EMBO Member’s Review

The making of a chloroplast

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Since its endosymbiotic beginning, the chloroplast has become fully integrated into the biology of the host eukaryotic cell. The exchange of genetic information from the chloroplast to the nucleus has resulted in considerable co-ordination in the activities of these two organelles during all stages of plant development. Here, we give an overview of the mechanisms of light perception and the subsequent regulation of nuclear gene expression in the model plant Arabidopsis thaliana, and we cover the main events that take place when proplastids differentiate into chloroplasts. We also consider recent findings regarding signalling networks between the chloroplast and the nucleus during seedling development, and how these signals are modulated by light. In addition, we discuss the mechanisms through which chloroplasts develop in different cell types, namely cotyledons and the dimorphic chloroplasts of the C₄ plant maize. Finally, we discuss recent data that suggest the specific regulation of the light-dependent phases of photosynthesis, providing a means to optimize photosynthesis to varying light regimes.

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Introduction

As a defining feature of plants, the chloroplast represents a marvel of evolution. Since its origin as a cyanobacterial symbiont about 1 to 1.5 billion years ago (Douzery et al., 2004; Yoon et al., 2004), this organelle has become fully integrated into the life cycle of photosynthetic eukaryotes and has essentially underpinned global ecosystems. Photosynthesis comprises two conceptually distinct phases that occur entirely within the chloroplast. The light-dependent reactions take place on the thylakoid membrane, in which light energy drives electron transport between a series of multi-subunit protein complexes. In two of these complexes, photosystem I (PSI) and photosystem II (PSII), protein-bound chlorophyll pigments are excited by light and initiate electron flow, so generating ATP and reducing equivalents. This chemical energy is then used in the light-independent reactions that take place in the chloroplast stroma, in which CO₂ is fixed by Rubisco to generate sugars. Subsequently, this carbohydrate is either immediately exported to the cytosol or is stored within the chloroplast as starch. Beyond photosynthesis, the chloroplast is also the site of fatty acid biosynthesis, nitrate assimilation and amino-acid biosynthesis. Given the importance of plant products to human beings, photosynthetic development and the biogenesis of chloroplasts have received intense scrutiny. In seed plants, chloroplasts develop from a non-photosynthetic form called the proplastid, which is transmitted between generations through the ovule and is maintained in meristematic stem cells. How does a chloroplast develop from a proplastid? How is photosynthetic competence reached and sustained? These are certainly complex and open questions, but two central themes emerge. First, the co-ordination and integration of multiple parallel processes, none of which operates in isolation, are absolutely necessary. This theme is most clearly shown by the fact that mutations in single chloroplast components can have major ramifications beyond the immediate process in question. Second, constant interorganellar crosstalk occurs both during the initial construction of the chloroplast and to maintain form and function in mature tissues. Coupled with the need to respond to a constantly variable environment, this crosstalk reflects the existence of two genomes and the need to regulate dynamically the relative input from each towards constituent parts of the chloroplast. This review covers some of the major cellular and developmental aspects of chloroplast biogenesis that encompass the above themes.

Light signalling during photomorphogenesis

In seed plants, light is a prerequisite for the synthesis of chlorophyll, and chloroplasts do not develop in the dark. Photomorphogenesis describes the developmental programme undertaken by seedlings exposed to light, and is typified by the inhibition of hypocotyl growth, the development of chloroplasts and the opening of cotyledons (in eudicotyledonous species). Light is perceived by a suite
of wavelength-specific photoreceptor proteins that undergo conformational changes to allow interaction with downstream signalling partners. The phytochromes, which perceive red and far-red light, and the cryptochromes, which respond to blue and UVA light, are the two varieties of photoreceptor responsible for photomorphogenesis (Jiao et al., 2007). In Arabidopsis, there are five phytochromes (encoded by PHYA to PHYE), of which primarily phyA and phyB act during seedling photomorphogenesis (Quail, 2002; Tepperman et al., 2006). Phytochromes exist in the cytosol in an inactive Pr form that is activated by light and is converted into the biologically active Pfr form, which translocates into the nucleus to initiate signalling (Quail, 2002). The phytochromes are represented by three proteins: cry1, which translocates from the nucleus to the cytosol on light activation; cry2, which is constitutively nuclear localized and cry3, which seems to be dual targeted to mitochondria and plastids (Kleine et al., 2003; Lin and Shalitin, 2003). A great deal of effort has been invested in clarifying the signalling and transcriptional networks that follow the perception of light, and the field has recently been reviewed thoroughly elsewhere (Jiao et al., 2007). Here, we offer a brief overview of the light signalling pathways that lead to the biogenesis of chloroplasts to provide a basis for introducing recent findings regarding signalling mechanisms.

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expression of genes involved in ribosome production, protein translation and cell proliferation before visible leaf emergence (Lopez-Juez et al., 2008). Genes involved in chloroplast biogenesis—primarily photosynthesis genes—are expressed subsequently, 6 to 24 h after light exposure (Lopez-Juez et al., 2008). Within the leaf primordium, phytochromes and cryptochromes bring about a myriad of changes that initiate chloroplast biogenesis, and a series of subsequent molecular events must occur in parallel to complete the process successfully. Obvious activities include the import of nuclear-encoded proteins, the ramping up of chlorophyll levels and the establishment of a thylakoid network complete with photosynthetic electron transport (PET) complexes. Table 1 and Figure 2 summarize the main functional processes that occur in making a chloroplast, along with examples of chloroplast components that perform those processes. Below, we discuss some aspects of this process in more detail.

