Chloroplast research in the genomic age

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Chloroplast research takes significant advantage of genomics and genome sequencing, and a new picture is emerging of how the chloroplast functions and communicates with other cellular compartments. In terms of evolution, it is now known that only a fraction of the many proteins of cyanobacterial origin were rerouted to higher plant plastids. Reverse genetics and novel mutant screens are providing a growing catalogue of chloroplast protein–function relationships, and the characterization of plastid-to-nucleus signalling mutants reveals cell–organelle interactions. Recent advances in transcriptomics and proteomics of the chloroplast make this organelle one of the best understood of all plant cell compartments.

Plastids are organelles characteristic of plant cells. As endosymbiotic remnants of a free-living cyanobacterial progenitor, plastids have, over evolutionary time, lost the vast majority of their genes. Indeed, depending on the organism, contemporary plastomes contain only 60–200 open reading frames (ORFs). The plastomes of green algae and flowering plants are remarkably similar in the sequences of their genes, whereas the organization of genes on the plastid chromosome differs drastically. Although identical plastome copies are contained in each cell of a plant, the organelles themselves can vary to a large extent in their morphology and function. In fact, proplastids can develop into green chloroplasts, red or yellow chromoplasts, or other variants specialized for the storage of starch, lipids or proteins. The photosynthetically active chloroplasts are characterized by high rates of transcription and translation, allowing the synthesis of large amounts of the enzyme ribulose bisphosphate carboxylase (Rubisco) and a rapid renewal (through turnover) of electron transfer components, features necessary for efficient photosynthetic CO_2 fixation. Besides photosynthesis, chloroplasts carry out other essential plant functions, such as the synthesis of amino acids, fatty acids and lipids, plant hormones, nucleotides, vitamins and secondary metabolites. Technological developments in the genomics of *Arabidopsis thaliana*, including the sequencing of its genome, have recently stimulated the initiation of projects aimed at systematically identifying the functions of chloroplast proteins and their encoding genes, and elucidating their evolution. This article highlights the new findings, focusing on screens for novel chloroplast mutants, systematic reverse genetics, bioinformatics, transcriptomics and proteomics, in *A. thaliana* and other plant species.

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Size, composition and phylogeny of the chloroplast proteome

The vast majority of chloroplast proteins are nucleus-encoded and, with the exception of the outer envelope proteins, require N-terminal presequences, termed 'chloroplast transit peptides' (cTPs), to target them to the chloroplast. Between 2100 and 3600 distinct proteins are estimated to be located in the *Arabidopsis* chloroplast [1,2]. These estimates are based on the computational identification of cTPs in the predicted protein-coding regions of the *Arabidopsis* genome. In general, for almost all chloroplast proteins identified by experimental proteomic analysis in pea or *Arabidopsis*, and for ~90% of orthologous proteins from other plant species, coding regions for precursor proteins have been correctly predicted by computer programs through the identification of a cTP [3–5].

Prediction of the intraorganellar location of these proteins, after import into the chloroplast and cleavage of the cTP, is more difficult. The proteins of the stroma, inner envelope and stromal side of thylakoid membranes, as well as most integral proteins of the thylakoid membrane, have no additional transit peptides. By contrast, proteins destined for the thylakoid lumen are targeted and translocated via a second N-terminal sequence, which is located directly C-terminal of the cTP (Box 1). Two different mechanisms are known to facilitate translocation of such lumenal proteins across the thylakoid membrane, the Sec pathway and the twin-arginine translocation (TAT) pathway. The latter requires a specific amino acid sequence, the TAT motif. In *A. thaliana*, the protein content of the thylakoid lumen has been characterized by two-dimensional electrophoresis and mass spectrometry, as well as by genome-wide searches for the TAT motif. Thirty to 35 lumenal proteins, identified by experimental proteome analysis, and 30–56 additional proteins encoded by the *Arabidopsis* nuclear genome and containing a tentative TAT motif, are listed by the groups of van Wijk and Kieselbach, respectively [4,5]. This allows the prediction that the thylakoid lumen should contain at least 80 different proteins [5]. Based on the annotation of the novel lumenal proteins, new functions, in addition to the accumulation of protons necessary for ATP synthesis and the equilibration of ion currents through the thylakoid membrane, can be associated with the thylakoid lumen. A large group of peptidyl-prolyl *cis-trans* isomerases (rotamases) and proteases, a family of proteins related to the P subunit

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Box 1. Chloroplast structure and intraorganellar sorting of thylakoid proteins

Chloroplasts comprise three different membranes (the double-membrane envelope and the thylakoid membrane), which enclose three distinct soluble phases (intermembrane space, stroma and thylakoid lumen) (Fig. I). The stroma is the site of carbon fixation, amino acid synthesis and many other pathways. In the thylakoid membrane system light is captured and ATP synthesized, whereas in the thylakoid lumen several extrinsic photosynthetic proteins, as well as polypeptides operating in the folding and proteolysis of thylakoid proteins (see main text) are housed.

