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Chromoplast-Targeted Proteins in Tomato (*Lycopersicon esculentum* Mill.) Fruit[1]

Susan D. Lawrence, Kenneth Cline, and Gloria A. Moore*

Department of Horticultural Sciences, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida 32611

The chloroplast to chromoplast transition during tomato (*Lycopersicon esculentum* Mill.) fruit ripening is characterized by a dramatic change in plastid structure and function. We have asked whether this process is mediated by an increase in the steady-state level of RNA for plastid targeted proteins. Assays for import of radiolabeled translation products into isolated pea (*Pisum sativum* L.) chloroplasts were used to monitor levels of chromoplast-targeted proteins at four stages of tomato fruit development. We have found striking increases during development in levels of translatable RNA for two such proteins. Additionally, the import of in vitro translation products was examined for seven individual cDNA clones known to encode RNA that increase during fruit ripening. Three of these clones produced in vitro translation products that were imported into pea chloroplasts. This implies that there is synthesis and import of new proteins during the transition from chloroplast to chromoplast and that the plastid conversion is an active developmental program rather than a simple decline in synthesis of the photosynthetic apparatus. Furthermore, our results demonstrate the utility of this method for identification of structural genes involved in plastid morphogenesis.

Electron micrographic analysis has shown that a massive reordering of plastid structure occurs during the transition from chloroplast to chromoplast (Thelander et al., 1986). In tomato (*Lycopersicon esculentum* Mill.) fruit, this is characterized by the overall breakdown of chloroplast-specific components, e.g. the thylakoid membrane system and starch, with the concomitant accumulation of membrane-associated deposits of the carotenoid lycopene (Thelander et al., 1986). The manner in which these events are regulated is largely unknown. An examination of nuclear-encoded, plastid-targeted, photosynthetic proteins has shown that their transcription stops 5 to 10 d before the fruit reaches full size (Piechulla et al., 1986). Analysis of plastid-encoded transcripts has demonstrated that some are greatly reduced, whereas others persist, and one appears to increase during chromoplast formation (Kobayashi, 1991; Richards et al., 1991). It has not been determined whether the breakdown of chloroplast structures is caused simply by the decrease in synthesis of photosynthetic proteins or whether increases in degradative enzymes actually enhance this process. Similarly, little has been ascertained about the function or expression of genes involved in the synthesis of chromoplast structures because few clones specific to this developmental stage have been available for analysis (Kuntz et al., 1992).

We are interested in determining how the transition from chloroplast to chromoplast is regulated, e.g. does a subset of plastid proteins increase during this process? In an initial examination of this question, we asked whether an increase in the accumulation of RNA for a subset of plastid proteins correlates with the chloroplast to chromoplast transition. Because transcription and/or translation of the plastid genome seems to diminish during chromoplast formation (Kobayashi, 1991), these proteins are probably encoded in the nucleus. Nuclear-encoded plastid proteins are synthesized in the cytosol and posttranslationally imported into plastids. The import of proteins into plastids is a highly specific process (Keegstra, 1989). The proteins are made as precursors with transient amino-terminal "transit peptides," which are both necessary and sufficient for the correct targeting into this organelle. It is this latter observation that makes the import into plastids a potentially diagnostic assay for the identification of chromoplast-associated proteins.

Our approach has been to monitor chloroplast import of in vitro translation products from RNA of tomato fruit isolated at four different ripening stages. We have also studied translation products programmed with transcripts from cDNAs (Slater et al., 1985) whose steady-state RNA levels increase during ripening. One of these clones, pTOM5, has recently been shown to encode a protein involved in the plastid-localized synthesis of the lycopene precursor, phytoene (Bird et al., 1991; Bartley et al., 1992). Import of translation products from total poly(A)$^+$ RNA as well as RNA derived from individual cDNA clones suggests that the transition is an active developmental process and that this type of analysis can successfully identify genes involved in this process.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill. cv Rutgers) were grown in a greenhouse in Gainesville, FL. Fruit were harvested at four ripening stages. MG1 was characterized by full-size green fruit with firm locular tissue, MG3 fruit had

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* Corresponding author; fax 1–904–392–5653.

