Tomato Allene Oxide Synthase and Fatty Acid Hydroperoxide Lyase, Two Cytochrome P450s Involved in Oxylipin Metabolism, Are Targeted to Different Membranes of Chloroplast Envelope[1]

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Allene oxide synthase (AOS) and hydroperoxide lyase (HPL) are related cytochrome P450s that metabolize a common fatty acid hydroperoxide substrate to different classes of bioactive oxylipins within chloroplasts. Here, we report the use of in vitro import assays to investigate the targeting of tomato (*Lycopersicon esculentum*) AOS (LeAOS) and HPL (LeHPL) to isolated chloroplasts. LeAOS, which contains a typical N-terminal transit peptide, was targeted to the inner envelope membrane by a route that requires both ATP and proteinase-sensitive components on the surface of chloroplasts. Imported LeAOS was peripherally associated with the inner envelope; the bulk of the protein facing the stroma. LeHPL, which lacks a typical chloroplast-targeting sequence, was targeted to the outer envelope by an ATP-independent and protease-insensitive pathway. Imported LeHPL was integrated into the outer envelope with most of the protein exposed to the inter-membrane space. We conclude that LeAOS and LeHPL are routed to different envelope membranes by distinct targeting pathways. Partitioning of AOS and HPL to different envelope membranes suggests differences in the spatial organization of these two branches of oxylipin metabolism.

Fatty acid hydroperoxides produced by lipoxygenase (LOX) are important intermediates in the synthesis of a diverse group of bioactive compounds known as oxylipins (oxygenated fatty acids). In one branch of oxylipin metabolism, allene oxide synthase (AOS) transforms 13-hydroperoxide linolenic acid (13-HPOT) to an epoxide intermediate (EOT) that converts spontaneously into α- and γ-ketols that have unknown function in plants. In the presence of allene oxide cyclase (AOC), however, EOT is converted to 12-oxophytodienoic acid (OPDA) and then to jasmonic acid (JA) (Fig. 1). JA and related cyclopentanone products of the AOS pathway are essential signals for plant defense against pest attack, mechanical responses, and developmental processes (Creelman and Mullet, 1997; Staswick and Lehman, 1999). An alternative pathway for 13-HPOT metabolism is initiated by fatty acid hydroperoxide lyase (HPL). The C_6 aldehyde products of HPL, together with their corresponding reduced alcohols, are important volatile constituents of the characteristic odor of fruits, vegetables, and green leaves (Hatanaka, 1993). The HPL-derived oxylipins are also implicated as antimicrobial toxins for host defense against pathogens (Croft et al., 1993; Blée, 1998), as well as signals for the regulation of defense-related genes (Bate and Rothstein, 1998). 13-HPOT is metabolized to other defense-related oxylipins by other plant enzymes including peroxygenase (Blée et al., 1993) and divinyl ether synthase (Grechkin et al., 1995).

Sequence analysis of cDNAs encoding AOS and HPL has led to the realization that these enzymes are related members of an atypical family of cytochrome P450s, designated CYP74. Unlike typical P450 monooxygenases, AOS (CYP74A) and HPL (CYP74B) have a low affinity for CO and do not require O_2 or NADPH-dependent cytochrome P450 reductase for their activity (Song and Brash, 1991; Lau et al., 1993; Shibata et al., 1995a, 1995b). AOS and HPL are also unusual with respect to their intracellular localization outside the endoplasmic reticulum or mitochondria. The deduced amino acid sequence of several AOS- and HPL-encoding cDNAs contains a typical N-terminal transit peptide for targeting to the chloroplast (Song et al., 1993; Laudert et al., 1996; Bate et al., 1998; Howe et al., 2000). This is consistent with localization of AOS and HPL activity to chloroplast membrane fractions and, more specifically, to envelope membranes (Vick and Zimmerman, 1987; Gardner et al., 1991; Hatanaka, 1993; Harms et al., 1995; Blée and Joyard, 1996; Zhuang et al., 1996). Relevant to our understanding of the localization of AOS and HPL, it is noteworthy that not all such enzymes contain a typical chloroplast targeting peptide. One example of this is a cytosolic form of AOS in guayule

[1] This work was supported by the Cell Biology Program of the National Science Foundation (grant no. MCB–9904524), the Division of Energy Biosciences at the U.S. Department of Energy, and the U.S. Department of Agriculture National Research Initiative (grant no. 9801335).

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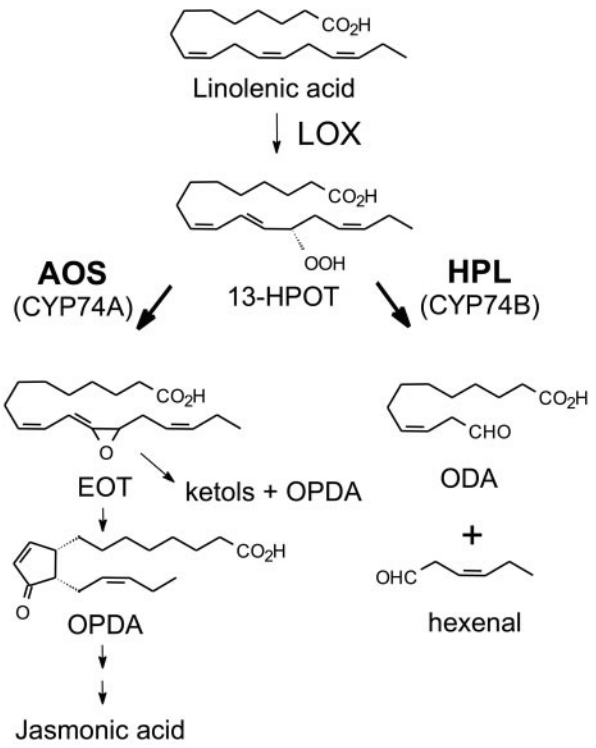

Figure 1. Metabolism of 13-HPOT by the cytochrome P450s AOS (CYP74A) and HPL (CYP74B). AOS transforms 13-HPOT to an unstable EOT (12,13-epoxyoctadecatrienoic acid) that is subsequently converted to the JA precursor OPDA by AOC. In the absence of AOC, EOT spontaneously converts to α- and γ-ketols and racemic OPDA. HPL cleaves 13-HPOT to produce a volatile C_6 aldehyde (cis-3-hexenal) and a C_{12} oxo-acid (ODA; 12-oxo-cis-9-dodecenoic acid). 13-HPOT is produced from the action of 13-LOX on linolenic acid.

that is associated with rubber biosynthetic particles (Pan et al., 1995). A second example is barley AOS, which despite the lack of any apparent chloroplastic transit peptide, accumulates in chloroplasts (Maucher et al., 2000). Taken together, these findings suggest considerable diversity in the intracellular location and targeting of members of the CYP74 family of P450s.

