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**Polyphenol Oxidases - Expression, Assembly and
Function**

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Abbreviations used in the report, in order:

PPO, polyphenol oxidase; CaMV35S, cauliflower mosaic virus 35S promoter; ROS, reactive oxygen species; AS, antisense; OX, overexpressors; WT, wild type; kDa, kilodalton; PS, photosystem; OE23 and OE33, the 23 and 33 kDa polypeptides of the oxygen evolving complex of PS II; CCCP, carbonyl cyanide m-chlorophenylhydrazinone; PC, plastocyanin; 'p' and 'i' as a prefix are used to denote the precursor and intermediate form of a protein, MeJA, methyljasmonate; LHCP, light harvesting chlorophyll binding protein of PS II; DTT, dithiothreitol.

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Refereed (published, in press, accepted)	1	3	1	5
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Master theses				
Ph.D. theses		1	1	2
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Cooperation consisted of consultation and regular exchange of information on results, techniques and ideas. The Cornell laboratory supplied seeds of transgenic tomato (antisense and over expressing) vital to experiments in Jerusalem and mutants, constructs and techniques were exchanged between the two groups. Emma Ne'eman visited the Cornell lab to acquire technologies for the preparation of transgenic tomato plants. Cooperation and even communication were however hampered to a certain extent when Dr. Steffens moved to 'Novartis' NC halfway into the project. A Sabbatical Dr. Steffens had planned to spend in Jerusalem was canceled for the same reason.

ABSTRACT

Polyphenol oxidases (PPOs) participate in the preparation of many plant products on the one hand and cause considerable losses during processing of plant products on the other hand. However, the physiological functions of plant PPO were still a subject of controversy at the onset of the project. Preliminary observations that suggested involvement of PPOs in resistance to herbivores and pathogens held great promise for application in agriculture but required elucidation of PPO's function if modulation of PPO expression is to be considered for improving plant protection or storage and processing of plant products. Suggestions on a possible role of PPO in various aspects of chloroplast metabolism were also relevant in this context. The characterization of plant PPO genes opened a way for achieving these goals. We reasoned that "understanding PPO targeting and routing, designing ways to manipulate its expression and assessing the effects of such modifications will enable determination of the true properties of the enzyme and open the way for controlling its activity".

The objective of the project was to "obtain an insight into the function and biological significance of PPOs" by examining possible function(s) of PPO in photosynthesis and plant-pest interactions using transgenic tomato plants; extending our understanding of PPO routing and assembly and the mechanism of its thylakoid translocation; preparing recombinant PPOs for use in import studies, determination of the genuine properties of PPOs and understanding its assembly and determining the effect of PPO's absence on chloroplast performance. Results obtained during work on the project made it necessary to abandon some minor objectives and devote the effort to more promising topics. Such changes are mentioned in the 'Body of the report' which is arranged according to the objectives of the original proposal.

The complex expression pattern of tomato PPO gene family was determined. Individual members of the family are differentially expressed in various parts of the plant and subjected to developmentally regulated turnover. Some members are differentially regulated also by pathogens, wounding and chemical wound signals. Wounding systemically induces PPO activity and level in potato. Only tissues that are developmentally competent to express PPO are capable of responding to the systemic wounding signal by increased accumulation of PPO mRNA. Down regulation of PPO genes causes hyper susceptibility to leaf pathogens in tomato while over expression results in enhanced disease resistance. An interesting and surprising feature of down regulation of PPO expression in tomato plants is their apparent increased tolerance to drought. Both the enhanced disease resistance conferred by PPO over expression and the increased stress tolerance due to down regulation can be used in the engineering of improved crop plants. Photosynthesis rate and variable fluorescence measurements in wild type, and PPO-null and over expressing transgenic tomato lines suggest that PPO does not enable plants to cope better with stressful high light intensities or reactive oxygen species. Rather, high levels of the enzyme aggravate the damage caused under such conditions. Our work suggests that PPO's primary role is in defending plants against pathogens and herbivores. Jasmonate and ethylene, and apparently also salicylate, signals involved in responses to wounding and defense against herbivores and pathogens, enhance markedly and specifically the competence of chloroplasts to import and process pPPO. The interaction of the precursor with thylakoid membranes is primarily affected. The routing of PPO shows other unusual properties: stromal processing occurs in two sites, resulting in intermediates that are translocated across thylakoids by two different mechanisms - a Δ pH- and a Sec-dependent one. It is suggested that the dual pattern of processing and routing constitutes a 'fail safe' mechanism, reflecting the need for a rapid and flexible response to defense challenges. Many of the observations described above should be taken into consideration when manipulation of PPO expression is contemplated for use in crop improvement.

ACHIEVEMENTS

The physiological function(s) of polyphenol oxidases (PPOs) in plants remained obscure until recently, in spite of more than a century of research. PPOs participate in the preparation of many plant products on the one hand and cause considerable losses during processing of plant products on the other hand. Hypotheses on the involvement of the enzyme in defense against herbivores and pathogens as well as in protection against reactive oxygen species and in regulating photosynthetic electron transfer had to be examined if manipulation of its expression is to be used for engineering improved crop plants. e.g. if down regulation was to be considered for improving storage and processing of plant products or over expression for improving plants protection. The findings in this project, along with results from our previous BARD grant (US-1870-90) present a detailed and complex, though still incomplete, picture of PPO's expression, regulation, routing and translocation.

