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Project Title: Enhancing Quality Attributes Of Potato And Tomato By Modifying And Controlling Their Oxidative Stress Outcome

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Keywords : Hydroperoxidation, phospholipids hydroperoxide glutathione peroxidase, lipoxygenase, catalase, regeneration, field performance, transgenic, expressed sequence tag, macroarray, gene expression, tobacco, polyamine

Abbreviations commonly used in the report, in alphabetical order:

CAT, catalase; EST, expressed sequence tags; LOX, Lipoxygenase;

PHGPx, Phospholipids hydroperoxide glutathione peroxidase;

SAMde, s-adenosine methionine decarboxylase

Budget: IS: \$136,000

US: \$164,000

Total: \$ 300,000

Signature
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Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)				
Submitted, in review, in preparation	1	3	1	5
Invited review papers				
Book chapters				
Books				
Master theses				
Ph.D. theses				
Abstracts	1	2		3
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Venu Perla (13 months) and Dr Alka Srivastva (9 months) were paid from this grant grants

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	0	2		2
Longer Visits (Sabbaticals)				

Description of Cooperation:

There was a great deal of exchange of both biological material and thoughts between the Israel and the USA groups. Both groups made chimeric gene constructs and provided to each other for potato and tomato transformation respectively. There was a constant exchange of thought using various communication means including email. During the third year, the Israeli collaborators made two short visits to Purdue to discuss overall progress of this project. Also, Dr Eshdat presented a wonderful seminar on this project to Purdue Faculty during his visit.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)				
Licensed				

ABSTRACT

General

The final goal and overall objective of the current research has been to modify lipid hydroperoxidation in order to create desirable phenotypes in two important crops, potato and tomato, which normally are exposed to abiotic stress associated with such oxidation. The specific original objectives were: (i) the roles of lipoxygenase (LOX) and phospholipid hydroperoxide glutathione peroxidase (PHGPx) in regulating endogenous levels of lipid peroxidation in plant tissues; (ii) the effect of modified lipid peroxidation on fruit ripening, tuber quality, crop productivity and abiotic stress tolerance; (iii) the effect of simultaneous reduction of LOX and increase of PHGPx activities on fruit ripening and tuber quality; and (iv) the role of lipid peroxidation on expression of specific genes. We proposed to accomplish the research goal by genetic engineering of the metabolic activities of LOX and PHGPx using regulatable and tissue specific promoters, and study of the relationships between these two consecutive enzymes in the metabolism and catabolism of phospholipid hydroperoxides.

USA

Significant progress was made in accomplishing all objectives of proposed research. Due to inability to regenerate tomato plants after transforming with 35S-PHGPx chimeric gene construct, the role of low catalase induced oxidative stress instead of PHGPx was evaluated on agronomical performance of tomato plant and fruit quality attributes. Effects of polyamine, that protects DNA from oxidative stress, were also evaluated. The transgenic plants under expressing lipoxygenase (LOX-sup) were crossed with catalase antisense (CAT-anti) plants or polyamine over producing plants (SAM-over) and the lines homozygous for the two transgenes were selected. Agronomical performance of these line showed that low catalase induced oxidative stress negatively affected growth and development of tomato plants and resulted in a massive change in fruit gene expression. These effects of low catalase activity induced oxidative stress, including the massive shift in gene expression, were greatly overcome by the low lipoxygenase activity. Collectively results show that oxidative stress plays significant role in plant growth including the fruit growth. These results also for the first time indicated that a crosstalk between oxidative stress and lipoxygenase regulated processes determine the outcome during plant growth and development.

Israel

Regarding PHGPx, most of the study has concentrated on the first and the last specific objectives, since it became evident that plant transformation with this gene is not obvious. Following inability to achieve efficient transformation of potato and tomato using a variety of promoters, model plant systems (tobacco and potato cell cultures, tobacco calli and plantlets, and *Arabidopsis*) were used to establish the factors and to study the obstacles which prohibited the regeneration of plants carrying the genetic machinery for overproduction of PHGPx. Our results clearly demonstrate that while genetic transformation and over-expression of PHGPx occurs in pre-developmental tissue stage (cell culture, calli clusters) or in completed plant (*Arabidopsis*), it is likely that over-expression of this enzyme before tissue differentiation is leading to a halt of the regeneration process. To support this assumption, experiments, in which genetic engineering of a point-mutated PHGPx gene enable transformation and over-expression in plants of PHGPx modified in its catalytic site and thus inactive enzymatically, were successfully carried out. These combined results strongly suggest, that if in fact, like in animals and as we established *in vitro*, the plant PHGPx exhibits PH peroxidase activity, these peroxides are vital for the organisms developmental process.

ACHIEVMENTS, USA

Significance of main scientific achievements or innovations

The primary goal of research at Purdue was to understand roles the cellular redox state plays in plant growth and development and gene expression during fruit development and ripening. The specific emphasis was to regulate oxidative stress to enhance quality attributes of tomato and potatoes. Although we were not able to transform tomato with PHGPx due to its effect on plant regeneration capacity, the use of catalase antisense line that modified the oxidative stress in developing tomato plants provided significant new information of great interest.

Our results for the first time demonstrated that catalase has a profound effect on plant growth and development. Several growth parameters including plant height, total fresh and dry weight gains of the whole plant or the shoot were negatively impacted in low catalase activity plants. Also, number of fruits and total fruit yield per plant were greatly reduced by lowering the catalase activity. Interestingly the under expression of catalase has a very strong effect on the fruit shape. Tomato fruit became more elongated and oval shaped compared to round fruits in the parental wild type plants. Low catalase fruit had significantly higher levels of total soluble solids, but little effect on the juice pH. The under expression of lipoxxygenase had little effect on any of the agronomical performance parameter evaluated. Remarkable novel results were obtained when the transgenic tomatoes harboring both antisense catalase and cosuppression causing lipoxxygenase truncated gene were evaluated under the field conditions. Almost all effects of low catalase activity, except its effect on fruit shape, disappeared, suggesting crosstalk between the low catalase-induced oxidative stress and lipoxxygenase regulated effects on plant growth and development. Although further understanding of the effects of catalase and lipoxxygenase on plant growth and development is needed, these results for the first time provide evidence that modifying and controlling oxidative stress would have significant effects on crop productivity.

To evaluate effects of oxidative stress on gene expression, a tomato cDNA macroarray, containing over 1000 unique cDNA, representing over 12 metabolic functional categories, was developed in the present investigation. This array was used to understand the effect of increased oxidative stress and reduced lipoxxygenase expression on metabolic activity of tomato cells during fruit ripening. Our results, again for the first time, provide evidence that increased oxidative stress imposed by higher H_2O_2 in low catalase plants causes a massive shift in the gene expression (over 37% of total gene showed significantly altered expression). Interesting, in the presence of low lipoxxygenase activity the deleterious effect of high H_2O_2 was ameliorated.

In summary, our results demonstrate that oxidative stress plays a significant role in plant growth development including fruit ripening. Results from both the agronomical performance and gene expression studies clearly show a cross talk between oxidative stress and lipoxygenase pathway. Further understanding of molecular mechanisms regulating these processes would allow create novel methods to modify plant growth and development and enhance crop productivity.

ACHIEVMENTS, ISRAEL

Significance of main scientific achievements or innovations

Two main objectives of the research project include the understanding of the role of lipoxygenase (LOX) and phospholipids hydroperoxide glutathione peroxidase (PHGPx) in regulating endogenous levels of lipid peroxidation in plant tissues, and of the role of lipid peroxidation on expression of specific genes. With respect to PHGPx, at the time when study was initiated three years ago, very little was known about its molecular properties and role **in plants**, while our previous study provided characterization of this enzyme regarding its *in vitro* peroxidase specificity towards phospholipids hydroperoxides (PH) and other substrates, of its catalytic active-site residue (Cys 41), of its antioxidative power supporting vitality of recombinant *E. coli*, and of its structure.