Abbreviations: MG1, mature green 1; MG3, mature green 3.

completely developed locular gel, the 20 to 50% ripe fruit were red over less than half of the outer pericarp, and >80% ripe fruit were red over 80% of the outer pericarp. The pericarp was frozen in liquid nitrogen immediately after harvesting.

Preparation and Translation of RNA

Total RNA was extracted by combining the protocols of McCarty (1986) and Grierson et al. (1985). Briefly, this involved grinding the frozen tissue with a coffee grinder and homogenizing in extraction buffer with a Polytron. The extraction buffer consisted of 100 mM Tris-HCl (pH 9.0), 200 mM NaCl, 5 mM DTT, 1% SDS, and 20 mM EDTA. The insoluble material was pelleted and reextracted with 0.5 volume of extraction buffer. The supernatant was extracted twice with phenol:chloroform (1:1) and once with chloroform. The final supernatant was brought to 0.1 M KCl and precipitated overnight with 2 volumes of ethanol at 4°C. The precipitate was washed several times as described by Grierson et al. (1985), and the soluble material was applied to a cellulose (Sigmacell type 50) column to remove substances that interfere with extraction of poly(A)$^+$ RNA (Grierson et al., 1985). Poly(A)$^+$ RNA was isolated from total RNA by one passage over oligo(dT)-cellulose (BRL).

Individual pTOM clones (Slater et al., 1985) were subcloned into the in vitro transcription vector pSport (Bethesda Research Laboratories) at the *Pst*I site. Plasmid DNA was isolated by the alkaline lysis method of Lee and Rasheed (1990). The plasmid DNA was linearized and transcribed with either SP6 polymerase or T7 polymerase as described by Cline (1988). The poly(A)$^+$ RNA and the in vitro transcribed RNA were translated (see figure legends for specific experiments) with either a wheat germ system (Cline, 1988) or a rabbit reticulocyte lysate system from Promega (Madison, WI) in the presence of [^{35}S]Met.

Chloroplast Isolation and Import Assay

Chloroplasts were isolated from pea (*Pisum sativum* L. cv Laxton's Progress 9) shoots by a combination of differential centrifugation and Percoll gradient centrifugation (Cline et al., 1989). Import assays were conducted as described by Cline (1988) except that translation products were diluted 1:3 with unlabeled Met in import buffer; 50 μL of diluted translation products were added to 100 μL of chloroplasts (1 mg Chl mL^{-1}). Import assays were conducted for 20 min at 25°C with white light and 10 mM Mg-ATP. After incubation, intact chloroplasts were reisolated by centrifugation through 35% Percoll with or without treatment of the protease thermolysin to remove surface-bound proteins (Cline, 1988). Repurified plastids were lysed in 30 μL of 10 mM Hepes/KOH (pH 8.0). Experiments testing the requirement of ATP were conducted in foil-wrapped tubes to maintain darkness.

Subfractionation and Treatment of Plastids after Import

In experiments in which import of translation products from total poly(A)$^+$ RNA was examined, the repurified plastids were lysed in 10 mM Hepes/KOH (pH 8.0) and subsequently subfractionated into stromal (soluble) and membrane (pellet) fractions by centrifugation for 10 min at 10,000*g*. In experiments with translation products from individual cDNA clones, the soluble fraction was isolated from the membranes by lysing the plastids in 10 mM Hepes/KOH (pH 8.0) on ice for 5 min and centrifuging for 30 min at 37,500*g*. The membranes were washed once with 1 mL of 10 mM Hepes/KOH (pH 8.0) and resuspended in the same solution. Alternatively, the membrane fraction was washed with 0.1 N NaOH (Cline, 1986). Envelopes were separated from thylakoid membranes as described by Cline (1986); however, 1.3 mL of plastids (1 mg Chl mL^{-1}) were used for each import reaction.

