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**Coding, Expression, Targeting, Import and Processing
of Distinct Polyphenoloxidase in Tissues of Higher
Plants**

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Cloning, Expression, Targeting, Import and Processing
of Distinct Polypeptide Kinase in Tissues of Higher
Animals

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ABSTRACT

Coding, Expression, Targeting, Import and Processing of Polyphenoloxidases in Higher Plants

J.C. Steffens, E. Harel, A.M. Mayer

Polyphenol oxidase (PPO) catalyzes the oxidation of phenols to quinones at the expense of O_2 . PPOs are ubiquitous in higher plants, and their role in oxidative browning of plant tissues causes large annual losses to food production. Despite the importance of PPOs to agriculture, the function(s) of PPOs in higher plants are not understood. Among other roles, PPOs have been proposed to participate in aspects of chloroplast metabolism, based on their occurrence in plastids and high K_m for O_2 . Due to the ability of PPO to catalyze formation of highly reactive quinones, PPOs have also been proposed to be involved in a wide array of defensive interactions with insect, bacterial, and fungal pests. Physiological and biochemical studies of PPO have provided few answers to the major problems of PPO function, subcellular localization, and biochemical properties.

This proposal achieved the following major objectives: cloning of PPO cDNAs in potato and tomato; characterization of the tomato PPO gene family; antisense downregulation of the tomato PPO gene family; and reduction in post-harvest enzymic browning of potato through expression of antisense PPO genes under the control of tuber-specific promoters. In addition, we established the lumenal localization of PPO, characterized and clarified the means by which PPOs are imported and processed by chloroplasts, and provided insight into the factors which control localization of PPOs. This proposal has thereby provided fundamental advances in the understanding of this enzyme and the control of its expression.

OBJECTIVES

The original objectives of this proposal were to:

- 1) Characterize and compare the coding sequences of cDNAs representing 45 and 59 kD PPOs, which appear to differ in their tissue and subcellular localization, and in their molecular properties;
- 2). Compare the properties of plant PPOs synthesized in *E. coli* with those exhibited by the same enzymes *ex planta* to determine the properties of the enzymes in the absence of endogenous phenolics;
- 3). Attempt to understand the function(s) of PPO by generating transgenic plants with increased or decreased PPO levels; and
- 4). Study the sorting of PPOs within the cell, how they are imported into their respective organelles and how they are targeted for insertion into membranes.

Because of the discrepancy between the requested and obtained level of funding, we revised the objectives in proportion to the resources provided. The revised objectives of this proposal included:

- 1). Characterization and comparison of the coding sequences of 45 kDa and 59 kDa PPO;
- 2). Comparison of properties of plant PPOs synthesized in *E. coli* with those of PPO isolated from plant tissue; and
- 3). To determine how PPOs are imported into organelles and how they are targeted for insertion into membranes.

Progress toward meeting these objectives is described below.

RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

cDNA cloning and expression of potato polyphenol oxidase

Using tomato PPO cDNAs isolated by screening a tomato cDNA expression library with anti *Solanum berthaultii* PPO antibody (1, 2), two PPOs were cloned from *Solanum tuberosum* leaves. These cDNAs exhibited 97% and 98% similarity at the DNA and deduced amino acid levels, respectively. Putative copper-binding regions of both cDNAs are very similar to those of bacterial, mammalian, and fungal tyrosinases. Both leaf PPO cDNAs appear to encode precursor polypeptides which are processed to a mature molecular weight of 57,000. No evidence was seen for genes encoding 45 kD PPOs. In potato leaves, petioles, roots, tubers and flowers, PPO is encoded by *ca.* 2 kb transcripts. Leaf PPO mRNA is developmentally regulated and only detectable in young foliage. In contrast, the protein profile of immunologically detectable PPO remains constant from the apical node through the eleventh leaf node (3).

Organisation of the tomato PPO gene family.

