Update on Chloroplast Research: New Tools, New Topics, and New Trends

Ute Armbruster[a], Paolo Pesaresi[b], Mathias Pribil[a], Alexander Hertle[a] and Dario Leister[a,1]

a Lehrstuhl für Molekularbiologie der Pflanzen (Botanik), Department Biologie I, Ludwig-Maximilians-Universität München, Großhaderner Str. 2, D-82152 Planegg-Martinsried, Germany
b Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli studi di Milano, I-20133 Milano, Italy

ABSTRACT Chloroplasts, the green differentiation form of plastids, are the sites of photosynthesis and other important plant functions. Genetic and genomic technologies have greatly boosted the rate of discovery and functional characterization of chloroplast proteins during the past decade. Indeed, data obtained using high-throughput methodologies, in particular proteomics and transcriptomics, are now routinely used to assign functions to chloroplast proteins. Our knowledge of many chloroplast processes, notably photosynthesis and photorespiration, has reached such an advanced state that biotechnological approaches to crop improvement now seem feasible. Meanwhile, efforts to identify the entire complement of chloroplast proteins and their interactions are progressing rapidly, making the organelle a prime target for systems biology research in plants.

Key words: Chloroplast biology; genetics; genomics; molecular biology; proteomics; transcriptome analysis.

INTRODUCTION

The chloroplast (cp), the characteristic organelle of plants and green algae, harbors its own tiny genome and is responsible for various essential functions, including photosynthesis, lipid metabolism, starch and amino acid biosynthesis (Finkemeier and Leister, 2010). Chloroplasts are descended from an ancient cyanobacterial endosymbiont and many of its functions have been conserved. However, most of the genes it brought with it have been transferred to the host nucleus during the subsequent evolution of the organelle (Timmis et al., 2004).

Early functional studies of chloroplasts depended largely on the use of biochemical and biophysical approaches. During the 1980s and 1990s, methods were developed for transforming chloroplasts by homologous recombination and for systematically disrupting nuclear genes by inserting transposons or T-DNAs. These advances markedly enhanced the utility of genetic approaches to the study of cp function. With the sequencing of entire genomes and the establishment of high-throughput tools for the analysis of their expression, cp research also entered the era of genomics (Leister, 2003). Functional genomics—the analysis of transcriptomes, proteomes, and metabolomes—opens immense possibilities for the elucidation of cp functions, serving both to characterize available mutants and to identify candidate loci for targeted mutagenesis.

At present, the green alga Chlamydomonas reinhardtii and the flowering plants Zea mays and Arabidopsis thaliana serve as the main workhorses in cp research. In this review, we focus on the impact of novel technologies and discuss some selected highlights and emerging trends in cp research, particularly in A. thaliana.

TOOLS

Forward and Reverse Genetics

Forward genetics—the isolation of mutants with specific phenotypes followed by the identification and analysis of the relevant genes—has long been the method of choice for identifying novel components that underlie plant functions of interest. Forward genetics is still an important tool in cp research, as evidenced by recent classical primary and suppressor mutant screens (Table 1), as well as screens based on the altered activity of a reporter gene in a wild-type or mutated genetic background (e.g. Baruah et al., 2009).

However, sequencing of the complete genomes of several photosynthetic organisms, generation of large collections of insertion mutants in A. thaliana (T-DNA insertion mutants) and Z. mays (endogenous transposons), and the advent of

[1] To whom correspondence should be addressed. E-mail leister@lrz.uni-muenchen.de, fax +49-89-2180-74599, tel. +49-89-2180-74550.

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Table 1. Selection of Genes for Arabidopsis Cp Proteins Identified by Forward Genetics in the Last Years.

Name	Mutant phenotype	Identified genes	Molecular function(s)	Reference(s)
Classical forward genetics				
Low PSII Accumulation (LPA)	Reduced PSII accumulation	LPA1#	D1 membrane integration	Peng et al., 2006
		LPA2	Efficient PSII assembly	Ma et al., 2007
		LPA3	Efficient PSII assembly	Cai et al., 2010
		LPA19	D1 precursor processing	Wei et al., 2010
		LPA66*	psbF editing	Cai et al., 2009
Non- Photochemical Quenching (NPQ)/Proton Gradient Regulation (PGR)	Reduced non-photochemical quenching	NPQ1/VPE	Xanthophyll cycle	Niyogi et al., 1998
		NPQ2/ABA1/ZEP	Xanthophyll cycle	Niyogi et al., 1998
		NPQ4/PSBS	Subunit of PSII	Li et al., 2000
		PGR1/PETC	Subunit of Cyt b_6/f complex	Munekage et al., 2001
		PGR3*	cp gene expression	Yamazaki et al., 2004
		PGR5	Cyclic electron flow around PSI	Munekage et al., 2002
Chlororespiratory Reduction (CRR)	No NDH activity	CRR1	NDH assembly or stability	Shimizu and Shikanai, 2007
		CRR2*	Expression of ndhB	Hashimoto et al., 2003
		CRR3	Subunit of NDH complex	Muraoka et al., 2006
		CRR4*	Site recognition factor in ndhD editing	Okuda et al., 2006
		CRR6	NDH assembly	Munshi et al., 2006
		CRR7	NDH assembly	Munshi et al., 2005
		CRR23	L subunit of NDH	Shimizu et al., 2008
High Chlorophyll Fluorescence (HCF)	High level of Chl a fluorescence	HCF101	[4Fe–4S] cluster assembly	Lezhneva et al., 2004; Stockel and Oelmuller, 2004
		HCF107#	Expression of psbH and CP47 synthesis	Felder et al., 2001; Sane et al., 2005
		HCF109	Translational termination	Meurer et al., 2002
		HCF136	Assembly of PSII reaction center	Meurer et al., 1998
		HCF152*	Processing of psbB-psbT-psbH-petB- petD transcript	Meierhoff et al., 2003
		HCF164	Transducing reducing equivalents to proteins in the thylakoid lumen	Lennartz et al., 2001; Motohashi and Hisabori, 2006
		HCF173	Initiation of psbA mRNA translation	Schult et al., 2007
		HCF208	Accumulation of Cyt b_6/f complex	Lyska et al., 2007
Suppressor screens				
Executer/Singlet Oxygen-Linked Death Activator (SOLDAT)	Suppression of singlet oxygen-mediated responses in flu mutants	EXECUTER1	Unknown	Wagner et al., 2004
		EXECUTER2	Unknown	Lee et al., 2007
		SOLDAT8	SIGMA6 factor of the plastid encoded RNA polymerase	Coll et al., 2009
		SOLDAT10	Plastid gene expression	Meskauskiene et al., 2009

TPR protein.
* PPR protein.

