The Plant Cell, Vol. 3, 1131–1140, October 1991 © 1991 American Society of Plant Physiologists

Developmental Regulation of the Plastid Protein Import Apparatus

Clas Dahlin and Kenneth Cline[1]

Fruit Crops Department, University of Florida, 1109 Fifield Hall, Gainesville, Florida 32611

Plastid development involves the programmed accumulation of proteins. Most plastid proteins are synthesized in the cytosol and imported into the organelle by an envelope-based protein import apparatus. Previous studies have shown that developmental rates of protein accumulation correspond to mRNA levels. Here, we examined the relationship between plastid development and the activity of the protein import apparatus. Developing plastids, primarily from wheat leaves, were analyzed for their protein import capability in vitro. Import capability, initially high in proplastids, declined as much as 20-fold as plastid development approached either the mature etioplast or the mature chloroplast. The observed decline was not due to senescence, nonspecific inhibitors, or protein turnover. Furthermore, the import capability of mature etioplasts, initially very low, was transiently reactivated during light-mediated redifferentiation into chloroplasts. These results suggest that plant cells regulate the import apparatus in concert with the protein demands of the developing plastids.

INTRODUCTION

The various plastids found in higher plants exhibit striking differences in structure and function (Kirk and Tilney-Basset, 1978). Despite such variation, all plastids are developmentally interrelated and ultimately derive from the small progenitor proplastid of embryonic and meristematic cells (Whatley, 1978). Biogenesis of plastids is accompanied by specific morphological and biochemical changes, such as increase in size and shape, formation of an extensive internal membrane system, and changes in pigmentation (Whatley, 1974; Mullet, 1988). Because each plastid type contains a complement of proteins that reflects its specific function, plastid biogenesis involves the accumulation of new proteins and, occasionally, the degradation of proteins specific to the progenitor. Formation of chloroplasts is the most thoroughly studied example of plastid-specific protein accumulation. Proplastids differentiate into chloroplasts during leaf development in the light (Mullet, 1988). During this time, the plastids accumulate thylakoid membrane proteins involved in the light reactions of photosynthesis and soluble proteins that participate in CO_2 fixation as well as other metabolic pathways. This is a major cellular process, as evidenced by the fact that in a mature leaf, chloroplasts can account for 75% of the total membranes (Forde and Steer, 1976) and greater than 50% of the soluble protein (Dean and Leech, 1982).

Several regulatory mechanisms appear to participate in chloroplast protein accumulation (Mullet, 1988). Approximately 80% to 85% of chloroplast proteins are encoded on nuclear genes. Nuclear genes for chloroplast proteins are transcriptionally regulated by developmental as well as environmental signals (e.g., Viro and Kloppstech, 1980; Gallagher and Ellis, 1982; Anderson, 1986). Plastid genes for the remaining 15% to 20% of chloroplast proteins are also transcriptionally regulated during chloroplast biogenesis (Baumgartner et al., 1989) and are further controlled at the translational level (Klein and Mullet, 1986, 1987). Finally, protein degradation can play a role in the steady-state level of plastid proteins (Bennett, 1981; Schmidt and Mishkind, 1983).

One process that has been overlooked when considering regulation of protein accumulation during plastid development is the plastid protein import apparatus. This translocation mechanism, located in the plastid envelope, is responsible for compartmentalizing nuclear-encoded plastid proteins. These proteins are synthesized in the cytosol as precursor proteins with amino-terminal "transit peptides" (Keegstra et al., 1989). They are imported by a process that involves binding to receptors on the plastid surface, ATP-dependent transport across the plastid envelope, proteolytic removal of the transit peptide, and assembly into functional structures. Assembly can involve further transport into or across the thylakoid membrane (Cline, 1986; Hageman et al., 1990; Payan and Cline, 1991).

In this paper, we address the possibility that the activity of the import apparatus is also regulated during chloroplast development. Our experimental approach involved isolating plastids from developing wheat leaves and analyzing

[1] To whom correspondence should be addressed.

their ability to import proteins in vitro. To test the generality of our findings, developing plastids from pea seedlings were also used in some experiments. The results demonstrated that import capability is correlated with plastid development; as plastids approach maturity, their import capability declines significantly. This decline was observed in light-grown plants as well as in dark-grown plants, where the proplastids differentiate into etioplasts. The down regulated import apparatus of mature plastids can be reactivated. This was shown during the "greening" of mature etioplasts into chloroplasts, where a transient reactivation occurred, apparently to accommodate the import of new protein necessary for photosynthesis.

RESULTS

Choice and Characterization of an Experimental System

Wheat and pea seedlings were chosen to study protein import in developing plastids. Developing wheat leaves have previously been used to study chloroplast development (e.g., Boffey et al., 1980; Dean and Leech, 1982). They offer the opportunity to obtain the entire developmental spectrum from the same leaf because the cell (and consequently the plastid) age increases from the basal meristem to fully expanded and mature leaf cells in the upper regions of the leaf (Esau, 1977). Developing plastids were also isolated from pea seedlings. Pea chloroplasts are commonly used for protein import studies, and most of our knowledge of this process applies to pea chloroplasts. However, it is more difficult to obtain a developmental range of plastids from pea plants because different leaves must be used as a source of different age plastids.