**Protein import**

The biogenesis of chloroplasts requires substantial protein import from the cytosol. Most chloroplast proteins are imported through the Toc/Tic complex, which both recognizes and transports nascent proteins across both envelope membranes (for a review, see Soll and Schleiff, 2004). Major components of the Toc/Tic complex are upregulated by light and even provide substrate specificity. For example, the Arabidopsis Toc33 knockout mutant, ppi1, is defective in the import and accumulation of photosynthetic proteins, but not of most non-photosynthetic proteins, and AtToc33 is most strongly expressed in young, light-grown seedlings (Kubis et al., 2003). Toc159, a GTP-dependent molecular motor that drives translocation, is also required for precursor protein recognition. The Toc159 subunits are encoded by four genes in Arabidopsis: AtToc159, AtToc132, AtToc120 and AtToc30. The atToc159 mutant is albino and does not survive past the cotyledon stage, implying that the other Toc159 family members cannot compensate for this defect (Bauer et al., 2000). Furthermore, overexpression of AtToc159 is unable to complement the pale green atToc132 atToc120 phenotype (Kubis et al., 2004). Together, these findings imply that each Toc159 isoform exhibits substrate selectivity. Expression of such different isoforms may provide an
Table I Examples of nuclear-encoded, chloroplast-localized components necessary for chloroplast biogenesis, grouped by functional class

| Protein import and suborganellar targeting | Molecular function | Mutant phenotype | Remarks | Reference |
|-------------------------------------------|--------------------|-----------------|---------|-----------|
| AtTOC33                                   | Protein translocation across outer envelope | Pale green, especially juvenile plants (ppi1) | Involved in import of photosynthetic proteins | Kubis et al (2003) |
| cpSRP43                                   | Subunit of stromal signal recognition particle | Pale green with reduced levels of thylakoid protein complexes (chaos) | Mediates insertion of proteins into thylakoid membrane | Klimyk et al (1999), Amin et al (1999) |

| RNA processing                           |                     |                 |         |           |
|------------------------------------------|---------------------|-----------------|---------|-----------|
| PPR4                                     | Splicing of plastid rps12 transcript | Embryo lethal (ppr4) | PPR family member required for plastid ribosome biogenesis | Schmitz-Linneweber et al (2006) |
| CRR2                                     | PPR-like protein; regulates RNA splicing between rps7 and ndhB transcripts | Impaired accumulation of NDH complex (crr2-1 and crr2-2) | NDH complex is involved in cyclic electron flow around PSI | Hashimoto et al (2003) |
| SVR1                                     | Pseudouridine synthase, RNA editing | Yellow-green; reduced stature (svr1-2) | svr1 is also a suppressor of var2 | Yu et al (2008) |

| Protein maturation and degradation        |                     |                 |         |           |
|------------------------------------------|---------------------|-----------------|---------|-----------|
| BSD2                                     | DnaJ-like protein chaperone | Pale green due to abnormal BS cell chloroplasts (Zea mays) | Required for post-transcriptional regulation of Rubisco large subunit (LSU) | Brutnell et al (1999) |
| FtsH2 (VAR2)                             | ATP-dependent metalloprotease | Variegated yellow-green leaves; cotyledons normal (var2) | Likely function in D1 protein turnover in photodamaged PSII | Chen et al (2000), Lindahl et al (2000) |
| ClpP6                                    | Stromal ATP-dependent Clp protease | RNAI lines exhibit chlorosis of younger leaves | Degrades a variety of stromal proteins | Sjogren et al (2006) |

| Plastid gene expression                   |                     |                 |         |           |
|------------------------------------------|---------------------|-----------------|---------|-----------|
| SIG6                                     | Sigma factor conferring promoter specificity to RNA polymerase | Delayed greening in cotyledons (sig6-1) | One of many sigma factors required for plastid gene transcription | Ishizaki et al (2005) |
| FUG1                                     | Plastid translation initiation factor | fug1-2 is embryo lethal | fug1 alleles suppress var2 | Miura et al (2007) |

| Thylakoid biogenesis and lipid biosynthesis |                     |                 |         |           |
|--------------------------------------------|---------------------|-----------------|---------|-----------|
| ArfTerC                                    | Unknown; required for early thylakoid biogenesis | Seedling lethal on light exposure | Similar to bacterial tellurite resistance proteins | Kwon and Cho (2008) |
| FZL                                        | Dynamin-like GTPase; membrane fusion | Pale green; disorganized granal thylakoids | May be involved in thylakoid remodelling | Gao et al (2006) |
| MGDG synthase                             | Catalyses final step in MGDG biosynthesis | Sucrose required for germination; albino; frequent inner envelope invaginations | Mutant phenotype supports budding hypothesis for thylakoid biogenesis | Kobayashi et al (2007) |
| VIPP1                                      | Possible function in membrane budding from inner chloroplast envelope | Viable with exogenous sucrose | Protein located on inner envelope and thylakoid membrane | Kroll et al (2001), Aseeva et al (2007) |