RNA interference approaches that allow the down-regulation of genes of interest in any species accessible to nuclear transformation have revolutionized plant genetics. In combination, these technical developments allow one to study gene functions starting from a specific gene of interest (reverse genetics). There are now numerous examples for cp functions identified and characterized by reverse genetics. Genes selected on the basis of their mRNA expression profiles or the localization of their products to the organelle have been popular targets of this approach (see sections on 'Chloroplast Proteomics: Cataloging and Characterizing Cp Proteins and their Dynamics' and 'Transcriptomics: Reaching the Next Level: Guilt-by-Association Approaches' below). Gene-tagging campaigns in maize and A. thaliana have created rich sets of localizable mutations in genes that affect chloroplast functions. Two related resources have been developed in Arabidopsis: The Chloroplast 2010 Project (www.plastid.msu.edu) (Ajjawi et al., 2010; Lu et al., 2008) and the Chloroplast Function

Database (http://rarge.psc.riken.jp/a/chloroplast/) (Myouga et al., 2010). Both resources have assembled phenotypic data for sequence-indexed T-DNA insertion mutants in experimentally validated or computationally predicted genes for chloroplast-localized proteins. The Chloroplast 2010 Project has focused on homozygous viable mutants and has scored phenotypes such as fatty acid composition, chlorophyll fluorescence, and chloroplast morphology in thousands of mutants (Ajjawi et al., 2010; Lu et al., 2008). The Chloroplast Function Database includes data on seedling-lethal and embryo-lethal phenotypes. In contrast to the Arabidopsis resources, the maize Photosynthetic Mutant Library (PML) was assembled by selecting mutants with defects in chloroplast biogenesis, as revealed by chlorophyll-deficient or high chlorophyll fluorescent phenotypes. The PML collection consists of ;2100 mutants, estimated to represent ;600 genes (http://pml.uoregon.edu/pml.html) (Stern et al., 2004; Williams-Carrier et al., 2010). The mutations are caused by Mutator transposons, which provide access to the disrupted genes. The abundance of the major photosynthetic enzyme complexes and the populations of chloroplast transcripts have been cataloged for many mutants in the PML collection. The PML collection has been particularly valuable for the discovery of novel protein classes involved in chloroplast gene expression, many of which are embryo-essential in Arabidopsis.

Chloroplast Proteomics: Cataloging and Characterizing Cp Proteins and their Dynamics

Various proteomic studies of the entire chloroplast (Ferro et al., 2010; Kleffmann et al., 2004; Rutschow et al., 2008; Zybailov et al., 2008) and its subproteomes have now been performed. These include analyses of the stroma (Ferro et al., 2010; Peltier et al., 2006; Rutschow et al., 2008), thylakoid membranes (Ferro et al., 2010; Friso et al., 2004; Giacomelli et al., 2006; Peltier et al., 2004; Rutschow et al., 2008), envelopes (Ferro et al., 2010, 2003; Froehlich et al., 2003), the thylakoid lumen (Peltier et al., 2002; Schubert et al., 2002), and plastoglobules (Ytterberg et al., 2006) (Table 2). A total of ;1750 different cp proteins have been identified so far (Table 2 and Figure 1), and the information garnered is publicly available in several databases, such as the Plant Protein Database (PPDB; Sun et al., 2009), the Subcellular Proteomic Database (SUBA; Heazlewood et al., 2007), and the Plastid Protein Database (plprot; Kleffmann et al., 2006). Advances in the semi-quantitative analysis of proteomes have resulted in the assignment of each cp protein to one or more of the three compartments stroma, thylakoids, and envelope membranes (Ferro et al., 2010). In addition, a publicly available AMT (accurate mass and time tags) database was established that enables application of this data for label-free quantification experiments.

Cp proteomics can also be employed to characterize post-translational protein modifications. Thus, numerous previously unknown substrates of cp kinases and phosphatases were recently identified by an analysis of the cp phosphoproteome (Reiland et al., 2009). In fact, the long-sought thylakoid phosphatase that dephosphorylates the light-harvesting complex II (LHCII) during state transitions (see section on 'Photosynthesis: New Insights into Non-Photochemical Quenching, Thylakoid Phosphorylation, and Alternative Electron Pathways' below), TAP38/PPH1 (Pribil et al., 2010; Shapiguzov et al., 2010), was first identified by cp proteomics (Zybailov et al., 2008).

Table 2. Number of Cp Proteins Identified by Proteomic Studies.

Cp subcompartment	Number of nucleus-encoded proteins		Reference
	Total	With predicted cTP*	
Envelope	351	270 (77%)	Froehlich et al., 2003
Envelope	125	91 (73%)	Ferro et al., 2003
Cp total	485	393 (81%)	Kleffmann et al., 2004
Cp total	916	788 (86%)	Zybailov et al., 2008
Cp total	1296	902 (70%)	Ferro et al., 2010
Thy membrane	179	161 (90%)	Friso et al., 2004
Thy membrane	221	204 (92%)	Peltier et al., 2004
Thy lumen and peripheral proteins	65	62 (95%)	Peltier et al., 2002
Thy lumen and peripheral proteins	95	86 (91%)	Giacomelli et al., 2006
Thy lumen	47	45 (96%)	Schubert et al., 2002
Stroma	234	205 (88%)	Peltier et al., 2006
Stroma	263	230 (87%)	Rutschow et al., 2008
Total	1816	1094 (60%)	All data combined
Final total**	1741	1093 (63%)	All data combined

* Predicted by TargetP and/or Predotar.
** After removal of obvious contaminants or proteins most likely associated with the cp outer envelope, such as 80S-type ribosome subunits. Cp, chloroplast; Thy, thylakoid.