Sensitivity of the imported proteins to thermolysin was tested as follows. A standard import assay was carried out and divided into two equal fractions. These fractions were either resuspended in import buffer or lysed with 10 mM Hepes/KOH (pH 8.0). Aliquots of 25 μL (1 mg Chl mL^{-1}) were incubated with 0 or 20 μg of thermolysin for 45 min at 4°C. EDTA was added to 5 mM, and the samples were immediately frozen. An equal volume of 2× SDS-PAGE buffer (Cline, 1986) was added to freshly thawed samples, and the tubes were heated to 98°C for 3 min.

Analysis of Import Products

Import products from total poly(A)$^+$ RNA were examined by SDS-PAGE and fluorography on 7.5 to 20% gradient gels (Cline, 1986). Gel lanes received equivalent amounts of protein as determined by Chl estimation of the recovered plastids. Import products from in vitro transcribed RNA were examined on 12.5% SDS-PAGE gels.

RESULTS AND DISCUSSION

Profiles of in vitro translation products of poly(A)$^+$ RNA differed markedly between ripe fruit and mature green tissue (Fig. 1A). The profiles of the two later ripening stages closely resembled one another, as did the translation products from the two samples of mature green tissue. Speirs et al. (1984) reported a similar result from a more detailed analysis of the polypeptide profiles of total mRNA from ripening tomato fruit.

Pea chloroplasts were chosen to analyze import into plastids because of the ease with which they are isolated. The isolation of tomato chromoplasts has been reported by others (Iwatsuki et al., 1984; Bathgate et al., 1985; Hunt et al., 1986; Wrench et al., 1987), but we were unable to isolate pure and intact fruit chromoplasts that withstand the handling required to demonstrate successful import. However, in vitro import of an amyloplast-specific protein into chloroplasts (Klosgen et al., 1989) and the in vivo import of a chloroplast protein into nongreen plastids in transgenic plants (deBoer et al., 1988) implied that chromoplast proteins should also be imported into pea chloroplasts.

Changes in the profile of imported proteins during chromoplast development were detected (Fig. 1B). A number of proteins decreased during this period (Fig. 1B). For example, 30- and 14-kD soluble fraction proteins diminished as rip-

ening progressed. Two imported translation products dramatically increased in the later ripening stages. A 26-kD protein fractionated with the soluble plastid proteins, whereas a 17-kD protein was associated with the membrane fraction. These results imply that synthesis and import of new proteins occur during the transition from chloroplast to chromoplast and that the plastid conversion may be an active developmental program rather than a simple decline in synthesis of the photosynthetic apparatus.

We next asked whether an import assay could be used to identify individual clones coding for chromoplast-targeted proteins. A specific cDNA clone, pTOM5, was selected to evaluate this system. Slater et al. (1985) originally isolated this cDNA because its RNA becomes more abundant as ripening proceeds. Armstrong et al. (1990) recognized that the deduced protein sequence shares approximately 25% identity with the predicted polypeptide of a bacterial prephytoene synthase. Bird et al. (1991) confirmed the involvement of pTOM5 in carotenoid synthesis by creating an antisense pTOM5 tomato transformant that produces yellow rather than normal red fruit. The transgenic fruit are blocked in the synthesis of phytoene from its immediate precursor, implying that pTOM5 encodes phytoene synthase (Bramley et al., 1992). We selected this clone because enzymological data suggest that synthesis of this nonpigmented carotenoid occurs in plastids (Lutke-Brinkhaus et al., 1982; Mayfield et al., 1986; Dogbo et al., 1987).

The major translation product of the pTOM5 transcript was 46 kD (Fig. 2, lane 1); this is in agreement with the size of the hybrid-selected translation product for pTOM5 identified by Slater et al. (1985). Minor lower molecular mass translation products were also produced from this transcript and may represent commencement of translation from internal Met's, because these products are not imported into plastids. Incubation with pea chloroplasts resulted in the appearance of a 41-kD polypeptide that fractionated with