| Chlorophyll biosynthesis                   |                     |                 |         |           |
|-------------------------------------------|---------------------|-----------------|---------|-----------|
| GUN4                                       | Enhances Mg-chieletase activity | Pale green (gun4-1, weak); yellow-white (gun4-2, null) | Essential under normal growth conditions | Larkin et al (2003) |
| CHLM                                       | Mg-protoporphyrin methyltransferase | chlm null mutants are albino and lack thylakoid protein complexes | Essential under normal growth conditions | Pontier et al (2007) |

| Metabolite transport                       |                     |                 |         |           |
|-------------------------------------------|---------------------|-----------------|---------|-----------|
| CUE1 (AIPPT1)                             | Imports phosphoenoipyruvate (PEP) into chloroplast stroma | Reticulate pale green leaves with dark green BS cells; perturbed M cell differentiation | PEP is required for fatty acid, amino acid and isoprenoid biosynthesis through the shikimate pathway | Li et al (1995), Streetfield et al (1999) |

| Photosystem assembly                      |                     |                 |         |           |
|-------------------------------------------|---------------------|-----------------|---------|-----------|
| LPA2                                      | Required for stability/assembly of PSII core | Pale green (ipa2); reduced PSII levels | Intrinsic thylakoid protein | Ma et al (2007) |

PPR, pentatricopeptide repeat protein; NDH, nicotinamide dinucleotide (phosphate) dehydrogenase; MGDG, monogalactosyldiacylglycerol, a non-phosphorous glycolipid of thylakoid membranes.

"Arabidopsis" unless otherwise specified.

The EMBO Journal VOL 28 | NO 19 | 2009

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First, when leaves are cooled to 12°C, vesicle-like structures contiguous with the inner envelope membrane accumulate in the chloroplast stroma (Morre et al., 1991). Second, direct connections between the inner envelope and thylakoid membranes have been reported, implying that the two compartments represent a partly contiguous, dynamic continuum (Shimoni et al., 2005). Third, the vip1 mutant is defective in thylakoid formation and does not form cold-induced vesicles (Kroll et al., 2001; Aseeva et al., 2007). Another mutant, thf1, exhibits a variegated phenotype, and affected chloroplasts contain profuse vesicles with no thylakoid membrane (Wang et al., 2004). VIPP1 is associated with both the thylakoids and inner envelope, whereas THF1 is found in the stroma and thylakoids; the presence of two suborganelar locations is consistent with a trafficking function for these proteins. Finally, chloroplast bioinformatics has revealed the presence of homologues of small GTPases with putative membrane fusion functions similar to those in the eukaryotic secretory pathway, such as ARF1 and Sar1 (Andersson and Sandelius, 2004). Recently, a dynamin-like GTPase called FZL has been identified that specifically affects thylakoid membrane structure in Arabidopsis. Again, FZL is localized to both the inner envelope and the thylakoid membranes (Gao et al., 2006). Although fzl mutant plants are not deficient in thylakoid formation per se, fzl chloroplasts are large and unusually shaped, they contain abnormal proportions of stromal and granal lamellae and they frequently accumulate small vesicles (Gao et al., 2006). These findings imply that FZL is a membrane-remodelling factor that is required for maintaining a dynamic thylakoid network, but the basis for abnormal chloroplast division is unclear.

### Chloroplast division

Once chloroplast biogenesis is underway, the chloroplasts must proliferate to match cell division and expansion: Arabidopsis mesophyll (M) cells can contain over 100 individual chloroplasts and the final count is tightly correlated with cell size (Pyke and Leech, 1994). The molecular nature of chloroplast division has been covered extensively in recent reviews (Maple and Moller, 2007; Yang et al., 2008), but one particular development is worth discussing here. As leaf development progresses, chloroplasts become progressively larger and dumb-bell-shaped plastids become less common, suggesting that division occurs early in chloroplast biogenesis (Pyke, 1999; Okazaki et al., 2009). Chloroplasts divide by binary fission, driven by two contractile protein rings that form on each side of the chloroplast envelope. The inner division ring forms first and is composed of the FtsZ1 and FtsZ2 proteins, which are homologous to bacterial fission proteins (Osteryoung and McAndrew, 2001). The constituents of the outer ring are not fully known, but the plastid division1 (PDV1) and PDV2 proteins in the outer envelope membrane recruit a cytosolic dynamin-like component, DRP5B, around the chloroplast exterior in alignment with the inner ring (Miyagishima et al., 2006). It has recently been shown that PDV1 and PDV2 are determinants of the rate and extent of chloroplast division, a question that has remained open for some time (Okazaki et al., 2009). pdv1 and pdv2 mutants had earlier been shown to contain large, deformed chloroplasts (Miyagishima et al., 2006), but when both PDV1 and PDV2 are overexpressed together, Arabidopsis M cells contain small chloroplasts that are twice as numerous as in wild type (Okazaki et al., 2009). PDV promoter activity is highest

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The EMBO Journal | VOL 28 | NO 19 | 2009 | 2865
around the meristem, in which proplastids are differentiating into chloroplasts. Crucially, the levels of PDV protein decrease in concert with the rates of chloroplast division as leaves aged, but FtsZ2 and DRPB5 levels remain at similar levels throughout development (Okazaki et al., 2009), tying in neatly with observed developmental patterns of chloroplast division and size. Constitutive expression of the cytokinin responsive transcription factor CRF2 and application of exogenous cytokinins both increase the activity of PDV2, linking cell division and chloroplast division and implying that the PDV proteins are primary mechanistic components in determining the cell’s chloroplast complement. This PDV-dependent mechanism seems to be evolutionarily conserved, holding true in the moss Physcomitrella patens, in common with other components of the PDV machinery such as FtsZ (Okazaki et al., 2009).

