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Review

Molecular biology of chloroplast biogenesis: gene expression, protein import and intraorganellar sorting

J. Bauer, A. Hiltbrunner and F. Kessler*

Institute of Plant Sciences, Plant Biochemistry and Physiology Group, Swiss Federal Institute of Technology, Universitätstrasse 2, CH-8092 Zürich (Switzerland), Fax ++ 41 1 632 10 44, e-mail: felix.kessler@ipw.biol.ethz.ch

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Abstract. The chloroplast is the hallmark organelle of plants. It performs photosynthesis and is therefore required for photoautotrophic plant growth. The chloroplast is the most prominent member of a family of related organelles termed plastids which are ubiquitous in plant cells. Biogenesis of the chloroplast from undifferentiated proplastids is induced by light. The generally accepted endosymbiont hypothesis states that chloroplasts have arisen from an internalized cyanobacterial ancestor. Although chloroplasts have maintained remnants of the ancestral genome (plastome), the vast majority of the genes encoding chloroplast proteins have been transferred to the nucleus. This poses two major challenges to the plant cell during chloroplast biogenesis: First, light and developmental signals must be interpreted to coordinately express genetic information contained in two distinct compartments. This is to ensure supply and stoichiometry of abundant chloroplast components. Second, developing chloroplasts must efficiently import nuclear encoded and cytosolically synthesized proteins. A subset of proteins, including such encoded by the plastome, must further be sorted to the thylakoid compartments for assembly into the photosynthetic apparatus.

Key words. Chloroplast biogenesis; light regulation; gene expression; protein import; protein sorting.

Introduction

Depending on host cell differentiation, plastids develop into functionally distinct organelles that are defined by specific gene expression programs and sets of proteins [1]. Undifferentiated plastids are termed proplastids and are present in meristematic tissue containing plant stem cells. Leucoplasts, lacking pigmentation, specialize in the storage of starch (amyloplast), lipids (elaioplast) and protein (proteinoplast), respectively. Chromoplasts, enriched in colored carotenoids, color fruits and flowers. Chloroplasts, due to the presence of chlorophyll, lend green pigmentation to the plant aerial tissues, leaves in particular. Light triggers the specific gene expression program that leads to the differentiation of chloroplasts from proplastids, the major subcellular event in photomorphogenesis [2]. Light signals are transduced by photoreceptors and interpreted (amongst other physiological responses) to induce expression of photosynthetic genes in both chloroplast and the nucleus [3, 4]. Apart from light, the expression of photosynthetic genes is also influenced by developmental signals of chloroplast origin. Chloroplast signals communicate the state of the organelle to the nucleus and modulate light-controlled gene expression accordingly. Nuclear encoded chloroplast proteins, containing a primary N-terminal targeting sequence (transit sequence), are synthesized on free ribosomes in the cytosol. The transit sequence mediates posttranslational import into the chloroplast via a protein import machinery located in the envelope membranes [5–7]. Upon cleavage of the transit sequence in the chloroplast stroma, an assortment of intrachloroplastic transport systems that recognize distinct secondary target-

* Corresponding author.

ing signals route proteins to the thylakoids for assembly into the photosynthetic machinery [5, 8, 9]. Chloroplast biogenesis therefore defines a process in which regulatory events give rise to extensive structural and functional changes of the organelle through a succession of steps involving light-controlled gene expression, protein transport and assembly. This review discusses the molecular events that govern chloroplast biogenesis. Both gene expression and transport/assembly of the prototypical chloroplast protein LHCP (light-harvesting chlorophyll a/b binding protein) have been extensively studied allowing to at least partially reconstruct the distinct steps of chloroplast biogenesis. An integrated view of chloroplast biogenesis will be given by covering aspects of light-regulated nuclear gene expression and trafficking of newly synthesized proteins from the cytosol to the thylakoid membrane as they pertain to LHCP.

Light regulation of photosynthetic genes

Plants are able to sense light both qualitatively and quantitatively and respond to it in many different ways throughout the life cycle [2]. The young seedling is directed to either a skotomorphogenic (dark) or a photomorphogenic (light) program depending on the availability of light. In the absence of light the seedling uses the resources stored in the seed to grow heterotrophically until exhaustion. These etiolated plants have an elongated axis, an apical hook and unopended cotyledons or rudimentary primary leaves. These morphological features are suited to growing through soil. Once in the light, the seedling de-etiolates and greens, indicating the onset of photoautotrophic growth. The rate of axis elongation is reduced, the apical hook disappears and cotyledons (or primary leaves) unfold, resulting in a seedling morphology that is optimized for photosynthesis [2]. As an integral part of de-etiolation, a specific genetic program is initiated that allows undifferentiated proplastids to develop into chloroplasts. Light-regulated nuclear genes, including those encoding chloroplast components, contain multipartite light-responsive elements, including the G-box, in their promoter regions. A host of protein factors bind to the regulatory elements within a photoregulated promoter, but in most cases their role in light-activated transcription still remains obscure [10]. HY5, a bZIP type transcription factor [11], is a positive regulator of photomorphogenesis [12]. It binds to the G-box and is essential for activating genes containing the G-box in their promoter in response to light [13, 14]. In the dark, transcriptional activation by HY5 is repressed. This has been shown elegantly by the analysis of *Arabidopsis* mutants (pleiotropic *cop* (constitutively photomorphogenic), *det* (de-etiolated) and *fus* (fusca) mutations), which develop the phenotype of light-grown plants in the dark. *cop*/*det*/*fus* plants have partially developed chloroplasts and express plastid and nuclear genes, which are normally light regulated, inappropriately in the dark [15, 16]. In wild-type plants, the nucleus-located COP1 protein binds directly to HY5 in the dark, thereby inhibiting transcriptional activation [14]. In the light, COP1 relocates to the cytosol, relieving repression of HY5 [17]. Several other COP/DET/FUS proteins are organized in the COP9 signalosome [18] that appears to regulate nucleocytoplasmic partitioning of COP1 [4]. Thus, light ultimately activates a default photomorphogenic program by relieving transcriptional repression by COP1. The recent identification of the transcriptional regulator CIP7 [19], a COP1 interacting protein that positively regulates multiple light-inducible genes including LHCP, suggests that COP1 functions as a master regulator that represses the activities of multiple transcription factors. Furthermore, light signals transduced by an assortment of photoreceptors decrease the nuclear concentration of COP1, identifying COP1 as a downstream target of multiple photoreceptor signaling pathways [20]. Biochemical studies and mutant analyses in *Arabidopsis* have revealed a panel of photoreceptors positioned at the beginning of light-signaling pathways that control photomorphogenesis. The phytochromes, phyA and phyB [3, 21], and the cryptochrome CRY1 [22, 23] are the best characterized of these and respond to far red, red and blue light, respectively. These photoreceptors have unique as well as overlapping functions, and therefore each may contribute to several photomorphogenetic traits. Expression of LHCP is induced by red light, implying phytochrome activity. Phytochromes form homodimers of a 120-kDa protein, each monomer being covalenty attached to a linear tetrapyrrole chromophore (phytochromobilin). Phytochromobilin accounts for the spectral properties of phytochrome and allows it to switch between red (Pr) and far red (Pfr) absorbing forms by cis, trans-isomerization [24]. Phytochrome has two domains. The N-terminal domain is sufficient and required for chromophore attachment and spectral responses [24], whereas the C-terminal signal transduction domain has structural similarity to bacterial histidine kinases [25] and may thus transmit signals through protein phosphorylation. Despite the resemblance of the C-terminal domain to histidine kinase, experimental evidence rather points to a serine-threonine kinase activity of phytochrome [26, 27]. Indeed, a cytosolic substrate of phytochrome kinase activity (PKS1) has been identified and shown to function as a modulator of phytochrome-dependent responses, such as hypocotyl (axis) elongation [26]. Because PKS1 is a cytosolic protein, it may directly affect cytosolic processes. A role of cytosolic PKS1 in gene expression implies downstream signal transduction events. These might involve signaling through heterotrimeric G-proteins, and subsequently cyclic GMP (cGMP) or Ca^{2+}/calmodulin. Ca^{2+}/calmodulin