Figure 1 depicts the tissues used for isolating the four fractions of plastids examined in these experiments. With wheat, the four fractions encompass a little over one-half of the length of the leaf. With pea, fractions 1 and 2 were obtained from the same leaf sample but differed in their migration through the Percoll gradient during the purification procedure (see Methods). The isolated plastids were analyzed to assess characteristics of their development, i.e., ultrastructure, chlorophyll content, chlorophyll *a*/*b* ratio, and protein content by SDS-PAGE. These criteria demonstrate that plastids in fractions 1 to 4 increase in developmental age.

Electron micrographs of isolated wheat plastids and isolated pea plastids possessed ultrastructural characteristics similar to those observed in the corresponding tissue sections (data not shown). Preparations from the basal region of wheat (fraction 1) contained a small percentage of proplastids; the remainder possessed some internal membrane with limited stacking. Some of fraction 1 plastids contained starch grains and prolamellar bodies, often present in the same plastid. Fraction 2 plastids were larger and contained more extensive internal membrane systems and fewer starch grains. Fraction 3 plastids contained extensive thylakoid membrane and numerous stacked regions with up to 11 thylakoids per granum. Fraction 4 contained mature chloroplasts and grana with up to 16 thylakoids. These results are generally consistent with other studies of plastids in developing cereal leaves (Baker and Leech, 1977; Wellburn et al., 1982). Differences between plastids in the four fractions were also apparent in the light microscope. For example, fraction 1 plastids were much smaller than fraction 4 plastids. This characteristic enabled us to discriminate fraction 1 from fraction 4 plastids in a mixture of the two (see below).

Biochemical characteristics also demonstrated a developmental gradient. Table 1 shows that the chlorophyll content increased and the chlorophyll *a*/*b* ratio decreased in plastids from sections 1 through 4. Similar increases were observed in the total protein as well as identifiable chloroplast-specific proteins, i.e., the small and large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the apoproteins of the light-harvesting complex of photosystem II (LHCP), as shown in Figure 2. Two proteins that have been implicated in the biogenesis of plastid proteins, the chaperonin 60 (cpn60, Lubben et al., 1989; Roy, 1989) and the chloroplast heat shock protein 70 (hsp70, Marshall et al., 1990), were examined by immunoblotting. The plastid cpn60 is known to be involved in the assembly of Rubisco and is thought to facilitate the folding and assembly of other plastid proteins (Lubben et al., 1989). A mitochondrial hsp70 protein facilitates import of mitochondrial proteins (Kang et al., 1990); the chloroplast hsp70 may perform a similar function. As seen in Figure 2, the amount of these assembly-related proteins is about the same per plastid in the four fractions.

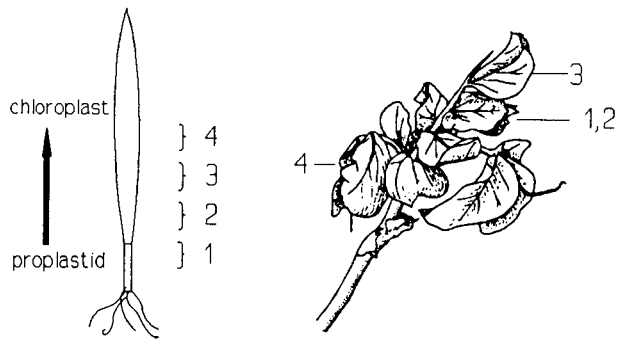

Figure 1. Sketches of Wheat and Pea Seedlings Showing Tissues Used for Plastid Isolation.

The wheat and pea seedlings were grown for 7 and 13 days, respectively. Segment 1 denotes the youngest tissue and segment 4 the oldest. The illustration of the pea seedling was adapted from Fish and Jagendorf (1982).

Table 1. Chlorophyll Content of Plastids Isolated from Different Developmental Sections of Wheat and Pea Plants[a]

Sample	pg Chl[b] per Plastid	Chl *a*/*b* Ratio
Wheat		
1	0.08	6.3
2	0.13	5.5
3	0.29	4.6
4	0.63	3.4
Pea		
1	0.13	5.4
2	0.21	4.9
3	0.87	3.0
4	1.2	2.9

[a] See Figure 1.
[b] Chl, chlorophyll.

However, when considered within the context of the total protein content of these plastids (cf. Figure 2), it is clear that hsp60 and hsp70 make up a larger proportion of the total proteins in immature than mature plastids.

Plastids in the four sections of pea seedlings showed a similar pattern of development. One difference between pea and wheat is that neither starch grains nor prolamellar bodies could be detected in pea plastids regardless of plastid age (data not shown). A second difference is that the pea plastids appeared to be more developed than the wheat plastids in corresponding fractions. In none of the fractions of either wheat or pea were any signs of plastid senescence observed.