The vast majority of chloroplast proteins are encoded in the nucleus, synthesized in the cytosol as precursors with a chloroplast transit peptide (cTP, indicated in Fig. I as a red rectangle), and guided by the so-called 'guidance complex' (not shown) to the Toc/Tic translocon (TT) [a]. After import into the chloroplast the cTP is cleaved by the stromal processing peptidase (indicated as black scissors). No additional transit peptides are required for the sorting of proteins to the stroma or the inner envelope. By contrast, for uptake into or passage through the thylakoid membrane four different pathways exist in higher plant chloroplasts (for an overview, see Ref. [b]), of which three depend on a second N-terminal sequence (indicated as ellipses), located directly C-terminal of the cTP and cleaved by the thylakoidal processing peptidase (indicated as white scissors). Substrates of the ATP-dependent Sec pathway contain a Sec-type thylakoid targeting signal (dark-green ellipse), interact with the stromal SecA protein, and are translocated in an unfolded state through the Sec translocon. The ΔpH-dependent twin-arginine translocation (Tat) pathway accomplishes the transport of fully folded precursors whose lumenal transit peptide carries a characteristic twin-arginine motif preceding the hydrophobic core (blue ellipse). The signal recognition pathway is thought to be reserved for integration of thylakoid membrane proteins such as the light-harvesting protein Lhcb1, and requires a stromal recognition particle (SRP), FtsY, Alb3, GTP and translocation machinery. Interestingly, the thylakoid targeting information (yellow circle) is located within the mature Lhcb1 protein. The fourth, so-called 'spontaneous', pathway mediates the insertion of selected thylakoid membrane proteins and also requires the presence of a second cleavable signal peptide (white ellipse), which is superficially similar to Sec-type signal peptides.

References

a Jarvis, P. and Soll, J. (2001) Toc, Tic, and chloroplast protein import. *Biochim. Biophys. Acta* 1541, 64–79

b Robinson, C. *et al.* (2001) Multiple pathways used for the targeting of thylakoid proteins in chloroplasts. *Traffic* 2, 245–251

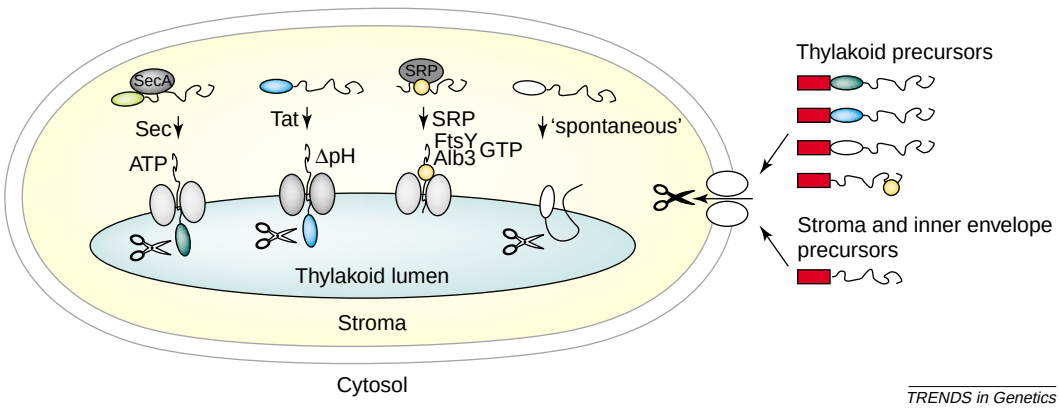


Fig. I.

of photosystem II (PSII), and a network of *m*-type thioredoxins, peroxidins and ascorbate peroxidases have been described, indicating that proteins of this compartment also operate in the folding and proteolysis of thylakoid proteins, as well as in providing protection against oxidative stress [4,5].

An estimate of the number of proteins present in the chloroplast (or in other compartments) that originated from the cyanobacterial endosymbiont has been provided by a BLAST analysis in which the protein complements of *Arabidopsis* and the cyanobacterium *Synechocystis* were compared. Between 1400 and 1500 *Arabidopsis* proteins of cyanobacterial origin were identified, about half of which are targeted to chloroplasts as judged by the presence of cTPs [1]. Other studies, based on phylogenetic analyses of the entire *Arabidopsis* genome or samples of it, support the conclusion that between 400 and 2200 *Arabidopsis* genes might be of cyanobacterial origin [2,6]. The most thorough analysis of the cyanobacterial heritage in the *Arabidopsis* nuclear genome compared it with the genomes of three cyanobacterial species, 16 other prokaryotes, and yeast [7]. Of 9368 *Arabidopsis* proteins sufficiently conserved for primary sequence comparison, 866 detected homologues only in cyanobacteria, whereas an additional 834 were grouped with cyanobacterial proteins in phylogenetic trees. Extrapolating from these data, it was suggested that ~4500 *Arabidopsis* genes descend from the cyanobacterial endosymbiont [7]. Surprisingly, of these only ~1300 encode proteins predicted to be chloroplast-targeted (Box 2). This supports the idea of a massive redistribution of cyanobacterium-derived proteins to other cellular compartments (Fig. 1). Of the three cyanobacterial species considered by Martin and co-workers, *Nostoc*'s gene complement is most closely related to that of the ancestor of plastids [7].

Reverse genetic analysis of plastome genes

Most ORFs encoded by the plastome DNA belong to two major classes: genes required for the maintenance and expression of the organelle's own genetic system (encoding

Box 2. From transit peptide predictions to subcellular targeting

The accuracy of prediction of protein targeting by the TargetP software [a], based on the protein's N-terminal amino acid sequence, is characterized by specificity and sensitivity. The specificity value indicates how many of the predicted targeting signals are real, whereas the sensitivity value refers to the probability with which any real targeting signal will be identified. To calculate, for example, the number of 'real' chloroplast transit peptides (cTP_{real}) encoded by the *Arabidopsis* genome ($n = 25\,678$ genes) from the number of predicted cTPs (cTP_{pred}, $n = 3856$), the specificity ($spec_{cTP} = 0.69$) and sensitivity ($sens_{cTP} = 0.85$) of TargetP prediction, the following equation is used:

$$cTP_{real} = \frac{cTP_{pred} - (1 - spec_{cTP}) \times cTP_{pred}}{sens_{cTP}}$$

$$= \frac{spec_{cTP}}{sens_{cTP}} \times cTP_{pred} = 3130 \quad [1]$$

In Table I the results of this calculation are listed for all four classes of targeting signals. Of 9368 *Arabidopsis* proteins sufficiently conserved for phylogenetic analysis, 1700 were calculated to come from cyanobacteria [b]. Their subcellular targeting was calculated by TargetP (Table II, col. 2), and extrapolation to the entire genome (25 678 genes) was then performed by multiplying by a factor of 2.74 (25 678/9368; col. 3). In a next step, the accuracy of the TargetP program was checked as described above (col. 4).

