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Plastid biogenesis, between light and shadows

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Abstract

Plastids are cellular organelles which originated when a photosynthetic prokaryote was engulfed by the eukaryotic ancestor of green and red algae and land plants. Plastids have diversified in plants from their original function as chloroplasts to fulfil a variety of other roles in metabolite biosynthesis and in storage, or purely to facilitate their own transmission, according to the cell type that harbours them. Therefore cellular development and plastid biogenesis pathways must be closely intertwined. Cell biological, biochemical, and genetic approaches have generated a large body of knowledge on a variety of plastid biogenesis processes. A brief overview of the components and functions of the plastid genetic machinery, the plastid division apparatus, and protein import to and targeting inside the organelle is presented here. However, key areas in which our knowledge is still surprisingly limited remain, and these are also discussed. Chloroplast-defective mutants suggest that a substantial number of important plastid biogenesis proteins are still unknown. Very little is known about how different plastid types differentiate, or about what mechanisms co-ordinate cell growth with plastid growth and division, in order to achieve what is, in photosynthetic cells, a largely constant cellular plastid complement. Further, it seems likely that major, separate plastid and chloroplast 'master switches' exist, as indicated by the co-ordinated gene expression of plastid or chloroplast-specific proteins. Recent insights into each of these developing areas are reviewed. Ultimately, this information should allow us to gain a systems-level understanding of the plastid-related elements of the networks of plant cellular development.

Key words: Chloroplast, light, photosynthesis, phytochrome, plastid.

Introduction

Chloroplasts, arguably, define plant life. The overall vegetative structure of a plant can be ultimately conceptualized as a set of organs (the leaves) occupied by cells filled with chloroplasts and exposed to light and the atmosphere, and accompanied by their 'ancillary' organs, the roots and stem. In other words, plants are, effectively, self-standing solar panels, with chloroplasts being the solar 'cells'. Chloroplasts, however, do not just carry out photosynthesis (photoreduction of carbon, nitrogen, and sulphur), but are central hubs in plant metabolism (Neuhaus and Emes, 2000). They manufacture fatty acids, aromatic and non-aromatic amino acids (essential for protein synthesis, but also for a vast array of plant secondary metabolites), purine and pyrimidine bases, isoprenoids (like carotenoids and sterols) and tetrapyrroles (like haem and chlorophyll). Most of these functions are essential for every cell type, and chloroplasts have integrated into cellular development pathways by differentiating into a variety of other, interconvertible, non-photosynthetic plastid types (Whatley, 1978; Waters and Pyke, 2004). In parallel with cellular differentiation, the range of plastid types even includes a slimmed-down, 'meristematic cell' equivalent, the undifferentiated proplastid.

Like mitochondria, plastids are double-membrane organelles derived from an engulfed endosymbiont, in their case a photosynthetic cyanobacterium. Its closest, well-characterized, known living relatives belong to the genus *Nostoc* (Dyall *et al.*, 2004; Martin *et al.*, 2002), whose genome encodes in excess of 5000 proteins (depending on strain). Plastids have retained a semi-autonomous character, a minimal genetic machinery, and genes for a small

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Abbreviations: NEP, nuclear-encoded RNA polymerase; PEP, plastid-encoded RNA polymerase; PS, photosystem; Tic, translocon of the inner membrane of chloroplast; Toc, translocon of the outer envelope of chloroplast.

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number of polypeptides, the expression of which needs to be directed by the nucleus at appropriate times. However, the majority of plastid proteins are encoded in the nucleus, translated in the cytosol, imported into the organelle and further targeted to one of its suborganellar compartments. Plastids also need to grow and multiply to keep pace with their 'host' cells, and increase their number by binary fission. Furthermore, plastids 'report' on their physiological status to the nucleus of the cell, to ensure co-ordination between the two genomes (Nott *et al*., 2006). This review will attempt to summarize the recent, and dramatic, progress in these areas. However, it intends to argue that some very important aspects of plastid biology remain very poorly understood, to summarize recent insights into some of the less-well-explored areas, and to suggest those areas in which increased effort of inquiry could be justified.

A recent comprehensive update on plastid biology is available (Møller, 2004). Excellent reviews on plastid genetics (Sugiura *et al*., 1998; Mache and Lerbs-Mache, 2001; Wakasugi *et al*., 2001), plastid protein import (Jarvis and Robinson, 2004; Bedard and Jarvis, 2005), division (Osteryoung and Nunnari, 2003), interorganellar communication (Nott *et al*., 2006), and the role of the ancestral organellar genome in the host (Timmis *et al*., 2004) have been published. A more extensive overview of plastid developmental processes, and of the evidence for roles of plastids in plant development, has been attempted elsewhere (López-Juez and Pyke, 2005).

Plastid biogenesis: the achievements

Endosymbiosis and the origin of plastid proteins

The evidence for an endosymbiotic origin of chloroplasts is overwhelming. Initial cell biological observations noted not only the morphological similarity of chloroplasts and free-living cyanobacteria but also the apparent relative autonomy of behaviour of chloroplasts within plant cells (Martin and Kowallik, 1999). This included physical intracellular movements, and even the production and subsequent retraction from the body of chloroplasts and other plastids, of transient projections, now called stroma tubules or stromules and the subject of renewed interest (Köhler and Hanson, 2000).

The endosymbiotic process has gained great new insights in recent years. The availability of entire nuclear and plastid genome sequences has made it possible to compare those of photosynthetic eukaryotes with cyanobacteria and non-photosynthetic eukaryotes (Martin *et al*., 2002; Richly and Leister, 2004; Timmis *et al*., 2004). Cyanobacterial genomes, of *Synechocystis* and of two species of *Nostoc*, are estimated to contain from 3000 to over 7000 genes. The chloroplast proteome has been estimated for *Arabidopsis* and rice, based on the presence of transit peptides in nuclear-encoded genes, predicted through a combination of computing algorithms (see below). The number of chloroplast proteins thus predicted, with an obviously large degree of uncertainty (Richly and Leister, 2004) ranges from 2100 (*Arabidopsis*) to 4800 (rice). Meanwhile chloroplast genomes encode around 130 genes in total, of which around 80 code for proteins (Martin *et al*., 2002; López-Juez and Pyke, 2005). It is obvious that (a) the majority of the chloroplast ancestral genome is now located in the nucleus of plant cells, and (b) the majority of plastid proteins are encoded in the nucleus. A major outcome of these studies, however, is the observation that an extensive amount of reshuffling has taken place (genes in these 'a' and 'b' sets overlap only by about 50%), with both pre-existing eukaryotic genes having been recruited into functions located in the plastids, and cyanobacterial genes having acquired novel plastid-unrelated functions in the nucleus of plant cells (Martin *et al*., 2002; Timmis *et al*., 2004). An example of the former is the carbon-reducing Calvin cycle, which is composed of a number of enzymes of both cyanobacterial and host origin. An example of the latter is probably the range of bacterial sensory histidine kinases now present in plants, which are absent in metazoans and very rare in fungi. A note of caution has been introduced by the analysis of the full genome of the unicellular red alga *Cyanidioschyzon merolae* (Matsuzaki *et al*., 2004). This tiny red alga (cell of 2 μm diameter, with a single chloroplast and a single mitochondrion) is predicted to have a minimal photosynthetic eukaryote genome, with fewer genes (5300) than *Nostoc*. *Cyanidioschyzon* does not, for example, contain a gene for phytochrome, the light sensor in plants derived from bacterial histidine kinases, and has no nuclear-encoded histidine-kinase response regulators. Whether this is a result of gene loss or reflects a smaller contribution of the chloroplast ancestor's genome than otherwise thought, remains an open question. Its chloroplast, however, appears to be derived from the same, single endosymbiotic event that gave rise to all extant chloroplasts.

