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**Production of Stress Tolerant Plants by
Overproduction of Enzymatic Oxyradical Scavengers**

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Enzymatic Oxyradical Scavengers***

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Abstract

Most of the objectives that were outlined in the original proposal have been met with two exceptions. Briefly, our goals were to:

(1) construct transgenic tobacco plants which overproduce one or more of the enzymatic oxyradical scavengers and associated ancillary enzymes, including superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, and monodehydroascorbate reductase; (2) evaluate the tolerance of these transgenic plants to oxidative stress; and (3) extend these studies to an agronomically important crop such as citrus.

As can be seen in the following pages, our original objectives (1) and (2) have been achieved, although transgenic lines overexpressing phospholipid hydroperoxide glutathione peroxidase (PHGPX) were not obtained and our evidence to date suggests that constitutive overexpression of the enzyme is probably lethal. However, transgenic tobacco expressing the antisense construct for PHGPX were obtained. Tobacco plants overexpressing ascorbate peroxidase and those sense-suppressing monodehydroascorbate reductase are more tolerant to oxidative stress, as mediated by the redox-cycling agent paraquat; in contrast, plants expressing the PHGPX-antisense construct are more sensitive to paraquat. Additional research is warranted on each of the six types of transgenic lines which we generated with regard to their tolerance to saline stress. Until recently, attempts to transform citrus were not very successful, and thus additional attention is currently being directed at objective (3). We are optimistic that use of the plant transformation vector, pBIN, will lead to stable transgenic citrus, as preliminary experiments demonstrate stable expression of the GUS reporter gene.

Other important contributions resulting from this BARD project include the biochemical characterization of the first plant phospholipid glutathione peroxidase and the biochemical and molecular analysis of another key antioxidant enzyme, monodehydroascorbate reductase. Overall this BARD-supported project was quite successful, and the biological resource of numerous transgenic lines which have altered levels of antioxidant enzymes should be valuable for years to come.

PRODUCTION OF STRESS TOLERANT PLANTS BY OVERPRODUCTION OF ENZYMATIC OXYRADICAL SCAVENGERS

I. Objectives of the Original Research Proposal

- A.** *To construct transgenic tobacco plants which overproduce one or more of the enzymatic oxyradical scavengers and associated ancillary enzymes, including superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, glutathione reductase, and monodehydroascorbate reductase.*
- B.** *To evaluate the tolerance of these transgenic plants to oxidative stress.*
- C.** *To extend these studies to an agronomically important crop such as citrus.*

II. Report Narrative and Evaluation of Research Achievements

A. Characterization of citrus glutathione peroxidase

Glutathione peroxidases (GPXs, EC 1.11.1.9) are a family of key enzymes involved in scavenging oxyradicals in animals. Only recently, indications for the existence of this enzyme in plants were reported. The cDNA encoding Cit-SAP, designated as *csa*, was cloned from a cDNA expression library prepared from mRNA isolated from *Citrus sinensis* salt-treated cell suspension (Holland et al., 1993). The amino acid sequence of the protein deduced from the nucleotide sequence of *csa* revealed considerable homology with the glutathione peroxidase superfamily, in agreement with the partial direct amino acid sequence analysis of tryptic peptides isolated from the plant protein. Among the members of this family, the highest homology, as well as the best fit in hydropathy character, was with the animal phospholipid hydroperoxide glutathione peroxidase (PHGPX).

In an early attempt to assign biological function to this protein, transformed *E. coli* cells expressing Cit-SAP were examined for their ability to tolerate free radicals formed by paraquat, a superoxide radical forming agent (Holland et al., 1994). In the presence of paraquat, the survival rate of the transformed bacteria expressing Cit-SAP was much higher as compared to the wild-type bacteria. These results suggest that the functional protein product encoded by *csa* is synthesized in the *E. coli* clone and support the assumption that Cit-SAP is a plant GPX-like protein which participates in the enzymatic system aimed at scavenging oxyradicals in plants.

The final proof that Cit-SAP is indeed a plant PHGPX was obtained in recent studies in our laboratory in Israel in which we have partially purified Cit-SAP from cultured citrus cells. By employing affinity chromatography, Cit-SAP was separated from the plant glutathione-S-transferase (GST). The expected specific enzymatic activity for PHGPX, *e.g.*, its ability to catalyze specifically the reduction of phosphatidylcholine hydroperoxide by glutathione, was observed in the protein fraction containing Cit-SAP devoid of any contamination contributed by GST (Beeor-Tzahar et al., 1995). The enzyme, as expected, catalyzes the reduction of lipid hydroperoxides, as does the animal enzyme. However, the rate of the catalysis of the plant protein was lower than that of the animal

enzyme, probably due to the presence of a catalytic cysteine residue in the plant PHGPX, instead of selenocysteine as typified by animal PHGPX. Except for the citrus enzyme, no other protein product encoded by a plant PHGPX gene has thus far been isolated.

Although the best substrates for the plant PHGPX *in vitro* are phospholipid hydroperoxides, it is not yet clear whether such phospholipids, that comprise biomembranes, are the natural substrates of the enzyme. The physiological role of the plant PHGPX is a subject of our future studies.

B. Characterization of pea monodehydroascorbate reductase

Monodehydroascorbate radicals are generated in plant cells enzymatically by the hydrogen peroxide scavenging enzyme, ascorbate peroxidase, and nonenzymatically via the univalent oxidation of ascorbate by superoxide, hydroxyl, and various organic radicals. Regeneration of ascorbate is achieved by monodehydroascorbate reductase (EC 1.6.5.4) using NAD(P)H as an electron donor or, alternatively, by a set of two coupled reactions requiring dehydroascorbate reductase, glutathione reductase, glutathione, and NAD(P)H. As monodehydroascorbate reductase (MDAR) is a key enzyme in maintaining reduced pools of ascorbate, an important antioxidant, we undertook this study to learn more about its structure, function, and regulation. With support from BARD, we succeeded in the molecular cloning and characterization of a cDNA encoding MDAR of pea, *Pisum sativum* L. (Murthy and Zilinskas, 1994). The cDNA encodes a 433-amino acid polypeptide that shows, respectively, 73 and 87% identity with peptide fragments from soybean and cucumber MDAR. It contains the NAD(P)H and FAD binding domains of other flavin oxidoreductases. The cloned enzyme lacks a transit peptide, but the sequence of the carboxyl terminus is Ser-Lys-Ile, similar to the targeting motif found in peroxisomal proteins. When expressed in *Escherichia coli* fused to the maltose-binding protein, MDAR has enzymatic properties comparable with the purified soybean and cucumber enzyme. Polyclonal antibodies were raised in rabbits against MDAR purified from the pea enzyme expressed in *E. coli*. Northern blot analysis shows that the 1.6 kb MDAR transcript is expressed at relatively low levels in all plant tissues examined and that oxidative stress results in elevated MDAR transcript levels.

