The nucleotide-binding sub-proteome of mustard chloroplasts and its involvement in plastid redox signaling

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Abbreviations

2D	two dimensional
ADP	adenosine diphosphate
Alpha-NAC-like	Alpha-nascent polypeptide associated complex like
AtECB1	Arabidopsis early chloroplast biogenesis 1
ATP	adenosine triphosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
CAC3	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha
CRU3	cruciferin 3
CSK	chloroplast sensor kinase
CSP41	chloroplast steem loop binding protein of 41 kDa
cTP	chloroplast transit peptide
Cytb_6f	cytochrome b6f
DBMIB	2,5-Dibromo-3-methyl-6-isopropyl-p-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea)
DNA	desoxyribonucleic acid
EF1-alpha4	Elongation factor 1-alpha4
EF-tu	Elongation factor tu
eIF	Eukaryotic translation initiation factor
EMSA	electrophoretic mobility shift assay
ESI-MS/MS	electron spray ionisation-tandem mass spectrometry
FabZ	Fatty acid biosynthesis z
FLN1	Fructokinase-like 1
FSD	Iron superoxide dismutase
HS	heparin Sepharose
Hsp	heat shock protein
Hsp70	heat shock protein of 70 kDa
IEF	isoelectric focussing
IF	initiation factor
kDa	kilo-Dalton
Lhc	light harvesting complex
lTP	luminal transit peptide
LTR	long term response
MDH	Malate dehydrogenase
MFP2	Multi functional protein 2
MLS	Malate synthase
MS	mass spectrometry
MurE-ligase	UDP-N-acetylmuamoylalanyl-d-glutamate-2,6-diaminopimelate ligase
NAD+/NADH	nicotinamide adenine dinucleotide (oxidized/reduced)
NADP+/NADPH	nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
NEP	nucleus-encoded plastid RNA-polymerase
NL	non linear
nm	nano meter
P5CR	Pyrroline-5-carboxylate reductase
PAGE	poly-acrylamide gel electrophoresis
PAP	PEP associated protein
PC	phosphocellulose

PEP	plastid-encoded plastid RNA-polymerase
PET	photosynthetic electron transport
PLP	pyridoxalphosphat
PQ	plastoquinone
PS I, II	photosystem I, II
PSAT	Phosphoserine aminotransferase
pTAC	plastid transcriptionally active chromosome
RbcS	RubisCO small subunit
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNAs	ribosomal ribonucleic acid
RubisCO	Ribulose-1,5-bisphosphate-carboxylase/-oxygenase
SDS	sodium-dodecyl sulphate
SHMT	Serine hydroxymethyltransferase
SpoU methylase	tRNA/rRNA methyltransferase
STN7	state transition 7 kinase
STR	short-term response
TCP1	T-complex protein 1
Tic	Translocon of the inner membrane
Toc	translocon of the outer membrane
tRNA	transfer ribonucleic acid
TRXz	Thioredoxin z
λ	wavelenght

1 Introduction

1.1 Chloroplasts as location for photosynthesis

The chloroplasts as typical organelles of plants and algae fulfill an enormous achievement by converting solar energy into a useable form for themselves and other organisms and thus ensure life on earth. The process of photosynthesis takes place in chloroplasts thylakoid membranes where light energy, carbon dioxide and water are transferred into organic material and oxygen.

Embedded in thylakoids are two photon-catching photosystems, photosystem II (PSII) and photosystem I (PSI), each consisting of a reaction center with light wavelength absorption maxima at λ=700nm (PSI) and λ=680nm (PSII) and associated light harvesting complexes (Lhc). These photosystems are crucial for capturing solar energy and transmit it to excite electrons. For linear photo-phosphorylation the photosystems are connected in series via the photosynthetic electron transport (PET) chain of several electron carriers. They transfer electrons generated by water oxidation on the manganese cluster of the oxygen evolving complex *inter alia* via plastoquinol and the cytochrome *b6f* complex to ferredoxin in order to reduce NADP+ to NADPH by the ferredoxin-NADPH-oxidoreductase and thus fix the energy. During electron transport a proton gradient between thylakoid lumen and stroma is generated which drives ATP synthesis by ATP synthase. This classical Z-scheme of photosynthesis may be diversified to the cyclic electron transport which involves only PSI. Instead of reducing NADP+ electrons were directly transferred back to the cytochrome *b6f* complex and the proton gradient for ATP synthesis is generated.

This so-called light reaction is complemented by carbon dioxide fixation and reduction in the Calvin-Benson cycle. In this dark-reaction the regeneration of ADP and NADP+ for reuse in the light reaction takes place (Aro and Andersson 2001; Blankenship 2002).

1.2 Plastids as endosymbiotic organelles

Plant cell organelles, mitochondria and plastids, have an endosymbiotic origin and evolved out of unicellular ancestors. Thereby chloroplasts progenitors are unicellular photosynthetic algae (Mereschkowsky 1905, Martin *et al*. 1999, Blankenship 2001, Willis and McElwain 2002, Martin *et al*. 2002, Dyall *et al*. 2004, Kutschera and Niklas 2005). Most of the ancestor genes are integrated into the nuclear genome of plant cells during evolution or even lost and thus organelles are strongly dependent on nuclear gene expression (Martin *et al*. 2002, Timmis *et al*. 2004). The comparison of the nuclear genome of *Arabidopsis thaliana* (L.) with prokaryotic, cyanobacterial and yeast genomes showed that approximately 18% of the *A. thaliana* genome originates from the cyanobacterial ancestor of plastids (Martin et al. 2004; Leister 2003). Nevertheless organelles retain their own gene expression machinery and genes for important organellar processes as relict of endosymbiosis and hence semiautonomy.

Even the gene expression machinery as well as the photosynthetic apparatus and all other structural and metabolic elements of chloroplasts comprise a mixture of plastid and nucleus encoded proteins. All in all the chloroplast proteome consists of about 3000 proteins but only about 80 of them are plastid encoded (Leister 2003; Richly and Leister 2004). The detailed composition of the plastid proteome and sub-proteomes are fragmentary but clarified within this work concerning the constitution of the plastid gene expression machinery.

The majority of plastid proteins is encoded by the nuclear genome, translated on cytosolic ribosomes and transferred into plastids across the organelle membranes via the Toc (translocon of the outer membrane) and Tic (Translocon of the inner membrane) translocation systems (Soll 2002; Jarvis and Soll 2002; Soll and Schleiff 2004; Inaba and Schnell 2008; Li and Chiu 2010; Shi and Theg 2013). For recognition and initialization of this translocation process nucleus encoded chloroplast proteins carry N-terminal transit peptides (cTP) as well as luminal transit peptides (lTP) when further on directed into the thylakoid lumen (Robinson *et al*. 1998; Bruce 2000). Organellar TPs share similarities which differentiate them to cytosolic proteins. TPs and their length are predictable by online tools like TargetP and ChloroP (Emanuelsson *et al*.

2000). That offers the possibility to distinguish between organellar and cytosolic plastid proteins in an easy and fast manner. Limitations of this TP prediction were discussed at several parts within this work (e.g. Schröter *et al.* 2010; Schröter *et al.* 2014).

1.3 Plastid gene expression

About 120 of original 3000-7000 cyanobacterial genes are maintained in plastids (Timmis *et al.* 2004). The plastid genome (plastome) encodes tRNAs, rRNAs and proteins for photosynthesis, gene expression and some other biological processes (Bedbrook *et al.* 1979; Ellis 1981; Dyer 1984; Sugiura 1992; Martin *et al.* 2002; Timmis *et al.* 2004). Two different types of RNA polymerases conduct transcription in plastids. The single-subunit and phage-type nucleus-encoded plastid RNA-polymerases (NEP) are particularly active during early developmental stages of plastids and the plastid-encoded plastid RNA-polymerase (PEP), as an *Escherichia coli* (*E. coli*)-like multisubunit protein complex, executes the main transcriptional activity (Hess and Börner 1999; Cahoon and Stern 2001; Lysenko and Kuznetsov 2005; Shiina *et al.* 2005; Liere *et al.* 2011; Yagi and Shiina 2014). The PEP consists of four different plastid encoded subunits 2α, β, β' and β'', creating the core enzyme, complemented by nucleus encoded σ factors for promoter recognition and hence nuclear control of plastid gene expression via PEP (Igloi and Kossel1992; Link 1996; Allison 2000; Shiina *et al.* 2005; Schweer *et al.* 2010; Lerbs-Mache 2011). For proper function the core enzyme is complemented further on by PEP associated proteins. The analysis of the PEP and its essential associated proteins is one main part of this work.

Founded in the endosymbiotic origin of plastids the translational process is performed on 70S ribosome with a strong sequence homology to eubacterial ribosomes. The plastid 50S subunit contains 33 subunits with 31 orthologues to *E. coli* and the two plastid specific subunits and the 30S subunit is composed of 21 *E. coli* orthologues and 4 plastid specific proteins with no homologues in other ribosomes (Yamaguchi *et al.* 2000). Also bacterial factors, acting at the stage of translation initiation, elongation and release, own a plastid counterpart in *Chlamydomonas rheinhardtii* and higher plant chloroplasts (Beligni *et al.*

2004). The posttranscriptional processes in plastids are of minor interest for this work and reviewed in excellent articles in detail elsewhere (Tiller and Bock 2014; Wobbe *et al*. 2008; Marin-Navarro 2007; Rochaix 1996).

1.4 The plastid gene expression apparatus

The plastome itself is present in a high copy number at about 20 to 300 copies per chloroplast and depending on tissue and chloroplast number of the plant cell up to multiple thousand plastome copies per cell (Kuroiwa *et al*. 1982; Bendich 1987; Coleman and Nerozzi 1999). Several plastome copies are associated with proteins and RNA to constitute nucleoids which inherent the gene expression activity of plastids (Sato *et al*. 2003; Sakai *et al*. 2004). Composition and structure of plastid nucleoids is quite divers and depends on age, activity of plant cells and plastids and environmental conditions on them (Bendich 2004; Krupinska *et al*. 2013).
Several studies analyzed the composition of the gene expression machinery of chloroplasts. Different purification procedures are effective for isolating different constitutions of it. The three main entities to be distinguished are whole nucleoids, the membrane attached plastid transcriptionally active chromosome (pTAC) and the soluble PEP.
Nucleoids are effective to isolate by differential centrifugation and are present in a huge amount in high molecular weight fractions of plastid preparations (Cannon *et al*. 1999; Olinares *et al*. 2010; Majeran *et al*. 2012; Huang *et al*. 2013). An inventory of proteins in nucleoid preparations for *A. thaliana* and *Zea mays* (L.) at different developmental time points and finally reference proteomes of this preparations are publicized (Olinares et al. 2010; Majeran et al. 2012; Huang et al. 2013). Nevertheless a notably amount of contaminating proteins is isolated together with nucleoids and it is hard to distinguish between proteins essential for gene expression, real components of nucleoids and remaining proteins with further functions.
The pTAC can be separated from other chloroplast components by gel filtration as shown for several species (Hallick *et al*. 1976; Rushlow *et al*. 1982; Reiss and Link 1985; Bülow *et al*. 1987; Krupinska and Falk 1994; Suck *et al*. 1996; Krause and Krupinska 2000; Pfalz *et al*. 2006; Melonek *et al*. 2012). Via modern

proteomic identification methods even the more recent examinations provide a detailed view in TAC composition.

The soluble PEP and its associated proteins were successful prepared via several purification methods. The isolation method is mostly based on anion exchange chromatography of plastid lysates. Heparin-Sepharose (HS) and Phosphocellulose (PC) were suitable column materials for separating PEP and nucleotide binding proteins as shown for several species (e.g. Burgess *et al*. 1969; Bottomley *et al*. 1971; Sun *et al*. 1986; Rajasekhar *et al*. 1991; Tiller and Link 1993; Pfannschmidt and Link 1994, 1997; Pfannschmidt *et al*. 2000; Suzuki *et al*. 2004). Within this work the isolation of plastid proteins by HS- and PC-chromatography was fundamental for further studies on the nucleotide binding sub-proteome of the organelles. The behavior, composition and quality of these preparations were analyzed here and complement the knowledge about plastid gene expression.

1.5 Communication signals of plant cells and chloroplasts

Stress response and acclimation processes to environmental, developmental and metabolic conditions often include changes in plastid protein composition and thus gene expression. Plastid protein complexes contain nuclear and plastid encoded subunits and demand a coordination of plastid and cytosolic gene expression. The communication between plant cell and organelle requires anterograde signals from nucleus to plastids as well as retrograde signals from plastid to nucleus (Taylor 1989; Mayfield 1990; Susek and Chory 1992; Goldschmidt-Clermont 1998; Rodermel 2001; Gray *et al.* 2003; Fey *et al*. 2005b; Dietzel *et al*. 2008b).

The anterograde signaling involves the import of nuclear encoded proteins into plastids and represents the obligatory influence of the plant cell to the organelles as tribute to endosymbiosis. The most obvious example for an influence in plastid gene expression by the nucleus are the sigma-subunits of the PEP. These are nucleus encoded and essential for plastid transcription *via* PEP.

Retrograde signaling is very divers and contains signals of plastid gene expression, signals from pigment biosynthesis and signals dependent on the

redox state. The latter involves effects of the PET and reactive oxygen species (ROS). The diversity of retrograde signals is reviewed in Dietzel *et al*. 2008b.

1.6 Redox signals as mediator in acclimation and stress responses

The variety of retrograde signals is vast representing a responding network which coordinates acclimation processes and connects the cellular compartments. For a detailed understanding of the whole network its single components and reaction cascades have to be analyzed separately. We concentrated on the redox signals and redox control arising from the redox state of components of the PET chain in this work (Pfannschmidt *et al*. 2008; Dietzel *et al*. 2008b; Pfalz *et al*. 2012).

Chloroplasts metabolism and especially photosynthesis has to adapt to external and internal changes in a fast and economic manner. Vastly important for chloroplasts activity is the quality and quantity of light. The divergence of absorption maxima in PS reaction center tends to a stronger activity of one of them under differing light conditions. Changes in illumination disturb the photosynthetic efficiency and result in imbalances of the electron transport. Hence an excess or deficit of electrons generates a reduced or oxidized electron transport chain respectively and triggers acclimation processes of the plastid, cytoplasm or nucleus (Pfannschmidt 2003; Baier and Dietz 2005; Buchanan and Balmer 2005; Pfannschmidt *et al*. 2008; Dietzel *et al*. 2008b). Also ROS like hydrogen peroxide or singlet oxygen may arise from over-excitation or as by-product of photosynthesis and were involved in redox signaling as well (Apel and Hirt 2004; Foyer and Noctor 2005; Dietzel *et al*. 2008b).

The best analyzed source of redox signals of the PET chain is the PQ pool. The oxidized PQ pool signals a less active PSII and a reduced PQ pool indicates minor activity of the PSI. The redox state displays the rate-limitation of one of the PS and initiates counterbalancing short-term (STR) and long-therm responses (LTR) (Pfannschmidt 2003; Baier and Dietz 2005; Buchanan and Balmer 2005; Pfannschmidt *et al*. 2008; Dietzel *et al*. 2008b).

The STR is performed as so called state transition and involves a phosphorylation/ dephosphorylation dependent movement of LhcII to the less

active PS-site for an increased antenna cross-section and thus stronger absorption of photosynthetic active radiation. (Bonaventura and Myers 1969; Murata 1969; Haldrup *et al.* 2001; Allen and Forsberg 2001; Wollman 2001). As signal transducer the thylakoid-associated kinase STN7 is required (Allen *et al.* 1981; Allen 1992; Allen *et al.* 2001; Bellafiore *et al.* 2005; Dietzel *et al.* 2008b; Pesaresi *et al.* 2009). The continuance of excitation imbalances for hours or days result in the LTR *via* an adjustment of the photosystem stoichiometry by plastid and nuclear gene expression (Allen and Pfannschmidt 2000; Dietzel et al. 2008). The redox state of the PQ pool as well as the kinases STN7 and CSK1 are involved in signal transmission for LTR (Bonardi *et al.* 2005; Puthiyaveetil *et al.* 2008; Pesaresi *et al.* 2009).
Beside the activity of the mentioned kinases STN7 and CSK1 only few is known about the redox signal transmission to the nucleus and within the plastids. Any hints for phosphorylation cascade dependent signal transmission exist as well as the idea of a direct passage of PQ molecules from plastids to the nucleus (Escoubas *et al.* 1995; Pfannschmidt 2003; Steiner *et al.* 2009; Shimizu *et al.*, 2010). A thioredoxin mediated signal transduction may involve the PEP associated proteins PAP6/FLN1 and PAP10/TRXz (Arsova *et al.* 2010; Steiner *et al.* 2011; Pfalz *et al.* 2012).
However regulatory proteins which directly interact with the gene expression machinery during LTR are so far unknown.

1.7 Generation and analysis of photosynthetic redox signals

In Pfannschmidt *et al.* 2008 the different experimental setups generating redox signals in plastids are described and discussed in detail giving an overview of the possibilities to analyze their effects and the advantages and disadvantages of the single approaches.
Inhibitors of the PET are useful for creating redox signals. The electron transport at the PSII may be blocked by DCMU (3-(30,40-dichlorophenyl) 1, 10-dimethyl urea) and causes an oxidation of the following PET chain (Trebst 1980; Pfannschmidt *et al.* 2008). DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone) blocks the PET at the Cytb_6f complex and thus creates a

reduced PET chain before it and an oxidized chain after the Cytb_6f complex (Trebst 1980; Pfannschmidt *et al*. 2008).

Photosynthesis mutants may be used for the identification of the origin of a redox signal and its effects. The PET is blocked at a distinct site caused by a mutation of the respective gene and thus a reduced PET chain before and an oxidized after the defect is generated (Yang *et al*. 2001; Sherameti *et al*. 2002; Frigerio *et al*. 2007; Pfannschmidt *et al*. 2008)

Another way for generating redox signals is the use of illumination effects by a shift between two opposed light situations. Dark-light, low-light to high-light and the illumination with light of defined wavelengths that favor PSI (PSI-light) or PSII (PSII-light) and a switch into the respective other light provokes excitation imbalances on the photosynthetic apparatus (Pfannschmidt 2003; Pfannschmidt *et al*. 2008). The PSI-PSII light switch system also generates the redox signal under low light condition and thus largely avoids the formation of ROS and its related reactions (Piippo *et al*. 2006; Wagner *et al*. 2008).

2 Aims

The knowledge about PET redox signal transmission to the responding genes as well as regulating components involved in gene expression is marginal. For a more precise understanding of the processes during redox signal mediated gene expression this work focuses on the analysis of the plastid gene expression machinery in detail, combining classical chromatographic purification procedures with polyacrylamide gel electrophoresis (PAGE) and mass spectrometry. The focus on the isolation and identification of the soluble PEP and proteins associated with opens a way to the complementation of the knowledge about plastid gene expression.

For these analyses mustard (*Sinapis alba* (L.)) is an excellent organism as shown before in divers studies about plastids gene expression (Oelmuller et al. 1986; Tiller and Link 1993; Pfannschmidt and Link 1994; Link 1996; Baginsky et al. 1997). The major advantage of *S. alba* is the fast growing and larger size of mustard cotyledons in comparison to *A. thaliana*. It delivers an enormous amount of biomass within 5-7 days of growing. This facilitates biochemical purification procedures substantial and is crucial when using multiple preparation and purification steps. Fortunately *A. thaliana* as well studied model organism is closely related to mustard. Hence data of *Sinapis* protein analysis are comparable with known data of *A. thaliana* and other *Brassicales*. It should be demonstrated within this work that mustard is a suitable organism for proteome analysis additionally to known model organisms.

For a part of the studies of this work the illumination with PSI and PSII light as well as a PSI-PSII switch light regime was used during mustard cotyledon growth. Thus a distinct redox signal in the electron transport chain and the plastoquinone pool was generated. *S. alba* was compared to *A. thaliana* concerning the physiological reactions to a induced redox signal. This is crucial for the adaption of *A. thaliana* based models and knowledge on mustard.

Further on the nucleic acid-binding sub-proteome of mustard plastids was isolated by HS-chromatography and used for analysis *via* different approaches in Steiner *et al*. 2009, Schröter *et al*. 2010 and Steiner *et al*. 2011 and represents the first purification step in Schröter *et al*. 2014. The quality of HS fractions with regards to contaminations of extra plastidic cell components was

tested in Schröter *et al*. 2010. Moreover a comparison of the isolated HS peak fractions in silver stained SDS polyacrylamide gels and 2D BN-PAGEs reveals an effect of the excitation imbalances on their protein composition (Steiner *et al*. 2009; Schröter *et al*. 2010). The use of PC chromatography in addition to HS chromatography creates a different protein composition again with a stronger focus on nucleotide binding ones as visible by the comparison of the HS and PC proteins in SDS polyacrylamide gels. A more detailed view on the composition of the PC fractions is apparent on 2D gels with isoelectric focussing in the first dimension followed by SDS-PAGE in the second dimension.

Promoter binding studies and the analysis of phosphorylation effects of the isolated HS fractions verify the participation of the isolated proteins on plastid gene expression and an involvement of phosphorylation events in redox signal transmission (Steiner *et al*. 2009).

Finally the proteins of the HS and PC protein fractions resolved on polyacrylamide gels were identified v*ia* electron spray ionisation-tandem mass spectrometry (ESI-MS/MS) and data analysis using *Brassicales* databases (Schröter *et al*. 2010; Steiner *et al*. 2011; Schröter *et al*. 2014). Thus this work contributes considerably to the elucidation of plastid gene expression and its components.

3 Overview of manuscripts

Manuscript I

Dietzel L, Steiner S, Schröter Y and Pfannschmidt T. Plastid-to-nucleus communication in plant cells: Retrograde signalling. In: The Chloroplast - Interaction with the Environment, Plant Cell Monographs 2008 Vol 13. 181-206

This review deals with retrograde signaling and summarizes the different signal sources and pathways from plastids to the nucleus. The signals depending on plastid gene expression, pigment biosynthesis and plastid redox state were discussed in detail and distinguished between the more developmental signals from undifferentiated plastids and the functional and physiological signals from mature plastids. Also the comprehensive view on the network of different signal types is depicted here as well as an integration of mitochondria as further organelles with retrograde signaling.
I was involved in writing the manuscript, preparation of figures and model generation as well as critical discussion of the scripts content. I contributed to 15% this work.

Manuscript II

Pfannschmidt T, Bräutigam K, Wagner R, Dietzel L, Schröter Y, Steiner S, Nykytenko A. Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding. Ann Bot 2009 Feb;103(4):599-607. doi: 10.1093/aob/mcn081. Epub 2008 May 20.

This article represents a review of the photosynthesis induced redox regulation of gene expression in photosynthetic cells. It gives an overview of known redox signal transmission cascades and further possible pathways within plastid and to the nucleus. Moreover approaches for experimental investigations of the redox signals and their targets were discussed, highlighting advantages and disadvantages of the single methods.
I contributed to 5% to this article by critical discussion of the content and counterchecking of the script.

Manuscript III

Steiner S, Dietzel L, Schröter Y, Fey V, Wagner R, Pfannschmidt T. The role of phosphorylation in redox regulation of photosynthesis genes psaA and psbA during photosynthetic acclimation of mustard. Mol Plant 2009 May;2(3):416-29. doi: 10.1093/mp/ssp007. Epub 2009 Feb 27.

In this research article it was investigated if mustard (*Sinapis alba*) is a suitable organism for examinations of redox signaling and long term responses (LTR) and whether it is comparable with *Arabidopsis thaliana* and known data of this plant. The LTR of mustard was examined using plastid-encoded plastid RNA polymerase (PEP) containing protein fractions isolated *via* heparin-Sepharose chromatography. These were analyzed concerning promoter recognition, phosphorylation state and kinase activity.
I contributed to 5% to this work by preparation of the plastid proteins as well as discussing the experimental results and manuscript content.

Manuscript IV

Schröter Y*, Steiner S*, Matthäi K, Pfannschmidt T. Analysis of oligomeric protein complexes in the chloroplast sub-proteome of nucleic acid-binding proteins from mustard reveals potential redox regulators of plastid gene expression. Proteomics 2010 Jun;10(11):2191-204.
* These authors contributed equally to this work

For this work the chloroplast nucleic acid-binding sub-proteome of *Sinapis alba* cotyledons was isolated using heparin-Sepharose chromatography and visualized by 2-D blue native PAGE. The quality of the isolated protein fractions was determined by western-immuno analysis, giving a basis for further studies. Ten oligomeric complexes and eleven further proteins were analyzed *via* ESI-MS/MS and identified using a *Brassicales* reference database and thus mustard could be established as additional model organism for further analysis and identification of plastid proteins.
I participated in protein isolation, mass spectrometry and protein identification, western analysis, knock out mutant characterization, preparation of figures, tables and manuscript writing to 35% to this work.

Manuscript V

Steiner S, Schröter Y, Pfalz J, Pfannschmidt T. Identification of essential subunits in the plastid-encoded RNA polymerase complex reveals building blocks for proper plastid development. Plant Physiol 2011 Nov;157(3): 1043-55. doi: 10.1104/pp.111.184515. Epub 2011 Sep 23.

In this article a catalogue of the essential subunits of the plastid-encoded plastid RNA polymerase was generated. Comparative analysis of biochemical purifications, mass spectrometric identification and the phenotypic characterization of knock out mutants are base of this work.
I contributed to 20% to this work *via* protein isolation, mass spectrometric identification of proteins, writing of the manuscripts and model generation.

Manuscript VI

Pfalz J, Liebers M, Hirth M, Grübler B, Holtzegel U, Schröter Y, Dietzel L, Pfannschmidt T. Environmental control of plant nuclear gene expression by chloroplast redox signals. Front Plant Sci 2012 Nov 19;3: 257. doi: 10.3389/fpls.2012.00257. eCollection 2012.

This review article summarizes the effects of redox signals of the photosynthetic electron transport and its generation by environmental influences. It includes the recent knowledge about signal transduction to the nucleus and thus complements the picture of this to date only poorly examined process. Genetic response pattern to the redox signals as well as an integration of photoreceptor mediated signaling complete the recent picture.
I was involved in critical reading and discussion of the manuscript content and contributed to 3% to this article.

Manuscript VII

Schröter Y, Steiner S, Weisheit W, Mittag M, Pfannschmidt T. A purification strategy for analysis of the nucleotide binding sub-proteome from chloroplasts of mustard cotyledons. Accepted for publication in Frontiers in Plant Science

Based on the HS preparations of Schröter et al. 2010 a second chromatographic step with Phosphocellulose was used in addition to HS for the isolation of the nucleotide binding sub-proteome of *S. alba*. Proteins were visualized on 2D-gels with isoelectric focusing for the first dimension, analyzed by ESI-MS/MS and identified by comparison with a *Brassicales* database. We achieved a stronger enrichment of gene expression related proteins as well as a reduction of contaminants and were thus able to draw a more detailed picture of the gene expression relevant proteome of mustard.

I contributed to 65% to this work by protein isolation, 2D-gelelectrophoresis, protein identification *via* mass spectrometry, manuscript writing and preparation of the figures and tables.

4 Manuscript I

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Plastid-to-nucleus communication in plant cells: Retrograde signalling.

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Retrograde Signalling

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Abstract Plastids are organelles typical for plant cells. They are a metabolic and genetic compartment that is involved in most aspects of the life of a plant. Plastids were acquired by plants via endosymbiosis of a photosynthetically active prokaryotic ancestor. Establishment of this endosymbiosis required communication between the endosymbiont and the nucleus of the host cell. During evolution a complex network evolved that embedded development and function of the new organelle into that of the cell. Today the nucleus controls most functions of plastids by providing the essential proteins. However, there exists a backward flow of information from the plastid to the nucleus. This "retrograde" signalling represents a feedback control reporting the functional state of the organelle to the nucleus. By this means extensive communication between the two compartments is established. This helps the plant to perceive and respond properly to varying environmental influences and to developmental signals at the cellular level. Recent observations have extended our understanding of retrograde signalling. Models are presented that provide an overview of the different known pathways.

1 Introduction

Plastids are organelles that are specific for plant and algal cells. They represent a distinct and indispensable biochemical and genetic compartment which is involved in many essential metabolic processes (Buchanan et al. 2002). Plastids originated from an endosymbiotic event in which a photosynthetic active cyanobacterium was engulfed by a heterotrophic eukaryotic cell. During the establishment of this endosymbiosis the cyanobacterium was step-by-step integrated into the cellular processes of the host cell. This was mainly achieved by the transfer

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of most but not all of the cyanobacterial genes to the nucleus of the host cell. This gene transfer gave the host cell control over development and function of this novel compartment since the organelle became functionally dependent on the coordinated expression and import of its nuclear encoded protein components. As a result of this evolutionary invention an autotrophic, eukaryotic cell evolved that was able to perform photosynthesis. From this chlorophyta and, finally, plants evolved 450–500 million years ago (Stoebe and Maier 2002). Today higher plants possess plastids that are typically surrounded by two membranes. The outer one originated from the engulfing host cell, the inner one from the cyanobacterial ancestor. Its further morphology and function exhibits a high plasticity and depend mainly on the tissue context of the respective cell. For instance in photosynthetic tissues cells contain green chloroplasts while in fruits or flowers cells contain coloured chromoplasts (Buchanan et al. 2002). Nevertheless, all plastid types contain an identical genome (the so-called plastome) with a size of around 120–200 kb. It encodes a relatively conserved set of 100–130 genes, which code mainly for components of the photosynthetic apparatus and the plastid-own gene expression machinery which controls the expression of the genetic information on the plastome (Sugiura 1992). However, the largest part of the plastid protein complement is encoded in the nucleus. Current estimates of plastid protein number range from 2,500–4,500 different proteins in these organelles depending on the plastid type (Abdallah et al. 2000; van Wijk 2000; Kleffmann et al. 2004).

All prominent multi-subunit protein complexes of plastids are comprised of a patchwork of plastid and nuclear encoded subunits. Therefore, establishment, assembly and maintenance of these complexes require the coordinated expression of genes in the two different genetic compartments. This coordination is established via extensive flow of information from the nucleus to the plastid (anterograde signalling), i.e. the import of nuclear-encoded plastid proteins. However, there exists a feedback control that signals information about developmental and functional state of the plastid toward the nucleus (retrograde signalling), which induces appropriate changes in the expression of the nuclear-encoded plastid proteins. By this means plastids can control their own protein complement and adapt it to the present developmental and functional state. This complex network of anterograde and retrograde signalling is a major component of plant cell signal networks and contributes to a large extent to plant development and environmental acclimation (Bräutigam et al. 2007). Retrograde signalling has attracted much interest in the last decade and a lot of excellent reviews have been written about it (Rodermel 2001; Jarvis 2001; Papenbrock and Grimm 2001; Gray et al. 2003; Strand 2004; Beck 2005; Nott et al. 2006; Pesaresi et al. 2007). Research data from the last years clearly demonstrated that several different plastid signals exist which are active under different developmental or functional conditions. These signals depend on (1) plastid gene expression (Fig. 1), (2) pigment biosynthesis (tetrapyrrole and carotenoid synthesis) (Figs. 2 and 3) and (3) plastid redox state (photosynthetic electron transport and reactive oxygen species (ROS) accumulation) (Fig. 4). Many of these signals are closely related or functionally linked. Furthermore, plant cells

contain a third genetic compartment, the mitochondria, which are also of endosymbiotic origin and contain (in plants) a genome with around 40–50 genes (Burger et al. 2003). Like plastids they import the majority of proteins from the cytosol and assemble them into multi-subunit complexes together with the organellar encoded proteins. Mitochondria are energetically coupled to chloroplasts, however, their signalling events to the nucleus and the potential interaction with plastids are rarely investigated. A number of recent reports demonstrated that this organelle contributes significantly to or interacts with the retrograde plastid signals and thus represent an important player in this signalling network (Pesaresi et al. 2007; Rhoads and Subbaiah 2007).

2 Plastid Signals Depending on Plastid Gene Expression

The first proposal of a retrograde influence by plastid protein synthesis on nuclear gene expression was based on studies with the *albostrians* mutants of barley (Bradbeer et al. 1979). The mutant does not form intact ribosomes in plastids of the basal leaf meristem. This blocks synthesis of plastid polypeptides (Fig. 1) and generates white striped or even completely white leaf tissue. Beside this cytoplasmic protein synthesis of nuclear-encoded plastid proteins, for example the small subunit of RubisCO was decreased despite the existence of intact ribosomes in the cytoplasm (Bradbeer et al. 1979). Furthermore, activities of phosphoribulokinase and NADPH-glyceraldehyde-3-phosphate dehydrogenase were found to be down-regulated in white tissues. These data suggested a retrograde control by plastids affecting nuclear gene expression (Bradbeer et al. 1979). Further studies demonstrated that the expression of various nuclear-encoded proteins involved in photosynthesis, photorespiration or nitrogen assimilation was decreased in un-pigmented *albostrians* mutants (Hess et al. 1991, 1994), whereas several genes encoding proteins for chlorophyll biosynthesis and plastid DNA replication exhibited equal or even an enhanced expression level in white vs. green tissues (Hess et al. 1992, 1993).

Similar mutants exist also in the dicot model organism *Arabidopsis thaliana*. The mutant *sco1* (snowy cotyledons) is mutated in the gene for the plastid elongation factor G (EF-G) (Albrecht et al. 2006), which diminishes chloroplast translation resulting in un-pigmented cotyledons and reduced transcript amounts of nuclear-encoded photosynthesis gene. This phenotypic effect, however, is restricted to the first days of seedling development when strong protein synthesis is required. The *apg3* (albino or pale green mutant 3) mutant of *Arabidopsis* is deficient in the chloroplast ribosome release factor 1. The mutant exhibits an albino phenotype, but can be maintained on agar plates with sucrose. Although 21-day-old plants do not posses detectable amounts of D1, RbcL (large subunit of RubisCO) and RbcS proteins, nuclear transcripts of photosynthesis genes were only slightly decreased (Motohashi et al. 2007). This indicates that (1) impairment of plastid translation *per se* does not cause a repression of nuclear gene expression and (2) that the repression might be restricted to early stages of plant development as seen in the *sco1* mutant.

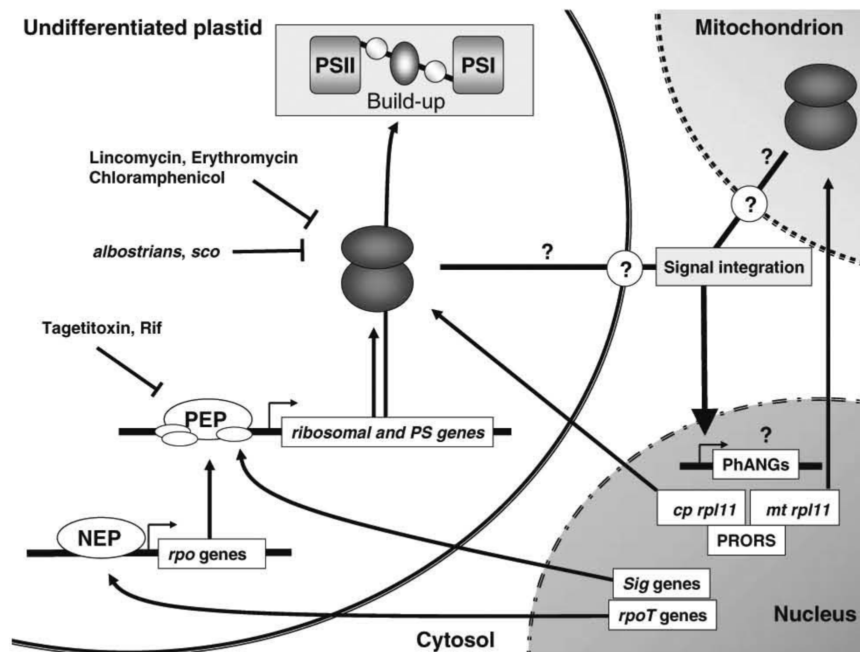

Fig. 1 *Plastid signals depending on organellar gene expression*. The plant cell compartments plastid (in an early undifferentiated form), mitochondrion, cytosol and nucleus are depicted schematically. Organellar ribosomes are given as *dark double ovals*, important enzymes are indicated by *white ovals*. The photosynthetic apparatus which has to be built up during chloroplast development is given schematically in a *separate box*. Genes are shown as *white boxes* labelled with the respective names (for identities compare text). Transcription start sites are indicated by a *small arrow* in front of the genes. Location of the encoded component is indicated by *thin black arrows*. Repressive effects of inhibitors or mutations are given as a *black line with a hammerhead*. Influences of organellar processes on nuclear gene expression are indicated by *thick black arrows*. Transduction of these signals within plastid and mitochondrion, over the respective membrane and through the cytosol (including integration given as a *black dot*) are not known and marked by *question marks*

The role of plastid translation was also extensively studied by application of antibiotics which selectively affect the prokaryotic-type 70S ribosomes (Fig. 1). Chloramphenicol treatment reduced the expression of the nuclear genes *RbcS* and *Lhcb* (encoding the light harvesting proteins of PSII) (Oelmüller and Mohr 1986). Streptomycin treatment reduced *RbcS* transcription in rice (Yoshida et al. 1998), and application of erythromycin and lincomycin resulted in decreased transcript accumulation of *Lhcb* and *RbcS* (Gray et al. 1995; Sullivan and Gray 1999). By this means, the rate of *Lhcb* and *RbcS* transcript accumulation became a kind of molecular marker for the action of the "plastid signal" or "plastid factor" (Oelmüller 1989). During these analyses it became apparent that the antibiotics operated only properly when applicated within the first 36–72 h after germination (Oelmüller and Mohr 1986; Gray et al. 1995). This supports the conclusion that the signal originating from plastid gene expression machinery might be restricted to this short time span.