Several pathways have been described for the targeting and insertion of proteins into the chloroplast envelope (for reviews, see Schnell, 1998; Keegstra and Cline, 1999). Insertion of proteins into the outer envelope membrane is thought to occur by one of two routes. The first pathway is used by proteins such as outer envelope membrane protein of 14 kD (OM14) that lack a typical transit peptide. Insertion of proteins into the outer envelope via the "OM14 pathway" does not require ATP or surface-exposed receptors (Salomon et al., 1990; Li et al., 1991; Fischer et al., 1994; Li and Chen, 1996; Chen and Schnell, 1997). In the second pathway, targeting of other proteins to the outer envelope is directed by an N-terminal transit peptide and requires components of the general import apparatus. For example, the mature region of Toc75 inserts into the outer envelope following removal of the N-terminal portion of a bipartite transit peptide by stromal processing peptidase (Tranel and Keegstra, 1996). Proteins destined for the inner envelope membrane follow yet a different pathway. These proteins require a typical N-terminal transit peptide, ATP, and surface-exposed receptors. However, the targeting signals that direct the final insertion of these proteins into the inner envelope membrane reside within the mature region of the protein. Several mechanisms have been proposed for the targeting of proteins to the inner envelope (Brink et al., 1995; Knight and Gray, 1995; Lübeck et al., 1997; Keegstra and Cline, 1999).

We recently reported the characterization of tomato (*Lycopersicon esculentum*) AOS (LeAOS) and HPL (LeHPL) P450s that metabolize 13-HPOT (Howe et al., 2000). It is interesting that LeAOS contained a putative chloroplast transit peptide, whereas LeHPL appeared to lack such a sequence. This observation, together with the recent description of chloroplastic AOSs that lack a transit peptide (Maucher et al., 2000), suggested that LeAOS and LeHPL might provide useful markers for investigating the pathways by which CYP74s are targeted to chloroplasts. Here, we report the use of in vitro chloroplast import assays to examine this question. Our results show that LeAOS and LeHPL use different pathways for targeting to the inner and outer membranes of the chloroplast envelope, respectively.

RESULTS

LeAOS and LeHPL Use Different Pathways for Targeting to the Chloroplast

The deduced N-terminal sequences of AOSs and HPLs, aligned with respect to conserved residues within mature proteins, are shown in Figure 2A. As previously reported, AOS from flax, Arabidopsis, and tomato contain typical transit peptides for chloroplast targeting (Song et al., 1993; Laudert et al., 1996; Howe et al., 2000; Sivasankar et al., 2000). A similar sequence was also noted at the N terminus of HPL from Arabidopsis (Bate et al., 1998). In contrast, the N terminus of tomato HPL (LeHPL) resembled that of chloroplastic forms of barley AOS that lack transit peptides (Fig. 2A).

We compared the ability of in vitro translated LeAOS and LeHPL to bind and target to isolated chloroplasts. Initial experiments focused on determining the import of LeAOS. In the presence of isolated pea (*Pisum sativum* var Little Marvel) chloroplasts and low concentrations of ATP, in vitro translated LeAOS was susceptible to proteolytic digestion by Thermolysin (Fig. 2B, lanes 1–4). This indicates that LeAOS precursors bind to the chloroplast surface but are not imported. In the presence of higher concentrations of ATP, LeAOS precursors became resistant to Thermolysin, indicating import into chloroplasts (Fig. 2B, lanes 5–8). Fractionation of import reactions into membrane and soluble compo-