Based of previous studies done on transformation of plant enzymes involved in redox metabolic reactions (like SOD, APx, and LOX), we believed, at the beginning of this study, that obtaining transgenic plants which will have the capacity to either over-express or to decrease the expression of PHGPx, independently or together with similar regulation of the expression of LOX, will be feasible, thus providing us a tool to study the role and to control important physiological pathways in two agriculturally important crops as tomato and potato. However, though we have constructed a large variety of plasmids which afforded several options to enable us to obtain successful plant transformation and over-expression of PHGPx; we were unable to obtain an efficient regeneration of transformed plants carrying this gene. These unexpected observations led us to assume that the putative peroxidation of PH may hamper one of the biochemical pathways that mediate successful regeneration. To identify and characterize such event, which will also shed light on the role of PHGPx in plants, we have carried out PHGPx transformation, devoid of regeneration stage, to yield transformed tobacco and potato cell cultures, tobacco calli, and *Arabidopsis* plants. In all these four systems, successful transformation and over-expression of PHGPx was achieved. These experiments not only demonstrated that over-expression of PHGPx in plants is possible and not harmful at the cell

level or in the mature plant, but also that such over-expression might hamper the differentiation processes occurring during plantlets development and regeneration.

To prove that the enzymatic activity of PHGPx is involved in the effect of the protein over-expression on the plantlets development, we performed a point mutation in the PHGPx gene (G¹²⁵ into C¹²⁵ in the ORF region) leading to exchange of the presumed catalytic residue Cys⁴¹ into Ser, a residue that is chemically devoid of reducing power as Cys but is structurally similar. The results obtained showed that plant transformation with the plasmid 35S-Ser⁴¹PHGPx bearing the mutated PHGPx gene, was efficient and resulted in over-expression of the protein.

To conclude, the results obtained in the present study clearly demonstrate for the first time that uncontrolled PHGPx over-expression in plants interferes with plantlets regeneration, probably during differentiation. Since abolishment of the putative catalytic activity of PHGPx diminishes such effect and enables plant development when over-expression of inactive PHGPx occurs, it is likely to assume that phospholipids hydroperoxidation may be essential for normal differentiation and plant development.

APPENDIX**TABLE OF CONTENTS****Publications**

1. Manuscript under preparation.....8
2. Abstracts.....8

Unpublished Data

1. USA.....9
2. Israel.....32

List of publications:

Manuscript under preparation: These manuscripts are at different stages of preparation. As soon these are accepted copies will be submitted to BARD.

1. Enhanced catalytic activity of phospholipid hydroperoxide glutathione peroxidase interferes with plant regeneration (Eshdat et al., Israel)
2. Oxidative stress modifies fruit shape and effect agronomical performance of tomato (Perla V. and Avtar K. Handa et al., USA)
3. Global gene expression profiling in tomato (*Lycopersicon esculentum* cv. Mill.) ripening fruit using cDNA macroarrays. Alka Srivastava, Tatsiana Datsenka, Lauren McIntyre and Avtar K Handa (USA)
4. Low expression of lipoxygenase overcomes deleterious effects of oxidative stress induced by impaired catalase expression Avtar K Handa, , Venu Perla, Tatsiana Datsenka, Lauren McIntyre and Alka Srivastava (USA)
5. Effects of impaired catalase, lipoxygenase on gene expression of ripening tomato fruits. Alka Srivastava, Tatsiana Datsenka, Lauren McIntyre and Avtar K Handa (USA)

Abstracts:

1. Enhanced catalytic activity of phospholipid hydroperoxide glutathione peroxidase interferes with plant regeneration (Abstract number 70), Plant Biology 2004: Friday, July 24 - Wednesday July 28, 2004 - Lake Buena Vista, FL USA (Presenter: Eshdat, Yuval) Faltin, Zehava, Perl, Avi, Velcheva, Margarita, Tsapovetsky, Marina, Holland, Doron, Roeckel-Drevet, Patricia, Handa, Avtar K , Abu-Abied, Muhamad, Friedman-Einat, Miriam, Eshdat, Yuval
2. Perla V., Datsenka T., Srivastava A., Handa AK. Physiological genetics of transgenic tomato harboring multiple transgenes. 2nd International Congress of Plant Physiology, New Delhi, January 8-12, 2003.
3. Srivastava A., Datsenka T, Perla V., McIntyre LM. and Handa AK. Characterization of global gene expression during tomato fruit ripening using cDNA-bacterial macroarray. 2nd International Congress of Plant Physiology, New Delhi, January 8-12, 2003.

APPENDIX – UNPUBLISHED DATA

EXPERIMENTAL, RESULTS AND DISCUSSION

1. USA

1.1. Tomato transformation with PHGPx under the control of CaMV 35S promoter: The chimeric gene construct 35S-PHGPx-pCambia2301, sent by our Israeli collaborator, was used to transform tomato following a protocol previously standardized in our laboratory (Tieman et al., Plant Cell 4:667-679 (1992)). Using this protocol we routinely obtain over 25 % rate of Agrobacterium based tomato transformation. However, we were unable to obtain transgenic plant using the 35S-PHGPx-pCambia2301 construct. In the same set of experiments, however, we were able create transgenic tomato plant expressing either vector alone or another vector containing a heat shock gene. As we understood later, based on the work done by Dr Eshdat group in Israel, this was primarily due to the inhibitory effect of PHGPx on regeneration of plant cells. These result, however, confirm the observation made by our Israeli collaborators that PHGPx plays significant role in plant cell regeneration potential.

1.2. Crossing of genetically engineered tomato with reduced lipoxygenase (LOX) with transgenic tomato with reduced catalase (CAT) and enhanced polyamine levels (SAMde): We have previously created transgenic tomato with reduced lipoxygenase activity by expressing a truncated gene for a tomato lipoxygenase under the control of 35S CaMV promoter. Since we were not able to create tomato line expressing PHGPx under the 35S promoter we used a line with reduced catalase activity by expressing an antisense gene for catalase. This line was created in Dr. Woodson laboratory at Purdue. We have generated earlier several lines that over express S-adenosine methionine decarboxylase under the control of a fruit specific promoter E8 (Mehta et al., 2002). These tomato lines (SAMde) accumulate several fold higher levels of spermine and spermidine. Since polyamines have been implicated in protecting DNA from oxidative stress we evaluated the role of enhanced polyamine in the presence and absence of LOX on the field performance of tomatoes.

Crosses were generated between the co-suppressed lipoxygenase and antisense catalase lines and over-expressing SAMdc line. Before crossing, lines homozygous for the each transgene were selected by analyzing the progenies from the segregating populations of the self crosses for each

of the transgene. The seeds from these crosses were grown in the green house and evaluated for presence of the truncated lox and of either anti-catalase or SAMde using PCR. Several independent plants having both genes were selected and selfed. The segregating selfed F1 progenies of the LOX-CAT and LOX-SAMde crosses were characterized for phenotypes and growth attributes to evaluate role of LOX and catalase impairment on plant growth, fruit set, fruit development and ripening and fruit shelf life (Table1.1 and 1.2). About 50 segregating progenies of each self cross were grown at the Purdue Research Farms and seeds from each progeny were collected for the homozygosis analysis. Seeds for each progeny were germinated in the green house and tested for the presence of various transgene by PCR (Table1.3 and 1.4). Lines homozygous for two transgenes were selected for evaluating the effect of simultaneous modification of expression of two genes.