signaling downstream of phytochrome has been implied in the control of LHCP expression [28]. The possible involvement of heterotrimeric G-proteins as well as of cGMP and Ca^{2+}/calmodulin in phytochrome signaling has been concluded from biochemical complementation and pharmacological studies of the phytochrome chromophore biosynthesis-deficient tomato mutant, *aurea* [28, 29], but has not yet been linked to genetic studies in *Arabidopsis*. Phytochromes enter the nucleus in response to red light and may therefore affect gene expression in a very direct manner [30, 31]. Recently, components of a phytochrome signaling pathway have been identifed that may directly regulate gene expression. PIF3, a phytochrome-interacting factor, is a novel member of the basic helix-loop-helix transcription factor family and is located in the nucleus [32]. It binds to the signal transduction domain of the Pfr form of phytochrome B in a photoreversible way [32, 33]. Plants with lowered levels of PIF3 showed reduced photoresponsiveness, including reduction of LHCP expression [32]. According to the direct regulation model, phytochrome enters the nucleus in response to red light, and binds to and mediates transcriptional regulation through PIF3. The direct regulation model has been substantiated by the finding that PIF3 binds directly to the G-box present in various light-regulated promoters [34]. Clearly, phytochrome signaling is highly complex because *Arabidopsis* mutant screens have identified a number of additional loci potentially defective in phyA and phyB signaling [4]. Thus multiple light-regulated pathways and factors likely contribute to the expression of LHCP and other light-regulated genes.

Developmental regulation of photosynthetic genes

Light is not the only factor that affects the expression of LHCP. The circadian clock [35], hormones [36] and, most pronouncedly, the state of the chloroplast modulate LHCP expression. Studies using the herbicide norflurazone revealed a connection between chloroplast integrity and nuclear gene regulation. Norflurazone, a herbicide that inhibits phytoene desaturase in carotenoid biosynthesis [37], causes bleaching of plants due to oxidative chloroplast damage. Chloroplast degeneration is accompanied by a strong reduction of light-regulated gene expression, including that of LHCP [38]. This finding implies a signaling pathway from the chloroplast to the nucleus. The existence of such a signaling pathway is supported by genetic evidence. Genetic screens in *Arabidopsis* intended to identify positive regulators of LHCP expression by scoring for mutants that underexpress LHCP in the light resulted in identification of *cue* mutants (*cue*: CAB (identical with LHCP) underexpressing mutant) [39, 40]. Surprisingly, the screens also yielded mutants that are affected in components unlikely to be directly involved in signal transduction. The gene product affected in *cue1* [39], a reticulate pale green mutant, has been identified as the phosphoenolpyruvate/phosphate translocator (PPT) localized in the inner chloroplast membrane [41]. The defect in this transporter molecule compromises synthesis of aromatic amino acids and their derivatives [41], suggesting that the main function of PPT is to funnel phosphoenolpyruvate into the shikimate pathway [42]. As a consequence of the lack of aromatic compounds, chloroplast biogenesis in leaf mesophyll cells cannot be completed. Defective chloroplast function in the *cue* mutants results in downregulation of light-induced genes similar to that observed upon norflurazone treatment [39, 40]. Another mutant, identified in a screen for *cue* mutants is *ppi1* (plastid protein import mutant 1) [43]. In *ppi1* (discussed below), TOC33, a gene encoding a component of the chloroplast protein import machinery, is disrupted by a T-DNA (transferred DNA) insertion. The resulting protein import defect is sufficient to retard chloroplast development in young seedlings and, furthermore, is coupled to a reduction of light-regulated expression of photosynthetic genes. The effects of both the *cue1* and *ppi1* mutations are therefore pleiotropic, in that they do not only impair the respective pathways their gene products operate in, but also act indirectly, through their detrimental effects on chloroplast function, in downregulating light-induced gene expression. Further genetic evidence for a signaling pathway from the chloroplast to the nucleus stems from a screen designed to reveal mutants expressing LHCP despite chloroplast damage [44]. The resulting *gun* (genomes uncoupled) mutants exhibit the wild-type phenotype under most conditions, but express light-induced photosynthetic genes aberrantly upon treatment with norflurazone or in the background of a genetic lesion resulting in chloroplast damage [44]. Thus, synthesis of metabolic signals or protein components of the chloroplast to the nucleus signaling pathway may be affected in the *gun* mutants. Furthermore, the evidence suggests that in both norflurazone- and genetically induced chloroplast defects the signal and signaling pathways resulting in reduced gene expression are identical. Gun gene products likely hold clues to the nature of the elusive signal (also called plastid factor) emanating from the chloroplast. Work done in *Chlamydomonas* indicates that chlorophyll precursors (Mg-protoporphyrin IX or its dimethylester) induce the accumulation of several messages of nucleus-encoded proteins and raises the possibility that chlorophyll precursors constitute the plastid factor [45–47]. In the future, it will be of interest to see at which point the light-dependent and chloroplast-to-nucleus signaling pathways converge. In chloroplasts, it appears that the two pathways cooperate to tune the metabolic and photosynthetic state of the organelle under varying internal and external conditions by regulating gene expression in an interdependent fashion (fig. 1).