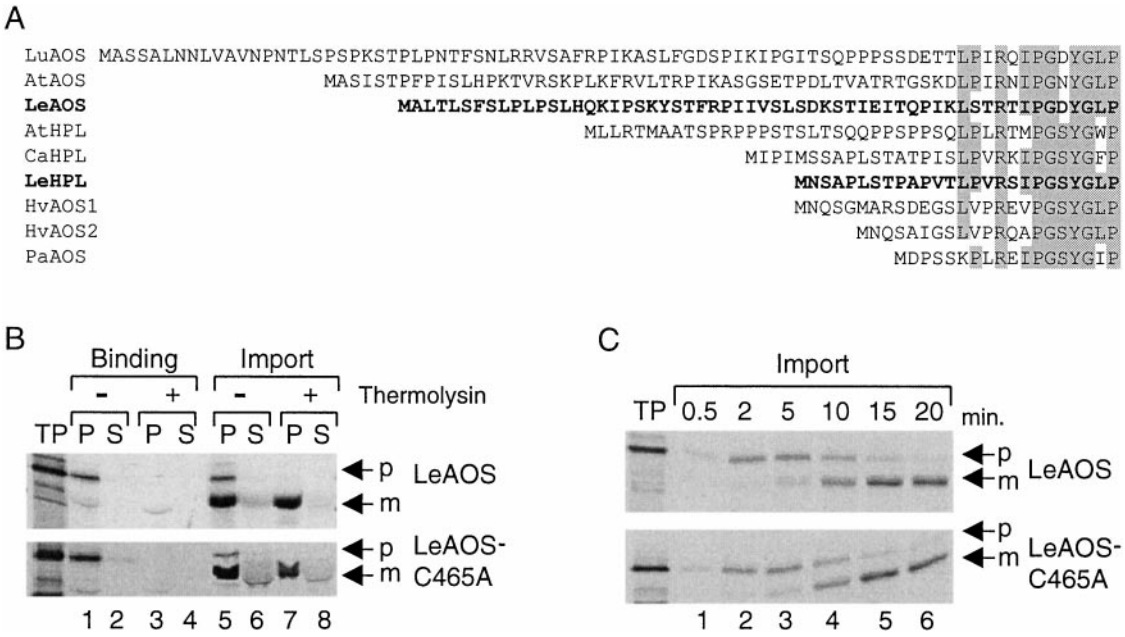

Figure 2. LeAOS import into isolated pea chloroplasts is ATP-dependent. A, The deduced N-terminal sequence of AOSs and HPLs, aligned with respect to conserved residues within mature proteins (gray shading). AOS sequences were from *Linum usitatissimum* (U00428), *Parthenium argentatum* (X78166), Arabidopsis (Y12636), *L. esculentum* (AF230371), and *Hordeum vulgare* (AJ250864 and AJ251304). HPL sequences were from *Capsicum annum* (U51674), Arabidopsis (AF087932), and *L. esculentum* (AF230372). B, Binding and import of LeAOS and mutant LeAOS-C465A to isolated pea chloroplasts. [^{35}S]-labeled LeAOS and LeAOS-C465A were bound to pea chloroplasts under low ATP conditions (lanes 1–4) or imported under high ATP conditions (lanes 5–8) for 30 min. After binding or import, chloroplasts were incubated with (+) or without (−) Thermolysin for 30 min at 4°C. Chloroplasts were recovered by centrifugation through a 40% (v/v) Percoll cushion and fractionated into a total membrane (P) and soluble (S) fraction. C, Time course of import of LeAOS and LeAOS-C465A into pea chloroplasts in the presence of 4 mM Mg-ATP. At the times indicated, intact chloroplasts were recovered and lysed. Total membrane fractions were isolated and analyzed by SDS-PAGE and fluorography. TP, Represents 10% of translated product added to a single import assay; p, precursor; m, mature form of AOS or AOS-C465A.

nents showed that imported LeAOS was associated with the total membrane fraction. The appearance of a lower M_r form of LeAOS after import suggested proteolytic removal of an N-terminal transit peptide. This form of LeAOS accumulated in a time-dependent manner (Fig. 2C), supporting the hypothesis that the gel mobility shift of LeAOS during import represents removal of a transit peptide. The import behavior of LeAOS into isolated tomato chloroplasts was identical to that observed when using pea chloroplasts (data not shown).

The C-terminal end of LeAOS contains a heme-binding domain that is highly conserved among cytochrome P450s (Howe et al., 2000). To assess the role of heme association with apoLeAOS during the import process, we examined the import behavior of a LeAOS mutant (LeAOS-C465A) in which the highly conserved heme-binding Cys at position 465 was changed to an Ala. Control experiments showed that LeAOS-C465A expressed in *Escherichia coli* does not bind detectable amounts of heme and is devoid of AOS activity (data not shown). Despite the inability of this polypeptide to associate with heme, import was indistinguishable from that of wild-type LeAOS (Fig. 2, B and C). These results indicate that the binding, import, and association of LeAOS with the membrane fraction do not require prior heme attachment.

We next examined the interaction of in vitro translated LeHPL with isolated chloroplasts (Fig. 3). The role of ATP in the binding of LeHPL to chloroplasts was assessed by depleting the import assay system of ATP by two methods: (a) Gel filtration was used to remove low-M_r compounds from wheat germ-translated precursor proteins (Olsen and Keegstra, 1992) and (b) Chloroplasts were pre-incubated with nigericin to reduce endogenous levels of ATP. The results show that LeHPL associated with chloroplastic membranes in the ATP-depleted system as it did in the presence of different concentrations of exogenous ATP (Fig. 3A). In further contrast to LeAOS, the gel mobility of chloroplast-bound LeHPL was identical to that of in vitro translated LeHPL. Thus, LeHPL does not appear to be proteolytically processed during targeting to the plastid. Import of LeHPL is reminiscent of OM14, a previously characterized protein that inserts into the outer envelope in an ATP-independent manner (Li et al., 1991). In contrast to OM14, however, chloroplast-bound LeHPL was resistant to Thermolysin (Fig. 3, A and B, lanes 1–4). This indicates that imported LeHPL was not

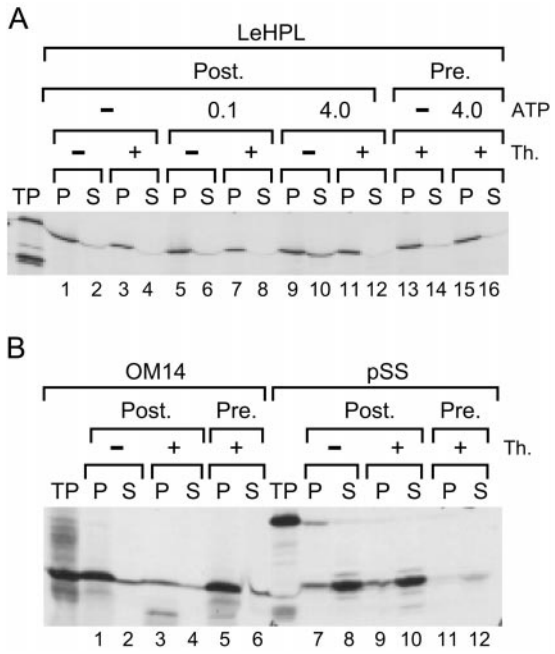

Figure 3. LeHPL is targeted to the outer envelope membrane by the OM14 pathway. A, [^{35}S]-Labeled LeHPL was incubated with isolated pea chloroplasts under ATP-depleted conditions (−) or in the presence of 0.1 or 4.0 mM Mg-ATP for 30 min. Chloroplasts were recovered by sedimentation through a 40% (v/v) Percoll cushion and then incubated with (+) or without (−) Thermolysin for 30 min at 4°C. Intact chloroplasts were again recovered by centrifugation through a 40% (v/v) Percoll cushion and fractionated into a total membrane (P) and soluble (S) fractions. [^{35}S]-Labeled LeHPL was likewise incubated with isolated pea chloroplasts that had been pretreated with Thermolysin. B, The import of OM14 and pSS served as controls for a protein using the OM14 pathway (ATP-independent), or the general import pathway (ATP-dependent), respectively. TP, Represents 10% (v/v) of translated product added to a single import assay.

exposed to the chloroplast surface, as was the case for OM14 (Li et al., 1991). The data shown in Figure 3 also addressed the question of whether or not proteinaceous components on the surface of the chloroplast are required for LeHPL targeting. For this analysis, chloroplasts were pretreated with Thermolysin to remove surface proteins, and then re-isolated prior to the addition of precursors. Under these conditions, import of the precursor of the Rubisco small subunit (pSS), which requires surface receptors, was significantly diminished (Fig. 3B). In contrast, OM14 readily associated with the Thermolysin-treated chloroplasts (Fig. 3B, lanes 5 and 6), as previously shown (Li et al., 1991). LeHPL likewise associated with Thermolysin-treated chloroplasts (Fig. 3A, lanes 13–16).