Beside inhibition of translation also inhibition of transcription was found to affect nuclear gene expression (Fig. 1). Treatments with tagetitoxin, nalidixic acid or rifampicin repressed accumulation of *RbcS* and *LhcB* mRNAs, whereas other nuclear genes remained unaffected. This effect was limited to the initial stage of establishment of the plastid transcription machinery since no effect of the drug could be observed in older leaves (Lukens et al. 1987; Rapp and Mullet 1991; Gray et al. 1995; Pfannschmidt and Link 1997). Essential parts of plastid ribosomes (16 and 23 S rRNAs, various ribosomal subunits) and a complete set of tRNAs are encoded on the plastome. Proper assembly and function of plastid ribosomes, therefore, requires a functional transcription. This explains why inhibition of plastid transcription results in the same effects as a treatment with translational inhibitors.

Interestingly, also mitochondrial translation plays a role in plastid-to-nucleus-communication (Fig. 1). Disruption of nuclear genes for ribosomal subunit L11 of both plastids and mitochondria in *Arabidopsis*, resulted in down-regulation of photosynthesis-associated nuclear genes (PhANGs). However, this was observed only when both genes were affected whereas the single mutants revealed no effect (Pesaresi et al. 2006). A similar result could be seen by down-regulation of the essential and dual-targeted prolyl-tRNA-synthetase 1 (PRORS1) (Pesaresi et al. 2006). This enzyme is required for translation in both organelles. While a knock-out of the PRORS1 gene is lethal because of arresting embryo development, leaky mutants exhibiting only down-regulation of the gene are viable. Interestingly, these mutants exhibited a decrease in PhANG expression like that observed in the *rpl11*-mutants. It could be shown that this regulation is light- and photosynthesis-independent and also not caused by oxidative stress. These data suggest a cooperative, synergistic role of translation in both organelles in the modulation of PhANG expression and add an important new facet to this research field.

3 Signals Depending on Pigment Biosynthesis Pathways

Tetrapyrrols and carotenoids are the major pigments of plants that are involved in absorption and quenching of light energy. The expression of the pigment binding proteins such as the Chl a/b binding proteins of the light harvesting complex II (Lhcb) are coupled to the biosynthesis of these pigments. The tetrapyrrole pathway (Fig. 2) plays a crucial role in primary metabolism of a plant and has to be strictly controlled for several reasons. (1) Tetrapyrroles are not only compounds for chlorophyll synthesis but also for heme, phytochrome, and enzymatic cofactors. Therefore, the flux of tetrapyrroles within the different biosynthetic branches has to be controlled. (2) All chlorophylls and its precursors are phototoxic. Once they are produced they have to be rapidly integrated into proteins. (3) All enzymes involved in pigment biosynthesis and light harvesting are plastid-localised while their corresponding genes are encoded in the nucleus. Coordination of pigment biosynthesis and nuclear gene expression, therefore, requires a bi-directional communication between plastids and nucleus (Rüdiger and Grimm 2006).

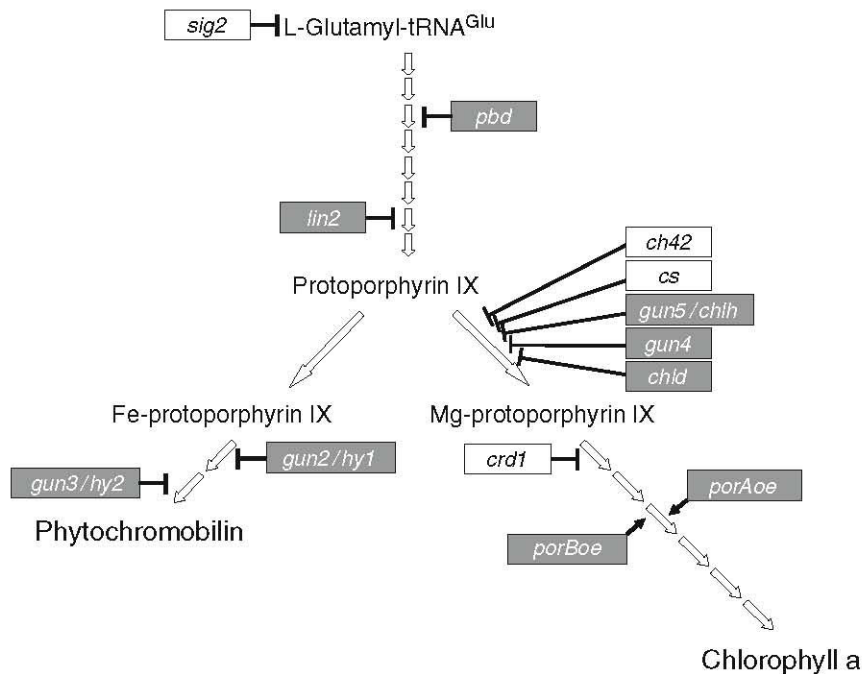

Fig. 2 *Tetrapyrrole biosynthesis pathway*. Only major components of the pathway are given by name. *White arrows* indicate steps of synthesis. Inhibition of single steps by mutational defects is indicated with *black lines with a hammerhead*. The respective mutated genes are given in *boxes* (for identities compare text). *Dark grey boxes* indicate mutants [or overexpressors (oe), *porAoe*, *porBoe* (McCormac and Terry 2004)] which exhibit a *gun* phenotype, *white boxes* mark mutants that do not display such a phenotype. *Lin2* (lesion initiation) encodes coproporphyrinogen oxidase (Ishikawa et al. 2001), *chld* (subunit D of Mg-chelatase) (Strand et al. 2003). The *sig2* mutant has been not tested for a *gun* phenotype

3.1 Tetrapyrrole Biosynthesis

The light-induced expression of Lhc proteins was found to coincide with the greening and maturation process of chloroplasts implying the action of a plastid signal. One potential signal could be attributed to the chlorophyll precursor Mg-Proto-Porphyrin-IX (Mg-Proto-IX). Feeding experiments with the iron chelator dipyridyl led to decreased *Lhcb* mRNA levels in *Chlamydomonas reinhardtii* (Johanningmeier and Howell 1984). The chelation of iron leads to an interruption of the heme feedback inhibition in the tetrapyrrole pathway which in turn causes accumulation of Mg-Proto-IX. This effect could be also observed in higher plants (Kittsteiner et al. 1991). Direct feeding of Mg-Proto-IX to *Chlamydomonas* cell cultures led to induction of nuclear heat-shock genes *HSP70a/b/c* (Kropat et al. 1997) supporting the notion that this chlorophyll precursor could mediate a retrograde signal which affects nuclear gene expression.

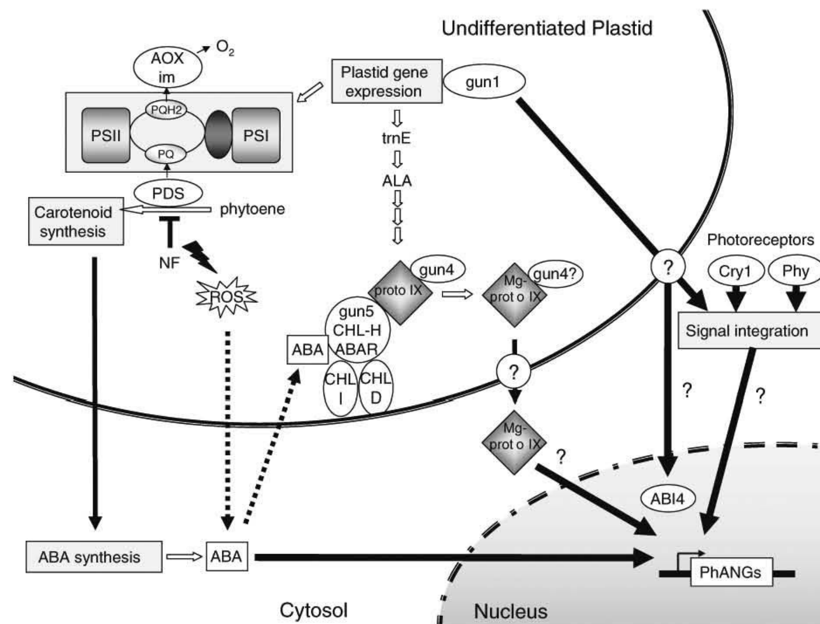

Fig. 3 *Plastid signals depending on tetrapyrrole and carotenoid biosynthesis.* Plastid, cytosol and nucleus are depicted schematically. The photosynthetic apparatus is given schematically in a *separate box*. Genes are shown as *white boxes*, transcription start sites are indicated by a *small arrow* in front of the genes. Protein components are given as *white ovals* (for identity compare text). Tetrapyrrols are represented by *grey squares*. Repressive effects are given as a *black line with a hammerhead*. Influences of organellar processes on nuclear gene expression are indicated by *thick black arrows*, putative diffusion by *dotted arrows*. Transduction of these signals are not known and marked by *question marks*

Another line of evidence for involvement of chlorophyll precursors in retrograde signalling came from studies on carotenoid-deficient plants. Maize seedlings with defects in carotenoid synthesis exhibited a decreased accumulation of *Lhcb* mRNA while other nuclear-encoded transcripts for cytosolic enzymes were not impaired (Mayfield and Taylor 1984). Alternatively, disruption of carotenoid biosynthesis by blocking the phytoene desaturase (PDS) (catalysing an early enzymatic step in this pathway) with the herbicide norflurazon (NF) (Chamovitz et al. 1991) led to comparable effects as the genetic defects. The resulting carotenoid deficiency of plastids led to reduced photosynthetic efficiency followed by photo-oxidative damage of thylakoid membranes due to the loss of non-photochemical de-excitation mechanisms. This photo-oxidative stress within the plastid prevented conversion of proplastids into mature chloroplast and resulted in a decreased expression of nuclear *Lhcb* and *RbcS* genes (Oelmüller and Mohr 1986). Thus, it was concluded that intact plastids are required for expression of nuclear photosynthesis genes and that a "plastid factor" is required for a correct build-up of the photosynthetic machinery (Oelmüller 1989; Taylor 1989).

Using this NF-mediated repression as a tool a genetic approach was performed to get deeper insights into the nature of the plastid signal (Susek et al. 1993). First, a transgenic *Arabidopsis* line carrying a fusion construct consisting of the *Lhcb1.2* (*CAB3*)-promoter (known to be down-regulated by NF treatment, see above) and reporter genes conferring hygromycin resistance and β-glucuronidase activity was created. Then the seed pool of this reporter line was mutagenised with ethyl methane sulphonate (EMS) and the resulting EMS mutant population was grown on plates with an NF-containing medium. By this means the seedling population was screened for individuals exhibiting a genetic defect which interrupted the down-regulation of the *Lhcb* expression under NF and, consequently, conferred hygromycin resistance. These mutants are regarded as defective in plastid signalling and, therefore, were named *genomes uncoupled* (*gun*) mutants. In the second screening step expression of the β-glucuronidase gene was tested and relative activity of the *Lhcb1.2* promoter was estimated. All *gun* mutants exhibited *Lhcb* expression whereas in wild-type plants *Lhcb* transcription was almost abolished. In total six different *gun* mutant lines (*gun1–gun6*) were found in this screen (Susek et al. 1993). Since *gun1* is different from *gun2–gun5* it is discussed in a separate section (Sect. 4).

The phenotype of the *gun* mutants varies from pale yellowish to undistinguishable from wild-type. The mutants *gun2-gun5* were mapped to the tetrapyrrole synthesis pathway (Fig. 2) (Surpin et al. 2002) and demonstrated reduced accumulation of Mg-Proto-IX under NF treatment which causes down-regulation of *Lhcb* expression in wild-type (Strand et al. 2003). The *gun2* and *gun3* mutant alleles were identified to encode the haem oxygenase and phytochromobiline synthase, respectively. The genetic lesions cause an overproduction of haem which activates a feedback loop that inhibits the *trnE*-reductase (HEMA), the first step of tetrapyrrole biosynthesis. This prevents accumulation of Mg-Proto-IX. Both mutants are allelic with *hy1* and *hy2* (hypocotyl) mutants found in a screen for photomorphogenesis mutant which is consistent with the function of tetrapyrroles as chromophores of phytochromes (Mochizuki et al. 2001). The mutants *gun4* and *gun5* were found to be directly involved in chelation of magnesium into protoporphyrin IX, the step which generates Mg-Proto-IX. *gun4* was found to encode an activator of the Mg-chelatase and *gun5* was affected in CHL-H, a subunit of Mg-chelatase (Fig. 3). GUN4 is a small soluble protein 22-kDa in size that can bind either the substrate proto-IX or the product of the chelation reaction, Mg-Proto-IX. The binding constant of GUN4 and Mg-Proto-IX was found to be lower than that of GUN4 and Proto-IX, however, only the latter couple is able to activate Mg-chelatase. By this means GUN4 could avoid accumulation of phototoxic Mg-Proto-IX and could control the chlorophyll biosynthesis pathway (Mochizuki et al. 2001; Larkin et al. 2003; Strand 2004).

In cyanobacteria GUN4 was shown to modulate enzyme activities of the Mg-chelatase and ferrochelatase that produces haem (Wilde et al. 2004). Thus, GUN4 may function as a global controller of the haem and chlorophyll branches. Since haem or its precursor Proto-IX is exported to mitochondria a control step at this point tetrapyrrole synthesis appears to be ideal for regulation and signalling.

Recently, the presence of Mg-Proto-IX in the cytosol could be visualised by confocal laser scanning technology. The actual low amount of Mg-Proto-IX in the

plant cell was increased by circumventing the HEMA feedback inhibition by direct ALA feeding of NF treated seedlings (Ankele et al. 2007). This favours the model that Mg-Proto-IX is directly transported into the cytosol (Strand 2004) over the model which involves a Mg-Proto-IX sensing protein (like GUN4) and a subsequent cytosolic signal transduction cascade (Larkin et al. 2003). How this can be reconciled with the high photo-toxicity of Mg-Proto-IX still has to be resolved.

The *gun5* mutant was found to possess a mutated allele of CHL-H which provides Mg-Proto-IX for the $[\text{CHL-I:CHL-D}]_x$ complex in which the Mg insertion into Proto-IX occurs (Willows and Hansson 2003). Mutations in any of the Mg-chelatase subunits resulted in decreased Chl level. Interestingly, mutations in the CHL-I do not result in a *gun* phenotype although these mutants produce even less amounts of Mg-Proto-IX than *gun5* (Mochizuki et al. 2001). This is consistent with the phenotype of a number of other mutants with defects in tetrapyrrole biosynthesis [*ch42* (chlorata), *cs*, *crd1* (copper response defect) (now called chl27)], which all exhibit no *gun* phenotype (Koncz et al. 1990; Tottey et al. 2003). *ch42* and *cs* accumulate less Mg-Proto-IX than wild-type, however, it has not been investigated if this occurs also upon NF treatment. In contrast, the *crd* mutant accumulates more Mg-Proto-IX compared to wild-type. Especially the observations with the *ch42* and *cs* mutants suggest that Mg-Proto-IX levels do not exclusively account for the tetrapyrrole-mediated signal. This is supported by a recent study on *Chlamydomonas* mutants with defects in the Mg-chelatase. These mutants exhibit reduced levels of Mg-tetrapyrroles but increased levels of soluble haem. It was shown that haem can mimic the activating role of Mg-Proto-IX on the induction of HSP70A promoter and other Mg-Proto-IX inducible genes. It was concluded that both tetrapyrroles can act as retrograde signals and that the respective signalling pathways converge at the same *cis*-elements (von Gromoff et al. 2008).

Further support for the idea that a developmental signal descends from the CHL-H (GUN5) subunit came from expression analyses of the nuclear transcripts of *AtSig1-6* genes. They encode sigma factors that are crucial for promoter recognition of the plastid encoded RNA-polymerase (compare Rolland et al. 2008). These factors were found to be repressed after NF-treatment in wild-type but not in the *gun5* background. A similar de-repression in the *gun5* mutant was also found for plastid transcripts depending on PEP activity like *psbA*, *psaA*, *psaC* whereas genes transcribed by the nucleus-encoded RNA-polymerase were not affected. This suggests that GUN5 might act via regulation of nuclear-encoded components of the plastid gene expression machinery in early plastid development (Ankele et al. 2007).

A recent publication reported that CHL-H might be a plastid localised ABA receptor (Fig. 3) (Shen et al. 2006). The authors found that the *Arabidopsis cch* (constitutive chlorina) mutant was deficient in ABA-related responses. The genetic lesion in this mutant was found to be a stronger allele of *gun5*. Therefore, the *cch* mutant displays a "*gun* phenotype". It could be further shown that direct ABA feeding to wild-type plants led to an increase in Mg-Proto-IX levels but to decreased Chl levels. This suggests that a component downstream of the Mg-chelatase plays an additional role in the tetrapyrrole and ABA crosstalk. Whether or not ABA deficiency caused by NF treatment is related to the putative ABA receptor function of the Chl-H subunit has to be studied in the future.

3.2 *Integration of Plastid and Cytosolic Signals on Promoter Level*

While plastid signal transduction mechanisms still remain elusive, some responsive promoter elements have already been identified. The first studies concluded that light and plastid signals act on the same *cis* elements (Bolle et al. 1996; Kusnetsov et al. 1996) and that these are a complex composition of known transcription factor binding sites (Terzaghi and Cashmore 1995; Puente et al. 1996). Subsequently, it was shown that combination of I- and G-box in a minimal *RbcS* promoter is sufficient to respond to NF triggered plastid signal, sugar, ABA and light (Acevedo-Hernandez et al. 2005). Furthermore, a G-Box and a related sequence motif called CUF (cab upstream factor) element in the *Lhcb1* promoter were shown to be essential for NF-triggered plastid signals (Strand et al. 2003). In addition the *Lhcb1* promoter carries a putative S- (sugar responsive) box which is responsive to ABA. Interestingly, ABI4 (ABA insensitive 4), an AP2-transcription factor, can bind to the respective S-boxes within *Lhcb1* and *RbcS* promoters (Acevedo-Hernandez et al. 2005; Koussevitzky et al. 2007) and the respective ABI4-deficient mutant exhibits a weak *gun* phenotype. This suggests ABA signals and other plastid signals interact at promoter level. This idea is further supported by the recent finding that an ABA (and high light) responsive promoter element within the *Lhcb1* promoter represses the *Lhcb* expression. This promoter element was neither influenced by phytochrome activation nor NF application (Staneloni et al. 2008). A further study in *Chlamydomonas* revealed a distinct *cis*-acting sequence responsive to Mg-Proto-IX and light. The authors concluded that light responsiveness of PhANGs in *Chlamydomonas* is mediated by Mg-Proto-IX (von Gromoff et al. 2006). However, again the light and plastid responsive *cis*-acting elements could not be separated. Further evidence for such an interaction of plastid and cytosolic light-signalling networks came from recent data that the cytosolic blue light photoreceptor *cry1* gene represents a weak *gun* allele (Ruckle et al. 2007) (Sect. 5).

3.3 *Carotenoid and ABA Biosynthesis*

Carotenoid and chlorophyll biosynthesis as well as *Lhc* gene expression are closely related (Anderson et al. 1995). Phytoene desaturation catalysed by PDS and the subsequent zeta-carotene desaturation are key steps in coordination of photoprotection, chloroplast development and nuclear gene expression. The PDS oxidises phytoene and requires plastoquinone (PQ) as an electron acceptor (Fig. 3). The excess electrons are transferred to oxygen via a plastid terminal oxidase (PTOX). Mutants that lack PTOX (called *immutans*) accumulate phytoene due to the high reduction state of PQ. These mutants show a variegated phenotype indicating that an early step in plastid development is blocked. Interestingly, a complete inhibition of plastid development occurs when PDS is blocked by NF. A similar effect could

be found when the subsequent enzyme in the pathway, zeta-carotene desaturase (ZDS), is mutated. A full knock-out of the *SPC1* gene encoding ZDS arrested chloroplast development in the mutant whereas a weaker allele caused only reduction of chlorophyll synthesis due to a down-regulation of genes for components involved in Chl biosynthesis like *PorB* and *CAO* (Dong et al. 2007). Furthermore, the mutation led to carotenoid or ABA deficiency. The ABA-insensitive phenotype of the mutant could be partially restored by exogenously applied ABA.

Although enzymes of the carotenoid and ABA pathway are initially expressed in a light-dependent manner certain enzymes of the xanthophyll cycle pathway are also regulated by the redox state of the photosynthetic electron-transport chain (PET). The application of PET inhibitors DCMU and DBMIB revealed a correlation between the redox state of the PQ pool and the expression of zeaxanthin epoxidase and beta-carotene hydroxylase. Furthermore, a down-regulation of violaxanthin de-epoxidase could be shown after blocking PET (Woitsch and Römer 2003).

Additionally, ABA synthesis was shown to be influenced by lumenal ascorbate availability. Ascorbate is limiting under high light stress conditions and in the *Arabidopsis vtc1* mutant (Pastori et al. 2003; Baier and Dietz 2005). Furthermore, cytosolic events may regulate ABA levels since the last steps of synthesis are cytosolic (Seo and Koshiba 2002). Taken together, ABA synthesis is closely connected to PET, plastid redox state and pigment synthesis. Therefore, ABA levels might be a good indicator for the plastid status during development and under stress. Since ABA is mobile and activates transcription factors it is a reasonable candidate for a retrograde signal.

4 Crosstalk of Signals from Gene Expression and Chlorophyll Biosynthesis

Among the isolated *gun* mutants (Sect. 3) *gun1* is unique since it exhibits de-repression of the *Lhcb* gene not only after NF treatment but also after inhibition of plastid translation with lincomycin or chloramphenicol (Susek et al. 1993). Thus, GUN1 was discussed as a factor potentially involved in both, plastid gene expression and chlorophyll biosynthesis (Fig. 3) (Nott et al. 2006). This was supported by findings that double mutants of *gun1* with either *gun4* or *gun5* exhibit a stronger *gun* phenotype. In addition, earlier microarray data exhibited different expression profiles with only a small overlap in de-regulated genes in *gun1* mutants when compared to *gun2* or *gun5* mutants pointing to two separate but partly redundant signalling pathways (Strand et al. 2003). Recently, a new *gun1* allele was isolated and the GUN1 gene was cloned (Cottage et al. 2007; Koussevitzky et al. 2007). The gene encodes a plastid localised pentatricopeptide repeat (PPR) protein containing a putative DNA binding small mutS related domain. PPR proteins are thought to be involved in interactions with RNA in processing, stability and translation but also with DNA (Saha et al. 2007). For the GUN1 protein, so far, only unspecific DNA binding activity could be demonstrated (Koussevitzky et al. 2007). Interestingly,

GUN1 co-localises with PTAC2, another PPR protein, which is part of the transcriptionally active chromosome of plastids (Pfalz et al. 2006). Until now the precise function of GUN1 is elusive. It was discussed that NF treatment could indirectly affect plastid gene expression (Gray et al. 2003), but treatment with dipyridyl showed that *gun1* mutants retain their *gun* phenotype also in the presence of Mg-Proto-IX suggesting a role of GUN1 downstream of Mg-Proto-IX accumulation and chlorophyll biosynthesis (Koussevitzky et al. 2007).

A novel aspect in retrograde signalling could be inferred from a completely different experimental line. The chlorophyllide a oxygenase (CAO), responsible for the conversion of chlorophyllide a (Chlide) to Chlide b, was found to be involved in the import of *Lhcb* precursors into the plastids (Reinbothe et al. 2006). This offers a potential explanation for transduction of a plastid gene expression derived signal to the nucleus without the need of an export of any signal molecule. Chlorophyll synthesis starts with glutamyl-tRNA which is transformed to δ-aminolevulinic acid (ALA) (Rüdiger and Grimm 2006). Transcription of the plastid encoded gene for glutamyl-tRNA, *trnE*, is exclusively performed by the PEP enzyme in combination with sigma factor 2 (Sig2) (Hanaoka et al. 2003). Thus, any perturbance of plastid transcription or translation by inhibitors or mutations will affect chlorophyll biosynthesis and subsequently signals originating from this pathway. Lack of CAO as in the *chlorina* mutant of *Arabidopsis* prevents the accumulation of Chl b and of the LhcB proteins. A recent study with plastids from the chlorine mutant demonstrated that the major portion of CAO is located at the inner envelope of plastids. It could be cross-linked to Tic40, Tic22 and Tic20 indicating an interaction with the protein import machinery of the plastid inner envelope to form a novel Tic sub-complex distinct from the known Ptsc52 translocon complex (Reinbothe et al. 2006). This complex was found to be responsible for the import of Lhcb1 and Lhcb4 (CP29) proteins but not for the import of a plastocyanin precursor. It was hypothesised that Chlide a binding to CAO and its conversion into Chlide b may prevent the Lhcb precursor from slipping back into the cytosol and supporting its import. On the basis of these data a simple feedback model for plastid signals from Chl biosynthesis was proposed (Bräutigam et al. 2007). Blocking expression of *trnE* either via plastid transcription or translation prevents formation of Chlide a, and thus, Lhcb import is drastically reduced. This would lead to accumulation of Lhcb precursors in the cytosol which could activate a feedback repression of nuclear transcription of PhANGs.

5 Interactions of Plastid and Light-Signalling Networks during Early Plastid Development

Plant photomorphogenesis is regulated by a complex signalling network that activates or represses genes essential for photomorphogenic development (Jiao et al. 2007). These processes include chloroplast biogenesis as well as control of PhANG

expression (Fig. 3). Thus, interaction and/or cooperation of retrograde plastid signals with light signals, for example at the level of promoter usage are likely (see above). The pea mutant *lip1* (light-independent photomorphogenesis) exhibits photomorphogenesis in the dark, containing partially developed chloroplasts and elevated levels of PhANG transcripts (Frances et al. 1992). Seedlings of *lip1* treated with inhibitors of plastid translation displayed a reduced accumulation of transcripts of PhANGs in the light. Similar effects were also shown for the *Arabidopsis* photomorphogenetic mutant *cop1-4*. Interestingly, even in the dark plastid translation is necessary for transcript accumulation of PhANGs in *lip1*. Hence, light is not an obligatory requirement for the plastid signal (Sullivan and Gray 1999). This supports the view that early plastid signals have a developmental origin which is distinct from light-induced signals. On the other hand observations exist that plastid signals may affect light-signalling networks. A screen for *cue* (cab under-expressed) mutants from *Arabidopsis* identified plants with defects in de-repression of *cab* (now *Lhcb*) gene expression in response to phytochrome activation. Thus, plastid-derived signals are closely linked to phytochrome control of PhANG expression (Lopez-Juez et al. 1998). In a recent study focussing on weak *gun* alleles a number of new *cryptochrome1* mutants were isolated suggesting that plastid signals may remodel light-signalling networks (Ruckle et al. 2007). Genetic experiments including *cop1-4*, *hy5* and *phy* mutants combined with different light quality treatment suggested that plastid signals are able to convert action of light signalling pathways from a positive into a negative manner by affecting HY5, a positive regulator of PhANG expression. Thus, plastid signals may be required to remodel light-signalling networks in order to integrate information about developmental state of plastids and the environmental light situation.

6 Signals Depending on Photosynthesis and Reactive Oxygen Species

Photosynthetic efficiency is highly dependent on environmental cues. Adverse conditions are, therefore, counteracted by so-called acclimation responses, the aim of which is to compensate for the unfavourable parameter (Anderson et al. 1995; Kanervo et al. 2005; Walters 2005). Many acclimation mechanisms include changes in gene expression both in plastids and nucleus which are controlled by a functional feedback loop via the reduction/oxidation state of components of the PET chain or coupled redox-active molecules (Fig. 4). Such changes in the redox poise are caused by the environment. By this means photosynthesis actively adapts processes in plastids, cytosol or nucleus to its own actual function and coordinates the required changes in the three compartments (Pfannschmidt 2003; Baier and Dietz 2005; Buchanan and Balmer 2005). In addition, reactive oxygen species produced either as a by-product of photosynthesis or as a result of distinct stresses play a major role in redox signalling between plastids and nucleus and add an additional level of regulation (Apel and Hirt 2004; Foyer and Noctor 2005).

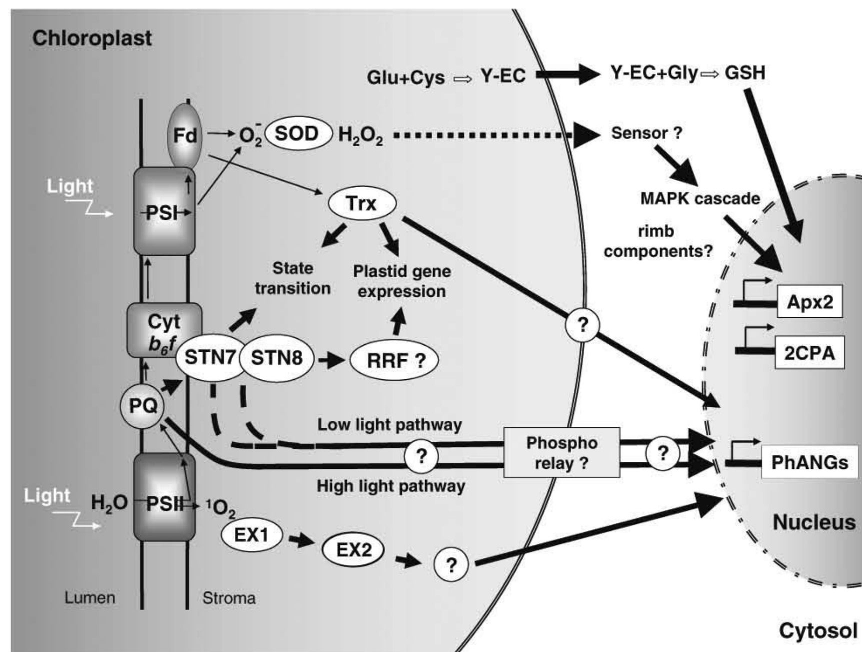

Fig. 4 *Retrograde signals from photosynthetic electron transport and ROS*. The plant cell compartments chloroplast, cytosol and nucleus are depicted schematically. Redox signals generated within the electron transport chain or by generation of ROS and scavenging mechanisms initiate signalling pathways which activate or repress specific target genes in the nucleus (for details see text). Electron flow is given as *very thin black arrows*. Redox signals influencing nuclear gene expression are given by *thick black arrows*. A *dotted arrow* indicates putative diffusion. Unclear or unknown steps or processes are marked by *question marks*. Fd: reduced ferredoxin, SOD: superoxide dismutase, H_2O_2: hydrogen peroxide, GSH: glutathione, $^1O_2$: singlet oxygen, EX1, EX2: Executer 1 and 2, RRF: redox responsive factor. For other abbreviations see text. The figure has been modified from Fig. 2 in Pfannschmidt et al. (2008) Potential regulation of gene expression in photosynthetic cells by redox and energy state – approaches towards better understanding. Annals in Botany (doi: 10.1093/aob/mcn081)

6.1 Signals Originating from Photosynthetic Electron Transport

The first evidence for influences of PET on nuclear gene expression came from experiments with the unicellular algae *Dunaliella tertiolecta* and *Dunaliella salina* (Escoubas et al. 1995; Maxwell et al. 1995). Further studies demonstrated that redox regulation by PET also exist in higher plants (Fig. 4). A study investigating acclimation of *Lemna perpusilla* to varying light intensities indicated that plastoquinone redox state regulates *Lhcb* transcript and LHCII protein accumulation (Yang et al. 2001). In another study potential combinatorial effects of plastid redox state and sugar on *Lhcb* gene expression of *Arabidopsis* were tested (Oswald et al. 2001). DCMU application was able to abolish an increase in *Lhcb* transcript

accumulation which usually occurs after sugar depletion. This implies a connection between PET and sugar signalling in *Arabidopsis*. In winter rye *Lhcb* gene expression was found to be regulated by redox signals from PET which were induced by varying light and temperature regimes (Pursiheimo et al. 2001). The authors concluded that the redox state of electron acceptors at the PSI (Photosystem I) acceptor site were a regulating parameter of nuclear gene expression under these conditions. In transgenic tobacco plants carrying a pea *PetE* gene construct it could be demonstrated that the *PetE* construct as well as endogenous *Lhcb1* transcripts decreased upon DCMU treatment. In contrast, nuclear run-on transcription assays indicated up-regulation of the pea *PetE* construct expression suggesting multiple parallel influences at different levels of gene expression (Sullivan and Gray 2002). Redox regulation of post-transcriptional processes was also uncovered. In transgenic tobacco a pea ferredoxin-1 gene construct exhibited light-induced transcript accumulation which could be observed even under the control of a constitutive promoter. Since this response could also be influenced by DCMU it was concluded that PET controlled the transcript accumulation of the transgene. Furthermore, the ribosome loading of the message was affected (Petracek et al. 1997; Petracek et al. 1998). The *Apx2* gene encodes a cytosolic ascorbate peroxidase that detoxifies hydrogen peroxide which accumulates under stress. This gene was found to be induced by high-light treatment, however, DCMU and DBMIB treatments suggested an involvement of the PQ redox state in this regulation at least at an early stage (Karpinski et al. 1997, 1999). This was supported by another study on transgenic tobacco (Yabuta et al. 2004). Other experiments indicated an involvement of leaf transpiration state and abscisic acid in *Arabidopsis Apx2* expression (Fryer et al. 2003). This is consistent with the role of ABA in stress signalling (see above). The *Arabidopsis* mutant *cue-1* (chlorophyll a/b binding protein underexpressing) lacks the phosphoenolpyruvate/phosphate translocator PP1 and exhibits a light intensity dependent under-expression of *Lhc* genes. Measurements of rapid induction kinetics of Chl a fluorescence suggest that a reduced PQ pool size and PET cause the *Lhc* under-expression (Streatfield et al. 1999).

In another experimental approach variations in light quality instead of light quantity were used to manipulate photosynthetic electron transport. Illumination of plants with light sources that preferentially excite either PSI or PSII allow controlled oxidation or reduction of the electron transport chain. This low-light system avoids stress-mediated side effects which may occur under high-light and mimics natural light quality gradients in dense plant populations. The excitation imbalance is counterbalanced in the short-term by state transitions and in the long-term by photosystem stoichiometry adjustment (Dietzel et al. 2008). The latter involves the controlled change of photosynthetic gene expression both in plastids and nucleus. Initial studies with transgenic tobacco together with DCMU and DBMIB treatments indicated PQ redox control of the PC promoter (Pfannschmidt et al. 2001). Further studies with the *nia2* (encoding the cytosolic nitrate reductase) promoter in *Lemna*, *Arabidopsis* and tobacco demonstrated that this control extends also to non-photosynthesis genes (Sherameti et al. 2002). Thus, it can be concluded that PQ redox control of nuclear gene expression occurs both under high- and low-light

conditions. Whether this involves two different signal transduction pathways has to be clarified in the future. A recent study with *Chlamydomonas* mutants with different defects in the cytb_6f complex demonstrated that light induction of nuclear genes for tetrapyrrole biosynthesis genes did not depend on PQ redox state but on the integrity of the cytb_6f complex (Shao et al. 2006). This suggests the existence of additional redox signals beside the PQ pool. Whether this regulation mode is also active in higher plants has to be elucidated in the future.

Recent array experiments investigated PET redox effects on nuclear gene expression in a more extended way. Using a macroarray containing genes for proteins with predicted chloroplast localisation (Kurth et al. 2002; Richly et al. 2003) it could be demonstrated that light quality shifts combined with DCMU treatment affect 286 nuclear genes in a redox-dependent manner in *Arabidopsis* (Fey et al. 2005). Identified genes encode proteins not only for photosynthesis but also for gene expression, metabolism and signal transduction indicating broad impact of PET redox signals. Another study used a different array with around 8,000 randomly selected *Arabidopsis* genes and investigated expression profiles in response to different light intensities and light qualities (Piippo et al. 2006). Under these conditions nuclear gene expression responded to redox signals from stromal components such as thioredoxin. Present data are not sufficient to resolve this contradiction arguing for further analyses of these complex regulation events.

6.2 *Transduction Pathways from PET Toward the Nucleus*

Transduction of redox signals over the plastid envelope and its transmission through the cytosol into the nucleus is not understood yet. So far, the PQ pool is the best-characterised source for redox signals. In a study with *Dunaliella tertiolecta* it could be demonstrated that phosphatase inhibitors were able to reduce the acclimation in response to the high- to low-light shift suggesting that the mediation of the signal might involve a phosphorylation cascade (Escoubas et al. 1995). In a working model a redox-sensitive kinase is proposed to phosphorylate a still unknown plastid protein. After transfer of the signal over the envelope a cytosolic kinase activity might be responsible for phosphorylation of a repressor protein which binds to the *Lhcb* promoter in the nucleus (Durnford and Falkowski 1997). Indeed, several different protein complexes could be observed to interact with the *Lhcb* gene promoter during photoacclimation (Chen et al. 2004). Interestingly this study found that also the trans-thylakoid pH gradient contributes to the *Lhcb* regulation suggesting the existence of several redox signals. Studies with transgenic tobacco lines demonstrated that the promoter for the nuclear gene PsaF (encoding subunit IV of PSI) is regulated by plastid redox signals (Pfannschmidt et al. 2001). Another study investigating the responsiveness of this promoter in more detail demonstrated that it can be activated by a cytosolic kinase activity even when plastid development is arrested by application of norflurazon (Chandok et al. 2001). This supports the idea of a kinase cascade in the transduction of plastid redox signals.