Chloroplasts represent only one of several interconvertible types of plastids. To assess the dynamics of plastid proteomes, other types of plastids, such as etioplasts (von Zychlinski et al., 2005), chromoplasts (Barsan et al., 2010), root plastids (Daher et al., 2010), proplastids (Baginsky et al., 2004; Brautigam and Weber, 2009), and the two types of specialized plastids in C4 plants (Brautigam and Weber, 2009; Majeran et al., 2008) have also been investigated by proteomics.

Previous estimates based on computational predictions and comparative genomics predict that between 2500 and 3000 proteins reside in chloroplasts (Figure 1). At the current rate of progress, cp proteomics appears to be well on the way to experimentally identifying the total inventory of cp proteins. Systematic characterization of the compartmentalization and post-translational modification of cp proteins and the dynamics of the plastid proteome during differentiation has already begun.

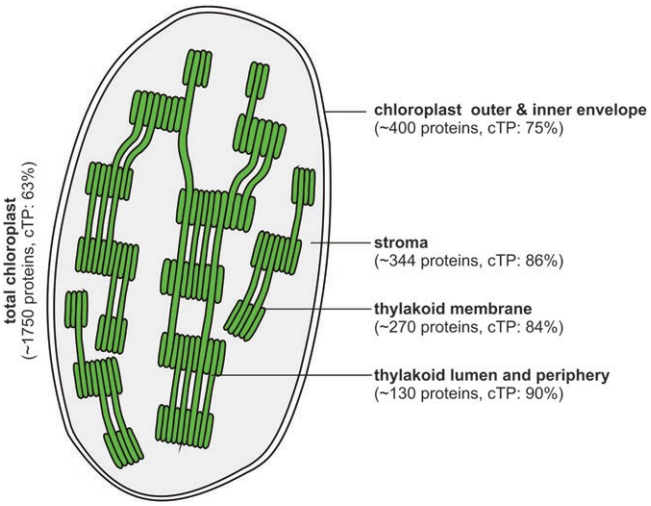

Figure 1. Compartments of Chloroplasts and Numbers of Experimentally Identified Cp Proteins.

Numbers and cTP predictions are based on experimental analysis of (sub)proteomes of the chloroplast according to Table 2. Because the sub-organellar location for some cp proteins is unknown or ambiguous, the numbers on the right add up to only 1141. The total number of cp proteins is estimated to be between 2000 and 3000 proteins (Abdallah et al., 2000; Leister, 2003; Richly and Leister, 2004).

Transcriptomics: Reaching the Next Level: Guilt-by-Association Approaches

It was initially expected that transcriptome analyses would directly elucidate the function of genes based on a simple comparison of the transcript profile of wild-type with that of any given mutant. It soon became clear, however, that the secondary and pleiotropic effects of mutations blur specific signatures, making it necessary to study transiently induced defects instead of stable mutations (see section on 'Post-Translational Modifications' below). Another application for data from transcriptome analyses, the 'guilt-by-association' approach, emerged recently and is based on the concept that genes with similar functions often display similar transcriptional profiles. At the level of nuclear genes for cp proteins, it was shown that genes for photosynthesis or the plastid gene expression machinery indeed exhibit a high level of co-expression at the transcript level (Biehl et al., 2005). This finding was exploited to systematically characterize by reverse genetics genes of unknown function that exhibited photosynthesis gene-like transcriptional profiles and led to the identification of PGRL1, a central component of cyclic electron flow around photosystem I (PSI) (DalCorso et al., 2008) (Table 3). In a similar way, putative subunits of the NAD(P)H dehydrogenase (NDH) complex (Takabayashi et al., 2009), in vivo targets of the cp 2-cysteine peroxiredoxin (2-CysPrx) (Muthuramalingam et al., 2009), a putative cp metabolite transporter (Sawada et al., 2009), and novel factors involved in Chl degradation (Ren et al., 2010) and sulfolipid biosynthesis (Okazaki et al., 2009) have been identified and functionally characterized (Table 3).

These results demonstrate that the transcriptomic guilt-by-association approach, in combination with reverse genetics, provides a powerful tool for elucidating cp functions. It appears that this strategy can be applied to diverse functional groups of cp proteins. For A. thaliana, patterns of co-expression can be analyzed with the ATTED-II (Obayashi et al., 2009),

Table 3. Selection of Genes with Functions Assigned by Transcriptomic (T) or Comparative Genomic (CG) Guilt-by-Association Analyses.

Gene	Approach	Function	Reference
PGRL1A, PGRL1B	T	Cyclic electron flow around PSI	DalCorso et al., 2008
BASS5	T	Putative glucosinolate intermediate transporter	Sawada et al., 2009
UGP3	T	Sulfolipid biosynthesis	Okazaki et al., 2009
CRN1	T	Chlorophyll degradation	Ren et al., 2010
RARE1	CG	Editing of the cp accD transcript	Robbins et al., 2009
NDF1 (NDH48)	T, CG	NDH complex subunit	Takabayashi et al., 2009
NDF2 (NDH45)	T, CG	NDH complex subunit	Takabayashi et al., 2009
NDF4	T, CG	NDH complex subunit	Takabayashi et al., 2009
NDF6	T, CG	NDH complex subunit	Ishikawa et al., 2008

Genevestigator (Zimmermann et al., 2004), Arabidopsis Co-expression Tool (ACT) (Manfield et al., 2006), and CressExpress (www.cressexpress.org) databases.

Comparative Genomics: Conservation of Genes and their Functions

Besides A. thaliana, the genomes of several photoautotrophic eukaryotes and cyanobacteria have been sequenced over the past several years (Table 4). Furthermore, large EST databases are now available for a number of other plant species. This wealth of information has paved the way for phylogenetic profiling—a bioinformatic approach that identifies functionally linked proteins based on their presence in species that display common physiological capabilities (Pellegrini et al., 1999).

Table 4. Sequenced Genomes of Photoautotrophic Eukaryotes.