Additional experiments were performed to support the idea that LeAOS and LeHPL use different pathways for targeting to the chloroplast. First, competition studies revealed that pSS competes with LeAOS but not LeHPL for entry into the general import pathway (Fig. 4A). Second, LeAOS is proteolytically processed by a peptidase present in stromal extract, whereas LeHPL is not (Fig. 4B, lanes 1–4). This finding supports the idea that a cleavable transit peptide on LeAOS directs the protein into the general import pathway. Finally, by using chloroplasts pretreated with Thermolysin, we further demonstrate that LeAOS but not LeHPL requires functional surface-exposed receptors for translocation into chloroplasts (Fig. 4C, compare lane 1 with lane 3).

Localization of Imported LeAOS and LeHPL within the Chloroplast Envelope

To more precisely determine the suborganellar localization of each protein, Suc step gradients were used to fractionate import reactions into the stroma, outer envelope, inner envelope, and thylakoid membranes (Fig. 5). Established markers for the outer envelope (pToc75; Tranel et al., 1995), inner envelope (tp110-110N; Lübeck et al., 1997), stroma (pSS), and thylakoid (pPC; Baurle et al., 1991) were used to confirm the identity of the various fractions. LeAOS co-fractionated almost exclusively with the inner envelope marker tp110-110N (Fig. 5D). In contrast, LeHPL co-fractionated exclusively with the outer envelope marker Toc75 (Fig. 5C). Thus, we conclude that imported LeAOS and LeHPL are localized to the inner and outer envelope membranes, respectively.

Interaction of Imported LeAOS and LeHPL with Envelope Membranes

We examined the physical interaction between envelope membranes and LeAOS or LeHPL by assessing the ability of various reagents to extract the imported cytochromes from envelope membranes (Fig. 6). Extraction of envelopes with high concentrations of NaCl removed only a small fraction of total imported LeAOS (Fig. 6A). Sodium carbonate, however, removed the majority of LeAOS from the envelope, as did detergent solubilization. This result indicates that LeAOS is peripherally associated with the inner envelope. The extraction profile of the LeAOS-C465A mutant was nearly identical to that of wild-type LeAOS (Fig. 6B). This finding suggests that heme binding to apoLeAOS is not required for peripheral association of the protein with the inner envelope. In contrast to LeAOS, neither NaCl nor sodium carbonate extracted significant amounts of LeHPL from envelope membranes (Fig. 6C). The extraction profile of LeHPL was identical to that of the outer envelope marker protein OM14 (Fig. 6C). Like OM14, only Triton X-100 proved effective in extracting LeHPL. We conclude that LeHPL is intrinsically associated with the outer envelope membrane, whereas LeAOS is peripherally bound to the inner membrane.

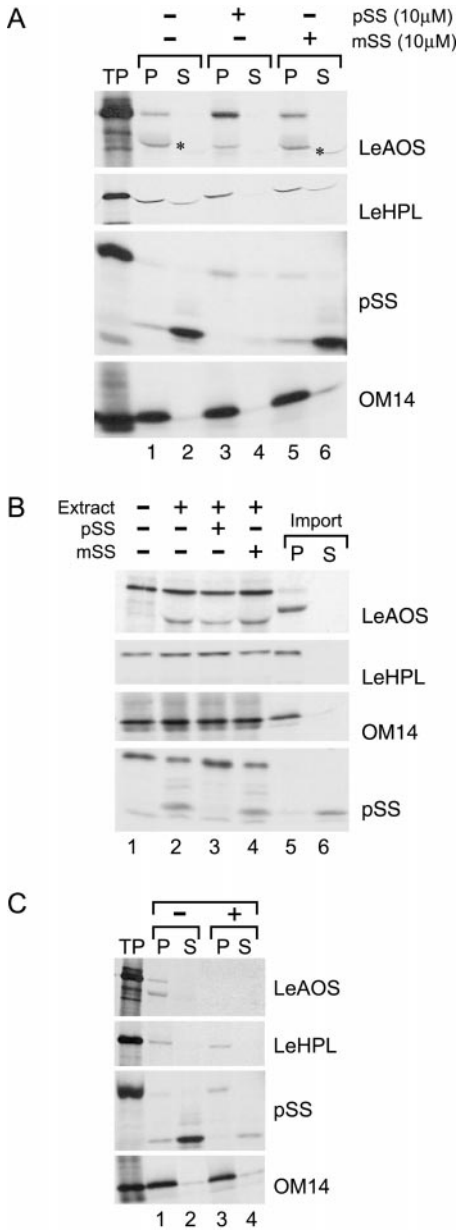

Figure 4. LeHPL and AOS use different pathways for targeting to the chloroplastic envelope. A, Targeting of LeAOS and LeHPL to isolated chloroplasts was done in the presence (+) or absence (−) of unlabeled pSS or mSS. Competition assays were incubated for 15 min at 25°C, after which chloroplasts were recovered by centrifugation through a 40% (v/v) Percoll cushion. Re-isolated chloroplasts were separated into a total membrane (P) and soluble (S) fraction. PSS and OM14 serve as controls for proteins that either use or not use, respectively, the general import apparatus. *, Identifies imported LeAOS. B, LeAOS and LeHPL were subjected to an in vitro stromal processing assay. [^{35}S]-Labeled LeAOS and LeHPL were incubated with isolated pea stromal extract for 90 min in the presence (+) or absence (−) of unlabeled pSS or mSS. Assays were quenched by the addition of 2× sample buffer and heated for 10 min at 100°C. An import assay (lanes 5 and 6) was likewise performed to generate authentic processed products. The migration of processed LeAOS (lanes 2 and 4) was distorted by the presence of Rubisco in the stromal processing assay. C, Chloroplasts were pretreated with (+) or without (−) Thermolysin to remove surface-exposed receptors. Thermolysin was quenched with 5 mM EDTA, and intact chloroplasts were recovered by centrifugation through a 40% (v/v) Percoll cushion containing 5 mM EDTA. Chloroplasts were washed with import buffer and then used in a standard 30-min import reaction. Chloroplasts were recovered by centrifugation through a 40% (v/v) Percoll cushion and separated into a total membrane (P) and soluble (S) fraction. All samples were analyzed by SDS-PAGE and fluorography. TP, represents 10% (v/v) of translated product added to a single import assay.