The major conclusion of our work is that PPO functions in protecting plants against pathogens and, in some cases, against herbivores. It also takes part in the response of plants to wounding. Variations exist as to the mechanism of PPO's involvement in plant defense e.g. constitutive or induced activity or the special case of potato and tomato trichomes. Since PPO is located in the thylakoid lumen, the contribution of PPO to plant defense will depend on the exposure of vacuole phenolics to luminal PPO and atmospheric oxygen upon disruption of the cell by wounding or herbivore or pathogen attack. This will result in formation of highly reactive quinones that covalently modify or cross-link cellular substances (26).

The hyper susceptibility to leaf pathogens caused by down regulation of PPO genes in tomato and the enhanced disease resistance brought about by over expression obviously suggest utilization of PPO in the engineering of improved crop plants. Up regulation of PPO in some plants and in tissues and developmental stages where its level is low might prove beneficial in some cases. However, substantiation of our findings in crops other than potato and tomato and for additional herbivores and pathogens should precede immediate application in plant breeding. Estimation of the economic benefits will obviously depend on the evaluation of PPO's distribution and the spectrum of pests involved.

Yet another approach where our findings may contribute to improvement of plant products is in down regulating the expression of PPO genes in products where browning is a problem. This proved to be simpler than over expressing the enzyme and has already been used in breeding potato for 'non-browning' tubers.

Several conclusions of our project should eliminate some doubts regarding the use of down regulation of PPO expression:

1. PPO does not fulfil a vital function in plants where it is active since antisense lines that possess no detectable activity are still viable.
2. PPO does not participate in regulating photosynthetic electron transfer, the Mehler reaction, protection from reactive oxygen species or other aspects of chloroplast metabolism.
3. PPO is not involved in the biosynthesis of phenolic compounds (e.g. hydroxylation).

However, several findings and observations made in this project should be kept in mind when either over expression or down regulation of PPO is contemplated:

1. The complex pattern of expression of the PPO gene family in tomato (and probably also in other plants). Individual members of the family are differentially expressed in various parts of the plant and subjected to developmentally regulated turnover. Some members are differentially regulated also by pathogens, wounding and chemical wound signals.
2. Only tissues that are developmentally competent to express PPO are capable of responding to signals by increased accumulation of PPO mRNA in some cases.
3. Down regulation could affect susceptibility to pathogens.
4. Up regulation might affect drought tolerance and aggravate damage caused by high light intensity, drought and possibly other stresses.

The surprising observation on increased tolerance to drought in tomato plants in which PPO has been down regulated deserves special attention. The potential of application for crop improvement is immense. In any case, the phenomenon should be examined in other crop plants and the possibility that susceptibility to pathogens could be increased at the same time should be kept in mind.

Several findings of the work on import, processing and assembly of PPO show unusual properties and can be regarded as novel or deserving further attention:

1. Most interesting, novel and possibly related to function is the demonstration that a defense related protein is not only induced by wounding and attack related signals but its import is scaled up too. Identification of the translocation apparatus components involved may help in improving the delivery of engineered proteins to their target compartment.

2. The novel stromal processing peptidase that cleaves the presequence at alternative sites and the fact that each of the two intermediate species formed is translocated across thylakoids by a different pathway, a feature that might ensure efficient routing in an emergency. It suggests that proteins could be engineered for transport by more than one pathway, a feature that might improve their routing.
3. PPO is the only available chloroplast precursor that can bind a cofactor with an apparent change of conformation. This makes it an important tool in thylakoid translocation studies and is also relevant to recent studies on protein export in bacteria.

Cooperation between the Cornell and Jerusalem laboratories consisted of consultation and regular exchange of information on results, techniques and ideas. The Cornell laboratory supplied seeds of transgenic tomato (antisense and over expressing) vital to experiments in Jerusalem and mutants and constructs and techniques were exchanged between the two groups. Emma Ne'eman visited the Cornell lab to acquire technologies for the preparation of transgenic tomato plants. Cooperation and even communication were however hampered to a certain extent when Dr. Steffens moved to 'Novartis' NC halfway into the project. A Sabbatical Dr. Steffens had planned to spend in Jerusalem was canceled for the same reason.

BACKGROUND, SCIENTIFIC AND AGRICULTURAL RELEVANCE OF THE PROJECT, METHODOLOGY, RESULTS AND DISCUSSION

The following is a summary of work performed in the laboratories of Dr. John C. Steffens in Ithaca N.Y. (Cornell University) and Dr. E. Harel in Jerusalem (The Hebrew University). The results and observations are reported according to the 'Objectives' of the original proposal. Scientific background and agricultural relevance comments are brought at the beginning of each objective and section.

