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Red Bell Pepper Chromoplasts Exhibit in Vitro Import Competency and Membrane Targeting of Passenger Proteins from the Thylakoidal Sec and ΔpH Pathways but Not the Chloroplast Signal Recognition Particle Pathway[1]

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Chloroplast to chromoplast development involves new synthesis and plastid localization of nuclear-encoded proteins, as well as changes in the organization of internal plastid membrane compartments. We have demonstrated that isolated red bell pepper (*Capsicum annuum*) chromoplasts contain the 75-kD component of the chloroplast outer envelope translocon (Toc75) and are capable of importing chloroplast precursors in an ATP-dependent fashion, indicating a functional general import apparatus. The isolated chromoplasts were able to further localize the 33- and 17-kD subunits of the photosystem II O_2-evolution complex (OE33 and OE17, respectively), lumen-targeted precursors that utilize the thylakoidal Sec and ΔpH pathways, respectively, to the lumen of an internal membrane compartment. Chromoplasts contained the thylakoid Sec component protein, cpSecA, at levels comparable to chloroplasts. Routing of OE17 to the lumen was abolished by ionophores, suggesting that routing is dependent on a transmembrane ΔpH. The chloroplast signal recognition particle pathway precursor major photosystem II light-harvesting chlorophyll *a/b* protein failed to associate with chromoplast membranes and instead accumulated in the stroma following import. The Pftf (plastid fusion/translocation factor), a chromoplast protein, integrated into the internal membranes of chromoplasts during in vitro assays, and immunoblot analysis indicated that endogenous plastid fusion/translocation factor was also an integral membrane protein of chromoplasts. These data demonstrate that the internal membranes of chromoplasts are functional with respect to protein translocation on the thylakoid Sec and ΔpH pathways.

Plastids are developmentally related organelles capable of interconversion among a variety of structurally and biochemically distinct forms in response to both environmental and tissue-specific cues (Whatley, 1978; Thomson and Whatley, 1980). Formation of chromoplasts in many fruits is one striking example of this plasticity. Heavily pigmented, photosynthetically inactive chromoplasts frequently develop from chloroplasts during ripening. This conversion involves dramatic changes in the organization and composition of the internal plastid compartment, which include the loss of proteins involved in carbon fixation in the stroma and replacement with chromoplast-specific proteins, the breakdown of the photosynthetic thylakoid membranes and loss of proteins involved in light capture and electron transfer, and, in some cases, the formation of new membranes (Spurr and Harris, 1968; Camara and Brangeon, 1981; Piechulla et al., 1987; Kuntz et al., 1989).

Chromoplast formation is an active rather than simply a degradative process. New proteins, specific to or enhanced in chromoplasts, are synthesized and compartmentalized in the plastid (Camara et al., 1995; Price et al., 1995). Most chromoplast proteins are predicted to be nuclear encoded, translated on cytoplasmic ribosomes, and posttranslationally imported into the plastid, as are nuclear-encoded chloroplast proteins. Import of chloroplast proteins occurs via a general import machinery that appears to mediate translocation of most or all proteins that are delivered to the chloroplast stroma, either as a final destination or as an intermediate location (Cline and Henry, 1996; Robinson and Mant, 1997; Schnell, 1998). Proteins are targeted to the general import pathway by an N-terminal extension that is cleaved upon import, resulting in the appearance of a processed protein of reduced M_r. Presumably, the import of proteins into chromoplasts is accomplished by the same machinery that is responsible for import of pro-

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Abbreviations: cpSRP54, the chloroplast homolog of the SRP 54-kD protein; Hsp70, 70-kD chloroplast heat-shock protein; LHC, light-harvesting complex; LHCP, PSII light-harvesting chlorophyll *a/b* protein; OE17, OE23, and OE33, the 17-, 23-, and 33-kD subunits of the PSII oxygen evolution complex, respectively; pXXX, iXXX, mXXX, full-length precursor, intermediate precursor, and mature-sized forms, respectively, of OE17, OE23, or OE33; Rbcs, Rubisco small subunit; SRP, signal-recognition particle; Tic110, the 110-kD component of the chloroplast inner envelope translocon; Toc75 and Toc34, 75- and 34-kD components of the chloroplast outer envelope translocon.

teins into chloroplasts, although this has never been directly examined.

In some chromoplasts an extensive set of internal membranes accumulates, replacing the thylakoids. For example, in the fibrillar-type chromoplast of bell pepper (*Capsicum annuum*), the photosynthetic membranes are replaced by membranous sheets and vesicles in addition to the carotenoid-rich plastoglobules and fibrils (Spurr and Harris, 1968; Laborde and Spurr, 1973; Camara and Brangeon, 1981; Deruere et al., 1994). The often extensive internal membranes are the site of synthesis of keto-xanthophylls, which constitute the major carotenoids of red fruit (Bouvier et al., 1994).

Our interests are in the biogenesis of the internal membranes of plastids, in particular the proteins that are integral to the bilayer, as well as those located in the luminal compartment formed by the bilayer. In chloroplasts, proteins destined for the thylakoid membrane or lumen are routed from the stroma into the thylakoid membrane and lumen by one of at least four distinct mechanisms: the ΔpH, chloroplast SRP, thylakoid Sec pathways, and an apparently spontaneous insertion mechanism (for review, see Cline and Henry, 1996; Robinson and Mant, 1997; Schnell, 1998). In view of the extensive internal membrane system of bell pepper chromoplasts, one would expect the presence of proteins and accompanying translocation machinery in these membranes. However, no chromoplast-specific proteins have been conclusively demonstrated to be either integral or luminal to these membranes.

One protein, Pftf (plastid fusion/translocation factor), predicted to be membrane anchored by sequence analysis, has been purified from the stromal compartment of pepper chromoplasts (Hugueney et al., 1995). This raised the possibility that mature chromoplasts lack the ability to localize proteins into/across internal membranes. To address this question we developed a method for isolating protein import-competent chromoplasts from bell peppers. Immunoblotting confirmed that these chromoplasts contain known translocation machinery components. Chromoplasts were assayed in vitro for their ability to import and localize passenger proteins from the three known protein-machinery-dependent thylakoid-targeting pathways. We found mature chromoplasts to be capable of membrane targeting of proteins that utilize the thylakoidal Sec and ΔpH pathways but not capable of inserting a membrane protein, LHCP, which utilizes the chloroplast SRP pathway. Pftf was inserted into the membranes of these chromoplasts in a manner similar to that observed in chloroplasts, and resident Pftf was also found to be integrally associated with chromoplast membranes. The precise role of these pathways in the formation of bell pepper chromoplasts remains to be fully elucidated.