The genome-wide extrapolation results in 4660 genes of cyanobacterial origin (col. 3), in contrast to the ≈4500 genes estimated by Martin *et al.* [b], who considered only 24 990 of the 25 678 *Arabidopsis* genes. Note that after the second extrapolation step (col. 4) the total number of cyanobacterial proteins only sum up to ≈4300. This is due to the slightly different composition of the dataset used to calculate the accuracy of TargetP prediction [a] and the dataset representing all protein-coding genes of *Arabidopsis*.

References

a Emanuelsson, O. *et al.* (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J. Mol. Biol.* 300, 1005–1016

b Martin, W. *et al.* (2002) Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl Acad. Sci. USA* 99, 12246–12251

Table I. Calculation of plant cell proteome fractions in *Arabidopsis thaliana*

Targeting signal	Number of genes identified by TargetP	Specificity according to Ref. [a]	Sensitivity according to Ref. [a]	Corrected genome-wide prediction considering TargetP accuracy
cTP	3856	0.69	0.85	3130
mTP	2856	0.90	0.82	3135
SP	4382	0.96	0.91	4623
none	14 584	0.78	0.85	13 383

cTP, chloroplast transit peptide; mTP, mitochondrial transit peptide; SP, signal peptide; none, no N-terminal targeting sequence.

Table II. Cyanobacteria-derived genes: calculation of subcellular targeting of their products

Targeting signal	Number of genes based on TargetP analysis of 9368 protein-coding genes [b]	Approximating the entire genome (× 25 678/9368)	Corrected genome-wide estimates considering TargetP accuracy
cTP + Cyano	571	1565	1270
mTP + Cyano	140	384	421
SP + Cyano	302	827	872
none + Cyano	687	1882	1727
Total Cyano	1700	4660	4290

cTP, chloroplast transit peptide; mTP, mitochondrial transit peptide; SP, signal peptide; none, no N-terminal targeting sequence; Cyano, cyanobacteria-derived gene.

rRNAs, tRNAs, ribosomal proteins, RNA polymerase subunits) and photosynthesis-related genes. The functions of relatively few hypothetical plastid ORFs (*ycf*s) are not precisely known. Efficient homologous recombination in chloroplasts allows wild-type *ycf* genes to be replaced by knockout alleles, and their phenotypes studied in homotransplastomic plants.

Novel proteins involved in photosynthesis

Targeted inactivation of *ycf*s in tobacco and in the green alga *Chlamydomonas reinhardtii* has, over the past few years, led to the discovery of novel subunits of the cytochrome b_6f complex (YCF7/PetL and YCF6/PetN) (Fig. 2) and assembly factors for photosystem I (PSI), such as YCF3 and YCF4 (reviewed in Ref. [8]). YCF3 is a chaperone that interacts directly and specifically with at least two of the PSI subunits during assembly of the PSI complex [9]. For the *ycf9* gene, which was knocked-out by several groups [10–14], Wollman and co-workers provided the first strong evidence for the localization of its gene product to PSII, and the protein is now designated as PSII-Z [14]. PSII-Z controls the interaction of the PSII core with its light-harvesting antenna; in its absence tobacco plants show severe growth defects and increased light sensitivity [14].

Small subunits of photosystems I and II

Recently, the plastid genes encoding the PSII subunits J and L were inactivated. Tobacco Δ*psbL* lines lack a functional PSII and can be propagated only under heterotrophic conditions, whereas absence of PSII-J leads to deregulation of both photosynthetic electron flow and assembly of the water-splitting complex [15,16]. Inactivation of *Chlamydomonas psaJ*, coding for the J subunit of PSI, does not prevent photoautotrophic growth,