It is clear that molecular-genetic approaches have been incredibly powerful tools in establishing what events are critical for chloroplast biogenesis. A notable point is that mutations in genes required for any one particular molecular process, such as chloroplast RNA processing, severely hamper the establishment of photosynthetic competence in general, as many mutants are pale green, albino or even embryo lethal (Table I). As such, many chloroplast processes are in some way interdependent: for example, the light-harvesting complex of PSII (LHCII) is comprised of several Lhc-binding proteins, which are only imported into the chloroplast and properly folded in the presence of chlorophyll synthesized on the inner envelope membrane (Espineda et al., 1999; Reinbothe et al., 2006). Similarly, defects in lipid biosynthesis severely compromise chlorophyll levels and PET complex assembly because thylakoid membranes cannot be generated (Kroll et al., 2001; Kobayashi et al., 2007). Such tight mutual dependence requires exquisite co-ordination between the chloroplast, in which the events are happening, and the nucleus, in which many of the protein components are encoded.

**Retrograde chloroplast-to-nucleus signalling**

In many respects, chloroplast biogenesis is rather nucleo-centric: the early events in light signalling dominate in the nucleus, and in Arabidopsis, the nucleus encodes about 2100 chloroplast proteins, compared with just 117 originating from the chloroplast genome (Richly and Leister, 2004; Cui et al., 2006). This forward, or anterograde, communication to the chloroplast is balanced by retrograde signals passing in the opposite direction. The existence of such signals has been well documented by a number of experimental approaches over the past 30 years (reviewed by Nott et al., 2006). When seedlings are treated with chemical inhibitors of chloroplast biogenesis, transcript levels of nuclear genes encoding photosynthetic proteins are reduced, implying the existence of a plastid-derived retrograde signal that can repress nuclear gene expression when chloroplasts are damaged. A genetic screen for mutants defective in such repression led to the isolation of five non-allelic nuclear loci called genomes uncoupled (gun) (Susse et al., 1993). All five of these loci have since been identified, of which four (gun2 to gun5) encode plastid-localized proteins that function in tetrapyrrole biosynthesis, a pathway that culminates in heme and chlorophyll production (Nott et al., 2006). This observation led to a substantial body of evidence that implicated a chlorophyll intermediate—specifically Mg protoporphyrin IX (MgProtoIX)—as the identity of a negative signal emanating from defective plastids to repress gene expression in the nucleus (Mochizuki et al., 2001; Strand et al., 2003; Ankele et al., 2007). However, two recent landmark papers have shown that there is no correlation between the steady-state levels of MgProtoIX, or indeed any of the chlorophyll biosynthetic intermediates, and the degree to which nuclear photosynthetic gene expression is repressed (Mochizuki et al., 2008; Moulin et al., 2008). Instead, it is suggested that the destruction of chloroplasts may trigger the generation of short-lived reactive oxygen species (ROS) from limited amounts of tetrapyrrole intermediates, several of which are phototoxic (Mochizuki et al., 2008; Moulin et al., 2008). This is consistent with the sensitivity of nuclear gene transcripts to singlet oxygen that results from increased levels of Pchlide (op den Camp et al., 2003). However, a direct link between ROS and photosynthesis-related transcripts has yet to be shown.

**Plastid gene expression pathway**

Among all the original gun mutants, gun1 is unique because it is the only one to respond similarly to both norflurazon, which inhibits carotenoid biosynthesis and induces photo-oxidative damage, and lincomycin, an inhibitor of plastid protein synthesis (Gray et al., 2003; Nott et al., 2006). Double mutant analyses have shown that GUN1 and GUN2–GUN5 define two distinct, but partially redundant signalling pathways that regulate overlapping groups of nuclear genes (Mochizuki et al., 2001; Strand et al., 2003). GUN1 is, therefore, required for a second signal that is triggered by defects in plastid gene expression (PGE). GUN1 was recently identified as a plastid-localized pentatricopeptide repeat protein that is associated with nucleoids, which are transcriptionally active complexes of plastid DNA, RNA and ribosomes (Koussevitzky et al., 2007). An abscisic acid-insensitive mutant, abi4, also exhibits a gun phenotype, showing that ABI4 is a further component of the PGE pathway (Koussevitzky et al., 2007). ABI4 is a nuclear transcription factor that binds to a sequence adjacent to or overlapping the G-box motif. GUN1 is also required for transmitting the ‘MgProtoIX’ signal described above, and for glucose-mediated repression of photosynthetic gene expression. As such, GUN1 acts as an integrator of several signals within the plastid (Koussevitzky et al., 2007). A model has, therefore, been proposed in which GUN1 is a master switch that generates or transmits an unknown signal, which in turn induces ABI4 to bind to promoter sequences and block photosynthetic gene expression in the nucleus, perhaps by inhibiting access of transcription factors such as HY5 to the G-box (Koussevitzky et al., 2007; Larkin and Ruckle, 2008). Key questions that remain are the mechanism by which GUN1 integrates multiple signals, one of which does not seem to be a chlorophyll intermediate after all, and the nature of the secondary signal that is transmitted subsequently.