Topology of LeHPL and LeAOS in the Envelope Membrane

The precise orientation of AOS and HPL within the envelope membrane system is likely to be an important factor in determining the extent to which these two metabolic pathways interact, for example by competing for a common pool of substrates. As a first step to investigate the subcellular organization of the oxylipin pathway, the topology of LeHPL and LeAOS within the envelope membrane was investigated by using proteases that selectively penetrate the outer envelope membrane. Thermolysin does not penetrate the outer envelope, whereas trypsin penetrates the outer but not the inner envelope (Cline et al., 1984; Jackson et al., 1998). Radiolabeled precursor proteins were first targeted to the envelope membrane. After import and treatment with Thermolysin or trypsin, intact chloroplasts were recovered and fractionated into total membrane and soluble fractions. These fractions were then analyzed by SDS-PAGE and fluorography for the presence of the radiolabeled protein. Membrane-targeted LeHPL was resistant to Thermolysin (Fig. 7A). However, LeHPL was degraded by Thermolysin when chloroplasts were solubilized with detergent prior to the protease treatment, indicating that Thermolysin was active in these experiments (Fig. 7B, lanes 5 and 6). In contrast, treatment of import reactions with trypsin resulted in complete degradation of LeHPL (Fig. 7A, lanes 7 and 8). The trypsin sensitivity profile of LeHPL mirrored that of the OM14 outer envelope protein (Fig. 7D). Taken together, these results suggest that LeHPL reside in the inter-membrane space attached to the inner leaflet of the outer envelope membrane.

Protease protection experiments showed that imported LeAOS, like LeHPL, was not susceptible to Thermolysin (Fig. 7B, lanes 1–4). Control experiments showed that imported LeAOS was digested when chloroplasts were solubilized prior to Thermolysin treatment (Fig. 7B, lanes 5 and 6). In sharp contrast to LeHPL, treatment of import reactions with trypsin had no effect on the level of imported LeAOS (Fig. 7B, lanes 7 and 8). The activity of trypsin in this experiment was confirmed by showing that LeAOS was degraded when chloroplasts were solubilized with detergent prior to trypsin treatment (Fig. 7, lanes 9 and 10). The protease susceptibility profile of LeAOS was identical to that of pSS, a stromal marker protein (Fig. 7C). We conclude that LeAOS

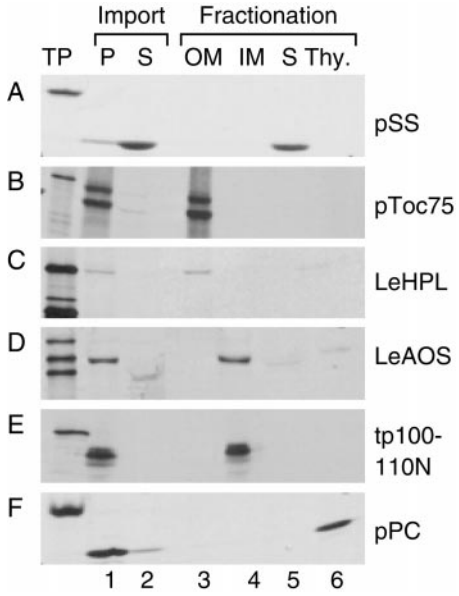

Figure 5. LeAOS is targeted to the inner envelope membrane and LeHPL is targeted to the outer envelope membrane of chloroplasts. [^{35}S]-Labeled precursor pSS (A), a stroma marker; pToc75 (B), an outer envelope marker; LeHPL (C); LeAOS (D); tp100–110N (E), an inner envelope marker; and pPC (F), a thylakoid marker were imported into isolated pea chloroplasts in separate translocation assays. After 30 min, an aliquot of each import assay was sedimented through a 40% (v/v) Percoll cushion. Intact chloroplasts were recovered, lysed, and separated into a total membrane (P) and soluble (S) fraction and analyzed by SDS-PAGE and fluorography (lanes 1 and 2). The remaining portions of the import assay was sedimented through a 40% (v/v) Percoll cushion. Recovered intact chloroplasts were lysed and fractionated by a Suc step-gradient. Stroma, outer and inner envelope membranes, and thylakoid fractions were recovered and analyzed by SDS-PAGE and fluorography (lanes 3–6). TP, represents 10% of translated product added to a single import assay.

associates with the inner envelope membrane with the bulk of the protein facing the stroma. Western-blot analysis, using polyclonal antibodies raised against recombinant LeAOS (Howe et al., 2000), revealed a similar localization and topology for endogenous pea AOS (data not shown).

DISCUSSION

Targeting of LeAOS

AOS and HPL are members of a novel family of cytochrome P450s that commit fatty acid hydroperoxides to the JA and C_6 aldehyde branches of oxylipin metabolism, respectively (Fig. 1). An increasing body of evidence indicates that both enzymes are localized to the chloroplast (Vick and Zimmerman, 1987; Hatanaka, 1993; Song et al., 1993; Harms et al., 1995; Blée and Joyard, 1996; Zhuang et al., 1996; Maucher et al., 2000). In the present study, we used in vitro import assays and chloroplast fractionation experiments to examine the specific location and topology of tomato AOS and HPL. A major conclusion drawn from our results is that LeAOS is targeted to the inner membrane of the chloroplast envelope. Several lines of evidence indicate that targeting of LeAOS to this location involves components of the general import pathway used for delivery of many nuclear-encoded proteins to the chloroplast: (a) Import of LeAOS into intact chloroplasts required ATP; (b) Import of LeAOS was reduced by the presence of excess amounts of pSS, a well-defined substrate for the general import pathway; (c) Surface-exposed receptors were required for efficient import of LeAOS; (d) In vitro translated LeAOS precursor was cleaved during import by a stromal processing protease. The latter finding is consistent with presence of a putative chloroplastic transit peptide at the N terminus of LeAOS (Fig. 2A; Howe et al., 2000). The presence of transit peptide sequences in the flax and Arabidopsis AOS suggest that import and localization of these P450s is similar to that of LeAOS. However, it is interesting to note that chloroplastic AOS from barley, the only monocot AOS reported to date, lacks a typical transit peptide (Maucher et al., 2000). This finding raises the possibility that monocots and dicots use different pathways for targeting of AOS. Alternatively, it is possible that the transit peptide-lacking form of barley AOS represents a subclass of CYP74A that either remains to be discovered or is not present in dicot plants. Recently it was shown that tomato encodes a second AOS that contains a predicted chloroplast targeting sequence (Sivasankar et al., 2000). It will be interesting to compare the subcellular location of this enzyme with that of the LeAOS reported here and to investigate the possibility that specific AOS isoforms serve different physiological roles. This hypothesis is in keeping with the