In tomato the PPO gene family consists of seven members (PPOs A, A', B, C, D, E, and F) which fall into three structural classes (I, II, and III) based on *Eco* R1 and *Hind* III restriction fragment length polymorphisms (RFLP). RFLP mapping and pulsed field gel electrophoresis demonstrated that the genes reside on chromosome 8, and are clustered within a region which does not exceed 165 kbp. Phage insert mapping demonstrated that PPO E and PPO F (both Class III), and PPOs B, D, and A (Classes I, II, and III, respectively) are grouped within two separate 12.4 kbp clusters. PPOs A' and C are found on separate phages. The complete nucleotide sequence was determined for each gene. No evidence was seen for the existence of genes encoding 45 kD PPOs. Comparison to cDNAs revealed that all seven tomato PPO genes lack introns. A transcript of about 2 kb is expected for each PPO. Each PPO possesses a region encoding a transit peptide characteristic of polypeptides targeted to the thylakoid lumen. Predicted precursor polypeptides range in mass from 66 to 71 kD and predicted mature polypeptides range from 57 to 62 kD. All the PPOs encode two putative copper-binding sites characteristic of bacterial, fungal and mammalian tyrosinases. Five of the seven genes possess divergent DNA sequences in their 5' promoter regions. These flanking sequences may regulate the differential expression of PPO genes (4).

Import and Processing of PPO by plastids

The import and targeting of PPO were studied in detail. Part of our observations were summarized in a paper (6). Other chapters, dealing with the stromal processing peptidase, the inhibition of uptake and processing by copper ions, etc., are being prepared for publication (10) or are being completed for this purpose. The two step routing of pPPO into chloroplasts, described below, is typical of thylakoid lumen proteins. It is consistent with the two-domain structure of the transit peptide, a feature of all plant PPO genes isolated to date. No evidence was found for the unorthodox routing mechanisms previously been suggested to be involved in the import of PPOs. However, this two-step routing mechanism may account for some of the multiplicity of PPO observed *in vivo*.

We subcloned the tomato PPO-B cDNA, Hy-19, into pGEM-7Z f(+). PPO B was transcribed *in vitro* using SP6 RNA polymerase and the mRNA obtained was translated with reticulocyte lysate in the presence of ³⁵S-methionine. The 67 kDa translation product obtained was used to study the import, targeting and processing of PPO by isolated plastids of tomato, pea, maize and mangold (*Beta vulgaris*) chloroplasts. The size and localization of the predominant processed form obtained depended on the plant species, growth conditions and developmental stage of the leaves from which plastids were isolated. In both pea and tomato chloroplasts, PPO was targeted to the lumen, as expected from the deduced sequence of the transit peptide. Routing took place in two steps. The precursor was first imported into the stroma by an ATP-dependent process in which its N-terminal portion was removed by a stromal peptidase. The resulting ca. 62 kDa intermediate

continued to the thylakoid lumen in a light-dependent reaction. The second processing step, which produces the mature 59 kDa polypeptide, occurred only in the presence of thylakoids.

Tomato chloroplasts processed the PPO precursor completely to its mature 59 kDa form. Pea plastids accumulated both the 62 kDa intermediate and the mature 59 kDa forms. The relative amounts of the two forms depended on leaf age, illumination regime and mineral nutrition. In maize leaf etiochloroplasts, only the intermediate 62 kDa stromal form was observed. It is not known whether either the precursor or processed forms have enzymatic activity.

The two processing steps involved in targeting pPPO into the thylakoid lumen have been studied in greater detail. The stromal processing activity, which converts the 67 kD precursor to the 62 kD intermediate, was partially purified by sucrose gradient centrifugation and HPLC size exclusion chromatography. The enzyme was inhibited by low concentrations of Cu^{2+} (1 μM and lower) and by some chelators, notably 1,10-orthophenanthroline. The stromal processing peptidase (SPP) catalyzed the processing of all the tomato PPO precursors examined (PPO A,B,C and E) although at different rates. Highest activity was observed with PPO A, where more than 90% conversion of the 67 to 62 kD was observed after 90 min incubation. The precursors of plastocyanin, Rubisco small subunit, and Subunit II of PS I reaction center were also processed by the purified SPP. However, the precursor of intrinsic thylakoid proteins, such as LHCP I, LHCVP II and phytoene desaturase were not processed.