C. Construction of transgenic plants

1. Overview

Transgenic *Nicotiana tabacum* cv Bel W3 plants were constructed in our Rutgers University laboratory to overproduce either superoxide dismutase (SOD), which scavenges superoxide radicals; ascorbate peroxidase (APX), which removes hydrogen peroxide; or monodehydroascorbate reductase (MDAR), which reduces monodehydroascorbate radical (MDA) to ascorbate (AsA). Five types of transgenic tobacco lines were constructed: (1) APXcyt plants that overproduce pea cytosolic APX in the cytosol; (2) APXchp plants that overproduce pea cytosolic APX in the chloroplasts; (3) Mn-SODchp plants that overproduce pea mitochondrial Mn-SOD in the chloroplast; (4) Cu/Zn-SODcyt plants that overproduce pea cytosolic Cu/Zn-SOD in the cytosol; and (5) MDARcyt plants that overproduce pea cytosolic MDAR in the cytosol. In addition, transgenic plants that overproduce both pea cytosolic APX and pea cytosolic Cu/Zn-SOD in the cytosol were also constructed using two different approaches, as described in detail below. In contrast, all attempts in Israel and the U.S.

to overexpress citrus phospholipid hydroperoxide glutathione peroxidase (PHGPX) in tobacco were unsuccessful (see below), although transgenic tobacco transformed with antisense *csa* constructs were obtained.

2. Construction of plasmid vectors

The full-length cDNA encoding pea cytosolic Cu/Zn-SOD (White & Zilinskas, 1991) was cloned into the unique *Xba*I and *Sac*I sites of the plant transformation vector pBI121, downstream of the CaMV35S promoter and upstream of the *nos* termination sequence, in place of the *uidA* reporter gene encoding β -glucuronidase (GUS).

The coding sequences of pea cytosolic APX, pea cytosolic MDAR, and citrus PHGPX were obtained using the respective full-length cDNAs (Mittler and Zilinskas, 1991; Murthy and Zilinskas, 1994; Holland et al., 1993) as templates. *Nco*I and *Xba*I sites were introduced respectively by PCR at the 5' and 3' termini to facilitate subsequent cloning steps. The coding sequence for the pea mitochondrial mature Mn-SOD (eliminating the mitochondrial targeting sequence) was obtained by PCR using pea Mn-SOD cDNA (Altomare, 1992) as template. *Nco*I and *Xba*I sites were introduced respectively by PCR at the 5' and 3' termini to facilitate cloning.

For expression in the cytosol, the above-described coding sequences were cloned into the *Nco*I and *Xba*I sites of the pST7 plasmid cloning vector (gift of Dr. P. Maliga, Rutgers University), downstream from the CaMV35S promoter and upstream from the CaMV35S terminator, replacing the *uidA* (GUS) reporter gene in pST7. The *Hind*III/*Eco*RI restriction fragment containing either the APX, MDAR or PHGPX expression cassette was then cloned into the plant transformation vector pBI121, replacing the GUS reporter cassette found within pBI121.

For expression in the chloroplast stroma, the APX and mature Mn-SOD coding sequences were cloned into the *Nco*I and *Xba*I sites of pTBA3 plasmid cloning vector (gift of Dr. P. Maliga, Rutgers University) in place of the *uidA* (GUS) reporter gene. This placed the APX or mature Mn-SOD coding sequence upstream from the CaMV35S terminator and downstream from the CaMV35S promoter and the chloroplast targeting sequence and transit peptidase processing site provided by the coding sequence for the RuBisCO small subunit transit peptide and the first five amino acids of the mature RuBisCO small subunit. The *Hind*III/*Eco*RI restriction fragment containing the APX or Mn-SOD plant expression cassette was then cloned into the plant transformation vector pBI121, replacing the GUS expression cassette.

A number of additional plasmid constructs were made in Israel with the objective of expressing the citrus *csa* cDNA in tobacco (as a model plant) and in citrus as the target cultivar. For this purpose, both sense and antisense *csa* configurations under the control of the 35S promoter were constructed (pJFS, pJFATS in Fig. 1). Initially, we cloned the full-length cDNA (clone c-17) into the binary vector pPCV002 which was modified to include the 35S promoter. After several dozen transgenic tobacco plants were obtained with no obvious phenotypes, we decided to change the strategy. Experiments performed in Zilinskas' laboratory indicated that the 5' untranslated region of the transcript could have a negative effect on translation. Therefore, we decided to delete the 5' noncoding region of the cDNA and clone only the *csa* coding region and its 3' flanking region. Two

types of plasmid vectors were used for this purpose. The first type included the pPCV002 cloning vector, similarly to our original cloning vector (pARO25, pARO26, Fig.2). However, pPCV002 requires use of a particular strain of *Agrobacterium* which is very difficult to eliminate by antibiotic treatment, and thus we decided to shift to other cloning vectors. Our choice for type II vectors (pARO31, pARO32 in Fig. 3) was to use one of the pPGTV series (Becker et al., 1992). In addition to their ease of use, they provide a wide range of selectable markers. This feature is particularly useful for the following reasons: 1. Our intention was to produce plants to overproduce at least PHGPX and APX. For this purpose, it would have been very useful to use two different selectable markers for construction of transgenic plants producing these two enzymes. 2. Selection of citrus transformants with kanamycin is not very effective, and other selectable markers could be beneficial.