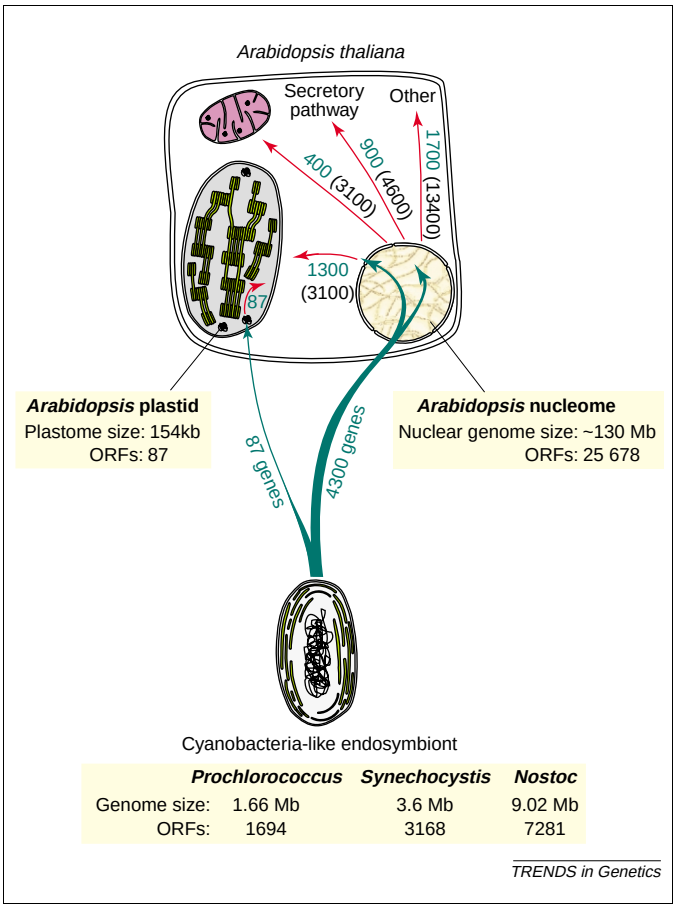


Fig. 1. Genes of cyanobacterial origin and the intracellular targeting of their products in *Arabidopsis*. Double-membrane-bound chloroplasts such as those in *Arabidopsis* descend from a cyanobacterial-like endosymbiont. Because the identity and gene content of this endosymbiont are still unknown, the three cyanobacterial species whose genome sequences are known (*Synechocystis* sp. PCC6803, *Prochlorococcus marinus* and *Nostoc punctiforme*) were used to reconstruct protein phylogenies [7]. Calculation of the number and intracellular targeting of cyanobacterium-derived gene products is illustrated in Box 2. Note that after calculation of their targeting sequences the number of nuclear genes of cyanobacterial origin totals ~4300. Green lines indicate the origin of the 87 plastid and ~4300 nuclear genes of cyanobacterial origin, whereas green numbers indicate the number of products (red arrows) of nuclear genes of cyanobacterial origin predicted to be targeted to the different cellular compartments. Black numbers in parentheses indicate the total number of predicted proteins targeted to the respective compartment.

but results in altered kinetics of oxidation of plastocyanin or cytochrome c_6 [17].

Essential proteins

For the two giant tobacco ORFs with unknown function, *ycf1* and *ycf2*, as well as for the tobacco and *Chlamydomonas clpP* genes, which encode a catalytic subunit of the Clp protease (functioning in the degradation of abnormal stromal proteins), homotransplastomic plants cannot be constructed [18–20]. This strongly suggests that these ORFs are essential for cell viability. It is interesting to note that for the *rpoA*, *rpoB* and *rpoC1* genes (encoding subunits of the RNA polymerase) homotransplastomic knockout lines can be obtained in tobacco, but not for the *rpoB1*, *rpoB2* and *rpoC2* genes in *Chlamydomonas* (reviewed in Ref. [8]).

Rubisco

In addition to the cases already mentioned, reverse genetics has been instrumental in the characterization of other plastid-encoded proteins. Targeted deletion of the *rbcL* gene coding for the large subunit of Rubisco, combined with its allotropic expression, was accomplished several years ago [21]. More recently, replacement of the hexadecameric tobacco Rubisco (which is made up of large and small subunits) by the less efficient homodimeric Rubisco from a red alga (without small subunits) resulted in the expected change in the properties of the photosynthetic machinery [22], an important step towards the direct alteration of net efficiency of photosynthesis.

Reverse genetic analysis for nuclear genes encoding plastid proteins

For reasons that are unclear, knockout of nuclear genes by homologous recombination is unfeasible at present in higher plants. In *Arabidopsis*, maize and rice, the targeted mutagenesis of nuclear genes coding for plastid proteins has been facilitated by the availability of large collections of insertion mutants – based on gene disruptions by T-DNA (*A. thaliana*), transposons (maize and *A. thaliana*), or mobilized retrotransposons (rice) – which can be systematically searched for mutations in genes of interest. The targeted inactivation of nuclear genes by antisense, co-suppression and RNA interference (RNAi) strategies has also made a significant contribution. In principle, the mutational saturation of all nucleus-encoded plastid proteins is feasible, but such a large-scale effort has not yet been launched. Current studies focus on the identification and characterization of nuclear mutations for a limited number of genes, although large-scale projects have recently begun. One example of a reverse genetics screen involved the knockout of more than a dozen genes for components of PSI in *A. thaliana* (Fig. 2). The PSI complex of this plant is a mosaic of plastid- and nucleus-encoded protein subunits; of the latter, three (PSI-D, -E and -H) are each encoded by two functional gene copies. Cross-species comparison of PSI raises the question of why the subunits D, E and F in higher plants contain N-terminal extensions not present in their cyanobacterial orthologues. A second question concerns the function of PSI-G, -H, -N and -O, which are absent in the PSI of cyanobacteria. In general, more information is needed on which subunits or domains are responsible for plant-specific PSI functions, such as state transitions or the interaction between the PSI core and its plant-specific antenna (LHCI, the light-harvesting complex of PSI).

Downregulation of individual PSI subunits by antisense or co-suppression strategies, combined with the identification of insertion mutants for most of the corresponding nuclear genes, has provided the basis for an analysis of the functions of almost all PSI polypeptides in photosynthetic electron transport, as well as the overall composition of this multiprotein complex (reviewed in Refs [23,24]). More recently it was shown that the mobile pool of LHCII, which can shuttle between PSI and PSII, docks to the H subunit of PSI [25]. The analysis of lines lacking PSI-N led to the identification of a novel PSI subunit (PSI-O) [26], whereas the molecular characterization of lines without PSI-K, PSI-G, or both, revealed that the stoichiometry of the four LHCI proteins is flexible [27–29].