Species	Nuclear genome size*	Chloroplast genome sizes**
Flowering plants		
A. thaliana	120 Mb/Arabidopsis Genome Initiative, 2000	154 kb
O. sativa	430 Mb/Goff et al., 2002; Yu et al., 2002	135 kb
A. lyrata	207 Mb/phytozome	Ns
C. rubella	250 Mb/JGI	Ns
B. distachyon	272 Mb/phytozome	135 kb
G. max	975 Mb/Schmutz et al. 2010	152 kb
M. guttatus	430 Mb/phytozome	Ns
P. trichocarpa	403 Mb/Tuskan et al., 2006	157 kb
C. sativus	203 Mb/phytozome	156 kb
V. vinifera	487 Mb/Jaillon et al., 2007; Velasco et al., 2007	161 kb
C. papaya	135 Mb/Ming et al., 2008	160 kb
R. communis	400 Mb/phytozome	Ns
S. bicolor	698 Mb/Paterson et al., 2009	141 kb
Z. mays	2061 Mb/Schnable et al., 2009	140 kb
Mosses		
S. moellendorfii	213 Mb/JGI	144 kb
P. patens	480 Mb/Rensing et al., 2008	123 kb
Algae		
C. reinhardtii	120 Mb/Merchant et al., 2007	204 kb
O. tauri	12.6 Mb/Palenik et al., 2007	72 kb
C. merolae	16.5 Mb/Nozaki et al., 2007	150 kb
T. pseudonana	32.4 Mb/Bowler et al., 2008	129 kb
D. salina	130 Mb/JGI	Ns
M. pusilla	23 Mb/Worden et al., 2009	42 kb

* The size of the sequenced nuclear genome, as well as the corresponding publication or website phytozome (www.phytozome.net/; JGI, www.jgi.doe.gov/genome-projects/) are provided.
** Sizes of plastomes were obtained from www.ncbi.nlm.nih.gov/genomes/. ns, not sequenced.

In the field of plant sciences, a comparative analysis of multiple plant and non-plant genomes has led to the identification of genes specific for, and conserved in, cp-containing photosynthetic organisms, the so-called 'GreenCut' (Merchant et al., 2007). Not surprisingly, most 'GreenCut' genes code for cp proteins, but many of these are of unknown function and their identification provides the starting point for further analyses of novel cp functions (Grossman et al., 2010).

Like the transcriptomic guilt-by-association approach, comparative genomics has enabled the tentative assignment of functions to novel genes. One example concerns the NDH complex, which is known to be derived from the cyanobacterial endosymbiont and is present in all green plant species except the chlorophyte algae (e.g. C. reinhardtii). Genes for novel NDH subunits were recently identified among the subset of Arabidopsis genes that have close homologs in cyanobacteria, but not in Chlamydomonas or other non-photosynthetic organisms (Takabayashi et al., 2009) (see section on 'Photosynthesis: New Insights into Non-Photochemical Quenching, Thylakoid Phosphorylation, and Alternative Electron Pathways' below and Table 3).

PROGRESS IN ELUCIDATING PHYSIOLOGICAL PROCESSES LOCALIZED IN CHLOROPLASTS

Substantial progress has been made in elucidating the metabolic processes that occur in chloroplasts, so that only a selection can be highlighted here. A more complete overview can be obtained by consulting the November 2009 special issue of Molecular Plant on chloroplasts.

Protein Uptake: Entering the Chloroplast without a Transit Peptide

Although most cp proteins are synthesized in the cytosol as precursor proteins containing a presequence (cTP), and post-translationally imported via the sequential action of the translocons of the outer and the inner cp envelope (Toc, Tic) (reviewed by Agne and Kessler, 2009; Benz et al., 2009; Oreb et al., 2008; Sommer and Schleiff, 2009), not all cp proteins possess a cleavable cTP. For instance, most proteins of the outer cp envelope membrane clearly lack a cTP and are directed to their destination by intrinsic targeting information (Jarvis, 2008; Soll and Schleiff, 2004). Furthermore, evidence for an ER-dependent targeting pathway for the Arabidopsis carbonic anhydrase 1 (CAH1) protein has been provided (Villarejo et al., 2005). Targeting of nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) to the chloroplast was later reported to follow a similar pathway (Nanjo et al., 2006). Based on its similarities to cp protein transport in many algal groups and apicomplexan parasites, the CAH1 pathway might represent an ancestral co-translational targeting mechanism that arose prior to the evolution of the now dominant post-translational Toc/Tic system (Villarejo et al., 2005). Yet another

potential mechanism of cp targeting has emerged with the finding that cytosolic mRNA encoding the eukaryotic translation factor 4E can be imported into chloroplasts (Nicolai et al., 2007).

A survey of data from large-scale proteomic studies implied that the fraction of cp proteins that are not imported by the canonical Toc/Tic machinery might be as large as ;30% (Armbruster et al., 2009). Extrapolations based on experimental validation of the sub-cellular location of putative non-canonical cp proteins suggest that the fraction of cp proteins that enter the inner compartments of the organelle, although they lack a cTP, might actually be in the region of 10–15% of the total cp proteome (Armbruster et al., 2009). Whether or not cytosolic proteins that associate with the cp outer membrane can account for inflated estimates of non-canonical cp proteins is still open. In vitro import studies with chloroplasts suggest that many proteins that are not imported into chloroplasts nevertheless can stably attach to the cytosolic side of the cp envelope (Armbruster et al., 2009), but a semi-quantitative analysis of cp subproteomes has concluded that true cytosolic contaminants make up only 0.3% of the envelope proteome (Ferro et al., 2010).