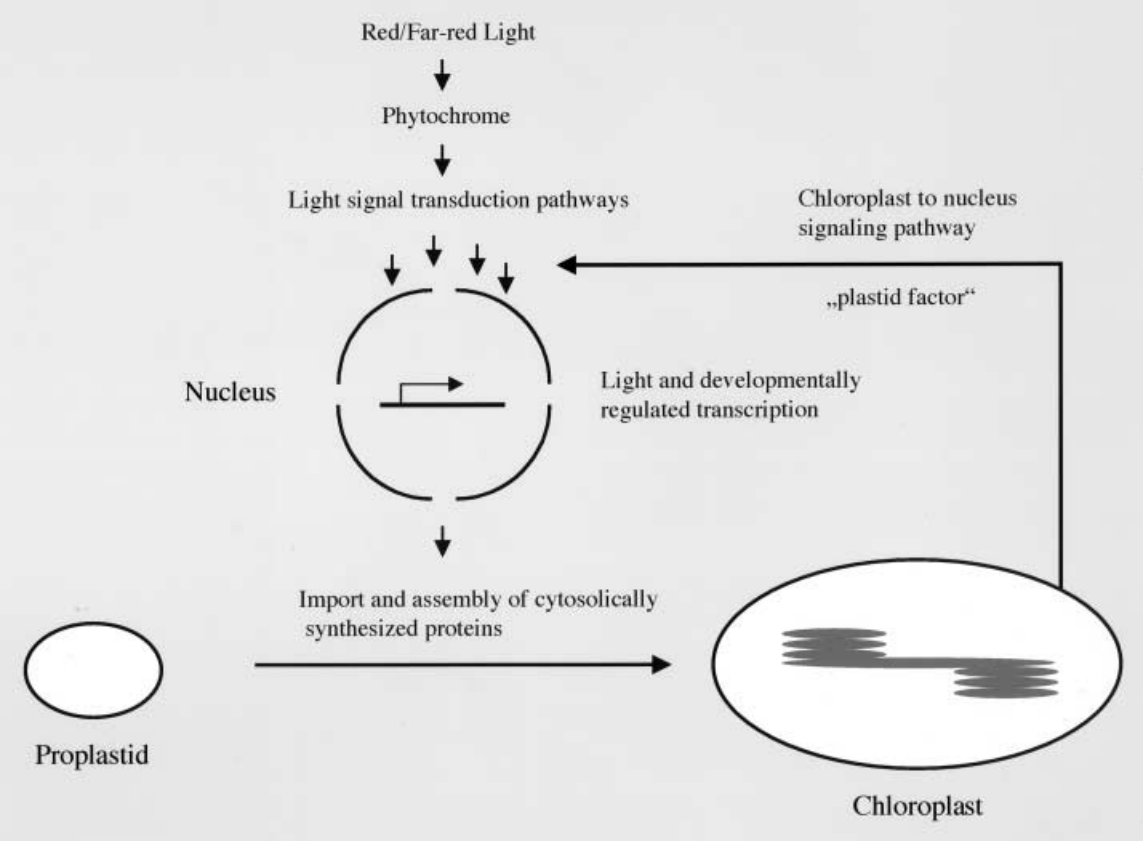

Figure 1. Schematic respresentation of chloroplast biogenesis. Chloroplast biogenesis is light dependent. Red and far-red light activate phytochrome-dependent signaling pathways, resulting in the transcription of photosynthesis-associated genes. Nuclear encoded proteins are synthesized in the cytosol and subsequently imported and assembled in the proplastid, allowing it to develop into a chloroplast. The developmental state of the chloroplast is tightly controlled. Its condition is relayed to the nucleus by a chloroplast-to-nucleus signaling pathway to modulate light-dependent gene expression accordingly.

Protein targeting to the chloroplast envelope membranes

Nuclear encoded chloroplast proteins such as LHCP are synthesized in the cytosol as precursors with an N-terminal transit sequence. Only after translation has been completed does transport across the chloroplast envelope membranes take place (posttranslational import). It seems improbable that newly synthesized precursors arrive at the chloroplast solely by diffusion. Transfer from the translation site to the chloroplast surface therefore implies a specific targeting step involving soluble cytosolic factors. Yet to date, no cytosolic factors involved in the targeting process to the chloroplast surface have been characterized in detail. Moreover, numerous protein import studies using isolated pea chloroplasts and precursors synthesized in a rabbit reticulocyte lysate negate a requirement for a plant-specific cytosolic factor in vitro. The finding that a soluble, chemically pure preferredoxin precursor can be imported into isolated chloroplasts indicates that no soluble factor at all is required in the case of this precursor [48]. However, in vitro experiments do not reproduce intracellular conditions, and specific targeting factors may well be required in vivo. In vitro synthesized precursors, to retain their import competence, are held in a soluble, unfolded state by chaperone proteins present in a reticulocyte lysate or wheat germ extract [49, 50]. Heat shock proteins of the Hsp70 family have emerged as a common cytosolic factor involved in protein transport to many organelles [49]. The role of Hsp70 proteins is to maintain the solubility of an unfolded precursor in the crowded cytosol rather than to guide it to a specific organelle [51]. Targeting factors specific to organelles operate in parallel to Hsp70. Organelle-specific cytosolic targeting factors have been identified for the endoplasmic reticulum (ER) and mitochondria. For cotranslational import (import during ongoing protein synthesis) into the ER, SRP (signal recognition particle) targets the ribosome nascent chain complex to the translocation site at the ER surface [52]. In the mitochondrial system, several cytosolic factors have been identified that affect import of precursors. Mft52 (mitochondrial fusion protein targeting factor) [53] and NAC (nascent chain-associated complex) [54] are required for faithful protein delivery to yeast mitochondria in vivo.

Presequence binding factor [55] and mitochondrial import stimulating factor MSF [56] both bind to presequences and stimulate import into isolated yeast mitochondria. MSF belongs to the 14-3-3 protein family [57]. In the chloroplast system, a heterooligomeric 200-kDa guidance complex consisting of 14-3-3 proteins and Hsp70 was recently reported [58]. The guidance complex binds to the transit sequence of a precursor protein in vitro [58]. To do so, the transit sequence must be phosphorylated at a motif that shows similarity to the phosphopeptide binding motif of 14-3-3 proteins [59]. The guidance complex targets the precursor to the chloroplast surface and stimulates its import into isolated chloroplasts. The primary structure of the 14-3-3 components of the guidance complex and its in vivo function remain to be determined.