Import of Photosynthesis-Related Proteins Declines during Plastid Maturation

Wheat plastids isolated from the above-described sections were assayed for their ability to import in vitro-synthesized precursor proteins, i.e., the precursors to the small subunit (SS) of Rubisco (pSS) and to LHCP (pLHCP). Because of the varying amount of chlorophyll in plastids of different ages, radiolabeled precursors were incubated with an equal number of plastids, and import activities were determined as the number of molecules imported per plastid. The pSS used in this experiment was in vitro-transcribed/translated from a pea cDNA clone (see Methods). The pLHCP was transcribed/translated from a wheat pLHCP clone (see Methods). As shown in Figure 3, immature plastids exhibited a very high import activity, which declined steeply as the plastids approached maturity. This trend was essentially the same with both precursors, but import of pLHCP exhibited the most pronounced effect. In some experiments, there was a 20-fold decrease in the ability of plastids to import pLHCP upon maturation. This dramatic effect did not appear to be related to thylakoid localization events that occur after import because subfractionation of plastids of either fraction 1 or fraction 4 after import revealed that more than 90% of the imported LHCP was present in the thylakoid membranes, correctly processed, and inserted into the bilayer (data not shown). Several other experiments have examined plastids from 1-cm sections apical to section 4, i.e., to just below the leaf tip. Plastids from these sections possessed import capability comparable with that of fraction 4 (data not shown).

A decline in import capability in developing pea plastids was also observed, as shown in Figure 4. Import of pLHCP was dramatically reduced in plastid from fractions 3 and 4, whereas import of pSS was significantly reduced only in fraction 4.

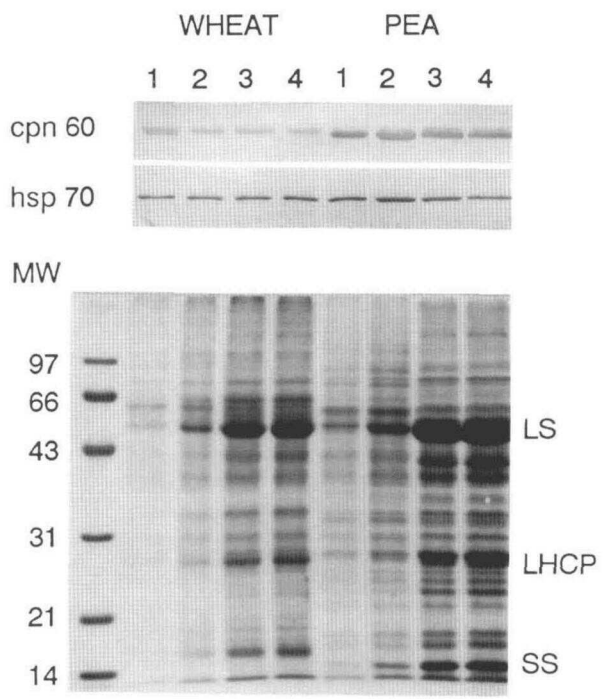

Figure 2. Polypeptide Composition of Isolated Plastids of Increasing Developmental Age.

Plastids were isolated from different sections representing different developmental stages of light-grown wheat and pea plants, as shown in Figure 1, and subjected to SDS-PAGE. Gels were either stained with Coomassie blue (bottom panel) or electroblotted and immunodecorated with antibodies to cpn60 or hsp70 proteins (top panels) as described in Methods. Each gel lane was loaded with 1×10^6 plastids, equivalent to the following amounts of protein: wheat fraction 1, 0.9 μg; fraction 2, 2.3 μg; fraction 3, 7.3 μg; fraction 4, 7.7 μg; pea fraction 1, 1.6 μg; fraction 2, 4.1 μg; fraction 3, 14 μg, fraction 4, 19.6 μg. MW, molecular weight of reference proteins. Reference proteins are phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). LS, large subunit; SS, small subunit.

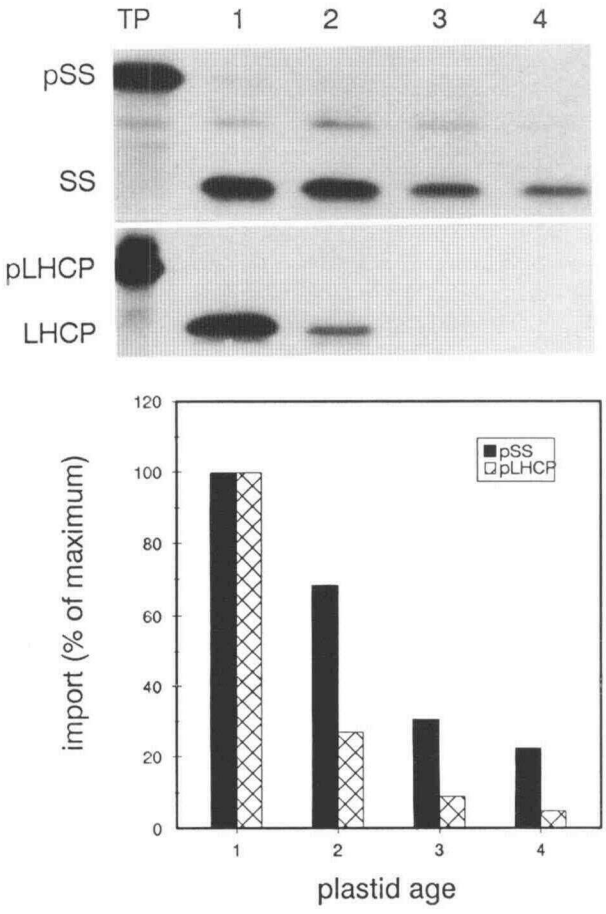

Figure 3. The Capability of Wheat Plastids To Import pSS and pLHCP Declines with Developmental Age.