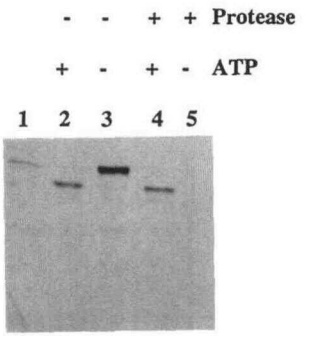

Figure 2. The 41-kD form of pTOM5 is protected from protease digestion by the plastid envelope, and its appearance requires the addition of ATP. Lane 1, In vitro transcribed RNA was translated with a rabbit reticulocyte lysate system in the presence of [^{35}S]Met. Import assays and postassay treatments were conducted as described in "Materials and Methods." Lanes 2 to 5, Import of pTOM5 in vitro translation products was conducted with (lanes 2 and 4) or without (lanes 3 and 5) 10 mM Mg-ATP in darkness. Intact chloroplasts were reisolated with (+, lanes 4 and 5) or without (−, lanes 2 and 3) protease posttreatment. Proteins from lysed plastids were separated on 12.5% SDS-PAGE gels and fluorographed.

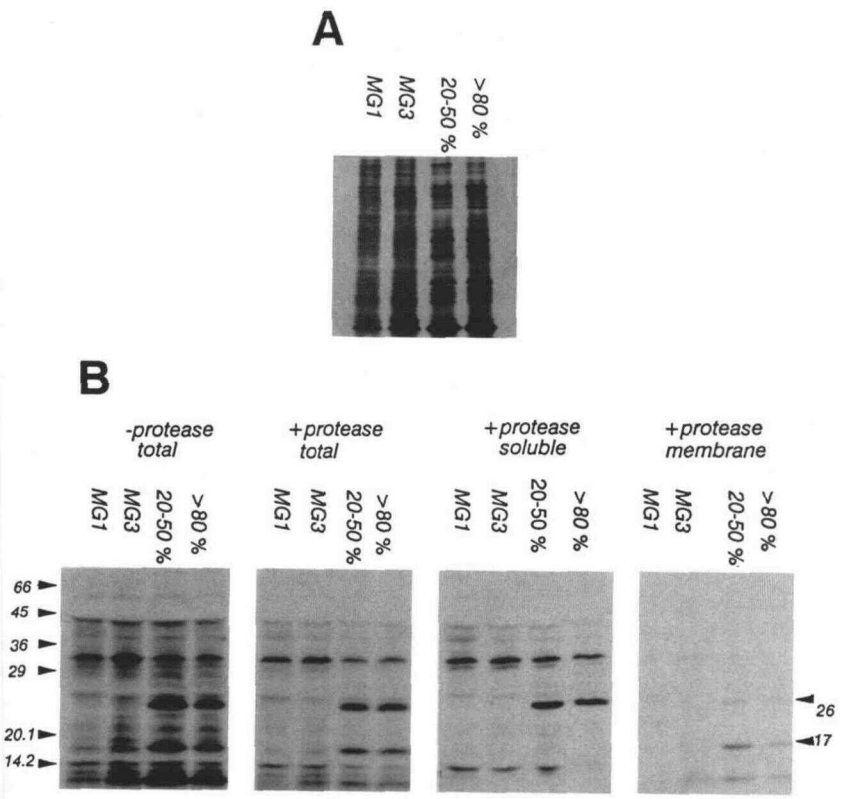

Figure 1. Profiles of in vitro translation products and import products are altered during tomato fruit ripening. A, In vitro translation of total poly(A)$^+$ RNA from four different ripening stages of tomato fruit pericarp. Total poly(A)$^+$ RNA (1 μg 50 μL^{-1} of reaction) was translated in vitro in a wheat germ system in the presence of [^{35}S]Met. Translation products were examined by SDS-PAGE and fluorography on 7.5 to 20% gradient gels. The four stages are MG1, MG3, 20 to 50% red over the outer pericarp, and >80% red over the outer pericarp. B, Import into pea chloroplasts with in vitro translation products of total poly(A)$^+$ RNA extracted from tomato fruit pericarp at four different ripening stages. Import assays were conducted and fractionated as described in "Materials and Methods." The soluble fraction was isolated from the membranes of the repurified lysed plastids by centrifugation for 10 min at 10,000g. Equivalent amounts of plastid protein (as determined by Chl estimation) from the + or − protease-treated plastids were separated on 7.5 to 20% SDS-PAGE gradient gels and fluorographed. ^{14}C-Labeled 10- to 70-kD molecular mass standards (Sigma) are indicated with arrowheads on the left. The size of the two import products were derived from a standard curve and are designated with arrowheads on the right.

intact plastids and was resistant to protease treatment of the chloroplasts (Fig. 2, lane 4). Protease resistance was not due to an inherent property of the 41-kD protein because it was degraded by thermolysin if the plastids were lysed before treatment (data not shown). This result is expected of a protein produced in the cytosol, imported into the plastid, protected from protease by the organellar envelope, and cleaved of its transit peptide.