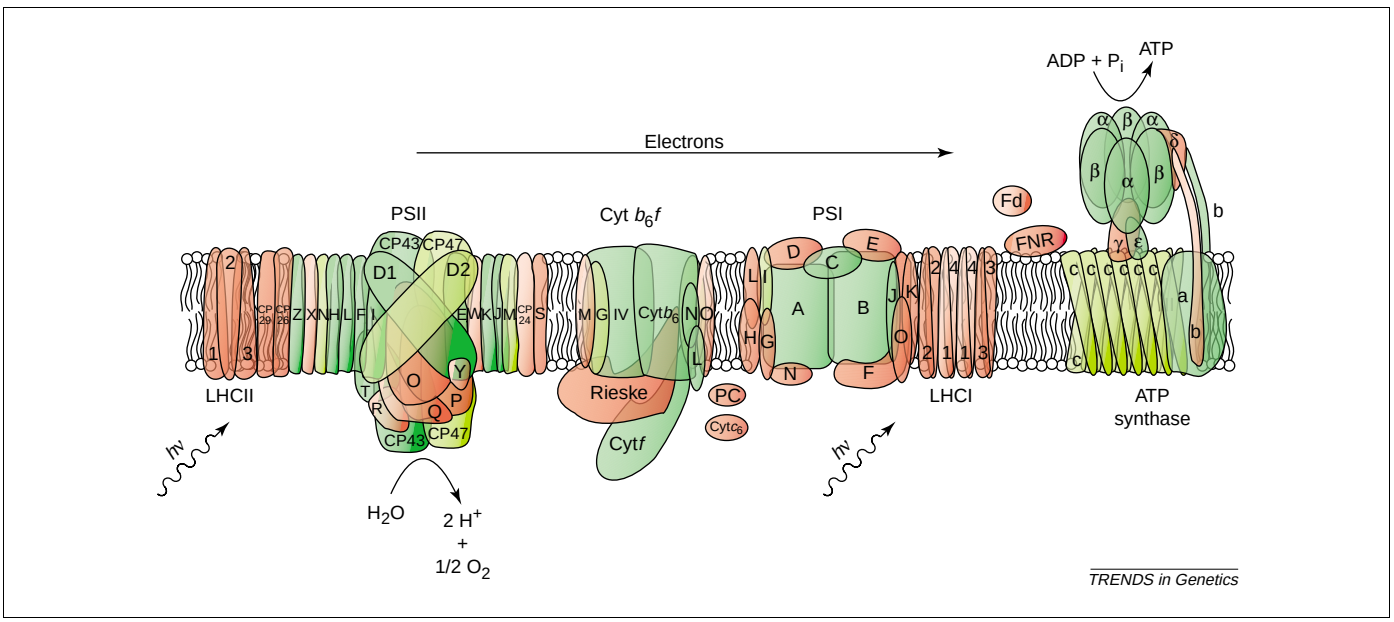


Fig. 2. Composition of the photosynthetic apparatus in the thylakoid membrane, and summary of the mutants available. Known structural data were used to illustrate the composition of multiprotein complexes, but the positions of subunits within the complexes are largely arbitrary. Plastome-encoded subunits are indicated in light green, or dark green if inactivation of the corresponding gene has been reported. Nucleus-encoded proteins are depicted in orange, or red if knockout or downregulation of the protein has been achieved. Note that although mutants from both *Arabidopsis* and *Chlamydomonas* are considered, the composition of the photosynthetic apparatus refers only to *Arabidopsis*, which in this regard differs in some respects from *Chlamydomonas*. YCF3 and YCF4 (not shown) are localized on the thylakoid membrane, but not stably associated with a multiprotein complex. PC, plastocyanin; Fd, ferredoxin; FNR, ferredoxin-NADPH reductase; LHC, light-harvesting complex; PS, photosystem.

This finding is supported by the direct analysis of co-suppression lines for two of the four LHCI proteins [30]. The phenotype of plants that do not express plastocyanin has refuted the dogma that in higher plants plastocyanin is the only electron donor to PSI [31]. Gupta and co-workers provided strong evidence that – as in cyanobacteria and algae – a functional cytochrome c_6 that can replace plastocyanin is present in *Arabidopsis*. Together the studies discussed above, combined with structural analyses in cyanobacteria, have greatly increased our understanding of function and function–structure relationships in PSI, making it the best-studied multiprotein complex of the chloroplast.

Another example of the successful application of reverse genetics in the characterization of chloroplast functions is the targeted mutagenesis of plant homologues of the bacterial cell division protein FtsZ. Plants seem to possess different *FtsZ* genes, encoded by two different gene families. Targeted disruption of the nuclear *FtsZ* gene from the moss *Physcomitrella patens* as well as antisense suppression of the *Arabidopsis* genes *AtFtsZ1* or *AtFtsZ2* inhibited plastid division, yielding cells having only a single large chloroplast [32,33]. In the chloroplast, FtsZ proteins form rings at the plastid division site, and co-localization of FtsZ1 and FtsZ2 in the chloroplast stroma indicates that such rings might also physically interact [34].

Forward genetics: mutant screens of a new quality and quantity

Which genes are important, or even essential, for chloroplast functioning? One tool that should be able to provide an answer to this question is forward genetics, which aims to identify and clone (mutant) alleles responsible for a phenotype of interest. At present, forward genetics is receiving a great deal of attention in chloroplast research, employing phenotypic screens for a wide spectrum of chloroplast functions.