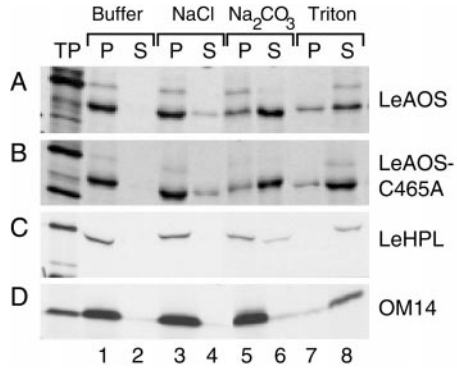

Figure 6. Extraction of imported proteins from envelope membranes. [^{35}S]-Labeled LeAOS (A), LeAOS-C465A (B), LeHPL (C), and OM14 (D) were incubated with isolated pea chloroplasts for 30 min. Intact chloroplasts were recovered by centrifugation through a Percoll cushion. Chloroplasts were lysed and a mixed envelope membrane fraction was obtained by using a Suc step gradient. Mixed envelopes were extracted with buffer, NaCl, or sodium carbonate and centrifuged at 100,000*g*. Membranes (P) and soluble (S) fractions were examined by SDS-PAGE and fluorography. D, OM14 control protein representing an outer envelope protein that is not extractable. TP, Represents 10% of translated product added to a single import assay.

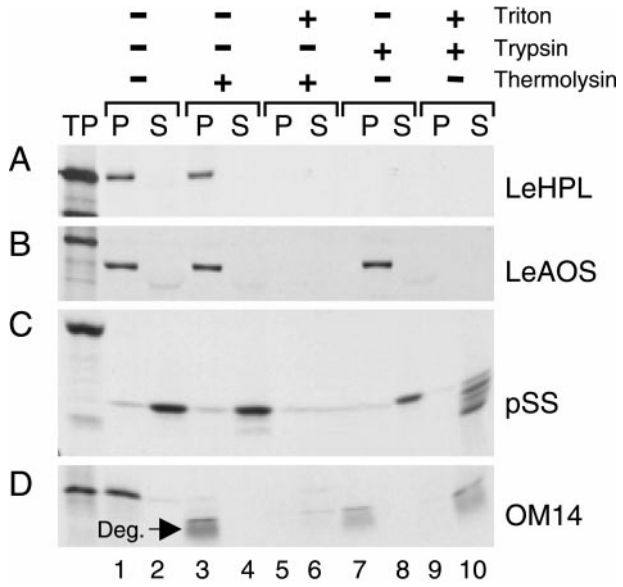

Figure 7. Topology of imported LeAOS and LeHPL. [^{35}S]-Labeled LeHPL (A), LeAOS (B), pSS (stroma control) (C), or OM14 (outer envelope control) (D) were incubated with isolated pea chloroplasts for 30 min. Chloroplasts were incubated with (+) or without (−) Thermolysin or trypsin in the presence (+) or absence (−) of Triton X-100. Intact chloroplasts were recovered by centrifugation through a Percoll cushion. Chloroplasts were lysed and total membrane (P) and soluble (S) fractions were isolated and analyzed by SDS-PAGE and fluorography. Likewise, Triton X-100-solubilized chloroplasts were ultracentrifuged to recover a pellet (P) and soluble (S) fraction and analyzed by SDS-PAGE and fluorography. TP, Represents 10% of translated product added to a single import assay.

proposal by Creelman and Mullet (1995) that there is more than one pathway for the initial steps of JA biosynthesis.

The topology of membrane-bound LeAOS was examined with proteases that selectively penetrate the outer membrane. The most straightforward interpretation of the results obtained is that LeAOS is bound to the inner leaflet of the inner envelope membrane with the bulk of the protein facing the stroma. The ability of sodium carbonate but not high salt to extract LeAOS from envelope membranes suggests that LeAOS is tightly associated with the membrane through a protein-lipid interaction. The protein determinants responsible for this interaction remain to be determined. Based on these findings, a likely pathway for the early stages of LeAOS import can be proposed. In the presence of low concentrations of ATP, pre-LeAOS binds to a receptor component of the general import apparatus. Increased levels of ATP promote translocation of the precursor across the double-membrane envelope, followed by removal of the transit peptide. The sequence of events that occurs between transit peptide removal and membrane association is less clear. The import behavior of the LeAOS-C465A mutant suggests that targeting of LeAOS to the inner membrane does not require heme attachment to the apocytochrome. Additional experiments are needed to elucidate the latter stages of LeAOS import, including the mechanism by which heme associates with the apoLeAOS.

Two models have been proposed to explain the pathway for protein targeting to the inner envelope. In the so-called "conservative sorting" model (Hartl and Neupert, 1990; Glick et al., 1992), proteins are first translocated to the stroma using the general import apparatus. Internal targeting signals within the mature region of the protein then re-direct the protein from the stroma to the inner envelope membrane. In the "stop-transfer" model (Blobel, 1980; High and Dobberstein, 1992), it is again proposed that proteins translocate via the general import pathway. However, a stop transfer domain within the protein halts the translocation process, allowing the transit peptide to be removed by the stromal processing peptidase. The mature protein then moves laterally out of the translocation apparatus and into the lipid bilayer to adopt a final topology. Additional experiments are needed to determine which of these models most accurately applies to the import and targeting of LeAOS. It is interesting to note that several aspects of LeAOS import, including cleavage of a transit peptide and an extrinsic membrane topology, resemble that of mitochondrial cytochrome P450s (Ogishima et al., 1985; Ou et al., 1986; Kumamoto et al., 1989).

Targeting of LeHPL

In vitro translated LeHPL was efficiently targeted to the outer envelope membrane of the chloroplast envelope. This finding is consistent with previous localization of HPL activity to chloroplast membranes in various plants (Hatanaka et al., 1987; Vick and Zimmerman, 1987; Gardner et al., 1991; Blée and Joyard, 1996; Zhuang et al., 1996). Insertion of LeHPL into the outer envelope proceeded through an ATP-independent pathway similar to that used by OM14. The inability of sodium carbonate to extract LeHPL from the envelope indicated that LeHPL, like OM14, is an intrinsic membrane protein. This interaction is reminiscent of microsomal P450s, which are anchored to the endoplasmic reticulum by an N-terminal transmembrane-spanning domain. However, the apparent lack of such a transmembrane sequence on LeHPL suggests that this P450 uses a different mechanism for membrane attachment.