Objective 1. Possible Functions of PPO

Work on objectives 1a and 1b has been performed primarily in the Cornell laboratory. However, the Jerusalem group observations on the effect of MeJA on the ability of chloroplasts to import and process PPO are highly relevant to objective 1a (see 3b). The expression pattern and turnover of members of the PPO gene family have been studied in order to get an insight into the biological function of the enzyme. In addition, the regulation of expression of individual PPO genes were studied in relation to various biotic and abiotic factors:

INDIVIDUAL MEMBERS OF THE POLYPHENOL OXIDASE GENE FAMILY ARE DIFFERENTIALLY EXPRESSED AND SUBJECTED TO DEVELOPMENTALLY REGULATED TURNOVER. Polyphenol oxidases (PPOs) are encoded by a highly conserved, 7-member gene family in tomato. Using gene-specific probes capable of differentiating between PPO A/C, PPO B, PPO D, and PPO E/F, we examined the spatial and temporal expression of this gene family during vegetative and reproductive development. RNA blot and *in situ* hybridization showed that although PPO expression is primarily confined to early stages of development. The steady-state mRNA levels of these genes are subject to complex patterns of spatial and temporal regulation in both vegetative and reproductive organs. Young tomato leaves and flowers possess the most abundant PPO transcripts. PPO B is most abundant in young leaves, whereas in the inflorescence PPO B and E/F transcripts are dominant. Differential expression of PPOs are also observed in various trichome types. PPO A/C are specifically expressed in Type I and Type IV trichomes. In contrast, PPO D is only expressed in Type VI trichomes. Type I, IV, and VI trichomes possess PPO E/F transcripts. Immunolocalization verified the translational activity of PPOs identified by *in situ* hybridization and suggested cell-type-specific, developmentally programmed PPO turnover. In addition, immunolocalization demonstrated the accumulation of PPO in specific idioblast cells of stems, leaves, and fruits (28).

DIFFERENTIAL REGULATION OF TOMATO POLYPHENOL OXIDASE F IS DIFFERENTIALLY REGULATED BY PATHOGENS, WOUNDING AND CHEMICAL WOUND SIGNALS. Tomato PPO genes are subject to complex patterns of differential expression during growth and differentiation. Antisense down-regulation or constitutive and induced PPO expression results in hyper-susceptibility to pathogens, suggesting a critical role for PPO-mediated phenolic oxidation in plant defense. However, the nature and extent of PPO induction, and its contribution to resistance are unclear. In this study we examined the inducibility of the tomato PPO gene family. In mature plants, PPO transcript levels systemically increased in young leaves (nodes 1-3) when mature leaflets (node 5) were injured. Transcripts hybridizing to PPO E/F-specific probes were the predominant wound-induced PPO mRNAs in young leaves. Analysis of PPO promoter:GUS fusion constructs shows that mechanical wounding and infection by fungal and bacterial pathogens induced transcription of PPO F. Different injuries, salicylic acid, ethylene, and jasmonates elicited distinct cell-specific and developmental stage-specific patterns of PPO F expression, whereas jasmonates and mechanical wounding significantly induced PPO F only in young leaves (nodes 1-3), and ethylene induced PPO F only in older leaves (node 7), salicylic acid induced PPO F in stems and foliage at all developmental stages. These results demonstrate that cis-element(s)

sufficient for PPO F inducibility reside in the 5' flanking region, and these elements are responsive to a broad range of signals (29).

1a. Altered PPO expression and insect herbivore performance.

The increase in PPO activity upon wounding is regarded as part of a 'protective response', that results in sealing the tissue against invasion by pathogens etc. The increase in PPO activity following herbivore grazing, which apparently reduces the 'attractivity' of the tissue for the invader, may be regarded as a related response. The proposed role of PPO as an antinutritive defense against herbivores depends on the generation of quinones by PPO which subsequently oxidatively modify (alkylate) protein or amino acid nucleophiles, thereby decreasing the nutritive quality of plant protein and the palatability of the plant (8). PPO is prominent amongst twenty different proteins that undergo a coordinated systemic induction following wounding or herbivore grazing of tomato plants (6, 7, 9). The response is mediated by the octadecanoid wound-signaling pathway and the short polypeptide systemin (22). Plants which overexpressed a prosystemin gene possessed PPO levels up to 70-fold higher than wild type ones (6) and wounding or treatment with systemin or methyl jasmonate, induced PPO to similar high levels (6).

WOUND INDUCIBILITY OF POLYPHENOL OXIDASE IN POTATO. Plant polyphenol oxidases (PPOs) have long been reported to be inducible upon biotic or abiotic wounding. However, observations of inducible PPO activity are frequently confounded by failure to distinguish PPO induction from loss of PPO latency, or PPO from peroxidase activity. We described the systemic induction of PPO activity, and increased steady-state levels of PPO and PPO mRNA in potato (*Solanum tuberosum* L.) in response to wounding. During normal growth and development, PPO is present throughout potato leaf maturation, from the apical leaf node through node 11. In contrast, PPO mRNA is only detectable in apical leaf nodes 1 to 3. Wounding of potato leaflets at nodes 6 to 8 results in 1.7-fold increase in PPO activity in apical leaf nodes 1 to 4 within 48 h after wounding. The increases in PPO activity are accompanied by comparable increases in PPO and PPO-specific mRNA. No PPO induction is observed in either leaf nodes 5 or 8. Our results suggest that only tissues which are developmentally competent to express PPO mRNA can respond to the systemic wounding signal by increased accumulation of PPO mRNA (27).