A potential candidate for a redox-sensitive plastid kinase involved in gene regulation is STN7 (Fig. 4). This thylakoid-membrane associated kinase was shown to be required for both state transitions and the long-term response to light-quality gradients (Bellafiore et al. 2005; Bonardi et al. 2005). It changes its activity in dependency on illumination demonstrating all requirements for redox-regulation by the PQ pool, however, its substrate specificity is not known yet. Gene regulation would require that the kinase phosphorylates either an additional protein beside the LHCII or that it initiates an additional signalling branch. Where and whether regulation pathways for state transitions and long-term response to light quality (LTR) form is still unclear. In our model we propose a down-stream redox-responsive factor (RRF) which may mediate the redox signals from the kinase (Fig. 4). Phosphorylation was shown earlier to be a crucial mechanism in controlling plastid gene expression during light-regulated etioplast–chloroplast transition (Tiller and Link 1993). The present picture is still not conclusive. Array analyses with a STN7-deficient mutant indicated only a minor role of STN7 in the regulation of nuclear and plastid gene expression (Bonardi et al. 2005; Tikkanen et al. 2006), but the lack of the orthologue kinase STN8 resulted in clear changes (Bonardi et al. 2005). Some data point to the possibility that the two kinases interact during photoacclimation and gene expression (Fig. 4) (Rochaix 2007; Dietzel et al. 2008), however, more experimental data are necessary to understand the transduction of plastid redox signals toward the nucleus.

6.3 Signals Mediated by Reactive Oxygen Species and Stress-Related Processes

Hydrogen peroxide (H_2O_2) is the principle ROS in plants. It is mainly generated at PSI under conditions when excitation exceeds energy usage by the dark reaction, e.g. under high light or in low temperature. Such conditions lead to over-reduction of the electron transport chain and to electron transfer from ferredoxin to oxygen generating superoxide (Fig. 4). This is detoxified by the superoxide dismutase (SOD) resulting in accumulation of hydrogen peroxide which is reduced to water by antioxidant enzymes such as APX. In this reaction ascorbate is used as electron donor and replenished by reduction via glutathione (Pfannschmidt 2003). Cytosolic APX enzymes are induced by oxidative conditions and therefore represent good markers for cellular stress (Shigeoka et al. 2002). In *Arabidopsis* high-light induction of nuclear genes *apx1* and *apx2* could be correlated to the action of H_2O_2 as a signalling molecule (Karpinski et al. 1997, 1999; Foyer and Noctor 1999). Interestingly *apx2* was also found to be regulated by the PQ pool pointing to a combined action of the two signals. This was confirmed by a recent study which showed that tobacco *apx2* is initially induced by the PQ pool while its later regulation occurred via H_2O_2 (Yabuta et al. 2004). The full impact of H_2O_2 on nuclear gene expression was elucidated by array analyses. 1–2% of the analysed genes exhibited responsiveness to the treatment. Among them many stress-related and defence genes were found (Desikan et al.

2001). More recent studies support these early observations (Vandenabeele et al. 2004; Davletova et al. 2005). How this regulation is performed, however, is currently being investigated. Working models assume that H_2O_2 pass the chloroplast envelope by diffusion and activate a cytosolic mitogen-activated protein kinase (MAPK) cascade (Kovtun et al. 2000; Apel and Hirt 2004) which links H_2O_2 accumulation and gene expression via a phosphorylation cascade. Studies with *Arabidopsis* suggest that H_2O_2 activates the MAPKK kinases ANP1 or MEKK1 which, in turn, activate other downstream MAPKs (Kovtun et al. 2000). A major problem in our understanding of ROS signalling currently is that H_2O_2 is produced under a number of quite diverse stresses, but that responses to these stresses are very specific. Differences in distribution and local concentrations of H_2O_2 as well as interaction with additional signals are discussed to confer specificity in these processes (Beck 2005).

Another important ROS in stress signalling is singlet oxygen ($^1O_2$), a non-radical ROS. It is continuously produced at PSII by energy transfer from triplet state P680 to oxygen, but its production increases under conditions of over-excitation (Fig. 4). Singlet oxygen possesses a very short half-life (~200 ns) and causes oxidative damage mainly at the site of its generation, i.e. in PSII (op den Camp et al. 2003). Nevertheless, it induces a number of distinct stress responses in the nucleus. Since excess excitation conditions produce several ROS in parallel it is difficult to discern the action of a specific ROS. However, the *Arabidopsis flu* (*fluorescent*) mutant provides a tool to circumvent this problem. The mutant accumulates free protochlorophyllide (Pchlide) when put into darkness and produces enhanced amounts of singlet oxygen upon re-illumination by energy transfer from the Pchlide. This leads to growth inhibition and cell death. However, under continuous illumination when Pchlide is not accumulated the mutant exhibits wild-type like development (Meskauskiene et al. 2001). Transcript profiling with the mutant indicated that around 5% of all genes changed their expression and that 70 genes were specifically activated by singlet oxygen (op den Camp et al. 2003). Surprisingly, the destructive effects of this ROS could be genetically suppressed in a second-site mutant screen of the *flu* mutant indicating that the cell death was not induced by the oxidative damage from singlet oxygen but was initiated by a response programme. In the double mutant *flu/ex1* the singlet oxygen-mediated stress responses were abrogated by the inactivation of a gene called *executer1* (*ex1*) (Wagner et al. 2004). It encodes a plastid-localised protein (EXECUTER1) with a still unknown function. This protein represents a potential sensor and/or mediator of singlet oxygen signals. A more detailed analysis of the cell death response in the *flu* mutant revealed that it is promoted by signalling pathway(s) dependent on ethylene, salicylic and jasmonic acid but that it is blocked by a jasmonic acid precursor (Danon et al. 2005). Recently, a second *executer1*-like gene called *executer2* was identified which is also implicated in singlet oxygen-dependent nuclear gene expression changes. The encoded protein EX2 is also confined to the plastid and appears to interact with EX1. In triple mutants *ex1/ex2/flu* up-regulation of singlet-oxygen regulated genes is almost completely suppressed suggesting that the two proteins are sufficient to confer singlet oxygen-mediated retrograde signalling (Lee et al. 2007). Interestingly, a mutated allele of the haem oyxgenase called *ulf3* can suppress the phenotype of the *flu*

mutant (Goslings et al. 2004). *ulf3* is allelic to *gun2* and thus point to a connection between tetrapyrrole and ROS signalling.

In another mutant screen using transgenic *Arabidopsis* carrying a 2-cysteine peroxiredoxin (2CPA)-promoter::luciferase construct several *rimb* (*redox imbalanced*) mutants (Fig. 4) were identified (Heiber et al. 2007). The 2CPA promoter is redox-sensitive and activated by redox signals from components at the PSI acceptor side to increase antioxidant capacity under conditions that could induce photooxidative damage (Baier and Dietz 2005). Thus, these mutants provide a tool for analysing retrograde redox signalling cascades which activate nuclear-encoded antioxidant enzymes. Future work will show whether these signalling pathways are different from that described above.

Reduced glutathione (GSH) is required for re-reduction of the primary ROS scavenger ascorbate. Recent observations indicate that GSH might act as a plastid signal during stress defence programmes (Fig. 4). For instance expression of stress-related genes *apx2* or *pr1* (pathogen related) were correlated with changes in cellular glutathione content (Mullineaux and Rausch 2005). The *Arabidopsis* mutant *rax1-1* (regulator of *Apx2*) is impaired in glutathione synthetase 1 (GSH1) and exhibits a decreased GSH content. As a consequence it exhibits a constitutive high expression of the *Apx2* gene suggesting that low GSH levels activate defence gene expression (Ball et al. 2004). Glutathione is synthesised from glutamate and cysteine forming γ-glutamylcysteine (γ-EC) followed by further addition of glycine. GSH1 catalyses the first step, GSH2 the second. Recent data suggest that in *Arabidopsis* step 1 is confined to the chloroplast while step 2 occurs predominantly in the cytosol (Wachter et al. 2005). This requires that the GSH precursor γ-EC must leave the chloroplast. Its amount, therefore, might well represent a plastid signal reporting potential stress in chloroplasts to the cytosol (Mullineaux and Rausch 2005).

7 Conclusions

In summary, known plastid signals can be classified into two major groups, signals from young, colourless and undifferentiated or damaged plastids and signals from mature and green chloroplasts. Arresting plastid development by inhibitors or mutations typically results in proplastid-like stages that are functionally very restricted (Sullivan and Gray 1999; Nott et al. 2006). The correlating repression of PhANG expression has been interpreted as a hint that the blocked plastid development generates a *negative* signal which represses nuclear gene expression. The observation that in *gun* mutants this repression can be interrupted genetically appears to support this assumption and suggest that tetrapyrrole biosynthesis intermediates might be involved in this negative signalling process. However, different models of negative regulation might also be possible and has been discussed here. A completely different interpretation of the data on inhibiting plastid differentiation would be that the block in plastid development leads to the lack of a *positive* plastid signal resulting in a negative feedback loop. Evolution integrated plastids deeply into the cellular

developmental networks. Blocking its biogenesis thus resembles a knock-out of an important hub in the network which subsequently blocks pathways connected to it but which can be disconnected genetically. Since plant development is highly regulated by photoreceptors a connection of plastid developmental signals with light-signalling pathways is a logical consequence and has been reported in several studies. Thus, studies on such undifferentiated plastids help us to learn more about plastid biogenesis and its integration into plant development as a whole. The role of tetrapyrroles as potential signals in green tissues has still to be elucidated. Variegation mutants containing both colourless undifferentiated and mature green plastids as well as transgenic plants with altered tetrapyrrole synthesis provide interesting models for further investigations of this topic (Sakamoto 2003; Alawady and Grimm 2005; Aluru et al. 2006). In contrast, signals from mature chloroplasts do not provide information about biogenesis but about actual function of the plastids. Here, plastids play the role of an active sensor for environmental changes in a mainly photoreceptor-independent manner. Coordination of photosynthetic acclimation and responses to various stresses are the predominant function of these plastid signals and are communicated by pathways to the nucleus which are completely different from that of the developmental signals. Therefore, studying plastid-to-nucleus communication covers two major fields of plant cell biology, understanding of (1) developmental cascades and (2) physiological acclimation to the environment. Detailed analyses of plastid-to-nucleus signalling pathways, therefore, are of greatest interest for many aspects in molecular plant research.

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5 Manuscript II

Pfannschmidt T., Bräutigam K., Wagner R., Dietzel L., Schröter Y., Steiner S., Nykytenko A.

Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding.

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REVIEW

Potential regulation of gene expression in photosynthetic cells by redox and energy state: approaches towards better understanding

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• *Background* Photosynthetic electron transport is performed by a chain of redox components that are electrochemically connected in series. Its efficiency depends on the balanced action of the photosystems and on the interaction with the dark reaction. Plants are sessile and cannot escape from environmental conditions such as fluctuating illumination, limitation of CO_2 fixation by low temperatures, salinity, or low nutrient or water availability, which disturb the homeostasis of the photosynthetic process. Photosynthetic organisms, therefore, have developed various molecular acclimation mechanisms that maintain or restore photosynthetic efficiency under adverse conditions and counteract abiotic stresses. Recent studies indicate that redox signals from photosynthetic electron transport and reactive oxygen species (ROS) or ROS-scavenging molecules play a central role in the regulation of acclimation and stress responses.
• *Scope* The underlying signalling network of photosynthetic redox control is largely unknown, but it is already apparent that gene regulation by redox signals is of major importance for plants. Signalling cascades controlling the expression of chloroplast and nuclear genes have been identified and dissection of the different pathways is advancing. Because of the direction of information flow, photosynthetic redox signals can be defined as a distinct class of retrograde signals in addition to signals from organellar gene expression or pigment biosynthesis. They represent a vital signal of mature chloroplasts that report their present functional state to the nucleus. Here we describe possible problems in the elucidation of redox signalling networks and discuss some aspects of plant cell biology that are important for developing suitable experimental approaches.
• *Conclusions* The photosynthetic function of chloroplasts represents an important sensor that integrates various abiotic changes in the environment into corresponding molecular signals, which, in turn, regulate cellular activities to counterbalance the environmental changes or stresses.

Key words: Photosynthesis, redox signals, gene expression, regulatory network, retrograde signalling, cross-talk, plastids, higher plants.

INTRODUCTION

Photosynthesis is the principle energy fixing process that sustains life on earth. It harvests sunlight to drive electron transport from the low-potential electron donor water to the high-potential electron end-acceptor $NADP^+$. During this reaction, energy (ATP) and reduction (NADPH) equivalents are generated that are used in subsequent steps of carbon dioxide fixation and reduction in the Calvin–Benson cycle, the dark reaction. Here ADP and $NADP^+$ are regenerated to be used again in the light reaction. Thus, the light-driven redox chemistry of the light reactions and the temperature-dependent enzymatic reactions of the dark reaction are tightly coupled. This coupling renders photosynthesis and its efficiency highly dependent on the prevailing environmental conditions. Changes in various abiotic factors such as the intensity and quality of the incident light (directly affecting the light reaction), the temperature and the nutrient and water availability (directly or indirectly affecting the dark reaction) have an immediate impact on photosynthetic efficiency and ultimately on plant yield. Changes in photosynthetic electron transport, however, have an effect on the components involved in it: the balance between reduced and oxidized forms is changed. Photosynthetic organisms including cyanobacteria, algae and higher plants have therefore evolved regulatory responses that acclimate the photosynthetic process to the prevailing environment and optimize photosynthetic electron transport (Aro and Andersson, 2001; Blankenship, 2002; Walters, 2005). So far as is currently known, most (if not all) acclimation responses are triggered by those changes in the redox state of photosynthetic components that are the target for the counteracting effect of the acclimation response. By this principle a feedback control is established, in which photosynthesis serves as a sensor that signals distinct changes in the environment and that controls respective acclimation responses (Arnon, 1982).

Acclimation responses to imbalances in excitation energy distribution between photosystem II (PSII) and photosystem I (PSI) are well studied. Since the two photosystems work electrochemically in series, any illumination situation in which one PS is favoured over the other results in either reduction or oxidation of the intersystem electron transport chain. Such imbalances are counteracted by two different mechanisms: state transitions and adjustment of photosystem stoichiometry. State transitions represent a short-term response that occurs in the order of minutes. It re-distributes excitation energy

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between the photosystems by variation of their relative antennae cross-sections (Allen and Forsberg, 2001; Haldrup *et al.*, 2001; Wollman, 2001). This is achieved by lateral movement of parts of the light-harvesting complex of PSII (LHCII). Upon reduction of the plastoquinone (PQ) pool, which carries the electrons from PSII to the cytochrome b_6f (Cytb_6f) complex, a redox-sensitive kinase is activated that specifically phosphorylates the mobile LHCII, resulting in its attachment to PSI, the so-called state II. Under PQ oxidizing conditions the kinase is inactive, LHCII is or becomes dephosphorylated (presumably by constitutively active phosphatases) and is relocated to PSII (state I). The mediation of the PQ redox signal toward the kinase is not understood yet; however, it involves the action of the PQ oxidation (Q_O) site at the cytb_6f complex (Vener *et al.*, 1997; Zito *et al.*, 1999). Recently kinases in the alga *Chlamydomonas rheinhardtii* (Depege *et al.*, 2003) and the higher plant *Arabidopsis thaliana* (Bellafiore *et al.*, 2005) were identified that appear to be essential for these processes. They were called Stt7 and STN7, respectively.

Photosystem stoichiometry adjustment is a long-term response (LTR) that requires hours to days. It re-directs the excitation imbalances by changing the relative amounts of the two photosystems (Anderson *et al.*, 1995; Melis *et al.*, 1996; Murakami *et al.*, 1997). In contrast to state transitions that represent a purely post-translational acclimation mechanism, adjustment in PS stoichiometry depends on targeted changes in the expression of photosynthesis genes both in the chloroplast and the nucleus (Pfannschmidt *et al.*, 1999, 2001). Interestingly, this acclimation response is also regulated by the redox state of the PQ pool. Most species investigated so far exhibit opposing expression changes in the reaction-centre genes of PSI and PSII. As a general model, it appears that upon reduction of the PQ pool expression of PSI genes is favoured while upon its oxidation expression of PSII genes is favoured. The molecular details may vary from species to species, but one general picture emerges: the redox state of the PQ pool indicates which photosystem is rate-limiting and initiates appropriate counterbalancing changes in gene expression (Pfannschmidt, 2003). Recent analyses have demonstrated that this also involves the action of the STN7 kinase (Bonardi *et al.*, 2005). This points to a functional coupling of state transitions and photosystem stoichiometry adjustment, which has been proposed earlier (Allen and Pfannschmidt, 2000; Rintamaki *et al.*, 2000).

The two responses described above are just an example of how photosynthesis can control acclimation via redox signals. Both typically occur under low-light conditions, e.g. in dense plant populations where the light intensity is low and the light spectrum is shifted towards the far-red wavelength range. Under different conditions resulting in high or excess excitation pressure other acclimation responses are activated, such as non-photochemical quenching, the D1 repair cycle or various stress-response programmes. These responses are also controlled via redox signals from photosynthesis involving the PQ redox state and signals from the PSI acceptor side, but also involve responses controlled by reactive oxygen species (ROS) such as hydrogen peroxide or singlet oxygen. This review does not aim to give a comprehensive overview on the existing literature and can not describe all redox-regulated mechanisms in detail. The interested reader, therefore, is referred to several excellent reviews focused on these topics (Noctor and Foyer, 1998; Niyogi, 2000; Apel and Hirt, 2004; Mittler *et al.*, 2004; Baier and Dietz, 2005; Fey *et al.*, 2005*a*; Mullineaux and Rausch, 2005). Here, we address central questions and experimental problems regarding the investigation of all these mechanisms. We explain typical limitations of various experimental set-ups and describe examples of how to circumvent at least some of these problems.

IDENTIFICATION OF NATURE AND ORIGIN OF PHOTOSYNTHETIC REDOX SIGNALS

For investigation of redox signals that control gene expression three general approaches have been applied using (1) inhibitors of photosynthetic electron transport, (2) diverse light regimes with effects on electron transport, and (3) mutants with genetic defects in the photosynthetic apparatus (Fig. 1; for reviews see Durnford and Falkowski, 1997; Rodermel, 2001; Pfannschmidt, 2003). Each of these approaches has its specific advantages but also its limitations, which are now briefly presented and discussed.

The most commonly used electron transport inhibitors are 3-(3′,4′-dichlorophenyl) 1, 1′-dimethyl urea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), which exhibit specific affinities to the Q_B binding site of the D1 protein of PSII and the plastoquinol oxidizing site at the Cytb_6f complex, respectively (Trebst, 1980). Application of DCMU, therefore, blocks the electron transport at PSII and oxidizes all following components of the transport chain, while treatment with DBMIB results in a block at the Cytb_6f complex that reduces the components before it and oxidizes those following it. An antagonistic effect of these inhibitors on the expression of a gene thus indicates that the redox state of the plastoquinone pool is the decisive determinant

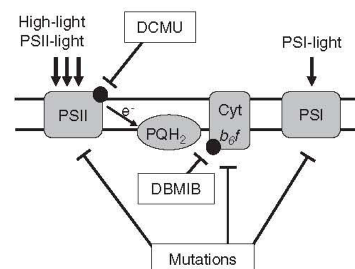

FIG. 1. Principle approaches to identify the nature and origin of photosynthetic redox signals. The photosynthetic electron transport chain is depicted schematically. Site-specific inhibitors act at different sites of the chain. Various light regimes can be used to manipulate the electron flow through the chain [PSII-light and high-light conditions lead to preferential or excess excitation of PSII and reduction of plastoquinone (PQH_2); PSI-light leads to presumed excitation of PSI and oxidation of the PQ pool]. Mutations may interrupt or affect the electron flow and generate redox signals (reduction of components before the genetic defect and oxidation after it). Combinations of these approaches can help to define the nature and origin of a redox signal. Negative effects are indicated by bars.

for its regulation. Equal effects of both inhibitors point to consecutive components of the Cytb_6f complex as regulators, for instance on stromal components reduced by PSI such as thioredoxins. These inhibitors thus represent useful tools in identifying the origin of the redox signal. Nevertheless, a number of pitfalls exist that should be avoided when working with it. DCMU is a very stable compound that easily penetrates tissues and closes the reaction centres completely for several days (usually the plant dies if not supplemented with sugar). When sprayed on *Arabidopsis* leaves its effect can be easily monitored by illumination with UV-light, since this light will be dissipated by the PSII antenna as visible red fluorescence generating a high chlorophyll fluorescence phenotype. This can be used to test the evenness of spraying (T. Pfannschmidt *et al*, pers. obs.). In contrast, DBMIB is unstable in tissues and looses its effect within hours (Pfannschmidt *et al.*, 2001). It is, therefore, desirable to repeat its application during the course of an experiment when working with plants. When working with photosynthetic organisms that can be cultured in liquid media such as algae or cyanobacteria, however, the inhibitor can be applied into the media, resulting in a permanent supply of the inhibitor (Alfonso *et al.*, 2000; Kovacs *et al.*, 2000). In *Chlamydomonas reinhardtii* it has been reported that stable inhibition at the Q_O site could be achieved by the plastoquinone analog stigmatellin, while comparable effects with DBMIB could only be observed when the compound was added in light but not in the dark (Finazzi *et al.*, 2001). The concentrations applied are another crucial point for a successful experiment. Concentrations that completely block the electron transport, in general, should be avoided in gene expression studies since they cause a number of side effects. In addition, applied in high concentrations DBMIB loses its specificity and binds also to the Q_B binding site, exacerbating the interpretation of results. Furthermore, a complete block of electron transport destroys the ability of photosynthesis for regulation, since a block prevents dynamic physiological changes of electron flow. Redox signals, however, are generated by perturbation in the redox balance of a compound that requires a permanent flow of electrons, but not by arresting it in a completely reduced or oxidized state. It is, therefore, useful to perform a careful titration of the concentration to be used for the final experiment of interest in order to determine the physiologically relevant range of regulation. In our experience, 30–50 % inhibition of electron flow was found to be sufficient to induce a significant response at the level of gene expression (Pfannschmidt *et al.*, 1999, 2001). Since it is difficult to determine the effective concentration within the tissue, the optimal concentration for this effect must be determined experimentally. This can be done by measuring Chl fluorescence before and after application of the inhibitor using a pulse amplitude-modulated fluorometer, which demonstrates the direct effect on the photosynthetic electron flow. It should be noted that – depending on leaf structure (thickness of cuticule and mesophyll) – the necessary concentration may vary between species (T. Pfannschmidt *et al*, pers. obs.).

Many studies have used changes in illumination, either alone or in combination with the inhibitors mentioned above, to induce redox signals. Dark–light shifts, low-light to high-light shifts or shifts between PSI- and PSII-light have commonly been used (reviewed in (Pfannschmidt, 2003). Each of these treatments induces different physiological responses that should be kept in mind when planning an experimental set-up. A dark–light transition of an etiolated seedling, for instance, will activate strong photomorphogenic effects controlled by photoreceptors, which include the build-up and assembly of the photosynthetic apparatus. Therefore, it is necessary to work with plants containing mature chloroplasts if studying photosynthetic redox signals. Typically, in dark–light illumination regimes plants grown in a defined light–dark cycle are used. With the onset of light, the activation of many light-dependent processes such as the Calvin–Benson cycle can be observed. Application of DCMU to control plants is helpful to distinguish between those processes activated by photoreceptors and those activated by photosynthetic electron flow, since only the latter are affected. Low-light to high-light shifts represent a different scenario, since under these conditions stress-dependent processes become activated. In general, the increase of incident light facilitates the generation of reactive oxygen species (ROS) which, in turn, activates antioxidant and repair processes. It is difficult to reconcile the responsible redox signals in these experiments directly as many different sites are possible for the generation of signals, e.g. reduced electron transport components, ROS and the antioxidant systems. However, considerable progress has been achieved in this field in recent years by using genetic strategies (see below). It should be mentioned that low-light to high-light shifts are strongly affected by the prevailing temperature (Huner *et al.*, 1998; Ensminger *et al.*, 2006). Low temperatures will decrease the activities of the Calvin–Benson cycle and the regeneration of the final electron acceptor $NADP^+$, which reduces the photosynthetic electron transport efficiency even under low-light conditions. Stable and controlled temperature conditions are therefore required in all such experiments. Another important environmental factor in this context is water supply. Plants under water stress close their stomata in order to reduce the loss of water. However, this results in decreased CO_2-uptake and hence slows down the Calvin–Benson cycle, which in turn affects the electron transport. Sufficient and stable watering is therefore a further requirement in order to study light-induced redox signals. Shifts between PSI- and PSII-light induce redox signals in the low-light range and are typically not connected to stress responses (Fey *et al.*, 2005*b*; Piippo *et al.*, 2006). The signals are generated within the photosynthetic electron transport and activate counterbalancing effects that are aimed to increase the efficiency of light absorption (see Introduction).

The use of photosynthesis mutants represents a third strategy to uncover possible origins of redox signals in the electron transport chain that control gene expression. In such mutants a genetic defect limits or blocks the electron transport at a distinct site, and thus generates reduced conditions before and oxidized conditions after the defect (Yang *et al.*, 2001; Sherameti *et al.*, 2002; Frigerio *et al.*, 2007). The use of mutants avoids many of the problems mentioned above, such as side effects of inhibitors or the ambiguity of influences by photoreceptors, since the known location of the genetic defect allows for direct conclusions regarding photosynthesis as a signal generator. On the other hand, mutants with strong

defects that directly affect the generation of the redox signal (e.g. absence of complexes of the photosynthetic electron transport) are of very limited use to study up- or down-regulation of gene expression. The reason for this is that the defect already exists in early development when the photosynthetic apparatus is established. Typically, mutants with a photosynthetic block display a pale-green or even white phenotype and have to be maintained on sugar-containing media (Leister and Schneider, 2003). Since photosynthesis in these mutants is disturbed in general, the electron transport is locked in a distinct state and the system loses its ability to sense environmental changes, and thus the ability for gene regulation (comparable to high concentrations of electron transport inhibitors; see above). Changes in gene expression observed in comparison with the wild-type actually show how the (remaining) signalling network of the mutant tries to compensate for the genetic defect. Furthermore, supplementing the mutants with sugar may cause cross-talk between carbohydrate and redox signalling (Oswald *et al.*, 2001). Such mutants, therefore, can be helpful to identify photosynthetic electron transport as the origin for signals regulating genes; however, since they are static with respect to their defect conclusions on the precise site of signal generation and the regulation mode require additional experiments.

Solutions for many of problems described above are inducible experimental set-ups in which signals can be generated at a given time point; this is a general requirement for all studies focussed on regulation. Controlled induction of a redox signal requires either a regulated environmental system or a genetic system that can be controlled physiologically. Examples of environmental systems are light-shift experiments as described above, since they enable redox signals to be induced at a given time. Another experimental possibility is to change the ambient CO_2 concentration, which enhances or represses the Calvin–Benson cycle and thus the electron flow through the photosynthetic transport chain (Wormuth *et al.*, 2006). An example for an inducible genetic system is represented by the *flu* (fluorescent) mutant of *Arabidopsis*. Under continuous illumination the mutant is undistinguishable from wildtype; however, it accumulates free protochlorophyllide (Pchlide) when put into darkness. Upon re-illumination the mutant then produces high amounts of singlet oxygen and can be used to study specifically the responses to this particular ROS (Meskauskiene *et al.*, 2001). Although the system is not very physiological, it provides a unique approach to test the impact of this particular ROS that is not possible with other systems such as high light since these also produce other ROS.

In summary, each of the strategies described above has its specific advantages or disadvantages. It is therefore highly recommended to combine several of these strategies in order to get as comprehensive a picture of the studied biological problem as possible.

POSSIBLE TRANSDUCTION OF REDOX SIGNALS

Transduction within plastids

While many studies have confirmed the existence of several redox signals controlling gene expression in plastids and the nucleus, little at present is known about the transduction of the signals to the level of gene expression. Within plastids redox signals have to be transduced only a short distance to the plastome (Bruick and Mayfield, 1999; Link, 2003; Pfannschmidt and Liere, 2005; Shiina *et al.*, 2005). As outlined in the Introduction, the LTR controlling chloroplast genes for photosynthetic core proteins involves the action of the thylakoid membrane kinase STN7 (Bonardi *et al.*, 2005). This kinase might therefore represent a crucial component for sensing PQ redox signals and transducing them via a (putative) phosphorylation cascade to the gene expression machinery (Fig. 2). The model of a phosphorylation cascade is consistent with models that describe the control of plastid transcription by phosphorylation of sigma factors of the RNA polymerase (Tiller and Link, 1993; Link, 1996). Whether sigma factors are involved in this regulation event or not is as yet unclear; therefore, we propose the existence of a putative redox-responsive factor (RRF, Fig. 2) that perceives the PQ redox signal. Another model for redox control of plastid gene expression involves thioredoxin regulation of thiol groups in the protein disulfide isomerase. It controls binding of a RNA-binding complex to the *psbA* mRNA and subsequent translation initiation (Danon and Mayfield, 1994; Kim and Mayfield, 1997). Because thioredoxins can move freely through the stroma they provide an easy way of signal transduction. The model has been proposed in *Chlamydomonas reinhardtii*, but some data suggest that it might also be valid for higher plants (Shen *et al.*, 2001).

Transduction into the nucleus

Transduction of photosynthetic redox signals that control nuclear gene expression appears to be more complex than that within plastids since such signals have to leave the plastid, pass the cytosol and enter the nucleus. They therefore represent a novel type of retrograde signals (Rodermel, 2001; Beck, 2005; Nott *et al.*, 2006; Bräutigam *et al.*, 2007; Pesaresi *et al.*, 2007). Two general types of signal-transduction mechanisms have been proposed: in the first the redox signal is sensed by a plastid-internal system and transduced over the envelope, whilst in the second a redox-regulated compound can leave the plastid directly.

Examples for the first scenario have been reported for thylakoid-located PQ molecules and short-lived singlet oxygen. The best candidate for sensing of PQ redox signals at present is the STN7 kinase (see above), but it is not clear whether it is involved in all mechanisms reported to be under PQ redox control. Some observations suggest a potential functional interaction of STN7 with its paralogue kinase STN8; however, further investigations are necessary in order to fully understand these relationships (reviewed in Dietzel *et al.*, 2008). In addition, a recent array study proposed that under varying light qualities of low intensity the redox state of stromal components (potentially thioredoxin) may affect the expression of nuclear genes via a still-unknown pathway (Piippo *et al.*, 2006). Beside the light-quality-dependent low-light pathway(s) mentioned above, another one has been described in *Dunaliella tertiolecta*, which represses *Lhcb* gene expression upon a high- to-low-light shift (Fig. 2). A current model assumes that a light-intensity-dependent

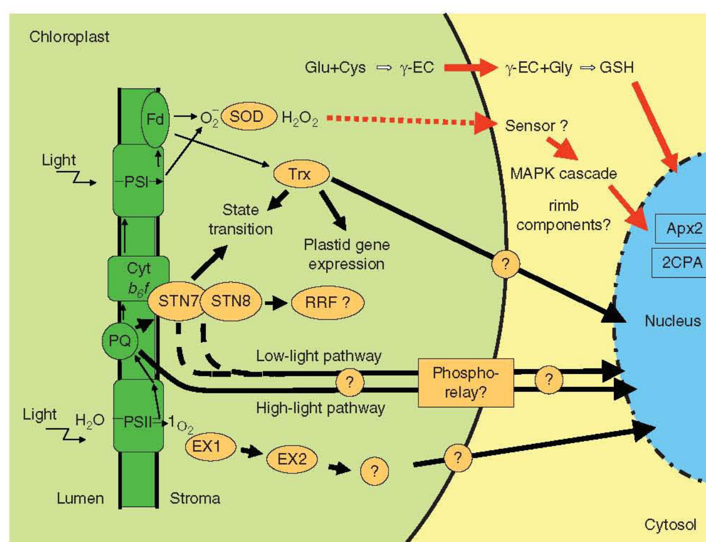

FIG. 2. Possible signal transduction pathways of photosynthetic redox signals. The three plant cell compartments are depicted schematically: chloroplast (light green), cytosol (light yellow) and nucleus (blue). Redox signals generated within the electron transport chain (dark green) or by generation of ROS initiate signalling pathways that activate or repress specific target genes in the nucleus (for details see text). Thin black arrows represent electron transport. ROS are generated as by-products of photosynthesis, e.g. by transfer of electrons from PSI or reduced ferredoxin (Fd) to oxygen-generating superoxide (Mehler reaction). This is detoxified by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). Hydrogen peroxide can be reduced to water by ascorbate peroxidases using ascorbate as the electron donor, and requiring glutathione (GSH) to restore the electron donor. Unscavenged H_2O_2 is able to diffuse across the chloroplast envelope and is thought to start MAP kinase cascades in the cytosol. Electrons from PSI are also transferred to thioredoxin (Trx), which can affect LHCII phosphorylation (state transitions) and plastid gene expression and possibly also nuclear gene expression. Glutathione synthesis might be another pathway by which stress signals can directly leave the plastid. Another ROS, singlet oxygen ($^1O_2$), is generated at PSII. Because of its short half-life it requires additional signalling components, Executer 1 and 2 (EX1, EX2). The plastoquinone pool is the origin for at least two redox signalling pathways that are active under low or high light and which are targeted to plastid and nuclear gene expression machineries. Perception of the redox signal requires a proposed redox responsive factor (RRF). An important sensor for the PQ redox state represents the thylakoid kinase STN7 (possibly in conjunction with STN8). Present data point to the involvement of a phosphorylation cascade in PQ redox signal transduction. Red arrows, redox signals by components that directly leave the plastid; black arrows, redox signals that are mediated by unknown components. Proteins are indicated by orange.

pathway exists that involves the action of cytosolic kinase activities that eventually control the action of *Lhcb* gene repressors (Escoubas *et al.*, 1995; Durnford and Falkowski, 1997; Chen *et al.*, 2004). A completely different signalling pathway has been characterized by analysis of the *flu* mutant mentioned above. It was demonstrated that the response to singlet oxygen can be blocked genetically and that the activation of the activated-stress programme involves plastid-localized proteins called Executer1 and 2 (EX1, EX2; Fig. 2; Wagner *et al.*, 2004; Lee *et al.*, 2007). Their precise function is not known and neither are the further-downstream components of a signalling cascade. However, the singlet-oxygen-induced response was found to be promoted by ethylene-, salicylic acid- and jasmonic acid-dependent signalling pathways, whilst it was blocked by a jasmonic acid precursor (Danon *et al.*, 2005).

Hydrogen peroxide is the principle ROS in plants that accumulates under various conditions such as excess excitation energy, drought, chilling and starvation. It activates a different response programme than singlet oxygen and is also more stable. It is therefore assumed that it can freely diffuse across the chloroplast envelope and that it activates a mitogen-activated protein kinase (MAPK) cascade in the cytosol that subsequently affects gene expression in the nucleus (Fig. 2; Kovtun *et al.*, 2000; Vranova *et al.*, 2002; Apel and Hirt, 2004; Mittler *et al.*, 2004). By this means it represents an example for the second type of signal transduction.

Recent observations have suggested that glutathione may also act as a plastid signal that controls expression of stress-defence genes. Glutathione is an important component of the scavenging machinery that counteracts ROS. In *Arabidopsis* it has been proposed that the first step of its synthesis [cysteine and glutamate fused to γ-glutamylcysteine (γ-EC)] is confined to plastids while the second step (γ-glutamylcysteine and glycine fused to glutathione) is performed in the cytosol (Wachter *et al.*, 2005; Rausch *et al.*, 2007). Plastid γ-EC must therefore leave the plastid. Since its synthesis is affected by changes in photosynthesis, it can potentially report such changes to the nucleus (Mullineaux and Rausch, 2005).

VELOCITY OF REDOX SIGNALS

The time frames in which acclimation responses occur have been measured at various levels, e.g. by determination of changes in photosystem number, Chl and protein accumulation (Kim *et al.*, 1993; Walters and Horton, 1994, 1995*a*, *b*; Pfannschmidt *et al.*, 2001). However, the speed at which the respective genes respond to the redox signals is less well investigated. To do this requires that a distinct redox signal is induced at a given time and that the changes in gene expression are then followed during the course of the response. Light stress experiments indicated that the nuclear genes *Apx1* and *Apx2* (encoding cytosolic ascorbate peroxidases) are activated within 15–30 min upon a low-light to high-light shift in *Arabidopsis* (Karpinski *et al.*, 1997); this was found to be part of a systemic response to excess excitation energy (Karpinski *et al.*, 1999). In another study, kinetic experiments with isolated plastids showed that chloroplast gene expression is affected within 15–30 min after inducing a redox signal by light quality shifts (Pfannschmidt *et al.*, 1999). First kinetic experiments suggest that the same redox signals induced by light quality are transduced to the nucleus within 30 min, which corresponds to the time frame within the plastids (K. Bräutigam and T. Pfannschmidt, unpubl. res.). These data suggest that the signal transduction cascades already exist when the signals are generated, and that the signals are processed as fast as other intracellular stimuli. It remains to be clarified as to how the components of the proposed signalling pathways (cf. Fig. 2) are integrated into the known parts of the signalling network.