We used clone c-17 for PCR amplification. One of the primers (20-mer) is based on the sequence immediately preceding the ATG initiation codon of c-17. The original sequence was slightly modified to include a *Bam*HI restriction site within the primer. The second primer was simply an oligo dT. The PCR fragment was cloned as a *Bam*HI fragment between the 35S promoter and the NOS terminator in a modified pPGTV vector. In addition, we cloned the *Bam*HI restriction fragment, containing the amplified *csa* coding region between the 2X 35S promoter and the NOS terminator of the modified plasmid pSUN18 (kindly provided by Sunford). The entire expression cassette was cloned as an *Eco*RI-*Hind*III fragment in pPGTV (BAR). For this purpose pPGTV was first digested with *Eco*RI and *Hind*III to remove the GUS region. Similarly an *Eco*RI-*Hind*III expression cassette containing the coding sequence for pea cytosolic APX under the control of the 35S promoter was cloned in pPGTV (BAR). The following plasmid vectors were used in subsequent plant transformations:

- a. pARO25 (Fig. 2): sense expression of *csa* under the control of the 35S promoter in vector pPCV002
- b. pARO26 (Fig. 2): antisense expression of *csa* under the control of the 35S promoter in vector pPCV002
- c. pARO31 (Fig. 3): sense expression of *csa* under the control of the 2X 35S promoter in vector pPGTV(BAR)
- d. pARO32 (Fig. 3): antisense expression of *csa* under the control of 2X 35S in vector pPGTV(BAR)
- e. pJFS (Fig. 1): sense expression of the full-length *csa* cDNA under the control of the 35S promoter in pPCV002
- f. pJFATS (Fig. 1): antisense expression of the full-length *csa* cDNA under the control of the 35S promoter in pPCV002.

3. Transformation of plants

a. Transformation of tobacco

In the U.S. we primarily used *Nicotiana tabacum* cv Bel W3 for transformation. This cultivar was chosen because we were interested in the role of antioxidant enzymes in mitigating the effects of the air pollutant ozone in plants (research supported by the U.S. Environmental Protection Agency), and Bel W3 tobacco is known to be very sensitive to ozone. We later also constructed transgenic tobacco Samson NN to facilitate anticipated crosses with Samson NN plants engineered in Israel. We also chose Samson NN for attempted transformation with the PHGPX expression vector, to address the problems encountered by our Israeli collaborators in their transformations with the *csa* sense constructs.

As described above, all plant transformation vectors constructed at Rutgers University were based on the pBI121 vector. The engineered pBI121 vector, which carries an *nptII* gene to allow for selection by kanamycin resistance, was mobilized into *Agrobacterium tumefaciens* LBA4404 by triparental mating. Leaf pieces of *Nicotiana tabacum* cv Bel W3 were transformed as described by Horsch et al. (1985) and regenerated on Murashige-Skoog basal medium (Sigma) solidified with 0.8% agar and containing 3% sucrose, 200 µg/ml kanamycin, 10 µM BA and 0.1 µM NAA. For non-transformed controls, leaf pieces were inoculated with *Agrobacterium*-free media, but were otherwise carried through identical steps of regeneration and propagation with the exception that they were not exposed to kanamycin.

For each construct in Bel W3, from 16 to 34 primary transformants (T_0) plants were obtained; these plants were screened for antioxidant enzyme overexpression by Western blot analysis (PHGPX, SOD, MDAR and APX); measurement of activity by spectrophotometric assays (MDAR) or by native gel assays (SOD and APX); or northern blot analysis (PHGPX and MDAR).

T_0 plants which overproduced APX and SOD were self-pollinated to produce T_1 generation seed which were plated on kanamycin-containing media to determine the number of T-DNA insertion loci by segregation analysis. Kanamycin-resistant T_1 plants were self-pollinated and T_2 lines, homozygous for the transgene as determined by kanamycin selection, were subsequently grown without further kanamycin selection. Where a homozygous T_2 seed lot was not available, either kanamycin resistant T_1 plants were used or individual T_1 plants grown from seed were tested for overexpression of APX or SOD before use. Sibling plants which were kanamycin-sensitive and did not express the antioxidant transgene were used as controls and were self-pollinated to produce controls with the same genetic background as the respective sibling transgenics. Where this type of control was not available, Bel W3 plants were used which were derived from the control transformation.

Homozygous Bel W3 plants containing a single insertion locus for APXcyt or Cu/Zn-SODcyt expression cassette were cross-fertilized to obtain seed hemizygous for each transgene. Selected T_2 lines of APXcyt and Cu/ZnSodcyt were used as either male or female. Seeds from each cross were tested by germination on kanamycin-containing medium and were shown 100% kanamycin resistant.

T₀ plants which overproduced or sense-suppressed MDAR were self-pollinated, and T₁ kanamycin-resistant plants were analyzed for MDAR expression. The response of these plants to oxidative stress were compared with F₁ progeny of several non-transformed Bel W3 control lines (see above).

Kanamycin-resistant primary transformants which had been transformed with the pBI121 vector designed to overexpress PHGPX were self-pollinated, and kanamycin-resistant T₁ progeny were examined for expression of the citrus PHGPX transgene. Northern blot hybridization and immunoblot analysis of these kanamycin-resistant plants showed no evidence of PHGPX expression. As all or our other efforts to overexpress other antioxidant enzymes using the identical strategy were successful, we interpret these results as constitutive overexpression of PHGPX is toxic. Therefore, no transgenics containing stable integration of this citrus PHGPX expression cassette were obtained, although stable integration of the *nptII* kanamycin-resistance gene was demonstrated by Southern hybridization.