Plastid genetics

Plastids possess a genome (plastome) of between 120 and 160 kbp that encodes between 120 and 135 genes (see the Organelle Genome Megasequencing Program, http://megasun.bch.umontreal.ca/ogmp, for a complete set of available genomes). Besides polypeptides, the genome encodes eubacterial-type ribosomal and transfer RNAs. Many plastome genes are organized in operons. Plastome-encoded proteins include subunits of the eubacterial-type RNA polymerase (rpo), further genetic machinery (splicing and ribosomal proteins), photosynthetic polypeptides, including several for the four main thylakoid complexes, NADH dehydrogenase genes, and a few polypeptides of other functions (Sugiura *et al*., 1998; De las Rivas *et al*., 2002). Plastids are highly polyploid, with plastomes probably arranged as concatenated, long, linear molecules rather than

small circles, and, in general, physically linked to the inner plastid envelope through Plastid Envelope DNA-binding (PEND) and other proteins (Sato *et al.*, 2003; Wycliffe *et al.*, 2005). Plastome copy number, at least in leaf cells, is very high (several thousand per nuclear genome), undergoes a phase of proliferation in young cells (Baumgartner *et al.*, 1989) and in spite of reports to the contrary, remains constant thereafter (Li *et al.*, 2006). One possible explanation for the highly polyploid nature of chloroplasts is the need to synthesize very large amounts of photosynthetic proteins. Another (not mutually-exclusive) reason is that it confers protection against mutations that, in asexually reproducing organisms (as these organelles are), accumulate without a possibility of repair through recombination during sexual reproduction. The occurrence of this protective gene conversion mechanism has recently been proven to occur in chloroplasts (Khakhlova and Bock, 2006).

Nuclear control of plastid gene expression

The expression of plastid-encoded genes is carried out by two RNA polymerases of different origin (Shiina *et al.*, 2005). One is the plastid-encoded polymerase (PEP), which has been retained from the ancestral endosymbiont, is composed of three subunits (encoded by *rpoA, B*, and *C* genes) and recognizes *E. coli*-like promoters (Suzuki *et al.*, 2004). The other is nuclear-encoded (NEP), phage-type, made of a single subunit, and probably derived from its homologue, the mitochondrial RNA polymerase (Hedtke *et al.*, 1997; Sato *et al.*, 2003). In fact, three phage-type polymerases are encoded in the nucleus of *Arabidopsis*, one targeted to mitochondria, one to chloroplasts, and one to both organelles (Hedtke *et al.*, 2000). A mutation in the dual-targeted NEP polymerase, *RpoT;2*, results in defects only in plastid gene expression, and delayed greening and leaf and root growth (Baba *et al.*, 2004). A mutation in the plastid-only NEP causes more severe defects in leaf development and gene expression (Hricová *et al.*, 2006), and the combination of both of these mutations results in very early seedling lethality (Hricová *et al.*, 2006). Although genes transcribed by both NEP and PEP polymerases exist, in general, a sequential action occurs: genes with NEP promoters are transcribed early in chloroplast development and are involved in housekeeping functions, primarily constituting the plastid genetic machinery, including the subunits of PEP. Thereafter PEP is involved in the expression of photosynthesis-related genes (Hajdukiewicz *et al.*, 1997). Interestingly, a developmental changeover seems to take place, with glutamyl-tRNA, a product of PEP transcription that is also a precursor for chlorophyll biosynthesis, actively binding and repressing NEP, and therefore ensuring that once a commitment to photosynthetic development has taken place, minimal resources are 'diverted' (Hanaoka *et al.*, 2005).

Although PEP is plastid encoded, the transcription of PEP-transcribed genes is also under nuclear control. This is a consequence of sigma-70 factors, which in eubacteria determine promoter specificity of the RNA polymerase, being encoded in the nucleus of plant cells (Isono *et al.*, 1997). In *Arabidopsis*, six sigma factors exist with partially discrete functions. For example, SIG6 is a sigma factor that acts early and generically in chloroplast development (Ishizaki *et al.*, 2005). SIG6 function is probably taken over by SIG3 and SIG1. SIG1 is a general sigma factor but is expressed later that SIG6 (Ishizaki *et al.*, 2005). SIG3 is constitutively expressed, but the protein associates with plastid internal membranes which are not well developed early on (Privat *et al.*, 2003). SIG2, meanwhile, is involved in transcription of tRNAs, including the precursor of chlorophyll, Glu-tRNA (Kanamaru *et al.*, 2001). One example of a regulatory circuit has recently been uncovered: a number of plastid-encoded photosynthetic genes, including *psbD*, are induced by intense blue and red light (Mochizuki *et al.*, 2004), probably as an adaptive response to the fact that the polypeptides they encoded are damaged by high light, and need to be turned over. The photoreceptors are the nucleo/cytoplasmic phytochrome A and cryptochromes, their primary action being the induction of *AtSIG5* gene expression. The SIG5 protein then activates the *psbD* blue light-responsive promoter (Nagashima *et al.*, 2004; Tsunoyama *et al.*, 2004).

The specific role of SIG5 beautifully illustrates how the nucleus holds control over plastid gene expression. As indicated earlier, this is one direction in a two-way traffic of control processes between the nucleus and the plastid. The physiological status of the plastids sets in motion signalling processes which, in turn, control the expression of nuclear-encoded, plastid-related genes (reviewed by Nott *et al.*, 2006).

Protein import and targeting

The endosymbiotic event, followed by transfer of genes to the nucleus, posed obvious challenges for the cell. One was the need to import proteins synthesized in the nucleo/cytoplasm into the organelle, i.e. across the double envelope. Second was the routing of these imported polypeptides, either to remain in the stroma, be targeted into the thylakoid membrane or the thylakoid lumen, or indeed end as components of either chloroplast envelope or its intermembrane space (a total of six possible destinations; Jarvis and Robinson, 2004). While the import stage, the uptake of proteins, is a novel process, the re-routing is equivalent to an ancient, pre-existing prokaryotic process, protein targeting or secretion, and indeed some pathways used by chloroplasts are shared with eubacteria.

The translocation of polypeptides across the envelopes is carried out by the Toc (translocon of the outer envelope of chloroplasts) and Tic (translocon of the inner envelope of chloroplasts) complexes (Fig. 1). The structure and evolution of these have been the subject of excellent reviews (Soll, 2002; Jarvis and Robinson, 2004; Bedard and

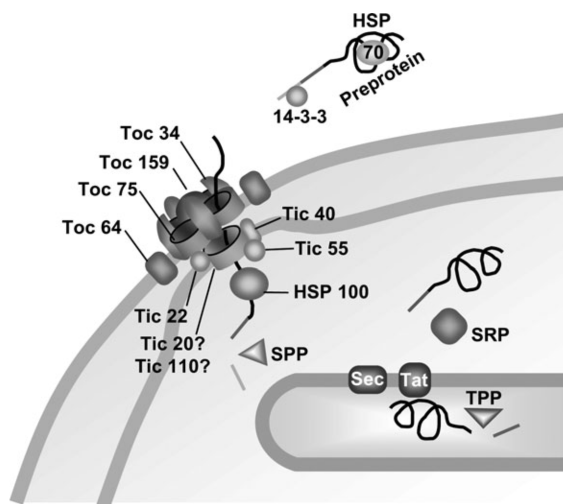

Fig. 1. Plastid protein import and thylakoid targeting mechanisms. Components of the translocon of the outer (Toc) and inner (Tic) membrane of chloroplast, and associated chaperones/heat shock proteins (HSP), 14-3-3 proteins, signal processing peptidase (SPP) and thylakoid processing peptidase (TPP) are shown. The signal recognition particle (SRP) is involved in the membrane integration of a few thylakoid proteins, while the secretory (Sec) or the twin-arginine or ΔpH (Tat) systems import proteins into the lumen. Adapted from López-Juez and Pyke (2005) and reprinted by kind permission of UBC press, Vancouver.