Leaf coloration and seedling lethality

The largest class of chloroplast-related mutations affects leaf coloration (bleached, pale green, etc.) and/or seedling viability. These phenotypes overlap to a significant extent and are easily identified by visual inspection of plants in the greenhouse. Mutants with altered leaf coloration frequently show pleiotropic effects. Most corresponding genes encode proteins located in the chloroplast that are related, among others, to protein translocation [35,36], proteolysis [37], enzyme activity [38,39], protein complex assembly [40], and metal ion homeostasis [41]. In maize, some 1700 independent transposon-induced mutants exhibiting altered pigmentation phenotypes have been collected by Barkan and co-workers (http://pml.uoregon.edu/). This population can be also screened by reverse genetics for transposon insertions into genes of known sequence with proposed roles in chloroplast biogenesis.

Scoring seedling lethality is a relatively nonspecific screening method for chloroplast functions. Seedling-lethal mutants are commonly observed in mutagenesis programmes, but only recently was a large-scale screen for such mutants combined with the molecular cloning of the corresponding genes [42]. This led Levin and colleagues to the isolation of hundreds of seedling-lethal mutants, most of them also affecting pigmentation and/or plant morphology. Sixty-four insertion-tagged lines were characterized, and the disrupted gene was identified for 39 of them. The tagged loci mostly encode plastid-targeted proteins, including a group previously shown to have a seedling-lethal phenotype: *CLA1* [43], *PAC* [44], and *TatC* [45]. In addition, mutant alleles of the *AtpD* gene coding for

Table 1. Mutant screens for plastid-related phenotypes and a selection of genes cloned

Mutant phenotype	Number of mutants	Genes cloned
Viability/pigmentation		
Leaf coloration	Several hundreds[a,b]	*AtCAO* [35], *AtVAR2* [37], *AtIM* [38,39], *AtALB3* [40], *AtHO1* [67,68], *ZmTHA4* [36], *ZmYS* [41]
Seedling lethality	505[a]	*AtCLA1* [43], *AtPAC* [44], *AtTatC* [45]; *AtAtpD*, *AtTIC40- LIKE*, *AtPetC*, and others [42]
Chlorophyll fluorescence		
hcf (high chlorophyll fluorescence)	67[a]	*AtHCF136* [50], *AtHCF164* [51], *AtVIPP1* [52], *AtHCF107* [55],
	Several hundreds[b]	*ZmHCF106* [53], *ZmCRP1* [54], *ZmPRPS17* [56]
npq (non-photochemical quenching)	119[a]	*AtZEAXANTHIN EPOXIDASE* [58], *AtVIOLAXANTHIN DEEPOXIDASE* [58], *AtPsbS* [60], *AtPetC* [61]
	150[c]	*CrNPQ5* (*Lhcbm1*) [80]
Φ_{II} (effective quantum yield of PSII)	63[a]	*AtPsaE1* [63], *AtPrpl11* [64], *AtIRT1* [65]
Plastid signalling		
gun (genome uncoupled)	9[a]	*AtGUN2/HY1* [67,68], *AtGUN3/HY2* [69], *AtGUN5* [70]
cue (chlorophyll *a*/*b*-binding protein underexpression)	38[a]	*AtCUE1/PPT* [72]
flu (fluorescence after etiolation)	4[a]	*AtFLU* [74]
laf (long after far-red)	20[a]	*AtLAF6* [71]

[a] *Arabidopsis*.
[b] Maize.
[c] *Chlamydomonas*.

the δ-subunit of the chloroplast ATPase, *PetC* encoding the Rieske protein of the cytochrome b_6f complex (see also Fig. 2), three genes involved in the plastid non-mevalonate isoprenoid biosynthetic pathway, and a gene similar to the pea *Tic40* [46], involved in chloroplast protein translocation, have been described.

Chlorophyll fluorescence mutants

Screens more specific for mutated chloroplast functions assess parameters of chlorophyll fluorescence as an indicator of photosynthetic performance. Alterations in chlorophyll fluorescence indicate defects in photosynthetic electron flow, due to perturbations in the thylakoid protein complexes. Depending on the specific chlorophyll fluorescence parameter monitored, different types of mutations are accessible, ranging from subtle defects without significant consequences for plant morphology or growth, to more dramatic structural changes such as the disappearance of entire multiprotein complexes of the thylakoids. The first systematic studies based on altered chlorophyll fluorescence characteristics considered the 'high chlorophyll fluorescence' phenotype (hcf), which is visually detected as red fluorescence in response to UV illumination of plants. *hcf* mutants have been identified in *C. reinhardtii*, maize and *Arabidopsis* [47–49] (Table 1), and in most lines, photosynthesis is severely affected, often resulting in seedling lethality. Corresponding HCF genes are involved in essential chloroplast functions, such as thylakoid biogenesis [50–52], protein translocation [53], mRNA processing [54,55] and translation [56].

The quantification of additional parameters of chlorophyll fluorescence using more complex fluorimeter systems allows the identification of mutations with relatively small alterations in photosynthetic performance, which do not necessarily result in seedling lethality. For this class of mutants, the parameters 'non-photochemical quenching' (NPQ), which reflects the energy dissipated as heat following energization of the thylakoid membrane due to lumen acidification, or the effective quantum yield of PSII (Φ_{II}), a measure of the overall efficiency of PSII reaction centres in the light, are monitored. NPQ mutants were first identified in *Chlamydomonas* [57], and more recently two laboratories have also identified *Arabidopsis* mutants that show changes in NPQ [58,59] (Table 1). The corresponding genes encode proteins involved in the xanthophyll cycle [58], or for subunits of the photosynthetic apparatus such as PSII-S [60] and the Rieske protein of the cytochrome b_6f complex [61]. *Arabidopsis* mutants with altered Φ_{II} [62] reveal defects in plastid and non-plastid proteins, including the E subunit of PSI [63], the plastid ribosomal protein PRPL11 [64], and the iron transporter IRT1 [65]. Of the 12 independent mutant alleles associated with changes in Φ_{II} identified so far, six encode plastid proteins and six for proteins without chloroplast targeting (D. Leister, unpublished results).