Additional criteria must also be demonstrated for the confirmation of import into plastids. Import is an energy-dependent process (Keegstra, 1989); white light or exogenous ATP must be provided for the successful import of proteins. Figure 2, lanes 2 and 3, shows that processing of the 46-kD protein into the 41-kD peptide required exogenous ATP. In the absence of ATP, the 46-kD putative precursor bound to the chloroplasts but was destroyed by the protease (Fig. 2, lanes 3 and 5). The import into plastids should also be time dependent; this was found to be the case for the production of the 41-kD protein (Fig. 3). Thus, the successful import of the pTOM5 translation product verified the utility of import assays for the identification of genes encoding chromoplast-targeted proteins.

Pea chloroplasts were fractionated into soluble and membrane fractions to allow analysis of the sublocalization of the 41-kD protein. The protein distributed between these two compartments (Fig. 3). Parallel import assays with the thylakoid-localized light-harvesting Chl *a/b* protein and the stromal small subunit of Rubisco were used to assess the quality of our subfractionation. Light-harvesting Chl *a/b* protein was found exclusively in the membrane fraction, whereas virtually all of the small subunit of Rubisco was recovered in the soluble fraction (data not shown). During preparation of this manuscript, Bartley et al. (1992) reported the isolation of a cDNA from a tomato fruit library by hybridization with a polymerase chain reaction fragment produced with oligonucleotides derived from the sequence of pTOM5. They found that their clone differs from pTOM5 in 19 amino acids that reside 5 amino acids away from the deduced carboxyl-terminal end. They also demonstrated that their in vitro translation product is imported via an ATP-requiring process and that their import product also appears in both stromal and membrane fractions of pea chloroplasts.

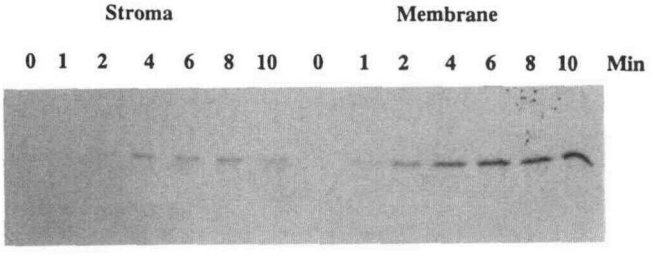

Figure 3. The production of the 41-kD form of pTOM5 was time dependent. This protein associated with both stromal and membrane fractions. pTOM5 import assays were conducted as described in "Materials and Methods." One-half of a standard import assay was immediately repurified on a 35% Percoll cushion after 0, 1, 2, 4, 6, 8, or 10 min of incubation. Chloroplasts were treated with protease, repurified, and subfractionated as described in "Materials and Methods." Samples were analyzed by SDS-PAGE and fluorography.

The membranes of pea chloroplasts were further subfractionated into envelopes and thylakoid membranes (Cline, 1986); upon fractionation of the membranes after import of pTOM5, virtually all of the mature protein was found to be associated with the thylakoid fraction (data not shown). A mild NaOH wash removed the pTOM5 protein from the membrane (data not shown). This suggested that the protein was only peripherally associated with this fraction (Cline, 1986).