In Israel, Samson NN was used for tobacco transformation. Transformation procedures were according to standard conditions. We were able to easily obtain tobacco transformants with all *csa* antisense constructs. Selection on kanamycin (pARO25) or selection on BASTA (pARO31, 33) was used. For each of the constructs, several dozen plants were obtained. However, we encountered difficulty when using the *csa* sense orientations. The problem did not result from the choice of vector or selectable marker because corresponding *csa* antisense constructs were not problematic. After several trials and repeated efforts, we were able to obtain a few kanamycin-resistant plants for construct pARO25 and for construct pARO31. However, Southern and northern analyses of these plants were negative, *i.e.*, we could not detect true transformants with the *csa* sense plasmid configurations. As stated above, we suspect that the expression of the *csa* cDNA in tobacco plants is toxic, and therefore the only plants that were obtained were probably plants that are escapes, *i.e.*, not true transformants, or that only the kanamycin or the BASTA resistance genes were integrated into the tobacco genomes while the intact *csa* coding region was not introduced. Unlike the sense plants, we were easily able to obtain plants with the antisense *csa* configuration. Transcription of the antisense *csa* was demonstrated by northern blot analysis (Fig. 4 (A,B,C and M,O)), and the presence of *csa* antisense construct within the plants was demonstrated by Southern analysis (Fig. 5).

Primary transformants testing positive in hybridization analysis were selfed, and T₁ and T₂ plants were obtained by growing the seeds on kanamycin-containing plates. None of the plants that were observed showed any unusual phenotype, except that some of the plants had abnormal anthers and petals in the first blooms. Later on, these phenotypes did not reproduce. Since we also observed that plants transformed with other genes occasionally had similar problems, we concluded that the abnormalities in petals and anthers resulted from the tissue culture or transformation procedures. For physiological experiments, kanamycin-resistant T₂ plants were analysed. Tobacco plants were also transformed with pARO33 (Fig. 3) which contained the *apx* expression cassette. BASTA-resistant plants were generated, and soluble proteins extracts were examined with antibodies raised against pea cytosolic APX.

b. Citrus transformation

Short stems (about 1 cm long) from Duncan grapefruit seedlings were cocultivated with *Agrobacterium tumefaciens* EHA105 that contains pARO25 (kanamycin vector, *csa* sense configuration) and pARO 31 (BASTA resistance, *csa* sense configuration). Regenerated plantlets from the cut surface were used to regenerate Duncan plants. We produced about 100 plants that were kanamycin-resistant and 30 plants that were BASTA-resistant. After a year, when the plants rooted and produced about 10 leaves, proteins were extracted from each individual plant. Western blot analyses were made using anti Cit-SAP antibodies (Fig. 6). Individual plants that showed high Cit-SAP expression were subjected to Southern analysis. None of the plants that were analyzed gave positive signals for recombinant *csa*. We concluded that all the plants produced were not true transformants. While in tobacco it is reasonable to suspect that *csa* expression may be toxic, in citrus it is less likely, as Cit-SAP is normally expressed in all tissues; nonetheless, the possibility still exists that overexpression of PHGPX is lethal also in citrus. In view of the reported difficulties with citrus transformation and the very high number of escapees on kanamycin selection, we suspect that in citrus the problem resides in the ability to transform the plants. We therefore realized that for citrus transformation one needs at least one additional visual marker for indication of successful transformation. We therefore used a new binary vector (kindly provided by Moshe Flaishmann). This vector contains the *nptII* gene conferring kanamycin resistance, the *bar* gene for BASTA resistance and the *uidA* gene (with an intron) under the control of the 35S promoter as a visual marker. In addition, we are constructing now an additional vector which is based on pBIN+. This particular vector is designed in such a way that the *nptII* gene is the last to be introduced from the bacterium to the plant, ensuring that once kanamycin-resistant plants are produced, they contain also the transgene of interest. Up until now, we have produced several plants that gave positive GUS signals (Fig. 7). This GUS signal is stable, and repeated staining of leaves after two months continued to show very strong labeling. After the plants grow to the ten leaf stage, Southern analysis will be performed to confirm integration of the transgene(s). Should our initial tests with our new cloning vectors prove to be successful, we will introduce *csa* into these vector in sense and antisense configurations.

D. Overexpression of the antioxidant enzymes in transgenic tobacco

1. Overexpression of ascorbate peroxidase

Following transformation of leaf explants and regeneration of tobacco with kanamycin selection, individual plants were tested for expression of the introduced pea transgene by APX activity gel assays. Comparison of enzyme activity in gels was made with the non-transformed tobacco control plants. Expression of the transgene was immediately obvious in the APX gel assay (Mittler and Zilinskas, 1993), where the apparent increase in APX activity ranged from 5- to 40-fold that of the endogenous tobacco APX for most of the kanamycin-resistant primary transformants analyzed. This is in contrast to our earlier attempts to express pea APX in transgenic tobacco where few plants overexpressed the enzyme and level of overexpression was never more than double that of the non-transformed control (Mittler and Zilinskas, unpublished data). In these previous experiments, the entire cDNA (including the 5' and 3' untranslated sequences) was present in the construct introduced into tobacco via *Agrobacterium tumefaciens*. Removal of the untranslated sequences using PCR

resulted in much higher levels of APX overproduction and a higher percentage of kanamycin-resistant plants that expressed the enzyme. As we have shown that pea APX expression is controlled post-transcriptionally, with probable regulation at the level of translation (Mittler and Zilinskas, 1994), the 5' untranslated leader probably limited the level of APX overproduction in our initial attempts to overexpress this enzyme in tobacco.

Targeting of pea cytosolic APX to the chloroplast and correct processing by the chloroplast transit peptidase was achieved by addition of the sequence encoding the transit peptide and first five amino acids of the mature RuBisCO small subunit to the 5' end of the APX coding sequence. Chloroplast targeting was verified for the APXchp plants by analysis of the isolated chloroplastic fraction on an APX activity gel. Comparisons of APXchp, APXcyt and Bel W3 plants showed much higher APX activity in the chloroplastic fraction of the APXchp plants. As assayed by native gels, the level of overexpression of APX in the chloroplast was similar to that observed in APXcyt transgenic plants. The slightly altered mobility in the gel can be accounted for by the additional five amino acids at the N-terminus of pea cytosolic APX; this small difference of ~550 d can be seen on high resolution SDS-PAGE/immunoblot analysis.