The observations on SPP were presented in a poster at the 4th International Congress of Plant Molecular biology (Amsterdam, June 1994; copy enclosed) and are now being prepared for publication (10).

We have prepared pPPOs [^3H]-labeled by specific amino acids (Val, Tyr, Leu or Arg). These were used to isolate the labeled 62 kD intermediate (iPPO) after incubation with purified SPP. The products were subjected to Edman degradation to determine the processing site. Preliminary results suggest that the cleavage site resembles the ones reported for some other lumen proteins of higher plants (Bassam et al., J. Biol. Chem. 266:23606, 12992) (see poster). However, since Edman degradation of radiolabeled peptides gives reliable results only for the first 10-12 residues, we believe that the results obtained for PPO A should be supported by additional data. We are presently analyzing pPPOE labeled by Val, Pro, or Lys. pPPE contains these residues in positions which should result in unambiguous assignment of the site of cleavage. When the *in vitro* cleavage site is determined with confidence we shall attempt to conclude this subject by determining the *in organello* processing site and comparing it to the *in vitro* one.

Initial attempts to establish an *in vitro* assay for the second, thylakoid-traversing step have

met with only partial success. Using the 62 kD intermediate, it was found that conversion of the latter to the mature form required thylakoids as well as prior unfolding of the 62 kD by urea. Interestingly, washed tomato thylakoids were able to import the 62 kD intermediate formed by the SPP purified from pea plastids. This supports our contention that the observations we have made with pea plastids represent the general pattern of events involved in the import and targeting of plant pPPOs.

Translocation required light and was enhanced by ATP. However, ATP is apparently not obligatory. Depletion of ATP after having accumulated the 62 kD in the stroma in darkness had no effect on subsequent thylakoid translocation in light. Such thylakoid translocation was abolished by nigericin and monensin, even in the presence of ATP. These observations suggests that ΔpH , probably without ATP, drives translocation across the thylakoid. Cu^{2+} does not inhibit thylakoid translocation, nor processing by the thylakoid processing protease (TPP). TPP is tightly bound to the thylakoid membrane and proved difficult to solubilize. Work on purifying and characterizing TPP was further complicated by non-specific proteolytic activity which resides in both thylakoids and lumen. We suspect that the TPP is part of the translocation complex, making it difficult to isolate in an active form.

Localization: lumen versus thylakoid-bound proteins

The product of the PPO B gene was found soluble in the lumen following import by intact pea chloroplasts. This corresponded well to the structure of the transit peptide and the predicted amino acid sequence of the mature part of the protein (6). However, only part of the native PPO(s) of tomato thylakoids was found soluble in the lumen by ^{125}I -immunoblotting or fractionation of enzymic activity. More than half of the activity remained bound to thylakoids after bursting in a Yeda press. This could be due to the presence of more than one gene product in the thylakoids studied. Of the seven members of the tomato PPO gene family, three have a C-proximal hydrophobic domain that can confer membrane association. Consequently, the various tomato PPOs may differ in their ultimate location in the thylakoid. We subcloned PPOs A, B, C, D, E and F (PPO A and A' differ only in their promoter region) in expression vectors and compared the import and localization of PPO A and C (which contain hydrophobic domains) to those of PPO B and E (which lack such regions). More than 70% of the 59 kD mature protein resulting from the import of all four precursors was found soluble in the lumen of pea thylakoids. PPO A appeared to be only slightly more membrane-bound even after prolonged incubation (2 h). We have also compared the import of these four precursors into tomato chloroplasts. The same picture was obtained. We conclude that the widely reported thylakoid location of PPOs is either the result of artifacts or of post-processing events. Of the four pPPO gene products whose import was studied so far, PPO A was imported most efficiently. PPO B and C showed similar rates while PPO E was least active. We have succeeded in establishing a reproducible import system with tomato plastids, using a local variety and introducing some modifications in the plastid isolation protocol. Minor differences were

found between the relative import efficiency in pea and tomato when the four precursors were compared. Contrary to earlier observations, the 62 kD intermediate was observed with all 4 precursors. There was evidence for proteolytic degradation of the 59 kD mature form by the thylakoids.

