologue. The fact that LeTPx1 a known homodimer can pull out from the two-hybrid screen its own self, the highly homologous to LeTPx2 and two type III (Tau) Glutathione S-transferases is a good indicator of the screen specificity. It should be noted that, unlike other GSTs, omega class appears to have an active site cysteine that can form a disulfide bond with glutathione.

With the aim of testing the ability of LeTPx1 and GST-omega to inhibit Bax lethality, characterization was made. Expression of the plant homologous to GSTO1 protein does not inhabit the lethality phenotype caused by Bax, in contrast with the human GST-omega that is an inhibitor of the Bax phenotype. It is well established that Bax protein is thought to decrease the intracellular levels of total glutathione, diminish the mitochondrial potential and alter organelle function by localizing to the outer mitochondrial membrane and forming an ion channel. In order to evaluate the protective effect conferred by the newly isolated LeTPx1, the Le GSTs proteins and Bcl-2 to cells under oxidative stress, the ability of cells to survive was assessed by establishment of in vivo survival assays. Hydrogen peroxide, tert-butyl hydroperoxide, and cumenehydroperoxideprooxidants provide reactive oxygen species that can react with a variety of biomolecules, altering or blocking their biological activity. The concentrations of the used prooxidants represent the minimal concentration and length of exposure that causes more than 90% death in EGY48 control cells. In these experiments, it was shown that expression of the LeGST proteins protected yeast cells from H2O2-induced oxidative stress to varying degrees ranging from complete absence of protection for LeGST-T1 and GST-T4 to very high levels from LeGST-T2 and LeGST-T3. TPxII and BI-GST show almost the same activity; meanwhile, Blc-2 appeared not to be active antioxidant. For the lipid soluble hydroperoxide, the most potent LeGST inhibitors were LeGST-T5 and BI-GST/GPx. TPxII and Bcl-2 showed significant antioxidant activity against organic hydroperoxide.

One of the interactors, the GST-Omega homologue, was expressed in E. coli and purified for further characterization. Purified Le GSTO1 showed two distinct bands of approximately 35 kDaon SDS-PAGE gels and western blots. This was probably due to protcolytic cleavage of this protein during expression in bacteria. Like its human counterpart, LeGSTO1 exhibited dehydroascorbatereductase activity. The specific activity of the tomato enzyme was 0,059 U/mg, which is similar to the activity of the human enzyme. In gel-filtration experiments, LeGSTO1 was found to exist both in the monomeric and dimeric form. This suggests that the tomato enzyme is weak dimer in solution in contrast to the human enzyme, which is found predominantly in the dimer form. Gel-filtration experiments failed to show any strong association between LeGSTO1 and LeTPx1. Thus, the interaction between these two proteins that was detected in the yeast two-hybrid assay is probably a transient one. Nevertheless, this interaction seems to be physiologically relevant, since LeGSTO1 is able to reduce the oxidized form of LeTPx1, as is manifested by the reduction of an inter subunit disulfide bond in LeTPx1 by the action of LeGSTO1.

Acknowledgement

The author would like to acknowledge several people for their helpful in the conceptualization and completion of this work. I am most grateful to Dr. AntoniosMakris (Coordinator of Natural Products and Biotechnology Department at MAICh), Dr. SotiriosKampranis, and Mr. Sami Al Heehfor Their cooperation, understanding, encouragement, and exceptional ideas during this research I am deeply indebted to them.

REFERENCES

Baier, M. and K. J. Dietz 1997. "The plant 2-Cys peroxiredoxin BAS1 is a nuclear-encoded chloroplast protein: its expressional regulation, phylogenetic origin, and implications for its specific physiological function in plants." *The plant journal.,* 12(1): 179-190.

Bewley, J. D. 1997. "Seed germination and dormancy." The plant cell 9(7): 1055.

Bowler, C. *et al*. 1992. "Superoxide dismutase and stress tolerance." *Annual review of plant biology* 43(1): 83-116.

Chae, H. Z. *et al*. 1994. "Thioredoxin-dependent peroxide reductase from yeast." *Journal of Biological Chemistry*, 269(44): 27670-27678.

Chase, M. W., *et al*., 1993. "Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene rbcL." Annals of the Missouri Botanical Garden: 528-580.

Dulhunty, A. *et al*., 2001. "The glutathione transferase structural family includes a nuclear chloride channel and a ryanodine receptor calcium release channel modulator." *Journal of Biological Chemistry,* 276(5): 3319-3323.

Elias, D. *et al*., 2001. "Curative treatment of peritoneal carcinomatosis arising from colorectal cancer by complete resection and intraperitoneal chemotherapy." *Cancer*, 92(1): 71-76.

Fields, S. and O.K. Song, 1989. "A novel genetic system to detect protein–protein interactions." *Nature.,* 340(6230): 245-246.

Kampranis, S. C. *et al*., 2000. "A novel plant glutathione S transferase/peroxidase suppresses Bax lethality in yeast." *Journal of Biological Chemistry,* 275(38): 29207-29216.

Kang, H.B. *et al*. 1998. "Epitaxial growth of ZnO films on (0001) sapphire at low temperatures by electron cyclotron resonance-assisted molecular beam epitaxy and their microstructural characterizations." *Japanese journal of applied physics,* 37(3R): 781.

Kilili, K. G. *et al*. 2004. "Differential roles of tau class glutathione S-transferases in oxidative stress*." Journal of Biological Chemistry,* 279(23): 24540-24551.

Kodym, R. *et al*. 1999. "The Cloning and Characterization of a New Stress Response Protein A MAMMALIAN Member Of A Family Of Θ Class Glutathiones-Transferase-Like Proteins." *Journal of Biological Chemistry,* 274(8): 5131-5137.

Loschen, G. *et al*. 1974. "Superoxide radicals as precursors of mitochondrial hydrogen peroxide." FEBS letters 42 (1): 68-72.

Neiman, R. S. *et al*., 1981. "Granulocytic sarcoma: a clinicopathologic study of 61 biopsied cases." *Cancer,* 48(6): 1426-1437.

Mc Garigal, K. *et al*., 2013. Multivariate statistics for wildlife and ecology research, Springer Science & Business Media.

Mukherjee, S. *et al*., 1999. "Endocytic sorting of lipid analogues differing solely in the chemistry of their hydrophobic tails." *The Journal of cell biology,* 144(6): 1271-1284.

North, T. *et al*., 1999. "Cbfa2 is required for the formation of intra-aortic hematopoietic clusters." *Development,* 126(11): 2563-2575.

Pan, X.R. *et al*., 1997. "Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance: the Da Qing IGT and Diabetes Study." *Diabetes care,* 20(4): 537-544.

Perez, E. F. *et al*., 1998. "Electrochemical sensor for hydrazine based on silica modified with nickel tetrasulfonated phthalocyanine." *Electroanalysis,* 10(2): 111-115.

Taiz, L. *et al*., 2015. Plant physiology and development, Sinauer Associates, Incorporated.

Turrens, J. F. *et al*., 1982. "The effect of hyperoxia on superoxide production by lung submitochondrial particles." *Archives of Biochemistry and Biophysics*, 217(2): 401-410.

Whitbread, A. K. *et al*., 2005. "Characterization of the omega class of glutathione transferases." Methods in enzymology401: 78-99.

Winston, G. W. *et al*., 1983. "The generation of hydroxyl and alkoxyl radicals from the interaction of ferrous bipyridyl with peroxides." *Biochemical Journal,* 216(2): 415-421.

****