Previous attempts by others to localize the site of phytoene synthesis within the plastid via enzyme assays have resulted in conflicting conclusions. Lutke-Brinkhaus et al. (1982) concluded that the production of phytoene is associated with the plastid envelope. However, Mayfield et al. (1986) and, independently, Dogbo et al. (1987) deduced that phytoene is synthesized in the stroma. The unexpected association of the protein with thylakoids in import assays and its peripheral association with the membranes demonstrated by the NaOH extraction may have provided additional evidence that this protein is functionally associated with the stroma. Alternatively, perhaps the 41-kD protein has not folded or localized correctly in pea chloroplasts. Bartley et al. (1991) reported the import of phytoene desaturase from soybean leaf into pea chloroplasts. Although this protein is imported effectively, Bartley et al. found that it does not also fractionate into the plastid compartment expected from biochemical analysis. Immunocytolocalization may clarify these contradictory results. Therefore, it appears that import assays can be useful for the identification of chromoplast-targeted proteins; however, it is currently unclear what conclusions can be made from the in vitro organellar sublocalization when using heterologous systems.

Six additional, ripening-enhanced pTOM cDNA clones of unknown function (Slater et al., 1985) were tested to determine whether they encode plastid-targeted proteins. These particular clones were selected because a comparison of the published sizes of the cDNA inserts with the size of their respective mRNA suggested that they might be full length (Slater et al., 1985; Maunders et al., 1987). In vitro transcription of the cDNAs was carried out in both directions because the orientation of the clones within the vector was unknown. In vitro translation products of transcript pairs of four of the six clones were not imported. Figure 4, lanes 1 to 7, shows examples of the translation products and lack of import products produced from these clones. However, because the transit peptide, which is required for successful import of the protein, is located on the amino-terminal end, and because in vitro translation can begin from any internally situated Met, any cDNAs that produced an in vitro translation product but not an imported product may simply not include the entire coding sequence. Translation products from pTOM41 and pTOM111 were imported successfully into pea chloroplasts (Fig. 4, lanes 9 and 11). The imported product from both proteins was smaller than the translation product, which is consistent with processing of the plastid transit peptide. These proteins were sensitive to protease treatment (data not shown) but resistant after import into plastids (Fig. 4). As with pTOM5, import of pTOM111 and pTOM41 translation

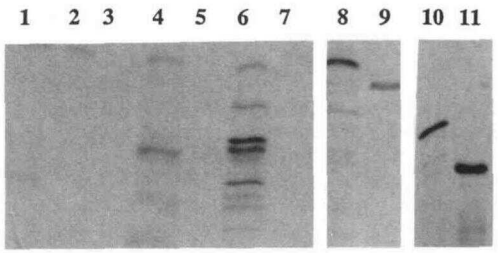

Figure 4. Translation products from transcripts of additional pTOM clones were tested for their ability to be imported into isolated pea chloroplasts. In vitro translation products from pTOM41 and pTOM111 were imported into isolated pea chloroplasts. In vitro transcribed RNA was translated with a rabbit reticulocyte lysate system in the presence of [^{35}S]Met. Import assays were conducted as described in "Materials and Methods." Samples were analyzed by SDS-PAGE and fluorography: in vitro translation with no added RNA (lane 1); pTOM92 transcribed in vitro with SP6 polymerase, translated in vitro (lane 2) and imported (lane 3); pTOM92 transcribed in vitro with T7 polymerase, translated in vitro (lane 4) and imported (lane 5); pTOM36 transcribed in vitro with T7 polymerase, translated in vitro (lane 6) and imported (lane 7); pTOM41 transcribed in vitro with T7 polymerase, translated in vitro (lane 8) and imported (lane 9); pTOM111 transcribed in vitro with T7 polymerase, translated in vitro (lane 10) and imported (lane 11).

products were ATP and time dependent (data not shown). These data support the idea that the cDNAs do indeed code for plastid-targeted genes. We have begun sequence analysis and antisense transformation experiments of these cDNAs to investigate their role in chromoplast function.