1.3. Field trial and data analysis: Field trial of transgenic tomato line with its wild genotype as a control was conducted at Meig's Farm, Purdue University during the summer with randomized block design concept. Each line tested consists of 3 replications with 20 plants in each replication. At about 40 days after sowing (DAS), seedlings of each tomato line from green house were hardened for about 10 days and transferred to raise beds spaced at 0.24 m distance with plastic mulch and drip irrigation. The spacing between plants in each row (bed) was maintained at 0.9 m. A common fertilization, weeding and plant protection operations were carried out as and when required during the study. Unless otherwise stated, all the observations were taken from four random plants from each replication at flowering (70 DAS, when most of the plants are at active flowering), ripening initiation (125 DAS, when $\leq 5\%$ of the fruits were at breaker and/or red stage), and harvesting (155 DAS, when $>70\%$ of the fruits were at breaker and/or red stage). Fruits with $\leq 10\%$ of orange color at the blossom end were considered as breaker fruits. Whereas, the fruits with $>90\%$ of red color were treated as red fruits. Many agronomical characteristics and fruit attributes were quantified, data presented is that obtained at the time of harvest (Table1.5 and 1.6)

Data collected was analyzed with Microsoft Excel software package and the significant differences were assessed with probability associated with Student's *t*-test with two-tailed distribution.

1.4. Effects of lipoxygenase and catalase under expression on the agronomical performance and fruit quality attributes in tomato plants: Tables 1.5 and 1.6 show the effects of under

expression of lipoxygenase, catalase or both on agronomical performance and fruit quality attributes of the genetically engineered tomato plants. Under expression of lipoxygenase had little effect on any of the agronomical performance parameter evaluated (table 1.5). However, under expression of catalase affected plant height, total fresh and dry weights accumulation of the whole plants or the shoots (Figures 1.1 and 1.2). Also number of fruits and total fruit yield were negatively impacted by lowering the catalase activity (Figures 1.3 and 1.4). Interestingly the under expression of catalase has a very strong effect on the fruit shape. As shown in Figure 1.6, fruit became more elongated and oval shaped. Catalase antisense fruits also had significantly higher levels of total soluble solids, but little effect on the juice pH (Figure 1.7).

The novel results were obtained when the transgenic tomatoes harboring both antisense catalase and cosuppression causing lipoxygenase truncated gene were field tested. Almost all effects of low catalase activity, except the effect on fruit shape, disappeared (Table 1.5 and 1.6: and Figures 1.1 -1.7). These results clearly demonstrate crosstalk between the oxidative stress induced and lipoxygenase regulated metabolic events. Further understanding of these processes would greatly help understanding the mechanisms that protect plants under the oxidative stress.

1.5. Effects of lipoxygenase under expression and SAMde over expression on the agronomical performance and fruit quality attributes in tomato plants: As stated previously we have created transgenic tomato plant that over-express a yeast S-adenosylmethionine decarboxylase (ySAMdc) gene under the control of a ripening- and ethylene inducible E8 promoter which results in several-fold increase in the levels of spermidine and spermine with about 3-fold increase in lycopene levels (Nature Biotechnology 20: 613-618 (2002)). Since polyamine are known to have anti-senescence properties, we were interested in determining the effects of increased spermine and spermidine on transgenic tomato with reduced membrane damage due to impaired lipoxygenase expression. Tables 1.5 and 1.6 show the effects of under expression of lipoxygenase, over expression of SAMde or both on various agronomical performance and fruit quality attributes of the genetically engineered tomato plants. Over expression of SAMde greatly influenced the growth potential of tomato plants. Increased plant height, better biomass accumulation and higher fruit yield were obtained in SAMde transgenic lines compared to wild type plants (Figures 1.1 to 1.7). However, most effects of increased polyamines on plant growth and development were not observed in genotype with reduced lipoxygenase background. It is likely the lox impaired lines act as dominant phenotype that overcome effects of introduced SAMde gene.

1.6 Development of tomato macroarray: For development of the tomato cDNA microarray, a normalized tomato fruit cDNA library was made from the poly A+ RNA isolated from the turning stage fruits. Over 5000 independent bacterial colonies having the tomato EST were printed on nylon membranes and hybridization to cDNA probes made to several stages of fruit development and ripening. Independent bacterial colonies harboring tomato ESTs that showed detectable signals were PCR amplified using T3 and T7 primers to create tomato cDNA macroarray. The cDNA array comprised of 1401 individual cDNAs; the remaining 135 spots included positive and negative control spots and the standards. The sequencing reaction failed to generate sequence output for 30 cDNAs thereby showing a 94% success. For the remaining 1371 cDNAs, the average length of the sequences was 500 bp. Sequences were compared to the public databases (NCBI, TIGR tomato gene index) using the BLASTX and BLASTN programs for homology and an e-value of 10^{-4} was used as a cut-off to select the sequence match. When compared to the sequences in the NCBI site, 8% of the sequences did not show homology to any of the available sequence in that database. Amongst the cDNAs that showed homology to the sequences in the databases, 60% showed similarity to known sequences. When compared to the TIGR tomato gene index, 124 sequences showed no homology whereas 293 sequences did not show significant homology to the sequences present in the TIGR tomato gene index database. Amongst the 1371 cDNA sequences analyzed, 1066 unique sequences were present on the array. The 525 sequences were grouped in 202 clusters/220contigs.

In an attempt to categorize the ESTs into functionally similar groups, the sequences were compared to the clusters of orthologous groups of proteins (COG) database and the keywords for the gene identity or the accession number of the top match for the sequence were searched against the TIGR and MIPS databases. The cut off e-value for selecting the best match was 10^{-4} . The ESTs were grouped in 14 different categories on the basis of sequence comparison with the COG, TIGR and MIPS databases. The ESTs that did not show homology to any of the sequences in the databases searched were designated as novel ESTs. The ESTs with no definite biological functions predicted were designated as unclassified. ESTs showing matches to sequences for proteins with unknown function in the public database and those that were below the cut off e-value of 10^{-4} were classified as unknown. We classified the proteins with known functional annotations into 12 categories. The ESTs assigned to the unclassified and unknown category abounded (48.6%), whereas those designated as novel comprised 7.5% of the library. A schematic representation of the ESTs with known functional classification revealed the largest group to be that of the ESTs assigned with metabolic function (23.4%), followed by those

involved in protein biosynthesis and degradation (16%) and signal transduction (12%). The ESTs were submitted to the NCBI database and have been assigned the database accession numbers CD002010 - CD003544.

1.7. Global transcriptional regulation of gene expression during fruit development and ripening:

To determine the effect of the introduced transgenes on fruit gene expression we used the tomato array developed at Purdue (see 1.6) to study the development and ripening process in tomato fruit and expression of fruit ESTs in leaf tissue. Among the 1066 unique and quantifiable genes present on the macroarray, 356 are differentially expressing ($p < 0.05$) at various stages of fruit development. The largest percentage amongst these genes are assigned to metabolism related functions (12 %) followed by those implicated in protein biosynthesis and degradation (11.8%). Surprisingly over 8 % ESTs represented defense and stress related genes indicating ripening analogous to a stress process. The efficacy of macroarray data was confirmed by northern and real time PCR analysis. Clustering of differentially expressed genes into five expression pattern groups revealed that 146 were differentially expressed during early development, 66 at the onset of ripening and 151 during ripening and senescence stages. Mature green stage was transcriptionally the most quiescent with only 9 ESTs showing more than two fold increases. Several ESTs with unknown biological functions showed similar expression profiles indicating that they play significant roles in fruit development and ripening. Taken together, our results provide a basis for identifying many additional genes that are either directly or indirectly play roles in fruit development and ripening.

1.8. Effect of modified oxidative stress on global gene expression: To evaluate the effects of modified oxidative stress on gene expression in ripening fruit we collected fruits from six stages of development and pericarp frozen at -70°C for quantifying gene expression using tomato diagnostic macroarray. Table 1.7 shows the effects of under expression of catalase and lipoxigenase on the expression of various classes of genes. Catalase has very noticeable effect on gene expression with about 37 % of all genes impacted by its low expression. Under expression of lipoxigenase also effected gene expression during fruit ripening but to a lesser extent. Interestingly simultaneous reduction in both catalase and lipoxigenase had much less effect indicating that reduction in lipoxigenase ameliorates the effect generated by low catalase activity. This result strongly support our earlier observation that reduced lipoxigenase can help elevate effects of oxidative stress (Table 1.5 and 1.6).