Radiolabeled pea pSS and wheat pLHCP, synthesized in a cellfree wheat germ extract, were imported into wheat plastids isolated from the different developmental sections shown in Figure 1. Plastids were recovered, protease treated to remove bound precursor, and analyzed by quantitative SDS-PAGE/fluorography (see Methods). The top panels show the fluorograms of import reactions with pSS and pLHCP, respectively. TP designates the translation product used for the reactions. The bottom panel displays the amount of imported molecules per plastid as a percentage of maximum import. One hundred percent corresponds to 2100 molecules of SS imported and to 562 molecules of LHCP. Assays received 4200 and 3600 molecules per plastid of pSS and pLHCP, respectively.

Decrease in Import Activity during Plastid Maturation Is Not Restricted to Photosynthesis-Related Proteins

The above results showed that the ability to import chloroplast-specific proteins declined with chloroplast maturation. To determine whether this was a general decline in import capability, i.e., not restricted to chloroplast proteins, import assays were conducted with a nonphotosynthetic protein, the precursor to hsp21 (pHSP21). As the name implies, this protein is synthesized and accumulates in plastids as a consequence of heat stress (Vierling et al., 1988; Chen et al., 1990). As seen in Figure 5, the import of pHSP21 protein exhibited the same pattern as import of pLHCP and pSS, suggesting a general down regulation in the ability to import plastid proteins.

Apparent Diminished Import Activity Is Not Due to Intraorganellar Degradation

It has been shown that imported proteins are rapidly degraded when intraorganellar assembly is incorrect or prevented (Bellemare et al., 1982; Schmidt and Mishkind, 1983; Cline et al., 1989; Kohorn and Tobin, 1989). It was conceivable that the observed decline of import was not due to an inactive import apparatus, but to increased degradation within the plastid. Reed et al. (1990) showed that the addition of $HgCl_2$ to in vitro import assays almost instantly stops import of precursor proteins and also arrests many reactions taking place inside the plastids. One of the reactions effectively inhibited is the degradation of imported proteins. When $HgCl_2$ stopping was combined with time-course analysis, it was possible to distinguish between reduced import and increased protein degradation (Reed et al., 1990). We used $HgCl_2$ at a final concentration of 3.3 mM to stop import at various times after

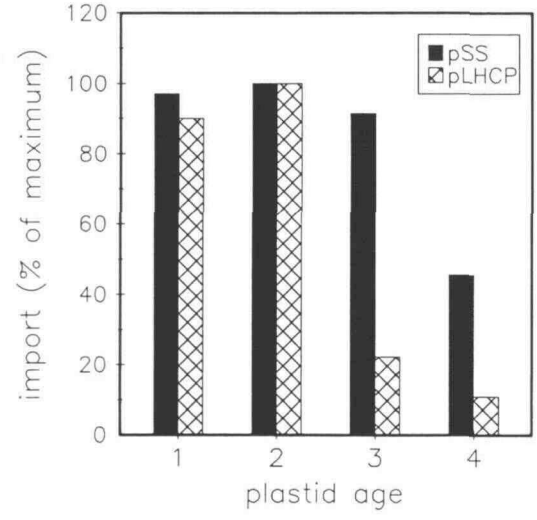

Figure 4. Pea Plastids also Exhibit a Developmental Decline in Their Ability To Import pSS and pLHCP.

Radiolabeled pea pSS and pea pLHCP were imported into pea plastids of the four different fractions shown in Figure 1. The amount of imported molecules is given as percentage of maximum, which for pSS is 2120 and for pLHCP is 630 molecules per plastid. Assays received 3600 and 3000 molecules per plastid of pSS and pLHCP, respectively.

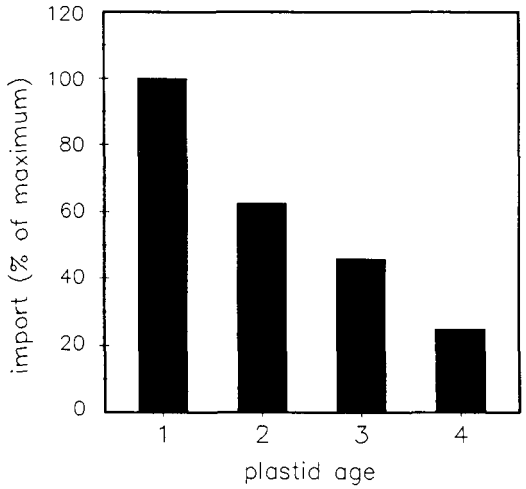

Figure 5. Developmental Decline of the Ability To Import the Nonphotosynthetic pHSP21.

Wheat plastids of different developmental ages (Figure 1) were assayed for import of the nonphotosynthetic protein pHSP21. The amount of imported molecules is given as percentage of maximum. One hundred percent corresponds to 816 molecules per plastid. All assays received 4800 molecules per plastid.

initiation of the reaction. The $HgCl_2$ stop technique was performed with plastids from sections 1 and 4 from pea as well as wheat plants, using the homologous pLHCP. As seen in Figure 6, the plastids accumulated radiolabeled proteins with time at the rates expected from Figures 3 and 4. This strongly suggests that the apparent differences in import activity are not due to altered levels of protein degradation within plastids.