Jarvis, 2005). The complexes recognize cytosolic proteins that carry a plastid transit peptide, an N-terminal sequence of between 20 and 100 amino acids, with surprisingly little conservation, except for a high content of hydroxylated and small amino acids and a low content of large and acidic amino acids (i.e. having a positive charge overall). Toc is composed of five transmembrane proteins. One, Toc75, which is likely to form the pore complex, has a transmembrane β-barrel structure and is part of an ancient family of outer membrane proteins (OMPs) present in eubacteria and organelles (Moslavac *et al.*, 2005). Two other core polypeptides, Toc 159 and Toc34, jointly contribute to the recognition of plastid transit peptides. These proteins function through a cycle of GTP hydrolysis, are of eukaryotic origin, and their mode of action has been extensively explored, although uncertainties remain as to whether, for example, the receptor Toc 159, in soluble form, directs plastid protein precursors from the cytosol towards the pore, or awaits them, as a membrane protein at the pore. The Tic complex is less fully understood, with conflicting evidence as to whether Tic20 or Tic110 form the pore (Bedard and Jarvis, 2005). The driving force for translocation is probably provided by chaperones and cochaperones part of, or associated with, Tic (Hsp93 and Tic40; Chou *et al.*, 2003). After import, the N-terminal transit peptide is removed by a stromal processing peptidase.

Not all plastid proteins are imported through Toc/Tic complexes. Envelope proteins have unique properties. Outer envelope proteins often do not contain transit peptides (Hofmann and Theg, 2005), and some inner envelope proteins have been shown to be routed by novel, Toc-independent pathways (Nada and Soll, 2004). One outcome of the systematic identification of plastid proteins is the large number of experimentally-determined envelope-associated proteins that, instead of containing transit peptides, are computationally predicted to be targeted to the endomembrane, secretory pathway (Kleffmann *et al.*, 2004). This prediction reflects the fact that they contain a nearly-N-terminal transmembrane domain, that resembles a cleavable signal peptide, but that is in fact used to target directly to the chloroplast envelope, and is never cleaved (Lee *et al.*, 2001; see below).

Diversity of import pathways

The most important recent insight into plastid protein import pathways has probably been that they are, to some extent, substrate-specific (Kessler and Schnell, 2006). This prevents low-abundance but essential proteins from being out-competed by the much more abundant photosynthesis-related ones, and is possible thanks to the existence of small gene families for most of the translocon components. For example, for the four homologues of Toc159 encoded in the *Arabidopsis* genome, a deficiency in AtToc159 causes a loss of photosynthetic proteins, and a phenotype which is most clear in leaves, while a double defect in AtToc132 and AtToc120 leads to low import of housekeeping proteins and defects in non-photosynthetic tissues (Kubis *et al.*, 2004). Similarly, loss of AtToc33 has a clear photosynthetic impact and reduces import of photosynthesis-associated proteins, while loss of the highly homologous AtToc34 does not reduce import of photosynthetic proteins, but instead affects root growth (Constan *et al.*, 2004). Although the study of this specialization of import receptors has been carried out primarily in *Arabidopsis*, it is likely to be a general phenomenon, at least in angiosperms (Voigt *et al.*, 2005). Even the outer envelope pore itself, Toc75, exists in two versions with different specificities (Baldwin *et al.*, 2005).

Once in the stroma, routing of polypeptides destined to cross into the thylakoid lumen requires a second transit peptide, and uses two possible routes, both homologous to secretion/export pathways in bacteria. The Sec pathway translocates unfolded proteins using ATP hydrolysis (Schuenemann *et al.*, 1999). The alternative, Tat pathway translocates fully folded proteins and uses the photosynthetic pH gradient as the source of energy (Jarvis and Robinson, 2004). Finally, among thylakoid membrane proteins, a few use components of the bacterial ‘signal recognition particle’ pathway for integration, but the majority are inserted without the assistance of any other known proteins, and so are thought to possess intrinsic biophysical properties that allow ‘spontaneous’ membrane insertion (Jarvis and Robinson, 2004).

Plastid division

Plastids cannot arise by *de novo* biogenesis due to the presence of their independent genome. Instead new plastids arise through the division of a pre-existing organelle. As with bacterial division, plastid division is easily observed under the microscope (López-Juez and Pyke, 2005). Recent years have seen dramatic progress in our understanding of plastid division as a result of successful forward and reverse genetic approaches (Osteryoung and Nunnari, 2003; Margolin, 2005; Fig. 2). A classical genetic approach (the isolation of plastid division mutants in *Arabidopsis*, and identification of the corresponding genes) was pioneered by Pyke and Leech (1992) and has yielded *Arc3* (Shimada *et al*., 2004), *Arc5* (Miyagishima *et al*., 2003), *Arc6* (Vitha *et al*., 2003), and *Crumpled Leaf* (Asano *et al*., 2004). Meanwhile a reverse genetics approach (the identification of homologues in plants of bacterial division genes) was pioneered by Osteryoung and Vierling (1995) and has yielded *FtsZ1, FtsZ2* (Vitha *et al*., 2001), and *MinE* (Itoh *et al*., 2001). A combined approach, in which a mutation was isolated and the mutant gene was identified by homology to prokaryotic division proteins, has yielded, *arc11/MinD* (Fujiwara *et al*., 2004), and *GC1/AtSuIA* (Maple *et al*., 2004; Raynaud *et al*., 2004).

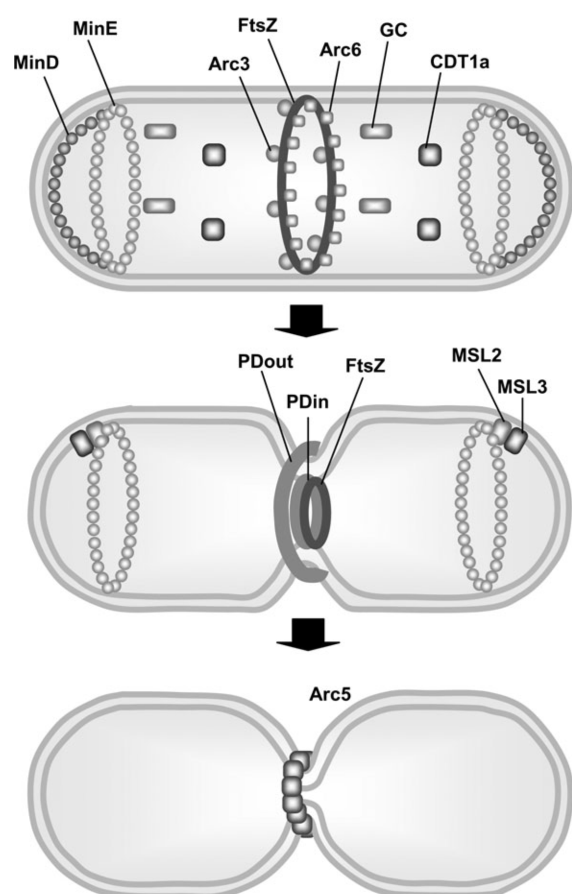

Fig. 2. Plastid division components. Components currently known to play a role in the plastid division process. MinD and MinE play a role in the location of the plastid division apparatus. FtsZ and the plastid division (PD) outer and inner rings physically carry out the constriction. Arc3 and Arc6 help assemble the FtsZ ring. GC plays a poorly-understood role. CDT1a may help co-ordinate plastid and nuclear division. MSL2 and MSL3 probably helps release ionic/hydrostatic pressure generated by the division. Arc5 carries out the final envelope separation. Adapted from hópez-Juez and Pyke (2005) and reprinted by kind permission of UBC press, Vancouver.