MATERIALS AND METHODS

Plant Material

Peppers (*Capsicum annuum*) were grown under field conditions in Gainesville, FL (cv Camelot X3R) and in a growth room with a light intensity of 200 μE m^{-2} s^{-1}, a daylength of 12 h, and a constant 26°C (cvs Yolo Wonder and Lemon King). For some experiments, red fruit was purchased locally.

Plastid Isolation

Chromoplasts were isolated from 200 g of red bell pepper fruit by a combination of differential and gradient centrifugation as previously described (Price et al., 1995) and modified as follows. Fruit was harvested and stored on ice for 2 h prior to use. Red pericarp was homogenized with a polytron in 700 mL of grinding buffer (0.33 M sorbitol, 50 mM Hepes/KOH, pH 7.5, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 5 mM sodium ascorbate, and 1% BSA). The homogenate was filtered through Miracloth (Calbiochem) and plastids were recovered by centrifugation at 800*g*. Plastids were resuspended in the same buffer and layered on a continuous Percoll (Pharmacia) gradient prepared by centrifugation of a 30% Percoll suspension in grinding buffer supplemented with 0.03 mg/mL glutathione for 30 min at 48,000*g* in a JA20 fixed-angle rotor. After the sample was centrifuged at 6,000*g* for 20 min using a JA13.1 swinging-bucket rotor, the lower band containing intact chromoplasts was recovered, washed in import buffer (50 mM Hepes/KOH, pH 8.0, and 0.33 M sorbitol), and finally resuspended in import buffer at a protein concentration of 5 to 10 μg/μL (equivalent to about 1 mL import buffer/200 g starting pericarp material). Chloroplasts were isolated from peas (*Pisum sativum* cv Laxton's Progress 9) as described before (Cline, 1986). Fourteen-day-old pepper seedling and green fruit chloroplasts were isolated in the same manner as pea chloroplasts.

Pigment Analysis of Chromoplasts and Chloroplasts

Chlorophyll concentrations were determined in 80% acetone extracts (Arnon, 1949). Relative chlorophyll and carotenoid levels were also analyzed by an absorption spectra of acetone extracts equivalent to 100 μg plastid protein/mL with a UV-160 spectrophotometer scanning wavelengths 350 to 750 nm (Shimadzu Scientific Intruments, Columbia, MD).

Electron Microscopy

Isolated chromoplasts were fixed with 3% glutaraldehyde in import buffer (1 h on ice), washed in 50 mM phosphate buffer, pH 7.5, and postfixed with osmium tetroxide using standard protocols for electron microscopy. Chromoplasts were viewed with a transmission electron microscope (model H-7000, Hitachi, Tokyo, Japan) by Karen Vaughn at the University of Florida Electron Microscopy Core Laboratory (Gainesville).

Preparation of Radiolabeled Precursors

Unless otherwise stated, plasmids for pLHCP, pOE33, pOE17, and pRbcs used for in vitro transcription and translation have been described (Cline et al., 1993). Cloning and analysis of DNA products was by standard molecular bi-

ology procedures (Sambrook et al., 1989). A transcription plasmid containing the Pftf-coding region (accession no. X80755) in pGEM4Z was synthesized by reverse transcriptase-PCR with total RNA from cv Yolo Wonder, as was the template and Moloney murine leukemia virus reverse transcriptase (SuperScript II, GIBCO-BRL), following the manufacturer's guidelines. The Pftf-coding region was amplified from the first-strand cDNA with Pfu DNA polymerase (Stratagene) following the manufacturer's guidelines, the products were ligated into pGEM-4Z (Promega) and transformed into *Escherichia coli* XL-1-Blue cells, and the resulting clones were sequenced entirely on both strands by the University of Florida Interdisciplinary Center for Biotechnology Research DNA-sequencing core. In vitro transcription with SP6 RNA polymerase (Promega) and translation with rabbit reticulocyte lysate (Promega) or with coupled transcription/translation in wheat germ extract (Promega) in the presence of [^{3}H]Leu (DuPont/NEN) was performed following the manufacture's guidelines. Translation products were diluted with 1 volume of 60 mM Leu in 2× import buffer prior to use.

Plastid Protein Import and Fractionation

Import of in vitro-translated precursors into intact chromoplasts (5.5 mg protein equivalent chromoplasts/mL) was carried out in import buffer in the absence or presence of 5 mM Mg ATP. Plastids and translation products for import in the absence of ATP were treated with 0.04 unit apyrase/μL for 10 min on ice prior to the start of the assay. For import in the presence of ionophores, plastids were preincubated for 10 min with nigericin and valinomycin (0.5 and 1.0 μM final concentrations, respectively, added from ethanolic stocks). Import reactions were conducted for 15 min at 25°C in room light and were terminated by transfer to an ice bath.

Following import, chromoplasts were recovered by centrifugation and washed two times with import buffer by resuspension followed by centrifugation. Unless otherwise stated, centrifugation of intact chromoplasts was for 5 min at 6,000*g*. Recovered chromoplasts were analyzed directly or following posttreatment with thermolysin for 40 min at 4°C (Cline, 1986). For subfractionation, recovered, untreated chromoplasts were lysed in 10 mM Hepes/KOH, pH 8.0, and the total membranes were separated from stroma by centrifugation for 15 min at 40,000*g*. The stromal fraction was further clarified by centrifugation for 20 min at 40,000*g* and the membranes were washed with import buffer, extracted with 0.1 M NaOH, or treated with thermolysin (Cline, 1986). Unless otherwise stated, total chromoplast membranes were recovered by centrifugation at 40,000*g* for 15 min. All samples were resuspended in 20 mM EDTA and stored at −20°C prior to analysis by SDS-PAGE and fluorography. Import of precursors into pea chloroplasts in the presence or absence of ATP and ionophores and chloroplast fractionations were as described by Cline et al. (1993).