Mature Tissue Does Not Contain Inhibitors of the Import Apparatus

Another possibility for the low apparent import capability in mature plastids was that tissue-specific factors, released during homogenization and plastid isolation, caused damage to the import apparatus. To address this possibility, section 1 and section 4 tissue segments from wheat leaves were combined and plastids isolated by the usual procedure. The composition of the resulting plastid preparation was determined by light microscopic examination based on the differing size of immature plastids and mature plastids. In all experiments, the plastid mixture contained between 40% and 50% immature and 60% and 50% mature intact plastids. This mixture was used in import reactions with pSS. After thermolysin treatment and Percoll cushion recovery of the plastids, the mixture contained approximately the same percentage of immature and mature plastids as before the assay. On a per-plastid basis, the mixture of plastids imported 92% to 94% of the expected number of molecules of pSS, calculated from import by plastids obtained from the individual sections 1 and 4. This result indicates that tissue-specific inhibitors are not responsible for the observed decline of import capability.

Developmentally Correlated Decline of Import Capability also Occurs in Dark-Grown Leaves

In dark-grown wheat leaves, proplastids develop into etioplasts. Etioplasts contain a large number of stromal proteins but lack most thylakoid proteins (Bradbeer, 1981). Although the developed etioplast is considered to be a mature plastid, it rapidly responds to light and differentiates into a chloroplast. This transition involves the accumulation thylakoid proteins, many of which are cytosolically synthesized and imported into the plastid. We were interested to learn whether the import activity of mature etioplasts was higher than that of mature chloroplasts. Plastids isolated from sections of dark-grown wheat leaves were used in

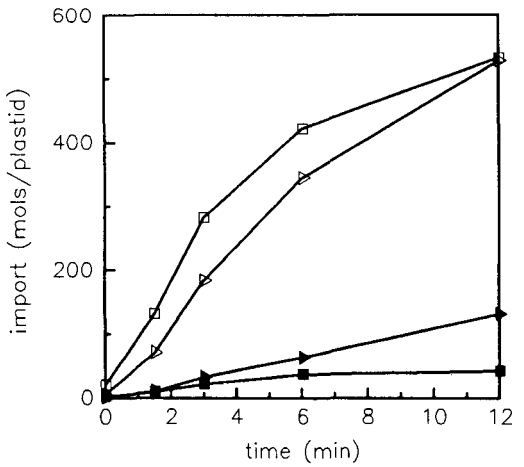

Figure 6. The Observed Decline Is Due to the Rate of Import rather than Intraplastid Degradation.

The assays were initiated with the addition of homologous pLHCP to wheat and pea plastids from fractions 1 and 4 (see Figure 1). At given times, reactions received 3.3 mM $HgCl_2$ to terminate import as well as intraplastidic reactions; the plastids were then protease treated, repurified, and analyzed for the amount of imported LHCP (see Methods). The amount of imported molecules is given as molecules per plastid. Squares, wheat; triangles, pea. Open symbols indicate fraction 1 and filled symbols indicate fraction 4 of the respective plants. Assays received 3700 molecules per plastid.

an import reaction with radiolabeled pSS. This protein was chosen because it has been shown that the LHCP imported into etioplasts in vitro fails to assemble into membranes (Chitnis et al., 1987) and, therefore, may be subject to degradation. As seen in Figure 7A, the import of pSS into plastids from dark-grown wheat leaves follows the same pattern as that observed with plastids from light-grown leaves. This is consistent with the concept that the down regulation of import capability is a general characteristic of plastid maturation.

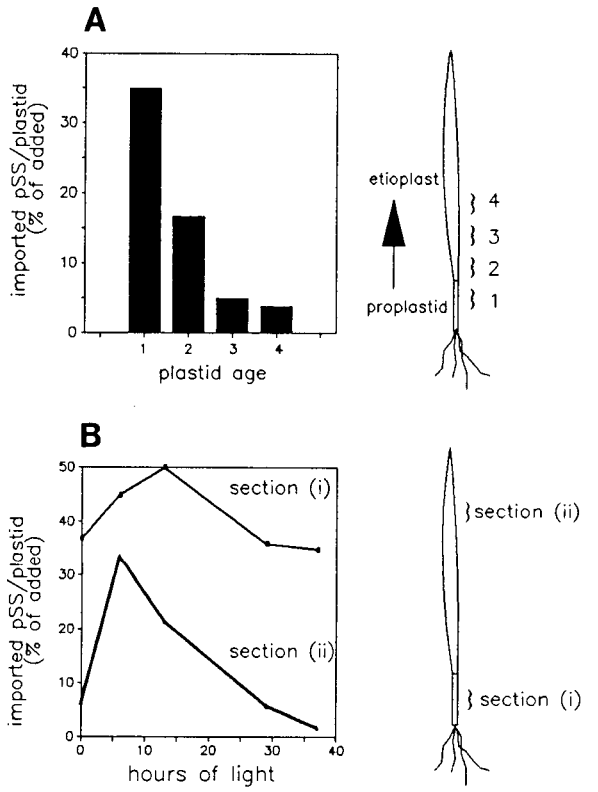

Figure 7. The Import Capability of Mature Etioplasts Is Transiently Reactivated upon Light-Mediated Differentiation into Chloroplasts.

(A) Import of radiolabeled pSS into plastids isolated from dark-grown wheat. Plastids were isolated from sequential 1.5-cm segments and assayed for their ability to import proteins. The amount of imported molecules is given as the percentage of added translation product. Each plastid received 6800 molecules of pSS during the import assay, and plastids isolated from fraction 1 imported 2373 molecules per plastid.