The results of protease sensitivity experiments suggested that the bulk of HPL is exposed to the intermembrane space. Proteolytic processing of LeHPL, either during import or in the SPP assay, was not detected. This finding is consistent with the lack of a typical transit peptide on LeHPL (Fig. 2A). Because of the inability of SDS-PAGE to resolve small differences in the M_r of proteins such as LeHPL (53,500), we cannot exclude the possibility that a small peptide is removed from LeHPL during targeting. Our results

also do not rule out the possibility that different isoforms of HPL are localized to membranes other than the outer envelope. For example, the deduced amino acid sequence of HPL from Arabidopsis contains a putative transit peptide for chloroplast targeting (Bate et al., 1998; Fig. 2A). It will be interesting to determine whether this protein is targeted to the chloroplastic envelope and, if so, to which of the two envelope membranes it associates with. An HPL-encoding cDNA sequence from pepper has also been reported (Matsui et al., 1996). Based on the high degree of sequence similarity between LeHPL and the pepper sequence (Fig. 2A; Howe et al., 2000), we predict that pepper HPL assumes a location and topology similar to that of LeHPL. The ATP-independent and protease-insensitive pathway used for targeting of LeHPL to the outer envelope membrane may also mediate the chloroplastic localization of transit peptide-lacking AOSs such as those recently described in barley (Maucher et al., 2000).

Oxylipin Metabolism in the Chloroplast Envelope

The localization of AOS and HPL to the chloroplast envelope (e.g. Blée and Joyard, 1996) raises the question of whether these enzymes use separate pools of fatty acid hydroperoxide substrate or whether they compete for a common pool. Lipophilic substrates and products of P450s are thought to enter and exit the active site of the enzyme directly from the membrane bilayer to which the P450 is attached (Williams et al., 2000). If this is the case for envelope-localized CYP74s, then our results suggest that LeAOS and LeHPL metabolize different pools of substrate. For example, LeAOS could obtain hydroperoxides from chloroplastic LOXs that act on inner envelope- or thylakoid-derived fatty acids, whereas LeHPL could obtain substrates generated by cytosolic LOX or vacuolar LOX released in wounded tissue. Sequestration of HPL and AOS to different membrane surfaces of the envelope may thus reflect differences in the spatial organization of these two branches of oxylipin metabolism. It is important to note that our results do not imply co-localization of AOS and HPL to the same cell or the same chloroplast in vivo. In fact, it can be hypothesized that the AOS and HPL pathways are partitioned in vivo by differential expression of the two P450s in different cell types. Previously it was shown that the expression pattern of *LeAOS* and *LeHPL* overlap in many of the same organs of tomato (Howe et al., 2000). However, it was also noted that stem and petiole tissues accumulate much more *LeAOS* mRNA than *LeHPL* mRNA. This raises the likelihood that LeAOS is expressed in vascular tissues, as was shown to be the case for AOS expression in Arabidopsis (Laudert and Weiler, 1998; Kubigsteltig et al., 1999). Conversely, expression of HPL in non-vascular cell types such as epidermal cells or trichomes would be consistent with the role of this enzyme in generating oxylipins that function to inhibit pathogenic microbes that invade the leaf surface.

Localization of LeAOS to the stromal side of the inner envelope suggests that this P450 metabolizes hydroperoxides produced by chloroplastic isoforms of LOX (Bell et al., 1995; Feussner et al., 1995). Determination of the substrate and regio-specificity of chloroplastic LOXs of tomato (Heitz et al., 1997), together with analysis of their cell type-specific expression, will facilitate the identification of specific LOX isoforms that metabolically interact with LeAOS. It will also be important to determine whether AOS physically interacts with LOX or other enzymes involved in JA biosynthesis. Analogous multi-enzyme complexes, such as the fatty acid synthase that channels acetate into long-chain fatty acids in the chloroplast envelope, have been described (Ohlrogge and Browse, 1995; Roughan and Ohlrogge, 1996; Shorrosh et al., 1996). One possibility is that AOS interacts with AOC, a soluble enzyme that metabolizes the epoxide product, 12,13-epoxy-octadecatrienoic acid (12,13-EOT), of AOS (Ziegler et al., 1997). The high instability of 12,13-EOT has led others to propose that AOS is localized in close proximity to AOC (Ziegler et al., 1997). Localization of AOS to the stromal face of the inner envelope indicates that a direct physical interaction between AOS and AOC would be possible if AOC is a stromal protein. It may be possible to identify such AOS-interacting proteins using immunoprecipitation techniques that have been successful in identifying protein components of the chloroplastic protein translocation complex (Akita et al., 1997; Nielsen et al., 1997). An equally attractive hypothesis, however, is that the 12,13-EOT product of AOS is channeled across the inner membrane to AOC located in the inter-membrane space (Fig. 8). The prod-

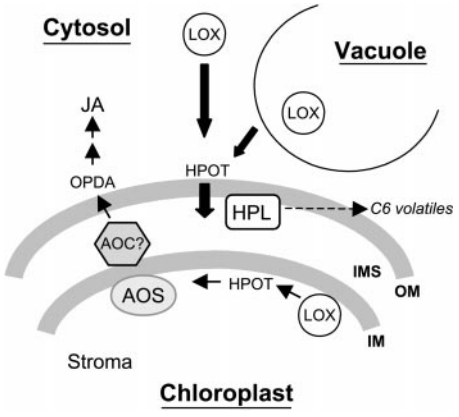

Figure 8. Proposed model for the compartmentalization of LeAOS and LeHPL within chloroplasts. The proposed location and topology of AOS and HPL is based upon the results of in vitro import experiments. The depiction of AOS and HPL within the same chloroplast is for simplicity, and is not meant to imply colocalization of the two enzymes in vivo. See text for detail. OM, Outer envelope membrane; IMS, intermembrane space; IM, inner envelope membrane.

uct of AOC (12-OPDA) is metabolized by OPDA reductase (OPR3), the next enzyme in the JA biosynthesis pathway. The gene encoding OPR3 was recently identified (Sanders et al., 2000) but the intracellular location of the enzyme remains to be determined. The final β-oxidation steps of JA biosynthesis presumably take place in the peroxisomes. An inter-membrane space location for AOC is also in keeping with the possibility that transit peptide-lacking AOSs, such as those recently described in barley (Maucher et al., 2000), are localized in a manner similar to LeHPL (i.e. inter-membrane space). Recently, a full-length cDNA clone coding for AOC was isolated from tomato (Ziegler et al., 2000). The protein was shown to contain an N-terminal transit peptide and was localized to the chloroplast. Future studies aimed at determining the precise subcellular localization of AOC and other octadecanoid pathway enzymes will provide important insight into the cellular organization of pathways for oxylipin metabolism and signaling.