EFFECTS OF ALTERED PPO EXPRESSION ON INSECT HERBIVORE PERFORMANCE The use of transgenic plants with modified PPO expression is a critical step to addressing the function of PPO in plants and to assess the validity of various hypotheses put

forward to explain its ubiquitous presence in plants. Previous studies seeking to establish a link between PPO and pest tolerance have depended upon correlation between species or non-isogenic hybrids differing in PPO activity (and presumably many other factors), insect feeding studies with artificial diets, or addition of PPO inhibitors to foliage (cf. 18, 58). These studies remained vulnerable to criticism that they either do not isolate PPO as a single differing factor in the analysis, or do not represent realistic tests of the hypothesis. The effect of antisense down regulation of PPO was therefore tested against two insect species. Since the antinutritive effect of quinone-mediated crosslinking and oxidation of dietary proteins is expected to be pH-dependent, we tested a lepidopteran tomato pest (fall army worm) and a coleopteran pest (a tomato-adapted strain of Colorado potato beetle), which possess highly basic and neutral to acidic midgut pH values, respectively. Neonate larvae were placed on transgenic tomato leaves and the following parameters recorded over eight days: developmental rate, weight gain, larval mortality and foliage consumption. In both test insects, mortality was lowest, and developmental rate and weight gain highest, on PPO-null plants expressing PPO in the antisense orientation. Mortality was highest, and developmental rate and weight gain lowest, on the PPO-overexpressing plants. Larval performance on nontransformed control plants was intermediate to the PPO over-expressing plants and the PPO-nulls. Although these results were consistent over the course of three repetitions, in each case the variance was high and statistical significance was not achieved. It seems likely that PPO affects the success of insect herbivores, and that its contribution may be significant with species which are not adapted to hosts, such as tomato, that possess high levels of PPO. However, it seems unlikely that engineering PPO overexpression in tomato could be used to increase resistance of this crop to insect pests.

1b. Role of PPO in resistance to foliar pathogens

DOWN REGULATION OF THE POLYPHENOL OXIDASE GENE FAMILY LEADS TO HYPER-SUSCEPTIBILITY TO PATHOGENS. To test the long-standing hypothesis that PPO functions in disease resistance in plants, induction of tomato PPO promoter-GUS fusion constructs by the foliar bacterial pathogen *Pseudomonas syringae* pv tomato was examined with each of the 7 members of the tomato PPO gene family. Only PPO F was responsive to this pathogen, and its expression was primarily localized around lesion sites, especially in young leaves. Localized induction was observed in leaf cells surrounding lesions. Very young leaf nodes lacking visible lesions showed systemic induction throughout the entire leaf. Tomato plants were also transformed with an antisense PPO construct. Plants expressing the antisense PPO lacked detectable

PPO and PPO activity, both constitutively and in response to inoculation with *P. syringae* results in loss of characteristic PPO-containing inclusions in thylakoids of cells known to be high in PPO (e.g., glandular trichomes). The phenotype was maintained into generations homozygous for the antisense construct. Leaves of homozygous antisense PPO-expressing plants inoculated with *P. syringae* supported 150-fold higher bacterial populations than inoculated control plants, showed 20-fold more lesions per unit leaflet area, and average lesion diameter was 5-fold larger in PPO-suppressed plants relative to controls. These studies provide the first genetic evidence supporting a role for PPO in plant disease resistance, and suggest that PPO overexpression may be an effective route to enhance such resistance. (Thipyapong et al., in preparation).

OVEREXPRESSION OF POLYPHENOL OXIDASE IN TRANSGENIC TOMATO RESULTS IN ENHANCED DISEASE RESISTANCE. A primary physiological role of PPOs is believed to be in plant defense against pests and pathogens. In order to assess the ability of PPOs to contribute to plant defenses, a *Solanum tuberosum* PPO cDNA was fused with a CaMV35S promoter and introduced into tomato plants by *Agrobacterium*-mediated transformation. Three lines of transgenic plants expressed up to 30-fold increases in PPO mRNA and 6-10 fold increases in PPO and PPO activity. Southern blot analysis showed that two to three copies of the PPO gene were inserted into the genome of the three transgenic lines. Extracts of PPO-overexpressing transgenic leaves oxidize endogenous phenolic substrate pools at higher rates than control plants. Chlorogenic acid (3-caffeoylquinic acid), the primary PPO substrate of tomato leaves was oxidized at a higher rate in extracts of transgenic PPO-overproducing leaves; rutin (3,3',4', 5,7-pentahydroxyflavone-3-rutinoside) which is not an effective PPO substrate, also underwent dramatically higher rates of co-oxidation in transgenic leaves. The three lines were inoculated with *P. syringae* pv. tomato at 2×10^7 cfu/ml. Seven days after inoculation with the virulent bacterial pathogen, the transgenic plants exhibited over 100-fold lower bacterial populations and over 15-fold fewer lesions than control plants. These data show that over expression of PPO resulting in increased phenolic oxidation rates can play a significant role in restricting disease development (Li and Steffens, in preparation).

Taken together with the results from the antisense plants, these finding show that PPO is not only a key part of plant resistance mechanisms, but that its engineering may be exploited to increase plant disease resistance as well.

1c. Does PPO affect the photosynthetic performance of intact plants or isolated plastids?