Other specialized screens: mutants affected in plastid-to-nucleus signalling, plastid division or other functions

Other more specialized screens designed for the large-scale identification of gene functions have the potential to uncover further and very specific chloroplast functions. Dissection of plastid-to-nucleus signalling was approached by screening for mutants that, in the absence of chloroplast development, express nuclear photosynthetic genes such as *Lhcb* (coding for LHCII) and *RbcS* (coding for the small subunit of Rubisco) – genome-uncoupled (*gun*) mutants. Other types of plastid-to-nucleus signalling mutants either underexpress light-regulated nuclear genes encoding chloroplast proteins – chlorophyll *a*/*b*-binding protein underexpression (*cue*) mutants – or show a change in both light-dependent morphological responses and gene expression – long after far-red (*laf*) mutants (reviewed in Ref. [66]). Some of the corresponding genes were recently cloned, and were found to have roles in porphyrin metabolism (*GUN2*/*HY1* [67,68], *GUN3*/*HY2* [69], *GUN5* [70]), and in the transport of porphyrins (*LAF6* [71]) or other metabolites (*CUE1* [72]).

Arabidopsis mutants affected in plastid division were identified by a microscope-based screen. Twelve *accumulation and replication of chloroplasts* (*arc*) mutants have been isolated so far (summarized in Ref. [73]). *arc* mutants

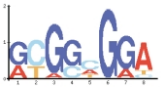
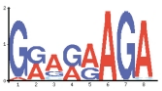
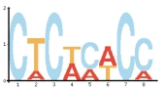
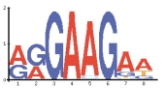
(a)	(b)	(c)	(d)
At1g55480	2.07	Unknown protein	
At1g74880	2.12	Unknown protein	
At2g14230	2.12	Hypothetical protein	
At2g01480	2.11	Similar to axi1 protein from *N. tabacum*	
At3g54920	2.12	Pectatelyase-like protein	
At4g39460	2.03	Mitochondrial carrier-like protein	
At2g33450	2.03	Chloroplast 50S ribosomal protein L28	
At5g51460	2.03	Trehalose-6-phosphate phosphatase	
At2g27860	2.01	Putatived TDP-glucose 4-6-dehydratase	
At1g51350	2.02	Hypothetical protein	
At5g23120	2.01	Photosystem II assembly factor HCF136	
At3g54660	–1.87	Gluthatione reductase, chloroplast precursor	
At4g10620	–1.92	Putative protein	
At4g27990	–1.90	Putative protein	
At1g31190	–1.79	Unknown protein	
At2g18470	–1.71	Putative protein kinase	
At2g44140	–1.71	Unknown protein	
At4g12740	–1.72	Adenine DNA glycosylase like protein	
At4g33460	–1.72	Putative ABC transporter	
At4g32540	–1.69	Dimethylaniline monooxygenase-like protein	
At3g44720	–1.66	Putative chloroplast prephenate dehydratase	
At5g55630	–1.67	Outward rectifying potassium channel KCO	

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Fig. 3. Differential expression and promoter analysis using a chloroplast-specific gene-sequence tag (GST) array. (a) Accession numbers of two classes of genes with similar expression ratios identified using the 1827-GST array in light- versus dark-grown seedlings [79]. (b) Log_2 values of their expression ratios (light versus dark). (c) Annotation of genes. (d) Consensus sequences of some conserved promoter regions identified by the *MotifSampler* program (http://www.esat.kuleuven.ac.be/~thijs/Work/MotifSampler.html). The size of each base printed in a logo is determined by multiplying the frequency of that base by the total information at that position. A value of two indicates that the nucleotide is present at this position in all eleven genes indicated to the left.

show a variety of different types of chloroplast division phenotypes, including lines having a larger number of smaller chloroplasts per cell than the wild-type (*arc1* and *arc7*), and *arc6* with a mean of only two very large chloroplasts per mesophyll cell – instead of the usual >100 chloroplasts.

Dark-grown (etiolated) *Arabidopsis* mutant seedlings that accumulate protochlorophyllide were identified by their red fluorescence when exposed to blue light (*flu* mutants), leading to the cloning of *FLU*, a negative regulator of chlorophyll biosynthesis [74]. State transition mutants in *C. reinhardtii*, which are deficient in the shuttling of LHCII between the two photosystems, were identified by chlorophyll fluorescence imaging systems [75,76], leading to the description of several independent mutant loci.

Chloroplast-related transcriptomics

DNA arrays containing plastid or nuclear genes

A macroarray containing PCR products derived from all 118 genes and 11 ORFs identified on the tobacco plastid chromosome has been generated recently and applied to study the effect of the knockout of the plastid-encoded RNA polymerase on plastid gene expression [77].