Two plastid division rings have been visualized under the microscope and shown to be made of constituents other than those mentioned in the paragraph above (Miyagishima *et al*., 2001). The overall division process involves the sequential assembly at the envelopes of FtsZ1/2 (the Z ring) and the plastid division rings, in the mid-organelle plane of a plastid. FtsZ1 and 2 are GTPases able to form polymers, and are related to tubulin. The central assembly location of these rings is determined by the MinD and MinE proteins, which act to prevent ring formation at the tips of the organelle. These proteins act together (Aldridge and Møller, 2005). The assembly of the rings is aided by Arc6 and Arc3 (Shimada *et al*., 2004; Maple *et al*., 2005). Constriction of the three rings begins, in the case of the Z ring, by removal of subunits. Once a substantial constriction has taken place, Arc5 assembles into a further ring. Arc5 is a dynamin-related protein. Dynamins are involved in vesicle formation and generally membrane 'pinching' in eukaryotes, including mitochondrial division. In keeping with this proposed function, the ring of Arc5, on the outer envelope of chloroplasts, forms at the stage of membrane constriction (Gao *et al*., 2003; Miyagishima *et al*., 2003; Fig. 2).

The membrane constrictions necessary during plastid division are likely to generate increased hydrostatic pressure inside the plastids. By the laws of dimensionality, two daughter plastids carrying the same amount of envelope membrane as their progenitor will always contain a lower combined volume. While this had not been given consideration before, Haswell and Meyerowitz (2006) have recently examined the function of two *Arabidopsis* proteins, MSL2 and MSL3, that are homologous to bacterial mechanosensing ion channels and have chloroplast transit peptides. Both proteins localize in patches on the chloroplast envelope associated with the division protein MinE. Simultaneous disruption of MSL2 and MSL3 results in heterogenous plastid populations, with some plastids becoming enlarged and spherical-shaped. The authors interpreted their observations as consistent with a role for MSL2 and MSL3 in the relief of turgor associated with division.

Are the remaining plastid components shared between daughter organelles simply by a process of random segregation brought about by physical separation? In the case of chloroplasts, specific processes dividing the thylakoid complex into two halves during organelle division seemed to be revealed by mutants in the *ARTEMIS* gene. In such mutants, chloroplasts showed separate and distinct thylakoid systems within incompletely separated organelles

(Fulgosi *et al*., 2002). The original *ARTEMIS* gene has recently been shown to be a mistaken merger of two separate open reading frames; one of them, *Alb4* (Gerdes *et al*., 2006), encodes a member of the Alb3/Oxa1/YidC family of proteins, involved in the insertion of other proteins in membranes of chloroplasts, mitochondria, and bacteria. The reason for the phenotype of the original *ARTEMIS* mutant is not fully understood. In terms of the segregation of the nucleoids, in which the plastid genome copies are contained, they arrange themselves before division into a reticulum associated with the envelope. This is assisted by the PEND protein, which probably allows their equitable partition (Terasawa and Sato, 2005).

Plastid biogenesis: the challenges

Do we know the majority of plastid proteins?

In spite of tremendous progress in recent years, and the availability of whole genome sequences, it is clear that we are nowhere near having a complete catalogue of the proteins that function in plastid biogenesis and, therefore, are far from a full list of plastid functions or developmental processes (Leister, 2003). There are two large areas of uncertainty: the list of plastid-localized proteins, and the catalogue of proteins necessary for plastid biogenesis, whether or not they are plastid localized.

Plastid-localized proteins have been catalogued on the basis of our understanding of plastid protein import. A number of algorithms predicting the presence of plastid transit peptides in user-supplied sequences have been developed, of which TargetP (Emanuelsson *et al*., 2000), which also predicts other subcellular destinations, appears to show the lowest rates of both false positive and false negative predictions. Use of TargetP on the full *Arabidopsis* genome led to a prediction of 3100 plastid-targeted proteins. Simultaneous use of a combination of algorithms (which decreases the number of false positive predictions, although it obviously increases the false negatives) predicts 2100 plastid-targeted proteins in *Arabidopsis* and, surprisingly, 4800 in rice, which has a similar total number of genes (Richly and Leister, 2004). Besides the uncertainty in bioinformatics predictions, a major caveat to the completeness of these lists has emerged from proteomics programmes, in which total proteins of diverse plastid types, or plastid fractions, are being detected by mass spectrometry methods (van Wijk, 2004, and references therein; Kleffmann *et al*., 2006). A major outcome is that a substantial number of proteins present in the outer chloroplast envelope, that often do not contain a plastid transit peptide, are predicted to be targeted to the secretory pathway. In the case of the homologous proteins OEP14 (pea) and OEP7 (*Arabidopsis*), it has been shown that the domain that TargetP wrongly identifies as the hydrophobic core of a signal peptide for the endomembrane system, in fact targets the protein to the outer chloroplast envelope and remains present, presumably as a transmembrane span, in the mature protein (Lee *et al*., 2001; Hofmann and Theg, 2005). The number of such proteins (predicted as targeted to the secretory pathway, but actually present in the chloroplast envelope) is larger than previously thought (Kleffmann *et al*., 2004). One further caveat to the use of bioinformatics to predict the final list of plastid proteins has been raised by the case of the *Arabidopsis* CAH1 carbonic anhydrase (Villarejo et al., 2005). This protein is correctly predicted to be targeted, through a cleavable signal peptide, to the secretory pathway, but only transiently while it is *N*-glycosylated on its way towards the chloroplast stroma, its final destination.

Do we know the majority of proteins/processes involved in plastid biogenesis?

The eventual catalogue of proteins required for plastid biogenesis, regardless of their location, shows an even greater uncertainty at present. A number of mutants detected on the basis of defects in chloroplast development have been analysed (López-Juez and Pyke, 2005). These include mutants defective in chloroplast and leaf mesophyll development, and variegated mutants (Sakamoto *et al*., 2003). A direct screen for defective *chloroplast biogenesis* (*clb*) mutants has been carried out by Gutiérrez-Nava *et al.* (2004), who searched among existing stock-centre collections for albino seedlings that do not result from photo-oxidative damage, and identified six such loci. The loci identified through these various mutant classes are involved in the assembly or function of the plastid genetic machinery (for example Pesaresi *et al*., 2006; Albrecht *et al*., 2006), including pentatricopeptide repeat proteins with RNA-binding roles (Lurin *et al*., 2004; Cushing *et al*., 2005; Gothandam *et al*., 2005), and the assembly or repair of protein complexes requiring proteolytic steps (Chen *et al*., 2005; Sakamoto, 2006). Novel roles for such classes of proteins probably remain to be uncovered, as shown by the role of a tetratricopeptide repeat protein in the regulation of transcription (Weber *et al*., 2006) or the possible signalling-related role of an intermembrane protease (Bolter *et al*., 2006). One class of mutations uncovered surprisingly frequently in screens for mutants with obvious chloroplast defects, affects genes involved in the methyl-erythritol 4-phosphate pathway of isoprenoid biosynthesis in plastids (Estévez *et al*., 2000; Rodríguez-Concepción and Boronat, 2002). Among the six *CLB* genes, the two thus far identified at the molecular level encode two enzymes in this pathway (Gutiérrez-Nava *et al*., 2004; Guevara-García *et al*., 2005). These mutations cause developing chloroplasts to arrest at the proplastid stage. The reason why defects in this pathway should be a frequent cause of albinism is unclear and, as mentioned, unrelated to loss of carotenoids and consequent photo-oxidative damage. Since both abscisic acid and gibberellins are isoprenoid

derivatives, one possibility is the existence of yet-undiscovered hormonal control of chloroplast development.