PRIMARY TARGET GENES OF REDOX SIGNALS

The identification of (a) primary target gene(s) of a certain signal helps us to understand which cellular processes are under redox control and provide (a) molecular tool(s) that can be used to analyse the transduction pathway and its components. One possibility is to fuse the target gene or its respective promoter to a reporter gene and to transform plants (typically *Arabidopsis*) with such a construct. This transgenic line then can be used to analyse the expression of the target gene in space and time *in planta*: this has led to the discovery of systemic acclimation to excess excitation energy (Karpinski *et al.*, 1999). In addition, after mutagenesis of such a stable transformed line, the progeny of it can be screened for a lack of a reaction of the reporter occurring to the signal of interest. The defective genes in mutants that are identified can be mapped and provide potential components of the signal transduction pathway, for instance in the case of *rimb* (redox imbalance) mutants (Heiber *et al.*, 2007). Here, the promoter of the nuclear 2-Cys peroxiredoxin-A (2CPA) gene was used to search for these mutants. The 2CPA gene was shown earlier to be redox-regulated under light stress conditions. Kinase inhibitor experiments indicated that the signal transduction involves the action of MAP kinases and serine/threonine kinases, in accordance with the stress signal transduction mentioned above. Furthermore the involvement of abscisic acid could be shown (Konig *et al.*, 2002; Baier *et al.*, 2004).

The identification of real primary target genes is a difficult task. Many recent studies have used array techniques in order to investigate gene expression changes in responses to various redox signals such as hydrogen peroxide, singlet oxygen or redox signals from the photosynthetic electron transport chain. These studies indicate that several hundred genes are responsive to many such signals (Desikan *et al.*, 2001; op den Camp *et al.*, 2003; Davletova *et al.*, 2005; Fey *et al.*, 2005*b*; Vanderauwera *et al.*, 2005; Piippo *et al.*, 2006). The major problem in all these studies is to distinguish between genes that are controlled directly by a given signal (a true primary target gene) and genes that are controlled indirectly (the secondary or tertiary target genes), e.g. by the effects resulting from expression changes in primary target genes. The most effective way to identify potential primary targets is to perform a kinetic study in which gene expression changes are determined at different times after application of a defined environmental signal. Such a kinetic should range from short (minutes) to middle (hours) and late (days) stages to cover the complete extent of the physiological response. After a first survey, this time range can be refined according to the results. As a general assumption we propose that those genes that exhibit expression changes first and in a strong manner are the primary target genes of a given signal. Modern array techniques provide the technical possibility to observe the full genome (i.e. from *Arabidopsis*) in such a study. However, it should be noted that most probably many genes are affected by several parameters at the same time and that primary target genes responsive to only one environmental factor might be rare.

Several bioinformatics approaches can be performed using the data from such a kinetic array analysis. Most useful is analysis of gene expression patterns, which can unravel groups of co-regulated genes (Biehl *et al.*, 2005). Such genes can have a common input signal for regulation, but do not necessarily need to have one. In any case, each potential target gene of interest should be checked for the reproducibility of its expression pattern by an independent experiment and approach (e.g. Northern analysis) before it is used as a target gene in a reporter-gene approach as mentioned above. However, array techniques provide the possibility to uncover potential expression signatures within a data set that are typical for a given redox signal. This signature can be compared with other array data and can lead to identification of genes that are only regulated under one certain condition. By this means redox-responsive promoters or promoter elements might be also identified.

DIFFERENTIATION AND INTEGRATION OF VARIOUS REDOX SIGNALS

A central question in plant cell biology is how the integration of various environmental signals at the same time is achieved in order to regulate expression of genes for appropriate responses (Bräutigam *et al.*, 2007). Understanding of this complex task includes several plant-specific but also some general problems of modern molecular cell biology (summarized in Fig. 3).

Under natural conditions many of the redox signals discussed in this article can occur at the same time or in a fast

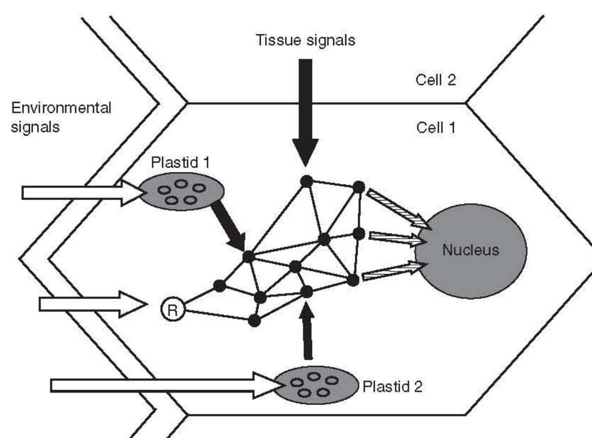

FIG. 3. Model of integration of various signals in a plant cell. A plant cell and its environment including neighbouring cells are shown. Various environmental influences that are detected by cytosolic receptors (circle marked 'R') or photosynthesis in different chloroplasts are represented by white arrows. Due to their different positions in the cell, the impact on the plastids may vary (plastid 1 and 2). The perceiving systems have different impacts (black arrows of different thickness) on the intracellular signalling network (represented as black dots connected by lines), which integrates these signals as well as signals from other cells, resulting in integrated signals (hatched arrows) that affect gene expression events. The 'gene-copy-number-problem' is indicated by the numerous ovals within the plastids, representing multiple copies of the plastome (see text for details).

consecutive manner. The nucleus has to recognize all these different redox signals and, in addition, to distinguish one from another as well as from other environmental signals (mediated for instance by photoreceptors). Such a specific signal recognition and initiation of respective responses requires sophisticated mechanisms and interactions within the signalling networks, which we are just beginning to understand. Furthermore, higher plants possess up to 100 single plastids per cell and not all of them necessarily send the same signals. For example, strong light-quality gradients that affect photosynthesis occur even within a leaf or a single cell, and therefore the redox signals of the plastids may vary depending on their individual localization within the cell (Terashima and Saeki, 1985). The large number of plastids also introduces another consideration, the so-called gene-copy-number problem (for a review see Bräutigam *et al.*, 2007). Higher plant plastids contain up to 100 copies of the plastid's own genome, the plastome. Since the photosynthetic apparatus and many other protein complexes in plastids are comprised of a patchwork of plastid and nuclear encoded protein subunits, up to a 10 000-fold excess of plastid over nuclear gene copies for one and the same protein complex has to be co-ordinated in expression. Interestingly, plastids of all known taxa universally encode the central proteins of the photosystems on the plastome, providing the possibility that the plastids themselves serve as the pacemakers in the co-ordination of photosynthesis gene expression within the two genetic compartments (Race *et al.*, 1999). Redox regulation of chloroplast gene expression has been hypothesized as the evolutionary reason for the maintenance of a plastid genome (Allen, 1993).

It is a challenging task to understand how environmental signals of different intensity and quality – which in addition vary temporarily, spatially and in rate – are integrated into a cellular response that helps the plant to deal with environmental fluctuations and stresses. The cytosolic network of interacting signalling components most likely does most of this job, resulting in a unique combination of transcription factors that activate or repress a combination of genes within the nucleus and plastids (and mitochondria) that is appropriate for the given environmental situation (Fig. 3). In addition, the data presently available clearly indicate that many other levels of gene expression are also included in signal integration, e.g. post-transcriptional and post-translational events. Only approaches at the level of systems biology will provide enough data that can be used to generate an integrated view of all cellular responses at the same time. However, such studies will only be successful if the basic experimental problems outlined above are solved.

CONCLUSIONS

Photosynthetic redox signals generated in the electron transport chain are connected to many environmental influences either directly via illumination or indirectly via the Calvin–Benson cycle. Thus they are able to signal in a very sensible manner the prevailing environmental conditions to the level of gene expression. Interactions with the energy state of the cell, which also fluctuates in response to many environmental conditions, are very likely. Further studies of redox signalling networks that also include mitochondria will unravel these connections (Rhoads and Subbaiah, 2007).

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6 Manuscript III

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The Role of Phosphorylation in Redox Regulation of Photosynthesis Genes *psaA* and *psbA* during Photosynthetic Acclimation of Mustard

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ABSTRACT The long-term response (LTR) to light-quality gradients improves performance and survival of plants in dense stands. It involves redox-controlled transcriptional regulation of the plastome-encoded genes *psaAB* (encoding the P700 apoproteins of photosystem I) and *psbA* (encoding the D1 protein of photosystem II) and requires the action of plastid-localized kinases. To study the potential impact of phosphorylation events on plastid gene expression during the LTR, we analyzed mustard seedlings acclimated to light sources favoring either photosystem I or photosystem II. Primer extension analyses of *psaA* transcripts indicate that the redox regulation occurs at the principal bacterial promoters, suggesting that the plastid encoded RNA polymerase (PEP) is the target for redox signals. Chloroplast protein fractions containing PEP and other DNA-binding proteins were purified from mustard via heparin-Sepharose chromatography. The biochemical properties of these fractions were analyzed with special emphasis on promoter recognition and specificity, phosphorylation state, and kinase activity. The results demonstrate that the LTR involves the action of small DNA-binding proteins; three of them exhibit specific changes in the phosphorylation state. Auto-phosphorylation assays, in addition, exhibit large differences in the activity of endogenous kinase activities. Chloroplast run-on transcription experiments with the kinase inhibitor H7 and the reductant DTT indicate that phosphorylation events are essential for the mediation of redox signals toward *psaA* and *psbA* transcription initiation, but require the synergistic action of a thiol redox signal. The data support the idea that redox signals from the thylakoid membrane are linked to gene expression via phosphorylation events; however, this mediation appears to require a complex network of interacting proteins rather than a simple phosphorelay.

Key words: Light-quality acclimation; redox control; protein phosphorylation; chloroplast transcription; *Sinapis alba*.

INTRODUCTION

Photosynthetic organisms possess a great number of molecular mechanisms that enable them to acclimate the photosynthetic process to a fluctuating environment (Aro and Andersson, 2001; Blankenship, 2002). Especially changes in incident light directly affect the photosynthetic electron transport and can dramatically reduce its efficiency. Acclimation responses maintain or restore the photosynthetic electron flux under such adverse conditions and, by such means, help to keep the net energy fixation as high as possible (Eberhard et al., 2008; Walters, 2005).

Naturally occurring light-quality gradients often result in a favored excitation of one photosystem over the other. Since the two photosystems work electrochemically in series, this induces an imbalance in excitation energy distribution between them. In the short term, this is counteracted by state transitions that occur in the order of minutes. In this acclimation response, the mobile part of the light harvesting complex of photosystem II (PSII) (LHCII) is phosphorylated through the action of a redox-sensitive kinase and subsequently migrates and attaches to photosystem I (PSI). The variation of the antenna cross-section redirects a part of the incident light

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between the photosystems and restores the excitation imbalance between them. The activity of this LHCII kinase is controlled by the redox state of the plastoquinone (PQ) pool, where its reduction results in activation and its oxidation in inactivation of the kinase (Allen and Forsberg, 2001; Haldrup et al., 2001; Wollman, 2001). Recently, kinases named STT7 and STN7 have been uncovered in *Chlamydomonas* and *Arabidopsis*, which were shown to be essential for the LHCII phosphorylation (Bellafiore et al., 2005; Depege et al., 2003). The redox-triggered regulation of the LHCII kinase activity is still not understood; however, mutation analyses demonstrated that it involves the Qo site of the cytochrome b_6f complex (cytb_6f), which serves as a sensor for the redox state of the PQ pool (Finazzi et al., 2001; Vener et al., 1997; Zito et al., 1999). In the long term, excitation imbalances are counteracted by a re-adjustment of photosystem stoichiometry, which occurs within hours to days, dependent on the species. This long-term response (LTR) results in the same but longer-lasting effect as the state transition and can be observed over a wide range of species, ranging from cyanobacteria over unicellular algae up to vascular plants (Allen and Pfannschmidt, 2000; Melis, 1991; Murakami et al., 1997; Pfannschmidt, 2003). In mustard and pea, it could be shown that this response is also regulated by the redox state of the PQ pool via a mechanism in which the expression of the core proteins of the two photosystems is oppositely regulated (Pfannschmidt et al., 1999a, 1999b; Tullberg et al., 2000). Under conditions resulting in a reduced PQ pool, transcription of *psaAB* (encoding the P700 apoproteins A and B of PSI) is enhanced, while this occurs for *psbA* (encoding the D1 protein of PSII) under conditions leading to an oxidized PQ pool. Comparable regulation mechanisms have been reported in the cyanobacterium *Synechocystis* PCC 6803 (Alfonso et al., 2000; Li and Sherman, 2000) and the green alga *Chlamydobotrys stellata* (Kovacs et al., 2000). In analogy to prokaryotic systems, it has been proposed that in vascular plants, redox signals from the thylakoid membrane might be transduced to the gene expression level via a phosphorylation cascade (Allen, 1993). Recent studies on *Arabidopsis* knock-out mutants lacking the kinase STN7 uncovered that these mutants are not able to perform a proper LTR, indicating that STN7 is responsible for the initiation of both short-term and long-term acclimation (Bonardi et al., 2005). This suggests that STN7 regulates or affects (a) pathway(s) toward the gene expression level. Whether this occurs directly via phosphorylation of an unknown substrate or indirectly via the phosphorylated LHCII is not understood yet. The present situation became even more complex with the recent identification of a further kinase found to be implicated in redox regulation of chloroplast gene expression, the chloroplast sensor kinase (CSK) (Puthiyaveetil et al., 2008). CSK is a prokaryotic-type kinase that is conserved in cyanobacteria, algae, and vascular plants. *Arabidopsis* CSK knock-out mutants display a severe disturbance in *psaA* transcript accumulation during the LTR, indicating that CSK is essential for proper redox regulation of *psaA*. How the functions of STN7 and CSK relate to each other is unknown to date.

Chloroplast transcription involves the activity of two different RNA polymerases—a single-subunit phage-type nuclear encoded polymerase that appears in chloroplasts in two different copies (RpoT1 and RpoT2) and a multi-subunit prokaryotic-type plastid encoded polymerase (PEP) (Cahoon and Stern, 2001; Hedtke et al., 1999; Liere and Maliga, 2001). The genes *psbA* and *psaAB* are predominantly transcribed by PEP, suggesting that it is the main target for redox regulation. Phosphorylation was also found to be a key event in the developmental regulation of chloroplast transcription initiation during the light-induced transition from etioplast to chloroplast stage. Phosphorylation of both PEP subunits and sigma factors has been shown to be important (Baginsky et al., 1997; Tiller and Link, 1993). This phosphorylation is catalyzed by a serine-specific kinase, termed plastid transcription kinase (Baginsky et al., 1999). The kinase belongs to the family of casein kinase II and is part of the PEP complex (Ogrzewalla et al., 2002). Its *in-vitro* phosphorylation activity was shown to be regulated by its own phosphorylation state (Baginsky et al., 1997) and by the redox state of glutathione (Baginsky et al., 1999). These results correlate with in-planta observations demonstrating that its activity is enhanced under high light stress when the glutathione redox state in chloroplasts turns to be more oxidized (Baena-Gonzalez et al., 2001).

Both the identification of STN7 and CSK as regulators of LTR as well as the general regulation of chloroplast transcription by phosphorylation strongly suggest that redox regulation during LTR is mediated via phosphorylation cascades. However, so far, it has been not experimentally addressed whether or not the LTR affects the phosphorylation of chloroplast protein fractions containing the plastid transcription machinery and its associated regulatory factors. To this end, mustard seedlings were acclimated to PSI- or PSII-light, chloroplasts were isolated and organellar RNA polymerase activity as well as functionally related factors such as DNA-binding proteins were enriched by heparin-Sepharose chromatography. Subsequently, biochemical properties of these fractions with respect to promoter recognition, aminoacid-specific phosphorylation and endogenous kinase activity were analyzed. The results were discussed with emphasis on the role of phosphorylation in the redox regulation of the LTR.

RESULTS

Purification of Chloroplast Protein Fractions with DNA Binding and PEP Activity

In order to study the biochemical properties of chloroplast proteins involved in gene expression, large amounts of uniform and transcriptional highly active leaf material are required. Since *Arabidopsis* is not well suited for such purposes, we used its close relative, mustard, for our study. Seedlings were grown for 7 d either continuously under PSI- or PSII-light (PSI or PSII

plants, respectively) or 5 d under PSI-light followed by 2 d in PSII-light and vice versa (PSI-II or PSII-I plants, respectively) as reported earlier (Pfannschmidt et al., 1999a, 1999b). Determination of various Chl fluorescence parameters using video imaging demonstrated that the cotyledons performed a well defined LTR without any phenotypic differences between the different growth conditions (Figure 1A and Table 1).

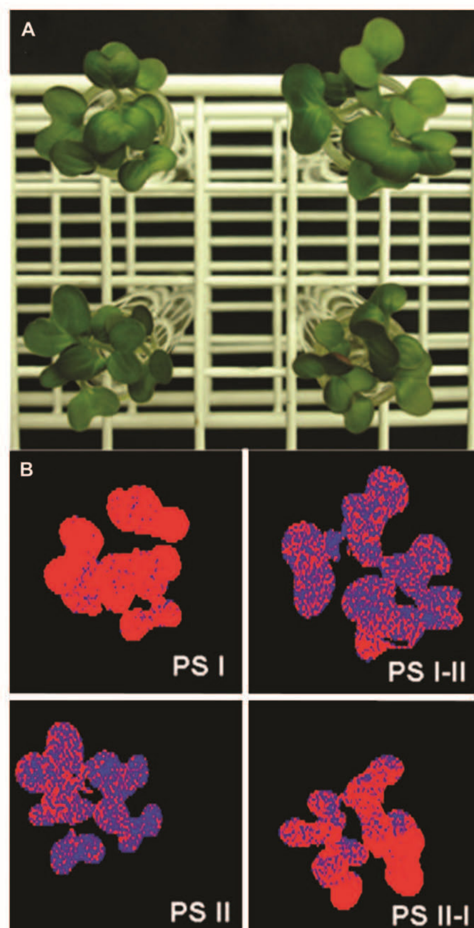

Figure 1. Video Imaging of Chl Fluorescence Parameter Fs/Fm in Mustard Seedlings Acclimated to PSI- or PSII-Light.

(A) Picture under white light of PSI, PSI-II, PSII, and PSII-I seedlings placed in one rack for simultaneous Chl fluorescence detection.

(B) Fs/Fm parameter of the same seedlings as in (A) shown in false colors. Red, high Fs/Fm; blue, low Fs/Fm. For integrated data of this picture, see Table 1.

The cotyledons revealed a reversible response with a low Fs/Fm value (blue) after acclimation to PSII-light and a high Fs/Fm value (red) after acclimation to PSI-light. The seedlings exhibited no stress symptoms (indicated by a high and constant Fv/Fm value) and displayed a higher effective quantum yield after acclimation to PSII-light. The 1-qP value indicates that the PQ pool was more reduced in the PSI-light than in PSII-light acclimated plants under the conditions of Chl fluorescence measurement. These data are consistent with the typical LTR of *Arabidopsis* (Wagner et al., 2008). For subsequent biochemical analyses, we used proteins from PSI-II and PSII-I seedlings, the two acclimation states that exhibited the strongest functional differences in earlier experiments (Pfannschmidt et al., 1999b). Cotyledons were harvested and chloroplasts were isolated by sucrose-gradient centrifugation. Isolated organelles were lysed in a buffer containing Triton X-100 and the resulting lysates were subjected to heparin-Sepharose (HS) chromatography. Bound proteins were eluted with a single high-salt step (Figure 2A). Such preparations have been extensively characterized earlier and contain a mixture of chloroplast proteins with affinities to nucleotides including chloroplast RNA polymerase PEP-A, sigma-factors, and other unknown proteins with DNA/RNA-binding activity, DNA polymerase activity, and plastid transcription kinase activity (Baginsky et al., 1997; Pfannschmidt and Link, 1994; Tiller and Link, 1993). The eluted fractions exhibiting transcriptional activity were identified with a standard test using heat-denatured calf thymus DNA as matrix (data not shown). Active fractions were pooled and further analyzed. Aliquots with equal protein amounts were subjected to SDS–PAGE followed by silver staining (Figure 2B). The protein composition of both preparations was apparently the same, covering at least 60–80 proteins ranging from 10 to 150 kDa in size. All visible proteins could be identified in comparable amounts, suggesting no major structural differences between the two samples.

Structure and Usage of the *psaA* and *psbA* Gene Promoters

Most plastid photosynthesis genes have promoters of prokaryotic-like structure. The *psbA* promoter of mustard has been analyzed in great detail and displays a relatively simple structure consisting of one –10 and one –35 region interspersed by

Table 1. Photosynthetic Parameters in Mustard Seedlings Acclimated to Different Light Qualities.

	Fv/Fm	Yield	Fs/Fm	1-qP
PSI	0.88 ± 0.01	0.72 ± 0.01	0.08 ± 0.004	0.14 ± 0.01
PSI-II	0.87 ± 0.01	0.77 ± 0.01	0.03 ± 0.001	0.06 ± 0.01
PSII	0.87 ± 0.01	0.79 ± 0.01	0.03 ± 0.004	0.05 ± 0.01
PSII-I	0.87 ± 0.01	0.74 ± 0.01	0.06 ± 0.004	0.11 ± 0.01

Room-temperature Chl fluorescence of PSI-, PSI-II-, PSII-, and PSII-I plants shown in Figure 1A was recorded by video imaging. Values represent the mean of five to seven seedlings.

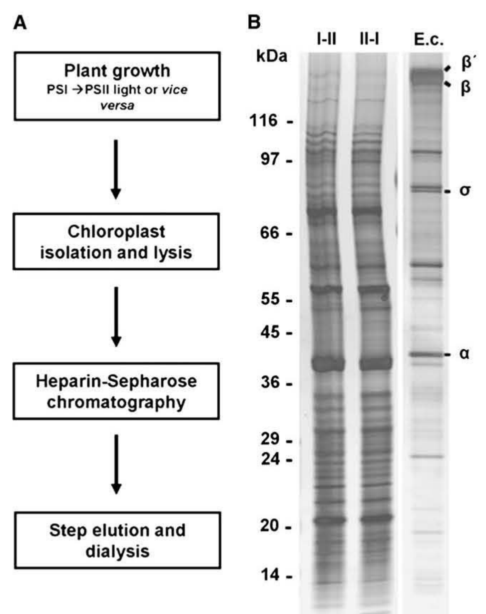

Figure 2. Purification and Electrophoretic Separation of Chloroplast Protein Fractions with RNA Polymerase Activity.

(A) Scheme of isolation procedure for chloroplast protein fractions with transcription activity.

(B) Peak fractions after heparin-Sepharose chromatography and high salt step elution. Equal protein amounts (15 μg) of the peak fractions were separated on a 6%–16% SDS gel and stained with silver. Sizes of marker proteins separated in parallel are given in the left margin; subunits of *E. coli* RNA polymerase (E.c.) are shown as additional control.

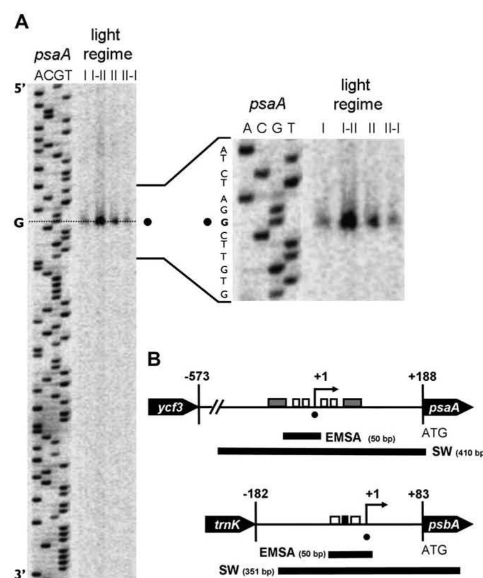

Figure 3. Redox Control at the *psaA* Promoter and Promoter Structures of Plastid Genes *psaA* and *psbA*.

(A) Primer extension assays at the *psaA* promoter. Plants were grown under PSI, PSI-II, PSII, and PSII-I growth light regimes and total RNA was isolated from each condition. A fluorescence dye-labeled primer was designed to anneal within the first 50 bp of the coding region of the *psaA* operon and was used both in a reverse transcription reaction with the isolated RNA and a sequencing reaction of chloroplast DNA fragments covering the *psaA* promoter region. Products were separated in parallel on a denaturing 4% acrylamide gel containing 7 M urea and detected by laser excitation in a Licor 4200 sequencer. A sequencer image of the primer extension analysis is shown. The DNA sequence within the *psaA* promoter is shown on the left, primer extension products on the right flanked by a magnification of the primer extension products. The detected 5′-end is marked by a dotted line; the respective transcription start nucleotide is given in bold letters.

(B) Schematic presentation of promoter structures of plastid genes *psaA* and *psbA*. Transcription start site is given as +1 and marked with the same black dot as in Figure 3A. All other positions are given relative to it. White boxes indicate –35/–10 boxes, gray-shaded boxes the position of the *psaA* region U (left of transcription start site) and region D element (right of transcription start site), black boxes a TATA-like element. Black bars indicate the position of probes used for EMSAs or Southwestern experiments (SW).

a single TATA-box-like element, resulting in a single defined transcription initiation site (Eisermann et al., 1990; Link and Langridge, 1984). Such –10 and –35 regions were also found in the *psaA* promoter of mustard, but in a tandem repeat (Summer et al., 2000). Furthermore, in the rice *psaA* promoter, additional *cis*-elements, called region D and region U, were identified, which are conserved also in the mustard promoter sequence. Despite this complex promoter structure, only a single 5′-transcript end of the *psaA* mRNA has been detected in white-light-grown mustard seedlings (Summer et al., 2000). In contrast, in *Arabidopsis*, which displays a comparable promoter structure, two 5′-transcript ends for the *psaA* mRNA were detected that both exhibited regulation under the same growth light regime as used here (Fey et al., 2005). To test whether such differential transcript initiation sites also occur at the mustard *psaA* promoter, we performed primer extension analyses with isolated RNA from PSI, PSI-II, PSII, and PSII-I plants under these conditions (Figure 3). Under all conditions, we found the same 5′-transcript end occurring at position –187 relative to the translation start site. The signal increased after a shift from PSI to PSII-light while it decreased in the reverse experiment, consistent with earlier results from Northern analyses (Pfannschmidt et al., 1999b). This indicates that redox regulation of the *psaA* gene occurs at a single transcript initiation site located directly behind typical PEP

promoter elements that is identical to the position detected earlier (see above). This suggests that these standard elements are sufficient to confer redox regulation in mustard while other *cis*-elements within the promoter region might be required under different conditions.

DNA Binding Proteins in HS Fractions

The protein composition of the PSI-II and PSII-I HS fractions was found to be highly comparable (Figure 2). Differential transcription initiation at the *psaA* and *psbA* promoters therefore requires very specific factors or protein complexes that are not detectable by simple protein comparison. In order to test whether such protein complexes are involved in promoter recognition, we performed electrophoretic mobility shift assays (EMSAs). Based on the promoter mapping data, synthetic fluorophore-labeled core promoter fragments covering respective transcription initiation sites and the 5′-upstream *cis*-elements (Figure 3B) were designed and used for *in-vitro* binding reactions with equal amounts of the two HS fractions. Generated protein–DNA complexes were separated on native polyacrylamid gels and identified by fluorescence scanning. Eight different protein complexes (Figure 4) could be observed. Complexes 1–6 were also present after addition of the unspecific competitor polydIdC, indicating that these DNA–protein complexes were of specific nature while complexes 7 and 8 were largely reduced upon the addition of the competitor. In addition, in competition experiments with an excess of the unlabelled promoter fragments, generation of

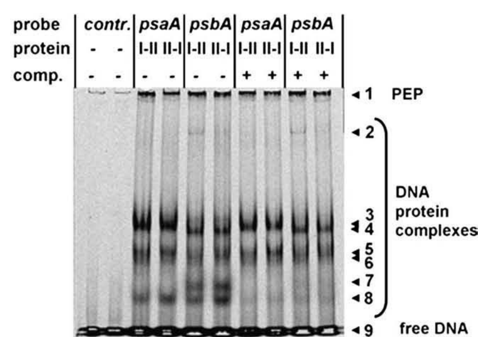

Figure 4. DNA-Binding Protein Complexes in HS Fractions.
A gray-scaled fluorescence image of the EMSA gel is shown. PSI-II or PSII-I heparin-Sepharose fractions were incubated with fluorescence-labeled core promoter probes for *psaA* and *psbA* (Figure 3B). After electrophoretic separation on a 6% native polyacrylamide gel, DNA–protein complexes were directly visualized using a fluorescence scanner. In order to test binding specificity, unspecific competitor (comp.) was added. The first two lanes represent negative probe controls (first lane: *psaA*; second lane: *psbA*). Signal 1 (PEP): binding signal of the plastid encoded RNA polymerase. Signals 2–8: seven different DNA-protein complexes. Signal 9: unbound, free DNA. Saturated signals in this area appear white/gray.

complexes 1–6 was repressed, providing further support for the conclusion that the observed interactions are specific (Supplemental Figure 1). The upper-most band (complex 1) occurred with both promoter probes and under all conditions tested. It represents the PEP-A complex described earlier, which migrates only very slowly in the gel matrix due to its high molecular weight (Pfannschmidt and Link, 1997). Complex 2 appeared only with the *psbA* promoter probe and predominantly in the PSI-II fractions. Complexes 3 and 4 differed slightly in size and occurred with either the *psbA* or the *psaA* probe only; however, the signal strength was the same, regardless of which HS fractions were used. Complexes 5 and 6 also differed slightly in size and occurred with both probes and both protein samples without exhibiting major differences in signal strength. This indicates the existence of at least three types of proteins interacting with the tested promoters in a (1) general (complexes 1, 5, 6), (2) promoter-specific (complexes 3, 4), and (3) promoter- and light-specific manner (complex 2). In control reactions with the promoter probes in a single stranded state, generation of a number of additional DNA-protein complexes could be observed, indicating the presence also of single strand binding proteins in the HS fractions. However, the binding properties of the HS fractions to such probes were very different from that to double-stranded probes, indicating that the involved proteins are different (Supplemental Figure 2). For transcriptional regulation, double-strand binding proteins are of major importance and our further studies were focused on these.

To characterize the promoter-binding proteins in a more direct way, we performed Southwestern analyses with the HS fractions. Equal amounts of the protein fractions were separated by SDS–PAGE and transferred to a nitrocellulose membrane. Blotted proteins were re-natured followed by incubation with fluorescent dye-labeled double-stranded DNA probes covering the *psaA* or *psbA* promoter regions (Figure 3B). After washing to remove excess DNA, the bound DNA was identified by a fluorescence scanner (Figure 5). With the *psaA* probe, we identified a set of ~20 DNA-binding proteins of 10–35 kDa in size, while the *psbA* probe was bound by a set of ~30 DNA-binding proteins of 10–85 kDa in size. While many signals in the range of 10–35 kDa were found with both promoter fragments, four signals at 39, 53, 56, and 85 kDa were exclusively detected with the *psbA* promoter. This indicates that the promoters of both genes are recognized by some common and also by different *trans*-acting factors, despite their similarity in *cis*-element structure. The principle DNA-binding proteins appeared to be identical in the two HS preparations; however, a few displayed differences in signal strengths. With the *psaA* promoter, only one signal was stronger in the PSI-II fraction (at 31 kDa, Figure 4), while six signals were stronger in the PSII-I fraction (at 13, 15, 21, 28, 29, and 32 kDa, Figure 4). In contrast, with the *psbA* fragment, we found five signals (at 11, 16, 17, 22, and 31 kDa, Figure 4) to be stronger in the PSI-II fraction and only one to be stronger in the PSII-I fraction (at 14 kDa, Figure 5). Since we found no differences in the

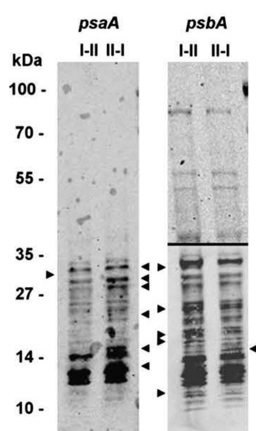

Figure 5. Southwestern Analysis of HS fractions.
30 μg of purified PSI-II and PSII-I fractions (I-II and II-I) were separated on a 6–16% SDS–polyacrylamide gradient gel, electro-blotted on a nitrocellulose membrane, re-natured and incubated with fluorescent dye-labeled *psaA* or *psbA* promoter probes. Signals indicate proteins able to bind the respective promoter fragment. Sizes of marker proteins separated in parallel are given in the left margin. The upper part of the *psbA* blot (indicated by a black line) is displayed with a higher contrast to visualize weaker signals.

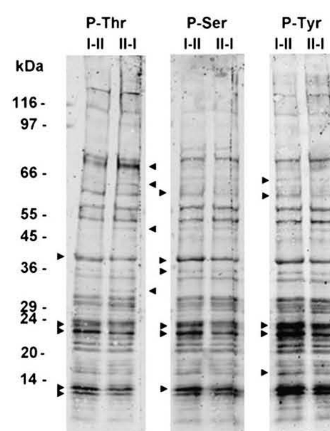

Figure 6. Long-Term Protein Phosphorylation State of HS Fractions.
Phosphorylation states were characterized by Western-immuno-detection after SDS–PAGE using anti-phospho-aminoacid antibodies directed against phospho-threonine (P-Thr), phospho-serine (P-Ser), and phospho-tyrosine (P-Tyr). Fluorescence-labeled secondary antibodies were detected using a fluorescence scanner. Black arrows mark proteins that exhibit a stronger signal in that sample to which the arrow is placed. Sizes of marker proteins separated in parallel are given in the left margin.

abundance of proteins within the samples, these differences in signal strength might be caused by other influences, such as post-translational modifications. In addition, the multiplicity of the small interacting proteins provides the potential base for generation of differing protein–DNA complexes as detected in the EMSA (Figure 4).

Protein Phosphorylation State of HS Fractions

Specific promoter recognition in plastids is known to be dependent on the phosphorylation state of sigma factors and DNA-binding proteins (Link, 1996; Tiller and Link, 1993). Therefore, we analyzed the protein phosphorylation state in the fractions by Western-immuno-detection using antibodies directed against phospho-serine (P-Ser), phospho-threonine (P-Thr) and phospho-tyrosine (P-Tyr)—a method usually used to test the phosphorylation state of mass proteins, such as in the thylakoid membrane. By this approach, we detected ~ 40 phosphorylated proteins in the chromatographically enriched HS protein fractions (Figure 6). Most proteins were found with all three antisera. Controls with defined phosphorylated substrates and removal of phosphoryl-groups by HCl treatment indicated that this result was caused by neither cross-reactivities between the antisera nor by unspecific binding of the antisera to unphosphorylated proteins (Supplemental Figures 3 and 4). This is supported by the observation that few proteins in the HS samples occurred in only one or two of the immuno tests (stars, Figure 6). The overall phosphorylation patterns for all three amino acids were similar in both fractions, suggesting only a minor effect of the LTR on the general phosphorylation state. However, a number of proteins exhibited a higher phosphorylation state in either one of the two samples (indicated by arrows in Figure 6). Such proteins were found with the P-Thr antibody in the PSI-II sample at 12, 13, 22, 23, and 39 kDa and in the PSII-I sample at 32, 52, 63, and 74 kDa. With the P-Ser antibody, preferentially phosphorylated proteins were identified at 13, 22, 23, 36, 39, and 60 kDa in the PSI-II sample. The test with the P-Tyr antibody resulted in the detection of preferentially phosphorylated proteins at 16, 22, 23, 60, and 64 kDa in the PSI-II sample. Thus, differential phosphorylation coincides with differential promoter binding activity at 13, 22/23, and 32 kDa (compare Figure 5), suggesting a specific role of phosphorylation on the DNA binding activity of such proteins. However, most phosphorylated proteins detected migrate at different sizes than proteins with differential binding activities, suggesting that other post-translational modification might also be involved in differential promoter recognition in the Southwestern experiment.

The phosphorylation state has been determined at the end of the LTR when the response to the redox signal is in equilibrium. Since the phosphorylation state of proteins is a very dynamic post-translational modification that can be changed within minutes after perception of a signal, we tested it in HS fractions purified from plants harvested just 1 h after

a PSI-II or PSII-I-light shift, respectively. The overall phosphorylation patterns for all three amino acids were again comparable in both fractions; however, the phosphorylation state of most proteins appeared to be higher in the PSI-II fractions. This was observable mainly with the P-Thr antiserum, but, to lesser extents, also with the P-Ser and P-Tyr antisera. We found at least seven phosphorylated proteins (at 9, 10, 14, 16, 26, 32, and 39 kDa, Figure 7) that did not occur in the long-term experiment while at least five proteins (at 52, 63, 74, 116, and 140 kDa) could not be detected that did occur in the long-term experiment. Thus, the phosphorylation state of fractions from short-term shift experiments displayed clear differences from that detected in the experiment above, indicating a dynamic reaction in response to redox signals.