One of the hypotheses regarding the function of PPO is based on PPO's location on the thylakoid membrane and its high K_m for O_2 . Mesophyll chloroplast PPO has been proposed to function in pseudocyclic photo-phosphorylation (30), in the Mehler reaction, or in regulation of plastidic oxygen levels (10, 19, 31, 32, 33). However, there is no evidence for a suitable PPO substrate in this compartment which could allow a PPO-based oxygen reduction cycle to operate. Never the less, the fact that plant PPOs are targeted to the thylakoid lumen, the ubiquitous presence of the enzyme in chloroplasts and a claim about its association with PSII suggest a role for PPO in some aspect of chloroplast metabolism.

SUPPRESSION OF PPOs INCREASES STRESS TOLERANCE IN TOMATO. PPO has been proposed to participate in the Mehler reaction (photoreduction of molecular oxygen by PSI). Under the reduced photosynthesis caused by drought stress, the Mehler reaction may provide a nondestructive sink for absorbed energy that is not used in photochemistry. We reasoned that plants with highly suppressed PPO levels, such as those expressing an antisense PPO construct, would be much more sensitive to the combined effects of withholding water under constant illumination. To our surprise, we found that plants with suppressed PPO levels possess greater tolerance of photooxidative conditions imposed by water stress and constant illumination. Transgenic plants with suppressed PPO levels exhibit less severe wilting at 4 days after withholding water, and delays both in stress-induced anthocyanin accumulation and chlorophyll degradation. In addition, PPO suppressed plants exhibit slower rates of declining water potential and stomatal conductance in response to water stress. Fluorescence measurements also indicate a decreased degree of photosystem damage in antisense plants compared to controls. These results suggest that PPO may have a role in the development of water stress and potential for photoinhibition and photooxidative damage, but not via effects on the Mehler reaction. As a preliminary attempt to understand the role PPOs may play in potentiating stress-induced damage, we examined the expression of PPOs during these experiments. Among the seven-member PPO gene family, we found that in response to water stress, two members, PPO B and D, are transcriptionally upregulated in abscission zones of leaf petioles. In addition, PPO B is induced in leaf veins and in stem vascular tissues, cortical and pith cells at all stages of development. Similarly, tissue printing of stem tissues following drought stress showed increased immunoreactive PPO in vascular tissues, cortical and pith cells of NT and OP plants. Foliar PPO activity of NT plants also

increased throughout development in response to water stress. Decompartmentalization of plastids in these tissues under water stress may act to release PPO, resulting in cellular damage, particularly to vascular tissues from PPO-induced quinone and reactive oxygen species formation (Thipyapong et al., in preparation).

DOWN REGULATION OF PPO EXPRESSION DOES NOT AFFECT THE PHOTOSYNTHETIC PERFORMANCE OF TRANSGENIC TOMATO PLANTS. In our research proposal we reasoned that "since the precise manner by which PPO might be involved in chloroplast metabolism is not clear, we will first compare the photosynthetic performance of control, antisense and PPO over-expressing tomato plants". Our working hypothesis was that if PPO is involved in anyway in removing reactive oxygen species (ROSs), regulation of photosynthetic cyclic electron flow etc., differences should be observed in the overall photosynthetic rates and other photosynthetic parameters at high light intensities and low CO₂ to O₂ ratios among the three types of plants. Since a pure PPO-over expressing PPO line was not yet available, over expressing seedlings were picked from a mixed population by assaying for PPO activity of a single leaf. Transient rates of photosynthesis in individual leaves were determined for WT, antisense (AS) and over expressing (OX) plants with an Irga computerized gas exchange system for analyzing CO₂ and H₂O gas exchange of intact plant leaves. No significant differences were observed between the three lines over a wide range of light intensities and CO₂ to O₂ ratios. Figure 1A shows the results for WT and AS eight week-old tomato leaves, with photosynthesis rates expressed as $\mu\text{mole CO}_2 \text{ m}^{-2} \text{ leaf area sec}^{-1}$. No difference was observed also in total leaf area between control, AS and OX 8-10 week old plants although control and OX plants were evidently bigger than AS ones at earlier stages of growth. No significant differences between the three types of plants were found in CO₂ exchange rates measured at high light intensity ($1450 \mu\text{E m}^{-2} \text{ sec}^{-1}$) and a wide range of CO₂ concentrations (0-950 ppm). More direct evidence of damage incurred to the photosynthetic apparatus was sought using the PAM 101/102/103 chlorophyll fluorescence system (Walz, Effeltrich, Germany) (23). The instrument measures steady state fluorescence under low intensity modulated light and after a flash of light (maximal fluorescence) in intact plants, in a non-destructive manner. Variable fluorescence was determined for WT and the two transgenic lines before and after exposure to photoinhibitory conditions (e.g. 4°C overnight followed by $1000 \text{ E m}^{-2} \text{ sec}^{-1}$ for 3 h), exposure to water deficit or high temperature. An example is shown in Fig. 1B. Variable fluorescence markedly declined in OX plants. We suggest that in OX plants, increased diphenol oxidation aggravates the damage caused to membrane integrity by some types of stress. AS plants did not seem to be

at a disadvantage under such stress conditions. Recovery from stress was also followed in some cases. It seemed that PPO-OX plants were slower to recover, compared with AS and WT plants. The Jerusalem results on variable fluorescence in transgenic tomato might be consistent with the Cornell laboratory observations on stress tolerance, where presence of PPO seems to enhance cellular damage that occurs during severe stress.