Two independent lines with similar activity in mid-high range for both the APXcyt and APXchp plants were selected for the bulk of further studies described below. These were lines APXcyt12 and APXcyt32 and APXchp2 and APXchp4. Segregation analysis demonstrated that all lines, with the exception of APXcyt32 were single locus insertions.

While APX activity gel assays proved to be very useful to quickly screen primary transformants and T₁ progeny for overexpression, we proceeded to measure APX activity spectrophotometrically following the procedure described by Amako et al. (1994) to enable us to quantitate the level of APX activity relative to the endogenous APX present in the cytosol and chloroplast. In doing so we found that a much lower level of pea APX was overexpressed relative to the endogenous tobacco APX than had previously been estimated by the gel assay. Typically, the activity of the introduced transgene was 3- to 5-fold the level of the endogenous enzyme. The apparent overestimation of the APX activity gel assay can be attributed to the very high stability of pea cytosol APX relative to tobacco APX (Mittler and Zilinskas, 1993). Subjecting leaf extracts to the gel assay apparently retains the pea enzyme activity, while the tobacco enzyme activity is diminished because of its lower stability. In the case of the APXcyt plants, the level of overproduction in the cytosol is approximately 4- to 5-fold that of the non-transgenic sibling siblings; the level of the endogenous chloroplastic APX is similar in the transgenic and non-transgenic siblings. For the APXchp plants, the overexpression of the pea cytosolic APX in the chloroplast is about 7-fold that of the endogenous chloroplastic APX as determined by both the Amako method and by direct measurement of activity in isolated chloroplasts. These elevated levels of APX activity are within the range of increases in APX activity reported in plants exposed to oxidative stress.

2. Overexpression of superoxide dismutase

After transformation of tobacco leaf pieces with *Agrobacterium tumefaciens* which contained a binary vector engineered to overexpress either pea cytosolic Cu/Zn-SOD or pea mitochondrial Mn-SOD in the chloroplast, leaf tissue or regenerated primary transformants (in the rosette stage of

development) was analyzed for SOD overexpression by native gel assay. Mn-SOD is normally located in mitochondria; however, the sequence encoding the mature Mn-SOD of pea was positioned in frame with the coding sequence for the transit peptide and first five amino acids of the mature RuBisCO small subunit in the case of MnSODchp plants.

As determined from SOD activity gel analysis in which activities of soluble leaf extracts from transgenic and non-transformed plants were compared, the pea Mn-SOD targeted to the tobacco chloroplast was present at about four times that of the endogenous tobacco chloroplast FeSOD. Pea Cu/Zn-SOD was overproduced in the tobacco cytosol at 1.5- to 3-fold that of the endogenous cytosolic Cu/Zn-SOD. The presence of the Cu/Zn-SOD was also verified by immunoassay (Pitcher and Zilinskas, 1996). Cu/Zn-SODcyt plants and Mn-SODchp plants chosen for phenotypic analysis were determined by segregation analysis to have a single locus T-DNA insertion.

3. *Overexpression of both superoxide dismutase and ascorbate peroxidase*

F₁ progeny of sexual crosses of homozygous T₂ generation transgenic plants that individually overexpressed cytosolic APX or cytosolic Cu/Zn-SOD were analyzed for expression of both antioxidant enzymes by native activity gel assay (for APX and SOD) and spectrophotometric assay (for APX). The APX activity and the SOD activity of the APXcyt-SODcyt “double transgenic” plants was about equal to that of the respective APX and SOD overproducing parental lines. We had also obtained transgenic plants by *Agrobacterium*-mediated transformation with a binary vector system in which both pea APX and pea Cu/Zn-SOD expression cassettes were linked in the same plasmid, along with the kanamycin-resistance marker. Although the binary vector was not pBI121, but rather a Monsanto (pMON) vector, the full-length Cu/Zn-SOD cDNA and the APX coding sequence were each cloned downstream from the CaMV35S promoter, as was the case with all our other constructs. Plants obtained with this “linked” vector expressed Cu/Zn-SOD at 1.5 to 3- fold the level of the endogenous enzyme, but APX overexpression was quite low, relative to that obtained with sexual crosses (described above). We therefore decided to abandon this strategy for overexpression of two antioxidant enzymes.

4. *Overexpression and co-suppression of monodehydroascorbate reductase*

Analysis of the primary transformants transformed with pBI121 containing the MDAR expression cassette showed that while most of the kanamycin-resistant transformants had substantially increased MDAR activity (up to 15-fold that of the non-transformed controls), several plants had barely detectable MDAR, as low as 15-fold less than the control plants. These plants typically had multiple insertions of the transgene, and appeared to be sense-suppressing the endogenous MDAR. Kanamycin-resistant T₁ progeny of the primary transformants which overexpressed MDAR continued to express the enzyme at high levels. On the other hand, kanamycin-resistant T₁ progeny of the sense-suppressors included those that continued to sense-suppress the endogenous MDAR and others in which the suppression had been unmasked and these T₁ progeny now overexpressed MDAR. These lines provided excellent material to assess whether altered levels of MDAR activity (both very high and very low relative to the “wild type”) affected oxidative stress tolerance.