Tables 1.8 and 1.9 show the genes that are either up regulated or down regulated by the introduced catalase antisense and lipoxygenase truncated cosuppression gene. It is note worthy that expression of a number of genes that were down regulated by catalase low expression returned to normal levels in the low-lipoxygenase background (Table1.9). Similar results were obtained for genes whose expression was impaired by low-lipoxygenase but returned to normal levels in the presence of low cat background. Collectively these results support hypothesis that there is a cross talk between cat-regulated and lipoxygenase regulated gene expression during fruit ripening.

Table 1.1: Selection of line homozygous for both LOX-cosuppression and CAT-antisense

Segregant #	LOX-PCR	CAT-PCR	BOTH-transgenes	Growth	Fruit set	Disease Symptoms
1	?					
2	?			Excellent	V. Good	NONE
3	+	+	YES	Excellent	V. Good	SV
4	+	+	YES	Excellent	Good	NONE
5	+	+	YES	Excellent	V. Good	NONE
6	+			Excellent	V. Good	NONE
7	+	+	YES	Excellent		SC
8		+		V. Good	V. Good	MV
9	+	+	YES	Excellent		NONE
10	+	+	YES	Excellent	V. Good	NONE
11		+		V. Good	Excellent	NONE
12	+	+	YES	V. Good	V. Good	NONE
13	+	+	YES	Excellent	Excellent	NONE
14	+			Excellent	Excellent	NONE
15	+			Excellent	V. Good	NONE
16	+	+	YES	Excellent	V. Good	MC
17	+			Excellent	V. Good	SC
18	+	+	YES	V. Good	V. Good	NONE
19	+	+	YES	Excellent	Good	NONE
20	+	+	YES			
21	+	+	YES	V. Good	Good	NONE
22		+		Excellent	Good	NONE
23	+	+	YES	Good	Fair	NONE
24	+	+	YES	Excellent	Good	NONE
25	+	+	YES	Excellent	V. Good	MC
26	+	+	YES	Excellent	V. Good	NONE
27	+			Excellent	V. Good	NONE
28	+	+	YES	V. Good	Good	NONE
29	+			V. Good	Good	HV
30	+			V. Good	Good	NONE
31		+		Excellent	Excellent	MC
32	+	+	YES	Excellent	V. Good	LC
33	+			Excellent	Excellent	NONE
34	+	+	YES	V. Good	V. Good	HV
35		+		Excellent	Excellent	HC
36	+	+	YES	V. Good	Good	NONE
37	+			Excellent	Excellent	MC
38	+	+	YES	Excellent	Excellent	SC
39	+	+	YES	Excellent	Good	NONE

40	+	+	YES	V. Good	Good	NONE
41	+			V. Good	Good	NONE
42		+		Excellent	Excellent	HC
43		+		Excellent	Excellent	HC
44	+			V. Good	Good	NONE
45	+			V. Good	Good	NONE
46	+			Excellent	Excellent	SC
47		+		Excellent	Excellent	NONE
48	+	+	YES	Excellent	V. Good	NONE
49	+	+	YES	Excellent	Excellent	MC
50	+	+	YES	Excellent	V. Good	HC

Table 1.2: Selection of line homozygous for both LOX-cosuppression and SAMde Over-expressed

Segregant #	LOX-PCR	SAM-PCR	Both transgenes	Growth	Fruit set	Disease Symptoms
1	+		No	V. Good	Excellent	M
2	?		No	Excellent	Excellent	none
3	+	+	Yes	Excellent	V. Good	none
4	+	+	Yes	Excellent	Excellent	none
5		+	No	Excellent	Excellent	none
6			No	V. Good	V. Good	none
7	+		No	Excellent	Excellent	none
8	+		No	Excellent	Excellent	none
9	+	+	Yes	Excellent	Excellent	L
10	+	+	Yes	V. Good	V. Good	M
11	+		No	Excellent	Excellent	none
12	+		No	Excellent	Excellent	M
13	+	+	Yes	V. Good	V. Good	none
14	+	+	Yes	Excellent	Excellent	none
15	+	+	Yes	Excellent	Excellent	none
16	+		No	Excellent	V. Good	none
17	+		No	Excellent	Good	none
18	+	+	Yes	V. Good	V. Good	none
19	+	+	Yes	Excellent	V. Good	L
20	+	+	Yes	Excellent	Excellent	none
21		+	No	Excellent	V. Good	L
22		+	No	V. Good	V. Good	M
23	+	+	Yes	Excellent	Good	none
24	+	+	Yes	Excellent	Excellent	none
25	+		No	Excellent	Excellent	M
26	+	+	Yes	V. Good	V. Good	M
27		+	No	Excellent	Excellent	none
28		+	No	Excellent	Excellent	M

29	+	+	Yes	Excellent	Excellent	L
30	+	+	Yes	Excellent	Excellent	none
31	+		No	Good	Fair	H
32	+	+	Yes	Excellent	Excellent	none
33	+		No	V. Good	V. Good	none
34	+		No	V. Good	Good	none
35	+	+	Yes	Excellent	V. Good	none
36	?		No	Excellent	V. Good	none
37	?		No	Excellent	Excellent	M
38	+	+	Yes	Excellent	Excellent	none
39		+	Yes	Excellent	Excellent	none
40		+	Yes	Excellent	V. Good	L
41	+	+	Yes	Excellent	Excellent	none
42	+	+	Yes	Excellent	Excellent	none
43	+	+	Yes	Excellent	V. Good	none
44	?		No	Excellent	Excellent	none
45	+		No	Excellent	Excellent	none
46	+	+	Yes	Excellent	Excellent	L
47	+	+	Yes	Excellent	Excellent	none
48	+	+	Yes	Excellent	Excellent	M
49	+	+	Yes	Excellent		none
50	?		No	Excellent	Excellent	none

Table 1. 3. Selection of line homozygous for both LOX-cosuppression and CAT-antisense transgene

Segregant #	Progenies Tested	LOX +	CAT +	Both genes	Homozygous
4	5	3	4	3	No
5	5	5	1	1	No
7	25	24	23	24	No
9	5	5	4	4	No
10	5	5	1	1	No
12	15	13	15	13	No
16	5	2	1	1	No
18	15	13	14	13	No
19	5	5	1	1	No
20	5	5	2	2	No
23	5	5	3	3	No
24	25	25	21	21	No
25	15	14	14	13	No
26	5	2	2	2	No
28	15	15	12	12	No
32	5	4	5	4	No

34	25	25	25	25	YES
36	10	9	5	9	No
38	5	5	3	3	No
39	5	5	4	4	No
48	5	5	1	1	No
49	5	5	0	0	No
50	5	5	4	4	No
Total	225	208	169	167	1

All segregant showing the presence of both CAT-antisense and LOX-cosuppression transgenes (Table1) were tested for homozygosis. Minimum of 25 seedlings were germinated for each of these lines. Sequentially five seedlings at a time for the each segregating line were evaluated for the presences of each transgenes until all seedlings (about 25) for a line were positive for the both transgenes. Those lines which showed seedlings with the absence of any gene were not further tested.

Please note only one line out of 50 segregating progenies of this cross turned out to be homozygous and were used for field evaluation.

Table 1. 4. Selection of line homozygous for both LOX-cosuppression and SAMde over expression

Segregant#	Progenies	LOX +	SAM +	Both genes	Homozygous
#	Tested				
3	20	19	19	18	No
4	25	25	25	25	Yes
5	5	4	3	1	No
9	15	14	15	14	No
10	5	4	5	4	No
13	5	3	5	2	No
14	9	7	5	7	No
15	5	5	3	3	No
18	9	9	4	8	No
19	5	3	5	3	No
20	5	3	3	1	No
23	5	3	4	2	No
24	5	2	4	1	No
29	5	4	4	23	No
30	5	4	3	2	No
35	24	24	5	24	Yes
38	24	24	5	24	Yes
41	23	23	5	23	Yes
42	5	4	3	2	No

43	9	6	5	6	No
46	17	16	5	16	No
47	5	4	5	3	No
48	5	3	4	2	No
Total	240	213	144	214	4

All segregant showing the presence of both SAMde-over expression and LOX-cosuppression transgenes (Table1) were tested for homozygosis. Minimum of 25 seedlings were germinated for each of these lines. Sequentially five seedlings at a time for the each segregating line were evaluated for the presences of each transgenes until all seedlings (about 25) for a line were positive for the both transgenes. Those lines which showed seedlings with the absence of any gene were not further tested.