1d. Is PPO involved in the hydroxylation of monophenols?

This direction was not pursued since preliminary experiments have indicated that there was no significant differences in the phenolics composition between WT and null-PPO lines of tomato.

Figure 1. CO₂ assimilation by WT and PPO-null plants in various light intensities. Leaf segments of 12 week old tomato WT (○) or PPO-null (◐) plants were monitored in a LCA-2 analyzer (ADC) and an IRGA, at 650 ppm CO₂. Mean and standard deviation for three plants.

B. Variable fluorescence of WT tomato and two transgenic lines exposed to high light intensity. Fluorescence was determined with the PAM 101/102/103 chlorophyll fluorescence system after exposure to 1000 μEi m⁻²sec⁻¹ for the time indicated. Steady state (Fs) and maximum (Fmax) fluorescence were determined and the variable fluorescence (VF) calculated as (Fmax-Fs)/ Fmax. Each point is the average of the four determinations.

It thus appears that PPO's primary role is in plant defense against pathogens and probably also herbivores. This is suggested by the fact that down regulation of the PPO gene family leads to hyper susceptibility to pathogens while over expression of PPO in tomato results in enhanced disease resistance. Our observations on the wound inducibility of PPO in potato, the effect of MeJA on plastid competence to import the enzyme (see below) and the insect feeding experiments suggest a role also in defense against herbivores. These conclusions are further strengthened by the finding that suppression of PPO increases, (rather than decreases) stress tolerance in tomato and by gas exchange and fluorescence measurements in wild type and transgenic plants.

Objective 2. Examining the effect on *Vicia faba* PPO latency when *Vicia* PPO is expressed in tomato.

This relatively minor objective has not been dealt with in project reported here since the other research lines appeared to be more promising and fruitful.

Objective 3. PPO Targeting, Processing and Assembly

This was the major objective of the Jerusalem lab's contribution. During a previous BARD grant to J.C. Steffens and E. Harel, PPO genes have been cloned, transcribed and translated *in vitro* and the ~ 67 kDa precursors (pPPO) obtained used to study the import, targeting and processing of PPO by isolated chloroplasts (24, 25). PPO was targeted to the lumen. Routing took place in two steps. The precursor was imported by an ATP-dependent process into the stroma where its N-terminal portion was removed by a processing peptidase. The resulting 62 kDa intermediate (iPPO) continued to the thylakoid lumen in a light-dependent reaction (24). In the present project we dealt with 4 major topics:

3a. The stromal processing peptidase (SPP) - two processing sites in pPPO. We purified the stromal processing peptidase (SPP) which converts pPPO to iPPO. The SPP cleaved various precursors of chloroplast proteins but was unable to process those of intrinsic thylakoid proteins (12). The SPP processed pPPO at a site identical to that observed *in organello* and the *in vitro* product of SPP action on pPPO was further processed and translocated by thylakoids. The enzyme is a metallo-endopeptidase, like other SPPs. However, it differs in substrate specificity, apparent size and most significantly, the major cleavage site of pPPO. While the processing sites of lumen proteins determined so far are relatively distant from the hydrophobic core of the thylakoid targeting domain, pPPO is first cleaved at a site (SCK-VTN) which closely resembles the intermediary cleavage site of pOE23, and at an additional one, immediately before the hydrophobic core (*cf.* Fig. 5 in 12). Cleavage at the second site removes the Arg-Arg motif characteristic of thylakoid targeting domains of lumen proteins which are translocated by the Δ pH-dependent pathway of thylakoid translocation (3). The significance of these observations becomes clear when the mechanisms of thylakoid translocation and the emerging function of PPO are considered. Translocation of lumen proteins takes place by two pathways: a Sec- and a Δ pH dependent ones (5, 21). Processing at the second site may serve to divert the PPO intermediate from the Δ pH- to the Sec-dependent pathway of thylakoid translocation. We suggest that under circumstances where rapid import of PPO is

required, e.g. wounding and insect or pathogen attack while a proton gradient is not readily formed (e.g. darkness), translocation is diverted from the ΔpH -dependent route to the ATP (Sec) dependent one. The major part of our observations on the PPO-processing SPP is described in (12).

3b. The second processing step and translocation across the thylakoid membrane

THYLAKOID TRANSLOCATION OF PPO: TWO PATHWAYS? Translocation of PPO across thylakoids has some unusual features. These could be related to the dual pattern of PPO's stromal processing described above and possibly also to the physiological function of the enzyme and its inducible expression in some tissues. Translocation of newly imported pPPO depended on ΔpH and was inhibited by nigericin and CCCP [Fig. 1 in (16), also (12, 24)]. pPPO possesses the twin Arg motif characteristic of precursors of lumen proteins that are translocated by the ΔpH -dependent pathway [cf. Fig. 5A in (12)]. However, it lacks the Lys residue next to the C-terminus of the hydrophobic core (HC), believed to confer 'Sec-avoiding' properties (1). The first cleavage site of pPPO by SPP results in the formation of a ΔpH -dependent precursor (iPPOI). However, the major cleavage site observed for pPPOA both *in organello* and with purified SPP *in vitro* [cf. Fig. 5A in (12)] creates a Sec-dependent precursor. Several lines of observations support the 'alternative pathways' suggestion: a. iPPOII that has accumulated in the stroma in darkness continued to the lumen and was processed to mature PPO when the inhibitor was removed and light turned on. Such translocation was sensitive to azide, a diagnostic inhibitor of the SecA dependent pathway (cf. Fig. 2 in 16). b. Pre-accumulated pPPO competed more effectively with Sec-dependent precursors (pPC and pOE33) than newly imported one (Figs. 4 and 5 in 16). c. Two iPPO forms were observed after short import incubations (24) or when PPO translocation was slowed down. d. Sec-dependent translocation of PPO could be observed after prolonged incubation in darkness. e. Modification of the first SPP cleavage site of pPPO by mutagenesis caused accumulation of an intermediate in the stroma that could be converted to mature PPO by isolated thylakoids (17).