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Tentoxin

Treatment of leaves with tentoxin, a toxin of fungal origin which inhibits CF1, causes loss of plastidic PPO. After tentoxin treatment, immunologically recognizable PPO remained associated with the plastid envelope and previous workers have suggested that tentoxin prevents the import and processing of PPO into an active form. We have confirmed this result in *in vitro* uptake experiments with tomato PPO. Low concentrations of tentoxin (10 μ M) prevented import of tomato pPPO into isolated plastids. This effect is specific to PPO, and the precursor accumulated at the plastid envelope.

Effect of Cu on PPO import and processing

The deduced amino acid sequence of all PPO genes isolated so far contains two highly conserved putative copper-binding domains CuA and CuB (see above) and a third histidine-rich domain (CuC) which may function as a copper binding site. CuA and CuB are highly homologous to the copper binding domains of fungal, bacterial and mammalian tyrosinases. Outside these domains there is little homology between tyrosinases and plant PPOs. We have determined that Cu^{2+} is not required for the import, targeting or processing of PPO. However, Cu^{2+} (1-5 μ M) strongly and specifically inhibits PPO import, leaving the precursor bound to the envelope. Import to the stroma was completely inhibited by 5 μ M Cu^{2+} , while import of plastocyanin or LHCP precursors was unaffected. Inhibition was prevented by addition of EDTA prior to Cu^{2+} . Cyanide, which removes Cu^{2+} from active PPO, relieved Cu^{2+} inhibition of pPPO import. Cu^{2+} has no effect on the import and processing of plastocyanin by isolated chloroplasts. Cu^{2+} prevents formation of the 62 kD PPO intermediate in intact plastids. Addition of Cu ion after formation of some 62 kD intermediate stops further import and 62 kD formation but allows the conversion of the 62 kD intermediate to the 59 kD and thylakoid translocation. Binding of Cu^{2+} to pPPO may prevent unfolding of the precursor, which is required for traversing the plastid envelope, but probably not required for crossing the thylakoid membrane.

Cu^{2+} inhibits the import of all pPPOs studied thus far: A,B,C and E, and inhibits processing by purified SPP of all pPPOs studied. Cu^{1+} also inhibits import at concentrations similar to those used with Cu^{2+} . None of the Cu-domain mutants - Cu A, B, C, AB, AC, BC or ABC - was insensitive to inhibition by Cu^{2+} for import or SPP activity. However, these mutants exhibit some interesting differences in rate or differential sensitivity of import or processing by the SPP which we are continuing to explore.

PPO gene family promoter and transit peptide gene constructs to determine cellular and subcellular patterns of expression and localization

A series of chimeric genes have also been constructed, consisting of putative regulatory regions of the tomato PPO genes, along with their transit peptides, fused in-frame to the reporter gene β -glucuronidase (GUS). The chimeric plasmids containing the PPO E,F, C and A/A' constructs (PPOs C and D have identical promoters, as do PPOs A/A') have been mobilized into *Agrobacterium tumefaciens* via electroporation and used to transform the tomato cultivar MoneyMaker (Newman et al., in preparation). We have adapted a transformation procedure for tomato cv. MoneyMaker based on hypocotyl and cotyledon explants. The procedure utilizes 7 day old axenic cotyledon and hypocotyl explants co-cultivated with *A. tumefaciens* (LBA4404) for 4 days and subsequently transferred to selective medium. Putative transformants appear within 2 to 6 weeks and are excised and transferred to selective rooting medium for 2 to 4 weeks. An apical cutting kept as a tissue culture backup is taken from each plant prior to transfer to the greenhouse. Plants transformed with the PPO A/A', B, C/D, E And F constructs (confirmed by Southern blotting) have been regenerated and grown in the greenhouse. Each shows discrete temporal and spatial patterns of expression in general agreement with earlier results of in situ hybridization using PPO gene-specific probes (Joel, Thipyapong and Steffens, in preparation). Further work will define both the cellular expression pattern of these PPOs as well as confirm the subcellular localization conferred by their putative transit peptides.