Please note four line out of 50 segregating progenies of this cross turned out to be homozygous and were used for field evaluation.

Table 1. 5: Effects of lipoxygenase under expression, catalase under expression and SAMde over expression either individually or in combination on agronomical performance of tomato plants

Parameter	Lox impaired	Cat-Impaired	SAMde Over-expression	CAT/LOX	LOX/SAM	Figure
Harvest index	NS	NS	NS	NS	NS	
Plant height, cm	NS	S – (p< 0.01)	S + (p< 0.01)	NS	NS	Fig. 1
Total plant wt	NS	S – (p< 0.03)	S + (p< 0.02)	NS	NS	Fig. 1
Shoot fresh weight	NS	S – (p< 0.03)	NS	NS	NS	Fig. 2
shoot Dry Wt of	NS	S – (p< 0.04)	NS	NS	NS	Fig. 2
# fruit/plant	NS	S – (p< 0.02)	S + (p< 0.01)	NS	NS	Fig. 3
# Red fruits	NS	NS	S + (p< 0.05)	NS	NS	Fig. 3
# Green fruits	NS	NS	NS	NS	NS	
# Breaker stage fruits	NS	NS	NS	NS	NS	
# ripening fruit	NS	NS	NS	NS	NS	
Total Fruit weight/plant	NS	S – (p< 0.04)	S + (p< 0.02)	NS	NS	Fig. 4
weight Red fruits	NS	NS	S + (p<0.04)	NS	NS	Fig. 4
Weight Green fruits	NS	NS	NS	NS	NS	

weight Breaker stage fruits	NS	NS	NS	NS	NS	
Avg Fruit wt	NS	NS	NS	NS	NS	

Wild-type parental (Ohio 8245), genetically engineered line impaired in lipoxygenase or catalase and over expressing SAMde, and crosses between LOX-impaired and cat-impaired and SAMde over expressing lines were grown at the Purdue research farms. All lines were homozygous for the introduced gene/genes.

Data is based on three replicates each consisting of 2-3 plants grown using a randomize field design.

S and NS represent significantly or not significantly different from the wild type where as the + and – indicate positive vs. negative effect of introduced gene /genes.

Data for parameters that showed significant differences is shown in indicated figure number.

Table 1.6: Effects of lipoxygenase under expression, catalase under expression and SAMde over expression either individually or in combination on tomato fruit attributes

Parameter	Lox impaired	Cat- Impaired	SAMde Over- expression	CAT/LOX	LOX/SAM	Figure
fruit weight, g	NS	NS	NS	NS	NS	
fruit length, mm	NS	S + (P<0.02)	NS	NS	S + (p<0.03)	Fig. 5
widest width, mm	NS	S - (P<0.02)	S + (P<0.02)	NS	S - (p<0.02)	Fig. 5
length between widest width and peduncle region, mm	NS	NS	NS	NS	NS	
Fruit pear shape index	NS	NS	NS	NS	NS	Fig. 6
Fruit oval shape index	NS	S - (P<0.003)	NS	S - (p<0.01)	S - (p<0.01)	Fig. 6
thickness of outer pericarp, mm	NS	NS	NS	NS	NS	
thickness of inner pericarp, mm	NS	NS	NS	NS	NS	
No of locules	NS	NS	NS	NS	NS	
TSS	NS	S + (p<000)	NS	S + (p< 0.02)	NS	Fig. 7
pH	S + (p< 002)	NS	S + (p<014)	NS	S + (p< 0.013)	Fig. 7

Wild-type parental (Ohio 8245), genetically engineered line impaired in lipoxygenase or catalase and over expressing SAMde, and crosses between LOX-impaired and cat-impaired and SAMde over expressing lines were grown at the Purdue research farms. All lines were homozygous for the introduced gene/genes.

Data is based on fruits randomly selected from at least three plants from three independent replicates. However, for fruit size and shape analysis only fruits having fresh weight between 35 and 45 g were used.

S and NS represent significantly or not significantly different from the wild type where as the + and – indicate positive vs. negative effect of introduced gene /genes.

Data for parameters that showed significant differences is shown in indicated figure number.

Table 1.7: Effect of introduced catalase antisense and lipoxygenase truncated gene (cosuppression) on global gene expression in ripening tomato fruits

Classification	No of ESTs on array	Cat regulated	LOX regulated	CATLOX regulated
		% total	% total	% total
Cell structure and maintenance	16	56	25	19
Cell wall	29	28	14	17
Defense /stress response	78	31	8	19
DNA replication repair recombination	11	55	64	27
Energy	41	46	17	24
Ethylene response	37	38	22	11
Metabolism	155	36	8	25
Protein biosynthesis/degradation	106	42	18	34
Signal transduction	79	43	28	25
Transcription	55	40	49	25
Transport	52	33	87	19
Unclassified	306	40	19	21
Unknown	291	45	24	23
Novel	113	35	16	19
Total	1371	37	21	21

Table 1.8: Differential up regulation of gene expression by introduced catalase antisense (CAT) and truncated lipoxygenase (LOX) at the breaker stage of ripening

Accession number	classification	CAT-up regulated	LOX-up regulated	CATLOX-up regulated
		Expression Ratio		
		CAT/WT	LOX/WT	CATLO/WT
Expression up regulated by CAT but not in the presence of LOX				
CD002592	cell structure and maintenance	1.51		
CD002307	cell structure and maintenance	1.55		
CD003209	defense/stress response	1.93		
CD003211	Defense/stress response	2.07		
CD003207	defense/stress response	2.13		
CD003221	Defense/stress response	2.27		
CD003002	DNA replication repair recombination	1.52		
CD002473	energy	1.52		
CD002859	energy	1.73		
CD002918	energy	1.81		
CD003216	ethylene response	1.54		
CD003218	Ethylene response	1.95		
CD003224	metabolism	1.92		
CD002836	metabolism	1.96		
CD002591	Novel	1.64		
CD002546	Novel	1.67		
CD002444	Novel	1.70		
CD002492	Novel	1.71		
CD002623	Novel	1.88		
CD003469	Novel	1.88		
CD002475	protein biosynthesis/degradation	1.75		
CD003183	protein biosynthesis/degradation	1.84		
CD002873	protein biosynthesis/degradation	1.95		
CD002594	transcription	1.71		
CD003232	transport	1.90		
CD003360	unclassified	1.69		
CD002536	unclassified	1.72		
CD003248	unclassified	1.87		
CD002868	unclassified	1.99		
CD003242	unknown	1.51		
CD002401	unknown	1.51		
CD002952	unknown	1.57		
CD003219	unknown	1.68		

CD002829	unknown	1.87		
CD003495	unknown	2.31		
Expression up regulated by LOX but not in the presence of CAT				
CD002843	transport		1.74	
CD003132	unknown		1.57	
Expression up regulated in the presence of both CAT and LOX				
CD002654	energy			1.60
CD002825	metabolism			1.68
CD003092	metabolism			1.57
CD002807	protein biosynthesis/degradation			1.80
CD003039	protein biosynthesis/degradation			1.58
CD003096	protein biosynthesis/degradation			1.97
CD002973	signal transduction			1.50
CD002808	transcription			2.32
CD002980	transcription			1.52
CD002982	unknown			1.64
Expression up regulated by CAT in the presence or absence of LOX				
CD002196	energy	2.35		1.56
Expression up regulated by LOX in the presence and absence of CAT				
CD003125	protein biosynthesis/degradation		1.54	1.81