Endogenous Kinase Activity in PSI-II and PSII-I Fractions

HS fractions of mustard chloroplast lysates had been shown to contain an endogenous kinase activity called plastid transcription kinase, which itself is under phosphorylation control (Baginsky et al., 1997; Tiller and Link, 1993). To test whether this kinase activity could also be detected in the HS fractions from PSI-II and PSII-I chloroplasts and, if yes, whether its activity is affected during the LTR, we performed autophosphorylation assays with the PSI-II and PSII-I samples using γ-^{32}P-ATP (Figure 8). Radioactively labeled proteins were visualized after electrophoretic separation and exposition to a phosphorimager. In both samples, we found phosphorylated proteins at 14–16, 23, 33, 36, 39, and 52 kDa, indicating the presence of the endogenous kinase activity; however, the radioactive signals in the PSII-I sample were much stronger than in the PSI-II sample (Figure 8, lanes 1, 2). In a control experiment, the endogenous kinase activity was heat-inactivated and the proteins were phosphorylated by exogenously added bovine heart kinase (PKA), which belongs to the class of serine/threonine kinases (Figure 8, lanes 5 and 6). This PKA treatment resulted in comparable phosphorylation patterns of both samples with the exception of signals at 14–16 and 23 kDa. In parallel, samples were pre-treated with a phosphatase before radioactive phosphorylation through PKA to display the maximum of phosphorylatable proteins (Figure 8, lanes 7 and 8). The resulting patterns were comparable with PKA treatment alone, indicating that neither a differing phosphorylation

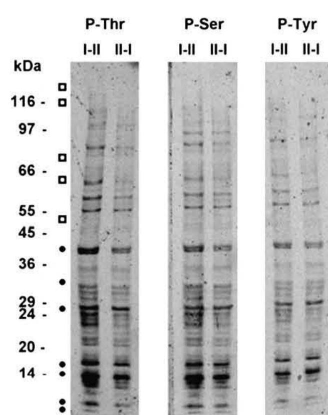

Figure 7. Short-Term Effect on Protein Phosphorylation State of HS Fractions.

HS fractions were isolated from plants harvested just 1 h after the light shift. Phosphorylation states were characterized by Western-immuno-detection after SDS–PAGE using anti-phospho-aminoacid antibodies directed against phospho-threonine (P-Thr), phospho-serine (P-Ser), and phospho-tyrosine (P-Tyr). Fluorescence-labeled secondary antibodies were detected using a fluorescence scanner. Black dots mark phosphorylation signals that were not detected after the long-term treatment; white squares indicate signals that were detected after the long-term shift but lacking after the 1-h treatment. Sizes of marker proteins separated in parallel are given in the left margin.

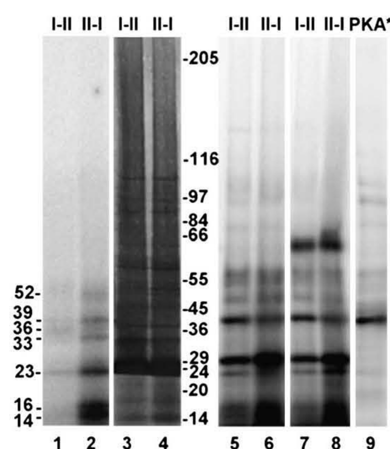

Figure 8. Characterization of Endogenous Kinase Activity in the Heparin-Sepharose Fractions.

Kinase activity from heparin-Sepharose purified proteins from PSI-II and PSII-I chloroplasts was assayed in *in-vitro* phosphorylation reactions using γ-^{32}P-ATP. Radioactively labeled proteins were separated by SDS–PAGE and detected by a phosphorimager. Lanes 1 and 2: autophosphorylation of peak fractions after heparin-Sepharose chromatography. Radioactive bands are marked by arrows in the left margin and the respective apparent molecular weight is given. Lanes 3 and 4: silver-stained proteins in the dried gel used for autoradiography shown in lanes 1 and 2 are presented as loading control. Sizes of marker proteins separated in parallel are given in the right margin. Lanes 5 and 6: peak fractions were heat-inactivated and subjected to phosphorylation by an exogenously added PKA. Lanes 7 and 8: peak fractions were dephosphorylated using shrimp alkaline phosphatase, heat-inactivated and finally radioactively labeled by PKA. The strong additional band at 58 kDa represents the deactivated phosphatase. Lane 9: PKA autophosphorylation control.

state nor varying substrate amounts caused the variations in signal strength. These data therefore point to varying kinase activities in the PSI-II and PSII-I samples and, in addition, to varying accessibilities of phosphorylation site(s) (in the case of the small proteins, around 14–16 kDa) as a reason for the pattern differences observed (Figure 5, lanes 1 and 2).

Cooperative Role of Phosphorylation and Thiol Redox State in the Regulation of Chloroplast Transcription

To study whether changes in phosphorylation events indeed affect chloroplast transcription during the LTR, we performed chloroplast run-on transcription experiments with chloroplasts from PSI-II plants and tested the impact of the serine/threonine kinase inhibitor H7 on the specific regulation of *psaA* and *psbA*. In PSI-II plants, it is expected that the thylakoid kinase STN7 is active due to the reduction of the PQ pool and, therefore, subsequent phosphorylation cascades controlling the LTR should be functional. In PSII-light-grown plants, *psaA* transcription is stronger than in PSI-light-grown plants, while, for *psbA*, the opposite reaction can be observed (compare controls, Figure 9). Surprisingly, addition of H7 did not affect the transcriptional rates of both genes (Figure 9) under these conditions, suggesting that phosphorylation is either not involved in the mediation of the redox signal or requires additional modifications to be active. We thus tested the impact of the reducing dithiol agent DTT. Exogenously applied DTT resulted in a decrease in the transcriptional rates of both genes. This decrease was abolished by the parallel addition of H7. This indicates that phosphorylation and thiol signals are required in a cooperative manner to influence chloroplast transcription.

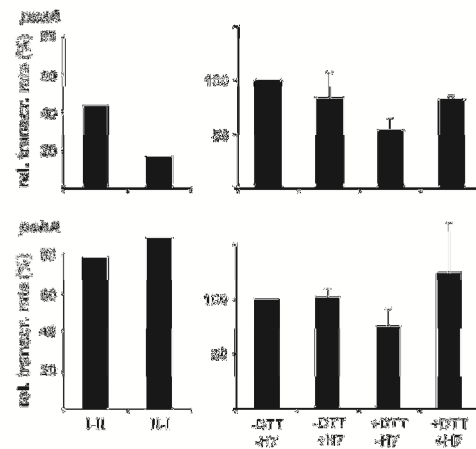

Figure 9. Chloroplast Run-On Transcription Assays.

Chloroplasts were isolated from PSI-II and PSII-I plants and used in either standard run-on transcription assays (left panels) or pre-incubation assays with H7 and DTT (right panels, only from PSI-II plants). Transcriptional activity of *psaA* and *psbA* was determined with a phosphorimager and normalized to 16S rRNA. Activities of *psaA* and *psbA* in the left panels can be directly compared. Variations were between 5 and 10%. In pre-incubation assays, plastids from PSI-II plants were incubated for 20 min in the presence of PSII-light and indicated agents. Activities were determined and normalized to the control (–DTT, –H7) which is set as 100%. Two independent experiments were done in triplicate. Standard deviation is given.

DISCUSSION

The main topic of this study was to investigate the potential impact of photosynthetic redox signals on occurrence and properties of plastid DNA-binding proteins at the biochemical level. This required the chromatographic enrichment of this low-abundant sub-proteome. We chose mustard as the subject of investigation for three reasons: (1) it provides sufficient biomass within a short growth period, (2) it performs a clearly defined LTR when grown under varying light-quality regimes (Table 1), and (3) mustard cotyledons provide highly homogeneous material for chloroplast protein purification (compare Figure 1). All observed changes in photosynthetic performance are consistent with earlier results obtained spectroscopically (Pfannschmidt et al., 1999a) and are in line with comparable experiments in *Arabidopsis* (Piipo et al., 2006; Wagner et al., 2008). By this means, mustard represents a suitable system for in-depth biochemical studies of the regulation events during the LTR.

We determined specific light-quality-dependent changes in the accumulation of a single *psaA* transcript 5'-end initiating directly behind a typical PEP promoter. The detected initiation point and the observed changes in accumulation correspond to earlier results obtained with *Arabidopsis* grown under a comparable light regime (Fey et al., 2005). Additional cis-elements as detected earlier in rice (Chen et al., 1990) appear to be of no importance in this physiological context. This confirms that redox control of *psaAB* transcription initiation is mediated at the standard PEP promoter, pointing to the PEP enzyme and its associated factors as the prominent target for redox regulation. To study the factors responsible for promoter recognition, we enriched chloroplast protein fractions with nucleotide binding activities including PEP by using protocols established for comparison of dark- versus light-grown plants (Pfannschmidt and Link, 1997; Tiller and Link, 1993). The protein compositions of the PSI-II and PSII-I samples after heparin-Sepharose chromatography were complex, but appeared very similar (Figure 2B) excluding major structural or compositional differences induced by the two growth regimes. Light-quality effects on the accumulation of minor proteins masked by more prominent proteins of the same size cannot be excluded at this stage of purification. For the present study, however, the multiplicity of proteins within these fractions was of advantage, since the experiments were aimed to provide a complete overview over all potentially redox-affected proteins. In our EMSA experiments, we observed several promoter- and double-strand-specific

DNA–protein complexes with both the *psaA* and the *psbA* promoter. Such complexes have not been described earlier, mainly because of the lower concentrated acrylamid matrix of the EMSA gels that results in a low resolution of small DNA–protein complexes (Pfannschmidt and Link, 1997; Tiller and Link, 1993). In this study, we used a higher concentration, leading to better resolution of these small complexes; however, high-molecular complexes such as PEP migrate only very poorly into such a matrix (compare Figure 4). The small complexes might be well suited for regulatory purposes such as enhancement or repression of transcription initiation by PEP. Since EMSA studies do not reflect the number and molecular weight of interacting proteins, we performed a Southwestern analysis to study which proteins in the HS fractions interact directly with the promoters. We observed 20–30 different proteins interacting with the two double-stranded promoter probes in a specific way (as indicated by the use of a competitor); most of them exhibited relatively small sizes, which is consistent with the occurrence of small DNA–protein complexes in the EMSA. Early Southwestern analyses with radioactively labeled *psbA* promoter probes uncovered at least seven interacting proteins in transcriptional active chromosome (TAC) fractions of white-light-grown mustard (Bülow et al., 1987). The sizes of the DNA-binding proteins were 14, 17, 19, 33, 57, 81, and 115–145 kDa, which resemble the sizes found in this study. Comparison of fractions from PSI- and PSII-light acclimated plants revealed several stronger binding signals with the *psaA* fragment under PSII-I conditions and with the *psbA* fragments under the PSI-II conditions. Higher binding affinities might repress transcription initiation as postulated for plastid sigma factors (Tiller and Link, 1993), which would fit with the observed lower transcription of the two genes under the respective conditions (Pfannschmidt et al., 1999b). However, since sigma factors do not possess DNA-binding activity of their own, it must be assumed that most of the binding proteins are of unknown nature. A number of biochemical approaches described mostly unidentified DNA binding proteins in plastids (Sato, 2001). Recent bioinformatics studies uncovered that up to ~100 transcription factors might be imported into plastids (Schwacke et al., 2007; Wagner and Pfannschmidt, 2006) that are not described at the biochemical level. Many of these genes encode relatively small proteins such as Why1 (24 kDa) (Grabowski et al., 2008; Krause et al., 2005). Thus, size and number of the DNA-interacting proteins detected here are of a realistic nature.

Preferential transcription of a gene under a given light regime requires specific recognition of its promoter. The primer extension analysis (Figure 3) clearly demonstrates that redox regulation of *psaA* occurs at the standard PEP promoter, which renders the existence of a specific redox-responsive *cis*-element rather unlikely. From this, it can be inferred that specificity is mediated by the action of *trans*-acting factors via variations in the interacting proteins themselves or modification of their activities by post-translational modifications. In analogy to bacterial systems, protein phosphorylation cascades have been hypothesized to be a likely regulatory mechanism in the mediation of redox regulation in chloroplast gene expression (Allen, 1993). Indeed, phosphorylation was shown to be very important for *psbA* promoter recognition in mustard (Tiller and Link, 1993) and for the blue-light-mediated *psbD* promoter recognition in barley (Kim et al., 1999). Furthermore, the Ser/Thr kinase STN7 was found to be necessary for the LTR (Bonardi et al., 2005) and the histidine sensor kinase CSK was found to be involved in the regulation of *psaA* transcript accumulation during light-quality acclimation (Puthiyaveetil et al., 2008). Despite this knowledge, the general phosphorylation state of the plastid subproteome of nucleotide binding proteins has never been assessed. Therefore, we analyzed the original phosphorylation state of the proteins using phospho-aminoacid-specific antibodies—an approach adopted from thylakoid membrane phosphorylation studies (Rintamäki et al., 1997). The technique was found to be very effective also with HS fraction and resulted in the detection of around 40 phosphorylated proteins, depending on the antiserum used. The high number of phosphorylated proteins was unexpected; however, this observation suggests that phosphorylation events are not restricted to a few proteins, but instead might be widespread in regulation of the plastid gene expression machinery. Interestingly, the general phosphorylation pattern for all three amino acids tested was very similar in both PSI-II and PSII-I samples. Control experiments clearly demonstrated that this observation was not due to cross-reactions between the different antisera, but that it represents the genuine phosphorylation state of the two states. This let us conclude that the majority of phosphorylation sites detected in this study are not involved in the regulation of the LTR. However, a number of proteins displayed differences in the strength of phosphorylation and, in three cases, this was correlated with differences in promoter binding and recognition (compare Figures 5 and 6). These proteins at 13, 22/23, and 32 kDa therefore represent likely candidates for redox-responsive factors (RRFs) controlling chloroplast transcription and are presently the target of further purification studies. Focusing on Thr phosphorylation, we found a generally high phosphorylation state of all proteins just 1 h after a shift from PSI- to PSII-light. This fits the assumption that STN7 is activated by PSII-light; however, it is unlikely that all proteins observed are true substrates of STN7. Rather, the data suggest the possible existence of a kinase network in which STN7 is an important primary hub. This is also consistent with the observation that most phosphorylated proteins carry phosphoryl-groups at Ser, Thr, and Tyr residues, which requires the action of several kinases with respective substrate specificities.

Earlier studies on heparin-Sepharose fractions from mustard chloroplasts identified a kinase activity of around 54 kDa in size, which was able to phosphorylate sigma-like factors *in vitro* (Baginsky et al., 1997). This plastid transcription kinase (PTK) co-purified in heparin-Sepharose chromatography because of its association with the PEP-A enzyme. It was found to be less active in a phosphorylated state and to be activated

antagonistically by the redox state of glutathione *in vitro* (Baginsky et al., 1997, 1999). The redox state of glutathione under PSI- or PSII-light, however, appears to be stable (Fey et al., 2005), excluding this type of regulation of kinase activity in the LTR. Therefore, it can be expected that under the physiological conditions tested here, only the phosphorylation state of PTK should be of importance for its activity. The endogenous kinase activity in the PSI-II fractions was found to be significantly lower than in the PSII-I fraction (Figure 8, lanes 1 and 2). It is tempting to speculate that the STN7 kinase that is activated under PSII-light (Wagner et al., 2008) directly or indirectly mediates the phosphorylation of PTK and thus its inactivation under PSII-light. Among the putative targets in the auto-phosphorylation assay, bands at 39 and 52 kDa appeared (Figure 8). These sizes correspond to those of biochemically characterized sigma-like factors of mustard, the target proteins of PTK (Baginsky et al., 1997, 1999), supporting the assumption that it is indeed PTK that is regulated under the different light regimes. Furthermore, we found bands that correspond in size to the three potential RRFs mentioned above. Together with sigma factors, these proteins would provide a possible mechanistic link between the redox state of PQ and the regulation of transcription initiation at PEP promoters via a signaling cascade involving STN7 and PTK. However, our run-on transcription experiments strongly suggest that the situation is more complex. Application of H7 alone had no effect on transcription, but, in combination with DTT, it prevents a decrease that is caused by the DTT application alone. This indicates that phosphorylation and dithiol sites act synergistically in the regulation of the assumed network. One possibility of how such an interaction could function is that reduction of disulfides might cause changes in protein conformation, which grants accessibility to an important phosphorylation site. On the other hand, both treatments are relatively unspecific and might affect multiple targets within the plastids. This could explain why any gene-specific regulation had been lost and *psaA* and *psbA* transcription was affected in the same manner.

In summary, our study demonstrates that numerous novel small DNA-binding proteins interact with the promoters of *psaA* and *psbA*. This multiplicity provides the potential base for different combinations of protein complexes recognizing the promoters in a specific and environmentally controlled manner, which then can affect transcription. The generation of such complexes might be controlled by phosphorylation; however, it is likely that this occurs in combination with other post-translational modifications such as reduction of dithiol groups. The high number of phosphorylated proteins in the HS fractions suggests the action of several kinases, such as STN7, STN8, CSK, and PTK. The respective impact of each of these kinases as well as the question whether further regulatory components exist will be a fascinating research field in the future. Taken together, the data are in line with the idea that redox signals from the thylakoid membrane are linked to gene expression via phosphorylation; however, this mediation most probably is performed by a complex network of interacting proteins rather than a direct signaling pathway. Such a network could provide both gene specificity and integration of multiple input signals that are known to affect plastid gene expression.

METHODS

Plant Material and Growth Conditions

Mustard seedlings (*Sinapis alba*, L., var. Albatros) were grown on soil for 7 d under continuous illumination at 20°C and 60% humidity. Developing seedlings were subjected to a growth light regime of 5 d under PSI-light followed by 2 d under PSII-light or vice versa, as described earlier (Pfannschmidt et al., 1999a). Control plants were grown for 7 d only under PSI- or PSII-lights. Lamps and filters for the light sources favoring PSI or PSII were as described (Fey et al., 2005). After 7 d, cotyledons were harvested under the respective growth light, placed on ice, and immediately used for preparation of chloroplasts or thylakoids.

Chlorophyll Fluorescence Measurements

In-vivo Chl *a* fluorescence parameters were determined at room temperature using a FluorCam 700F (Photon System Instruments, Brno, Czech Republic). All program parameters were essentially as described earlier (Wagner et al., 2004). Five to seven seedlings of each condition were taken for a simultaneous measurement. For optimal focusing of all cotyledones, seedlings were cut directly prior to the experiment; hypoctyls were put into water-containing vials and placed under the camera with the same distance to the objective. The determined Chl fluorescence changes were comparable to plants on soil. The optimum quantum yield Fv/Fm was calculated as Fm-Fo/ Fm (van Kooten and Snel, 1990). The steady-state fluorescence Fs was calculated as $Ft - Fo' = Fs$. Fluorescence quenching parameter qP (photochemical quenching) was calculated as $qP = (Fm' - Fs)/(Fm' - Fo)$ (Schreiber, 1986). The effective quantum yield of PSII (ϕ PSII) was calculated as ϕ $PSII = (Fm' - Fs)/Fm'$ (Genty et al., 1989).

Preparation and Characterization of Protein Fractions with RNA Polymerase Activity

Chloroplasts were isolated by differential centrifugation followed by sucrose gradient centrifugation as described (Bottomley et al., 1971; Reiss and Link, 1985). The isolated plastids were lysed and subjected to a heparin-Sepharose CL-6B (HS) chromatography as described earlier (Tiller and Link, 1993), except that chloroplast lysate was adjusted to 80 mM $(NH_4)_2SO_2$ and 10 mM $MgCl_2$. After extensive washing, the bound proteins were eluted with a single step of 1.5 M $(NH_4)_2SO_4$. Collected fractions containing the elution peak were identified by a Lowry protein test and enzymatically by determination of the *in-vitro* transcription activity of the fractions using a standard protocol described earlier

(Pfannschmidt and Link, 1994). For SDS–PAGE, proteins were solubilized in SDS-sample buffer (1% SDS, 5% glycerol, 3.67 mM 2-mercaptoethanol, 10.7 mM Tris-HCl, pH 6.8, 0.2% bromophenol blue), separated on 6–16% acrylamid gradient gels and stained with silver. Identified peak fractions were dialysed against storage buffer (50 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% Triton X-100 and 50% glycerol) and stored at –20°C until further use.

Primer Extension Analyses

For RNA isolation, 2 g of leaf material was harvested and frozen in liquid N_2 under the respective light source. Total RNA was isolated using the TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) following a protocol described earlier (Chomczynski and Sacchi, 1987). Concentration and purity of RNA samples were determined spectroscopically in a Biophotometer (Eppendorf, Hamburg, Germany). Intactness was proven by ethidium bromide staining of rRNA species after electrophoretic separation of aliquots on denaturing 1.2% agarose gels containing formaldehyde (Sambrook et al., 1989). Isolated RNA was stored at –80°C until further use. Primer extension analyses (Ghosh et al., 1978) were then carried out essentially as described earlier (Fey et al., 2005). The gene-specific primer for the *psaA* operon was: 5′-IRD 700, 5′-CCCATTCCTCGAAAG-3′ (sequence position +65 to +79 relative to ATG). The same primer was used to sequence the respective region of mustard chloroplast DNA using a cycle sequencing kit (MBI Fermentas, St Leon-Roth, Germany).

Electrophoretic Mobility Shift Assay

To find native protein complexes capable of binding the *psaA* or *psbA* core promoter elements, fluorescence-based electrophoretic mobility shift assays were performed as described (Steiner and Pfannschmidt, 2008). Complementary fluorescence-labeled oligonucleotides spanning the core promoters of *psaA* or *psbA*, respectively, were annealed for 5 min at 95°C and subsequently cooled down to room temperature, resulting in double-stranded DNA probes (*psaA*: 5′-DY-781-GGGTCCGTTGAGCACCCTATGGATATGTCATAATAGATCCGAAC ACTTGC-3′; 5′-DY-781-GCAAGTGTTCGGATCTATTATGACATAT-CCATAGGGTGCTCAACGGACCC-3′; *psbA*: 5′-DY-781-CATTG-GTTGACATGGCTATATAAGTTGTTATACTGTTTCATAACAAGC-3′, 5′-DY-781-GCTTGTTATGAAACAGTATAACATGACT TATATAGC-CATGTCAACCAATG-3′). Heparin-Sepharose fractions were concentrated to 1 μg μl $^{-1}$ protein using Amicon Ultra filter devices (MWCO 10 kDa) (Millipore, Schwalbach, Germany). For EMSA, 25 μg of the respective HS fraction was incubated for 20 min at RT with 0.3 pmol of the respective fluorescence-labeled promoter probe in binding buffer (30 mM Tris/HCl, pH 7, 5 mM β-mercaptoethanol, 0.5 mM EDTA, 10 mM $MgCl_2$). To test specificity, 1 μg of unspecific competitor poly (dIdC•dIdC) (Sigma, München, Germany) was added to the reaction mixture before adding the protein. After incubation, the mixture was separated electrophoretically on a native 6% acrylamide gel (29:1) at 4°C with 20 mA for 1 h and 30 mA until the end of the run. Note that the acrylamide concentration was increased in comparison to earlier studies with mustard HS fractions (Pfannschmidt and Link, 1997; Tiller and Link, 1993) to improve the resolution of the smaller protein–DNA complex in the lower part of the gels. After the run, the gel was scanned between the glass plates using an Odyssey Infrared Laser Imaging System (LI-COR, Bad Homburg, Germany).

Southwestern Analysis

Fluorescence-labeled promoter probes spanning the *psaA* or *psbA* promoter regions, respectively, were generated by PCR (*psaA* promoter: 410 bp; primer: forward 5′-IRD800-CCGTTGAGCACCCTATGGATATGTCATAATAGATCC-3′, reverse 5′-IRD800-GGATCT ATTATGACATATCCATAGGGTGCTCAACGG-3′; *psbA* promoter: 351 bp; primer: forward 5-IRD800-TTGGTTGACATGGCTATATAAGTCATGTTATACTGT-3′, reverse 5′-IRD800-ACAGTATAACATGACTTATATAGCCATGTCAACCAA-3′) and purified using Wizard SV PCR Clean up System (Promega, Mannheim, Germany). To identify single proteins capable of binding promoter elements, 30 μg of each of the HS fractions were separated on a 6–16% SDS polyacrylamid gradient gel and electroblotted onto a nitrocellulose membrane, which subsequently was blocked with 5% BSA in TBS-T. Blotted proteins were re-natured following a protocol described earlier (Handen and Rosenberg, 1997). Proteins were incubated with 4 pmol of the respective fluorescence-labeled promoter probe and a 100-fold excess of the unspecific competitor poly (dIdC•dIdC) (Sigma, München, Germany) in binding buffer on a shaker at 4°C overnight. After washing with a buffer containing 300 mM NaCl to remove unbound DNA, the blots were scanned with an Odyssey Infrared Laser Imaging System (LI-COR, Bad Homburg, Germany) to detect bound DNA.

Western-Immuno Analyses of Phospho-Aminoacids in Heparin-Sepharose Fractions

To examine the *in-vivo* phosphorylation state of the heparin-Sepharose fractions, 30 μg of the fractions were subjected to SDS–PAGE using 6–16% gradient gels as before. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Roth, Karlsruhe, Germany) by electroblotting and the membrane was blocked with 5% bovine serum albumin. Phospho-proteins were immuno-detected using polyclonal rabbit anti-phospho-threonine, polyclonal rabbit anti-phospho-serine, and polyclonal rabbit anti-phospho-tyrosine antibodies (Zymed Laboratories, San Francisco, USA) according to Rintamäki et al. (1997). Visualization was performed using a secondary fluorescence-labeled anti-rabbit IgG antibody (Rockland, Philadelphia, Pennsylvania, USA) and an Odyssey Intrared Laser Imaging System (LI-COR, Bad Homburg, Germany). Specificity of the antisera was tested with BSA chemically linked to either P-Thr, P-Ser, or P-Tyr (Sigma, München, Germany). Three nitrocellulose membranes with 50 ng of each type were generated by transfer using a slot-blot apparatus followed by immuno-analysis with the three different antisera according to the protocol given above. As additional control,

30 μg of the HS samples were treated for 30 min at 30°C with 0.3 N HCl to remove all phosphoryl groups before separation by SDS–PAGE followed by the immuno Western analysis with the three antisera.

Phosphorylation and Dephosphorylation Assays

Phosphorylation/dephosphorylation reactions were performed as described earlier (Baginsky et al., 1999; Tiller and Link, 1993) with slight modifications. 100 μg protein of the heparin-Sepharose fractions were phosphorylated either by endogenous kinase activity or by adding 30 U of the catalytic subunit of the cAMP-dependent protein kinase of bovine heart (PKA) in a final volume of 60 μl. The reaction mixture contained final concentrations of 130 mM Tris-HCl, pH 8.0, 40 mM KCl, 4.5 mM $MgCl_2$, 2 mM $CaCl_2$, 3.5 mM 2-mercaptoethanol, 0.5 mM DTE, 0.4 mM EDTA, 0.1 mM ATP, and 0.1 mM $MgCl_2$ and were incubated for 30 min at 30°C. For radioactive labeling of proteins, 0.74 MBq γ-^{32}P-ATP in 5 μl containing 3.3 μM ATP and 3.3 μM $MgCl_2$ were added. Radioactive PKA phosphorylation was performed after heat-deactivation of endogenous proteins (5 min at 90°C). For dephosphorylation, 100 μg protein of the heparin-Sepharose fractions were incubated for 30 min at 30°C in the same reaction mixture as for phosphorylation but in the presence of 1.2 U shrimp alkaline phosphatase (Roche). After the phosphatase treatment, the proteins were heat-denatured and subjected to radioactive PKA phosphorylation (see above). For visualization of phosphorylated proteins, the protein samples were heat-denatured for 5 min at 90°C in the presence of SDS-sample buffer (see above) and subjected to SDS–PAGE on a 7.5–15% acrylamide gradient gel. After electrophoresis, the gel was silver-stained and dried on a vacuum gel dryer. Radioactive signals were detected using a phosphorimager (Storm820, Molecular Dynamics) and the ImageQuant 5.0 software (GE Healthcare, München, Germany).

Chloroplast Run-On Transcription Experiments

Cotyledons (~20 g) from mustard plants acclimated to PSI or PSII-light were homogenized in a waring blender in ice-cold isolation buffer (300 mM sorbitol, 5 mM $MgCl_2$, 5 mM Na-EDTA, 10 mM $NaHCO_3$; Hepes pH of 8.0). The homogenate was filtered through six layers of muslin and a nylon membrane and the debris was removed by centrifugation for 1 min at 500 *g*. The supernatant containing the intact chloroplasts was centrifuged for 4 min at 2000 *g*. The resulting pellet was carefully re-suspended in homogenization buffer with a cut 5-ml tip. The run-on transcription was essentially carried out as described earlier (Mullet and Klein, 1987) with the following modifications: prior to lysis and run-on transcription, 1×10^7 plastids were pre-incubated for 20 min under PSI or PSII-light, respectively, in 1 ml isolation buffer containing no additional reagents (control), 0.3 mM DTT (+DTT), 20 μM H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine (Sigma, München, Germany); +H7) or both (+DTT/+H7). The plastids were spun down for 4 min at 2000 *g* and then immediately transferred to the run-on reaction mixture (50 mM Hepes KOH, pH 8.0, 10 mM $MgCl_2$, 25 mM potassium acetate, 10 mM DTT, 125 μM CTP, GTP, and ATP each, 10 μM UTP, 200 μCi α-^{32}P-UTP, 0.5 μg heparin ml^{-1}). After 8 min the reaction was stopped with phenol/chloroform. To the upper phase, Ribolock RNase-Inhibitor (Fermentas, St Leon-Roth, Germany) was added followed by treatment with RNase-free DNase-I (Fermentas, St Leon-Roth, Germany) for 20 min at 37°C. The samples were phenol/chloroform extracted and then precipitated with 1/10 vol. 3 M sodium acetate (pH 5.2) and 2 vol. of pure ethanol, washed two times with 70% ethanol and vacuum-dried for 2 min in a speed vac. The RNA was re-suspended in water, denatured at 65°C for 5 min and then transferred to hybridization tubes containing nylon membranes with spotted probes for various chloroplast genes. The hybridization was carried out overnight at 55°C in a buffer containing 250 mM sodium phosphate (pH 7.2), 1 mM Na-EDTA, 7% SDS and 1% BSA. Thereafter, the membranes were washed three times for 10 min at 55°C with washing buffer (20 mM sodium phosphate, pH 7.2; 1% SDS; 2 mM Na-EDTA). The hybridization signals were detected using a phosphorimager (Storm820, GE Healthcare, München, Germany) and quantified via the ImageQuant 5.0 software. As probes, we used purified PCR products of approx. 500 bp in length, obtained with following primer pairs: *psaA* forward 5′-AGGCTTCCACAGTTTTGGTTT-3′, *psaA* reverse 5′-CCCAAACATCTGACTGCATTT-3′; *psbA* forward 5′-ATTCATTGCTGCTCCTCC-3′, *psbA* reverse 5′-GCCCGAATCTGTA-ACCTTC-3′; *rrn16* forward 5′-TCATGGGAGAGTTCGATCCTG-3′; *rrn16* reverse 5′-GCTTTACGCCCAATCATTCC-3′; *18S rDNA* forward 5′-AACCCCGACTTATGGAAG-3′; *18S rDNA* reverse 5′-TAAGACCAGGAGCGTATC-3′. The probes were heat denatured for 10 min at 95°C in 0.5 M NaOH and then transferred to nylon membranes (Rotiplus 45 μm, Roth, Karlsruhe, Germany) using a slot-blot apparatus. The probes were UV cross-linked at 254 nm wavelength for 5 min.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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7 Manuscript IV

Schröter Y.*, Steiner S.*, Matthäi K. and Pfannschmidt T.

Analysis of oligomeric protein complexes in the chloroplast sub-proteome of nucleic acid-binding proteins from mustard reveals potential redox regulators of plastid gene expression.

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RESEARCH ARTICLE

Analysis of oligomeric protein complexes in the chloroplast sub-proteome of nucleic acid-binding proteins from mustard reveals potential redox regulators of plastid gene expression

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Photosynthetic light quality acclimation in plants involves redox-controlled changes in plastid gene expression. To study proteins potentially involved in this regulation, we isolated low-abundant chloroplast nucleic acid-binding proteins from the crucifere mustard (*Sinapis alba*) and investigated if photosynthetic redox signals affect their composition and/or oligomeric structure. We purified chloroplasts from plants subjected to light quality shifts and applied organelle lysates to heparin-Sepharose chromatography followed by 2-D blue native PAGE. We studied accumulation and structure of oligomeric protein complexes and applied MS/MS to identify them. We found ten oligomeric protein complexes of higher order and eleven smaller protein complexes or spots including plastid-encoded RNA polymerase (PEP), plastid transcriptionally active chromosome proteins, RNA-binding proteins, ribosomal subunits and chaperones. A translation elongation factor was found to be the only protein displaying major differences in its amounts in response to the growth lights. Furthermore, we found a novel thioredoxin as a subunit of the PEP, a 2-Cys-peroxiredoxin complex and a (soluble) ferredoxin:NADP-oxido-reductase, which represent potential redox regulator of plastid gene expression. A T-DNA knock-out line of the thioredoxin from *Arabidopsis* exhibits a yellowish-pale phenotype, demonstrating that this novel PEP subunit is essential for proper plastid development.

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Abbreviations: 2-CP, 2-Cys-peroxiredoxin; **γ-TIP 1,** γ tonoplast intrinsic protein 1; **BN-PAGE,** blue native PAGE; **CL,** chloroplast lysate; **Cyt f,** cytochrome f protein; **HS,** heparin-sepharose; **LTR,** long-term response; **PEP,** plastid encoded polymerase; **PSI,** photosystem I; **PSII,** photosystem II; **PTAC,** plastid transcriptionally active chromosome; **RbcS,** Rubisco small subunit; **TL,** total cell extract; **Trx,** thioredoxin; **UGPase,** UDP-glucose pyrophosphorylase; **VDAC1,** voltage-dependent anion-selective channel protein 1

1 Introduction

Chloroplasts are plant-specific cell organelles which are the site for photosynthesis and many other important biosynthetic pathways [1, 2]. A special feature of plastids is its own genetic system, which includes a plastid chromosome, the so-called plastome, and a fully functional transcriptional and translational apparatus for the expression of the genetic information on it. In higher plants, the plastome encodes a set of 100–120 genes mainly for components of the photosynthetic and gene expression machineries including photosystem genes, genes for the RNA polymerase (*rpo* genes), genes for ribosomal subunits and RNAs

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as well as for tRNAs [3]. Despite this, the majority of chloroplast proteins is imported from the cytosol [4]; therefore, the organelle is regarded as genetically semi-autonomous. Current estimates in *Arabidopsis* predict around 2100–4500 different proteins to be imported into chloroplasts [5–8]. This represents 15–25% of the total genome capacity, indicating that the chloroplast proteome is a major sub-proteome of the plant cell. Such predictions, however, were based on the detection of chloroplast transit peptides, which exhibit high false discovery rates because of their limited sensitivity and/or low specificity [9]. Furthermore, a number of chloroplast proteins have been experimentally identified, which either do not exhibit any recognizable transit peptides or possess ambiguous transit peptides for dual targeting into both mitochondria and chloroplasts [10, 11]. A recent study integrated available public data from various databases resulting in 1808 reliable and 5784 putative chloroplast proteins by generating a protein interaction network. Based on these predictions, 160 novel chloroplast proteins could be annotated [12]. However, the current studies clearly imply that experimental evidence for "true" chloroplast localization is necessary for all predicted candidate proteins.

Recent proteomic analyses of *Arabidopsis* chloroplast proteins have investigated the mass proteins in envelope, stroma, thylakoid and lumen [10, 13–20]. In these studies, around 1300 proteins were identified, which are involved in the major biosynthetic functions of the chloroplast including photosynthesis, amino acid and carbohydrate metabolism (compare entries in the plant plastid database and Plastid protein database (Plprot)) [21, 22]. These proteins constitute the great majority of the total chloroplast protein mass but represent only a minor part concerning the total predicted number and, thus, location of around 3000 putative chloroplast proteins remains to be clarified. These proteins presumably exist in only sub-stoichiometric amounts, suggesting that they possess mainly regulatory functions. Identification of such proteins therefore is difficult and requires enrichment of specific sub-proteomes according to their respective functional category.

The plastid transcription apparatus is one protein complex, which attracted much interest for biochemical purification and subunit identification by MS or Edman degradation. Beside the expected RNA polymerase subunits (encoded by the *rpo* genes), a number of additional proteins with functions not directly related to transcription such as kinases, pentatricopeptide-repeat proteins, RNA-binding proteins, superoxide dismutases and others were identified [23–26]. Furthermore, several biochemical studies reported the existence of a number of yet unidentified DNA-binding proteins and recent bioinformatics work suggests the existence of up to 100 different transcription factors in plastids, which may regulate plastid transcription [27–29]. However, this might represent just the tip of the iceberg of regulatory proteins in plastid gene expression since extensive regulation has been found at all gene expression levels such as RNA splicing and maturation, RNA editing, translation initiation and elongation [30–33], which suggest the existence of several hundreds of regulatory protein factors. However, most of them are still not identified and biochemical purification and identification by MS would facilitate our understanding of plastid gene expression in many aspects.