In addition we have carried out immunogold localizations of PPO at the electron microscopy level. These studies suggest that the products of different PPO genes are localized in plastids in two different ways. PPOs which are expressed at high levels accumulate as the dominant constituent of protein bodies within the lumen of plastids lacking thylakoid organization typical of photosynthetic plastids. PPOs expressed at lower levels in photosynthetic plastids are typically localized on the thylakoid membrane. We note that PPOs C and A/A' possess highly hydrophobic membrane-spanning C-termini which may serve to anchor the protein within the thylakoid membrane. In contrast, PPOs E and B, which lack these hydrophobic extensions, are soluble enzymes within the thylakoid lumen (Joel et al., in preparation).

Differential import/processing of precursors of different pPPOs by plastids: developmental regulation

We observed marked differences in the overall efficiency of import and fate of the pPPO

precursor (62 versus 59 kD product) by plastids of various species, developmental stages and growth conditions history. It seemed possible that the various tomato PPO genes differ in the efficiency with which their products are imported and processed by various plastid types. We are presently conducting a comparative study of tomato pPPOs, using chloroplasts from leaves of different ages (pea), tissues (tomato-cotyledons; leaves; hypocotyls, which are rich in developing trichomes), etioplasts and chloroplasts (pea, maize), leucoplasts (etiolated pea and castor bean hypocotyls) and developing chromoplasts (*Capsicum*). Of the preliminary observations obtained, the behaviour of pPPO C is interesting. It was imported and processed only by very young leaves, not by older ones, unlike pPPOs A, B and E. This study will be concluded when the results on the import of POs D and F are added to the picture.

PPO was reported to be localized in grana thylakoids and to co-fractionate with PS II (Lax and Vaughn, 1991). We observed that the import of PPO takes place primarily into stroma lamellae from which the mature form migrates to the stacked areas. The distribution of PPO import and processing thus resembles the pattern described for LHCPII (Yalovsky et al., 1992).

Expression of PPO in *E. coli*

The PPO B cDNA (HY-19) was subcloned into the *E. coli* expression vector pMAL (New England Biolabs). The PPO cDNA was prepared by removal of sequences encoding the transit peptide and then fused in frame with the *malE* gene of *E. coli* which encodes a maltose-binding protein. *MalE* is under the control of *lacZ* which allows inducible expression of the PPO gene in response to inducers such as IPTG. Large amounts of the PPO-maltose binding protein fusion product were accumulated upon IPTG induction. The fusion protein was purified by binding to an amylose column, released with 10 mM maltose, and the maltose-binding protein cleaved from PPO using factor Xa protease. Although the resulting PPO was freely soluble, possessed a mass of 59 kDa and retained immunogenicity, we have so far been unable to reconstitute enzymatic activity. Experiments in progress include expression as a periplasmic fusion protein to maximize correct formation of disulfide bonds, and attempts to renature the enzyme to an active form.

PPO A and B produced in the pGEM *E. coli* vector system accumulated as insoluble inclusion bodies which can be solubilized in the presence of 6M urea and DTT. The solubilized denatured proteins were made import-competent by dialysis in the presence of an extract of leaf proteins (see Waegmann et al., 1990). The precursor accumulated in plastids in a time-dependent manner (up to 90 min) but was not processed. Purified SPP was inactive towards the *E. coli*-expressed precursor. Since four additional amino acid residues (Met, Arg, Gly, and Ser) were added while constructing the expression vector, we suspected that these might have interfered with processing. We have constructed another vector which contains pPPO A with no additional amino acids and are now examining its over-expression as well as the import and processing of the protein. We are also trying to

over-express the intermediate (62 kD) and mature forms of PPO A in *E. coli*.