Table 1.9: Differential down regulation of gene expression by introduced catalase antisense (CAT) and truncated lipoxygenase (LOX) at the breaker stage of ripening

Accession number	Classification	Expression ratio		
		CAT-down regulated	LOX-down regulated	CATLOX down regulated
		CAT/WT	LOX/WT	CATLOX/WT
Expression impaired by CAT but not in the presence of LOX				
CD002954	cell structure and maintenance	0.47		
CD002855	cell wall	0.34		
CD002820	defense/stress response	0.39		
CD003093	Defense/stress response	0.47		
CD002937	defense/stress response	0.50		
CD002606	defense/stress response	0.51		
CD002740	defense/stress response	0.64		
CD002021	energy	0.52		
CD002720	energy	0.63		
CD002177	energy	0.64		

CD002044	metabolism	0.36		
CD003077	metabolism	0.38		
CD003003	metabolism	0.40		
CD002850	Metabolism	0.46		
CD002802	metabolism	0.47		
CD003483	metabolism	0.51		
CD002892	metabolism	0.52		
CD002804	metabolism	0.62		
CD002528	Novel	0.43		
CD002939	Novel	0.48		
CD002913	protein biosynthesis/degradation	0.36		
CD002521	protein biosynthesis/degradation	0.36		
CD002805	protein biosynthesis/degradation	0.41		
CD003000	protein biosynthesis/degradation	0.50		
CD002203	protein biosynthesis/degradation	0.52		
CD002890	protein biosynthesis/degradation	0.58		
CD002814	Signal transduction	0.36		
CD003104	signal transduction	0.47		
CD003102	signal transduction	0.48		
CD002894	signal transduction	0.54		
CD002292	signal transduction	0.55		
CD002205	signal transduction	0.64		
CD002935	signal transduction	0.65		
CD002210	transcription	0.62		
CD002818	transcription	0.40		
CD002388	transcription	0.60		
CD003103	transport	0.39		
CD003099	transport	0.46		
CD002718	unclassified	0.45		
CD002994	unclassified	0.33		
CD002284	unclassified	0.45		
CD002979	unclassified	0.51		
CD003012	unclassified	0.52		
CD003110	unclassified	0.53		
CD003047	unclassified	0.54		
CD002715	unclassified	0.56		
CD003051	unclassified	0.57		
CD002281	unclassified	0.58		
CD002154	unclassified	0.60		
CD002915	unclassified	0.62		
CD002364	unclassified	0.64		

CD002806	unclassified	0.67		
CD002366	unknown	0.26		
CD002925	unknown	0.28		
CD002857	unknown	0.29		
CD003066	unknown	0.29		
CD003008	unknown	0.43		
CD002900	unknown	0.44		
CD002813	unknown	0.44		
CD002902	unknown	0.44		
CD002970	unknown	0.46		
CD003109	unknown	0.48		
CD003033	unknown	0.48		
CD002993	unknown	0.48		
CD002181	unknown	0.49		
CD002042	unknown	0.50		
CD003015	unknown	0.51		
CD003055	unknown	0.51		
CD002919	unknown	0.52		
CD002978	unknown	0.54		
CD003098	unknown	0.54		
CD003107	unknown	0.56		
CD003049	unknown	0.57		
CD003065	unknown	0.57		
CD003020	unknown	0.58		
CD003088	unknown	0.58		
CD003044	unknown	0.58		
CD002878	unknown	0.59		
CD003091	unknown	0.60		
CD002849	unknown	0.60		
CD002989	unknown	0.60		
CD002811	unknown	0.61		
CD002958	unknown	0.62		
CD002726	unknown	0.64		
CD003031	unknown	0.65		
CD003087	unknown	0.66		
CD002861	unknown	0.67		
Expression impaired by LOX but not in the presence of CAT				
CD002167	metabolism		0.52	
CD002927	metabolism		0.53	
CD002951	metabolism		0.60	

CD002967	metabolism		0.62	
CD003113	metabolism		0.65	
CD002447	Novel		0.49	
CD003016	protein biosynthesis/degradation		0.59	
CD002133	protein biosynthesis/degradation		0.61	
CD002529	signal transduction		0.55	
CD002705	signal transduction		0.58	
CD002480	signal transduction		0.64	
CD002360	transcription		0.60	
CD002126	transcription		0.62	
CD003265	unclassified		0.57	
CD003070	unclassified		0.60	
CD002204	unclassified		0.65	
CD003373	unclassified		0.66	
CD002968	unknown		0.47	
CD002956	unknown		0.49	
CN550646	unknown		0.58	
CD002860	unknown		0.62	
CD003301	unknown		0.67	
Expression impaired by CAT both in the absence and presence of LOX				
CD002438	Novel	0.51		0.55
CD002969	signal transduction	0.37		0.60
CD002165	unclassified	0.61		0.62
Expression impaired by LOX both in the absence and presence of CAT				
CD002482	DNA replication repair recombination		0.63	0.54
CD002923	protein biosynthesis/degradation		0.47	0.56
CD002483	unclassified		0.59	0.65
Expression impaired by the presence of either CAT or LOX				
CD002856	DNA replication repair recombination	0.53	0.63	
CD002479	metabolism	0.58	0.41	
CD002441	Novel	0.63	0.49	
CD002166	unclassified	0.40	0.51	
CD003005	unclassified	0.66	0.60	
CD002442	unknown	0.46	0.61	0.46
Expression impaired by the simultaneous presence of both LOX and CAT				
CD002522	cell wall			0.63
CD002690	Defense/stress response			0.66
CD002910	energy			0.66

CD002967	metabolism			0.64
CD003427	Novel			0.53
CD002123	protein biosynthesis/degradation			0.65
CD003467	Signal transduction			0.67
CD003298	transcription			0.59
CD003419	transport			0.52
CD002187	unclassified			0.59
CD002500	unclassified			0.51
CD003276	unclassified			0.63
CN550591	unknown			0.60

Figure 1.1: Effects of lipoxigenase under expression, catalase under expression and SAMde over expression either individually or in combination on plant height and fresh weight at the time of harvest. Other details as in Table 5

Figure 1.2: Effects of lipoxigenase under expression, catalase under expression and SAMde over expression either individually or in combination on shoot fresh and dry weight at the time of harvest. Other details as in Table 5

Figure 1.3: Effects of lipoxigenase under expression, catalase under expression and SAMde over expression both individually or in combination on fruit set and ripening at the time of harvest. Other details as in Table 5

Figure 1.4: Effects of lipoxigenase under expression, catalase under expression and SAMde over expression either individually or in combination on total and ripe tomato fruit weight at the time of harvest. Other details as in Table 5

Figure 1.5: Effects of lipoxxygenase under expression, catalase under expression and SAMde over expression either individually or in combination on fruit length and maximum width. Other details as in Table 6

Figure 1.6: Effects of lipoxxygenase under expression, catalase under expression and SAMde over expression either individually or in combination on fruit oval and pear shapes. Pear shape was determined by dividing fruit length with the length between widest width and peduncle region. The Oval shape index was determined by dividing widest width with maximum length of fruit. Other details as in Table 6

Figure 1.7: Effects of lipoxxygenase under expression, catalase under expression and SAMde over expression either individually or in combination on total soluble solid and pH of the raw juice. Other details as in Table 6

APPENDIX – UNPUBLISHED DATA

EXPERIMENTAL, RESULTS AND DISCUSSION

2. ISRAEL

2.1 Plasmids Prepared for citrus-PHGPx Transformation in Plants

A few plasmids, all containing the same selection marker *NPTII* (encoding Neomycin phosphotransferase) but different promoters and/or target genes, were prepared and used throughout the study. These plasmids include:

35S-PHGPx: The open reading frame of *cit-PHGPx* (formally named *csa*, accession No. X66377), encoding the citrus PHGPx (cit-PHGPx), was cloned in an expression cassette (Shaul and Galili, 1992) between 35S Ω and OSC terminator (Fig 2.1.A).