Will these classes of mutations encompass the majority of the processes required for plastid biogenesis? This is probably far from the case. Evidence for this is provided by a large-scale mutant screen carried out by Budziszewski *et al.* (2001). They identified, in a population of 38 000 insertional mutant lines, over 500 seedling-lethal mutants. Interestingly, ~85% of the mutants exhibited albino, yellow or pale cotyledons, indicative of defective chloroplasts. For 23 mutants, 18 of which showed an albino or pale phenotype, the mutated gene was identified. Seven of those 18 genes encoded proteins whose function was either unknown or of unsuspected plastid relevance (like pyrimidine permease or Zn-finger protein). A simple extrapolation, ignoring the possibility of multiple alleles at single loci, would estimate 165 genes of unsuspected plastid-related function to be detected by this mutant screen alone. This gives an indication of the scale to which our understanding of plastid biogenesis is incomplete. A much smaller screen, for defects in expression of the nuclear photosynthetic gene *Lhcb* (*cue* mutants; Li *et al.*, 1995; López-Juez *et al.*, 1998; Vinti *et al.*, 2005), has identified seven mutant loci causing defective plastids. While *CUE1* encodes a plastid envelope transporter (Streatfield *et al.*, 1999; Voll *et al.*, 2003), progress towards the cloning of *CUE8* has narrowed it to a region of chromosome 5 containing 20 genes, none of which was previously suspected to play a role in plastid biogenesis or function (D Maffei, JR Bowyer, E López-Juez, unpublished results).

The evidence above suggests that, as has been proposed (Leister, 2003), large-scale screens for plastid biogenesis-defective mutants are still justified. One systematic existing approach is the maize Photosynthetic Mutant Library (http://chloroplast.uoregon.edu). Such genetic approaches, however, would still only uncover genes of non-redundant function. Clearly we are still a long way from a comprehensive catalogue of plastid biogenesis genes.

How are plastids integrated into the plant developmental programmes?

In spite of many years of observations, our knowledge about the make-up and development of different types of plastids, other than chloroplasts, is still limited (Neuhaus and Emes, 2000; Waters and Pyke, 2004; Kleffmann *et al.*, 2006). Figure 3 shows plastids visualized using a RecA transit peptide-targeted red fluorescent protein (Haswell and Meyerowitz, 2006). An *Arabidopsis* seedling probably contains plastids with many different functions (photosynthetic, large chloroplasts in leaf and cotyledon mesophyll and in hypocotyl cortex, smaller plastids in epidermis and in roots), but also different cells of an *Arabidopsis* seedling are occupied by plastids to very different extents. For example, in cotyledons, epidermal cells have a very small plastid complement compared with mesophyll cells, and in roots the pericycle cells have a larger complement than cortical or epidermal cells (Fig. 3). Interestingly, a gene expression atlas of the *Arabidopsis* root has identified a substantial level of expression of photosynthesis-related genes in pericycle cells (Birnbaum *et al.*, 2003). Clearly both 'plastid type' and 'cellular plastid compartment size' are characteristic of the cell type, and its stage of differentiation.

Very little work is being carried out to understand the mechanisms and possible signalling pathways presumably underlying these plastid differentiation responses, or the relative contributions to them of the nucleus and the plastid. One recent insight has come from the analysis of plastid protein import mechanisms. As described earlier, different isoforms of components of the Toc complex have different specificities, and as a result it is likely that separate Toc complexes primarily import either photosynthetic or non-photosynthetic, housekeeping proteins (Kessler and Schnell, 2006). This has been confirmed directly through an analysis of knock-out mutants of individual isoforms. One unexpected observation was the correlative change in protein import and in expression of the corresponding gene. For example *AtToc33* or *AtToc159* mutants show defects both in the rate of import of photosynthetic proteins and in the expression of photosynthesis-related nuclear genes (Kubis *et al.*, 2003, 2004). By contrast *AtToc34* or *AtToc132/120* mutants display defects in the import of non-photosynthetic proteins and in the expression of their genes (Constan *et al.*, 2004; Kubis *et al.*, 2004). This brings about a positive feedback mechanism that could potentially play an important role in plastid differentiation. For example, a cell type expressing low levels of *Toc33* and high levels of *Toc34*, would import photosynthetic proteins with low efficiency and eventually, as a consequence, would express low levels of photosynthesis-related genes, which would in turn lead to the development of non-photosynthetic plastids.

The extent of the integration of plastids into the biology of the cell is manifest in the roles played by plastids in whole plant development and phenotype. Many lines of evidence support these roles, but the underlying reasons are very poorly understood. Inhibition of plastid translation in sectors of chimeric plants (Ahlert *et al.*, 2003) causes developmental arrest, including an inhibition of cell division, in the sectors affected. The same consequences follow the targeted knockout of essential plastome genes, *clpP1* and *AccD* (Kuroda and Maliga, 2003; Kode *et al.*, 2005). Global blockage of plastid translation can be achieved through mutations in non-redundant aminoacyl-tRNA synthetases, and this (when observed in segregating populations of heterozygous plants) leads to arrests at the middle, transition stage of embryogenesis (Berg *et al.*, 2005). The import of nuclear-encoded proteins, including the RNA polymerase, is essential even earlier for embryo survival, because a defect in the outer envelope main pore

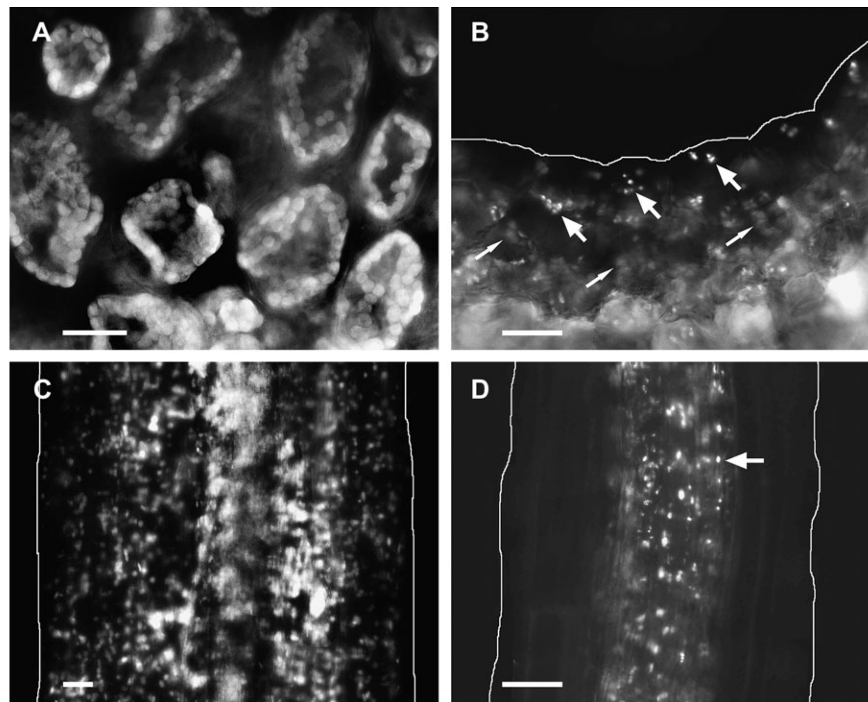

Fig. 3. Diversity of plastids in different cells. (A) Chloroplasts in spongy mesophyll cells of the first leaf. (B) Epidermal cell plastids (wide arrowheads), above chloroplasts in the mesophyll (narrow arrowheads) in a cotyledon side view. (C) Chloroplasts in hypocotyl. (D) Plastids in root (note that they are clearly visible primarily in the pericycle cells, arrowhead, surrounding the central vascular strand). All fluorescence micrographs obtained from an *Arabidopsis* line containing plastid-targeted dsRed fluorescent protein. The contour of the tissue has been highlighted in each case. Scale bars 25 μm.

component, AtToc75-III, causes arrest of embryogenesis at the two-cell stage (Baldwin *et al.*, 2005).