**Remodelling of retrograde signals by light**

It is clear that during photomorphogenesis, developing chloroplasts are subject to a combination of positive and negative signals resulting from light and plastid status. How might these conflicting signals be integrated into an appropriate
gene expression response? It has recently been shown that light and plastid signals can modulate one another. In a genetic screen to identify new components in plastid signaling, Ruckle et al (2007) recovered four mutants with a subtle gun phenotype, all of which turned out to be cry1 mutant alleles. This is surprising, because cry1 is usually considered to be a positive regulator of Lhcb expression. An even more surprising result was that cry1 gun1 double mutants showed much stronger derepression of Lhcb when grown on lincomycin than either single mutant. This suggests that cry1 and GUN1 act synergistically to effect most, if not all, of Lhcb repression under blue light when chloroplast biogenesis is blocked, and that a plastid signal can convert cry1 from a positive into a negative regulator of Lhcb. This observation is true in blue light and white light, but not in red light, suggesting that when chloroplast biogenesis is blocked, maximum repression of Lhcb expression requires photo-activated cry1. Consistent with the function of cry1 acting through COP1 to regulate HY5, cop1-4 is epistatic to cry1 (i.e. cry1 cop1-4 double mutants do not exhibit derepression of Lhcb expression when treated with lincomycin), and hy5 is a subtle gun mutant (Ruckle et al, 2007). Furthermore, a hy5 cry1 double mutant is indistinguishable from either single mutant in blue light, suggesting that both cry1 and HY5 operate in the same pathway. This implies that in healthy seedlings, cry1 acts through HY5 to promote Lhcb expression, but in the presence of dysfunctional plastids, a GUN1-independent signal converts HY5 into a negative regulator (Larkin and Pyke, 1998). This is in agreement with additional data suggesting that HY5 alone is insufficient to regulate transcription, and that HY5 can act as both a positive and a negative regulator (Lee et al, 2007).

Curiously, RbcS expression is not derepressed in lincomycin-treated cry1 or cry1 gun1 mutants, suggesting that cry1 only induces RbcS expression and cannot repress it (Ruckle et al, 2007). Furthermore, under the conditions used by Ruckle et al (2007), HY5 does not induce RbcS in blue light because hy5 mutants accumulate similar levels of RbcS transcripts as wild type. This implies the existence of another cry1/COP1-regulated transcription factor that does promote RbcS transcription. However, HY5 can still repress RbcS when chloroplast biogenesis is blocked as it does for Lhcb, provided the Gun1-mediated plastid signal is inactivated (i.e. in a gun1 mutant background) (Ruckle et al, 2007). This observation fits neatly with the model in which the Gun1 signal induces ABI4 to bind immediately upstream of the G-box of photosynthesis-related genes, thus preventing access by HY5 (Koussevitzky et al, 2007). There is also good evidence that phyB contributes to the repression of Lhcb, but not RbcS, when chloroplast development is blocked (Ruckle et al, 2007). Overall, these data imply that genes such as Lhcb and RbcS are regulated by complex and distinct mechanisms, incorporating a Gun1-independent plastid signal that can convert positive regulators into negative ones when plastids are damaged. Importantly, it is clear that this signalling network is crucial for efficient chloroplast biogenesis. gun1, cry1 and hy5 mutants are all more susceptible than wild type to photo-oxidative damage induced by high intensity light, with gun1 cry1 and gun1 hy5 double mutants being particularly badly affected (Ruckle et al, 2007). Finally, plastid signals dependent on cry1 and Gun1 influence several other aspects of seedling development, including anthocyanin biosynthesis, cotyledon expansion and inhibition of hypocotyl elongation (Ruckle and Larkin, 2009).

Cell-specific chloroplast development

During evolution, cell specialization within the photosynthetic organs of angiosperms has resulted in distinct chloroplast subtypes with varying functions. In Arabidopsis and tobacco, the plastids of epidermal pavement cells contain chlorophyll, but are small and underdeveloped in comparison to those in M cells, reflecting the function of the leaf epidermis as a protective, transparent cell layer (Dupree et al, 1991; Pyke and Page, 1998). However, the chloroplasts of stomatal guard cells are fully developed, suggesting that photosynthetic activity is necessary for efficient stomatal function (Lawson, 2009). Such cell-specific plastid development implies distinct developmental programmes that may result from a combination of positive and inhibitory cell-autonomous factors. Besides the function of the COP/DET/FUS family of photomorphogenic regulators inhibiting chloroplast development in roots, little is known about cell-specific chloroplast biogenesis. Here, we discuss some progress on this front using two examples.