Site-directed mutagenesis of PPO B copper-binding domains

Unlike PPOs from non-plant sources, plant PPOs possess three, rather than two, histidine-rich domains capable of coordinating the two copper ions which constitute the active site of the enzyme. We have employed site-directed mutagenesis to modify each of these putative copper-binding domains (individually and in combination) in the PPO B cDNA. These mutants are being used for two purposes. First, low (micromolar) concentrations of copper ion specifically inhibit the import of PPO into plastids. These mutants, after transcription and translation, are used to ascertain whether this effect is due to binding of copper by the precursor protein, and if so, by which of the copper-binding domains. Second, when the *E. coli* expression system is optimized, analysis of the gene products of these mutants will allow identification of which two of the three putative copper-binding domains are responsible for copper binding and enzymatic activity.

Point mutations, replacing His by Asn residues, singly or in combinations, had little effect on the ability of Cu^{2+} to inhibit pPPO import. We are therefore preparing another series of mutants which involve replacing two His residues or deleting 4- 10 amino acid residues in each of the Cu-binding domains of PPO A. These mutants will be tested for inhibition of import and processing by Cu^{2+} .

Conformational changes?

In vitro translated pPPO binds to DEAE-cellulose at 50 mM Hepes-KOH pH 8. The 62 kD intermediate, prepared from pPPO A by processing with purified SPP, does not. It is difficult to rationalize how removal of 47 amino acids (including 8 positively charged residues but no negatively charged residues) will cause such a change unless processing induces a major conformational change. This may have important implications with regard to import mechanisms. We intend to pursue this matter once an *E. coli* - expressed precursor cleavable by SPP is available.

A somewhat related observation was obtained when the *in vitro* translated pPPO A was subjected to controlled proteolysis by trypsin or thermolysin. The first product obtained was a 57 kD species. However, when proteolysis was performed in the presence of 5-10 μM Cu^{2+} , proteolysis was arrested after formation of a 59 kD form. Since the 57 kD and 59 kD forms were neither bound nor imported by plastids, nor processed by SPP, it would appear that cleavage took place at the N-terminus. It seems that binding of Cu^{2+} induced a conformation which is relatively inaccessible to proteases. It will be interesting to try such limited proteolysis on *E. coli* -expressed pPPO in the presence of Cu^{2+} and to examine the resulting 59 kD polypeptide for enzymic activity. The product should closely resemble the mature PPO which arrives at the lumen.

Wound inducibility of potato PPO

Inducibility of PPO activity by herbivores or pathogens has been demonstrated in several species as has the increased activity of PPO during senescence or deterioration of organelles. These studies have not determined whether this increase in enzymic activity was due to an increase in the amount of PPO or is due instead to the loss of PPO latency which is associated with limited proteolysis or changes in plastid membrane integrity. We have shown that PPO activity is inducible severalfold by wounding. Immunoblotting and ELISA show that inducible activity is accompanied by comparable increases in PPO, and Northern blots show that the PPO-specific steady-state mRNA level increases as well. When leaflets at node 8-9 are wounded, PPO induction is only detectable in apical leaf nodes 1-4. Interestingly the apical 4 leaf nodes are the only leaf tissues in which PPO mRNA is detectable in unwounded potato (8).

Antisense downregulation of the tomato PPO gene family

Despite the wide distribution of PPOs among angiosperms, an *in planta* function for PPOs has not been determined. Roles in plant defense, phenylpropanoid biosynthesis, plastidic oxygen regulation and electron transport have been hypothesized. To gain insight into PPO function(s), we introduced a chimeric antisense PPO cDNA into tomato. Oxidation of caffeic acid, the dominant *o*-diphenol of tomato foliage, was decreased 39-fold by antisense expression of PPO in transgenic tomato. Immuno-reactive PPO and PPO-specific mRNA and immunoreactive PPO were not detectable in the transgenic plants. These traits were maintained through the R₂ generation and segregated in a Mendelian fashion with the PPO antisense T-DNA. Growth and development were phenotypically normal under greenhouse conditions. However, epidermal leucoplasts displayed an abnormal morphology in antisense plants. PPO-containing protein bodies conspicuous in nontransformed plants were absent or reduced in antisense PPO plants and plastids did not label with antibodies directed against PPO. Comparison of phenylpropanoid profiles of antisense and nontransformed plants suggests that PPO does not contribute to 4-hydroxylation of *p*-coumaric acid to yield caffeic acid. However, insect herbivore performance is slightly improved on transgenic tomatoes possessing antisense PPO constructs, indicating that PPO may play a role in plant defense against herbivores (9).