Fig. 2.1: Schematic presentation of three of the plasmids used. The other 4 plasmids were based on the above constructs with the exchange of the promoter only.

2.1.1. 35S-LP-PHGPx. In order to target PHGPx to the chloroplast, *cit-PHGPx* was cloned, in the same expression cassette, in frame with the leader peptide (LP) of the Rubisco small subunit (Fig. 2.1B, Shaul and Galili, 1992).

2.1.2. Pat-PHGPx. A plasmid containing the inducible promoter of class I patatin gene B33 (accession A08215), which is strictly associated with potato tuber development, was prepared (Fig. 2.1C). Patatin glycoproteins contribute up to 40% of the tubers protein content.

Several other constructs were prepared based on the plasmids described in Fig. 2.1A & B:

2.1.3. PR1-PHGPx. In this construct the promoter of Pathogenesis Related Protein-1a (PR1) replaces the 35S+ Ω promoter in plasmid A (Fig. 2.1); it is strongly induced during the onset of systemic acquired resistance. The promoter (1kb, from base – 898 to base +19, accession X12737) was amplified from genomic DNA of *Nicotiana tabacum* cv. *Xanthi*.

2.1.4. RD29A-PHGPx and RD29A-LP-PHGPx. In this plasmid, the promoter of the stress gene RD29A 1a replaces the 35S+ Ω promoter in plasmid A (Fig. 2.1), which is induced in cold, drought, and salt stress. The promoter was amplified from genomic DNA of *Arabidopsis thaliana* (from base – 862 to +75, accession D13044). This promoter was also cloned instead the 35S+ Ω promoter in plasmid B (Fig. 2.1) containing the LP-PHGPx cassette to give RD29A-LP-PHGPx plasmid.

2.1.5. 35S-Ser⁴¹PHGPx. This plasmid carries a point mutation which leads to exchange of the catalytic amino acid cysteine 41 to serine 41 (TGT into TCT), inserted in the **35S-PHGPx** expression cassette (Fig. 2.1A), by oligonucleotide-directed site specific mutagenesis according to Kukel et al.1987. Such a change in the active site of pig PHGPx was shown to enzymatically inactivate PHGPx (Maiorino et al, 1995).

All these cloning cassettes were cloned in the binary vector pPZP111 and electroporated to *Agrobacterium* EHA105, and were used for the transformation experiments.

2.1.6. The following additional constructs were prepared as well, but they are used in current experiments which are still in progress and have not yet yielded conclusive results:

35S-APHGPx, in which *csa* is cloned in the 35S expression cassette (Fig. 2.1A) in the antisense orientation, and **35S-ALOX** in which tomato LOX, isolated in A. Handa laboratory, was also cloned in the antisense orientation in the same expression cassette. We

also plan to carry out experiments using **pER8-PHGPx** and **pER8-LOX**, in which PHGPx or LOX are cloned in pER8, provided that permission and plasmid will be kindly given from Prof. N.H. Chua (Rockefeller Univ.). This XVE binary vector is strictly regulated by estradiol.

2.2. PHGPx Transformation

2.2.1. Potato Plants

Potato plants (cv. Desiree), grown in a controlled greenhouse, were used for transformation with 35S-PHGPx and Pat-PHGPx. These vectors, constitutive and inducible, were the first to be chosen for the purpose of over-expressing of PHGPx. Following co-cultivation with *Agrobacterium* bearing these plasmids, the potato leaves were cultured on MS media supplemented with 3% sucrose, 2mg/l zeatin riboside, 0.02mg/l NAA and 0.02mg/l GA3 for shoot induction. Kanamycin (50mg/l) was used for selection and claforan (500mg/l) for *Agrobacterium* elimination. These experiments gave unexpected results since no regenerated plants were obtained using these constructs (while the usual number of regenerated plantlets was obtained when the same transformation procedure was done using the pPZP vector lacking PHGPx).

The failure in obtaining successful transformation in potato using 35S-PHGPx and Pat-PHGPx, led us to decide to examine, in model plant systems, fundamental questions which may explain possible restraints associated with PHGPx transformation and over-expression. Moreover, we designed additional inducible vectors (RD-29A and PR-1) for over-expression of PHGPx. RD-29A and PR-1 were selected based on the fact that these promoters are induced by a number of environmental and hormonal signals. RD-29A, a strong promoter, was shown to be rapidly induced by salt, drought, cold, ABA and is, like PHGPx, expressed in all tissues. PR-1, (like both LOX and PHGPx) is induced in pathogen infection, as well as by jasmonic acid and salicylic acid (SA) directly.

Hence, we have used these two, as well as the previous constructs, in attempt to establish ways to obtain transformation and gene-expression of *PHGPx* in plants, and to study possible physiological obstacles which may prevent the over-production of its encoded enzyme in the transformed plants. As a first model system we have chosen the tobacco as an obvious plant model.

2.2.2. Tobacco as a Model Plant for PHGPx Transformation

Transformation of tobacco leaves was modified according to the leaf disc method (Horsch et al 1985). The regeneration medium contained 2mg/l kinetin, 0.8 mg/l IAA and 1mg/l zeatin, supplemented with kanamycin as selection agent and carbenicillin for *Agrobacterium* elimination.

The transformation experiments with the 35S-PHGPx promoter yielded transformed rooting plants on the selective media though at a very low yield, about 5% relative to that obtained for transformation with the control plasmid lacking the PHGPx gene. Moreover, none of the transformed plants (out of 40 plants), tested by Western-blotting (WB) analysis showed expression of the citrus PHGPx (which could be distinguished from the endogenous tobacco enzyme, see Fig. 2.2), though all were shown to contain the NPTII gene and exhibited, of course, kanamycin resistance.

Fig. 2.2. Distinction between tobacco and citrus PHGPx. Western Blot of tobacco leaf extract stained by the ECL method using anti-citrus PHGPx antibodies (I). Following stripping of the citrus antibodies, colorimetric staining of the blot was done using anti-tobacco PHGPx antibodies and alkaline phosphatase (II). (1), tobacco leaves; (2), mixture of tobacco leaves extract and protein extract of recombinant *E. coli* expressing citrus-PHGPx; (3), recombinant citrus-PHGPx. Arrows point to the migration place of citrus PHGPx (A) and tobacco PHGPx (B), as determined by the reaction with specific antibodies.

Similar results were obtained when transformation of the tobacco plants were done using 35S-LP-PHGPx plasmid containing a chloroplast leader sequence (Fig. 2.1B), PAT-PHGPx (Fig. 2.1C), RD29A-PHGPx and RD29A-LP-PHGPx. In these experiments as well, a very low number of transformed plants were observed, none expressing cit-PHGPx.

A different result was obtained when PR1-PHGPx plasmid was used. This experiment yielded a similar number of tobacco plants transformed either with PR1-PHGPx or the control plasmid (lacking the PHGPx coding region). **However**, out of 45 transformed plants that were examined, **only one** plant revealed over-expression of PHGPx following induction of detached leaves with salicylic acid, which could not be detected in its F1 progeny.

Since one transformed plant that over-expressed PHGPx was obtained with this promoter, transformation of **potato** using this construct was done and indeed yielded transformed plantlets. However, out of 40 transformed plants that were examined, **none was proved to over-express the PHGPx enzyme**.