(B) Wheat seedlings were grown in darkness for 6 days, illuminated (100 μE m^{-2} sec^{-1}), and then used for plastid isolation. Plastids were isolated 0 to 1.5 cm from the base (i) and 2 to 3.5 cm from the tip (ii) and assayed for import as in **(A)**. Equal amounts of fresh translation product were added in each import assay.

Import into Mature Wheat Etioplasts Is Transiently Activated during Redifferentiation to Chloroplasts

It seemed likely that during etioplast redifferentiation into chloroplast, the import apparatus would have to reactivate to accommodate the increased requirement for protein import. We tested this possibility with a "greening" wheat system. For this experiment, plastids from two different sections of the leaf were used. In accordance with Boffey et al. (1980), our previous experiments showed that neither cell division nor cell elongation occurred in the apical approximately two-thirds of 7-day wheat leaves. Consequently, a segment at a fixed distance (2 to 3.5 cm) from the tip of the leaf is representative of the same plastid population during redifferentiation. This part was used for plastid isolation at different times after the onset of illumination. Plastids were also isolated from the basal 0 to 1.5 cm. These plastids represent the same stage of development during the experiment and, in accordance with the above results, were not expected to show major differences in import capability. Figure 7B shows that when dark-grown leaves were exposed to light, plastids from the lower section of the leaf, section (i), exhibited a transient ~20% increase in import activity. On the other hand, the import capability of plastids isolated from the upper portion of the leaf , section (ii), increased more than 400% during the early stages of greening and then diminished again to low levels as the system approached the fully greened status. This pattern of in vitro import is in accordance with the in situ kinetics of protein accumulation during greening. LHCP of greening plastids in section (ii) was quantified by immunoblot analysis on a per-plastid basis (data not shown). The rate of accumulation correlated with the import activity (cf. Figure 7B) that was measured during etioplast-to-chloroplast transition. Plastids in section (i) also showed a small but significant accumulation of LHCP during greening, possibly explaining the slight stimulation of import capability of plastids from this lower section. Thus, it appears that the plastids have the ability to reactivate or resynthesize their import machinery to accommodate the biogenetic needs of the plastid.

DISCUSSION

This study has shown that the import capability of leaf plastids is modulated during their development. Thus, as etioplasts or chloroplasts approach the fully mature form, their ability to import a variety of different proteins diminishes. This loss of import capability was observed with both the monocotyledonous wheat and the dicotyledonous pea. It is not due to senescence because the plastids in these assays showed no signs of senescence, such as loss of lamellar alignment or thylakoid breakdown (cf. Whatley, 1974). Furthermore, the chlorophyll content per developing chloroplast increased and the chlorophyll *a/b*

ratios decreased even as the import capacity was decreasing (Table 1). An even more interesting observation was that mature etioplasts, when induced with light to differentiate into chloroplasts, experienced a transient reactivation of their import capability that subsided when differentiation was complete.

Although these basic observations were made by measuring the ability of isolated plastids to import precursor proteins, we do not believe that this is an artifact of the in vitro system for several reasons. First, the decline in import activity is not due to heterologous precursor-plastid combinations. The precursors used with pea chloroplast were all pea precursors. The greatest decline observed with wheat plastids was with the wheat pLHCP, and the pattern was essentially the same when pea pLHCP was used with wheat plastids and vice versa. Second, the possible presence of tissue-specific inhibitors was ruled out by isolating plastids from a mixture of mature and immature tissue. In this case, the measured import was equal to the predicted sum of the import measured with plastids isolated separately. If inhibitory substances had been released from the more developed tissue, the import capability of the immature plastids would have been inhibited. Finally, the fact that the import capability of mature etioplasts reactivated upon illumination argues against the possibility that mature plastids are in some way more susceptible to structural damage to the outer envelope membrane during isolation. We have provided evidence that protein degradation within the plastids does not account for the lower amount of imported proteins in mature plastids. Thus, we interpret these results to mean that the import apparatus is itself regulated.

These results have practical implications for in vitro studies of protein import because they demonstrate that when entire seedlings are used for plastid preparation, a high percentage of import-incompetent plastids will be obtained. This is most apparent for wheat seedlings. Using the data on plastids per wheat leaf section given by Dean and Leech (1982), our results suggest that plastid preparations from the basal 6 cm of a wheat leaf would consist of approximately 75% inactive plastids. Preparing plastids from the rapidly developing regions of the leaf would not only guarantee high levels of import activity but would also obviate any potential masking of biogenesis-specific elements by the larger and more protein-, lipid-, and pigment-rich inactive plastids.

Of more importance is the physiological significance of modulated import capability. The most likely interpretation of these results is that the cell adjusts the import capability in concert with the protein demands of developing plastids. The results with greening etioplasts most closely fit this view because LHCP accumulated at a rate approximating the import activation/deactivation curve shown in Figure 7B (see above). LHCP is the predominant nuclear-encoded thylakoid protein and is thus representative of the general situation. Of interest is the observation by Chitnis et al. (1987) that the capability to insert LHCP into the thylakoid bilayer is also regulated during the differentiation of etioplasts into chloroplasts. Thus, it appears that for some proteins, both the import and the intraorganellar assembly machinery are modulated during greening.