Chloroplast Gene Expression: Many More Regulators than Target Genes/Transcripts

Basic features of the structure and expression machinery of cp genes, including the organization of cp genes in operons, their polycistronic expression, and post-transcriptional processing, reflect the prokaryotic ancestry of the organelle. However, unlike bacterial genes, some cp genes possess introns and RNA editing also takes place. Both inherited and newly acquired characteristics of cp gene expression require regulatory proteins. These proteins are encoded by the nucleus, enabling it to exercise 'anterograde' control over cp gene expression (which is itself primarily regulated at post-transcriptional and translational levels). One interesting aspect of the nuclear control of organellar gene expression involves the action of diverse families of eukaryotic RNA-binding proteins including PPR (pentatricopeptide repeat), CMR (cp RNA splicing and ribosome maturation), and PORR (plant organelle RNA recognition) proteins (Kroeger et al., 2009; Schmitz-Linneweber and Small, 2008; Stern et al., 2010) within the organelle. During the evolution of flowering plants, the PPR protein family (which appears to have originated from the tetratricopeptide repeat (TPR) domain that otherwise serves to bind other proteins) in particular has greatly expanded. PPR proteins are involved at multiple stages in cp gene expression (for instances, see Table 1) and are highly specific: each of them binds to only one to three transcripts in chloroplasts (Schmitz-Linneweber and Small, 2008). A further group of nucleus-encoded proteins of eukaryotic origin that are involved in cp gene expression is represented by the cp ribonucleoproteins (cpRNPs). These proteins, too, are involved in multiple cp RNA processing steps. However, in contrast to most PPR proteins, they seem to bind multiple RNA targets (Tillich et al., 2009).

Photosynthesis: New Insights into Non-Photochemical Quenching, Thylakoid Phosphorylation, and Alternative Electron Pathways

Non-Photochemical Quenching

To protect the photosynthetic apparatus from oxidative damage, xanthophyll pigments (see also section on 'Metabolic Pathways Located in Chloroplasts: Novel Insights into Compartmentalization and Regulation' below) are involved in the quenching of excited chlorophyll and reactive oxygen species. Quenching of excited chlorophyll molecules results in harmless dissipation of excitation energy as heat and is measured as non-photochemical quenching (NPQ) of chlorophyll fluorescence. The multiple roles of xanthophylls in photoprotection have been addressed on the basis of identifying mutants with decreased NPQ, leading to the identification of lines impaired in the xanthophyll cycle enzymes violaxanthin deepoxidase (NPQ1, VDE) and zeaxanthin epoxidase (ABA1, NPQ2, ZEP) (Niyogi et al., 1998; Table 1). In addition to specific xanthophylls, PsbS, a LHC protein, is necessary for NPQ in vascular plants (Li et al., 2000). Interestingly, the green alga C. reinhardtii uses LHCSR, a different member of the LHC protein superfamily, to dissipate harmful excess light energy instead of PsbS (Peers et al., 2009). This indicates that vascular plants and green algae employ different LHC proteins to regulate photosynthetic light harvesting in excess light.

Thylakoid Phosphorylation

Changes in incident light elicit alterations in thylakoid protein phosphorylation, which results in a reorganization of the photosynthetic machinery (Dietzel et al., 2008; Eberhard et al., 2008; Ruban, 2009; Steiner et al., 2009; Tikkanen et al., 2010, 2008, 2006). The effect is to balance the distribution of energy between the photosystems and thereby optimize photosynthetic efficiency. In the short-term response (state transitions), redistribution is mediated by reversible phosphorylation and migration of LHCII proteins between photosystems (Rochaix, 2007). If unbalanced excitation persists for many hours, the long-term response (LTR) sets in, which alters the molar ratio of the photosystem complexes according to light quality (Dietzel et al., 2008; Pfannschmidt et al., 1999, 2001). It was previously shown that the redox-dependent regulation of psaA and psbA expression, which is essential for the LTR (Pfannschmidt et al., 2001), involves changes in the phosphorylation state of small plastid DNA-binding proteins (Steiner et al., 2009). In land plants, both State Transitions and the LTR require the thylakoid protein kinase STN7 (Bellafiore et al., 2005; Bonardi et al., 2005; Pesaresi et al., 2009). Only recently, the long-sought LHCII phosphatase TAP38/PPH1 was identified in A. thaliana (Pribil et al., 2010; Shapiguzov et al., 2010). Loss of TAP38/PPH1 leads to a permanent block in State 2 and an irreversible increase in PSI antenna size. Moreover, under certain light conditions, thylakoid electron flow and growth are enhanced in lines lacking TAP38/PPH1 (Pribil et al., 2010; Shapiguzov et al., 2010). Chlorophyll

fluorescence lifetime imaging microscopy aimed to visualize phospho-LHCII dissociation from PSII during State Transitions in live cells of C. reinhardtii revealed that the dissociated phospho-LHCII formed energy-dissipative aggregations, and it is tempting to speculate that such a pool of unbound energy-dissipative LHCII might also be involved in other photoacclimation modes (Iwai et al., 2010b).

The role of PSII core protein phosphorylation mediated by the kinase STN8 is less clear and still under debate (Bonardi et al., 2005; Tikkanen et al., 2008). It is currently thought that PSII protein phosphorylation regulates the degree of folding of the thylakoid membranes, modulating the lateral mobility of the proteins therein (Fristedt et al., 2009).

Alternative Electron Pathways

In cyclic electron flow (CEF), a transthylakoid pH gradient is generated without the involvement of photosystem II (PSII). Two CEF pathways are known, one (antimycin A sensitive) dependent on ferredoxin (Fd), the other on the NAD(P)H dehydrogenase complex (NDH). Whereas the latter pathway is found in all photosynthetic organisms except conifers and green algae, some components of Fd-dependent CEF are not encoded in cyanobacterial genomes. The two pathways functionally overlap, as indicated by the more severe phenotype of mutants that lack both (Munekage et al., 2004).

Fd-dependent CEF requires a thylakoid complex that contains the PGR5 and PGR5-like1 (PGRL1) proteins (DalCorso et al., 2008; Munekage et al., 2002). The corresponding genes were identified by classical forward genetics (Table 1) and the guilt-by-association approach (Table 3), respectively. The existence of a supercomplex consisting of PSI, Cyt b_6/f, PGRL1, and PGR5 was postulated on the basis of split-ubiquitin interaction data (DalCorso et al., 2008), and indeed such a complex has since been tentatively identified in C. reinhardtii (Iwai et al., 2010a). Besides its role in CEF, Chlamydomonas PGRL1 was also found to be up-regulated under iron deprivation, to bind iron and to play an important role in thylakoid rearrangement (Petroutsos et al., 2009).