SDS-PAGE and Immunoblotting

Total membrane and total stromal samples for SDS-PAGE and immunoblotting were prepared as described for the import reactions, except that the stromal fractions were further clarified of membranes by centrifugation at 100,000*g* for 1 h. Immunoblot analysis was performed on 5 μg (1 μg for immunoblots with anti-LHCP) of chromoplast and chloroplast proteins after separation by SDS-PAGE and transfer to nitrocellulose, as described before (Payan and Cline, 1991). Immunoblots with horseradish peroxidase-conjugated 2° antibody (Bio-Rad) were developed with the ECL (Amersham) procedure according to the manufacturer's guidelines. The following sources and dilutions of primary antibodies were used: anti-Rbcs, 1:7,000 (E. Tobin, University of California, Los Angeles); anti-LHCP, 1:20,000 (Payan and Cline, 1991); anti-SecA, 1:5,000 (Yuan et al., 1994); anti-Pftf, 1:10,000 (Hugueney et al., 1995); anti-OE23, 1:20,000 (Fincher et al., 1998); anti-Hsp70, 1:5,000 (Yuan et al., 1993); anti-cpSRP54, 1:10,000 (R. Henry and K. Cline, unpublished results), anti-Toc75, anti-Toc34, and anti-Tic110, all at 1:7,000 (D. Schnell, Rutgers University, Newark, NJ).

Miscellaneous

Proteins were quantified using the bicinchoninic acid method (Pierce). Unless indicated, chemicals were purchased from Sigma. Transmembrane prediction of the Pftf amino acid sequence was performed with the TMpred program (ISREC Bioinformatics, Epalinges, Switzerland) (http:www.isrec.isb-sib.ch/software/TMPRED_form.html).

RESULTS

Isolation of Intact, Mature Red Bell Pepper Chromoplasts

Intact chromoplasts from mature red bell pepper were isolated by a combination of differential and Percoll density gradient centrifugation. Fully developed chromoplasts, uncontaminated by chloroplasts, were required for this study. The identity of these plastids was confirmed by structural and biochemical methods. The chromoplasts appeared intact and uncontaminated by other organelles, as assessed by electron microscopy. They contained the typical internal membrane system that includes, using the terminology of Spurr and Harris (1968), lamellae and vesicles, which are frequently observed to be associated with the inner envelope (Fig. 1; Spurr and Harris, 1968; Laborde and Spurr, 1973; Bouvier et al., 1994). These plastids also contained nonmembranous, but characteristic, internal structures that include plastoglobules and fibrils (Fig. 1).

Red bell pepper chromoplasts are known to be enriched in carotenoids and depleted of chlorophylls. As shown in Figure 2, the plastids utilized in the protein-targeting assays exhibited this pigment composition profile. A typical absorption spectrum of an extract of chromoplasts used here is shown in comparison with an extract of an equivalent protein weight (100 μg) of pea chloroplasts. Chloroplasts typically contain 90 μg chlorophyll/mg total plastid

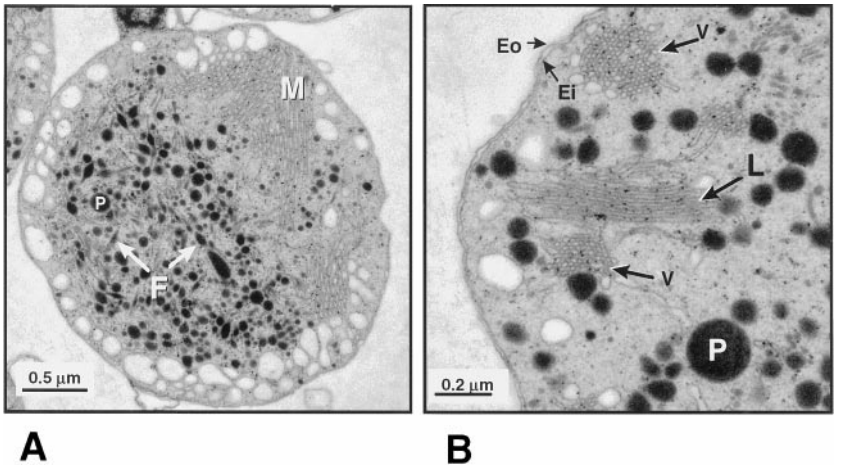

Figure 1. Electron micrograph images of isolated red bell pepper chromoplasts. A, Representative chromoplast showing internal membranes (M), osmiophilic plastoglobules (P), and fibrils (F). B, Detail of chromoplast internal membrane compartments. Note the inner and outer envelope membranes (Ei and Eo) and the internal membranes organized into vesicles (V) and lamellae (L).

protein (Chen, 1992). Chromoplasts contained from 0.4 to 4.2 μg chlorophyll/mg total plastid protein.

The isolated chromoplasts and chromoplast subfractions also displayed the characteristic protein profiles previously reported for pepper chromoplasts, as determined by SDS-PAGE. Total chromoplasts (Fig. 3, lane 1) contained the two major chromoplast proteins, ChrA (53 kD) and ChrB (35 kD), which comprise 30% and 10% of red pepper chromoplast protein, respectively (Hadjeb et al., 1988; Newman et al., 1989). In agreement with the model that ChrB is peripherally associated with the lipid monolayer of fibrils, ChrB cofractionated with stroma and membranes, with the majority in the stroma (Fig. 3, lane 2). ChrA, which has been observed to be tightly membrane associated, fractionated with the membranes (Fig. 3, lane 3). However, it was almost entirely removed by alkaline extraction (Fig. 3, lane 4), indicating that ChrA is not anchored in the membrane, as previously suggested (Cervantes-Cervantes et al., 1990). Several proteins, in particular one unidentified protein of a slightly higher M_r than ChrA, remained associated with the membrane fraction following extraction with 0.1 M NaOH, suggesting the existence of integral proteins in chromoplast internal membranes (Fig. 3, lane 4). This protein profile is strikingly different from that of chloroplasts of pea or pepper (Fig. 3, lanes 5–8), in which the 55-kD large Rubisco subunit and 15-kD small Rubisco subunit (Rbcs) are the major stromal polypeptides (Fig. 3, lane 6) and the LHCP is the major membrane polypeptide (Fig. 3, lane 7).