During the normal development of plastids in the light, the import capability is highly active in advance of the maximum rate of protein accumulation. Several studies have addressed the question of plastid protein accumulation in developing wheat seedlings. Lamppa et al. (1985) found that the maximum rate of LHCP accumulation occurred in the lower one-third of the light-grown wheat leaf. This generally agrees with the observations on the accumulation of LHCP in barley seedlings (Viro and Kloppstech, 1980). Rubisco appears to accumulate somewhat later in development than does LHCP (Dean and Leech, 1982; Topping and Leaver, 1990). To compare directly import capability with protein accumulation, we examined the accumulation of plastid proteins in our own experiments by SDS-PAGE/Coomassie Brilliant Blue R 250 staining/densitometry of an equal number of plastids isolated from six 1-cm segments sequentially excised from the base of the leaf, i.e., up to just below the leaf tip. We found that polypeptides of 25 to 28 kD, predominantly LHCP, maximally accumulated between fractions 2 and 3, and the accumulation slowed dramatically thereafter. Similarly, polypeptides migrating at the locations of the large and small subunits of Rubisco accumulated slightly later than LHCP but still with maximum accumulation between fractions 2 and 3. Again, the rate diminished dramatically after fraction 3. Thus, while the accumulation of these proteins was rapid early in leaf development, maximum rates occurred after the peak import capability, i.e., fractions 1 and 2. One interpretation of these results is that the import activity of very young plastids is high to accommodate the flux of proteins that will shortly occur.

The developmental modulation of import capability also shows parallels with the transcription of genes for plastid proteins. Baumgartner et al. (1989) showed that transcription of plastid genes was highest in the rapidly developing region of barley leaves and declined precipitously as the plastids matured. Similarly, Lamppa et al. (1985) showed that accumulation of RNA for LHCP was maximal in the lower third of developing wheat leaves. At present, it is only possible to draw parallels between import activity and the activity of other processes related to plastid biogenesis. It is not yet technically possible to determine whether the level of import capability influences either the rate or the final amount of protein accumulated. This will require the ability to manipulate the levels of import activity in vivo.

Recently, another example of regulated protein import was reported. Chiang and Schekman (1991) described regulation of a yeast vacuolar import system that is responsible for targeting fructose-1,6-bisphosphatase for

degradation during catabolite repression of the gluconeogenic pathway. Thus, it is possible that import modulation is a general phenomenon that accompanies or controls metabolic and developmental transformations. Whether or not modulation of import capability is characteristic of other types of plastid interconversions or even in response to environmental signals that result in the accumulation of cytosolically translated proteins is yet to be determined. Other types of plastids have been used for protein import experiments (e.g., Halpin et al., 1989), but analyses of import activity versus development have not been conducted. Because some plastid transformations require sequential maturation of plastid forms, e.g., the formation of fruit chromoplasts, the full developmental process may involve cycles of import activation/deactivation.

The question of how regulation is manifested in the import machinery is not easily addressed. At present, little is known of the mechanisms involved in plastid protein transport, nor have the components of the import machinery been identified. Current evidence indicates that some precursor proteins may initially interact with cytosolic factors such as hsp70 chaperone proteins to maintain transport competence (Waegemann et al., 1990). Precursors then bind to receptor proteins on the envelope and are translocated into the stroma by a mechanism that requires hydrolysis of ATP. Analogy with the mitochondrial import apparatus suggests that there may be several other envelope proteins as well as a stromal hsp70 involved in the translocation process (Kang et al., 1990; Neupert et al., 1990). The results presented here strongly suggest that the observed regulation must occur by changes in the envelope. Any necessary cytosolic factors are provided by the translation mixture in the in vitro assays conducted here. Furthermore, the immunodecoration assays shown in Figure 2 demonstrate that comparable amounts of hsp70 are present in immature as well as mature plastids. We envision two potential means of regulating the envelope import apparatus. The first would simply involve the balance between synthesis and degradation of translocator proteins. The second might involve a modification-type activation/deactivation process. In the case of yeast vacuole import (Chiang and Schekman, 1991), regulation appears to be accomplished by synthesis of protein component(s) of the import apparatus. Our efforts are currently focusing on understanding the basis for relative levels of plastid import activity and the use of light-mediated activation as a means of identifying envelope translocator proteins.

METHODS

Plant Material

Seeds of wheat (*Triticum aestivum* cv Florida 302) and pea (*Pisum sativum* var Laxtons' progress 9) were soaked in tap water overnight and grown in vermiculite at 20 ± 1°C for 6 and 13 days, respectively. The plants were grown under fluorescent light (100 μE m^{-2} sec^{-1}) in 16:8 hr light:dark cycles or in darkness (wheat only).