Inhibition of potato bruising by antisense expression of PPO in tubers

Spoilage of foods caused by post-harvest enzymatic browning is a problem of considerable importance to growers, processors and retailers. Potatoes are susceptible to a number of post-harvest syndromes in which PPO is responsible for significant crop losses. In particular, blackspot bruising is responsible for significant annual yield losses for both tablestock and processed potato products. Blackspot bruise results from physical impacts on the tuber which occur during mechanical harvesting or in post-harvest handling and storage. Localized discoloration initially appears at the sites of impact some hours after the event, changing from pink to black within 24 h. The discoloration is due to formation of melanin, as PPO released from plastids by tissue damage, converts tyrosine and

chlorogenic acid to quinones, which autoxidize to form various dark-colored melanins. Although susceptibility to blackspot bruising is cultivar-dependent and is also in part dependent upon environmental effects, in general higher soluble solids content (which results in greater hardness of the tuber) contributes significantly to bruising susceptibility. In addition, cutting of tubers prior to processing often results in melanization which can only be controlled with sulfite applications or heat treatments to inhibit or inactivate PPO.

Tuber melanization is apparently due to PPO activity, with little data available to support a role for either laccase or peroxidase. Therefore, downregulation of PPO could be expected to decrease the rate and extent of melanization in response to physical impacts or cutting. In addition, tuber melanization (and blackspot bruising in particular) is frequently not associated with visible tissue damage. Therefore the syndrome can be considered essentially a cosmetic one caused by a relatively simple biochemical process. Successful inhibition of tuber PPO could significantly decrease the frequency and severity of bruising or melanization and result in significantly improved post-harvest tuber quality.

We explored the consequences of PPO downregulation by antisense in potato, using either the CaMV 35S, patatin, or granule-bound starch synthase promoters driving expression of a potato PPO cDNA. Minitubers of over 1000 transformants were assayed for PPO activity (catechol oxidase) in a spectrophotometric assay. Of these some 100 were found to possess low to undetectable levels of PPO activity. The strongest suppression of PPO activity occurred in constructs utilizing the CaMV 35S or granule-bound starch synthase promoters. Patatin- driven antisense PPO expression was in general less effective in inhibiting PPO. Transgenic minitubers exhibiting decreased PPO expression were transplanted to the field in summer of 1992 and cultivated and harvested at maturity following commercial growing practices. After harvest and a period of storage, the tubers were subjected to an assay which involves application of a standard impact sufficient to cause bruising in standard cultivars. The results were in agreement with results derived from spectrophotometric PPO assays: plants with low or null PPO activity failed to develop melanin under conditions favoring development of blackspot bruises (7).

DESCRIPTION OF COOPERATION

The collaboration developed and grew stronger throughout the funding period. Professor Harel spent his sabbatic months of October 1992 through March 1993 at Cornell. For several weeks during this period he was joined by Mr. Amos Sommer, a doctoral student who has spearheaded much of the work of the Jerusalem group. During this time both worked closely with members of the Cornell lab. Among the more fruitful exchanges conducted during these visits were transfer of chloroplast uptake technology and expertise from the Hebrew University workers to the Cornell lab, testing of Cornell-generated PPO mutants in the chloroplast processing system, and subcloning genomic clones representing additional members of the tomato PPO gene family for analysis of their import and

processing characteristics.

Shortly after Professor Harel's departure for Israel, Professor Mayer visited Cornell to review progress and to explore possible future directions. Professor Steffens travelled to Israel for two weeks in Spring 1994 to continue the exchange of ideas and materials. Mrs. Emma Ne'eman, a member of the Jerusalem team, visited Cornell in Summer 1994 to acquire expertise in transgenic plant technology and to discuss recent findings. Finally in August 1994 Professor Harel visited Cornell for concluding discussion of results and to formulate objectives and approaches for a continuation proposal.