E. Evaluation of the tolerance of the transgenic plants to oxidative stress

1. Tolerance/sensitivity to paraquat

a. Overview

To determine whether altered levels of these various antioxidant enzymes affected oxidative stress tolerance, we utilized methyl viologen (also known as the herbicide paraquat). This redox-cycling agent intercepts light-driven electron flow through photosystem I, donating electrons to O_2 , thereby producing superoxide radicals. These, in turn, undergo SOD-catalyzed dismutation to generate H_2O_2 and O_2 . Superoxide and H_2O_2 together can lead to hydroxyl-radical production which causes damage to proteins, lipids and DNA. We assessed paraquat-mediated oxidative damage by measuring electrolyte leakage through damaged membranes.

b. APXcyt and MDAR sense-suppressed plants are protected from paraquat-induced oxidative stress

Preliminary tests indicated that seven out of ten independent APXcyt primary transformants tested were resistant to paraquat (Pitcher and Zilinskas, 1994). Two lines (12 and 32) were selected for further extensive work using the T_1 generation. These plants were grown on soil without benefit of kanamycin selection, then tested for the expression of the transgene by activity gel assay or immunoblot analysis. Siblings which showed 'wild-type' activity or protein were used as controls. Disks from recently fully-expanded leaves of bolting, preflowering plants were placed individually in petri dishes in 3 ml paraquat (PQ) and vacuum-infiltrated. After 16 h dark incubation at 25°C, the disks were illuminated for 5 h at $650 \mu Em^{-2}sec^{-1}$. Conductivity of the solutions was measured to assess electrolyte leakage due to PQ-induced membrane damage. Maximum electrolyte content was determined by measurement of conductivity of the replicate disks autoclaved in 3 ml of H_2O .

Four independent APXcyt and APXchp lines were tested; three independent Cu/Zn-SODcyt lines were tested; and two Mn-SODchp lines were tested. Disks from APXcyt plants showed strikingly effective protection against solute leakage even at paraquat concentration as high as 100 μM . APXchp plants showed a tendency to a relatively small degree of protection in some experiments at low PQ concentrations; however, when all experiments were averaged together no convincing protection was seen in these plants. Neither Mn-SODchp nor Cu/Zn-SODcyt plants showed protection from paraquat-mediated damage. All lines within a transgenic type behaved similarly. Figure 8 shows representative results for one line of each type.

APXcyt-Cu/Zn-SODcyt overexpressors produced by sexual crosses also showed protection approximately equal to that shown by the parental APXcyt overexpressor line.

Relative to non-transformed control lines, MDAR-overexpressing transgenic tobacco lines were not significantly protected against PQ-induced damage, as measured by ion leakage, while unexpectedly, the MDAR sense-suppressing lines were tolerant to PQ at concentrations as high as 50 μM (Figs. 9 and 10). Paraquat assays performed on T_1 siblings that had overcome sense-suppression, along with T_1 siblings that were still sense-suppressors, showed a clear correlation between sense-suppression of MDAR activity and PQ-induced damage (Figs. 9 and 11). In collaboration with Dr. Stephen Grace at Australia National University, we were able to measure the *in vivo* levels of

monodehydroascorbate radical (MDA) by EPR spectroscopy. Leaves were subjected to oxidative stress by infiltrating them with 1 mM PQ and exposing them to $800 \mu\text{Em}^{-2} \text{sec}^{-1}$ white light. MDA concentration was much higher in MDAR sense-suppressors than in control non-transformed plants. In turn, MDA concentration was higher in controls than in MDAR overexpressing tobacco plants (Fig. 12).

c. Tobacco expressing the csa antisense are more sensitive to paraquat

Sensitivity to PQ was tested on young, large leaves of tobacco plants. Leaf disks were placed in petri dishes (four for each plate) that contained 10 ml of H_2O with and without PQ. Preliminary experiments indicated that significant damage occurred when 2 mM PQ was present in the solution. The experiments were therefore conducted with 1 mM PQ. The plates were placed in the dark for 24 h and subsequently were exposed to about $50 \mu\text{Em}^{-2}\text{sec}^{-1}$ white light. Samples were taken periodically for detection of ion leakage by measuring conductivity of the bathing solution. After conductivity was assayed, the leaves were returned to the solution held in petri dishes. Usually after 24 h in the dark, no significant ion leakage from leaf disks was observed. Figures 13 and 14 show data obtained with antisense T_2 plants 23 and 24. A marked difference between wild-type plants and some of the antisense plants was observed 7-15 hours after exposure of leaf disks to light. The differences in conductivity were also observed upon visual inspection of the leaf disks (Fig. 15). We concluded from the outlined experiments that expression of the antisense *csa* configuration in some of the tobacco Samson NN lines might reduce the ability of these tobacco plants to cope with oxidative stress.

In addition, we measured electron transport and ΔpH in isolated chloroplasts under oxidative stress induced by PQ. Although most of the preliminary data with PHGPX indicated that the protein is localized in the cytoplasm, we wanted to monitor photosynthetic parameters within the chloroplast. The rationale behind this decision was that damage caused by oxidative stress in the cytoplasm should also be reflected in the chloroplasts as well. If antisense plants were more sensitive to oxidative stress, then their chloroplasts should also be more stressed and this should be reflected in photosynthetic parameters. For this purpose, we developed a modified assay to follow damages caused by PQ. In addition, we used a novel approach to measure ΔpH and electron transport. The method measures simultaneously and separately ΔpH and electron transport. The method is not published yet and was developed by Dr. Yoav Evron during his PhD in the laboratory of Prof. Uri Pick. Essentially, the method makes use of the fact that emission spectra of the fluorescence of the fluorescent reagent 9-aminoacridine largely overlaps the absorption spectrum of ferricyanide but not that of ferrocyanide. Thus, in the presence of ferricyanide, fluorescence will gradually increase if ferricyanide is reduced due to electron transport. Measurement of electron transport is obtained from the difference in fluorescence before and after illumination. ΔpH is obtained from the difference in fluorescence when the light is turned off.

In order to use the above-mentioned methodology, we developed the following assay. Tobacco leaves were placed in darkness for a week at 4°C . Then the leaves were sliced and immersed in either H_2O or PQ and illuminated for 12 h. Chloroplasts were isolated from the sliced leaves and brought to a concentration of 2-3 mg Chl/ml, according to Evron et al. (1990). Electron transport and ΔpH were measured in the presence of 9-aminoacridine and ferricyanide. Fluorescence was measured in a SUN Amico fluorimeter. Figure 16 clearly demonstrates that exposure of tobacco wild-type to PQ severely damages ΔpH of chloroplasts but has a much less pronounced effect on

electron transport. However, in antisense plants both parameters are severely affected, and the calculated difference in electron transport between wild-type and antisense plants is about one order of magnitude (Figure 16). The results strongly suggest that sensitivity to PQ is reflected strongly in the ΔpH , while damage to electron transport is much less pronounced. PHGPX activity might have an important role in the integrity of thylakoid membranes under oxidative stress. This conclusion is further supported by the preferred substrate specificity of Cit-SAP towards phospholipid hydroperoxides (Beeor-Tzahar et al., 1995). Despite the fact that Cit-SAP is most probably a cytoplasmic protein, its substrate could be peroxides of membrane phospholipids that were created under stress conditions. These results could also explain why Cit-SAP might be important for chloroplastic integrity under stress conditions.