Chloroplast biogenesis in cotyledons

In epigeous seedlings, germination takes place beneath the soil surface, and hypocotyl elongation pushes the cotyledons into the light. The cotyledons initially act as storage organs to support seedling growth, and only later become photosynthetic. Although true leaves are generated post-embryonically from the shoot apex, development of the cotyledons largely takes place during embryogenesis when tissue types and growth axes are specified (Aida et al, 1999; Stoyanova-Bakalova et al, 2004). Likewise, the chloroplasts in leaves develop from meristematic proplastids as the leaf primordia emerge, but chloroplasts in cotyledons develop from etioplasts that are already present in M tissue within the embryo. These etioplasts are primed for rapid conversion to chloroplasts on light exposure. The prolamellar body, a crystalline agglomeration of Pchlide, Pchlide oxidoreductase and fragments of prothylakoid membranes, provides the structural framework for the incipient photosynthetic apparatus (Sundqvist and Dahlin, 1997). The etioplast state does not normally occur in leaves because the shoot apex and leaf primordia are routinely exposed to light. Consistent with their different cytological origins, several lines of genetic evidence suggest that chloroplasts in cotyledons develop through distinct mechanisms from those in leaves.

Genetic screens have revealed mutants in which the greening of cotyledons and leaves are differentially affected. The snowy cotyledon (sco) mutants exhibit pale green or white cotyledons, but normal, green leaves. In sco1-1 mutants, germination is delayed and the cotyledons are initially white, but occasionally the cotyledons eventually are green and the seedlings survive if provided with exogenous sucrose (Albrecht et al, 2006; Ruppel and Hangarter, 2007). Immature Arabidopsis embryos contain photosynthetically active chloroplasts (Ruuska et al, 2004), but during seed dehydration and maturation, the chlorophyll and thylakoid membranes are lost, resulting in white embryos. Immature green sco1-1 embryos dissected from siliques germinate normally and do not exhibit white cotyledons, suggesting that sco1-1 mutants
are defective in the re-greening process (Ruppel and Hangarter, 2007). However, it is unknown if etioplast development is normal in sco1-1 mutants, so the defect could occur before or during seed maturation. Indeed, the sco1-2 and sco1-3 null alleles confer embryo lethality, suggesting that SCO1 is essential for early embryogenesis as well as de-etiolation (Ruppel and Hangarter, 2007). SCO1 encodes a chloroplast-localized elongation factor G (EF-G) and is thought to bind to the ribosomal complex to support plastid translation in a manner similar to that in Escherichia coli (Mohr et al., 2002). The Arabidopsis genome encodes just two other EF-Gs, which appear to be weakly expressed compared with SCO1 and may be dual targeted to the plastid and mitochondrion (Ruppel and Hangarter, 2007), and it is possible that these EF-Gs support plastid translation in other tissue types. The sco1 embryo-lethal phenotype emphasizes the importance of plastid translation on all stages of plant development (Ahlert et al., 2003).

Another mutant specifically defective in cotyledon chloroplast biogenesis is cyo1. This mutant has recently been characterized in detail and is allelic to sco2 (Shimada et al., 2007; Albrecht et al., 2008). Unlike sco1, null cyo1 mutants complete embryogenesis and germinate normally, and etioplasts resemble those of wild type (Shimada et al., 2007). However, similar to sco1, precocious germination of cyo1 mutants rescues the pale green cotyledon phenotype (Albrecht et al., 2008). Thus, the defect is specific to the generation of chloroplasts from etioplasts in cotyledons. The CYO1 protein includes a predicted zinc-finger domain similar to E. coli DnaJ, possesses disulphide isomerase activity, and is localized to the thylakoid membrane (Shimada et al., 2007). CYO1 may be required for the folding of cysteine-rich thylakoid-resident proteins, such as those comprising the photosystems, during de-etiolation when rapid arrangement of the photosynthetic apparatus is critical. Presumably, this function is either unnecessary in leaf chloroplasts or is performed by another unidentified chaperone. It is notable that DnaJ-like proteins have been shown to have several functions in plastid biogenesis: in maize, BSD2 is required for the assembly of the Rubisco holoenzyme in bundle sheath (BS) cells (Brutnell et al., 1999), and in cauliflower, the gain-of-function or mutation triggers the accumulation of carotenoids in the plastids of otherwise colourless tissues (Lu et al., 2006).

The plastid genome is transcribed by both a nuclear-encoded RNA polymerase and a plastid-encoded, eubacterial-like RNA polymerase (PEP). Promoter specificity of PEP is mediated by nuclear-encoded sigma factors, of which there are six in Arabidopsis (Allison, 2000). Of these, SIG2 and SIG6 are thought to have a specific function in the de-etiolation of cotyledon chloroplasts. Although the cotyledons of SIG2 antisense plants are chlorophyll deficient, the leaves are dark green (Privat et al., 2003); similarly, sig6-1 null mutants exhibit delayed greening of cotyledons, but otherwise normal leaves (Ishizaki et al., 2005). In vitro, SIG2 strongly binds to rbcL and PbsA promoters, whereas SIG1 does not, even though both sigma factors are expressed in young seedlings (Privat et al., 2003). This observation suggests that differential promoter recognition may be partly responsible for different paths of plastid biogenesis. The degree of redundancy in the SIG family has yet to be determined; complementation tests, promoter swaps and analysis of mutant combinations will help elucidate tissue specificities and functional divergence.