Pepper Chromoplasts Contain Low Amounts of Chloroplast-Specific Proteins, but Substantial Quantities of Those Are Involved in Protein Metabolism

Chromoplasts were subjected to immunoblot analysis to assess their content of residual chloroplast proteins involved in photosynthesis and for the presence of plastid proteins known to be involved in protein import, assembly, and routing (Fig. 4). Rbcs was present in the chromoplast stroma at a reduced level compared with chloroplasts (Fig. 4A). In some cases, the majority of the chromoplast Rbcs was associated with the membrane fraction (data not shown). Rbcs association with plastid membranes, in particular envelope fractions, has been previously reported

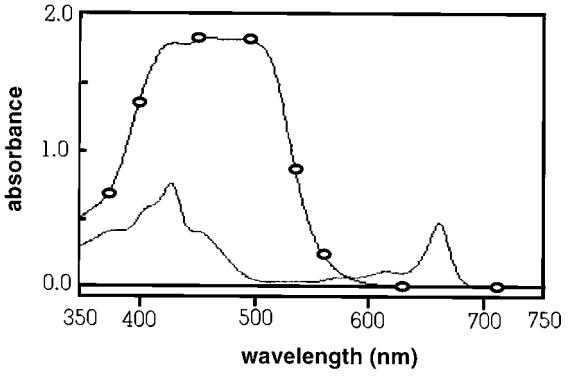

Figure 2. Pigment composition of chromoplasts and chloroplasts. Chlorophyll and carotenoid levels of chloroplasts and chromoplasts were analyzed by absorption spectra of 80% acetone extracts of 100-μg protein equivalents of plastids using a spectrophotometer scanning wavelengths of 350 to 750 nm. —○—, Red bell pepper chromoplast extract; ———, green pea chloroplast extract. A_{652} of chromoplasts = 0.012; A_{652} of chloroplasts = 0.256

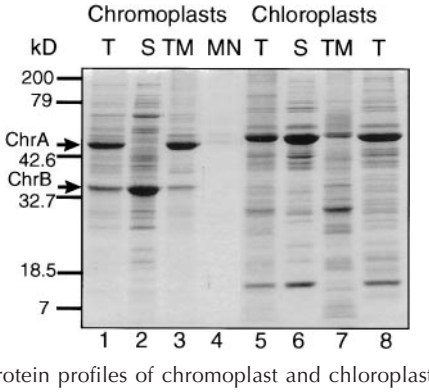

Figure 3. Protein profiles of chromoplast and chloroplast fractions. SDS-PAGE (12.5%) and Coomassie blue staining of fractionated red bell pepper fruit chromoplasts (lanes 1–4), pea seedling chloroplasts (lanes 5–7), and pepper seedling chloroplasts (lane 8). Lanes T, Total proteins; lanes S, stromal proteins; lanes TM, total membrane proteins; and lanes MN, NaOH-extracted membranes. Each lane contained 10 μg of protein except MN, which is equivalent to M prior to extraction. Positions of ChrA and ChrB are indicated.

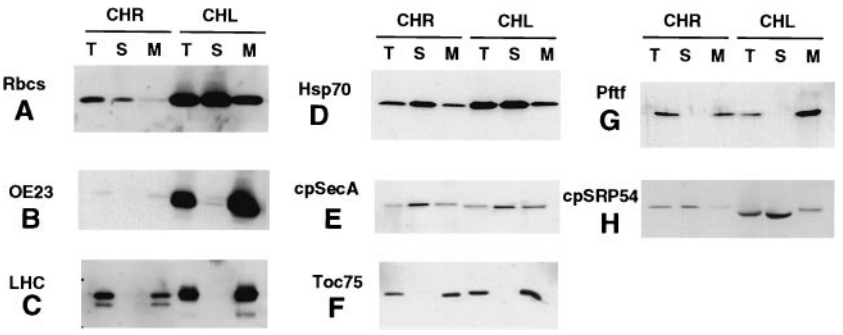

Figure 4. Detection of photosynthetic and protein assembly-specific proteins in chromoplasts and chloroplasts. Isolated pea chloroplasts (lanes CHL) and red pepper chromoplasts containing 3 μg chlorophyll/mg protein (lanes CHR) representing total plastid (lanes T), soluble (stromal) plastid (lanes S), and membrane plastid (lanes M) fractions were subjected to immunoblot analysis (see "Materials and Methods"). Immunoblots were probed with antibodies against: Rbcs (A), OE23 (B), LHCP (C), Hsp70 (D), cpSecA (E), Toc75 (F), Pftf (G), and cpSRP54 (H) and visualized by enhanced chemiluminescence.

(Werner-Washburne et al., 1983). The large Rubisco subunit and Rbcs have previously been detected immunologically in red bell pepper chromoplasts (Kuntz et al., 1989). Two thylakoid-associated photosynthetic proteins, OE23 and LHCP, were also detected in chromoplasts. OE23 was barely detectable on the immunoblot (Fig. 4B). Two LHCP immunoreactive bands were detected in the membrane fractions of fruit containing 3% of the chlorophyll content of a chloroplast (Fig. 4C) at levels as high as 10% of LHCP found in chloroplasts, as determined by a comparison of the signal to that in a dilution series of chloroplasts (data not shown). Amyloplasts from the white pericarp of unripe pepper cv Lemon King, a cultivar whose fruit never develop chloroplasts and when unripe lacks significant levels of either carotenoids or chlorophylls (0.4 μg chlorophyll/mg protein), also contained membrane-associated LHCP immunoreactive bands of identical mobility at levels 2% of that detected in chloroplasts (data not shown).