Besides being essential for cellular development, organellar genomes (plastids and mitochondria) can, in spite of their high degree of conservation and slow evolution, contribute substantially to plant phenotype later in development. This is shown by lines of maize bearing cytoplasms of distantly-related teosinte species (Allen, 2005). How such influence takes place, and earlier in development what the nature of the essential plastid function is (it is unlikely to be simply the loss of essential metabolites; Ahlert *et al.*, 2003; Gutiérrez-Nava *et al.*, 2004) remains unknown.

How do plastids respond to their cell's circumstances?

Two plastid types of major crop and biotechnological importance are starch-storing amyloplasts (Andon *et al.*, 2002; Balmer *et al.*, 2006) and carotenoid-storing chromoplasts (Bramley, 2002). In both cases their differentiation is under endogenous, hormonal control. For example, chromoplasts in tomato fruit differentiate from chloroplasts during fruit ripening, which is partially ethylene-dependent (Alba *et al.*, 2005). Ethylene must play a role in chromoplast differentiation, as judged from the phenotype of the Never Ripe ethylene receptor mutant (Wilkinson *et al.*, 1995). However, there is also evidence that plastid-derived signals are involved in the intriguing plastid conversion circuitry, perhaps in a feedback loop. For example, a chloroplast-localized heat shock protein is required for the transition from chloroplasts to chromoplasts, and elevating its expression accelerates the conversion (Neta-Sharir *et al.*, 2005). A role for the carotenoid accumulated in chromoplasts, lycopene, as a signal has been postulated (Barr *et al.*, 2004).

One further aspect of the integration of plastids within their host cells is the ability to respond to environmental signals that the cell responds to. Perhaps the clearest example of this is the biogenesis of chloroplasts in photosynthetic cells only in the light, and the continued fine-tuning of chloroplast composition to the prevailing conditions. Evidence described earlier demonstrated the way in which cytosolic photoreceptors can control the expression of plastid genes, by regulating the nuclear expression of a plastid-targeted sigma-70 factor. The rapid biogenesis of chloroplasts is a major response during seedling establishment, and this is reflected by the fact that the first dark-to-light transition of seedlings (the 'de-etiolation' process) brings about massive gene expression reprogramming (Ma *et al.*, 2001; Tepperman *et al.*, 2001), and around 50% of the genes eventually affected encode plastid-targeted proteins. Some evidence supports the notion that the subset of chloroplast development responses to light is, to an extent, separable from other light responses. For example, defects or mutations in plastid biogenesis affect the light response of photosynthesis-related genes specifically (López-Juez *et al.*,

1996, 1998; Vinti *et al*., 2005). However, a screen to identify components of the pathway of light control of chloroplast development has been performed based on reporting *Lhcb* expression in a light perception-deficient background (Hills, 2002; A Hills, T Shindo, Y Niwa, E López-Juez, unpublished results) and the two mutants that have been identified to date have defects in both photosynthesis-related genes and de-etiolation in general.

When/how are plastid growth and division controlled?

Given that a specific plastid subtype (the proplastid) has evolved to facilitate inheritance of plastids, it is reasonable to presume that instead of plastid inheritance being left to chance, mechanisms to co-ordinate plastid and cell division would also have evolved. This, indeed, appears the case, but the mechanisms remain very poorly understood. In *Cyanidioschyzon*, the 'minimal' red alga, the connection between cell and organelle division is tight and inescapable, since each cell has a single mitochondrion and a single plastid (Nishida *et al*., 2005), although inhibition of DNA synthesis, surprisingly, acts as a signal for multiple chloroplast divisions. In plants the situation is necessarily more complex, as cell growth and division are not obligately coupled, and because different cells carry different plastid complements (Fig. 3). It has long been known that in cereal leaves (Hashimoto and Possingham, 1989; Baumgartner *et al*., 1989) plastid proliferation and plastome replication take place rapidly in post-meristematic cells. In young dicot leaves, light that causes chloroplast differentiation but not full cell enlargement, activates plastid DNA replication, but not an increase in plastid numbers (DuBell and Mullet, 1995). This suggests separate links between differentiation and plastid growth, and between cell enlargement and plastid division.

Mesophyll cells observed under a microscope show a remarkably constant degree of occupancy by chloroplasts, even when cell size changes markedly, for example, as a response to the environment (Weston *et al*., 2000). A strict correlation appears to exist between the size of the cell and that of its plastid compartment (Pyke, 1997), even when comparing mesophyll cells of multiple species (Pyke, 1999) or examining cells vastly enlarged as a result of a prevention of mitosis (Jasinski *et al*., 2003). Obviously mechanisms for sensing plastid density, controlling both plastid growth and division, would explain this behaviour, but the nature and action of such mechanisms remain poorly understood. One suggestion put forward by Pyke (2006) is that MscS-like (MSL) proteins, the mechanosensing plastid envelope ion channels (Haswell and Meyerowitz, 2006), play a role in such density-sensing, at least in mesophyll cells, where chloroplasts occupy the cytoplasm in one layer until they can touch, even compress each other. Such mechanosensors could play a similar role in other cells with a low density of plastids, if they acted in stromules, the tubules that protrude from plastids more frequently in such cell types (Pyke, 2006). If this model were to hold true, it would need to link plastid/cell size ratios to plastid growth, because the constancy of the chloroplast compartment can be seen even when plastid division is prevented, for example, in the *arc6* mutant (Pyke, 1999). As to the processes underlying chloroplast growth, photosynthetic components obviously accumulate, but the increase in plastid genome copy number seems to precede this accumulation (Baumgartner *et al*., 1989). Importantly, and perhaps unsurprisingly, an increase in plastid ribosomes and the capacity for protein synthesis also precedes the build-up of the photosynthetic apparatus (Harrak *et al*., 1995). This may be of importance, since genes involved in plastid protein synthesis may play regulatory and rate-limiting roles in photosynthetic development (Pesaresi *et al*., 2006). It is interesting that genes for cytoplasmic ribosomal and translation-related proteins seem to play important, equivalent roles in cellular growth (Li *et al*., 2005). Perhaps a greater understanding of the regulation of the synthesis of the plastid translation machinery would help to uncover fundamental mechanisms of plastid growth control.