For the 3856 nuclear genes of *A. thaliana* that encode proteins containing a real or predicted cTP (Box 1), database searches show that for 1412 (or 37%) of them neither a biochemical function nor a sequence similarity to an orthologous protein with known function has yet been identified. Quantification of the mRNA expression levels under different genetic or environmental conditions could allow a function to be assigned to these genes. In *A. thaliana*, a set of 1827 nuclear genes coding for chloroplast proteins has been amplified from genomic DNA by PCR and spotted on nylon membranes to generate arrays of gene-sequence tags (GSTs) [78,79]. This 1827-GST array was employed to compare mRNA levels in dark- versus light-grown seedlings, as well as in wild-type versus *prpl11-1* mutant plants. In the *prpl11-1* mutant, the nuclear gene coding for the L11 subunit of the plastid ribosome is disrupted, severely affecting plastid protein synthesis, which in turn causes a drop in the levels of components of the photosynthetic apparatus and Rubisco, as monitored by PAGE and western analyses [64]. *prpl11-1* mRNA profiling shows that transcript levels of nuclear genes coding for proteins of the plastid ribosome, of the photosynthetic apparatus and of the small subunit of Rubisco were upregulated in the mutant [79], clearly demonstrating that the plant is able to monitor the altered physiological state of the chloroplast and to react by upregulating appropriate nuclear genes. This supports the idea that plant cells operate regulatory networks that can sense the levels of key proteins in the chloroplast and transmit a signal to the nucleus, which then acts to compensate for the relevant deficit. In the case of the photosystems and of Rubisco, which contain nuclear- and plastome-encoded subunits in a fixed stoichiometry, however, the upregulation of particular nuclear genes cannot repair the structural defect of *prpl11-1*, because the noted decrease in the level of plastome-encoded proteins seems also to limit the amount of nucleus-encoded protein subunits.

In our laboratory, the 1827-GST array was recently replaced by a 3500-GST array, which covers almost all nuclear *Arabidopsis* genes encoding chloroplast-targeted proteins, and is currently being used to analyse mRNA

expression under a variety of conditions, as well as to characterize mutants. As an example of the identification of promoter motifs based on the definition of co-regulated genes (regulons), expression profiles of dark- and light-grown seedlings [79] were used to search for promoters with common sequence motifs (Fig. 3).

Expression profiling for the analysis of plastid-to-nucleus signalling

The analysis of *gun* and *cue* mutants, as well as of the *prpl11* mutant (see above), has shown that plastid-to-nucleus signalling has a role in the coordinated expression of chloroplast and nuclear genes. Plastid signals that provide information on the state of photosynthetic metabolism and are mediated by porphyrins (see above), reactive oxygen intermediates or carotenoids, are known to regulate the transcription of several nuclear genes coding for plastid proteins (reviewed in Ref. [66]). Furthermore, the developmental and/or metabolic state of the plastid also affects the expression of nuclear genes for non-plastid proteins, as well as the expression of mitochondrial genes, supporting the conclusion that plastid signalling participates in the control of cellular metabolism and development. The concept of plastid signalling is, however, almost entirely based on the study of the expression of a few genes, such as *RbcS* and *Lhcb* genes (see above). Analysis of the behaviour of thousands of plastid protein genes under the influence of stimuli relevant to plastid signalling, or in chloroplast-related mutants (Table 1) should contribute to characterizing overlaps in the responses to different signals and hierarchies of different plastid signals.

Distinguishing between primary and secondary transcriptome changes

A major question concerns the evaluation and interpretation of transcriptomics data: how can one recognize the physiological meaning of expression profiles, or distinguish between primary and secondary effects of a mutation or treatment on the transcriptome? Programs such as *The Pathway Tools Overview Expression Viewer* (http://www.Arabidopsis.org:1555/expression.html) superimpose expression data onto the metabolic overview diagram for an organism, which allows interpretation of the transcriptomics data in a pathway context. Separating primary from secondary effects requires the careful selection of informative genetic or environmental conditions to be compared in transcriptome analyses. The comparison of mutants having unrelated primary defects but common phenotypic effects should contribute to identification of secondary effects. In other cases, analysis comparing particular mutants with wild-type plants that are treated so that they mimic the mutant phenotype might provide valuable information.

Outlook and conclusions

For the chloroplast, novel mutant screens and reverse genetics are currently identifying additional protein–function relationships in *Arabidopsis thaliana*, and in the two other model species for chloroplast research, maize and *Chlamydomonas*. These analyses indicate that many essential functions are housed in the chloroplast, and these show a high degree of interdependency. A general question concerns the number of genes that encode proteins essential for chloroplast functions, and can be identified in mutant screens. In the case of seedling lethality, a number between 320 and 480 genes has been extrapolated from available data in *A. thaliana* [42]. Assuming that the screens for abnormal leaf coloration, chlorophyll fluorescence and other plastid-related phenotypes might identify an equal number of independent gene functions, as many as 1000 genes can perhaps be identified by saturating mutagenesis.

Physiological and transcriptomic analyses of photosynthetic mutants show that photosynthetic lesions can result in changes in many other, and apparently unrelated, chloroplast functions. Vice versa, mutations of non-plastid proteins can cause defects in photosynthesis. Photosynthesis also has a role in the communication between the chloroplast and other cellular compartments, and one can expect that chloroplast-wide mRNA profiling will open a new chapter in plastid signalling research. The ability to predict the chloroplast localization of proteins based on their amino acid sequence is playing a crucial role in chloroplast functional genomics, as is, for instance, demonstrated by the analysis of thylakoid lumenal proteins. A further important step in chloroplast research will be the assignment of proteins to the distinct subcompartments of the chloroplast – inner and outer envelope, stroma, thylakoid membrane and lumen. Improved computational prediction programs, and systematic screens for protein interactions, such as those now available for the entire proteome of yeast, should further extend the range of tools available for automated experimental proteomics in the future. Last, but not least, the sequencing of the *Chlamydomonas* genome, of which a draft sequence will be announced at the end 2002, will stimulate forward and reverse genetics of nuclear genes for the dissection of chloroplast functions in this species, which has been for a long time a classic example for the reverse genetics of chloroplast genes.

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