Proteins involved in plastid biogenesis and protein translocation were present in chromoplasts at levels comparable to those of chloroplasts. The stromal chaperone Hsp70 and the stromal component of the thylakoidal Sec pathway, cpSecA, were both present is substantial amounts in chromoplasts, with the bulk of the protein in the stroma and a significant subfraction associated with the membranes, similar to their distribution in chloroplasts (Fig. 4, D and E). A component of the chloroplastic outer envelope general import apparatus machinery, Toc75, and a chromoplast homolog of bacterial FtsH, Pftf, accumulated to similar levels in the membrane fraction of both plastid types (Fig. 4, F and G). Chromoplasts also contained cpSRP54, a component of the chloroplast SRP pathway (Li et al., 1995). The level of this protein was lower than that present in chloroplasts (Fig. 4H).

The analyses in Figure 4 were conducted on red fruit (containing 2.2 μg chlorophyll/mg protein) chromoplasts and pea chloroplasts. Virtually identical results were obtained from immunoblots of red fruit chromoplasts and seedling chloroplasts isolated from bell pepper cv Yolo Wonder (data not shown), confirming that the differences observed do not reflect variation in affinity of the antibodies for pea or pepper proteins. Antibodies directed against pea Toc34 and Tic110 failed to cross-react with pepper chloroplast or chromoplast proteins (data not shown), raising the possibility that these proteins are more divergent than Toc75 among different plant species.

Isolated Chromoplasts Exhibit Plastid and Subplastid Protein-Targeting Activity

To determine whether chromoplasts possess the same protein import and routing capability as chloroplasts, isolated chromoplasts were assayed for their ability to import and localize four chloroplast-specific precursors (pRbcs, pLHCP, pOE33, and pOE17) and one known chromoplast-targeted precursor (pPftf; Fig. 5). All five precursors readily imported into the isolated chromoplasts in the presence, but not in the absence, of ATP. This was determined by the appearance of the processed (mature) forms of the respective proteins that were resistant to thermolysin treatment of the intact plastids (Fig. 5, lanes 2–5). In contrast, the full-length precursors present in the total chromoplast and total membrane preparations were not imported into the chromoplast, as assessed by their sensitivity to exogenous protease. These results also demonstrate the presence of a functional stroma-processing peptidase in chromoplasts.

To assess the subplastid localization of proteins following import, recovered chromoplasts were ruptured by osmotic shock and separated by centrifugation into the pellet (membrane-containing) and supernatant (stroma-containing) fractions. The membrane fraction includes both the envelope and the internal membranes. Imported Rbcs, a stromal protein in chloroplasts, localized with the soluble fraction of chromoplast (Fig. 5A, lane 6). The full-length precursor was associated with the membrane fraction (Fig. 5A, lane 7). Since the chromoplasts used for subfractionation were recovered and lysed prior to protease treatment, this precursor was most likely associated with the plastid surface, because it was available for protease degradation (Fig. 5A, compare lane 2 with lane 3) and this association was peripheral, because it was removed by NaOH extraction (Fig. 5A, compare lane 7 with lane 8), which is known to remove extrinsic proteins.

OE17 is a thylakoid lumen resident protein. In chloroplasts the bipartite signal peptide of OE17 is cleaved in two steps. First, the stroma-targeting domain is removed by a stromal peptidase either during or immediately after import into the stroma, creating intermediate OE17 (iOE17). Second, the lumen-targeting domain is removed by the thylakoid-processing peptidase following targeting into the lumen, resulting in the accumulation of mature OE17 (mOE17). Fractionation of chromoplasts following import of pOE17 demonstrated that the imported protein was distributed between the stromal and membrane fractions

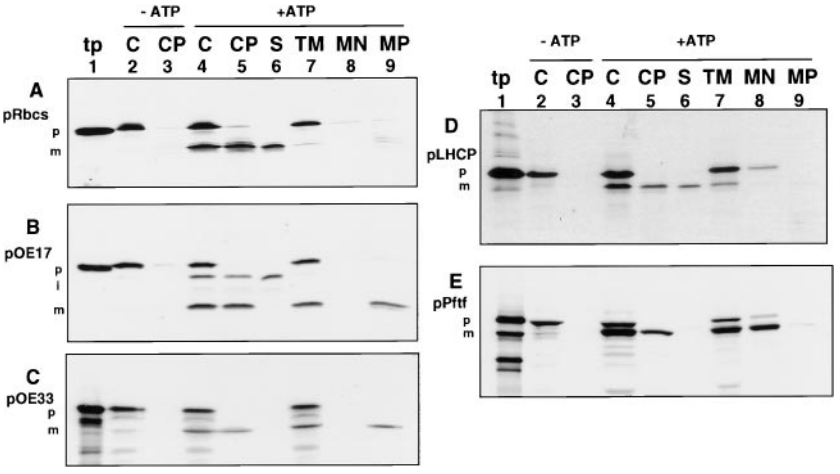

Figure 5. Import and subplastid localization of precursors. Isolated chromoplasts containing 2.0 μg chlorophyll/mg protein were incubated with radiolabeled in vitro translation products (lanes tp) in the presence (+ATP) or absence (−ATP) of ATP, as described in "Materials and Methods." Following import, chromoplasts were recovered without protease posttreatment (lanes C) or with protease posttreatment (lanes CP). Untreated chromoplasts were subfractionated into stroma (lanes S) and total membranes (lanes TM). Equivalent aliquots of membranes were extracted with NaOH (lanes MN) or treated with protease (lanes MP). One microliter of radiolabeled translation product and 15 μL of each sample (representing 7.5 μL of the original radiolabeled translation product added to the import reaction) were analyzed by SDS-PAGE (A–D, 12.5%; E, 7.5%) and fluorography. Precursors utilized for import were: Rbcs (A), OE17 (B), OE33 (C), LHCP (D), and Pftf (E). p, pOE17; i, iOE17; m, mOE17.

(Fig. 5B, lanes 6–9). The iOE17 was found in the stromal compartment (Fig. 5B, lane 6) and fully processed mOE17 co-localized with the membranes. Membrane-associated mOE17 was resistant to thermolysin treatment of the membrane fraction but was extracted from the membranes by NaOH (Fig. 5B, lanes 7–9). NaOH extraction not only removes peripheral proteins but it opens membrane vesicles and exposes the luminal compartments. Taken together, these data suggest that the mOE17 was localized in a luminal compartment.