MATERIALS AND METHODS

Materials

Percoll, ATP, and Thermolysin were obtained from Sigma (St. Louis). [^{35}S]-Met was purchased from DuPont/NEN (Boston). Pea (*Pisum sativum* var Little Marvel) seeds were supplied by Olds Seed Company (Madison, WI). Tomato (*Lycopersicon esculentum* var Castlemart) seedlings used for chloroplast isolation were grown as previously described (Howe et al., 2000).

Isolation of Pea and Tomato Chloroplasts

Intact chloroplasts were isolated from 8- to 12-d-old pea seedlings or 10-d-old tomato seedlings and purified using a Percoll gradient as previously described (Bruce et al., 1994). Intact pea or tomato chloroplasts were re-isolated and resuspended in import buffer (330 mM sorbitol, 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]/KOH, pH 8.0) at a concentration of 1 mg of chlorophyll/mL.

Site-Directed Mutagenesis of LeAOS

Mutagenesis of *LeAOS* was carried out using the Transformer Site-Directed Mutagenesis Kit from CLONTECH Laboratories (Palo Alto, CA). Two phosphorylated primers were used for the synthesis of the mutant DNA strand. The mutagenic primer (5′-GAAAAGGCCGGCGGACAATAAGGGG-3′) was designed to replace Cys-465 (TGC) with Ala (GCG), which is underlined. The selection primer (5′-GTGACTGGTGAGGCCTCAACCAAGTC-3′) was designed to replace a *Sca*I site within the pBluescript vector with a *Stu*I site, which is also underlined. The mutant plasmid was selected using series of enzyme digestions by *Sca*I. Selected clones were confirmed by DNA sequence analysis.

In Vitro Translation of Precursor Proteins

The plasmid containing pSS (Olsen and Keegstra, 1992) was linearized with *Pst*I and transcribed with SP6 RNA polymerase. Plasmids containing the coding region for LeAOS and LeAOS-C465A were both linearized with *Xho*I and transcribed with T7 RNA polymerase. The plasmid containing the coding region for LeHPL (Howe et al., 2000) was linearized with *Xho*I, transcribed with T7 RNA polymerase, and translated with a nuclease-treated rabbit reticulocyte lysate system using the suggested protocol of the manufacturer (Promega, Madison, WI). LeAOS and LeAOS-C465A were likewise translated using either a nuclease-treated rabbit reticulocyte lysate translation system or a wheat germ translation system as previously described (Bruce et al., 1994). The plasmids pToc75 (Tranel et al., 1995), tp110-110N (Lübeck et al., 1997), and pPC (Baurle et al., 1991) were all translated in a nuclease-treated rabbit reticulocyte lysate. All proteins were radiolabeled using [^{35}S]-Met.

Binding and Import Assays

Binding or import reactions (adapted from Bruce et al., 1994) received 500,000 dpm of radiolabeled translation product and 0.1 mM ATP for binding or 4 mM ATP for translocation following the addition of intact chloroplasts corresponding to 25 μg of chlorophyll in a final volume of 150 μL. Binding and translocation reactions were incubated for 30 min at room temperature. Intact chloroplasts were then recovered by sedimentation through a 40% (v/v) Percoll cushion. The pellets were resuspended in lysis buffer (25 mM HEPES-KOH, pH 8.0, 4 mM $MgCl_2$) and incubated on ice for 15 min. After ultracentrifugation at 100,000*g*, total membrane and soluble fractions were obtained. All fractions were analyzed by SDS-PAGE (Laemmli, 1970) and fluorography. Treatment of binding or import reactions with Thermolysin was performed as described by Cline et al. (1984). Likewise, treatment of binding and import reactions with trypsin was performed as described by Jackson et al. (1998). Competition studies using unlabeled pSS and mSS were performed as described by Tranel et al. (1995).

Isolation and Extraction of Mixed Envelope Membranes from Pea Chloroplasts

Following import, chloroplasts were fractionated using the method of Perry and Keegstra (1994) with the following modifications: chloroplasts were hypotonically lysed in 25 mM HEPES-KOH, pH 8.0, 4 mM $MgCl_2$, for 20 min on ice. The sample was layered on top of a Suc step gradient (0.5 mL of 1.2 M Suc and 0.5 mL of 0.46 M Suc; all Suc solutions were in 25 mM HEPES-KOH, pH 8.0). The gradient was centrifuged as described in Perry and Keegstra (1994). A mixed outer and inner envelope membrane fraction was isolated at the 0.46 M/1.2 M Suc interface and analyzed by SDS-PAGE (Laemmli, 1970) and fluorography. Mixed envelope membranes were extracted with high salt or sodium carbonate as described by Tranel et al. (1995).

Isolation of Purified Outer and Inner Envelope Membranes

Following import, chloroplasts were hypertonically lysed in 0.6 M Suc in 25 mM HEPES-KOH, pH 8.0, 4 mM $MgCl_2$ for 20 min on ice and then frozen at −20°C for at least 1 h. After one freeze thaw cycle, the solution was adjusted to 0.3 M Suc by diluting with 25 mM HEPES-KOH, pH 8.0, 4 mM $MgCl_2$. Samples were centrifuged at 4,500*g* for 15 min to remove most of the thylakoid membranes. The supernatant was recovered and applied to a Suc step gradient containing: 0.5 mL of 1.0 M Suc, 0.5 mL of 0.8 M Suc, and 0.5 mL of 0.46 M Suc; all Suc solutions were in 25 mM HEPES-KOH, pH 8.0. Centrifugation was performed as described by Tranel et al. (1995). Fractions at the 0.46 M/0.8 M Suc interface (outer envelope) and 0.8 M/1.0 M Suc interface (inner envelope) were collected and analyzed by SDS-PAGE (Laemmli, 1970) and fluorography.

ACKNOWLEDGMENTS

We thank Dr. Anton Sanderfoot and Robyn Perrin for critical reading of the manuscript. We likewise thank Marlene Cameron and Kurt Stepnitz for preparation of figures. We thank Dr. D. Schnell (Rutgers University, New Brunswick, NJ) for the gift of antibodies to Tic22 and Dr. M. Akita for providing purified overexpressed pea precursor SS and mature SS protein.

Received May 30, 2000; modified July 19, 2000; accepted August 15, 2000.

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