2. *Tolerance/sensitivity to other stress conditions*

a. *Overview*

While the most detailed analyses were made with paraquat-induced oxidative stress, as this redox cycling agent is known to produce reactive oxygen and it is relatively easy to reproduce treatment conditions, we also made preliminary measurements with the various transgenic lines for their tolerance to several other environmental stress conditions. These included: photoinhibitory stress induced at low temperatures and high intensity light; saline stress; and ozone stress. The latter was supported by the U.S. EPA and will not be reported here.

b. *Photoinhibitory stress*

Plants were used at a bolting, preflowering stage; 3.5 cm diameter disks were taken from the most recently fully-expanded leaf and placed on a iceblock under $1500 \mu\text{Em}^{-2} \text{sec}^{-1}$ light for varying time periods as described by Sen Gupta et al. (1993). For recovery periods of varying times, disks were floated on water maintained at 25°C under $700 \mu\text{Em}^{-2} \text{sec}^{-1}$ light. Photosynthetic oxygen evolution from the leaf discs was measured in saturating CO_2 with a Hansatech oxygen electrode.

Several preliminary tests were conducted to establish appropriate testing conditions. In these preliminary tests, the duration of exposure of leaf tissue to high intensity light and cold temperature was varied such that at its greatest, no recovery was seen after relieving the stress, and at its least, no decrease in photosynthesis was seen after the recovery period. In none of these preliminary experiments were any reproducible differences noted between any of the transgenic SOD or APX overproducing lines tested and their non-transformed controls. Final conditions were chosen wherein photosynthesis declined at about 80- 90% immediately at the end of the stress period, but recovered to at least 25% of pre-stressed levels during a 0.5 to 1 h recovery period. Two independent lines each of the APXcyt, AXPchp, and MnSODchp overproducers were tested. No significant differences were noted between the transgenic plants and their non-transformed controls (Fig. 17).

c. *Salt stress*

Our salt tolerance experiments were done with tobacco seedlings. Seeds from *csa* antisense T_1 plants were germinated on plates of Murashige-Skoog basal media that contained either kanamycin with

NaCl or kanamycin alone. Experiments were also carried out on plates that did not contain kanamycin, and the seedlings were transferred from kanamycin-containing plates to plates containing NaCl a few weeks after the seeds had germinated. Initial experiments indicated that the effective concentration of NaCl for wild-type Samson NN plants was around 100-200 μ M (Fig. 18). Under the conditions specified, we did not observe any reproducible differences between antisense and wild type plants, although occasionally we saw that antisense plants were slightly more sensitive to NaCl. To date, none of the other transgenic lines that were generated at Rutgers University have been tested for salt tolerance.

F. Salt and oxidative stress in citrus

Salt damage to plants has been attributed to a combination of several factors including mainly osmotic stress and the accumulation of toxic ions. Recent findings in our laboratory showed that phospholipid hydroperoxide glutathione peroxidase (PHGPX), an enzyme active in the cellular antioxidant system, was induced by salt in citrus cells and mainly in roots of plants. Following this observation we studied the two most important enzymes active in elimination of reactive oxygen species, namely, superoxide dismutase (SOD) and ascorbate peroxidase (APX), to determine whether a general oxidative stress is induced by salt. While Cu/Zn-SOD activity and cytosolic APX protein level were similarly induced by salt and the redox-cycling agent, paraquat, the response of PHGPX and other APX isozymes was either specific to salt or paraquat, respectively. Unlike PHGPX, cytosolic APX and Cu/Zn-SOD were not induced by exogenously added abscisic acid. Salt induced a significant increase in SOD activity which was not matched by the subsequent enzyme APX. We suggest that the excess of H_2O_2 interacts with lipids to form hydroperoxides which in turn induce and are removed by PHGPX. Ascorbate peroxidase seems to be a key enzyme in determining salt tolerance in citrus as its constitutive activity in salt-sensitive callus is far below the activity observed in salt-tolerant callus, while the activities of other enzymes involved in the defence against oxidative stress, namely SOD, glutathione reductase and PHGPX, are essentially similar (Gueta-Dahan et al., 1997).

III. Description of Cooperation

Antibodies raised against pea APX and pea Cu/Zn-SOD by BAZ and those raised against citrus PHGPX by YE have been exchanged and used to examine the response of pea and citrus to paraquat-induced oxidative stress and salt stress. These antibodies were also used to evaluate the expression of transgene in the transgenic tobacco plants. Similarly, cDNA clones for APX and PHGPX expression were exchanged between DH and BAZ for construction of binary plasmid vectors for *Agrobacterium*-mediated transformation of tobacco and citrus. DH visited BAZ on two occasions at Rutgers University to discuss experimental strategies, emerging data and future directions. YE visited BAZ at Rutgers in the fall of 1996 to exchange scientific ideas, plan experiments and interpret data. BAZ and GB-H met in the summer of 1993 in Denmark during the Third International Symposium on Plant Peroxidases which provided them with the opportunity to discuss methodology and project plans; a co-authored paper was recently published as an outcome of those early discussions.