In chloroplast-import experiments, thylakoid targeting is so efficient that iOE17 is not observed unless it is inhibited by ionophores or by competition with overexpressed precursor proteins (Cline et al., 1993). In the chromoplast-import experiments significant levels of iOE17 accumulated in the stroma, suggesting that thylakoidal targeting is less robust in chromoplasts than in chloroplasts. These results also demonstrate the presence of a functional thylakoid-processing protease.

In chloroplasts OE17 is routed to the thylakoid lumen by the ΔpH-dependent protein-targeting pathway. This pathway is characterized by the absolute transport requirement for a *trans*-thylakoid pH gradient. To determine whether chromoplastic OE17 membrane targeting is ΔpH dependent, simultaneous import assays into chromoplasts and chloroplasts were conducted in the presence or absence of ionophores that dissipate the ΔpH (Fig. 6). In the control experiments without ionophores, protease-protected mOE17 was present in the membrane fraction of chromoplasts and chloroplasts (Fig. 6, lanes 4 and 11). When import was conducted in the presence of ionophores, iOE17 was detected in the stroma (Fig. 6, lanes 6 and 13) of chromoplasts and chloroplasts, and mOE17 levels were decreased in the membrane fractions (Fig. 6, lanes 7 and 14). These data indicate that OE17 membrane targeting in chromoplasts is dependent on the presence of a transmembrane ΔpH.

OE33 is routed into the thylakoid lumen on the thylakoid Sec pathway in chloroplasts (Yuan et al., 1994) by a bipartite signal peptide consisting of a stroma-targeting and lumen-targeting domain that is cleaved in two steps in a similar fashion as the ΔpH pathway signal peptide. Following import and fractionation of OE33 in chromoplasts, processed mOE33 was recovered in the membrane subfrac-

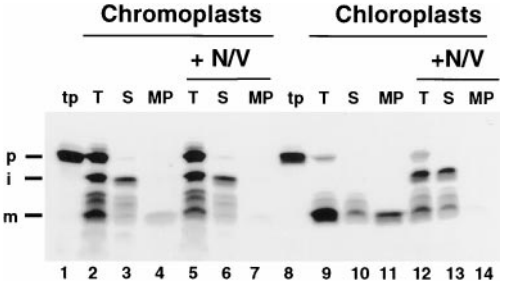

Figure 6. Ionophores abolish the luminal localization of OE17 in both chromoplasts and chloroplasts. Import of radiolabeled pOE17 into isolated chromoplasts (containing 0.4 μg chlorophyll/mg protein) and chloroplasts was performed in the absence or presence (+N/V) of nigericin and valinomycin at final concentrations of 0.5 and 1.0 μM, respectively, as described in "Materials and Methods." Following import, total plastids were recovered (lanes T) and fractionated into stromal (lanes S) and membrane fractions, and the membranes were treated with protease (lanes MP). One microliter of radiolabeled precursor (lanes tp) and 15 μL of each sample (representing 7.5 μL of the original radiolabeled precursor added to the import reaction) were analyzed by 12.5% SDS-PAGE and fluorography. p, pOE17; i, iOE17; m, mOE17.

tion (Fig. 5C, lane 7). As with OE17, the membrane-associated mOE33 was resistant to protease treatment of the membranes but susceptible to base extraction (Fig. 5C, lanes 8 and 9). This pattern of association is identical to that observed in chloroplasts and suggests that the thylakoid Sec pathway is functional in chromoplasts. This result is consistent with the earlier observation that chromoplasts contain cpSecA (Fig. 4E). In contrast to OE17, no intermediate form of OE33 accumulated in chromoplasts, suggesting that the cpSecA pathway is more efficient in chromoplasts than the ΔpH pathway.

LHCP is an integral thylakoid membrane protein in chloroplasts. pLHCP contains an N-terminal stroma-targeting domain that is cleaved by the stroma-processing peptidase (Cline et al., 1989). The mature-sized protein mLHCP is integrated into the thylakoid membrane by a third targeting pathway, a chloroplast homolog of the ER and bacterial SRP pathways (Li et al., 1995). Unlike results with the previous precursors, the localization of imported LHCP in chromoplasts differed markedly from that observed following import into chloroplasts (Fig. 5D). The mature-sized LHCP accumulated primarily in the stromal fraction (Fig. 5D, lane 6). A significant amount of pLHCP and mLHCP was present in the membrane fraction, but this was extracted by NaOH and was completely digested by thermolysin (Fig. 5D, lanes 7–9). This indicates that the imported LHCP was not integrated into the chromoplast membranes (Yuan et al., 1993). In contrast, immunoblot analysis of these samples showed that the endogenous LHCP was resistant to NaOH extraction and showed characteristic partial protease degradation, indicating that the endogenous LHCP is correctly membrane integrated (data not shown).

Pftf, the fifth precursor tested, is an endogenous protein of bell pepper chromoplasts (Hugueney et al., 1995). Following import into chromoplasts, the 73-kD pPftf was processed and associated with the membrane fraction (Fig. 5E, lanes 4–9). The 65-kD mPftf was resistant to NaOH extraction (Fig. 5E, lane 8); however, it was predominantly sensitive to proteolysis (Fig. 4E, lane 9).