Protein translocation across the chloroplast envelope membranes

Protein translocation across the chloroplast envelope membranes functions according to general principles that also govern transport across the bacterial plasma membrane, the ER and mitochondria [49]. The process of protein translocation across the chloroplast envelope membranes can be resolved into at least three stages. Once a precursor protein has reached the chloroplast surface, its transit sequence engages components of the outer membrane translocation machinery termed the Toc-complex (translocon at the outer membrane of the chloroplast) [60]. Then, transport across the inner membrane requires the additional activity of the Tic-complex (translocon at the inner membrane of the chloroplast) [5, 60].

The Toc-complex

The Toc-complex of pea chloroplasts consists of three major components [61, 62]: two homologous surface-exposed GTPases (Toc159 and Toc34) [63–67] and Toc75 [61, 68], a protein with sequence similarity to solute channels in the outer membrane of Gram-negative bacteria and cyanobacteria [69, 70]. In a first energy-independent and reversible step, Toc159 recognizes the transit sequence [62, 71] (fig. 2). Furthermore, addition of antibodies against Toc159 to isolated chloroplasts in an in vitro im-

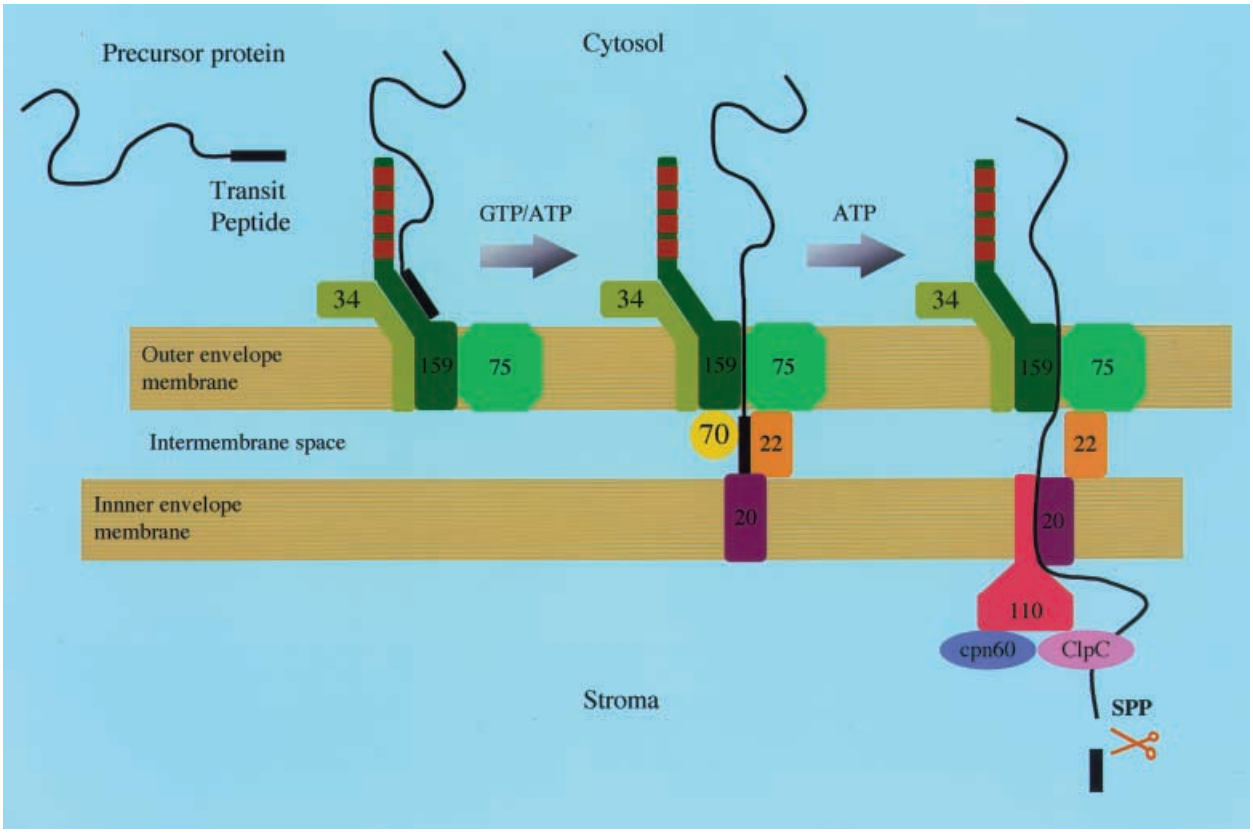

Figure 2. Functional model of the chloroplast protein import machinery: In a first energy-independent reaction, the precursor interacts with Toc159 of the trimeric Toc complex. In a second step that requires GTP and 0.1 mM ATP, the precursor inserts across the outer membrane and binds to components of Tic complex at the inner membrane surface (Tic22, Tic20). Full translocation of the precursor requires 1 mM ATP. At this stage the precursor spans both membranes and has engaged Tic110, which recruits chaperones (cpn60, ClpC) to the import site. Such chaperones may assist in folding newly imported proteins or drive import. The transit sequence is cleaved by stromal processing peptidase (SPP).

port experiment blocks precursor binding to the chloroplast surface [65]. The sum of evidence suggests that Toc159 functions as a protein import receptor. The precursor is irreversibly bound to the chloroplast in a process requiring the hydrolysis of both GTP and ATP at low concentrations (0.1 mM) [72, 73]. At this stage, termed the early intermediate, the precursor has fully engaged the trimeric complex and traversed the outer membrane but not the inner [61, 74] (fig. 2). Toc75 forms the hydrophilic channel, consisting of a porin-type β-barrel structure, through which translocation proceeds [75]. Portions of Toc159 possibly contribute to the protein-conducting channel, as the precursor is also in direct contact with Toc159 at this stage of translocation [62]. GTP hydrolysis by Toc34 appears to be required for the progression of the import reaction across the outer membrane [67]. It has been proposed that Toc34 does so by transiently binding to the precursor and handing it down from Toc159 to Toc75 [76]. The ATP requirement in the early intermediate stage is in the intermembrane space [73]. An integral outer membrane protein of the Hsp70 class, facing the intermembrane space, has been identified as a component of the early intermediate [61]. It likely serves to stably bind the precursor and thereby prevent its retrograde slippage.