**Chloroplast dimorphism in C₄ photosynthesis**

In C₄ plants such as maize, photosynthesis is spatially divided between two cell types in the leaf: the M and the BS. Carbon is initially fixed in M cells before being shuttled to the BS cells in which the Calvin cycle operates. The chloroplasts of M and BS cells of maize are morphologically and biochemically distinct: M cell chloroplasts contain grana, accumulate PSI and lack starch, whereas BS cell chloroplasts lack grana, accumulate Rubisco and contain multiple starch granules (Nelson and Langdale, 1992). A proteomic analysis of the stromal proteins of each chloroplast subtype has revealed in detail the degree to which each chloroplast type is specialized (Majeran et al., 2005). One of the more interesting findings of this study was that homologues within gene families are differentially expressed in each chloroplast type, consistent with models predicting functional divergence after selection at gene regulatory regions (Sage, 2004). Nevertheless, relatively few maize mutants have been characterized with defects specific to M and BS cells. One is BS defective2, which lacks the BSD2 DnaJ-like protein discussed above and, therefore, exhibits perturbed BS cell chloroplasts (Brutnell et al., 1999). Another mutation, golden2 (g2), also leads to aberrant BS chloroplasts, but in this case the mutated gene encodes an Myb family transcription factor that now defines a family of golden2-like (GLK) genes present in diverse groups of land plants (Rossini et al., 2001; Yasumura et al., 2005). Each species contains at least two GLK genes, and in Arabidopsis and moss, each gene acts largely redundantly to promote nuclear photosynthetic gene expression in all photosynthetic cell types (Fitter et al., 2002; Yasumura et al., 2005; Waters et al., 2009). This redundancy also seems to be true in rice, as a pale green phenotype only results once the activity of both GLK genes is knocked down (P Wang and JAL, 2009, unpublished data). In maize, however, g2 specifically disrupts photosynthetic development in BS cells, leaving M cell chloroplasts unaffected (Langdale and Kidner, 1994). Accordingly, G2 is expressed in BS cells, whereas its homologue ZmGLK1 is expressed most strongly in M cells (Rossini et al., 2001). This has led to the intriguing speculation that, as transcriptional activators, G2 and ZmGLK1 each has a central function in the series of events that leads to BS and M cell differentiation. However, attempts to recover a zmglk1 mutant to test this hypothesis have been unfruitful, which may indicate an essential function for ZmGLK1 in early photosynthetic development of the maize seedling.

A recently characterized maize mutant that is specifically deficient in M cell function has provided some insight into how differential gene expression across the two cell types might be achieved. ZmHcf136 is a seedling lethal mutation, which leads to loss of PSII activity and the absence of grana in M cell chloroplasts, whereas those in BS cells are unaffected (Covshoff et al., 2008). This phenotype is consistent with the earlier assigned function of HCF136 in Arabidopsis as a PSII stability or assembly factor (Plücke et al., 2002). Accordingly, ZmHCF136 transcripts accumulate only in M cells. Analysis of BS and M transcriptomes revealed that each cell type responded independently to the ZmHcf136 mutation: generally, genes that are normally differentially expressed in the BS became less so, and M-enriched transcripts became...
more abundant than in wild type (Covshoff et al., 2008). Such transcripts include those encoding the C₄ enzymes carbonic anhydrase (relatively more M-enriched in Zmhef136 compared with wild type) and phosphoenolpyruvate carboxykinase (relatively less BS-enriched). In the light of the extensive feedback signalling between the plastid and nucleus as described above, Covshoff et al. (2008) suggest that much of the differential gene expression associated with the C₄ state may result from modifications of the cell environment—such as plastid redox profiles and energy metabolite gradients—without invoking the need for extensive innovation at multiple gene regulatory regions. This might help explain how C₄ photosynthesis has evolved independently from the basal C₃ state at least 45 times in the angiosperms (Kellogg, 1999).

**Photosynthetic acclimation to the light environment**

Plants must balance the energy required for growth with that obtained through photosynthesis. However, the light levels experienced by different leaves and even different cells within a leaf vary substantially over time. To cope with these fluctuations, plants acclimate to their environment by dynamically adjusting the proportion of light energy used to drive photosynthesis (Walters, 2005). Under conditions in which light availability limits photosynthetic rate, *Arabidopsis*, like most plants, invests a greater proportion of resources into the light-capture stages of photosynthesis relative to carbon fixation (Walters and Horton, 1994) and grows broader, thinner leaves to maximize light interception (Anderson et al., 1995; Weston et al., 2000). In addition, low-light-grown plants decrease the relative ratio of the two different chlorophyll pigments (Chl a to Chl b) and possess larger grana (Weston et al., 2000). Plants accustomed to high light intensities exhibit the opposite characteristics. Although much research has focused on short-term photosynthetic acclimation brought about by state transitions (Allen, 2005; Bellafiore et al., 2005), relatively little is known about the regulation of the longer-term adaptations described above, all of which require substantial developmental changes. Long-term acclimation is likely brought about by redox signals from the chloroplast (Pfannschmidt et al., 2009). When harvested light energy consistently does not match metabolic requirements—such as the ATP and NADPH demands of the Calvin cycle—the overall redox state of the PET chain is altered. By an unknown pathway, these redox signals are

![Figure 3](image)