Chloroplast gene expression is highest in cotyledons or young leaves where the photosynthetic apparatus is built up and dramatically declines in mature and older leaves [34, 35]. Biochemical analysis of proteins involved in this process thus is best done in these early stages of plant development. Since *Arabidopsis thaliana* does not provide much material at this stage, we used the related crucifere mustard (*Sinapis alba*), which represents a well-analyzed model plant in the fields of photomorphogenesis and plastid gene expression. It is well studied at the physiological and biochemical level and provides much leaf material at the seedling stage after only a few days of growth [36, 37].

Mustard was also found to be highly suitable to analyze redox control of plastid gene expression during photosynthetic light quality acclimation [38–40]. Long-term excitation imbalances between the two photosystems, which occur in dense plant populations, are counterbalanced by the readjustment of photosystem stoichiometry resulting in redistribution of excitations energy [41]. This readjustment involves changes in the expression of the plastid-encoded photosystem core proteins D1 and PsaA/B [38, 39]. This photosynthetic long-term response (LTR) can be studied by using artificial light sources which preferentially excite either photosystem I (PSI) or II (PSII) (so-called PSI- and PSII-light). Plants grown under these lights exhibit either preferentially oxidized or reduced electron transport chains and by shifting plants between the two light regimes, it is possible to induce distinct reduction or oxidation signals within the transport chain without inducing large amounts of reactive oxygen species [42]. Mediation of this photosynthetic redox control toward the level of plastid gene expression requires the kinases STN7 and CSK [43, 44]; however, regulatory proteins that directly affect the gene expression machinery during the LTR are completely unknown.

Here, we combined the physiological question if and how photosynthetic redox signals affect proteins involved in chloroplast gene expression with a MS approach. Since these proteins represent a low-abundant minor fraction of the total chloroplast proteome, a biochemical enrichment prior to MS was required. Heparin-Sepharose (HS) chromatography is one suitable method to enrich such a sub-proteome of nucleic acid-binding proteins from chloroplasts. A recent study demonstrated that redox signals do not initiate large changes in composition of chloroplast proteins purified by HS [40]; however, differences in phosphorylation were observed which suggest a potential impact at the post-translational level which might affect the oligo-

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meric structure of proteins. This question was followed here by applying 2-D blue native PAGE (BN-PAGE) to HS fractions in order to study whether light quality acclimation induces structural changes in protein oligomers within the HS fractions. Besides this, the study demonstrates that mustard can be used as a model plant complementing *Arabidopsis* in MS approaches, which are difficult to tackle in the latter because of its small biomass production.

2 Material and methods

2.1 Plant material and growth conditions

Mustard seedlings (*S. alba*, L., var. Albatros) were grown on soil for 7 days under continuous illumination at 20°C and 60% humidity. Seeds were subjected to a growth light regime of 5 days under PSI-light followed by 2 days under PSII-light or *vice versa*. Lamps and filters for the light sources favoring PSI or PSII were as described [45]. After 7 days, cotyledons were harvested under the respective growth light, placed on ice and immediately used for preparation of chloroplasts. An *Arabidopsis thaliana* T-DNA insertion line (SALK_08162C) for thioredoxin (Trx) was identified in the mutant collection from the SALK institute [46] and seeds were obtained from the Nottingham Arabidopsis Stock Center. Seeds were grown on MS medium as described before [47] and homozygous plants were identified by PCR using the gene specific primers LP (5′-CCAACTACGCGACAAGGTATC-3′) and RP (5′-TTTTCCACACCTCAACACTCC-3′) and the T-DNA-specific primer LBb1.3(5′-ATTTTGCCGATTTCGGAAC-3′).

2.2 HS chromatography of chloroplast proteins

Cotyledons were homogenized in ice-cold isolation buffer using a Waring Blender and filtrated through three layers of muslin and one layer of nylon. Chloroplasts were isolated by differential centrifugation followed by sucrose gradient centrifugation as described [48, 49] with the exception that the sucrose gradients ranged from 30 to 55% sucrose to separate the band of broken plastids. The isolated plastids were lysed and subjected to a HS CL-6B (HS) chromatography as described earlier [40, 50]. After washing the bound proteins were eluted with 1.2 M $(NH_4)_2SO_4$. The elution peak was identified (i) by a protein quantification assay (RC-DC, BioRad) and to proof functionality of obtained enzymes (ii) by determination of *in vitro* transcription activity using a standard protocol described earlier [51]. Identified peak fractions were pooled and dialyzed against storage buffer (50 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% Triton X-100 and 50% glycerol) and stored at −20°C until further use.

2.3 2-D BN-PAGE

For 2-D gel electrophoresis of HS peak fractions, first dimension was performed as BN-PAGE in 4–12% or 10–15% acrylamide gradient gels followed by SDS PAGE in 7–17% acrylamide gradient gels as a second dimension. BN-PAGE was performed according to a protocol described by Herranen *et al.* [52] with the exception that no β-dodecylmaltosid was used.

2.4 Tryptic in-gel digestion and LC/ESI-MS/MS analysis

Peptide generation of proteins from silver stained gels was performed by tryptic in-gel digestion of cut-out spots using a described protocol [53, 54] with minor modifications.

Peptides from digested proteins were analyzed by LC/ESI-MS/MS using a LCQ™-DecaXP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Nano-LC was performed using an UltiMate™ Nano LC and Famos™ Autosampler HPLC unit and a reverse phase C18 PepMap™100, 3 μm, 100 Å Nano-column (75 μm id × 15 cm) (Dionex, Sunnyvale, CA, USA). Peptides were eluted using a three step gradient with mobile phases A (0.1% HCOOH and 5% ACN in water) and B (0.1% HCOOH and 80% ACN in water). The mobile phase flow was 27 μL/min with 5% B for the first 8 min, followed by 5–50% B in the next 17 min, 50–95% B for 0.5 min, held 95% B for 18 min, switched back to 5% B in 0.5 min and held 5% B for 16.5 min. The ion signals from the eluted peptides were collected using a data-dependent scan procedure with four cyclic scan events. The first cycle was comprised of a full MS scan of the mass range *m/z* 450–1200, followed by three MS/MS scans for the three most abundant ions. Sample run and data acquisition was performed using the Xcalibur™ software (Version1.3 © Thermo Finnigan 1998–2001).

2.5 MS data analysis

For peak list generation, the Ceate DTA tool of TurboSEQUEST® (v. 27 (rev. 12) © 1999–2002 Molecular Biotechnology Univ. of Washington J.Eng/S.Morgan/J.Yates Licensed to ThermoFinnigan) was used with default settings. Database search was conducted with TurboSEQUEST® (v. 27 (rev. 12) © 1999–2002 Molecular Biotechnology Univ. of Washington J.Eng/S.Morgan/J.Yates Licensed to ThermoFinnigan) against a *Brassicales* protein database of NCBI (NCBI *Brassicales* 2008.09.09.; 154 464 sequences). The enzyme specificity was set to trypsin strict and no missed cleavages were permitted. As variable modifications, the carboxyamidomethylation of cysteine (57.0293), oxidation of methionine (15.9949) and phosphorylation of serine, threonine and tyrosine (79.9663) were included. The mass tolerance for precursor ions was set to

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1.5 and 0 Da for fragment ions. Calculated cross-correlation (X_{corr}) values for significantly matching sequences had to be equal or above 1.5, 2.0, or 3.5 for singly, doubly, or triply charged precursor ions, respectively, and the ΔCorr values had to exceed 0.1. Proteins were accepted as identified with two ore more different significant matching peptides. The database used is highly redundant and consequently peptides match to several equivalent proteins of *A. thaliana* and other brassicales. Therefore, the protein entry of the first complete sequence of *A. thaliana* within the list of matching entries is given in the results. Alternatively, a representative species is given in the case that the *A. thaliana* sequence is not matching.

2.6 Western immuno-analyses

Western immuno-analyses were done as described previously [40]. Thirty microgram of the fractions were subjected to SDS-PAGE using 10% acrylamide gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Roth, Karlsruhe, Germany) by electroblotting and the membrane was blocked with 5% skimmed milk powder. Marker proteins for cytosol, mitochondria, thylakoids and stroma were immunodetected using polyclonal rabbit antibodies against UDP-glucose pyrophosphorylase (UGPase), voltage-dependent anion-selective channel protein 1 (VDAC1), cytochrome f protein (Cyt f) and Rubisco small subunit (RbcS) (AgriSera, Vännäs, Sweden). For detection of nuclei a polyclonal mouse anti-human DNA topoisomerase 1 antibody was used (BD Pharmingen, Franklin Lakes, NJ, USA). The tonoplast membrane was detected using an anti-γ tonoplast intrinsic protein 1 (γ-TIP 1) antibody [55]. Visualization of VDAC1, Cyt f, RbcS and γ-TIP 1 was performed using a secondary fluorescence labeled anti-rabbit IgG antibody (Rockland, Philadelphia, PA, USA) and an Odyssey® Infrared Laser Imaging System (LI-COR, Bad Homburg, Germany). UGPase was detected using an anti-rabbit IgG peroxidase conjugate (Sigma-Aldrich, München, Germany) and DNA topoisomerase 1 using an anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, München, Germany) and the ECL detection system.

3 Results

3.1 Basic biochemical purification procedure

To enrich protein complexes involved in plastid gene expression, we used 7-day-old mustard seedlings for preparation of chloroplasts. To test, in parallel, the impact of photosynthetic redox signals, we acclimated the plants to PSI- or PSII-lights and shifted them between these sources to induce either reduction (PSI-II) or oxidation (PSII-I) signals as reported earlier (Fig. 1A) [38–40, 56]. Intact chloroplasts were isolated by sucrose gradient centrifugation (Fig. 1B), lysed in a buffer containing the non-ionic detergent Triton X-100 and lysates were loaded on a HS column. Bound proteins were eluted with a single high-salt step. Comparable preparations from white-light grown seedlings have been partially characterized earlier and contain RNA polymerases, sigma-factors, unknown DNA- and RNA-binding proteins, DNA polymerase and kinase activities [50, 51, 57]. Therefore, these preparations are ideal to test potential redox effects on composition and oligomeric structure of nucleic acid-binding proteins. The elution peak was identified by protein content and transcriptional activity, pooled and subjected to further analyses. SDS-PAGE of aliquots followed by silver staining (Fig. 1C) revealed highly similar protein patterns within the two samples demonstrating that (i) the biochemical purification procedure is highly reproducible and (ii) photosynthetic redox control of chloroplast gene expression does not result in major changes of protein composition of HS fractions suggesting other mechanisms of regulation.

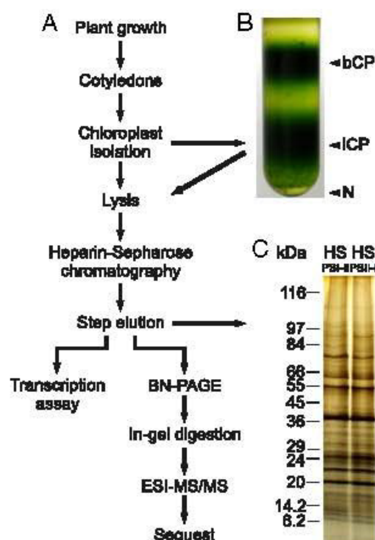

Figure 1. Scheme of purification, separation and identification of oligomeric protein complexes in chloroplast protein fractions with RNA polymerase activity. (A) Scheme of isolation procedure for chloroplasts, enrichment of nucleic acid-binding proteins and identification by ESI-MS/MS. (B) Sucrose gradient after separation of cell organelles. bCP: broken chloroplasts; iCP: intact chloroplasts; N: nuclei. A representative gradient with material from PSI-II plants is shown. (C) Eluted peak fractions after HS chromatography of proteins from PSI-II and PSII-I plants. Equal protein amounts (20 μg) were separated on a 7–17% SDS acrylamide gel and stained with silver. Sizes of marker proteins separated in parallel are given in the left margin.

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3.2 2-D BN-PAGE and protein identification

Redox control of gene expression often is mediated by post-translational modifications, which may affect the three-dimensional properties of proteins leading to changes in their interaction with other proteins. To identify such potential changes, we analyzed oligomeric complexes in the HS fractions by BN-PAGE followed by denaturing SDS-PAGE in a second dimension (Fig. 2). We observed ten oligomeric protein complexes of higher order (ranging from approx. 1000–250 kDa) and eleven smaller protein complexes/spots (below 200 kDa) (Fig. 2, top gels; compare Table 2). The resolution of the 2-D BN-PAGE in the low molecular range was relatively poor and this region was separated more extensively on additional gels using a first dimension ranging from 10–15% acrylamide (Fig. 2, bottom gels).

To identify the complexes, protein spots were cut out, subjected to in-gel tryptic digestion and peptide masses were determined by ESI-MS/MS. For mustard, no genomic sequence information is available; however, we could identify most proteins by the masses of the homologue peptides in the *Arabidopsis* sequence or other species in the *Brassicales* database (Table 1). In total, we measured 79 spots and identified 53 proteins covering 47 non-redundant sequences. We observed a certain bias for the dominant proteins to be identified. Spots belonging to the top 30% on the gels (based on normalized spot volumes) were significantly identified in 88% of all cases, while these success rates declined to 48 and 21% for moderate- and low-abundant proteins.

We found a number of proteins involved in chloroplast gene expression. Complex 2 represents the plastid-encoded RNA polymerase (PEP) as identified by the core subunits β, β′, â″ and α. The complex is around 1000 kDa in size and exhibits the typical subunit composition as described earlier for the soluble PEP A enzyme from mustard [23, 51]. An additional subunit known to be associated with PEP from tobacco is a phospho-fructokinase-like enzyme [25]. Other

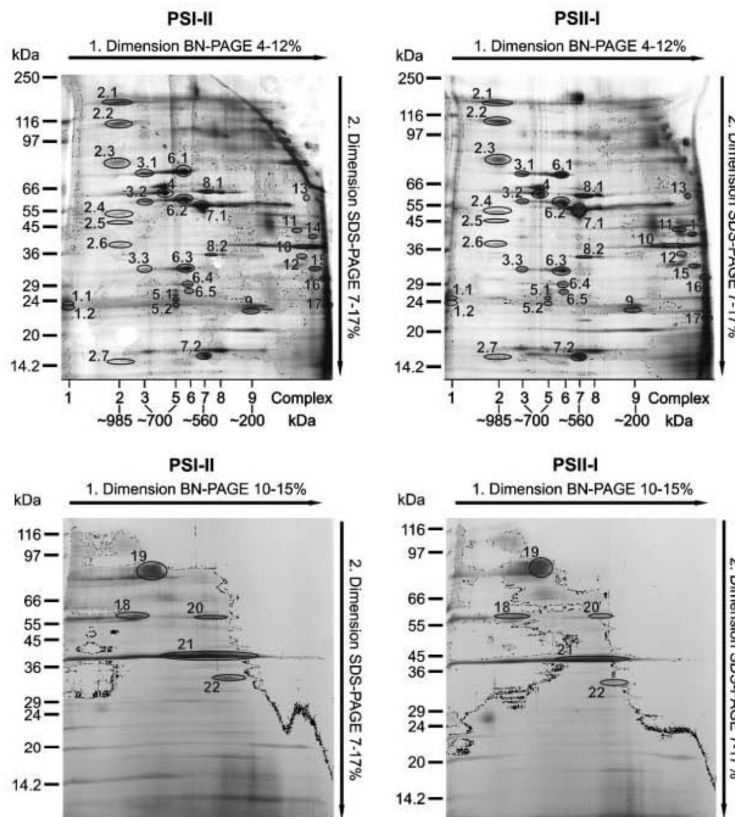

Figure 2. 2-D BN-PAGE with HS peak fractions. Identity of separated protein samples (140 µg each) is given on the top. Running directions of first and second dimensions are indicated by arrows. Sizes of marker proteins separated in parallel on the same gel are given in the left margin. High molecular weight protein complexes identified by MS of their subunits are marked by numbers on the bottom of the top gels starting with the largest complexes (compare Table 1). Single subunits within these complexes which gave significant hits in the databases are indicated by consecutive numbering. For identity and detailed data of MS, see Table 2 and Supporting Information Table 1.

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Table 1. Proteins after 2-D BN-PAGE significantly identified by ESI-MS/MS

Spot	Peptides	Accession	Identity	cTP	*S. alba*
1.1	4	gi|15235029 At4g10340	LHCB5	cTP 0.892 RC 2	This work
1.2	2	gi|115783 At1g29910	Chlorophyll a-b binding protein 2	cTP 0.778 RC 3	This work
	3	gi|15219941 At1g61520	LHCA3	cTP 0.891 RC 1	This work
2.1	10	gi|6685905 AtCg00170	RNA polymerase β″ subunit	–	[23]
	2	gi|12229937	RNA polymerase β″ subunit	–	[23]
2.2	13	gi|6686323 AtCg00190	RNA polymerase β subunit	–	[23]
	2	gi|12644277	RNA polymerase β subunit	–	[23]
	9	gi|15229259 At3g04260	PTAC3	cTP 0.971 RC 1	[24]
2.3	4	gi|145332787 At3g48500	PTAC10	cTP 0.791 RC 3	[24]
	7	gi|75194055 At1g74850	PTAC2	cTP 0.821 RC 2	[24]
2.4	4	gi|15232415 At3g54090	Phospho-fructo-kinase-like pfkB-2	cTP 0.624 RC 3	[24]
	2	gi|42566980 At4g20130	PTAC14	cTP 0.945 RC 1	[24]
2.5	3	gi|15232415 At3g54090	Phospho-fructo-kinase-like pfkB-2	cTP 0.624 RC 3	[24]
2.6	2	gi|6685903 AtCg00740	RNA polymerase alpha subunit	–	[23]
2.7	3	gi|15230779 At3g06730	Thioredoxin family protein	cTP 0.858 RC 3	[24]
3.1	14	gi|79321468 At1g78900	Vacuolar ATP synthase catalytic subunit A	–	This work
	2	gi|2493122	Vacuolar ATP synthase catalytic subunit A	–	This work
3.2	7	gi|79326468 At4g38510	Vacuolar ATP synthase catalytic subunit B2	–	This work
3.3	2	gi|12643432 At4g11150	Vacuolar ATP synthase catalytic subunit E	–	This work
4	7	gi|79537402 At5g56500	Chaperonin 60 β	cTP 0.830 RC 2	This work
	3	gi|30695947 At1g55490	Chaperonin 60 β	cTP 0.979 RC 1	This work
	2	gi|134104	Chaperonin 60 β	cTP 0.960 RC 2	This work
5.1	3	gi|145325425 At1g53850	PAE1 (20S proteasome alpha subunit E1)	–	This work
	2	gi|15223537 At1g56450	PBG1 (20S proteasome β subunit G1)	–	This work
5.2	2	gi|82581522 At4g31300	PSB6 (20S proteasome β subunit A1)	–	This work
6.1	18	gi|79321468 At1g78900	Vacuolar ATP synthase catalytic subunit A	–	This work
	3	gi|2493122	Vacuolar ATP synthase catalytic subunit A	–	This work
6.2	16	gi|79326468 At4g38510	Vacuolar ATP synthase catalytic subunit B2	–	This work
	4	gi|42571561 At1g20260	Vacuolar ATP synthase catalytic subunit B3	–	This work
	2	gi|79321468 At1g78900	Vacuolar ATP synthase catalytic subunit A	–	This work
6.3	6	gi|12643432 At4g11150	Vacuolar ATP synthase catalytic subunit E	–	This work
	3	gi|79326468 At4g38510	Vacuolar ATP synthase catalytic subunit B2	–	This work
6.4	6	gi|15231126 At3g58730	Vacuolar ATP synthase catalytic subunit D	–	This work
6.5	5	gi|15231126 At3g58730	Vacuolar ATP synthase catalytic subunit D	–	This work
7.1	13	gi|27752799 AtCg00490	RubisCO large subunit	–	This work
	9	gi|7525040 AtCg00480	AtpB chloroplast ATP synthase CF1 β subunit	–	This work
7.2	2	gi|27752799 AtCg00490	RubisCO large subunit	–	This work
8.1	9	gi|6685244 AtCg00120	AtpA chloroplast ATP synthase alpha subunit	–	This work
	3	gi|79537402 At5g56500	Chaperonin 60 β	cTP 0.830 RC 2	This work
8.2	2	gi|461550 At4g04640	AtpC1 chloroplast ATP synthase gamma chain 1	cTP 0.963 RC 1	This work
9	3	gi|1783308 At3g11630	2-Cys peroxiredoxin	cTP 0.988 RC 1	This work
10	2	gi|186478427 At1g12900	GAPDH subunit A	cTP 0.874 RC 2	[77]
	4	gi|22655450 At3g26650	GAPDH subunit A	cTP 0.850 RC 2	[77]
	3	gi|75313128 At1g09340	CSP41b	–	This work
11	3	gi|21431766 At3g54050	Fructose-1,6-bisphosphatase	cTP 0.941 RC 1	This work
	2	gi|22565 At4g20360	Translation elongation factor Tu	cTP 0.975 RC 1	[24]
12	4	gi|30686476 At5g17710	Co-chaperone GrpE family	cTP 0.788 RC 2	This work
13	10	gi|145334127 At4g24620	PGI1 chloroplastic phospho-glucose-isomerase	cTP 0.949 RC 1	This work
14	5	gi|20455491 At1g42970	GAPDH subunit B	cTP 0.674 RC 4	[77]
	7	gi|125576 At1g32060	Phospho-ribulokinase	cTP 0.739 RC 3	This work
	2	gi|18391442 At1g12840	Vacuolar ATP sythase subunit C, DET3	–	This work
15	3	gi|126633666	Ferredoxin-NADP reductase	cTP 0.945 RC 1	This work
16	3	gi|152112421 At4g09000	GF14 chi (GRF1), 14-3-3 like superfamily	–	This work
	2	gi|13447104	GF14 omega (GRF1), 14-3-3 like superfamily	–	This work
17	2	gi|145558858 At5g06290	2-Cys peroxiredoxin	cTP 0.973 RC 1	This work
	10	gi|75277355 At1g05190	50S Ribosomal protein L6	cTP 0.495 RC 5	This work
	2	gi|79595462 At2g42740	Ribosomal protein large subunit 16A	–	This work
18	10	gi|51701455 At4g37930	Serine/glycine hydroxymethyltransferase	mTP 0.673 RC 4	This work
19	9	gi|15231317 At3g06860	MFP2 enoyl-CoA hydratase	–	This work
20	11	gi|139389425 AtCg00480	ATP synthase subunit β, chloroplastic	–	This work

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Table 1. Continued

Spot	Peptides	Accession	Identity	cTP	*S. alba*
	2	gi|190358677	ATP synthase subunit β, chloroplastic	–	This work
	2	gi|56675678	ATP synthase subunit β, chloroplastic	–	This work
21	12	gi|75313128 At1g09340	CSP41b	–	This work
	8	gi|15229384 At3g63140	CSP41a	cTP 0.983 RC 1	[23]
22	3	gi|75313128 At1g09340	CSP41b	–	This work

Numbers refer to the respective spots shown in Fig. 2. Peptides: number of significantly matching peptides; accession: gi protein identification number and At gene accession number; identity: protein identity and function; cTP: probability of a transit peptide and reliability class (RC). *S. alba*: references with first identification of the respective protein in mustard.

proteins identified here as PEP subunits were plastid transcriptionally active chromosome (PTAC) 2, 3, 10, 14 and a Trx-domain containing protein, which were also described to be part of the transcriptionally active chromosome [24]. All these PTAC proteins have also been found in the tobacco PEP, however, not the Trx-like protein which therefore represents a novel PEP subunit. Complex 4 was identified as the chaperon Cpn60 complex and other chaperones or proteins with chaperone-like functions or members of the 14-3-3 family could be identified in spots 12 and 16. Among the smaller complexes, we identified ribosomal proteins (spot 17), the RNA-binding proteins CSP41a and CSP41b (spots 10, 21, 22) and a translation elongation factor (spot 11). The latter revealed strong enrichment in the PSII-I fraction which was not observable on the 1-D SDS gels indicating that some specific effects on protein composition do exist. The RNA-binding proteins CSP41a and b were remarkable in that they generate a highly abundant protein complex of largely varying size leading to a protein band ranging from more than 200 kDa to their final size of 38 or 41 kDa, suggesting the existence of various oligomeric structures of these small proteins. In addition, we found several photosynthesis related proteins in the HS fraction such as light harvesting complex proteins (complex 1), RubisCO (complex 7), ATPase (complex 8), glyceraldehyde phosphate dehydrogenase (GAPDH) (spots 10 and 14), fructose-1,6-phosphatase (spot 11), phosphoglucose-isomerase (spot 13), phosphoribulokinase (spot 14) and the ferredoxin:NADP oxido-reductase 1 (FNR1) (spot 15). We also identified a number of unexpected proteins. Complexes 3 and 6 were identified as V-type ATPases which usually are part of the tonoplast membrane and complex 5 consists of endopeptidases which are typically found in the 20S complex of the 26S proteasome. Complexes 9 and 17 contain 2-Cys-peroxiredoxin (2-CP), spot 18 is a mitochondrial serine-glycine hydroxymethyltransferase and spot 19 is a multi-functional protein (MFP2) associated with peroxisomes.

We analyzed the identified proteins for presence of chloroplast transit peptides using the prediction program TargetP (http://www.cbs.dtu.dk/services/TargetP/), ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) and Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html). Twenty-five of the 47 proteins exhibited a clear chloroplast transit peptide, seven are plastid-encoded proteins and 15 seem to belong to other cell compartments or do not possess a clear transit peptide (Table 1); thus, at minimum, 68% of all identified proteins are true chloroplast components. Twenty percent of them revealed DNA- or RNA-binding or interaction domains, 20% belong to photosynthesis and 20% are involved in translation, protein assembly and chaperone function (Table 1). Further 20% are ATPases of various origin and 7% are proteins with functions in redox regulation.

3.3 Purity of organelle preparation

Our MS data identified some proteins which usually are not plastid-localized. It is known that the purity of isolated plastids can be compromised by co-purifying sub-cellular fractions [58]. We therefore tested various protein fractions at different steps of our purification procedure for their content in specific cellular compartments, *i.e.* nuclei, mitochondria, chloroplast thylakoids, chloroplast stroma and tonoplast membrane by Western immuno-analyses. We tested aliquots from (i) total cell extract (TL) before separation on the gradients, (ii) lysate of intact chloroplasts (CL; the most important fraction with respect to the goal of the study) and (iii) peak fractions after HS chromatography (Fig. 3A). As positive control for nucleus marker proteins, we used the sucrose gradient pellet which contains the cell nuclei (N). Clear enrichment in chloroplast mass proteins (RubisCO and Lhcb) could be observed in CL *versus* TL while these proteins disappeared after HS chromatography and completely different proteins appeared as dominant bands. The strong difference between CL and HS is indicative for the selective enrichment of the nucleic acid-binding proteins on the HS matrix. The nucleus fraction was clearly distinguishable from TL, CL and HS and the Western immuno-analysis (Fig. 3B) indicated high amounts of nuclei in the pellet and much lower traces in the total leaf extract. Chloroplast and HS fractions were free of nucleus proteins. The cytosol marker protein UGPase was detected only in TL but not in CL or HS. The thylakoid membrane marker Cyt f displayed enrichment in CL but no traces in HS while the stroma marker RbcS gave

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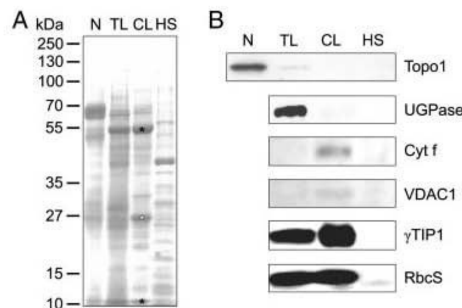

Figure 3. Western immuno-analysis of representation of subcellular compartments within protein fractions at different steps of the purification procedure. (A) Membrane after electroblotting and staining with Ponceau S. Equal protein amounts (30 μg) were separated on a 7–17% SDS acrylamide gel, blotted on a nitrocellulose membrane and stained with 5% Ponceau S. N: nuclei from gradient bottom; TL: total extract before separation on gradient; CL: chloroplast lysate of intact plastids; HS: HS peak fractions. Black stars mark the large (RbcL) and small (RbcS) subunits of RubisCO and the white star marks the light harvesting complex proteins (Lhcb). After scanning the membrane was destained and subjected to immuno-analysis. (B) Western immuno-analysis with antisera directed against proteins specific for cellular compartments. Topo1: DNA topoisomerase 1 (nucleus); UGPase: UDP-glucose pyrophosphorylase (cytosol); Cyt f: cytochrome f protein (thylakoid membrane); VDAC1: voltage-dependent anion-selective channel protein 1 (mitochondrial outer membrane); γ-TIP 1: γ-tonoplast intrinsic protein 1; RbcS: RubisCO small subunit (chloroplast stroma).

strong signals in TL and CL and a very weak signal in HS. The mitochondrial marker protein VDAC1 indicated a co-purification of some mitochondria with the plastids. The same result could be observed for the tonoplast membrane marker γ-TIP 1, which indicates strong enrichment of tonoplast membranes in the chloroplast fraction. Thus, our chloroplast fraction is free of cytosolic or nuclear proteins but contains few mitochondria as well as a considerable amount of the tonoplast explaining the identification of V-type ATPase in the HS fractions (Fig. 2). For the principle goal of the study, these preparations are of sufficient quality to allow further characterization.

3.4 Oligomeric state of identified protein complexes

To analyze the oligomeric state of the protein complexes, we counted the apparent molecular weight of all individual subunits in one complex obtained from the second denaturing dimension and compared it with the apparent migration in the first dimension. Much larger sizes than the approximate sum of the single subunits usually are indicative of oligomerization. Complex 1 consists of LHC proteins, which formed very large complexes and did migrate only

Table 2. Identity and approximate size of oligomeric protein complexes identified in HS peak fractions after 2-D BN-PAGE

Complex number	Complex identity	Subunit count	Sum of subunit sizes in the gel	Sum of subunit sizes in literature	Complex size denoted in literature	State of oligomerisation	Reference
2	Plastid encoded RNA-polymerase	19	~1000	~985	~985	Heteromultimer	[23]
3	ATPase vacuole	16	~872	440–1580	>700	Multiheteromer of eleven different subunits	[78]
4	Chaperonin 60 β	2	~120	~120	800	Tetradecamer	[13, 79]
5	20S Proteasome	10	~270	50–490	700–750	Multiheteromer of up to 14 different α- and β-typ subunits	[59]
6	ATPase vacuole	9	~459	440–1580	>700	Multiheteromer of eleven different subunits	[78]
7	RubisCO	7	~574	62–80	~560	Hexadecamer L_8S_8 +associated proteins	[13, 80]
8	ATPase chloroplast	5	~214	~228	~550	Multiheteromer $I_1II_1III_{12}IV_1\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$	[81]
9	2-Cys Peroxiredoxin	3	~60	40–60	40–200	Monomer, dimer, Decamer	[13, 60, 77]
10	CSP41	1	~40	38–79	38–79	Monomer, Heterodimer, Multiheteromer	[13, 82]
10+14	GAPDH	2	~79	~80	79–600	Tetramer	[83, 84]

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very poorly into the BN-PAGE matrix. They are, most probably, artificial nature generated by their high hydrophobicity. Complex 2 was identified as the PEP enzyme which is relatively well characterized at the biochemical level. Subunit composition and sizes as well as the migration behavior in the BN-PAGE correspond well to earlier reports (Table 2), suggesting that this complex does not form oligomers. Complexes 3 and 6 represent two different forms of the V-type ATPase. The major subunits are identical in both, but many of the minor regulatory subunits are lacking in complex 6, explaining its higher mobility. Complex 4 is a chaperonin complex and exhibits the subunit composition and complex size as reported for *Arabidopsis* [13]. The peptide masses identified complex 5 as a 20S proteasome that displays also the corresponding subunit composition and complex size [59]. Complexes 7 and 8 are RubisCO and chloroplast ATPase exhibiting their well-known complex sizes of 560 and 550 kDa, respectively. Complex 9 represents the peroxiredoxin complex for which a decameric structure was described [60], which corresponds to the size found here. Complex 10 contains CSP41, which occurs also in spot 21. CSP41 forms many different oligomers of varying sizes which corresponds to reports in *Arabidopsis* [13]. Finally, in complexes 10 and 14, GAPDH as tetramer was identified. In summary, the native sizes of the all complexes described above are in line with earlier observations indicating that the solubilization of the membranes with Triton X-100 during lysis of the chloroplasts did not affect the general native state of the proteins.

3.5 PEP associated Trx is an essential subunit

The Trx found in the PEP complex was an interesting finding concerning the physiological question of this study, *i.e.* redox regulation of plastid transcription. The biochemical preparation excluded the determination of differential redox states of the Trx domain because of the reductive agents necessary for plastid preparation. We thus followed a complementing genetic approach with *Arabidopsis* and identified a knock-out line in the SALK T-DNA collection [46]. Seed material was ordered and homozygous lines were isolated (Fig. 4) with the basic assumption that such lines could be tested for their responsiveness to our light system. If the identified Trx plays a decisive role in plastid redox regulation, we would expect that such lines would not display the typical LTR after growth in PSI- or PSII-light (or shifts between them) as described earlier [47, 61]. The phenotype, however, was very severe exhibiting white cotyledons and pale-green or yellowish following leaves. Plants were only viable on medium supplemented with sugar, indicating that they were completely devoid of photosynthesis. Thus, these lines were too strongly affected to be tested in a physiological approach; however, the phenotype indicates an unexpected important role of the identified Trx for plant development.

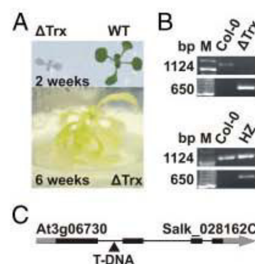

Figure 4. Isolation of a T-DNA knock out line for the PEP-associated Trx. (A) Homozygous Trx mutant line (ΔTrx) grown for 2 wk in comparison to Col 0 wild-type (WT) in MS medium under dim white light (30 μE PAR) and further grown until the age of 6 wk. (B) On top PCR product (intact gene) in WT and ΔTrx using gene specific primers LP and RP (1124 bp), on bottom PCR product (T-DNA tagged gene) using the T-DNA specific primer LBb1.3 and the gene specific primer RP (650 bp). (C) Gene model of At3g06730. Black bars represent exons (positions: W/2124276-2124506, 2124822-2124944, 2125552-2125632, 2125729-2125845). Position of T-DNA insertion SALK_028162C is indicated (C/2124454-2124704).

4 Discussion

The primary goal was to investigate whether photosynthetic light acclimation has an impact on structure and/or composition of proteins in HS fractions of isolated chloroplasts. Since these are low-abundant proteins, a MS approach is difficult to perform in *Arabidopsis* because large amounts of plant material are required. We circumvented this problem by using mustard, which belongs to the order of *Brassicales*. Its genes share a high homology to genes from *Arabidopsis* or other *Brassicacea* that lie between 92 and 98% amino acid identity (see Supporting Information Fig. 1). It was not known if this is sufficient to match mustard peptides against predicted *Arabidopsis* proteins in a quantitative way. A recent study also identified mustard proteins by MS but the corresponding proteins of *Arabidopsis* were purified in parallel and used as reference in the database searches [24]. Our results demonstrate that even independent from such reference proteins mustard proteins could be identified. Thus, mustard represents a suitable alternative to tackle difficult biochemical questions.

The biochemical purification scheme produced a highly reproducible protein fraction. We identified mainly its dominant proteins at this stage of purification. Most likely, this is caused by the higher amounts of peptides, which can be obtained and measured. Since our approach includes a heterologous database search for peptide identification, a certain number of mustard peptides will not match the *Arabidopsis* counterparts. This may identify differences in proteins of high abundance and might miss regulatory proteins of lower abundance as well as small proteins, which provide only a limited number of peptides. Additional

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purification steps are currently established to enrich also such proteins of lower abundance in HS samples for identification.

Thirty-two of the identified 47 proteins exhibit a clear chloroplast transit peptide or are plastid-encoded proteins confirming that the principle approach is applicable. However, we also identified proteins which obviously belong to other cell compartments and our Western analyses confirmed that tonoplast membranes and mitochondria co-purify with our plastid preparation. Since these cell compartments do not possess the same density as intact chloroplasts, they cannot co-migrate with the chloroplast band in the sucrose gradient, suggesting that its appearance is caused by physical association between these three compartments. In many electron micrographs, plastids and mitochondria (and peroxisomes) can be found side by side often paralleled by the tonoplast, which is mainly due to the manifold metabolic interactions of these compartments. Thus, it is conceivable to assume that the three compartments associate by, *e.g.* hydrophobic interactions of membranes or interaction of metabolite transporters during the process of cell rupture. Comparable observations have been made with envelope preparations of *Arabidopsis* [16], indicating that co-purification of mitochondria and tonoplast membranes represent a general phenomenon in plastid isolation. Contaminations by cytosolic or nuclear proteins could be excluded in our study and thus the presence of endopeptidases usually associated with the proteasome remains an interesting finding (see below).

We found a number of photosynthesis related proteins. Lhc proteins and RubisCO likely represent contaminants most probably because of their high amounts in the starting material and despite extensive washing steps during column purification trace amounts might remain in the fractions. For the RubisCO of *Chlamydomonas reinhardtii*, however, a RNA-binding activity has been reported [62]. If this property is conserved also in the plant enzyme, it might cause this co-purification on the HS matrix. ATPases (both from chloroplast and tonoplast), however, might have co-purified on the HS matrix because of their affinity to negatively charged substrates, *i.e.* to ATP in their ATP-binding cassettes. The same might be true for GAPDH, fructose-1,6-bisphosphatase, phosphoglucose-isomerase or GRF1, all of which bind phosphorylated substrates.