The cp NDH complex is of cyanobacterial origin and contains both cp- and nucleus-encoded subunits. The discovery of new subunits and assembly factors for the cp NDH complex is a still ongoing process (Battchikova et al., 2005; Hashimoto et al., 2003; Ishida et al., 2009; Ishikawa et al., 2008; Kotera et al., 2005; Okuda et al., 2009; Peng et al., 2008; Rumeau et al., 2005; Shikanai, 2007; Shimizu et al., 2008; Shimizu and Shikanai, 2007; Takabayashi et al., 2009; Tillich et al., 2009), to which forward genetics and guilt-by-association approaches have contributed (see Tables 1 and 3). Like PGR5/PGRL1, the cp NDH complex is associated with PSI (Peng et al., 2009, 2008). Open questions regarding the function of the NDH complex relate to the fact that not all cp counterparts of the cyanobacterial subunits of the electron donor binding subcomplex have yet been identified, and the actual substrate of the cp NDH complex is still under debate (Munekage et al., 2004; Rumeau et al., 2005).

In addition to linear and cyclic electron, also the plastid terminal oxidase (PTOX) is thought to modify the redox state of the plastoquinone (PQ) pool by accepting electrons from PQ and transferring them to oxygen to produce water. PTOX has been suggested to act as an electron safety valve that would prevent overreduction of PSII acceptors and avoid photoinhibitory damages at PSII (reviewed in Rumeau et al., 2007). In contrast to this idea, the reduction state of the PQ pool, measured as 1-qP, is increased and decreased in overexpressors and loss-of-function mutants, respectively, of the IMMUTANS (IM) protein, the tentative PTOX (Rosso et al., 2006). Recently, the relationship of CEF and IM function was analyzed by double and triple mutant analyses in A. thaliana (Okegawa et al., 2010). CEF mutations seem to suppress the im phenotype and vice versa, allowing the conclusion that PSI cyclic electron transport is already operating in early chloroplast development and that the im defect alleviates stromal overreduction in the PSI cyclic mutants (Okegawa et al., 2010). Because alternative electron pathways also operate in organisms that constitute the phytoplankton, the PTOX pathway has also implications for how phytoplankton acclimates to the conditions in the open ocean and for how phytoplankton primary productivity can be reliably assessed by chlorophyll fluorescence measurements (Zehr and Kudela, 2009).

Metabolic Pathways Located in Chloroplasts: Novel Insights into Compartmentalization and Regulation

Recent advances in proteomics and biochemistry have enhanced our understanding of how metabolic processes in chloroplasts are regulated and compartmentalized. Some of these are discussed in the following.

Tetrapyrroles and Carotenoids

All the major chloroplast compartments—thylakoid, envelope, and stroma—are involved in the synthesis and breakdown of chlorophylls (Joyard et al., 2009a). All steps required for biosynthesis of the intermediate protoporphyrinogen IX occur in the stroma. Enzymes that catalyze subsequent modifications of this compound are associated either with the envelope membranes or with the thylakoids, probably to facilitate channeling into chlorophyll, heme, or phytochromobilin biosynthesis. Proteomics studies also suggest that all three compartments participate in the breakdown of chlorophyll (Joyard et al., 2009a).

The enzymes of the xanthophyll cycle (see also section on 'Photosynthesis: New Insights into Non-Photochemical Quenching, Thylakoid Phosphorylation, and Alternative Electron Pathways above), violaxanthin deepoxidase (VDE) and zeaxanthin epoxidase (ZEP), are both localized at the thylakoid membrane. ZEP catalyzes the synthesis of violaxanthin, which is subsequently converted to neoxanthin. In addition to its function as a photosynthetic pigment, violaxanthin also serves as the C_{40} precursor of ABA synthesis, and ZEP is also found at the cp envelope membrane (Joyard et al., 2009a). This implies that the xanthophyll cycle and the synthesis of neoxanthin and

ABA precursors from zeaxanthin are spatially separated and that the two pathways probably do not interact. It therefore is questionable whether ABA synthesis is regulated by the availability of its xanthophyll precursors through the xanthophyll cycle.

Galactolipid Biosynthesis

Cp membranes contain distinctively high levels of the galactoglycerolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and both are synthesized within the organelle. MGDG is produced by the action of MGDG synthase, which transfers galactose from UDP-galactose to diacylglycerol and resides in the envelope membranes (Joyard et al., 2009b). MGD1, the major isoform, is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis (Kobayashi et al., 2007). Recent analyses of this enzyme have shown that it is allosterically activated by phosphatidic acid and phosphatidylglycerol, suggesting that it represents a key regulatory link between phospholipid and galactolipid synthesis in plants (Dubots et al., 2010). For DGDG synthesis, two mechanisms have been reported: the addition of galactose from UDP-galactose to MGDG is catalyzed by the DGDG synthase, while the transfer of a galactose from one MGDG molecule to another (with the release of diacylglycerol) is mediated by GGGT (Joyard et al., 2009b). Two DGDG synthases, DGD1 and DGD2, have been described, both of which reside in the cp envelope. mgd1 and dgd1 mutants are strongly deficient in photosynthesis (Dörmann et al., 1995). One of the functions of MGDG in photosynthesis was recently elucidated: it promotes de-epoxidation of the xanthophyll pigment violaxanthin (Schaller et al., 2010), an important step in the feedback de-excitation of excess light energy.

Starch Biosynthesis and Breakdown

ADP-glucose pyrophosphorylase (AGPase) catalyzes the first committed step in starch synthesis in the plastid, converting glucose 1-phosphate to ADP-glucose. ADP-glucose is subsequently used by starch synthases and branching enzymes to elongate the glucan chains of the starch granule. AGPase activity has been shown to be coupled to the redox state of the chloroplast via thioredoxins (Kolbe et al., 2005). More recently, it was shown that this enzyme is additionally regulated by the NADP-thioredoxin reductase C (NTRC), a single polypeptide chain which contains both an NADP-thioredoxin reductase and a thioredoxin domain. This enzyme can utilize metabolically generated NADPH as an alternative to photo-reduced Fd for the reduction of target proteins. Modulation of AGPase activity by NTRC represents one means by which the non-photosynthetic amyloplasts can regulate starch synthesis in accordance with the energy needs of the whole plant (Michalska et al., 2009).