Links between plastid and cell division also exist, even though uncoupling of these is obviously possible (Jasinski *et al*., 2003). Multiplication of plastids can be triggered by cytokinin (but not auxin) in dark-grown cotyledons, and under those conditions a simultaneous increase in *FtsZ* expression can be observed (Ullanat and Jayabaskaran, 2002). *FtsZ* expression also mimics that of a cell cycle-associated gene in cell cultures (El-Shami *et al*., 2002). *AtCDT1a* and *AtCDT1b* are genes encoding related forms of a key component of the complex of proteins needed to initiate nuclear DNA replication (the pre-replication complex). Interestingly *AtCDT1a* contains sequence for a predicted chloroplast transit peptide, fusion proteins with a green fluorescent protein-reporter are targeted to both nuclei and plastids, and a double mutant for both *CDT1* genes shows, besides cell cycle-related defects, pale leaves whose mesophyll cells contain large, unevenly divided chloroplasts (Raynaud *et al*., 2005). Thus AtCDT1 proteins are strong candidate constituents of the link between plastid and cell division.

Can the size of the plastid compartment be altered?

Given the roles of plastids as subcellular factories, the ability to manipulate the size of their cellular complement could have important implications for the biology of the plant and for its biotechnological exploitation. Mesophyll cells appear to have reached close to a maximum in chloroplast occupancy, but in other cell types increases in the plastid complement are physically possible. It has been seen earlier that both chloroplast biogenesis and leaf development are tightly regulated by light signalling pathways. A number of proteins are known to play positive and negative regulatory roles in light signalling, as revealed

primarily by the phenotype of defective mutants. While genetic studies of light signalling (Schäfer and Bowler, 2002) have focused on seedling morphology in *Arabidopsis*, mutant tomato plants have been particularly useful in uncovering plastid-related phenotypes. In tomato, a *high pigment-2* mutant (*hp-2*), with fruit enriched in the chromoplast pigment lycopene, is defective in *LsDET1*, the orthologue of *Arabidopsis* light-signalling negative regulator *DET1* (Mustilli *et al*., 1999). Another mutant with a similar fruit-pigment phenotype, *hp-1*, shows an elevated plastid DNA copy number (Yen *et al*., 1997), and displays fruit pericarp cells with a small but significant increase in plastid cell index (the ratio between plastid and cell projected areas) of between 13% and 29% (Cookson *et al*., 2003). A greater increase in plastid cell index in *hp-1* occurs in leaf palisade mesophyll cells, whose morphology resembles that observed in high light-grown leaves (Weston *et al*., 2000). *hp-1* is defective in the tomato *UV-damaged DNA-binding protein 1, LsDDB1,* gene (Liu *et al*., 2004). DDB1a and DET1 physically interact and act together in *Arabidopsis* (Schroeder *et al*., 2002). It appears, therefore, that exaggerated light signalling causes increased fruit plastid pigment content (Liu *et al*., 2004) and increases in the cellular plastid complement which, although small, are significant as they demonstrate that such plastid complement is not developmentally fixed, since it can be influenced by environmental signals.

Are there chloroplast master controllers?

The radically different phenotype of plastids in photosynthetic compared with non-photosynthetic tissues could most easily be explained if mechanisms existed that simultaneously controlled a large number of genes encoding chloroplast proteins. Arguably, however, plastids play central roles, other than in photosynthesis, in the biology of specific cell types. A biologically economical way of building such plastids would be to have co-ordinated control of genes encoding plastid proteins, irrespective of whether they are directly related to photosynthesis. In other words, the existence of both 'chloroplast-specific' and 'plastid-generic' master switches could be anticipated. Evidence has emerged that is consistent with the existence of both kinds of centralized regulation of gene expression.

A common phenotype of the *Arabidopsis det1* and the tomato *hp-2* mutants is the partial development of chloroplasts in cells or under conditions where they would not normally appear. This includes semi-developed chloroplasts instead of etioplasts in dark-grown cotyledon cells (Mustilli *et al*., 1999) but also, crucially, instead of proplastids in root cells (Chory and Peto, 1990; Mustilli *et al*., 1999). One possible interpretation is that control elements for the transcription of genes involved in chloroplast biogenesis are common to both light and tissue-specific signals. Several photosynthesis-related gene promoters have been extensively analysed for the presence of light-regulated elements. Different versions of one such element, called G-box, are common and have been shown to, in combination with other elements, confer both light responsiveness and plastid-dependent expression (Puente *et al*., 1996). A short, single element (CMA5) in the tobacco *RbcS8B* promoter is sufficient to confer light-dependent expression to a reporter gene in *Arabidopsis* (Martínez-Hernández *et al*., 2002). Importantly, the reporter gene is expressed in leaf, but not in root tissue, and disappears from leaves when chloroplasts are photodamaged by treatment of seedlings with norflurazon. Again this suggests the existence of photosynthetic gene-regulatory mechanisms shared by light and tissue-specific signals, and by signals of plastid-to-nucleus communication. Similar commonality has been found in the regulatory signals of other photosynthesis-related genes, even in cases where they act at the post-transcriptional rather than transcriptional level (Helliwell *et al*., 1997; Sullivan and Gray, 2002).

Large-scale gene expression profiling experiments have also provided evidence in this direction. Richly *et al.* (2003) used a custom-made array of gene-sequence tags to examine the expression of 3292 genes, greatly enriched for those encoding chloroplast-targeted proteins. They monitored changes in gene expression caused by 35 different environmental or genetic conditions. They observed three different broad classes of regulation: in two (their classes one and three) the majority of genes were up- or down-regulated, respectively. Conditions that caused a class one response included high light, the *gun1* and *gun5* mutations that lead to expression of photosynthesis-related nuclear genes in the absence of functional plastids (Susek *et al*., 1993; Mochizuki *et al*., 2001), and the *ppi1* mutation (causing reduced plastid protein import; Kubis *et al*., 2003). Conditions that caused a class three response included the *cue1* mutation, which causes defective plastid biogenesis, and loss of *Lhcb1* expression (Streatfield *et al*., 1999). Richly and collaborators interpreted their data as evidence for the existence of a 'master switch'. This acted in a binary mode (on in class one/off in class three), and controlled genes for plastid-targeted proteins (regardless of their relation to photosynthesis, as proved by the 'on' condition in the *ppi1* mutant). An extension of this analysis to a total of 101 conditions (Biehl *et al*., 2005) provided further insights: a total of 23 distinct clusters of co-expressed genes, known as regulons, could be identified. Two of those clusters showed the tightest co-regulation, and contained primarily genes for either the photosynthetic light reactions or for plastid protein synthesis (the largest number being chloroplast ribosomal proteins). These two clusters escaped the previous 'plastid master switch' model, but they themselves, in turn, on the basis of their tight co-regulation, uncovered a second master switch, this one specific for 'chloroplast' function.

Evidence suggesting the existence of large-scale co-regulation of genes for chloroplast biogenesis has also been obtained. Global gene expression changes taking place specifically in shoot apical meristematic regions, and separately in cotyledons when etiolated *Arabidopsis* seedlings are first exposed to light, have been examined (E Dillon, G Beemster, L Bögre, E. López-Juez, unpublished data). Broad clusters of genes showing co-ordinated expression were identified, and in a small number of these clusters genes associated with 'plastid', 'chloroplast', or 'thylakoid' descriptions (gene ontology terms) were statistically over-represented. The small number of clusters and relatedness of expression pattern within each of them was such that just a few upstream regulatory factors could, in theory, be responsible for the co-ordinated induction of a large number of chloroplast function-related genes.