These results, obtained for both potato and tobacco plants while using a large variety of different types of plasmids, clearly demonstrated that unlike many other genes, routine transformation and over-expression of PHGPx in plants is not obvious. . It should be noted that such difficulties in PHGPx transformation and expression in several plants were recently observed in other labs as well. These difficulties, which have hampered our progress in achieving the original objectives of the research, namely the biochemical and physiological interrelationship between LOX and PHGPx in tomato and potato, raised, however, a very interesting question regarding the role and biological function of PHGPx itself which is probably linked to the inability of the plant to over-express this enzyme under the common conditions in which most plants do. One likely explanation to the "negative" results we obtained is that over-expression of PHGPx at a certain stage of the regeneration process, is harmful to the development of the transformed plantlet and leads to its death. We therefore decided to try and characterize PHGPx transformation, expression and function also in more defined cell systems. We have utilized such experiments in tobacco BY2 cell line, potato cell cultures produced by us, tobacco leaf that was directed solely towards callus development following transformation, and *Arabidopsis*, in which the transformation and gene expression can be examined while avoiding the regeneration stage. In addition, we have examined the possible effect of the catalytic ability of PHGPx as an enzyme on this disability to obtain transformed plants over-expressing this protein. For this purpose, transformation with **35S-Ser⁴¹PHGPx plasmid**, in which a point mutation which leads to exchange of the catalytic amino acid cysteine 41 to serine 41, shown to inactivate the enzyme catalytic power, was inserted in the **35S-PHGPx** expression cassette.

2.2.3. BY2 Cell Line as a Model Cell System for PHGPx Transformation

BY2 cells were transformed with 35S-PHGPx, RD29A-PHGPx and Pat-PHGPx according to Shaul et al (1996). The cells were re-cultured in fresh medium every 7 days (while reaching stationary phase). 35S-PHGPx transformed cells were harvested 6 days after culturing. Induction of pat-PHGPx and RD29A-PHGPx transformed cells was done on the 4th day of culturing. Pat-PHGPx transformed cells were induced with 10% sucrose or with 100 mM proline, and harvested 24h and 48h later. RD29A-PHGPx transformed cells were induced by addition of NaCl and ABA (0.2M and 0.05mM final concentrations, respectively), and were harvested 8h, 24h and 48h after induction.. PHGPx expression was detected by WB analysis for all three constructs (Fig. 2.3).

Fig. 2.3. Expression of citrus-PHGPx in BY2 tobacco cells. Western blots were analyzed using anti-citrus PHGPx polyclonal antibodies employing the ECL detection method. Citrus-PHGPx migrated as a single band (apparent MW=20 kD, B1 & C7) and the tobacco-PHGPx as a double band (approximately 16 & 18 kD, C1&2). Expression of cit-PHGPx in BY2 cells transformed with 3 different plasmids: **A:** BY2 cells transformed with plasmid containing **35S-PHGPx**. **B:** BY2 cells transformed with plasmid **Pat-PHGPx**. Lane 1, *E. coli* transformed with *citrus-PHGPx*; lane 2, control plasmid lacking *citrus-PHGPx*, induced with sucrose for 24h; lane 3, induction with sucrose for 24h; lane 4, induction with sucrose for 48h; lane 5, no induction; lane 6, control plasmid induced with proline for 48h; lane 7, proline induction for 48h. **C:** BY2 cells transformed with plasmid containing **RD29A-PHGPx**. Lane 1, untransformed BY2 cells induced with NaCl & ABA for 24h; lane 2, untransformed cells induced for 48h; lane 3, no induction; lane 4, induction for 8h; lane 5, induction for 24h; lane 6, induction for 48h; lane 7, *E. coli* transformed cells expressing citrus-

PHGPx; lane 8, control plasmid lacking *citrus-PHGPx* with no induction; lane 9, control plasmid induced for 24h; lane 10, control plasmid induced for 48h.

2.2.4. PHGPx Transformation in *Arabidopsis*

Arabidopsis plants were transformed by the infiltration method done according to Clough and Bent (1998), in which the developing flora are dipped in *Agrobacterium* solution and the developed seeds are germinated on selective medium for the isolation of transformants. About 90% of the plants showed PHGPx expression (14 out of 16 examined) and more than 50% exhibited high levels of PHGPx (as demonstrated in Fig. 2.4, lanes 1 & 2).

Fig. 2.4. WB analysis of *Arabidopsis* plants transformed with 35S-PHGPx. Lane 3, cit-PHGPx marker (from recombinant *E. coli*); lane 8, Non-transformed tobacco plant (control); lanes 1 & 2, and 4-7, different transformed *Arabidopsis* plants.

2.2.5. PHGPx and Ser⁴¹PHGPx Transformation in Potato Cells

Potato cell lines were induced from shoot and leaf segments of micro-propagated potato plants, cultured on solidified MS medium supplemented with 3% sucrose, 2mg/l 2,4 D and 0.2 mg/l BAP(PCIM). The callus cells were suspended in PCIM liquid medium and sub-cultured every ten days. Transformation with **35S-PHGPx** and **35S-Ser⁴¹PHGPx** was carried out by co-cultivation of the cells with the appropriate plasmid-bearing *Agrobacterium* and subsequent selection on paromomycin (75mg/l) and claforan (500mg/l) containing PCIM. The selection was done on solidified agar plates and resistant calli clusters emerged after three weeks. High level of expression of PHGPx was detected on a Western blot (Fig. 2.5) for both constructs.

Fig. 2.5. WB analysis of potato callus derived from cells transformed with **35S-PHGPx** (A) and **35S- Ser⁴¹PHGPx** (B). Two different calli clusters (1 & 2) are shown for each plasmid.

2.2.6. Transformation of Tobacco Leaves Directed for Callus Development

Tobacco leaves were transformed as described (2.2.) with **35S-PHGPx** and **35S- Ser⁴¹PHGPx**, on a callus inducing MS media containing 3% sucrose, 1mg/l 2,4-D, 2 mg/l NAA, kanamycin and claforan. The developing calli around the cut leaves were cultured on a medium containing 3mg/l NAA and 1 mg/l BAP. Several calli clusters from both constructs were examined for PHGPx expression by WB analysis (Fig. 2.6). Most (83%) of the calli clusters transformed with **35S- Ser⁴¹PHGPx** expressed the mutated protein (5 out of 6 examined) while only 22% (4 out of 18 examined) of those transformed with **35S-PHGPx** were found to express the enzyme.

Fig. 2.6. WB analysis of tobacco calli clusters derived from transformed leaves with **35S-PHGPx** (lanes 2-8) and **35S- Ser⁴¹PHGPx** (lanes 9-10). Lane 1, cit-PHGPx marker. For details see text.

2.2.7. Tobacco Plant Transformation with Mutated PHGPx

Transformation experiments with the mutated **35S- Ser⁴¹PHGPx** and with **35S-PHGPx** were carried out with tobacco leaves on a medium that enables shoot formation (described in 2.2.). As

can be seen in Fig. 2.7, many shoots were regenerated from tobacco leaves transformed with the mutated Ser⁴¹ PHGPx gene and only few in the transformed native cit-PHGPx.

Fig.2. 7. Regeneration of shoots from tobacco leaves transformed with (A) **35S-Ser⁴¹PHGPx** and with (B) **35S-PHGPx**.

The fact that tobacco plantlets and rooted plants in tissue culture transformed with the mutated Ser⁴¹ PHGPx indeed over-expressed the mutated protein, while no expression could be detected in the few plantlets recovered from the 35S-citPHGPx transformed plants, was demonstrated by Western blot analysis (Fig. 2.8). 63% of the checked plants were positive (12 out of 19 checked)

Fig.2. 8. WB analysis of tobacco plants transformed with **35S-Ser41PHGPx**. 1, **cit-PHGPx** marker (from *E. coli*); 2, **Non-transformed tobacco plant (control)**; 3-13, **different transformed tobacco plants**. A, the location of **cit-PHGPx**; B, the location of **tobacco PHGPx**.

2.3. Concluding Remarks

The results obtained in the present study clearly demonstrate for the first time that uncontrolled PHGPx over-expression in plants interferes with plantlets regeneration, probably during differentiation. Since abolishment of the putative catalytic activity of PHGPx (Eshdat et al., 1997) diminishes such effect and enables plant development when over-expression of inactive PHGPx occurs, it is likely to assume that phospholipids hydroperoxidation may be essential for normal differentiation and plant development.

2.4. References

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