What is the physiological significance of such alternative routing? We suggest that in normal conditions, when the ΔpH pathway is sufficiently active, the first intermediate (iPPOI) is promptly translocated and processed by TPP. Under conditions where ΔpH -driven translocation is relatively slow (e.g. darkness), further cleavage, past the twin-Arg motif forming iPPOII, enables Sec-dependent transport to take place. The significance of the ability of pPPO to cross the thylakoid by both the pathways could

also lie in iPPO becoming enzymatically active upon binding Cu^{2+} . This would be undesirable due to the reactive nature of the products of PPO action. Most importantly, high rates of PPO synthesis and import might be important upon pathogen or herbivore attack or following wounding (6, 26, 28). Some of our observations on the mechanism of thylakoid translocation of PPO are summarized in two manuscripts, drafts of which are appended (16, 17).

COMPETENCE FOR PPO IMPORT IS MARKEDLY ENHANCED BY EXPOSING TOMATO PLANTS TO METHYLJASMONATE AND ETHYLENE. The expression of some PPO genes is increased by wounding in tomato and potato plants (27, 29) and PPO appears to be involved in the protection of some plants against grazing by insects (26). Constitutive expression of prosystemin in tomato and treatment of plants with methyl jasmonate (MeJA) resulted in a 40-70 fold increase in the level of leaf PPO (6). This agrees with viewing PPO as part of an insect-detering arsenal, taking into account the systemin-proteinase inhibitors system worked out by C.A. Ryan's group. We asked whether treatment with MeJA will also increase the ability of plastids to import and process pPPO, being particularly interested in what appears to be the limiting step in the process - the translocation across thylakoids. Exposing tomato plants to MeJA 12-48 hours before isolation of plastids markedly increased the rate of import and processing of pPPO by intact organelles. Wounding or exposure to ethylene had no effect and pea was indifferent to MeJA treatment. The effect persisted also in assays with isolated thylakoids. Thylakoids from MeJA treated plants bound most of the pPPO or iPPO presented to them while those from control plants remained practically indifferent (*cf.* Fig. 4 in 15). This suggested that thylakoid translocation is the step involved in the response to MeJA. Very high levels of a 90 kDa protein co-fractionated with chlorophyll containing membranes from MeJA tomato plants. It was not observed in pea or tobacco, nor after wounding tomato plants or exposing them to ethanol. Microsequencing showed that the protein is multicystatin, an inhibitor of papain. Thus, the protein we had hoped was part of the thylakoid import complex turned out to be yet another member of the anti-insect arsenal which happened to co fractionate with thylakoids. In an attempt to identify the thylakoid protein(s) involved in the specific interaction with pPPO, we crosslinked radiolabelled pPPO to thylakoids using glutaraldehyde. A 120 kDa crosslinked protein was observed only in membranes from MeJA treated plants and was absent after thylakoids have been subjected to mild digestion by proteases. Further work is evidently required to isolate and characterize the protein. Some of the observations on this subjected are summarized in Koussevitzky et al. (15).

3c. The effect of Cu ion on the import and processing of pPPO

In work initiated during the previous BARD project and completed during the present one we have described a complex picture of the effect of Cu^{2+} on PPO import and routing. Cu^{2+} was not required for the import, targeting or processing of PPO. Furthermore, $5\text{ }\mu\text{M Cu}^{2+}$ blocked pPPO import into the stroma while that of plastocyanin, also a lumen copper protein, and LHCP (24) was not affected. Binding of Cu^{2+} to pPPO probably prevented unfolding of the precursor that was required for crossing the plastid envelope. Using a selection of mutants carrying changes in the three copper sites of PPOA and PPOB (25) it was demonstrated that inhibition by Cu^{2+} involved binding to at least two of the three Cu-binding domains of pPPO. Translocation into the lumen was enhanced by the ion, suggesting that iPPO interacted with the thylakoid in a folded form. These conclusions were substantiated by further experiments in which the effect of various concentrations of Cu^{2+} was quantified for the WT and a selection of Cu site mutants (13). Thus, in some (single site) mutants, translocation was blocked and the intermediate accumulated in the stroma with an optimum which depended on Cu^{2+} concentration. This suggested that the site(s) involved in inhibiting the first import step differ from the ones responsible for folding the protein into a form that interacts with thylakoids. We also demonstrated that iPPO that had accumulated in the stroma continued to the lumen in a Cu^{2+} dependent process, when inhibition of the second step was lifted (12). Interestingly, it was shown that some proteins can cross envelopes and thylakoids in a folded state (4).