Perhaps one of the best examples of close cooperation between the Israel and U.S. laboratories was the concerted effort to overexpress PHGPX in tobacco. When with repeated efforts and various strategies the Israeli group failed to obtain transgenic lines containing the *csa* sense construct,

Zilinskas' laboratory proceeded to use a strategy which she used successfully for many different transgenic lines generated in her laboratory. When neither group was able to identify transgenic lines with stable integration of the PHGPX expression cassette, it became quite clear that constitutive overexpression of PHGPX is most probably toxic. This concerted effort of the two laboratories, although having a negative outcome with respect to a major project objective, has resulted in interesting new avenues of research to explore with regard to the apparent lethality resulting from constitutive overexpression of PHGPX.

IV. List of Publications

A. Journal articles

Holland, D., Z. Faltin, A. Perl, G. Ben-Hayyim and Y. Eshdat (1994) A novel plant glutathione peroxidase-like protein provides tolerance to oxygen radicals generated by paraquat in *Escherichia coli*. *FEBS Lett.* 337:52-55.

Murthy, S.S. and B.A. Zilinskas (1994) Molecular cloning and characterization of a cDNA encoding pea monodehydroascorbate reductase. *J. Biol. Chem.* 269:31129-31133.

Beeor-Tzahar, T., G. Ben-Hayyim, D. Holland, Z. Faltin and Y. Eshdat (1995) A stress-associated citrus protein is a distinct plant phospholipid hydroperoxide glutathione peroxidase. *FEBS Lett.* 366:151-155.

Eshdat, Y., D. Holland, Z. Faltin and G. Ben-Hayyim (1997) Plant glutathione peroxidases. *Physiol. Plantarum* 100:234-240.

Gueta-Dahan, Y., Z. Yaniv, B.A. Zilinskas and G. Ben-Hayyim (1997) Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. *Planta* 203:460-469.

Pitcher, L.H. and B.A. Zilinskas. Oxidative stress in tobacco as affected by overproduction of ascorbate peroxidase and superoxide dismutase. *Plant Physiol*: to be submitted in December, 1997.

Murthy, S.S., L.H. Pitcher, S. Grace, and B.A. Zilinskas. Characterization of transgenic tobacco with altered levels of monodehydroascorbate reductase and their response to oxidative stress. *Plant Physiol*: to be submitted in January, 1998.

B. Published abstracts

Murthy, S.S. and B.A. Zilinskas (1994) Molecular cloning and characterization of a cDNA encoding pea monodehydroascorbate reductase. *Plant Physiol.* 105S:107.

Pitcher, L.H., P. Repetti and B.A. Zilinskas (1994) Overproduction of ascorbate peroxidase protects transgenic tobacco against oxidative stress. *Plant Physiol.* 105S:116.

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VI. Figures

- Figure 1: pJFS, pJFATS
- Figure 2: pARO25, pARO26
- Figure 3: pARO33
- Figure 4: Northern analysis of tobacco transformed with *csa* antisense construct
- Figure 5: Southern analysis of tobacco transformed with plasmids, pARO24 and pARO31
- Figure 6: Citrus Western analysis
- Figure 7: Citrus GUS experiment
- Figure 8: Paraquat-induced ion leakage in four representative T₂ tobacco transgenic lines overexpressing APX in the cytosol or chloroplast or MnSOD in the chloroplast or Cu/Zn-SOD in the cytosol
- Figure 9: Fold-change in MDAR activity in T₁ transgenic plants relative to the mean activity of 7 independent non-transformed control lines. Plants labeled S,D and U are siblings from overexpressing lines. Primary transformants I and V were co-suppressors, and their T₁ siblings (shown here) include both suppressors as well as overexpressors.
- Figure 10: Paraquat-induced ion leakage in T₁ transgenic tobacco overexpressing MDAR (Panel A) and in transgenic tobacco sense-suppressing MDAR (Panel B)
- Figure 11: Paraquat-induced ion leakage in T₁ siblings of MDAR co-suppressing tobacco lines, V and I that had overcome sense-suppression (Panel A) and those that continued to sense-suppress (Panel B).
- Figure 12: *In vivo* EPR spectra of MDAR overexpressing and sense-suppressing transgenic tobacco relative to non-transformed control tobacco
- Figure 13: Paraquat-induced ion leakage in *csa* antisense tobacco plants
- Figure 14: Paraquat-induced ion leakage in *csa* antisense tobacco plants
- Figure 15: Paraquat-induced leaf damage in *csa* antisense tobacco plants
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- Figure 17: Effect of photoinhibitory stress on oxygen evolution in APXcyt, APXchp, and MnSOD overproducers and their controls
- Figure 18: Salt sensitivity of *csa* antisense tobacco plants

Fig. 1

Fig. 2

Fig. 3

Northern analysis of tobacco plants transformed with *csa* in antisense configuration

- Samples A-J were extracted from putatively tobacco plants transformed with antisense configuration (plasmid pARO32)
- Sample K is a positive control (probe on itself)
- Samples L-O: antisense configuration with plasmid pARO26

Fig. 5

Southern analysis of tobacco plants transformed with
plasmids pARO24 and pARO31

1 2 3 4 5 6 7 8 9 10 11 12

Samples 3,4: plants
transformed with pARO26

Samples 1,2,5,6,7,8,9:
plants transformed with
pARO32

Sample 12: *csa*

Sample 10: non transformed
plant

Fig. 6

Western analysis of putative citrus plants
transformed with *csa*

- Samples 1-11 from putatively transformed plants were reacted with anti-Cit-SAP antibodies

Fig. 7
0

Duncan Grapefruit transformed with the *uidA* gene and stained for GUS activity

Fig. 8

Fig. 9

Fig. 10

Fig. 11

A.

B.

Fig. 12

Leaves infiltrated with 1 mM paraquat/ PFD at leaf surface=800 $\mu\text{mol m}^{-2} \text{s}^{-1}$

Fig. 13

Fig. 14

1 uM methyl viologen

antisense 24

antisense 23

NN

Fig. 96

ANTISENSE TOBACCO + H₂O

B CONTROL TOBACCO + H₂O

CONTROL TOBACCO + 24M METHYL VIOLGEN

ANTISENSE TOBACCO + 24M METHYL VIOLGEN

Fig. 17

Fig. 18

הספריה המרכזית
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בית דגן