The search for plastid master switches

What could the nature of such master switches be? Genetic approaches have uncovered a number of genes worth exploring as candidates. Not surprisingly, studies of light signalling provide several of them. The *Arabidopsis hy5* mutant shows limited and delayed responses to light, including reduced expression of photosynthesis-related genes (McCormac and Terry, 2002). While the reduction is not dramatic, the fact that HY5 encodes a G-box binding bZIP transcription factor makes it of significance. Maize *Golden 2* was first identified as a bundle-sheath specific chloroplast defective mutant, but it was later found that both maize and *Arabidopsis* contain two *Golden 2-like* (*GLK*) genes, and when both are simultaneously mutated, defective chloroplasts, showing a severe reduction in thylakoids, are seen in every photosynthetic cell (Fitter *et al*., 2002). *GLK* genes are attractive candidates for being regulatory switches for 'chloroplasts' in photosynthetic cells because they encode transcription factors conserved in all lineages of land plants, including bryophytes, but not in photosynthetic single-cell organisms (Yasumura *et al*., 2005). However, their role cannot be fundamental since the chloroplast development defect in double mutants is, again, not strong. Transcription co-activators may be as important as transcription factors in controlling or co-ordinating states of activity of transcriptional complexes. The *Arabidopsis HAF2* gene encodes a protein homologous to TATA box-binding factors in yeast, and has histone acetyl-transferase activity (Bertrand *et al*., 2005). Interestingly, HAF2 knockouts show an obvious 'slow greening' phenotype, similar to the one in virescent *cue* mutants (see above), and reduced/delayed expression of photosynthesis-related genes.

Overall, then, there are some leads towards the uncovering of 'chloroplast master switches', but they are far from having been identified. Recently, an elegant genetic screen was designed in an attempt to identify chloroplast regulatory factors directly (Niwa *et al*., 2006). Kobayashi, Niwa and collaborators generated activation-tagged mutations on callus tissue that had been previously transformed to express a herbicide detoxifying activity under the control of a photosynthetic gene promoter. Normally such promoters are silent in colourless callus, but the activation tagging procedure can, potentially, turn them active and cause herbicide tolerance. In this way the *ces* (callus expression of *RbcS*) *101* mutant was successfully identified. The nature of the *CES101* gene, a receptor-like kinase, raised the possibility of proteins involved in the sensing of extracellular (potentially intercellular-communication) signals being critical activators of chloroplast biogenesis. What such signals could be is currently in the realm of speculation, but both the results of this work and its experimental approach seem highly promising.

Towards plastid systems biology

Successfully uncovering such master regulatory genes would not be the end of the road. Such 'switches' would need to be integrated into overall networks that together underlie the growth and development of plants. It is known that such integration is necessary, and phenomena that have been described earlier, such as the existence of plastid 'developmental signals', are either mechanisms or manifestations of the fact that the integration does take place. Already over 15 years ago it was postulated that chloroplast biogenesis could be nothing but a manifestation of a 'leaf mesophyll cell' initiation programme, with chloroplast and leaf development activities tightly intertwined (Chory and Peto, 1990).

A powerful method to uncover co-ordination of wide biological processes has become available with the ability to monitor, through microarray experiments, global gene expression. Such a technique is being used to address many separate environmental or developmental situations, in many different laboratories, but through only a small number of technology platforms, making it possible to compare results from hundreds, even thousands of experiments, at least in the *Arabidopsis* model. Bioinformatic tools to monitor such data are being developed and refined (Zimmerman *et al*., 2004; Toufighi *et al*., 2005). Figure 4 shows a comparison of the expression profiles of a number of key genes playing a role or potentially regulating aspects of plastid biology referred to earlier, across *Arabidopsis* individual tissues or developmental stages. The comparison uses Expression Browser (Toufighi *et al*., 2005). It is clear that genes for processes biochemically very distinct, such as plastid division, plastid gene expression, and expression of nuclear photosynthetic genes, show very tight co-regulation patterns, while within a single process, such as plastid protein import, homologous components clearly associate with distinct process (see *Toc33* or *Toc159* association with *FtsZ1*, *MinD* and *Arc6*, a subset of chloroplast division genes, while *Toc34* or *Toc 132* associate much more closely with mitotic

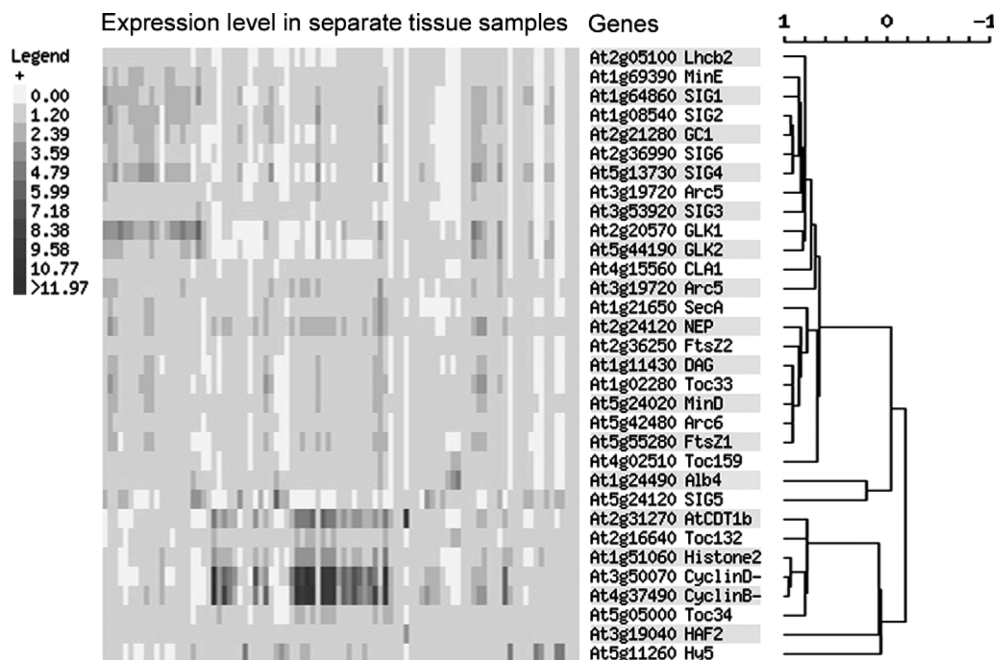

Fig. 4. Cluster tree showing similarity in expression pattern across development of genes representative of processes or candidate regulators discussed in the text. The tree was elaborated using the Expression-browser tool of the Botany Array Resource (http://bbc.botany.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi). Columns of the 'heat map' represent samples of different tissues or developmental stages across the *Arabidopsis* life cycle. The scale represents the ratio of each tissue's expression level relative to median expression levels in all tissues. Note, for example, the similarity in expression pattern between a photosynthetic protein, plastid division proteins, and the import receptors AtToc33 and AtToc159, while those of the isoforms of the import receptors, AtToc34 and AtToc132 respectively, resemble much more closely the expression pattern of cell cycle-related genes like Histone or cyclins, suggesting a role for these in plastid maintenance in rapidly-dividing cells.

and DNA-synthesis *cyclins* and *Histone 2A*, cell division genes of high activity in both shoot and root apical meristems). The associations may reflect the sharing of transcriptional regulatory mechanisms. While many key transcription factors playing roles in embryo development in *Xenopus* have been known for a number of years, it is only now that networks that link them together into biological processes are being drawn (Longabaugh *et al*., 2005). Only such interlinking networks can achieve full descriptive and predictive power, the ultimate aim of 'systems biology'.

We are clearly in the infancy of a 'plastid systems biology' era. Our understanding of how diverse cells differentiate distinct plastid types, particularly chloroplasts, of the correct type, to an extent that constitutes an appropriately-sized cellular plastid complement, is only beginning. Such an understanding could bring obvious rewards (for example, to manipulate the size of the plant's 'plastid factory'), and can only be expected to expand dramatically in the next few years.

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