The major focus of our study was on proteins which are related to gene expression. We found numerous of them including the PEP core subunits and PTAC proteins 2, 3, 10 and 14 [23–25, 63]. Spot 2.7 represents a novel Trx domain-containing protein. The protein has been described before as a 13-kDa subunit of the PEP complex, however, was not identified so far [23, 25, 51]. It possesses the conserved WCGPC motif but in *Arabidopsis* it is still not characterized and its localization is unclear [64]. Interestingly its orthologue in tomato, Trx CI, has been reported to be an adaptor protein between the receptor-like protein CF-9 and the Ser/Thr kinase ACIK1. It acts as a negative regulator of pathogen-induced cell death; however, the redox properties of the Trx are not required for this interaction [65]. In case the Trx in the PEP complex possesses the same adaptor capability, this would provide a potential molecular mechanism for linking redox regulation to the PEP complex. We observed changes in chloroplast run-on transcription experiments after adding DTT to isolated plastids, which can be abolished by the simultaneous application of the kinase inhibitor H7 [40]. This suggests that a phosphorylation-dependent and thiol-dependent pathway converge or interact in the regulation of plastid gene expression. An adaptor Trx as PEP subunit would be the ideal target for these regulation mechanisms. The ΔTrx line exhibited a very severe phenotype, which is very unusual since all known knock-outs for other Trx genes displayed no visible phenotype due to the high redundancy of the gene products. This observation points to a unique role of this specific Trx in plant development and is consistent with the phenotypes of various mutants deficient in other subunits of the PEP complex [24, 66, 67]. More detailed studies demonstrated that this Trx indeed possesses Trx activity and that its lack in the mutant lead to clear changes in chloroplast gene expression (Dr. F. Börnke, personal communication) supporting our assumptions above. Interestingly, we found also 2-CP, a redox active protein complex with multiple functions in antioxidant responses and detoxification, which is reduced by Trx [60, 68]. It is unclear how this protein interacts with the HS matrix; however, within spot 17, additional ribosomal subunits could be detected, which suggest a putative interaction of 2-CP and ribosomes resulting in co-purification on the column. Another possibility is a co-purification because of its redox properties. Whether it is involved in the specific redox regulation investigated here requires further experimentation, but such a role is not unlikely since various functions in redox signal transduction have been discussed as additional role of peroxiredoxin [69]. Even more enigmatic in the context of redox regulation is the finding that spot 15 represents the ferredoxin:NADP oxido-reductase 1. Usually, this enzyme is located at PSI and transfers electrons from ferredoxin to $NADP^+$ serving photosynthetic electron transport. However, it was reported that it dissociates from the thylakoid membrane upon oxidative stress serving unknown purposes [70]. Maybe it possesses functions in redox regulation of gene expression causing its co-purification by protein interaction. Dissociation from the thylakoid membrane upon a redox signal would render it into an ideal redox mediator. However, this would imply that its electron transport properties are not restricted to $NADP^+$ but include also other targets. This provides a number of novel possibilities for redox regulation in chloroplast which will be investigated in future.

We found a number of regulatory proteins related to protein stability/assembly and to translation such as chaperones or 14-3-3 proteins, RNA-binding proteins, ribo-

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somal subunits and a translational elongation factor. Of special interest here are the proteins CSP41a and b. Originally identified as a RNA stem-loop binding endonuclease, it was also found as a subunit of the PEP complex [23, 25, 71]. Accumulating evidence suggest a multi-functionality of this protein acting both in transcription and translation [72, 73]. CSP41a/b is a very dominant protein in our HS fractions but there are no visible redox effects on its amounts. Interestingly, it generates a very broad protein band in the BN-PAGE, suggesting that it forms many diverse complexes of varying sizes. This corresponds to observations with *Arabidopsis* proteins separated by CN-PAGE in which three different complexes of >1000 kDa, 224 kDa and 126 kDa were identified [13]. In our system, we found only the two smaller ones but not in clear distinct complexes but in a continuous transition. Using isoelectric focussing as a first dimension, we observed multiple different isoelectric forms of these proteins suggesting that they are post-translationally modified (Schröter and Pfannschmidt, unpublished observations), which might cause the complex diversity. The high abundance of these proteins further suggests that they perform basic structural roles rather than specific regulation events. In contrast to this, the translation elongation factor EF-Tu was found as one protein, which is strongly affected in its accumulation by the light system here. Chloroplast translation initiation has been demonstrated to be under tight redox control; however, this process seems to be mediated by RNA-binding proteins [74, 75]. Thus, our observation alone does not help to understand photosystem-specific gene expression changes in our particular light system, but it provides an interesting starting point for further analyses.

We have identified a number of unexpected proteins in the HS fractions. The V-type ATPase and the serine/glycine hydroxymethyltransferase could be well attributed to the co-purifying tonoplast membranes and mitochondria during organelle preparation. It should be noted that the great majority of identified non-chloroplast proteins belong to this V-type ATPase complex, thus 68% true plastid proteins most probably reflect a too low value for protein origin in the total fraction and we expect that this value will increase with the identification of lesser abundant proteins. The appearance of proteasome-associated endopeptidases is much more difficult to explain. These proteins do not exhibit a defined chloroplast transit peptide and usually the proteasome is located in nucleus or cytosol [76], cell compartments which are not present in our chloroplast preparations. This suggests that these peptidases might be true components of the plastid; however, our approach does not distinguish between components of the inner and outer sides of the chloroplast. It is either possible that these proteins are imported *via* unusual pathways or are located on the chloroplast outer surface providing interesting possibilities for regulation of chloroplast protein degradation and maturation during import. Further investigations are necessary to confirm and understand this observation in detail.

In summary, we were successful in analysing oligomeric protein complexes in HS fractions and could identify interesting novel candidates potentially implicated in redox regulation of plastid gene expression. Our study demonstrates that mustard is suited as an additional plant system for proteomic studies in which structural questions are combined with light biology and plastid gene expression.

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The authors have declared no conflict of interest.

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Proteomics 2010, *10*, 2887 2887

Erratum

Analysis of oligomeric protein complexes in the chloroplast sub-proteome of nucleic acid-binding proteins from mustard reveals potential redox regulators of plastid gene expression

By Y. Schröter *et al.*, vol. 10, pp. 2191–2204

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Owing to qualitative deficiencies in the original, Fig. 2 in this paper should appear as shown:

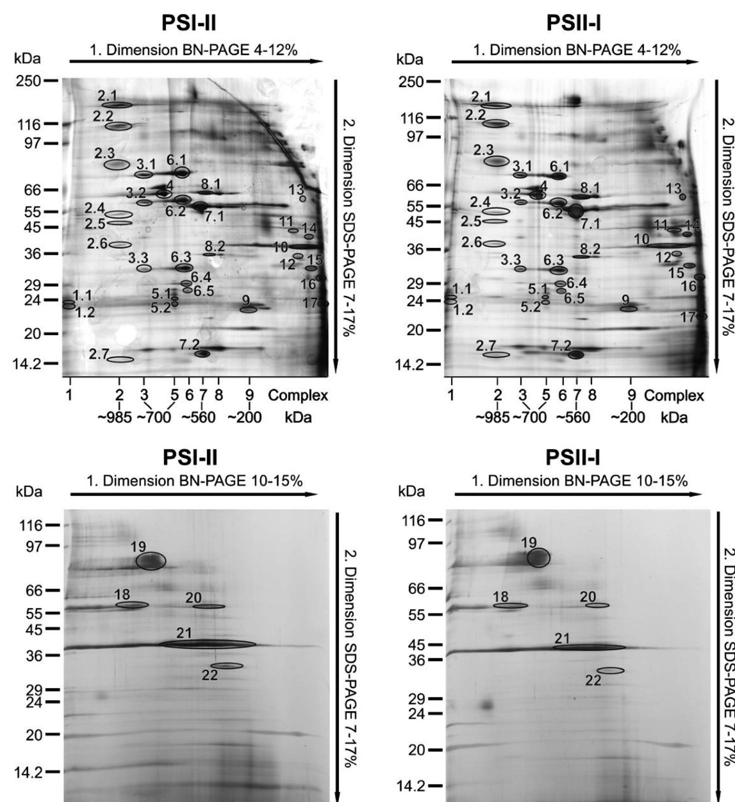

Figure 2. 2-D BN-PAGE with HS peak fractions. Identity of separated protein samples (140 μg each) is given on the top. Running directions of first and second dimensions are indicated by arrows. Sizes of marker proteins separated in parallel on the same gel are given in the left margin. High molecular weight protein complexes identified by MS of their subunits are marked by numbers on the bottom of the top gels starting with the largest complexes (compare Table 1). Single subunits within these complexes that gave significant hits in the databases are indicated by consecutive numbering. For identity and detailed data of MS, see Table 2 and Supporting Information Table 1.

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8 Manuscript V

Steiner S., Schröter Y., Pfalz J., Pfannschmidt T.

Identification of essential subunits in the plastid-encoded RNA polymerase complex reveals building blocks for proper plastid development.

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Identification of Essential Subunits in the Plastid-Encoded RNA Polymerase Complex Reveals Building Blocks for Proper Plastid Development[1][C][W][OA]

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The major RNA polymerase activity in mature chloroplasts is a multisubunit, *Escherichia coli*-like protein complex called PEP (for plastid-encoded RNA polymerase). Its subunit structure has been extensively investigated by biochemical means. Beside the "prokaryotic" subunits encoded by the plastome-located RNA polymerase genes, a number of additional nucleus-encoded subunits of eukaryotic origin have been identified in the PEP complex. These subunits appear to provide additional functions and regulation modes necessary to adapt transcription to the varying functional situations in chloroplasts. However, despite the enormous progress in genomic data and mass spectrometry techniques, it is still under debate which of these subunits belong to the core complex of PEP and which ones represent rather transient or peripheral components. Here, we present a catalog of true PEP subunits that is based on comparative analyses from biochemical purifications, protein mass spectrometry, and phenotypic analyses. We regard reproducibly identified protein subunits of the basic PEP complex as essential when the corresponding knockout mutants reveal an albino or pale-green phenotype. Our study provides a clearly defined subunit catalog of the basic PEP complex, generating the basis for a better understanding of chloroplast transcription regulation. In addition, the data support a model that links PEP complex assembly and chloroplast buildup during early seedling development in vascular plants.

Chloroplasts are the typical organelles of green plant cells, which originated from a cyanobacteria-like ancestor during endosymbiosis (Blankenship, 2002; Buchanan et al., 2002). They still possess many remnants of this prokaryotic origin, including its own genetic system. This consists of a plastid chromosome, the so-called plastome, and a fully functional transcriptional and translational apparatus for the expression of the genetic information on it. In vascular plants, the plastome contains a largely conserved set of 100 to 120 genes, including genes for photosynthesis proteins, genes for the RNA polymerase (*rpo* genes), and genes for ribosomal subunits and RNAs as well as for tRNAs (Sugiura, 1992). The vast majority of chloroplast proteins, however, are encoded in the nucleus and must be imported from the cytosol (Abdallah et al., 2000; Soll and Schleiff, 2004). As a result, all multiprotein complexes in plastids are composed of a patchwork of plastid- and nucleus-encoded subunits. The core proteins of large complexes (for instance, of the photosystems) are usually encoded in the plastome, while peripheral subunits typically appear to be encoded in the nucleus. This distribution reflects two evolutionary tendencies that occurred during the establishment of endosymbiosis. First, most genes from the cyanobacteria-like ancestor were lost to the nucleus of the host cell, and essential proteins had to be reimported and assembled into the complexes. During evolution, this was easier achieved for peripheral than for core proteins, which usually represent the pacemakers for complex assembly. Second, the organelle also gained novel proteins from the eukaryotic host cell, which conferred new properties to the prokaryotic multienzyme complexes of the endosymbiont. Both strategies led to the transfer of a large proportion of developmental and functional control from the symbiont to the nucleus of the host cell and, by this means, led to a complete integration of the organelle into the cell (Martin et al., 2002; Stoebe and Maier, 2002; Herrmann et al., 2003; Greiner et al., 2011).

The evolutionary patchwork of chloroplast protein complexes becomes especially obvious in the plastid transcription machinery. Multiple lines of evidence indicate that the transcription of plastomic genes depends on the activity of a phage-type, single-subunit, nucleus-encoded plastid RNA polymerase (NEP) and

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a prokaryote-type, multisubunit, plastid-encoded RNA polymerase (PEP; Hess and Börner, 1999; Cahoon and Stern, 2001; Lysenko and Kuznetsov 2005; Shiina et al., 2005; Liere et al., 2011). In Arabidopsis (*Arabidopsis thaliana*), NEP is encoded by two nuclear gene copies (*rpoTp* and *rpoTmp*), each with a different target sequence directing the encoded protein either to plastids or, via dual targeting, to plastids and mitochondria. A third gene product encoded by *rpoTm* is directed exclusively to mitochondria (Hedtke et al., 2000). The PEP subunits, in contrast, are encoded by a set of plastome-located genes (*rpoA* and the $rpoB/C_1/C_2$ operon) that exhibit approximately 26% to 50% sequence homology to corresponding genes from cyanobacteria, generating the so-called core enzyme (Igloi and Kossel, 1992). This core enzyme is supplemented by a number of nucleus-encoded σ-factors that provide the necessary promoter specificity to the complex (Link, 1996; Allison, 2000; Schweer et al., 2010). PEP is the major RNA polymerase activity in mature chloroplasts and represents the predominant target for environmental regulation, such as light-induced redox control of chloroplast transcription (Link, 2003; Pfannschmidt and Liere, 2005).

Initially, the structure, identity, and subunit composition of the chloroplast transcription machinery were mainly investigated by biochemical means. In plastids, the DNA and its associated or interacting proteins (including the RNA polymerase) are organized in so-called nucleoids or plastid nuclei, very large structures that represent bacteria-like assemblies of several plastome copies and numerous proteins with various functions in nucleoid structure and gene expression. Recently, a microscopic study using a plastid envelope DNA-binding protein-GFP fusion described in detail the localization and distribution of nucleoids in plastids from different plant cell types (Terasawa and Sato, 2005). Purified nucleoids were very useful in the determination of gene-specific transcription activities, but due to the high number of proteins within the complex, a detailed subunit analysis of the RNA polymerases was not feasible (Sakai et al., 2004). Therefore, a number of different biochemical purification procedures were developed aiming to enrich more distinct RNA polymerase complexes from chloroplasts. Basically, two types of plastid RNA polymerase preparations can be distinguished. The first represents an insoluble RNA polymerase preparation called transcriptionally active chromosome (TAC), which can be precipitated by ultracentrifugation. It represents a high-M_r DNA/RNA-protein complex containing approximately 40 to 60 proteins that is capable of in vitro transcription, resembling the nucleoids in this respect (Hallick et al., 1976; Reiss and Link, 1985; Little and Hallick, 1988; Krause and Krupinska, 2000; Pfalz et al., 2006). The second type of preparation usually includes a detergent treatment, resulting in a soluble RNA-polymerase activity that requires externally added DNA for transcriptional activity. Many studies concentrated on these soluble preparations, since these allowed a precise molecular analysis of the promoter specificity and cis-element usage of the purified transcription complex (Bradley and Gatenby, 1985; Lerbs et al., 1985; Rajasekhar et al., 1991; Lakhani et al., 1992; Pfannschmidt and Link, 1997).

Various biochemical purification procedures yielded highly purified RNA polymerase preparations that were able to recognize specifically the typical prokaryotic 10 and 35 promoter boxes of many plastid genes. However, these RNA polymerases did not exhibit the expected subunit structure 2α, β, β', and β'', resembling that of the *Escherichia coli* enzyme (2α, β, β'), but a much more complex structure composed of around 20 to 30 subunits. This apparent contradiction was resolved with the identification of the prokaryotic core subunits α, β, β', and β'' in various soluble RNA polymerase preparations by using various experimental approaches, including western analysis, Edman degradation, and mass fingerprints (Hu and Bogorad, 1990; Hu et al., 1991; Pfannschmidt et al., 2000). Interestingly, these subunits were also found in the TAC, indicating that TAC and soluble RNA polymerases represent two different biochemical preparations of the same complex rather than two separate RNA polymerase classes, as originally assumed (Little and Hallick, 1988; Suck et al., 1996). In addition, it turned out that the PEP enzyme undergoes a structural reorganization during light-dependent chloroplast maturation. In etioplasts or young greening chloroplasts, PEP displays the expected *E. coli*-like structure but is reorganized into a much more complex "eukaryote"-like RNA polymerase in mature chloroplasts (Hu and Bogorad, 1990; Hu et al., 1991; Pfannschmidt and Link, 1994; Pfannschmidt et al., 2000). This probably involves a number of still unknown posttranslational modifications of the *rpo* subunits, since (1) these exhibit differences in the apparent M_r between etioplast and chloroplasts and (2) the PEP enzyme appears to change its promoter recognition properties during the etioplast-chloroplast transition (Pfannschmidt and Link, 1997). The recruitment of further subunits with additional enzymatic activities has been interpreted as an evolutionary adaptation of the RNA polymerase complex and its functions to the specific conditions in the chloroplast (Link, 1996; Pfannschmidt and Liere, 2005). This, so far, is the best explanation for why even highly purified RNA polymerase preparations from chloroplasts of several species exhibit approximately 10 to 15 proteins in addition to the *rpo* subunits (Rajasekhar et al., 1991; Khanna et al., 1992; Lakhani et al., 1992; Pfannschmidt and Link, 1994; Rajasekhar and Tewari, 1995; Boyer and Hallick, 1998; Suzuki et al., 2004). The general criticism that these additional proteins may simply represent contaminants of the biochemical purification procedures has been recently invalidated by an elegant transplastomic approach in which the *rpoA* gene was fused to a His tag. The PEP enzyme from tobacco (*Nicotiana tabacum*) chloroplasts could be then purified via nickel-affinity

chromatography. Even this affinity tag-purified RNA polymerase preparation revealed a highly complex subunit composition, indicating that the additional subunits copurify due to an interaction with the *rpo* subunits and/or associated non-*rpo* subunits and therefore belong directly to the complex (Suzuki et al., 2004).

To understand chloroplast transcription and its regulation, it is necessary to identify all additional PEP subunits and characterize its potential functions. Using modern mass spectrometry, a number of the non-*rpo* subunits have been identified in the last few years, but several subunits still remained unknown (Pfannschmidt et al., 2000; Suzuki et al., 2004; Schröter et al., 2010). Furthermore, the highly varying subunit composition of the different transcription complexes described above suggests that the RNA polymerase represents a dynamic protein complex with many subunits only transiently attached. This raises the question of which subunits represent true and essential components of the basic RNA polymerase complex. In order to answer it, we have performed mass spectrometry with all subunits of PEP preparations from mustard (*Sinapis alba*) chloroplasts after heparin-Sepharose (HS) chromatography and blue native two-dimensional gel electrophoresis. We aimed to determine those subunits that can be reproducibly purified in order to distinguish between permanent and transient protein components. We identified all *rpo* subunits, including one novel variant of RpoC1 and 10 additional proteins. Combining these biochemical data with phenotypic analyses of corresponding knockout mutants from Arabidopsis, we could define the essential subunits of the basic PEP complex and present a comprehensive catalog of its components. A potential role of PEP subunit assembly as a decisive checkpoint in chloroplast development is discussed.

RESULTS

Basic PEP Subunit Composition as Defined by Biochemical Purification and Mass Spectrometry

We used 7-d-old white light-grown mustard seedlings as a source for PEP preparations, as reported earlier (Tiller and Link, 1993). Intact chloroplasts were isolated from cotyledons by homogenization and Suc gradient centrifugation, lysed in a buffer containing the nonionic detergent Triton X-100, and transcriptionally active fractions were subsequently enriched by HS chromatography. Comparable preparations have been partially characterized earlier and contain RNA polymerases, σ-factors, several DNA- and RNA-binding proteins, DNA polymerase, and kinase activities (Tiller and Link, 1993; Pfannschmidt and Link, 1994; Baginsky et al., 1997). The PEP enzyme was then further purified from such fractions by two-dimensional (2D) blue native (BN)-PAGE, as recently described (Schröter et al., 2010). We took advantage of the observation that the PEP complex possesses a size of more than 1,000 kD, being by far the largest protein complex in the HS fractions. Due to this large size, the protein complex displays very slow migration behavior in BN-PAGE. No other proteins or protein complexes from the HS fractions were observed to migrate in this area of the gel. The subunit composition of the PEP complex was revealed by subsequent separation on a denaturing second dimension, producing a distinct ladder of protein subunits (Fig. 1) that can be clearly distinguished from background bands or staining artifacts due to its perpendicular arrangement and characteristic spot shape. Theoretically, some single proteins could be accidentally retained in this gel area because of technical inconsistencies, such as unspecific retardation within the PEP complex during the separation or because of biological variations in the plant material. In order to exclude these possibilities, we analyzed three different protein purifications, each prepared from an independent biological replicate. Only proteins that reproducibly occurred in all preparations were regarded as candidates for true components of the complex. In addition, this list of subunits was compared with that of

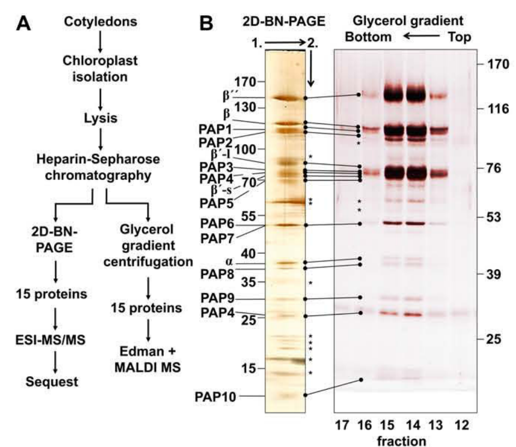

Figure 1. Comparison of subunit composition of the plastid RNA polymerase from mustard after 2D BN-PAGE and glycerol gradient centrifugation. A, Purification schemes and resulting proteins. B, PEP subunit composition obtained by 2D BN-PAGE (left, large gel, 7%–17%) and SDS-PAGE after glycerol gradient centrifugation (right, mini gel, 5%–15%). Two representative gels are shown. Total protein (150 μg) was separated and fixed, and proteins were stained with silver. Running directions of the first and second dimensions are indicated by arrows. Sizes of marker proteins separated in parallel on the same gel are given in the margins. Single subunits within the PEP complexes that gave significant hits in the databases are indicated by consecutive numbering. Corresponding proteins within the two preparations are connected by lines. Asterisks mark proteins not reproducibly found in the complexes. For identity and detailed data of mass spectrometry, see Table I and Supplemental Table S1. MALDI, Matrix-assisted laser-desorption ionization. [See online article for color version of this figure.]

highly purified PEP preparations after glycerol gradient centrifugation (Pfannschmidt and Link, 1994). In glycerol gradient centrifugation, the large PEP complex exhibits the fastest sedimentation of all protein complexes in the HS fractions and, therefore, can be easily separated from smaller complexes or single proteins. Only protein bands that appeared in both preparations were regarded as permanent PEP subunits. By this means, 15 different protein bands were reproducibly identified in the PEP complex (Fig. 1), which were then analyzed by mass spectrometry. The respective protein spots were cut out, subjected to in-gel tryptic digestion, and peptide masses were determined by electrospray ionization-tandem mass spectrometry (ESI-MS/MS).

We identified the mustard proteins by the masses of the homolog peptides in the Arabidopsis sequence or other species in the Brassicales database (Table I; Supplemental Table S1). In total, we measured 15 spots and identified 16 distinct protein sequences. We could confirm the identification of all subunits recently found by mass spectrometry in the mustard PEP (Loschelder et al., 2004; Schröter et al., 2010) but also found three novel components not described yet as PEP subunits. In particular, we found all *rpo* gene products (α, β, β', β'') representing the "classical" core of the PEP complex. The β''-subunit (encoded by *rpoC*$_2$) was the largest subunit at 141 kD, followed by the β-subunit (encoded by *rpoB*) at 118 kD. The β'-subunit (encoded by *rpoC*$_1$) was found at around 85 kD and, unexpectedly, in a second, smaller variant at about 72 kD. All RpoC$_1$ peptides detected in our mass spectrometric measurements were found for both proteins, with only one exception. This special peptide occurred only among those detected from the larger β'-variant and is located approximately in the middle of the RpoC$_1$ protein sequence (Fig. 2). This and the wide distribution of the identified peptides in the sequence suggest that the smaller β'-variant is a genuine gene product rather than a result of degradation. For a defined assignment, we named these two variants β'-l and β'-s (for large and small, respectively). The α-subunit (encoded by *rpoA*) was identified at 38 kD, which matches precisely the predicted size of 38 kD (Igloi and Kossel, 1992).

A second group of proteins identified here is composed of PTAC2, -3, -6, -10, -12, and -14, at apparent masses of 107, 110, 37, 76, 70, and 52 kD, respectively. All were described to be part of the transcriptionally active chromosome (Pfalz et al., 2006). PTAC2, -3, -10, and -14 contain a number of diverse functional domains related to DNA/RNA binding or interaction. These domains, however, are mainly characterized by domain prediction, and true functional assignments based on experimental evidence are lacking. PTAC6 is the most enigmatic PEP subunit, since it contains no known protein motif and any experimental clue to its potential function is missing (Table I). PTAC12 has not been described yet as a subunit of the soluble PEP complex. It has been reported to be potentially involved in protein degradation (Table I; Chen et al., 2010); however, this function was mainly attributed to its nuclear localization. In all cases, the apparent M_r values were close to the predicted theoretical ones.

Table I. *RNA polymerase subunits identified by ESI-MS/MS after 2D BN-PAGE*

Subunit, PAPs as given in Figure 1; AGI Accession No., Arabidopsis Genome Initiative gene accession numbers; Mass (kD; theor. cTP/app.), theoretical molecular mass without chloroplast transit peptide and apparent molecular mass observed on the gel; Identity/Protein Domain, identity of PAP and its predicted protein domain(s) as obtained by the Conserved Domain Database (Marchler-Bauer et al., 2011); Function, subunit functions predicted from subdomains or proposed/shown by experiment (ex.); Reference, source for functional classification.

Subunit	AGI Accession No.	Mass (kD; theor. – cTP/app.)	Identity/Protein Domain	Function	Reference
RpoC$_2$	AtCg00170	156/141	β'' subunit	DNA binding	Igloi and Kossel (1992)
RpoB	AtCg00190	121/118	β subunit	RNA synthesis	Igloi and Kossel (1992)
PAP1	At3g04260	110/110	pTAC3/SAP domain	DNA/RNA binding	Prediction
PAP2	At1g74850	89/107	pTAC2/PPR repeat, SMR domain	RNA metabolism	Prediction
RpoC$_1$	AtCg00180	80/85	β'-l subunit	Unknown	Igloi and Kossel (1992)
PAP3	At3g48500	77/76	pTAC10/S1-like domain	RNA binding	Prediction
PAP4	At5g23310	26/74	Iron superoxide dismutase 3	Superoxide detoxification (ex.)	Myouga et al. (2008)
RpoC$_1$	AtCg00180	80/72	β'-s subunit	Unknown	Igloi and Kossel (1992)
PAP5	At2g34640	57/70	pTAC12-HEMERA/RAD23 domain	Probably involved in ubiquitin-mediated proteolysis (ex.)	Chen et al. (2010)
PAP6	At3g54090	49/52	FLN1/pfkB-2 fructokinase	Fru phosphorylation; substrate specificity not detectable (ex.)	Prediction; Arsova et al. (2010)
PAP7	At4g20130	49/52	pTAC14/SET domain, Rubisco substrate binding	Lys methyltransferase, interaction with histones or Rubisco	Prediction
RpoA	AtCg00740	38/38	α-Subunit	Complex stabilization	Igloi and Kossel (1992)
PAP8	At1g21600	31/37	pTAC6	Unknown	Unknown
PAP9	At5g5110	29/29	Iron superoxide dismutase 2	Superoxide detoxification (ex.)	Myouga et al. (2008)
PAP4	At5g23310	26/26	Iron superoxide dismutase 3	Superoxide detoxification (ex.)	Myouga et al. (2008)
PAP10	At3g06730	12/13	TRX-z/thioredoxin WCGPC motif	Redox regulation (ex.)	Arsova et al. (2010)

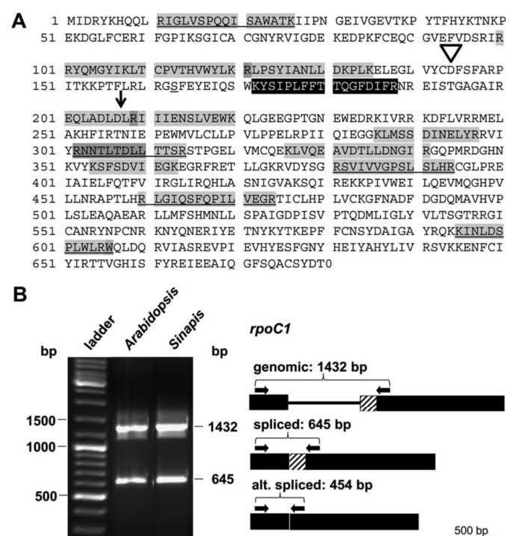

Figure 2. Analysis of the novel β'-s subunit. A, Detected peptides of the two β'-subunits. Peptides identified by mass spectrometry are given within the $RpoC_1$ amino acid sequence. Gray background, peptides identified in both subunits. Overlaps between neighboring peptides are indicated in dark gray. Gray background underlined, peptides of β'-s. Black background with white letters, peptide solely identified in the β'-l subunit. Positions of the spliced intron (triangle), the editing site (underlined S), and the proposed alternative splice acceptor site (arrow) are indicated. B, Results of RT-PCR analysis and corresponding gene models indicating potential splice variants of *$rpoC_1$*. Left panel, ethidium bromide-stained RT-PCR products using Arabidopsis and *Sinapis* cDNA and *$rpoC_1$*-specific primers. The top band represents a product generated from the genomic DNA, as depicted in the right panel. The bottom band represents a product generated from the spliced RNA. Right panel, gene model and splice variants. The coding sequence is given as the thick black bar and the intron as the thin black bar. A potential alternatively spliced area is given as the hatched bar.

A third group of subunits is composed of proteins that exhibit functions not directly related to gene expression. At 72, 29, and 26 kD, we identified the two iron superoxide dismutases FSD3 and FSD2. FSD2 at 29 kD has been found in earlier studies by Edman degradation of mustard PEP subunits, and both enzymes were detected by antibody reactions in nucleoids (Pfannschmidt et al., 2000; Myouga et al., 2008). However, so far, FSD3 has never been described as a PEP subunit. It appears in two bands at 72 and 26 kD. The large one differs from its theoretical size of 26 kD, while the small one fits precisely. This suggests that FSD3 generates a stable, probably trimeric complex that can only be partly resolved by the change to the second dimension SDS-PAGE.

The protein band at 52 kD always displayed a characteristic stronger staining intensity than other proteins. Our mass spectrometry data indicated that it contains two proteins of identical size, PTAC14 and a protein corresponding to a potential kinase with a domain typical for the phosphofructokinase family. An orthologous protein was also found in the tobacco PEP (Suzuki et al., 2004), and a corresponding ortholog called FLN1 was recently characterized in *Nicotiana benthamiana*. In a yeast-two-hybrid screen, FLN1 interacted with TrxZ (Arsova et al., 2010), a novel thioredoxin-like protein identified as a 13-kD subunit of the mustard PEP complex here and recently (Schröter et al., 2010). All these non-*rpo* subunits were regarded as essential components of the PEP complex and therefore were named PEP-associated proteins (PAP) 1 to 10.

Our biochemical approach identified neither σ-factors nor cpCK2, CSP41, and an annexin-like protein identified earlier in the mustard PEP complex by Edman degradation and mass spectrometry (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002). Sigma factors likely interact very shortly with the RNA polymerase during promoter recognition and probably exist only in substoichiometric amounts, exacerbating their biochemical identification (Schweer et al., 2010). In addition, biochemical observations demonstrated that the plastid transcription kinase (representing cpCK2) can dissociate from the RNA polymerase complex (Baginsky et al., 1999). This suggests that all these proteins likely represent transient or loosely attached components of the PEP complex, which are excluded under our stringent search conditions.

Phenotypic Effects of PAP Gene Knockouts in Arabidopsis Mutants

The association of proteins into a multisubunit protein complex is usually reflected in a common functional commitment of these proteins. Here, we studied the composition of the plastid PEP complex; therefore, one would expect that, besides the *rpo* subunits, the non-*rpo* subunits also exhibit functions that are somehow related to transcription. However, the functional assignments of only four subunits (PAP1, PAP2, PAP3, and PAP7) are related to gene expression, while those of PAP4, PAP5, PAP6, PAP8, PAP9, and PAP10 are difficult to reconcile with this function and appear unnecessary and/or dispensable for transcription. In order to understand the structural involvement of PAPs into the PEP complex, we screened Arabidopsis knockout mutant collections for the presence of PAP-deficient lines. Isolated knockout lines could potentially indicate the importance of the respective PAP if phenotypic effects are caused by the respective protein deficiency. *Sinapis* and Arabidopsis are related crucifers, and the combination of biochemical and genetic data from both species provides a useful tool for analyzing the functions of novel proteins, as demonstrated recently (Schröter et al., 2010). The screening for potential mutants was further complemented by a survey of literature and databases for descriptions of potential phenotypic effects in PAP knockout mutants.

We found studies and database entries describing detailed phenotypes of knockout lines for most of the non-*rpo* subunits (Table II), with the exception of PAP3, PAP6, and PAP7. For these subunits, we isolated homozygous knockout lines from respective collections, tested the repression of PAP transcript accumulation by reverse transcription (RT)-PCR, and finally checked the phenotypic appearance of confirmed knockout lines in petri dishes on standard Murashige and Skoog medium (Fig. 3). This provided a complete survey of the phenotypes for knockout mutants of all PAPs in Arabidopsis. For PAP1/PTAC3, the reported knockout lines exhibited an albino phenotype, while those for PAP2/PTAC2 displayed a slightly greenish phenotype that was also reflected in the plastid ultrastructure (Pfalz et al., 2006; Myouga et al., 2010). For PAP3/PTAC10, the isolated T-DNA insertion line exhibited an albino-like phenotype in the seedling stages and turned into an ivory phenotype in later stages (Fig. 3C). Knockout lines for PAP4 and PAP9 (FSD2 and FSD3) were reported to exhibit pale-green phenotypes in single knockout lines (with leaves being paler in FSD3 than in FSD2) and a full albino phenotype in the double mutant (Myouga et al., 2008). PAP5/PTAC12 seedlings were found to be white (Chen et al., 2010), while older plants turned into an ivory phenotype (Pfalz et al., 2006). For PAP6/FLN1, we isolated an Arabidopsis knockout line that also displayed an albino phenotype. Recently, the orthologous gene was analyzed in *N. benthamiana* by virus-induced gene silencing. Intriguingly, down-regulation of FLN1 expression resulted in white sectors in the affected leaves, while the same experiment with the paralogous protein FLN2 (which we did not identify as PAP) produced no apparent phenotypic variations (Arsova et al., 2010). For PAP7/PTAC14, we isolated an Arabidopsis T-DNA insertion line and again observed an albino phenotype. For PAP8/PTAC6, a knockout line was described that exhibited an albino phenotype (Pfalz et al., 2006). Finally, PAP10/TrxZ has recently been reported to be the first thioredoxin whose knockout results in a visible phenotype, again with an albino appearance (Arsova et al., 2010; Schröter et al., 2010).

Thus, knockouts of all subunits defined as PAPs by our biochemical approach result either in a complete block or a severe retardation of chloroplast development. In all cases, the developmental deficiencies were so strong that the mutants were only viable on Suc-supplemented medium. This makes the results highly comparable even if they were generated in different laboratories. It should be noted that we used the phenotypic description "albino" as it was found in the literature. A more detailed phenotypic analysis indicated in most cases that the albino turned into an ivory phenotype, usually clearly visible as yellowish coloring in the older stages (indicated in Table II). An ivory phenotype indicates carotenoid biosynthesis and, therefore, active and dividing plastids, which, however, cannot perform the transition into fully developed chloroplasts. This is consistent with the electron micrographs available for many PAP mutants, displaying plastids without thylakoid membrane systems and high accumulation of plastoglobuli (Table II). It also coincides with the analyses describing the respective plastid gene expression profiles, which in all cases investigated revealed a NEP-dependent transcript accumulation pattern. All these observations correspond to observations in transplastomic tobacco lines in which the *rpo* subunits had been knocked out. These exhibited an albino-like phenotype, indicating the necessity of the PEP enzyme for early chloroplast development. Typically, such plants were viable when grown on medium supplemented with Suc and displayed increased expression of the NEP-transcribed genes of the plastome, while PEP-transcribed genes were largely reduced (Allison et al., 1996; Hajdukiewicz et al., 1997; De Santis-MacIossek et al., 1999). These data indicate that regardless of the predicted function, knockout of PAPs results in the same appearance as *rpo* gene knockout lines.