The Tic-complex

At the early intermediate stage the precursor encounters components of the Tic-machinery [74]. Tic22, an extrinsic membrane protein that resides on the surface of the inner membrane binds to the transit sequence [74, 77]. Based on its inner membrane surface location [78] and its ability to interact with the transit sequence, Tic22 likely functions as a transit sequence receptor component at the inner membrane and may also be involved in the formation of contact sites between the inner and outer envelope membranes [5]. Tic20, an integral protein of the inner membrane, also interacts with the precursor transit sequence [74,77]. The predicted topology of Tic20, allowing for three transmembrane helices, makes this protein a candidate for a component of the protein-conducting channel at the inner membrane [77]. Full import of a precursor requires millimolar concentrations of ATP, which is hydrolyzed in the stroma [79]. When under these conditions an in vitro import reaction is arrested by chilling on ice, a late translocation intermediate is formed by a trapped precursor spanning both membranes [61]. In addition to the components of the trimeric Toc-complex, this complex contains two more major proteins: A protein of 36 kDa, still to be characterized, and Tic110 [61]. Tic110 is an integral protein of the inner chloroplast membrane anchored by a hydrophobic domain close to the N-terminus containing two neighboring putative transmembrane helices [80, 81]. Its large C-terminal soluble domain faces the stroma [80, 82]. This domain appears to play a role in chaperone recruitment to the stromal exit of the import machinery. Association of Tic110 with both cpn60 (chaperonin 60) [80] and clpC (the regulatory subunit of the clp protease) [83] has been reported. The recruitment of chaperones may serve two purposes: on the one hand chaperones assist in the folding and assembly of newly imported proteins. It may therefore be efficient to dock cpn60 to the import site to couple membrane translocation to subsequent protein folding [80]. On the other hand, chaperones are able to drive import, as is the case for Hsp70 in yeast mitochondria and ER [49]. It has been proposed that clpC, which can partially substitute for Hsp70 in yeast mitochondria, might fulfil the task of reeling the precursor into the stroma through its interaction with Tic110 [83, 84]. Other candidate Tic- and Toc-proteins, such as Tic55 and Toc64, have been identified, but their functions not yet established [85, 86].

The work reviewed so far in this section is largely based on the analysis of an in vitro import system employing isolated pea chloroplasts. Whereas this system has allowed the biochemical identification and characterization of a set of Toc and Tic proteins that likely form the core of the chloroplast protein import machinery, it is not amenable to a molecular genetic approach.

Chloroplast protein import in *Arabidopsis thaliana*

The *A. thaliana* system provides the opportunity to test the in vivo functions of Tic and Toc proteins, identified in pea, by using reverse genetics. Novel components of the chloroplast import machinery can be identified by means of genetic screens and mutant analysis. Furthermore, *Arabidopsis* genomic sequences and EST (expressed sequence tag) collections greatly simplify the identification of homologues to known Tic and Toc proteins. Homology searches of the *Arabidopsis* databases have revealed a picture of chloroplast protein import that differs significantly from that in pea: in pea all evidence points to an import mechanism that relies on a single general import machinery consisting of unique components. In *Arabidopsis*, homologues of the unique components of the pea chloroplast import machinery exist. Excitingly, in the case of Toc34 [43] and Toc159 [87], small gene families encode multiple homologues in *Arabidopsis*. Concerning the functions of the multiple homologues, at least two possibilities appear plausible [88]. On the one hand, the homologues may represent tissue- or plastid-specific isoforms. In this scenario, individual members of the Toc gene families may be adapted to the specific requirements of different plastid types. On the other hand, the family members may represent substrate-specific isoforms. Such homologues may coexist in different plastid types but would be specialized in the import of specific substrates. Progress towards answering these questions has already been made.

The *ppi1* mutant

Presently, sequences of two *Arabidopsis* homologues of pea Toc34, i.e. atToc33 and atToc34, are present in the databases. Plants lacking atToc33 (*ppi1*: plastid protein import mutant 1) were identified in a genetic screen for loci affecting the expression of nucleus-encoded photosynthetic proteins [43]. The *ppi1* plants appear uniformly pale during the first 2 weeks after germination, whereas in mature plants the oldest leaves resemble those of the wild type. Although chlorophyll levels are reduced at all developmental stages, the extremely low chlorophyll levels in 2-day-old seedlings suggest that atToc33 functions very early in chloroplast biogenesis during cotyledon expansion. Chlorophyll levels in *ppi1* increased throughout the life cycle of the mutant, except during days 5–10, suggesting that atToc33 also plays a role during the formation of the primary leaves. In in vitro experiments using isolated *ppi1* plastids, protein import was reduced in 10-day-old mutant plants, but was indistinguishable from wild-type in plastids isolated from mature plants. These data correlate well with the expression of atToc33 and atToc34. Since atToc33 RNA levels were highest in young tissues, import capacity may be coupled to the expression of translocon components. Indeed, it has been shown that plastid protein import is regulated developmentally and is maximal during the early stages of leaf expansion [89]. The observation that mature *ppi1* plants have a wild-type phenotype and normal chloroplasts at the ultrastructural level indicates that the lack of atToc33 can be completely compensated for. Indeed, the ectopic expression of either atToc33 or atToc34 was able to rescue *ppi1*. These data indicate that atToc33 and atToc34 are functionally equivalent and suggests that in mature *ppi1* plants atToc34 substitutes for atToc33.

The *ppi2* mutant

A more complex situation exists in the case of Toc159. The *Arabidopsis* databases contain full coding sequences of three Toc159 homologues: atToc159, atToc120 and atToc132. The proteins of the Toc159 family have a tripartite structure apparent from their primary sequence [66, 67, 87] and experimentally confirmed by controlled proteolysis [65, 67]: The C-terminal M-domain (membrane domain) anchors the protein in the outer membrane. The central G-domain (GTP binding domain) contains conserved GTP binding motifs and binds GTP specifically [63]. Both the G-domain and the acidic N-terminal A-domain (acidic domain) are exposed to the cytosol [66, 67]. The A-domains of atToc159/132/120 vary strongly both in sequence and length. In addition, the A-domains of atToc159 and atToc132 each contain several distinct conserved repeats [66, 67]. It has been proposed that possible functional variations amongst atToc159/132/120 might stem from their divergent A-domains. The intact A-domain of Toc159 appears to be required for optimal precursor binding and translocation in isolated pea chloroplasts but is not essential [66, 67]. Of the three homologues in *Arabidopsis*, atToc159 is expressed 5–10 times more strongly than atToc120/132 at the transcript level, suggesting, with due caution, that atToc159 may function as the major chloroplast protein import receptor [87]. Using a reverse genetic approach, T-DNA insertional mutants lacking atToc159 were identified and termed *ppi2*. Strikingly, *ppi2* plants have an albino phenotype. Due to the complete lack of chlorophyll, *ppi2* plants are unable to photosynthesize. TOC159 is therefore an essential gene, and its disruption lethal at the seedling stage. At the ultrastructural level, *ppi2* plastids appear as small, undifferentiated proplastids without the thylakoid membranes and starch granules typical of photosynthetically active chloroplasts (fig. 3). Apparently, *ppi2* plants are unable to initiate chloroplast