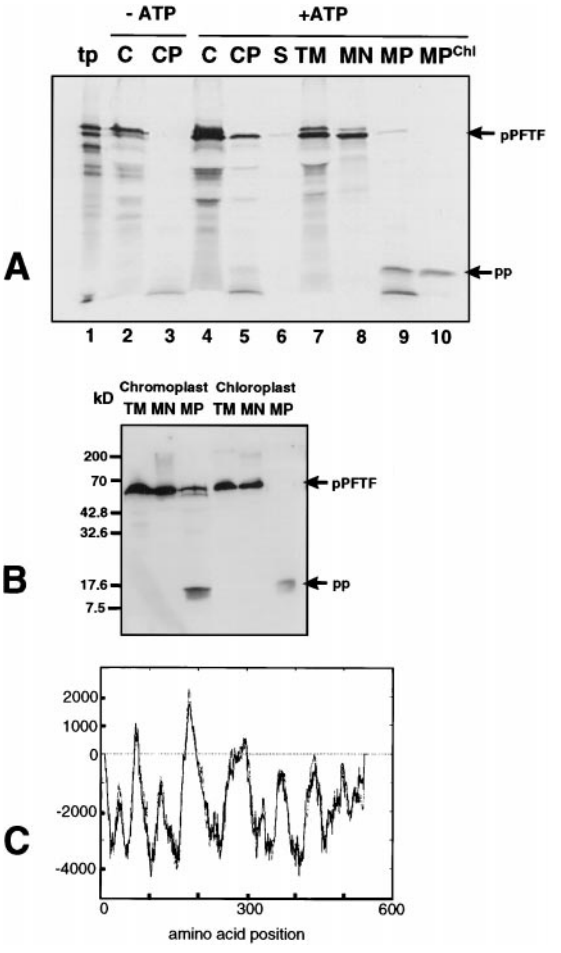

Figure 7. Endogenous and imported Pftf is integrally associated with membranes of both pepper chromoplasts and pea chloroplasts. A, Analysis of identical samples from Figure 5E and the protease-treated thylakoid fraction following import into pea chloroplasts (lane MP^{Chl}) by 12.5% polyacrylamide SDS-PAGE and fluorography. The 65-kD mPftf and 13-kD protease-protected (pp) bands are indicated by arrows. B, Immunoblot analysis of chromoplast (containing 0.4 μg chlorophyll/mg protein) and chloroplast membranes. Proteins from chromoplast and chloroplast total membranes (lanes TM), NaOH-extracted membranes (lanes MN), and protease-treated membranes (lanes MP) were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, probed with anti-Pftf, and visualized by enhanced chemiluminescence. The 65-kD mPftf and 13-kD protease-protected (pp) bands are indicated by arrows. C, Transmembrane prediction of the amino acid sequence of Pftf generated by the TMpred program. More positive values indicate residues more likely to occupy a transmembrane domain.

Pftf Is an Integral Membrane Protein in Chromoplasts and Chloroplasts

Analysis of the samples in Figure 5E by electrophoresis on a 12.5% (Fig. 7A) instead of a 7.5% SDS-PAGE gel prior to fluorography revealed an approximately 13-kD band in the protease-treated membrane fraction (Fig. 7A, lane 9). A band of identical mobility was present in protease-treated thylakoids following import of pPftf into pea chloroplasts (Fig. 7A, lane 10).

To determine whether endogenous Pftf is associated with the membranes of chromoplasts and chloroplasts in a manner similar to that of in vitro-imported Pftf, immunoblot analysis was performed on total, base-extracted, and protease-treated chloroplast and chromoplast membranes (Fig. 7B). These results indicate that endogenous Pftf is resistant to extraction with NaOH and that proteolysis of the membranes results in the appearance of an approximately 13-kD immunoreactive band, as it does in the import assays.

This approximately 13-kD protease-protected band corresponds well with the transmembrane and amino-terminal domains predicted by analysis of the Pftf amino acid sequence (Fig. 7C). These data suggest that Pftf is anchored by an N-terminal transmembrane domain, and that the bulk of the protein is exposed to the stroma in chromoplasts, as it is in the chloroplasts (E.J. Summer and K. Cline, unpublished data).

DISCUSSION

Differentiation of all, or virtually all, plastids requires new synthesis and import of proteins, some of which are probably common to all plastid types (e.g. Hsp70) and others of which are specific to one plastid type (e.g. photosystem proteins or ChrA). The majority of data suggest that a common import pathway is functional in all plastid types (de Boer et al., 1988). These data are predominantly based on the observation that any plastid type can import and localize to the stroma precursors destined for any other plastid type. In vitro studies demonstrated that isolated castor bean leukoplasts and sycamore amyloplasts can import a number of chloroplastic precursors, although there may be precursor-specific variations in efficiency (Strzalka et al., 1987; Halpin et al., 1989; Wan et al., 1996). Conversely, tomato chromoplast-targeted and corn amyloplast-targeted proteins are imported into isolated pea chloroplasts (Klosgen et al., 1989; Lawrence et al., 1993). One example of specific regulation of import has been well characterized: the differential import of pre-protochlorophyllide oxidoreductase A. Pre-protochlorophyllide oxidoreductase A translocation across the envelope of etioplasts requires protochlorophyllide, and therefore chloroplasts, which do not accumulate protochlorophyllide, are unable to import pre-protochlorophyllide oxidoreductase A (Reinbothe et al., 1997). It is not known whether this plastid variation in import reflects a difference in the composition of the general import apparatus. Our data showing that bell pepper chromoplasts are also able to efficiently import chloroplastic and chromoplastic proteins in an ATP-dependent fashion supports the hypothesis that import is functional and usually not precursor selective in different plastid types. It is assumed but not demonstrated that the other plastid types possess the same import machinery as chloroplasts. Here we have demonstrated that chromoplasts contain at least one component of the import machinery, Toc75, at levels similar to those found in chloroplasts.

There is more evidence for plastid-specific variation in routing of proteins to internal membranes. Etioplasts lack both stromal and membrane factors required for integration of LHCP, and it has been recently demonstrated that chlorophyll is the limiting membrane component (Chitnis et al., 1987; Kuttkat et al., 1997). However, etioplasts do correctly localize the ΔpH pathway proteins OE17 and OE23, as well as the thylakoid Sec pathway substrate plastocyanin (Voelker et al., 1997). Etioplasts develop when tissues that usually contain chloroplast are grown in the absence of light; therefore, this represents an example of an environmental effect on plastid protein targeting. Most nonphotosynthetic plastids are the result of tissue-specific developmental cues. In the one normally nongreen plastid type in which protein targeting to internal membrane compartments has been addressed, castor bean leukoplasts, very little subplastid localization of proteins (thylakoid Sec pathway substrates OE33 and plastocyanin) was detected (Halpin et al., 1989; Wan et al., 1996). Precursors that utilize the other known targeting pathways were not tested. Although it was not determined whether these leukoplasts contain cpSecA, the simplest explanation for the lack of detectable membrane targeting in these plastid types is the lack of significant amounts of internal membranes.