EVALUATION OF RESEARCH ACHIEVEMENTS

The revised objectives of this proposal included 1). Comparison of the coding sequences of 45 kDa and 59 kDa PPO; 2). Comparison of properties of plant PPOs synthesized in *E. coli* with those of PPO isolated from plant tissue; and 3). To determine how PPOs are imported into organelles and how they are targeted for insertion into membranes.

As described in the earlier Annual Scientific Reports, Objective 1 has been completed (Hunt et al. 1993; Newman et al. 1993; Steffens et al., 1994). Objective 2 has proven successful in terms of producing large amounts of soluble PPO in *E. coli*. Recently, this protein has also been successfully rendered import-competent. However, considerable work remains to be done toward optimizing conditions for obtaining enzymatic activity from this product. Continuation of this objective has been requested in the recent submission of a continuation proposal for this project. Objective 3 is also complete (Sommer et al., 1994). As described above, we continue to explore and define this system using other cloned members of the tomato PPO gene family. Complementary information is now becoming available from analysis of reporter gene constructs employing the promoters and transit peptides of individual PPO genes.

Clearly, this project has met or exceeded the original goals and timetables proposed at the outset of the funding period. An important contribution to agriculture is described in (7), in which antisense PPO genes were used to inhibit the expression of PPO in potato tubers. This work demonstrates that enzymic browning is due exclusively to PPO, and that enzymic browning can be controlled by antisense expression of PPO. Furthermore, in (9) we showed that antisense downregulation of the entire PPO gene family does not cause deleterious effects on plant growth and development. Therefore, antisense expression of PPO genes may represent a general approach to control of post-harvest enzymic browning in a wide array of fruit, vegetable and other crops of interest to the agriculture of both the U.S. and Israel.

LIST OF PUBLICATIONS RESULTING FROM RESEARCH PROJECT

1. Kowalski, S.P., Eannetta, N.T., Hirzel, A.T., and J.C. Steffens (1992). Purification and characterization of polyphenol oxidase from glandular trichomes of *Solanum berthaultii*. *Plant Physiol.* 100: 677-684.
2. Yu, H., Kowalski, S.P., and J.C. Steffens (1992). Comparison of polyphenoloxidase expression in glandular trichomes of *Solanum* and *Lycopersicon*. *Plant Physiol.* 100: 1885-1890.
3. Hunt, M.D., Newman, S.M., Eannetta, N.T., Yu, H. and J.C. Steffens (1993). Cloning and expression of potato polyphenol oxidase. *Plant Molec. Biol.* 21: 59-68
4. Newman, S.M., Eannetta, N.T., Yu, H., Prince, J., De Vicente, C., Tanksley, S.D., and J.C. Steffens (1993). Sequence and organization of the tomato polyphenol oxidase gene family. *Plant Molec. Biol.* 21: 1035-1051.
5. Steffens, J.C., E. Harel, and M.D. Hunt (1994). Polyphenol Oxidase. *In* Genetic Engineering of Secondary Metabolism, B.E. Ellis, G.W. Kuroki, H.A. Stafford, eds., Recent Advances in Phytochemistry, Vol. 28, pp.275-312.
6. Sommer, A., Ne'eman, E., Steffens, J.C., Mayer, A.M., and E. Harel (1994). Import, targeting and processing of polyphenol oxidase. *Plant Physiol.* 105: 1301-1311.
7. Bachem C.W.B., van der Linde P.C.G., Hunt M.D., Steffens J. C. and M. Zabeau (1994). Antisense expression of polyphenol oxidase genes inhibits enzymatic browning in potato tubers. *Bio/Technology* 12:1101-1105.
8. Thipyapong, P., Hunt, M.D., and J.C. Steffens (1994). Systemic wound induction of polyphenol oxidase is controlled by transcriptional regulation. Submitted to *Plant Physiol.*
9. Hunt, M.D., and J.C. Steffens (1995). Antisense downregulation of polyphenol oxidase in transgenic tomato. In preparation.
10. Sommer, A., Ne'eman E., Paran N., Steffens J.C., and E. Harel (1995). A stromal processing peptidase involved in routing polyphenol oxidase to the thylakoid lumen. In preparation.