3d. Association with the thylakoid membrane

PPO has long been considered to be a thylakoid-bound enzyme (19, 33). However, Only two of the tomato PPO genes, PPOA and C, possess domains which can confer binding to the thylakoid membrane although all six genes are targeted to the lumen. The mature forms of all PPOs were found soluble in the thylakoid lumen following import into isolated pea and tomato plastids, irrespective of whether they possessed an α -helical domain capable of anchoring the protein in the thylakoid membrane (20, 24). Only a small proportion of PPOA and C, which possess such hydrophobic C-terminal domains, remained bound to thylakoids after rupture by a Yeda press. The same was true for intact chloroplasts isolated from PPO-null tomato plants. The properties of mature ^{35}S -PPOs A and C, isolated from pea plastids following import were compared to membrane bound PPOs from tomato thylakoids by mixing the radiolabelled and native proteins and assaying for radioactivity and enzymatic activity following HPLC. No significant differences were observed between radiolabeled mPPO and the enzyme extracted from tomato thylakoids. Although association with the

thylakoid membrane might takes place after routing into the lumen is completed, it appears that binding is mostly an artifact of cross linking by oxidized phenols following cell disruption.

Objective 4. Assembly, Enzymatic Activity and Properties of pPPO Expressed in *E. coli*, Baculovirus or Yeast The importance of recombinant PPO for this project was two fold: for use in competition experiments etc., while studying translocation mechanisms and for studying the genuine properties, of the enzyme. During our previous BARD project, cloning mature PPOB in *E. coli* using the pMAL vector produced large amounts of a soluble 59 kDa protein which retained immunogenicity. However, attempts to renature the enzyme to an active form were unsuccessful. Attempts to over express either the precursor or mature PPO in Baculovirus or yeast (using *Pichia*) during the present project failed to produce significant amounts of the desired proteins. We have expressed the precursor, two intermediate forms (iPPOI and iPPOII) and mature PPO as well as several mutants in *E. coli*. All accumulated in inclusion bodies. The precursor was made import competent by dilution of the dissolving urea and DTT and addition of cytosolic proteins which probably contain chaperones. The course of *E. coli* expressed pPPO routing into the lumen resembled that of the *in vitro* translated precursor. The recombinant pPPO proved therefore suitable for competition experiments etc. Its properties, import and some of the competition results are described in (14) and (16). However, no enzymic activity was observed after import into chloroplasts from pea or antisense (null-pPPO) tomato plants. Nor were we able to renature recombinant pPPO, iPPOI, iPPOII or mature PPO into an active form. We are still trying to express pPPO and iPPOI in a *tat* mutant of *E. coli* which can export proteins to the periplasmic space and processes them to the mature form (2). This avenue requires further work.

Objective 5. Does Absence of PPO affect Chloroplast Performance? In our proposal we reasoned that since PPO reaction products affect the performance of enzymes and organelles isolated from plant tissues, it will be interesting to examine plastidic activities which are highly sensitive to plastid integrity and 'well being'. We were able to devote only limited time to this subject. Studies with chloroplasts from antisense tomato lines showed no significantly better performance compared with WT plastids in protein import assays nor were they able to convert imported recombinant PPO into an active enzyme. However, they were superior to plastids from PPO-over expressing plants. The subject deserves further attention.

Additional Topics

IMPORT, PROCESSING AND THYLAKOID TRANSLOCATION OF TRUNCATED PRECURSORS

The relatively slow rate of PPO import and the difficulty we encountered in establishing a reliable thylakoid assay for PPO could have resulted from the large size of the mature protein - the largest lumen protein reported so far (11). We examined therefore the performance of two truncated precursors in which 19 or 37 kDa C-terminal portions have been deleted. The two truncated precursors were imported and processed at significant, though somewhat lower, rates compared with WT. Their performance in thylakoid assays were no better than WT.

THYLAKOID ASSAY IN TOMATO Since pea does not have active PPO, we reasoned that it could be responsible for the fact that pea plastids showed poor performance in thylakoid assays. Tomato thylakoids did indeed translocate pPPO and iPPO and processed it to the mature form. However, thylakoids from transgenic null-PPO tomato plants did not convert imported recombinant PPO into an active enzyme.

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* References resulting from this project are numbered in **bold** both here and in the text.

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**1. Sommer A, Ne'eman E, Koussevitzky S, Hunt MD, Steffens JC, Mayer AM, Harel E (1995) The inhibition by Cu^{2+} of the import of polyphenol oxidase into chloroplasts. In: Photosynthesis: from Light to Biosphere, (P. Mathis ed.), Vol III, pp 827-830, Kluwer Academic Publishers, The Netherlands

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a. Thipyapong P, Hunt MD, Steffens JC (1999) Down regulation of the polyphenol oxidase gene family leads to pathogen hyper-susceptibility.

b. Li L, Steffens JC (1999) Overexpression of polyphenol oxidase in transgenic tomato results in enhanced disease resistance.

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e. Koussevitzky S, Ne'eman E, Harel E (1999) Modification of the stromal processing site of PPO results in a Sec dependent precursor. (Manuscript enclosed).

** Publications completed during the present project but in which most of the work has been done under a previous BARD projects US 1870-90.