*Figure 3* A model for long-term photosynthetic regulation by GLK proteins. Under light-limiting conditions (left), the PET chain cannot supply sufficient ATP and reducing equivalents to the Calvin cycle, and, therefore, tends to be in an oxidized state. This prompts a chloroplast-derived signal to the nucleus (dashed arrow) that upregulates transcription of GLK genes. GLK proteins in turn bind to promoter sequences of genes that function in light harvesting, such as *Lhcb* and key chlorophyll biosynthetic genes. Transcript levels of these GLK target genes increase, leading to higher levels of the corresponding protein (*Lhcb* in this case), as depicted by the thicker arrow. Upregulation of chlorophyll biosynthesis and LHC assembly leads to higher specific chlorophyll levels, a lower Chl a/b ratio and more abundant grana (stacked discs of thylakoids), as observed in 3SS:GLK transgenic plants. Increased grana abundance is associated with LHC trimers forming highly organized photosystem supercomplexes (Allen and Forsberg, 2001; Kovacs et al., 2006). When light is plentiful or even at inhibitory levels (right), the rate of CO₂ fixation is insufficient to use all of the output of the light-harvesting reactions, resulting in an overly reduced PET. This triggers a negative signal (and/or absence of a positive signal) that leads to lower rates of GLK transcription. The accompanying decrease in *Lhcb* and chlorophyll-related gene transcripts eventually results in a fall in the light-harvesting components in the thylakoid membrane and lower chlorophyll levels. In turn, there are fewer, less stacked grana and a higher proportion of non-stacked, stromal lamellae, as observed in glk1 glk2 mutants. Together, these changes help to redress the imbalance between light absorption and CO₂ fixation. Note that glk1 glk2 mutants are always paler than WT plants, suggesting that some degree of GLK activity is required under all conditions.
transduced to the cell nucleus in which gene expression is modified accordingly through transcription factors (Bonardi et al., 2005). Recent work has shown that GLK transcription factors are prime candidates for modifying the capacity of the light-dependent stages of photosynthesis.

Arabidopsis gkl1 gkl2 double mutants are pale green and contain chloroplasts with non-stacked thylakoids and reduced levels of PET complexes (Fitter et al., 2002). Furthermore, they have an unusually high ratio of Chl a to Chl b: grown under identical conditions, the ratio in wild-type plants is ~3.5 and in mutants ~5.5 (Waters et al., 2009). This alteration is likely to result partly from reduced levels of LHC proteins, to which Chl b is exclusively bound (Green and Dunford, 1996). When GLK genes are overexpressed in a mutant background, the total chlorophyll content is greater than in comparable wild-type plants, and the Chl a/b ratio is reduced to wild-type levels or lower, suggesting that GLK proteins act to promote chlorophyll synthesis and LHC assembly (Waters et al., 2008, 2009). The GLK1 transcription factor acts directly on the promoters of genes encoding LHC proteins, especially those of LHCII, and key enzymes of the chlorophyll biosynthetic pathway (Waters et al., 2009). Accordingly, in GLK-overexpressing plants, transcript levels of these genes are significantly higher than in the wild type; crucially, however, genes encoding enzymes of the Calvin cycle are unaffected. Together, these findings imply that GLK proteins may be responsible for regulating the balance between the light-dependent stages of photosynthesis and carbon fixation. As GLK proteins regulate a large suite of genes involved in light-harvesting and thylakoid protein complexes, they represent a potent control point in the nucleus. Consistent with this notion, levels of GLK transcripts are sensitive to plastid-derived retrograde signals, at least one of which is GUN1 independent (Waters et al., 2009). In addition, GLK proteins act as cell autonomously, providing a means by which the specific photosynthetic requirements of each cell across the leaf can be regulated independently (Waters et al., 2008). Although it has yet to be established that whether redox-dependent retrograde signals affect GLK expression in mature plants, we propose a model in which GLK proteins act as key photosynthetic regulators as part of plant acclimation to variable environmental circumstances (Figure 3).

Concluding remarks

Clearly, chloroplast development is a complex and highly regulated process. The data reviewed here have placed a strong emphasis on the function of PGE—incorporating transcription, mRNA editing, translation and protein complex assembly—in mediating the critical early steps of chloroplast biogenesis. Nevertheless, given the swathe of information on the molecular biology of plastids obtained from recent studies, it is surprising that broader aspects of chloroplast biogenesis remain largely unaddressed. For example, considering that etiolation is an evolutionarily derived state, is chloroplast development the default pathway that is, therefore, continually repressed in non-photosynthetic tissues? What factors determine such cell-specific plastid development? Why do some cell types contain hundreds of plastids, and others very few? In light of the clarification of PGE proteins in regulating PGE, addressing this latter question will now be much easier, and it will be particularly interesting to see whether PGE overexpression leads to excess PGE in normally sparsely populated cells. Further mechanistic factors may be uncovered with suppressor/enhancer screens, screens for PGE-interacting protein partners and microarray mining to discover regulatory pathways. In addition, the basis for the developmental changes induced during long-term photosynthetic acclimation are still poorly understood. Even the developmental changes beyond the photosynthetic apparatus—such as palisade cell elongation—are likely to be driven by chloroplast-derived signals, given the influence plastids have on cell and organ development (Lopez-Juez and Pyke, 2005). It will be interesting to determine whether photosynthetic mutants and chloroplast signalling mutants exhibit defects in the different aspects of acclimation. Elucidation of the sources and nature of chloroplast redox signals will be paramount to moving forward our understanding of this aspect of plant biology.

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Conflict of interest

The authors declare that they have no conflict of interest.

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