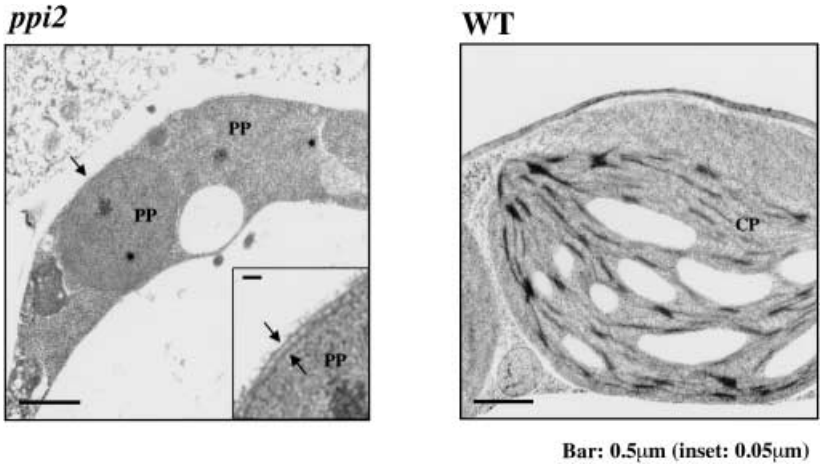

Figure 3. Plastid morphology. WT: Wild-type chloroplasts (CP) contain thylakoids and starch granules. *ppi2*: Plastids (PP) of the protein import mutant *ppi2* lack thylakoids and starch granules and are smaller than wild-type chloroplasts and therefore resemble proplastids.

biogenesis, presumably because the mutant plastids are compromised in their ability to import proteins. However, *ppi2* plants do not accumulate nonimported precursors in the cytosol. On the contrary, in these plants the transcription of both nucleus- and chloroplast-encoded photosynthetic proteins is repressed, indicating a negative effect of the defective *ppi2* plastids on gene expression (fig. 1). Thus, *ppi2* can be classified as a strong *cue* mutant. Nevertheless, photosynthetic proteins can be detected by Western blotting. Furthermore, both the nucleus-encoded small subunit of Rubisco and LHCP appear to be processed to their lower molecular mass mature forms, suggesting that the two proteins are imported into and processed in *ppi2* proplastids, though at strongly reduced levels. Immunogold electron microscopy confirmed the presence of the small subunit in *ppi2* proplastids. These data indicate that even though TOC159 is essential for chloroplast biogenesis, *ppi2* proplastids are still able to import proteins from the cytosol. As atToc120 and -132 are expressed in *ppi2* plants [D. J. Schnell, and F. Kessler, unpublished results], it appears possible that the two Toc159 homologues partially compensate for the absence of atToc159. The presence of several less abundant, nonphotosynthetic imported proteins in *ppi2* plastids was also confirmed: atTic110 and atToc75, two components of the import machinery targeted to the chloroplast via the Toc-pathway, were present at wild-type levels in *ppi2* plants and processed to their mature forms, suggesting plastid localization. Thus it appears that some proteins can be normally imported in the absence of atToc159, suggesting the existence of alternative import pathways in vivo. A model accommodating these findings has been proposed. According to the model, different import receptors with different, but possibly overlapping specificities recognize different classes of precursors. Such specificity may be mediated by the N-terminal domains of atToc159, -132 and -120, where the three vary most. Although transit peptides may determine the efficiency of precursor import into various plastid types [90], distinct classes of transit peptides cannot be identified based on primary structure. Another plausible model would emphasize the quantitative aspect of precursor protein import rather than the qualitative differences in putative classes of transit peptides. In this model, atToc159, the most abundant of the three receptor proteins, is required for the rapid, large-scale import of highly abundant photosynthetic proteins early during chloroplast biogenesis. In the absence of atToc159, large-scale protein import cannot be sustained, leading to a breakdown of chloroplast biogenesis, although the alternative import receptors, atToc132 and atToc120, maintain a basal level of import. The defective state of such chloroplasts is communicated to the nucleus, where this information effects the subsequent repression of photosynthetic genes. Genes of precursors not under chloroplast control and generally less abundant continue to be expressed, and their protein products accumulate in the plastid by means of alternative import pathways that might require the function of atToc132/120. How, in the wild type, competition between highly abundant and scarce import substrates for a limited number of import sites can be avoided remains an open question. Both atToc33 and atToc159 are members of protein families. Disruption of the corresponding genes in both cases does not totally abolish protein import into chloroplasts, yet in the case of *TOC159*, disruption is still lethal at a very early developmental stage. Furthermore, disruption of *TOC159* specifically affects photosynthetic proteins, whereas expression and import of other proteins does not appear to be changed. It is therefore likely that apart from photosynthesis, most essential plastid functions are retained. It will be of interest to analyze *Arabidopsis* lines that are disrupted in genes of unique components of the protein import machinery. Tic110 is a candidate for such a component, as the *Arabidopsis* Genome Initiative has identified only a single gene encoding a Tic110 homologue. If atTic110 is indeed unique, alternative import pathways relying on different Toc159/Toc33 homologues may converge at this component which, facing the stroma, serves in the later stages of protein import. In contrast to atToc33 or atToc159, disruption of a gene encoding a unique component of the protein import machinery should result in a complete block of protein import. In such a mutant, virtually all plastid functions relying on imported proteins would be affected, potentially leading to a complete loss of plastid function. In this scenario, gene disruption may result in embryo lethality and thus the inability to isolate homozygous plants.

Intrachloroplast sorting of imported proteins

The subcompartmentalization of the chloroplast requires further intraorganellar sorting of thylakoid proteins once they have reached the chloroplast stroma [5, 8]. Mutant analysis and an in vitro translocation assay, using isolated thylakoids, in which experimental conditions can be adjusted to determine requirements of energy and stromal factors, have greatly increased our knowledge of the transport mechanisms operating in the thylakoids. A variety of pathways has been uncovered along which proteins are transported either to the thylakoid membrane or the thylakoid lumen, the soluble compartment within (fig. 4). Considering the highly variable nature of the transport substrates, including soluble, lumenal proteins, and single and multiple spanning integral membrane proteins, the existence of multiple sorting pathways is not surprising. Three of the four known pathways operate with components conserved from prokaryotic protein transport machineries that export proteins to the bacterial plasma