Many nongreen plastid types, such as bell pepper chromoplasts, do contain elaborate internal membranes, which raises the possibility that they might utilize membrane-targeting machinery. The origin, organization, and function of these membranes is unclear, although the enzymes involved in the final steps of carotenoid biosynthesis are membrane associated, although probably not integral. Analysis of the sequence of pepper phytoene dehydrogenase (accession no. 117513), lycopene synthase (accession no. 999411), and capsanthin-capsorubin synthase (accession no. 522120) for potential hydrophobic transmembrane domains indicates that these are not likely to be integral membrane proteins (data not shown). It has been repeatedly observed in electron microscopic images that chromoplast development in red fruit initiates with a massive appearance of vesicles associated with the inner envelope and the apparent degradation of the granal lamella, suggesting that new membrane synthesis is occurring (Camara et al., 1995). It is also possible that at least some of the membrane structures observed in mature fruit is the result of reorganization of the existing thylakoid sheets (Spur and Harris, 1968). The chromoplast membrane fraction contains numerous, abundant proteins (Oren-Shamir et al., 1993; Price et al., 1995), most of which were removed by NaOH extraction, indicating that they are extrinsic or contained in the luminal compartment (Fig. 3). Nevertheless, our results show that two, possibly three, of the pathways for localizing thylakoid proteins are functional in chromoplasts. Membrane luminal targeting of OE33 demonstrated the activity of the thylakoid Sec pathway in chromoplasts, and this was correlated with the presence of cpSecA, the stromal protein required for thylakoid Sec pathway membrane targeting in chloroplasts.

Membrane luminal targeting of OE17 demonstrated the activity of the ΔpH pathway in chromoplasts, and this was further shown to be abolished by dissipation of the transmembrane pH gradient. The mechanism by which chromoplasts can maintain a ΔpH is unknown. In photosynthetic thylakoids the ΔpH is generated primarily by the release of protons into the lumen during photosynthetic electron transport. ATP in the absence of photosynthetic electron transport can also be hydrolyzed to generate a ΔpH by the ATPase-coupled proton-pumping activity of the coupling factor$_1$/coupling factor$_0$ ATP synthase complex (van Walraven and Bakels, 1996). The potential role of the ATP synthase complex in chromoplasts is further supported by the observation that mRNA levels of a chloroplast-encoded subunit of the ATP synthase complex, *atpA*, are up-regulated during green-to-red fruit development, whereas mRNA for the photosystem subunits *psaA* and *psbA* are down-regulated (Kuntz et al., 1989).

Unlike OE17 and OE33, the chloroplast SRP pathway substrate LHCP failed to integrate into chromoplast membranes and, instead, the processed protein accumulated in the stromal fraction following import. Accumulation of processed LHCP in the stroma has been observed previously in chloroplasts following import under conditions where integration into the thylakoids is inhibited by un-

couplers (Cline et al., 1989). The level of cpSRP54 was reduced but not abolished in chromoplasts compared with chloroplasts. The failure of LHCP to integrate into chromoplast membranes is possibly due to the lack of chlorophyll rather than a defect in the SRP pathway per se.

In view of the complete inability of chromoplasts to integrate newly synthesized LHCP, the presence of endogenous membrane-associated LHCP at levels up to 10% of that present in chloroplasts is puzzling, whereas another photosystem protein, OE23, was almost completely absent. Previous reports suggested that pepper chromoplasts lack LHCP (Kuntz et al., 1989); however, this was based on the absence of a discernible band in stained gels and not by highly sensitive enhanced chemiluminesence immunoblotting. There are several possible explanations for the discrepancy. One possibility is that the immunoreactive LHC might be a different LHC than the major LHCP precursor utilized in the import assays. The LHC gene family is rather large and antigenically related (Jansson, 1994). Only one member, PSII-S, is known to be stable in the absence of chlorophyll (Funk et al., 1995). Another possibility is that LHCP is not capable of integrating in chromoplasts, and the LHCP detected is present in only a subset of membranes derived from reorganization of the thylakoids. If so, LHCP could serve as a biochemical marker to distinguish reorganized thylakoids from newly synthesized membranes.

Pftf was the only precursor in our study that was originally isolated from bell pepper chromoplasts. Pftf is a member of the AAA (ATPase associated with a variety of cellular activities) protein family, related to bacterial FtsH, mitochondrial Yta10/Yta12, and eukaryotic NSF (Hugueney et al., 1995; Patel and Latterich, 1998). FtsH and Yta10/Yta12 are membrane-anchored proteins that share dual chaperone and proteolytic activities (Suzuki et al., 1997). NSF is a soluble protein required for vesicle fusion (Woodman, 1997). Pftf was originally isolated in an assay for the transfer of capsanthin capsorubin synthase activity in vitro from red to yellow bell pepper chromoplast vesicles (Hugueney et al., 1995). The ability to promote capsanthin capsorubin synthase activity in trans is consistent with chaperone or membrane fusion activities, and thus the protein was designated Pftf for plastid fusion and/or protein translocation factor. In our assays Pftf was found to be an integral membrane protein, as is predicted by sequence analysis in both chloroplasts and chromoplasts, which is at variance with the originally assigned stromal localization. A related chloroplast FtsH has been cloned from Arabidopsis and demonstrated to be an integral membrane protein with proteolytic activities in chloroplasts (Lindahl et al., 1996; Ostersetzer and Adam, 1997). Pftf shares with FtsH and Yta10/Yta12 the presence of only one AAA domain, a zinc metalloprotease-binding domain, and a membrane-anchoring domain (E. J. Summer and K. Cline, unpublished data). In contrast, NSF contains two AAA domains, contains no zinc metalloprotease domains, and is a soluble protein (Woodman, 1997). These data indicate that Pftf is structurally more similar to FtsH and Yta10/Yta12 and thus would be expected to have proteolytic and/or protein-translocation activity rather than membrane-fusion activity, although this has not been demonstrated directly. It is clear, however, that bell pepper chromoplasts contain both the internal membranes and the mechanism by which to properly localize Pftf.

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