The initial steps in starch breakdown involve phosphorylation of the starch granule surface, probably to disrupt the semi-crystalline structure of amylopectin. However, dephosphorylation also plays an important role: mutants deficient in either of the protein phosphatases Starch Excess4 (SEX4) and Like SEX4 (LSF1) have substantially higher amounts of starch in their leaves than do wild-type plants (Comparot-Moss et al., 2010; Kötting et al., 2009). In subsequent steps, amylopectin is degraded by a set of glucan hydrolases, and glucose is produced from maltotriose and other maltose-oligosaccharides by disproportionating enzyme (DPE). The two starch metabolites exit the chloroplast via distinct transporters (MEX1 and pGlcT, respectively). Analysis of mex1 and mex1/dpe mutants has indicated that the accumulation of maltose and maltose-oligosaccharides in the chloroplast elicits a retrograde signal (see section on 'Plastid-to-Nucleus Signals: One, Many, None?' below) that triggers cp degradation (Stettler et al., 2009).

Plastid-to-Nucleus Signals: One, Many, None?

Although chloroplasts have their own genome, the vast majority of their proteins are encoded by the nuclear genome, making it necessary to convey information from the chloroplast to the nucleus. This plastid-to-nuclear or retrograde signaling is thought to serve to regulate nuclear gene expression according to need of the organelle and to ensure efficient assembly of multi-protein complexes consisting of cp- and nucleus-encoded subunits. It is also one of the most controversial issues in cp research. Depending on the signal source, four main sources of retrograde signals have been postulated: (1) tetrapyrrole biosynthesis, (2) organellar gene expression (OGE), (3) organellar redox state, and (4) reactive oxygen species. In addition, sugar sensing might overlap and interact—at least in certain tissues—with other retrograde signaling pathways and could therefore modulate the response of nuclear genes to retrograde signals. For a more detailed and critical discussion of retrograde signaling, the reader is referred to Kleine et al. (2009).

To Be or Not Be a Plastid Signal? Mg-Protoporphyrin IX

Pioneering work in plastid signaling came from studies of the so-called genomes uncoupled (gun) mutants. Unlike the wild-type, gun mutants express nuclear genes for the cp proteins Lhcb1 and the small subunit of RubisCO even when chloroplasts are photo-bleached by treatment with the herbicide norflurazon. Four of the corresponding genes (GUN2–GUN5) code for proteins involved in tetrapyrrole biosynthesis. Their characterization led first to the proposal that ChlH, a subunit of the Mg-chelatase, might mediate plastid signaling (Mochizuki et al., 2001), but later the tetrapyrrole pathway intermediate Mg-protoporphyrin IX (Mg-proto IX) was proposed to act directly as a signaling molecule and to traverse the cytosol (Strand et al., 2003). More recent analyses argue against the idea that a tetrapyrrole like Mg-proto IX could operate as a signaling molecule that leaves the chloroplast (Mochizuki et al., 2008; Moulin et al., 2008) and indicate that the gun mutants with defective tetrapyrrole biosynthesis have an enhanced ability to express certain cp genes when treated with norflurazon (Voigt et al., 2010), which links their defect in tetrapyrrole biosynthesis to altered organellar gene expression (see below). Why gun2–gun5 mutants accumulate more LHCB1

and RBCS transcripts than wild-type plants when treated with norflurazon remains enigmatic, but it is tempting to speculate that the drop in chlorophyll biosynthesis might make these mutants more resistant to photo-oxidative stress in chloroplasts, which might indirectly lead to altered plastid signaling.

GUN1: A Novel Key Player

Because treatment with lincomycin, which inhibits translation in chloroplasts, does not repress nuclear photosynthesis gene expression in gun1 mutants as it does in the wild-type, GUN1 may be involved in the transfer of signals derived from altered cp OGE to the nucleus (Gray et al., 2003). Cloning of GUN1 showed that the encoded product belongs to the PPR family (Koussevitzky et al., 2007)—a finding that supports a role in regulating plastid gene expression (see section on 'Chloroplast Gene Expression: Many More Regulators than Target Genes/ Transcripts' above).

What Happens in the Nucleus?

The concept of plastid signaling includes the postulate that following the generation of the retrograde signal(s), the information arrives in the nucleus, where a re-orchestration of nuclear gene expression takes place (Pesaresi et al., 2007). Transcriptomics analyses in A. thaliana have suggested that different layers of transcriptional control over nuclear cp genes exist, including a 'master-switch' that acts in a binary mode to induce or repress the same large set of genes (Biehl et al., 2005; Richly et al., 2003). Recently, the AP2-type transcription factor ABI4 has been proposed to act as a component of this master-switch (Koussevitzky et al., 2007). Moreover, several lines of evidence suggest that GUN1 and ABI4 act in the same signaling pathway. GUN1-dependent signaling might also be involved in coordinating the expression of photosynthesis-related nuclear genes with the efficiency of cp protein import (Kakizaki et al., 2009).

FUTURE TRENDS IN CHLOROPLAST RESEARCH

Adopting Experimental Tools from Other Fields

Other fields in plant and animal research may serve as sources of advanced methods for cp research. In particular, the area of redox regulation of cp functions is emerging as a hot topic in cp research, making it necessary to establish redox markers and in vivo sensors in cp research (Dietz, 2008; Nagahara, 2010). Redox sensitive GFP (roGFP) has already been successfully targeted as sensor of the glutathione redox potential to chloroplasts of Arabidopsis (Meyer et al., 2007; Schwarzländer et al., 2008). HyPer, a hydrogen peroxide sensor tested in cytosol and mitochondria of HeLa cells (Belousov et al., 2006), and Redoxfluor, a redox sensor for cytosol and peroxisomes of yeast and Chinese hamster ovary (CHO) cells (Yano et al., 2010), might represent alternative genetically based redox sensors.