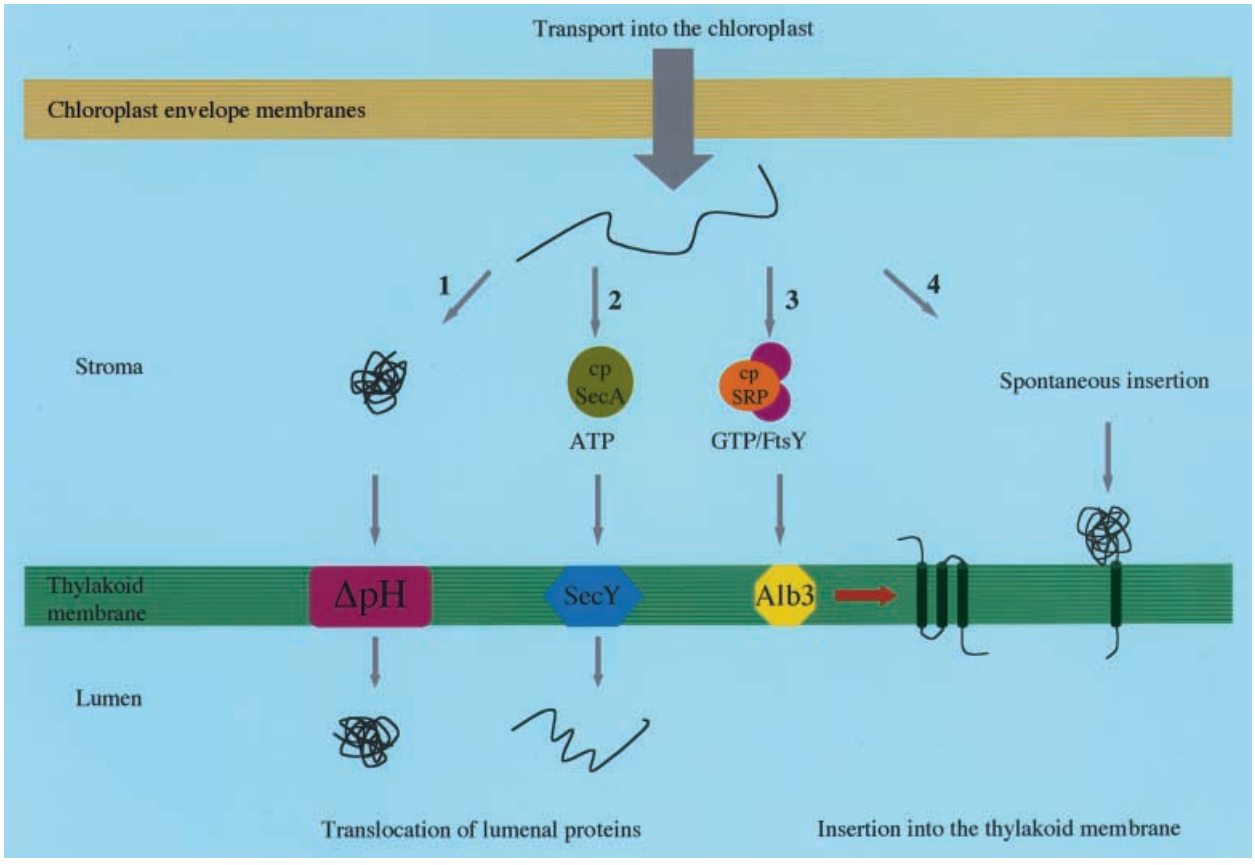

Figure 4. Intrachloroplastic protein sorting: Four pathways target proteins into or across the thylakoid membrane. (1) The ΔpH pathway transports twin-arginine motif proteins, possibly in a folded state, into the thylakoid lumen. The thylakoidal proton gradient drives the process. (2) The parallel Sec-pathway requires stromal SecA and ATP and the integral membrane protein SecY. (3) Insertion of polytopic thylakoid membrane proteins, such as LHCP, requires stromal chloroplast signal recognition particle (cpSRP), FtsY and GTP, the integral membrane protein Alb3 and likely other uncharacterized membrane proteins. (4) Finally, some proteins may spontaneously insert into the thylakoid membrane without a requirement for stromal or thylakoid membrane components.

membrane or to the periplasmic space. This close relationship is plausible, considering that the chloroplast originated in evolution from an endosymbiontic prokaryote. All nuclear encoded thylakoid proteins are synthesized with a stromal transit sequence that promotes import into the stroma where the transit sequence is cleaved. Subsequent 'expor' from the stroma to the thylakoids requires additional targeting information, most often located adjacent to the transit sequence. Thus, the transit sequence and the thylakoid signal sequence together form a bipartite targeting domain. Export to the thylakoids can be resolved into at least two stages, i.e. formation of a transient stromal intermediate and transport into or across the thylakoid membrane, respectively (fig. 4).

Spontaneous insertion of membrane proteins into the thylakoid membrane

A pathway which has no known counterpart in bacteria has been elucidated for subunit II of the CF_0 component of the H^+-ATPase. Subunit II is made up of a single transmembrane region and a large stromal domain. The protein is synthesized with a bipartite targeting sequence, the second part of which resembles typical Sec-type signal peptides (discussed below) and is processed by the thylakoid processing protease (TPP) located in the thylakoid lumen. Subunit II inserts into the thylakoid membrane in the absence of stromal factors, nucleotides, thylakoidal ΔpH or any protease sensitive components at the thylakoid surface. Based on these findings it has been proposed that subunit II spontaneously inserts into the thylakoid membrane. The role of the Sec-type signal sequence of subunit II in spontaneous insertion is unclear, but it may aid membrane partitioning as it is partially hydrophobic [91, 92].

The Sec and ΔpH pathways transport proteins to the thylakoid lumen

In lumenal proteins, cleavage of the stromal transit sequence exposes an adjacent thylakoid targeting sequence

resembling a bacterial signal peptide [93]. Within the lumen, TPP cleaves the signal sequence. Despite the common denominators, lumenal proteins fall into two groups. Plastocyanin and the 33-kDa subunit of the oxygen-evolving complex (OE33), amongst other proteins, are transported by a mechanism resembling the bacterial Sec-pathway. In an in vitro translocation system using isolated thylakoids, the transport of these proteins requires the presence of stromal factors, i.e. the chloroplast homologue of SecA, cpSecA, and ATP [94]. At the thylakoid membrane, chloroplast homologues of the bacterial SecY and SecE proteins are likely to be involved in protein translocation [95]. Mutations in either cpSecA [96] or cpSecY [97] result in severe thylakoid defects and confer a seedling lethal phenotype to maize. In contrast to plastocyanin and OE33, transport of the 17-kDa (OE17) and 23-kDa (OE23) subunits of OE33 do not require stromal factors or ATP [98], but instead is strictly dependent on thylakoidal proton gradient (ΔpH) for membrane translocation [99, 100]. Selectivity between the Sec and ΔpH pathways resides in the thylakoidal targeting signals. Proteins targeted to the ΔpH pathway contain a typical twin arginine motif in their lumenal targeting signal [98] which otherwise is structurally indistinguishable from a Sec-type lumenal targeting sequence [101]. The maize mutant *hcf106* is defective in the ΔpH-dependent pathway and accumulates OE23 and OE16 stromal intermediates [96]. Hcf106 encodes a thylakoid protein consisting of a large globular domain facing the stroma and a single transmembrane region [102]. The membrane topology of the protein suggests that hcf106 may function as a thylakoidal surface receptor component of the ΔpH translocation machinery. A ΔpH-related protein transport pathway, the Tat-pathway, exists in bacteria [102]. The export of *E. coli* twin arginine proteins is linked to this pathway involving the hcf106 homologues TatB [103] and TatA/TatE [104]. In bacteria, proteins containing the twin arginine motif are often attached to redox factors (FeS-centers, molybdopterin) and are at least partly folded in the cytosol [105]. Based on the nature of the bacterial twin arginine motif proteins, it seems probable that the ΔpH pathway of chloroplasts and the corresponding Tat-pathway of bacteria are able to transport proteins in a partially folded state [106].