Isolation of Plastids

Primary leaves of wheat were gently pulled until the blades broke close to the meristem. The leaves were cut in four 1-cm pieces, in which section 1 denotes the nearly white segment adjacent to the meristem and section 4 denotes the oldest segment. Plastids were isolated using a modification of the method described by Baumgartner et al. (1989) by a combination of differential centrifugation and Percoll gradient centrifugation. Because of the smaller size of the younger plastids, Percoll gradients used to purify plastids from sections 1 and 2 were centrifuged at 7700*g* in a Beckman SW 13.1 rotor for 15 min, whereas those used for fractions 3 and 4 were centrifuged at 1900*g* in a Beckman Accuspin centrifuge for 15 min. Plastids of dark-grown plants were obtained from sections taken at 1.5-cm intervals from the base. Isolation was performed as above, except that all handlings, including the import reactions, were performed in dim green light. For the isolation of pea plastids, the youngest, the folded, and the oldest leaves were used (cf. Fish and Jagendorf, 1982). The pea plastids were isolated as above. Fractions 1 and 2 were collected from the youngest leaf. Fraction 1 was collected as a distinct lighter band above fraction 2 in the same Percoll gradient. This band was generally found at a gradient position that corresponded to an approximate density of 1.068 g/mL.

Preparation of Radiolabeled (Precursor) Proteins

Capped RNA for the various precursors was prepared in vitro with SP6 polymerase (Cline, 1988). The plasmid pSP65-pLHCP (Lamppa, 1988) containing the coding sequence for wheat pLHCP was kindly provided by Dr. G. Lamppa, University of Chicago, and was linearized before transcription with HindIII. The psAB80XD/4 plasmid containing the AB80 gene coding for the pLHCP of pea (Cline et al., 1989) was linearized with EcoRI. The plasmid containing the coding sequence for pHSP 21 of pea (Vierling et al., 1988) was kindly provided by Dr. E. Vierling, University of Arizona, Tucson, and was linearized with BamHI. The plasmid pSMS64 (Anderson and Smith, 1986) containing the coding sequence for the pSS of Rubisco was kindly provided by Dr. S. Smith, University of Edinburgh, and was linearized with EcoRI. RNAs were translated in a wheat germ system using ^{3}H-leucine (Du Pont-New England Nuclear) as described by Cline (1988). Before use, the translation mixture was diluted sixfold and adjusted to 30 mM unlabeled leucine in import buffer (0.33 M sorbitol, 50 mM Hepes/KOH, pH 8.0).

Assays of Import

Import reaction mixtures (300 μL) contained 80 × 10^{6} plastids, 10 mM Mg-ATP, and 50 μL of adjusted translation mixture. Import reactions were carried out at 25°C under laboratory light for 10 min. Termination of assays, thermolysin treatments, and reisolation of intact plastids were performed as described by Cline et al.

(1985). In import experiments using $HgCl_2$, the experimental procedure was performed essentially as described by Reed et al. (1990).

Gel Electrophoresis and Fluorography

Electrophoresis was carried out by SDS-PAGE on 12.5% or 15% gels. Gels were loaded with protein equivalent to 1×10^6 plastids per lane and run at a constant voltage (200 V) for approximately 1 hr. Gels were analyzed and radiolabeled proteins further quantified by liquid scintillation counting of proteins extracted from gel bands (Cline, 1986).

Protein Gel Blotting

Proteins were separated by SDS-PAGE through 10% SDS gels and were electroblotted to nitrocellulose membrane according to standard procedures. Immunodecoration of membranes was performed by the method of Harlow and Lane (1988) with alkaline phosphatase-linked second antibody and bromochloroindoyl phosphate/nitro blue tetrazolium development. The antibodies to LHCP have been described (Payan and Cline, 1991). Antibodies to cpn60 were prepared in rabbit to the *Escherichia coli* groEL protein, and antibodies to hsp70 were prepared in rabbit to the *E. coli* dnaK protein. These antibodies were kindly provided by John McCarty and Caroline Donnelly (Massachusetts Institute of Technology, Cambridge).

Electron Microscopy

Electron microscopy was performed by the University of Florida Interdisciplinary Center for Biotechnology Research Electron Microscopy Facility, Gainesville. Isolated plastids were fixed in 2% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.33 M sorbitol, 25 mM KH_2PO_4, and 2 mM $MgCl_2$, pH 7.5. The samples were rinsed in 0.16 M sorbitol and then in 0.1 M cacodylate buffer without sorbitol. Samples were postfixed in 1% OsO_4 for 1 hr and dehydrated in an ethanol series followed by 100% acetone. The samples were embedded in araldite, sectioned, and poststained with uranyl acetate for 10 min, followed by lead citrate for 4 min.

Miscellaneous

Chlorophyll was determined spectrophotometrically in 80% acetone according to Lichtenthaler and Wellburn (1983). Plastid concentrations were counted in a hemacytometer.

ACKNOWLEDGMENTS

The authors thank Gayle Lamppa, Elizabeth Vierling, and Steven Smith for the gift of clones for precursor proteins. We thank Ron Barnett for supplying the wheat seeds and John Mullet for providing a laboratory protocol for plastid isolation. We are grateful to John McCarty and Caroline Donnelly for the gift of antibodies to *E. coli* groEL and dnaK proteins. We thank Bonnie O'Brien and Gregory Erdos of the University of Florida Interdisciplinary Center for Biotechnology Research Electron Microscopy Facility for the microscopic analysis of the plastid and tissue samples. The authors also thank Mark Schulle for excellent technical assistance and Chris Chase, Charlie Guy, and Jianguo Yuan for critical review of the manuscript. This work was supported in part by National Science Foundation Grant No. DCB-8718560 to K.C. This paper is Florida Experimental Station Journal Series No. R-01781.

Received June 7, 1991; accepted August 20, 1991.

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