Post-Translational Modifications

While reversible phosphorylation of thylakoid proteins is a well characterized post-translational modification in chloroplasts (Pesaresi et al., 2010), during the last years, protein S-nitrosylation has emerged as the most important mechanism for transduction of the bioactivity of nitric oxide, and also several cp proteins have been described to become S-nitrosylated (for a review, see Lindermayr and Durner, 2009). Glutathionylation is a more recently described redox post-translational modification and represents the major form of S-thiolation in cells by formation of a mixed disulfide between a free thiol on a protein and a molecule of glutathione (Rouhier et al., 2008). Glutathionylation is thought to occur under oxidative stress and can protect cysteine residues from irreversible oxidation, and alter positively or negatively the activity of diverse proteins. In A. thaliana, several cp proteins have been described to be glutathionylated (Rouhier et al., 2008), including thioredoxin f during redox signaling (Michelet et al., 2005). A systematic proteomic approach in C. reinhardtii identified 25 glutathionylation targets, mainly chloroplastic, involved in various metabolic processes (Michelet et al., 2008).

The further improvement of methods to detect S-nitrosylation and glutathionylation with high specificity and sensitivity will be crucial for the full dissection of the impact of these post-translational modifications on the regulation of cp processes.

Prospects of Transcriptome Analyses

Application of deep sequencing approaches towards transcript quantification may provide a cheaper alternative to hybridization-based microarray platforms (Lister et al., 2009). The analysis of inducible systems that allow one to transiently generate or complement lesions in organellar properties, thus enabling reconstruction of effects on nuclear and plastid gene expressions with high temporal resolution (Pesaresi et al., 2007) promises to extend the power of transcriptomics. One emerging system is represented by riboswitches, which are natural RNA sensors that control gene expression via their capacity to bind small molecules (metabolites). They fold into RNA secondary structures whose conformation switches between an 'on' state and an 'off' state in response to ligand binding. Recently, a synthetic translational riboswitch controlled by the ligand theophylline was successfully employed in tobacco chloroplasts (Suess et al., 2004; Verhounig et al., 2010). The ability to activate and shut off specific cp genes at will provides new opportunities for functional genomics and for plastid biotechnology (see section on 'Towards the Chloroplast Interactome' below).

Towards the Chloroplast Interactome

The most direct approach to elucidating protein interactions is the preparative isolation and fractionation of native protein complexes, using one- or multidimensional electrophoreses, chromatography, or density-gradient centrifugations alone or in combination. Matrix-assisted laser desorption-ionization–time-of-flight (MALDI–TOF) tandem mass spectrometry

(MS–MS) (Liu et al., 2008) can then identify the composition of isolated complexes. Although this approach is straightforward, it has been utilized in only a relatively small number of studies of plant protein complexes so far and should be more widely explored.

Identification of protein–protein interactions typically relies upon purification of a bait protein and interacting prey proteins, using either a custom antibody specific for the bait or a commercially available antibody to a peptide or protein epitope tag fused to the N or C terminus of the bait (Berggard et al., 2007; Morsy et al., 2008). Alternatively, multiple affinity tags might be employed, allowing two consecutive or 'tandem' affinity purification (TAP) steps, usually under mild and selective elution conditions that reduce the risk of contamination (Rigaut et al., 1999). The intact complexes can then be easily eluted by proteolytic cleavage of the affinity-bound tag, resulting in protein preparations of suitable purity for MS/MS based protein identification approaches (Kocher and Superti-Furga, 2007). High-throughput protein microarrays have great potential for obtaining large-scale experimental interactome data (Hall et al., 2007; LaBaer and Ramachandran, 2005). These arrays consist of full-length proteins or protein domains immobilized onto the surface of a glass slide, allowing interactions to be detected using fluorescence or chemiluminescent probes.

Interaction networks can be predicted on the premise that orthologous proteins that are known to interact in one organism can interact in the system under study (Sharan et al., 2005). This approach was used recently to predict the interactome of Arabidopsis (Geisler-Lee et al., 2007; Yu et al., 2008). Predictions are still at an early stage (Leister and Kleine, 2008), but are expected to improve rapidly as more interactome data become available in plants. The STRING database combines physical and functional protein–protein interactions from 630 organisms (Jensen et al., 2009).

Systems Biology

The combinatorial analysis of multiple (transcriptomics and other 'omics') datasets will be essential for the in silico reconstruction of regulatory networks (Nacu et al., 2007). Eventually, a cp development or functional state-driven nuclear gene expression network would be built based on genome-wide identification of transcription factors via yeast one-hybrid assays. Additionally, their direct and indirect target genes will be identified using chromatin immunoprecipitation with an antibody against such transcription factors followed by a deep sequencing approach and identification of genes whose expression levels in the loss-of-function mutant background are affected (Jung and Chory, 2010).

Tailored Chloroplasts

The ability to transform chloroplasts by homologous transformation and drive high-level expression of transgenes in chloroplasts, coupled with their maternal mode of inheritance in most species of interest, make chloroplasts a prime target for biotechnological improvement of crop plants. However, commercial varieties harboring transgenic chloroplasts have not been generated yet (Bock and Warzecha, 2010; Maliga, 2003).

One promising target for modifying chloroplast functions is photorespiration. Photorespiration results from the oxygenase reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase and serves as a carbon recovery system. It comprises enzymatic reactions distributed in chloroplasts, peroxisomes, and mitochondria (Maurino and Peterhansel, 2010). The Escherichia coli glycolate catabolic pathway has been introduced into A. thaliana chloroplasts to reduce the loss of fixed carbon and nitrogen that occurs in C_3 plants when phosphoglycolate, an inevitable by-product of photosynthesis, is recycled by photorespiration (Kebeish et al., 2007). The resulting transgenic plant produced more biomass, giving rise to the hope that the manipulation of photorespiration and/or photosynthesis (TAP38/PPH1, see section on 'Photosynthesis: New Insights into Non-Photochemical Quenching, Thylakoid Phosphorylation, and Alternative Electron Pathways' above) can indeed be used to improve agronomic performance.

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