SRP-dependent insertion of proteins into the thylakoid membrane

The insertion of multiple membrane-spanning thylakoid proteins, such as LHCP, requires yet another protein-sorting pathway. This pathway involves a chloroplast version of the signal recognition particle (SRP), but it functions in a posttranslational manner, whereas SRP in eukaryotes and bacteria functions in cotranslational protein transport [52]. Eukaryotic SRP consists of six polypeptides, among them SRP54, a GTPase that binds directly to the signal sequence, and a 7S RNA involved in ribosome binding. SRP targets nascent proteins bearing a signal sequence to the ER membrane while they are attached to the ribosome. Bacterial SRP, which functions primarily in the cotranslational insertion of plasma membrane proteins, consists of a single protein, a SRP54 homologue, and an RNA molecule, and requires SecY for final membrane insertion. Chloroplast SRP, cpSRP, is yet another variation on the theme. CpSRP is trimeric, consisting of two cpSRP43 molecules and a cpSRP54 molecule [107] but an RNA molecule has not been identified [52]. Instead, cpSRP43 may replace the RNA in the posttranslational mode of thylakoid targeting [108]. In posttranslational membrane insertion, LHCP, and likely other polytopic membrane proteins, assemble into a soluble transit complex upon import into the stroma [109]. In the transit complex, LHCP binds tightly to the 54-kDa subunit of cpSRP [110]. However, cpSRP alone is not sufficient to fully reconstitute insertion of LHCP into isolated thylakoids. In addition to cpSRP, cpFtsY (a homologue of the SRP receptor) and GTP [109] are required to mediate membrane insertion [107]. Since GTP is not required for binding of LHCP to cpSRP [110], it is probably needed for the targeting of SRP-bound LHCP to the thylakoid membrane via cpFtsY [107]. Thylakoid membrane components that participate in the insertion of LHCP into the thylakoid membrane are less well characterized. The only clearly identified membrane component involved in LHCP membrane insertion is the Alb3 protein [111, 112]. Alb3 appears to be directly involved in LHCP membrane insertion but not in docking of the LHCP transit complex to the thylakoid surface [112]. In bacteria and eukaryotes, SRP-mediated protein transport requires SecY or its homologues [52], suggesting that the cpSecY protein is involved in the membrane insertion step of LHCP. Indeed, a bacterial homolog of Alb3, YidC [113] is a subunit of the Sec complex in *E. coli* [114]. In the analogous scenario in chloroplasts, therefore, the cpSRP and cpSec transport pathways converge at the membrane-bound cpSecY protein. Alternatively, an entirely different transport machinery may have evolved that works in conjunction with Alb3 in chloroplasts. *Arabidopsis* mutants of cpSRP54, cpSRP43 and Alb3 have been identified. All of these mutants show more or less pronounced chloroplast defects. Mutants with low levels of cpSRP54 are yellowish early in development but later develop fully functional chloroplasts [115]. CpSRP43 is encoded by the CAO gene [116]. Mutation of the CAO gene gives rise to the *chaos* mutant (chlorophyll a/b-binding protein harvesting organelle specific) which is pale green throughout the life cycle and contains less than half the wild-type levels of LHCP. Finally, plants carrying the *alb3* mutation are albino to yellowish and die at the seed-

ling stage [111]. Although LHCP and other light-regulated genes are expressed in *alb3*, the respective Messenger RNA(mRNA) levels are reduced. Thus, all three mutants have in common that they interfere with chloroplast biogenesis at the level of LHCP membrane insertion, which in turn results in the reduction of expression of light-induced photosynthetic genes and a pale green *cue* mutant phenotype.

Conclusion and outlook

The data reviewed in this paper reveal a considerable complexity of chloroplast biogenesis. Yet the drastic morphological, physiological and ultrastructural changes taking place during biogenesis make the chloroplast an attractive model system for organelle development. Expression of LHCP is used as an established marker of chloroplast development and light signaling. Transport and assembly of the protein are well but not nearly fully understood. An interesting observation is that transport and assembly of LHCP are intimately entwined with the expression of the corresponding gene. Mutant plants defective at any stage of transport or assembly of LHCP exhibit reduced gene expression mediated by a chloroplast-to-nucleus signaling pathway. The chloroplast-to-nucleus signaling pathway is not restricted to LHCP but has a general function in relaying the state of the chloroplast to the nucleus. A host of mutants defective in a wide range of chloroplast developmental and metabolic processes exhibit similar pale green to albino phenotypes accompanied by the reduced expression of LHCP and other genes associated with photosynthesis. A unique plastid factor of yet unknown nature appears to integrate dissimilar deleterious primary defects into the modulation of gene expression normally under the control of light. Thus, the plastid factor may be viewed as a quality control signal by which the organelle and hence the plant prevents the unproductive synthesis of extremely abundant proteins in view of their limited usefulness in a defective background. Although a clear molecular picture of chloroplast biogenesis is emerging, many more pieces of the puzzle need to be discovered and put in place. The factors and mechanisms of light-regulated gene expression are rapidly being unraveled. The plastid factor involved in chloroplast-to-nucleus signaling may soon be identified. The core components of the chloroplast protein import machinery have probably been identified, but the exact functions of most of them remain to be determined. Furthermore, mechanisms of precursor targeting to the chloroplast surface and the molecular basis of the energy requirements of chloroplast protein import are still poorly understood. Research on intrachloroplastic sorting has benefited tremendously from the similarities to bacterial protein export systems. In turn, homologues of the components of the ΔpH pathway, first identified in plants, have been found to also operate in the bacterial Tat-pathway. The integral membrane components of thylakoid transport systems have not been characterized to the same extent as the stromal factors involved in the process and will require attention. Of particular interest are the membrane-bound components of the chloroplast SRP-pathway which bring the journey of LHCP to conclusion.

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