We wondered whether our initial examination of import from total poly(A)$^+$ translation products (Fig. 1B) might underestimate the number of chromoplast-targeted proteins. Therefore, we asked whether the pTOM import products could be recognized among the total poly(A)$^+$ RNA import products. The pTOM5 protein co-migrated with a band shown to increase in the import products from the membrane fraction of late-stage fruit (Fig. 5, lanes 5 and 6). It is interesting that the 26-kD soluble import product (Fig. 1B) migrates similarly to the pTOM111 import product (Fig. 5). However, analysis of these two proteins on several gels suggested that the pTOM111 product was slightly larger. Consequently, it is unclear whether pTOM111 represents a different isozyme or a completely dissimilar protein. Because the import of the in vitro translation product of pTOM41 associates with the stromal fraction, this was compared with the soluble fraction of total poly(A)$^+$ import products. No protein appears to increase in the later stages of ripening fruit that co-migrates with this band (Fig. 5, lanes 2 and 3). We concluded, therefore, that not all of the chromoplast-targeted proteins could be detected in our analysis of total poly(A)$^+$ RNA import products.

It is presently unclear how many plastid-targeted proteins increase during the chloroplast to chromoplast transition. The data presented in Figures 4 and 5 clearly demonstrate that our original examination of chromoplast-targeted proteins shown in Figure 1 underrepresents the number of proteins that increase during this process. In fact, this is not surprising when considering the limited sensitivity of import of total poly(A$^+$) translation products. Therefore, we believe that individual testing of ripening-enhanced cDNA clones, with the inherent increase in the quantity of the individual protein product, is required to clearly identify all of the chromoplast-targeted proteins whose RNA increases during this developmental process.

Little detailed analysis of the regulation of ripening-enhanced genes has been undertaken. Lincoln and Fischer (1988) and DellaPenna et al. (1989) concluded that increases in transcription rate were mainly responsible for the increases in steady-state RNA levels for the ripening-enhanced genes they have analyzed. However, they also invoked posttranscriptional mechanisms to explain differences between the rate of transcription and accumulation. Although we cannot exclude posttranscriptional mechanisms for the increase in steady-state levels of the RNAs we have analyzed, the simplest interpretation of our data that is consistent with that for other ripening-enhanced genes is that the processes involved in the development of the chromoplast are directed at a transcriptional level in the nucleus.

Utilizing an in vitro assay for the import of proteins into plastids, we have identified two proteins from in vitro translation products of total poly(A)$^+$ RNA that increase during the ripening of tomato fruit. Additionally, we have shown that the in vitro translation products from three individual ripening-enhanced cDNA clones are imported into plastids. Therefore, the transition from chloroplast to chromoplast coincides with an increase in steady-state levels of RNA for proteins destined to this organelle. This suggests that this

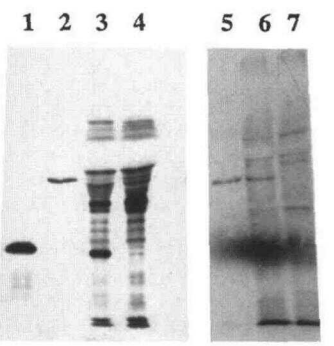

Figure 5. Import products corresponding in size to those for pTOM111 and pTOM5 were enhanced in late-stage tomato fruit. However, no polypeptide that coincided with the pTOM41 import product was seen. pTOM41, pTOM5, and pTOM111 were transcribed in vitro with T7 polymerase. RNA was translated with rabbit reticulocyte lysate in the presence of [^{35}S]Met. In vitro translation, import assays, and subfractionation were conducted as described for analysis of individual translation products in "Materials and Methods." Lane 1, Soluble fraction of the pTOM111 import product. Lane 2, Soluble fraction of the pTOM41 import product. Lane 3, Soluble fraction of imported in vitro translation products of total poly(A)$^+$ RNA from >80% ripe fruit. Lane 4, Soluble fraction from imported in vitro translation products of immature green total poly(A)$^+$ RNA. Lane 5, Membrane fraction of the pTOM5 import product. Lane 6, Membrane fraction from imported in vitro translation products of total poly(A)$^+$ RNA from >80% red ripe fruit. Lane 7, Membrane fraction from imported in vitro translation products of immature green total poly(A)$^+$ RNA.

process is not a simple decline in the synthesis of photosynthetic proteins that causes a breakdown of the thylakoid membranes and a shunting of substrates into the carotenoid pathway but an active developmental program.

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