Table II. *Phenotyping of Arabidopsis PAP knockout mutants*

Growth phenotype, Developmental appearance of knockout mutants (Suc, viable only on Suc-supplemented medium); Plastid Structure, plastid morphology in knockout/silenced mutants (t., thylakoids; p., plastoglobuli enrichment); Molecular Phenotype, NEP expression profile of plastid transcript accumulation; Reference, source of phenotypic descriptions; n.d., not described. Classification as "ivory" is based on our own observations.

Subunit	Growth Phenotype	Plastid Structure	Molecular Phenotype	Reference
PAP1	Albino/ivory; Suc	n.d.	n.d.	Myouga et al. (2010)
PAP2	Pale-yellow/green; Suc	Impaired; few t.; p.	NEP	Pfalz et al. (2006)
PAP3	Albino/ivory; Suc	n.d.	n.d.	This work
PAP4	Pale-green; Suc	Impaired; no t.; p.	NEP	Myouga et al. (2008)
PAP5	Albino/ivory; Suc	Impaired; no t.; p.	NEP	Pfalz et al. (2006); Chen et al. (2010)
PAP6	Albino/ivory, Suc	n.d.	n.d.	This work
PAP7	Albino/ivory; Suc	n.d.	n.d.	This work
PAP8	Albino/ivory; Suc	Impaired; no t.; p.	NEP	Pfalz et al. (2006)
PAP9	Pale-green; Suc	Impaired; no t.; p.	NEP	Myouga et al. (2008)
PAP10	Albino/ivory; Suc	Impaired; no t.; p.	NEP	Arsova et al. (2010); Schröter et al., 2010)

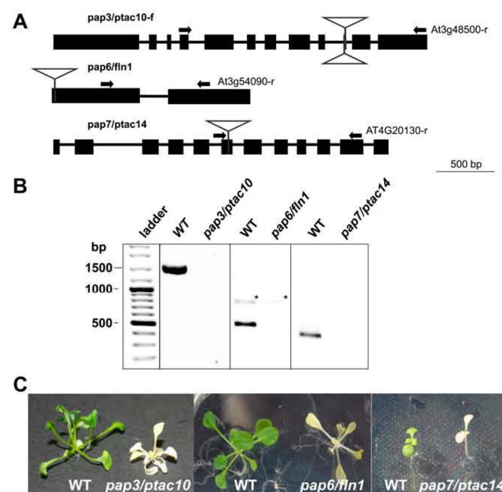

Figure 3. Identification and analysis of homozygous *pap3/ptac10*, *pap6/fln1*, and *pap7/ptac14* T-DNA insertion mutants. A, Schematic presentation of the corresponding genes showing the positions of the T-DNA insertions as confirmed by PCR and subsequent sequencing. B, RT-PCR amplification of the *pap3/ptac10*, *pap6/fln1*, and *pap7/ptac14* genes using gene-specific primers given in A. Lines homozygous for *pap3/ptac10*, *pap6/fln1*, and *pap7/ptac14* T-DNA insertion fail to express the wild-type (WT) allele. Asterisks indicate bands derived by genomic DNA. C, Wild-type and homozygous *pap3/ptac10*, *pap6/fln1*, and *pap7/ptac14* plants germinated on petri dishes with medium. Seeds of the T-DNA insertion line were surface sterilized and placed on sterile agar plates containing Murashige and Skoog medium supplemented with 2% Suc.

The phenotypic commonalities suggest that the PAPs are related to each other in a structural and/or developmental context. In order to obtain further support for such a potential relation, we determined coexpression patterns of the genes for PAPs using the Arabidopsis coresponse database (Steinhauser et al., 2004; Lisso et al., 2005; Usadel et al., 2005). The etioplast-chloroplast transition during photomorphogenesis is a major step in seedling development that involves parallel changes in thousands of nuclear genes (Ma et al., 2001). In order to distinguish PAP expression patterns from these light-induced developmental changes, we used nuclear genes encoding plastid protein components not involved in photosynthesis (e.g. RNA metabolism, metabolic pathways) as controls. Coexpression patterns within array data from AtGenExpress were obtained (Supplemental Fig. S1). In the category "developmental series," PAPs exhibited strong coregulation (average r_s of 0.901), which was clearly different from the controls. This indicates that PAP expression appears to be coregulated, supporting the notion that PAPs are related in a structural/developmental context.

DISCUSSION

The PEP Core Enzyme

Our mass spectrometry data identified all *rpo* gene products in the PEP complex and are in good accordance with earlier reports (Hu and Bogorad, 1990; Hu et al., 1991; Pfannschmidt et al., 2000; Suzuki et al., 2004). The identification of the β'-s variant, however, is an unexpected and novel finding. Earlier studies probably missed this subunit because it migrates in the mass range between 70 and 80 kD, where at least five different PEP subunits of similar size are located, which may mask each other if not separated in a high-resolution gel system as used here. The *rpoC$_1$* gene is the only *rpo* gene with an intron that exists, however, only in dicot plants (Igloi et al., 1990). It is conceivable that the two proteins simply represent translation products from spliced and nonspliced variants. However, the intron sequence encodes several stop codons distributed over the complete intron (Supplemental Fig. S2), making it unlikely that unspliced transcripts are translated. This conclusion is confirmed by observations in the *otp70* mutant of Arabidopsis, which displays a defect in *rpoC$_1$* splicing. This defect results in PEP deficiency of the mutant, implying that a complete splicing of *rpoC$_1$* transcripts is essential for the generation of a functional PEP complex (Chateigner-Boutin et al., 2011). An alternative possibility for two RpoC$_1$ variants originates from early characterizations of the *rpoBC$_1$C$_2$* transcript maturation in spinach (*Spinacia oleracea*) via S1 mapping analyses. These suggested the existence of a second splice acceptor site within exon 2 of the *rpoC$_1$* gene, giving rise to a second, smaller version of the transcript and its resulting protein product (Hudson et al., 1988). This smaller product would fit the apparent molecular mass of 72 kD of β'-s. We detected a peptide from this variant covering the alternative splice site by a few amino acids but not one within the area between the two splice acceptor sites. Instead, we detected a single β'-l peptide. The analyses in the Arabidopsis mutant *otp70* indicated that exactly in that area, an editing site exists that requires the action of the PPR protein OTP70 to be maturated. Unspliced *rpoC$_1$* transcripts appear to be preferentially edited; therefore, rapid splicing in the wild type eventually prevents *rpoC$_1$* transcripts from being fully edited (Chateigner-Boutin et al., 2011). This could generate two pools of transcripts with differing sequence at *rpoC$_1$* residue 21,806, coding either for Ser or Leu (Chateigner-Boutin and Small, 2007), which eventually could affect translation or posttranslational events. Alternatively, one could speculate that the binding of the editing factor redirects the splicing machinery toward the second splice acceptor site, resulting in a smaller transcript and hence a smaller translation product. However, our RT-PCR approach did not detect alternative *rpoC$_1$* splice variants, suggesting that the β'-s subunit is likely generated by posttranslational modification of the β'-l

subunit. The type of modification and its effect on functionality require further investigation.

As a side aspect, we observed that the α-subunit band did not exhibit an increased staining strength, as observed for the 52-kD band containing PTAC14 and FLN1. This observation is just a hint but suggests that the α-subunit does not necessarily exist in two copies per complex, as predicted from simple adaptations of the *E. coli* structure $2\alpha, \beta, \beta'$. It is equally likely that the structure of the PEP core enzyme could be an α, β, β'-l, β'-s, β'' assembly. Probably, only structural work, including crystallography, will be of sufficient resolution to fully understand the composition and structure of the PEP core complex.

PAPs

We could reproducibly identify 10 essential non-*rpo* protein subunits of the PEP complex, which can be roughly divided into two functional groups. One group consists of PAP1, PAP2, PAP3, and PAP7, with domains or motifs likely involved in gene expression/regulation (SAP, PPR, S1, SET, respectively; Table I). The other group consists of PAP4 to -6 and PAP8 to -10, which all are related to or involved in redox-dependent processes or regulation. The specific functions of PAP1, PAP3, and PAP7 are based only on protein domain predictions, while all other PAPs have been, at least in part, functionally characterized. PAP2, PAP5, and PAP8 are also known as PTAC2, PTAC12, and PTAC6, and the corresponding knockout mutants all display a specific PEP-deficient plastid gene expression phenotype (Pfalz et al., 2006). Intriguingly, the same has been observed in knockout mutants for PAP4 and PAP9 as well as for PAP6 and PAP10, and it is reasonable to expect a similar expression pattern also in the uncharacterized PAP1, PAP3, and PAP7 mutants, since they exhibit comparable phenotypes. PAP5 or PTAC12/HEMERA is special among all these proteins, since it has recently been demonstrated to be dual targeted to nucleus and plastids (Chen et al., 2010). In the nucleus, it appears to be located in so-called nuclear bodies and seems to act in phytochrome signaling, probably in ubiquitin-mediated proteolysis, since it exhibits some similarities to the yeast RAD23 protein. Dual localization in nucleus and plastids within the same plant cell was first demonstrated for the RNA-binding protein Whirly1/PTAC1 (Grabowski et al., 2008), and further analyses suggested that this dual subcellular distribution occurs also for other plant cell proteins (Krause and Krupinska, 2009). Whether PAP5 is involved in plastid protein degradation, however, is not known yet. PAP4 and PAP9 are two superoxide dismutases, the first of which, to our knowledge, has been identified here for the first time as a PAP, while the second was described earlier (Pfannschmidt et al., 2000). A recent independent study could show that these two proteins interact in a yeast two-hybrid assay and that both are located within plastid nucleoids (Myouga et al., 2008). These observations are consistent with our data. Interaction in a yeast two-hybrid assay also could be demonstrated for PAP6 and PAP10. PAP6 is also called FLN1, and its sequence suggests that it belongs to the class of the phosphofructokinases; however, it could be shown that this enzyme lost its ability to recognize this type of substrate (Arsova et al., 2010). The interacting PAP10 is also called TrxZ and represents a novel type of thioredoxin. It still functions as a "true" thioredoxin in the insulin activation assay (Arsova et al., 2010), but it is the only thioredoxin that apparently cannot be replaced by another one, since the knockout results in an albino phenotype (Arsova et al., 2010; Schröter et al., 2010). Nevertheless, despite these investigations, little is known about the true PAP functions, and further characterizations will be necessary to unravel the specific roles of the distinct PAPs in the RNA polymerase complex.

Impact of PAP Gene Knockouts on Plastid Development

The major common feature of all PAPs, regardless of their predicted/detected functions, is that a knockout of the corresponding gene always results in a severe defect in chloroplast development. In knockout mutants of PAP2, PAP4 to -6, and PAP8 to -10, this is accompanied by high NEP-dependent and low PEP-dependent transcript accumulation (Table II and refs. therein). This suggests that PAP knockouts cause a block of PEP activity that prevents the transition of plastid transcription from a NEP-dependent to a PEP-dependent mode in the same manner as could be observed for *rpo* gene knockout mutants of tobacco (Allison et al., 1996; Hajdukiewicz et al., 1997; De Santis-MacIossek et al., 1999) and *rpo* knockdown mutants of Arabidopsis (Chateigner-Boutin et al., 2008, 2011; Zhou et al., 2008).

For *S. alba*, extensive biochemical data exist that describe the subunit composition of the soluble PEP enzyme in etioplasts, greening chloroplasts, and mature chloroplasts (Pfannschmidt and Link, 1994; Pfannschmidt et al., 2000; Ogrzewalla et al., 2002; Loschelder et al., 2004). The combination of these data with the biochemical results reported here suggest an explanation for the observed phenotypes in the Arabidopsis PAP knockout mutants (Fig. 4). In early seedling development, the *rpo* subunits of PEP are expressed by the nucleus-encoded NEP enzyme, representing a first essential checkpoint in the establishment of the plastid gene expression machinery. In combination with some PEP starter molecules inherited from the parent plant (Demarsy et al., 2006), these first PEP complexes provide effective transcription of PEP-dependent plastid genes in the very early stages of plastid development. Data from etioplasts and greening chloroplasts suggest that these complexes exhibit the basic prokaryote-like PEP structure, representing a core complex consisting of only the *rpo* gene products (PEP-B; Hu and Bogorad, 1990; Hu et al., 1991; Pfannschmidt et al., 2000). With the onset of photomorphogenesis, this PEP-B enzyme is reconfigured into a much more complex eukaryote-like enzyme complex, the PEP-A enzyme. This most likely

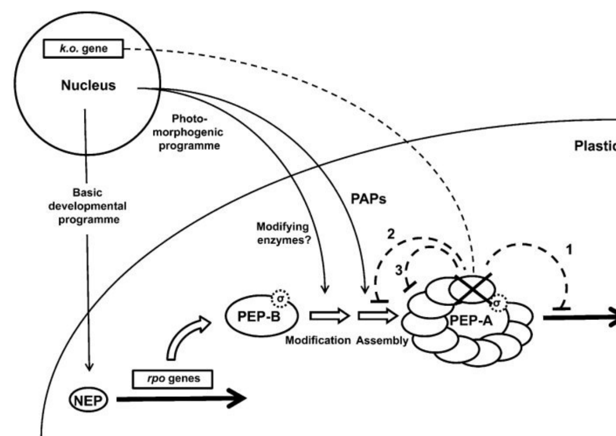

Figure 4. Checkpoint model describing the reconfiguration of the plastid RNA polymerase complex as an essential step in plastid development. The scheme depicts the structural assembly of the plastid-encoded RNA polymerase, starting with the expression of the *rpo* genes by the NEP enzyme, resulting in the formation of the basic PEP-B enzyme. Interaction with σ-factors is assumed. Upon initiation of photomorphogenesis, it is modified first by posttranslational changes of *rpo* subunits (via unknown modifying enzymes) and second by the addition of PAPs, generating the structurally more complex PEP-A. White arrows indicate the flow of events required for PEP-A buildup. Thin black arrows indicate the action or involvement of nucleus-encoded proteins delivered in a fixed sequence that follows a distinct developmental program in the nucleus. Thick black arrows indicate transcription activity. Dotted lines indicate the possible impact of a PAP gene knockout in the nucleus on PEP-A. The lacking subunit is indicated by a cross, its inhibitory feedback by dotted lines. Numbers refer to discussed possibilities causing the observed phenotypes of PAP knockout mutants. For further details, see text.

involves first a posttranslational modification of the PEP-B *rpo* subunits, since the complex changes subunit sizes and promoter recognition properties (Pfannschmidt and Link, 1997), which allow in a second step the assembly of the nucleus-encoded PAPs. The time range for this reconfiguration of the PEP complex parallels the etioplast-chloroplast transition and requires only a small time window of about 16 to 48 h, depending on the growth conditions of the seedlings (Hu and Bogorad, 1990; Hu et al., 1991; Pfannschmidt and Link, 1994). During normal plastid development without an intermediate etioplast stage, this complex conversion likely takes place faster, making it very difficult to resolve this process in a temporal manner. The addition of PAPs to the PEP core complex is the second essential checkpoint in the establishment of the plastid gene expression machinery, and the strong phenotypes of the Arabidopsis mutants indicate that it represents an irreplaceable step in chloroplast development.

While the latter observation is indisputable, the reason for it remains obscure. In principle, there are three possibilities why lack of any PAP results in a block or severe disturbance of plastid development (Fig. 4). First, PAP functions are essential for the transcriptional activity or regulation of the complex; second, the proteins are required for the assembly or attachment of further proteins; third, the proteins are required for the integrity of the complex itself. One could imagine that failure in each one of these processes leads to inactivity of the PEP-A complex and, hence, to a disturbance in plastid development. However, not all possibilities are equally likely. The basic PEP-B complex is already capable of faithful transcription, rendering it unlikely that the addition of PAPs is essential for the transcriptional activity of PEP-A. Thus, it appears more reasonable that PAPs modulate or regulate transcriptional activity, and at least four subunits seem to confirm this assumption, because their predicted functions are potentially involved in gene expression (Table II). The NEP expression profile observed in some mutants, however, points more to a complete inactivation of the PEP-A complex, which is unlikely if just one regulatory event is affected. Knockouts for most known regulators of chloroplast transcription do not result in such strong phenotypes and usually display just a few gene-specific changes or minor general effects of limited impact (Bollenbach et al., 2009; Schweer et al., 2010; Barkan, 2011; Lerbs-Mache, 2011). This suggests that structural effects are potentially the reason for the observed phenotypes. If one or more of the PAPs are lacking, the whole complex could become unstable and either breaks apart or its further assembly is retarded or blocked. This could result in an inactive PEP complex, causing the observed NEP expression phenotype of the chlorotic knockout mutant lines. The ivory phenotype of many of the mutants indicates that the plastids, although PEP deficient, are still active, producing

carotenoids and also being able to divide, since older leaves of plants grown on Suc-supplemented medium appear yellow. This suggests that the plastids are arrested in an early developmental stage, being unable to reach the next step of development despite the presence of light. This model would be consistent with the phenotypic effects in the knockout lines of PAP5/PTAC12/HEMERA. The lack of PAP5 leads to a block in PEP complex assembly in developing plastids and causes chlorosis. In parallel, it prevents photomorphogenic responses of the young seedlings due to its lack in the nucleus, as apparent from its lacking a response to red/far-red shift experiments (Chen et al., 2010). Since chloroplast development is an intrinsic part of photomorphogenesis, one and the same expression program of PAP5/PTAC12/HEMERA could serve both genetic compartments.

CONCLUSION

We generated a comprehensive and complete catalog of the subunits of the chloroplast RNA polymerase from mustard, comprising five subunits encoded by plastid *rpo* genes and 10 subunits called PAPs encoded by nuclear genes. We identified three novel protein subunits, β'-s, FSD3, and PTAC12, not described yet in the soluble PEP complex. Combining these biochemical data with observations from reverse genetics, we could establish that PAPs represent essential components of the PEP complex. These components display a coexpression pattern that is mainly determined by developmental programs, pointing to the reconfiguration of the PEP complex as an essential step in plastid and plant development. Our checkpoint model explains the white/ivory/pale-green phenotypes of PAP knockout mutants as the likely result of an interruption in this PEP complex reconfiguration. Although we now have a clear picture of the subunit composition of the PEP complex, our knowledge of their precise functions is still rudimentary, and further studies are required to fully understand the processes involved in plastid transcription and its regulation. Complementation of PAP knockout lines with corresponding full-length genes carrying modified functional domains provides a useful tool for this future goal.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Mustard seedlings (*Sinapis alba* var Albatros) were grown for 7 d on soil under continuous white light illumination at 20°C and 60% humidity. Cotyledons were harvested under the growth light, placed on ice, and immediately used for preparation of chloroplasts.

HS Chromatography of Chloroplast Proteins

Two kilograms of cotyledons was homogenized in ice-cold isolation buffer using a Waring blender and filtered through three layers of muslin and one layer of nylon. Chloroplasts were isolated by differential centrifugation followed by Suc gradient centrifugation, lysed, and subjected to HS CL-6B chromatography as described earlier (Tiller and Link, 1993; Steiner et al., 2009). Bound proteins were eluted with a single high-salt step of 1.2 M $(NH_4)_2SO_4$. The elution peak was identified by a protein quantification assay (RC-DC; Bio-Rad). RNA polymerase activity was determined in an in vitro transcription activity assay (Pfannschmidt and Link, 1994). Identified peak fractions were pooled and dialyzed against storage buffer (50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% Triton X-100, and 50% glycerol) and stored at −20°C until further use.

2D BN-PAGE

HS peak fractions were subjected to 2D gel electrophoresis using as a first dimension BN-PAGE with 4% to 12% acrylamide gradient gels followed by denaturing SDS-PAGE on 7% to 17% acrylamide gradient gels as a second dimension, as described recently (Schröter et al., 2010).

Tryptic In-Gel Digestion and Liquid Chromatography/ESI-MS/MS Analysis

Peptide generation of proteins from silver-stained gels was performed by tryptic in-gel digestion of cutout spots using a described protocol (Mørtz et al., 1994; Stauber et al., 2003) with minor modifications. Peptides from digested proteins were analyzed by liquid chromatography/ESI-MS/MS using a LCQ-DecaXP ion trap mass spectrometer (Thermo Finnigan). Nano-liquid chromatography was performed using an UltiMate Nano liquid chromatograph with a Famos Autosampler HPLC unit and a reverse-phase C18 PepMap100, 3-μm, 100-Å Nano column (75 μm i.d. × 15 cm; Dionex). Peptides were eluted using a three-step gradient with mobile phases A (0.1% HCOOH and 5% acetonitrile in water) and B (0.1% HCOOH and 80% acetonitrile in water). The mobile phase flow was 27 μL min^{-1} with 5% B for the first 8 min, followed by 5% to 50% B in the next 17 min, 50% to 95% B for 0.5 min, held at 95% B for 18 min, switched back to 5% B in 0.5 min, and held at 5% B for 16.5 min. The ion signals from the eluted peptides were collected using a data-dependent scan procedure with four cyclic scan events. The first cycle was composed of a full mass spectrometry scan of the mass-to-charge ratio range 450 to 1,200, followed by three MS/MS scans for the three most abundant ions. Sample run and data acquisition were performed using Xcalibur software (version 1.3; Thermo Finnigan).

MS Data Analysis

For peak list generation, the Ceate DTA tool of TurboSEQUEST (version 27 [revision 12]; Molecular Biotechnology, University of Washington, licensed to Thermo Finnigan) was used with default settings. Database search was conducted with TurboSEQUEST (version 27 [revision 12]) against a Brassicales protein database of the National Center for Biotechnology Information (NCBI Brassicales 2008.09.09; 154,464 sequences). The enzyme specificity was set to trypsin strict, and no missed cleavages were permitted. As variable modifications, the carboxyamidomethylation of Cys (57.0293), oxidation of Met (15.9949), and phosphorylation of Ser, Thr, and Tyr (79.9663) were included. The mass tolerance for precursor ions was set to 1.5 D and 0 D for fragment ions. Calculated cross-correlation values for significantly matching sequences had to be equal to or above 1.5, 2.0, or 3.5 for singly, doubly, or triply charged precursor ions, respectively, and the delta correlation (ΔCorr) values had to exceed 0.1. Proteins were accepted as identified with two or more different significant matching peptides. The database used is highly redundant; consequently, peptides match to several equivalent proteins of Arabidopsis and other Brassicales species. Therefore, the protein entry of the first complete sequence of Arabidopsis within the list of matching entries is given in "Results." Alternatively, a representative species is given in the case in which the Arabidopsis sequence is not matching.

rpoC$_1$ Transcript Analysis

rpoC$_1$ transcript splice sites were analyzed by RT-PCR using cDNA from Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) and mustard generated from total RNA with random hexamer oligonucleotides. Primers were *rpoC1*-fwd (5′-AATTGGCTTAGTTTCTCCTCAG-3′) and *rpoC1*-rev (5′-CCCTTCTTCTCCTAATTGTTTCC-3′). Preparation of total RNA for cDNA synthesis,

PCR, and RT-PCR programs followed standard procedures described at The Arabidopsis Information Resource (http://www.arabidopsis.org).

In Silico and Genetic Analyses of Arabidopsis PAP Knockout Mutant Plants

Gene identification numbers of the determined proteins obtained from SEQUEST searches as well as trivial names of identified proteins/enzymes were used to screen the PubMed literature database at the NCBI (http://www.ncbi.nlm.nih.gov/pubmed/) for publications concerning either the encoded proteins or corresponding knockout or knockdown mutant lines in various plant species. In addition, Arabidopsis T-DNA knockout mutant collections at The Arabidopsis Information Resource (http://www.arabidopsis.org) were screened for respective tagged lines. Identified knockout lines were compared with published data and checked for phenotypic descriptions in research reports, the Chloroplast Function Database (Myouga et al., 2010; Table II), and the Chloroplast 2010 database (http://www.plastid.msu.edu; Ajjawi et al., 2010). Seeds of unpublished lines CS16115 for PAP3/PTAC10, GK-443A08 for PAP6/FLN1, and SAIL_566_F06 for PAP7/PTAC14 were ordered, and homozygous plants were isolated. Primers for the detection of T-DNA locations were as follows: LB3 (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′), CS16115-(pap3/ptac) forward (5′-TCAGGGAGCGTTTGTTGACAT-3′), CS16155-(pap3/ptac10) reverse (5′-GGTGATCAGAGAGCAGCCCTT-3′), GK-443A08-forward (5′-CAAATAGCGAGTCCTCAGGTG-3′), GK-443A08-reverse (5′-GATCAATTCCCACAAGGAAGC-3′), SAIL_566_F06-forward (5′-AGAAGGTCCAGATGGTTTTGG-3′), and SAIL_566_F06-reverse (5′-TGCAGAGAATGATCAATCGTG-3′). Primers for RT-PCR were as follows: CS16155-f (5′-TCAGGGAGCGTTTGTTGACAT-3′), At3g48500-r (5′-TCAGTCTGTCAAGACTTGAG-3′), pap6-fwd (5′-CAAGATCAAGTTTAAGGACGG-3′), pap6-rev (5′-GTTCCATCAGTCACAACCAG-3′), pap7-fwd (5′-CAACAACAGAAACGAATCCT-3′), and pap7-rev (5′-CAGAGAACTTTATAGCATCCCA-3′). PCR and RT-PCR programs followed standard procedures described at The Arabidopsis Information Resource (http://www.arabidopsis.org). The phenotypic appearance of the homozygous lines was tested on Murashige and Skoog medium containing 2% Suc, as described recently (Schröter et al., 2010).

Coexpression Analysis of PAPs

For the analysis of coexpression, we used the Arabidopsis coresponse database (http://csbdb.mpimp-golm.mpg.de; Steinhauser et al., 2004) and searched for expression correlations of all PAPs to each other in the transcript profiles of the AtGenExpress stress series, developmental series, and miscellaneous. The nonparametric Spearman's ρ rank correlation r_s (ranging from +1 to −1) was obtained for each pair, linked to a value-dependent color code for visualization, and given as a matrix (Supplemental Fig. S1).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AtCg00170, AtCg00190, At3g04260, At1g74850, AtCg00180, At3g48500, At5g23310, AtCg00180, At2g34640, At3g54090, At4g20130, AtCg00740, At1g21600, At5g51100, and At3g06730.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Coexpression analysis of PAPs.

Supplemental Figure S2. Computer translation of the unspliced *rpoC*$_1$ gene of Arabidopsis.

Supplemental Table S1. Primary data of mass spectrometry for *rpo* and non-*rpo* subunits.

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9 Manuscript VI

Pfalz J., Liebers M., Hirth M., Grübler B., Holtzegel U., Schröter Y., Dietzel L., Pfannschmidt T.

Environmental control of plant nuclear gene expression by chloroplast redox signals.

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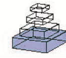

Environmental control of plant nuclear gene expression by chloroplast redox signals

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Plant photosynthesis takes place in specialized cell organelles, the chloroplasts, which perform all essential steps of this process. The proteins involved in photosynthesis are encoded by genes located on the plastid and nuclear genomes. Proper function and regulation of light harvesting and energy fixation thus requires a tight coordination of the gene expression machineries in the two genetic compartments. This is achieved by a bi-directional exchange of information between nucleus and plastids. Signals emerging from plastids report the functional and developmental state of the organelle to the nucleus and initiate distinct nuclear gene expression profiles, which trigger responses that support or improve plastid functions. Recent research indicated that this signaling is absolutely essential for plant growth and development. Reduction/oxidation (redox) signals from photosynthesis are key players in this information network since they do report functional disturbances in photosynthesis, the primary energy source of plants. Such disturbances are caused by environmental fluctuations for instance in illumination, temperature, or water availability. These environmental changes affect the linear electron flow of photosynthesis and result in changes of the redox state of the components involved [e.g., the plastoquinone (PQ) pool] or coupled to it (e.g., the thioredoxin pool). Thus, the changes in redox state directly reflect the environmental impact and serve as immediate plastidial signals to the nucleus. The triggered responses range from counterbalancing reactions within the physiological range up to severe stress responses including cell death. This review focuses on physiological redox signals from photosynthetic electron transport (PET), their relation to the environment, potential transduction pathways to the nucleus and their impact on nuclear gene expression.

Keywords: photosynthetic acclimation, electron transport, redox signaling, gene expression, environmental sensing

INTRODUCTION

Plants are sessile and, therefore, cannot escape from unfavorable conditions in their environment. During evolution they developed a number of responses which help them to deal with varying and adverse environmental cues. These responses cover several time scales acting at the physiological level within minutes, at the developmental level within days and at the seasonal level within months. Most of these responses work at the molecular level including regulation of gene expression networks, adjustment of metabolic pathways, or nutrient allocation. One important sensing system of plants for changes in the environment is photosynthesis. Its unique combination of light-dependent light harvesting processes and temperature-dependent carbon fixation reactions makes it ideal for precise and rapid detection of abiotic environmental changes since for optimal photosynthesis both parts needs to work in a fine-tuned balance. Changes in temperature, light intensity or quality, or in the availability of water, CO_2, or nutrients may disturb this balance resulting in less efficient photosynthesis. In all cases the immediate effect is a change in photosynthetic electron flux which affects the reduction/oxidation (redox) state of the components involved in it. In many cases this change in redox state initiates acclimation responses which help the plant adapting the photosynthetic process to the changed environment (Anderson et al., 1995; Kanervo et al., 2005; Walters, 2005; Dietzel et al., 2008a; Eberhard et al., 2008). However, since photosynthesis is the ultimate source of energy for plants it is tightly connected with many other physiological and metabolic processes. The redox signals regulating photosynthesis, thus, lead to a systemic response also in non-photosynthetic processes. It is important to note that the type of response depends highly on strength and duration of the environmental disturbance and its effect on photosynthesis. In recent years laboratory experiments have uncovered a number of strategies how plants cope with the environment, but we are far away from understanding these responses under free-fluctuating conditions in nature which can vary in an unpredictable way. It

becomes increasingly clear that many redox signals occur at the same time or in varying combinations when observed under natural or variable experimental conditions. It is, therefore, reasonable to assume the action of redox signaling networks rather than that of single signaling pathways. Nevertheless, for building networks it is essential to understand the immediate molecular mechanisms initiated by a distinct redox signal. Thus, in future a combined strategy of experiments with a single changing parameter and experiments with two or more changing parameters will be required.

IMPORTANT OPERATIONAL REDOX SIGNALS FROM PHOTOSYNTHESIS

Photosynthesis, in simple terms, is the light-driven transfer of electrons and protons from water to $NADP^+$, the formation of ATP using the trans-thylakoidal proton gradient generated during this transfer and, subsequently, the use of these reduction and energy equivalents in the fixation of CO_2 to produce carbohydrates as chemical energy source for growth and development of the organism (Buchanan et al., 2002). This complex process contains many reduction and oxidation steps and, therefore, the components involved change their redox status depending on the efficiency of photosynthesis. Two of them, plastoquinone (PQ) and thioredoxin, are of special importance as they fulfil not only a function as redox-active molecules but also initiate signaling cascades which control molecular responses acclimating photosynthesis to the environment (Aro and Andersson, 2001; Foyer et al., 2012). This includes also the control of nuclear gene expression and, thus, represents an example for operational control in retrograde signaling (Pogson et al., 2008) which will be the focus of this review.

PQ is the intermediate electron carrier which connects photosystem (PS) II and the cytochrome (cyt) b_6f complex in the photosynthetic electron transport (PET) chain. This location makes this molecule pool very sensitive to any imbalance in the relative activities of PSII and PSI, especially since the PQ oxidation represents the slowest step in linear electron transport (Allen, 2004). Under conditions favoring PSII PSI becomes rate-limiting and the PQ pool receives more electrons from PSII than it can deliver to PSI resulting in a reduction of PQ. Under conditions favoring PSI the opposite situation is established and the pool becomes oxidized. These redox changes occur almost immediately and can be induced either physiologically by environmental changes or chemically by treatment with electron transport inhibitors (Pfannschmidt et al., 2009). DCMU irreversibly binds to the Q_B binding site of PSII and prevents any electron transport from PSII to subsequent acceptors resulting in oxidation of the PQ pool. DBMIB binds to the plastoquinol-oxidation site of the cyt b_6f complex resulting in PQ reduction (Trebst, 1980). It must be noted that DBMIB is a labile compound which becomes easily inactivated if not re-supplied. Furthermore it can loose its specificity since at high concentrations it also binds to the DCMU binding site. Experiments based on such inhibitor treatments, therefore, essentially require titration controls and must be checked for side effects (Pfannschmidt et al., 2009).

In photosynthesis the PQ redox state controls phosphorylation of light harvesting complex proteins of PSII (LHCII) and the relative allocation of the mobile antenna to the two PS. This short-term response (half-time ~10 min) balances excitation energy distribution between the PSs (state transitions) and requires the action of the thylakoid-bound kinase STN7 (Rochaix, 2007). In the long-term (half-time 1–2 days) PQ redox state controls the adjustment of PS stoichiometry which requires a tight control of both plastid and nuclear encoded photosynthesis genes (Allen and Pfannschmidt, 2000). The latter requires the transduction of the PQ redox signal toward the nuclear compartment and represents an important plastidial signal.

Thioredoxins are a family of small proteins with a size of ~12 kDa which possess a redox-active dithiol group in a conserved WCGPC amino acid motif (Schurmann and Buchanan, 2008). In *Arabidopsis* 44 different thioredoxins have been identified and a large number of them are active in the chloroplast (Meyer et al., 2005). These receive their electrons from PSI *via* the action of an enzyme called ferredoxin–thioredoxin oxidoreductase (FTR). In the dark thioredoxins are usually oxidized but become rapidly reduced upon illumination when the PET chain is activated. In their reduced state they are able to reduce regulatory thiol groups especially in the enzymes of the Calvin–Benson cycle and, by this means, control production of carbohydrates in the carbon reduction cycle of photosynthesis. They also functionally separate the reductive and oxidative pentose-phosphate pathway avoiding futile cycling of common substrates (Buchanan et al., 2002).

In recent years a number of additional thioredoxin targets have been identified for instance in the lumen (Buchanan and Balmer, 2005; Buchanan and Luan, 2005). Most recently a novel thioredoxin-like protein designated as TrxZ has been identified as subunit of the plastid RNA polymerase complex potentially linking redox regulation and plastid transcription (Arsova et al., 2010; Schroter et al., 2010). Inactivation of its gene in *Arabidopsis* creates an albino phenotype indicating that it cannot be replaced by any other thioredoxin, a property which is unique in this group of reductive proteins.

As an unavoidable side reaction of oxygenic photosynthesis reaction centres transfer electrons not only to their primary acceptors but also to molecular oxygen in their immediate surrounding generating reactive oxygen species (ROS) such as superoxide (mainly at PSI *via* ferredoxin) or singlet oxygen (mainly at PSII *via* triplett state chlorophylls) (Apel and Hirt, 2004). ROS induce oxidative damages to proteins or membrane lipids and, therefore, are harmful for all kinds of biological molecules including the photosynthetic apparatus itself (e.g., during photoinhibition). Plant cells and chloroplasts, therefore, possess a sophisticated antioxidant network consisting of reductive low-molecular weight components (glutathione, ascorbate, and α-tocopherol) (Szarka et al., 2012) as well as various enzymatic activities (superoxide dismutases, ascorbate peroxidises, peroxiredoxins, and glutaredoxins) (Dietz, 2011; Zaffagnini et al., 2012) which scavenge and detoxify ROS and a number of recently discovered reactive nitrogen species (NOS) such as nitric oxide (NO) (Navrot et al., 2011). However, ROS and NOS possess a dual role and act also as important signaling molecules in stress responses including pathogen defense and programmed cell death. This includes a number of redox-dependent protein modifications

such as glutathionylation or nitrosylation. This area of research is rapidly expanding and has been extensively reviewed elsewhere. This review will include only that work which is essential for understanding of physiological redox signals from the PQ and Trx pools and the reader interested in details of stress responses is referred to the reviews mentioned above and the references therein.

ENVIRONMENTAL INDUCTION OF PHOTOSYNTHETIC REDOX SIGNALS

The processes of light energy absorption, transfer within the antenna, and charge separation depend mainly on the biophysical properties of the reaction centres and their antenna structure. These processes are largely independent from ambient temperature; however, the amounts of photons absorbed and the efficiency of their transport are directly influenced by the quantity and the quality of the incident light (see also below). In addition, in natural habitats the illumination of plants is not constant but fluctuates within seconds and minutes, as well as daily and seasonal periods, having multiple effects on electron transport efficiency. In contrast, subsequent metabolic reaction such as the Calvin–Benson cycle, N- and S-reduction reactions or other plastid biosynthesis pathways are not directly affected by light but are strongly influenced by the environmental temperature as well as by availability of substrates, nutrients, carbon, or water. Nevertheless they require sufficient amounts of energy and reduction equivalents (i.e., ATP and $NADPH_2$) from the light reaction for optimal activity. Thus they are also dependent on the functionality of PET *via* its production of ATP and $NADPH_2$. On the other hand efficient PET is possible only if a sufficiently high concentration of final electron acceptors (i.e., $NADP^+$) is available. If metabolic activities and, thus, the use of ATP and $NADPH_2$ are down-regulated (for instance by low temperatures), the availability of these final acceptors can become limiting leading to a feedback restriction in electron transport efficiency. In conclusion, there exists a delicate balance between photosynthetic light reaction and subsequent metabolism leading to a mutual dependency which is largely determined by the residing environmental condition (**Figure 1**). This makes photosynthesis a very sensitive system for environmental changes in the surrounding of the plant

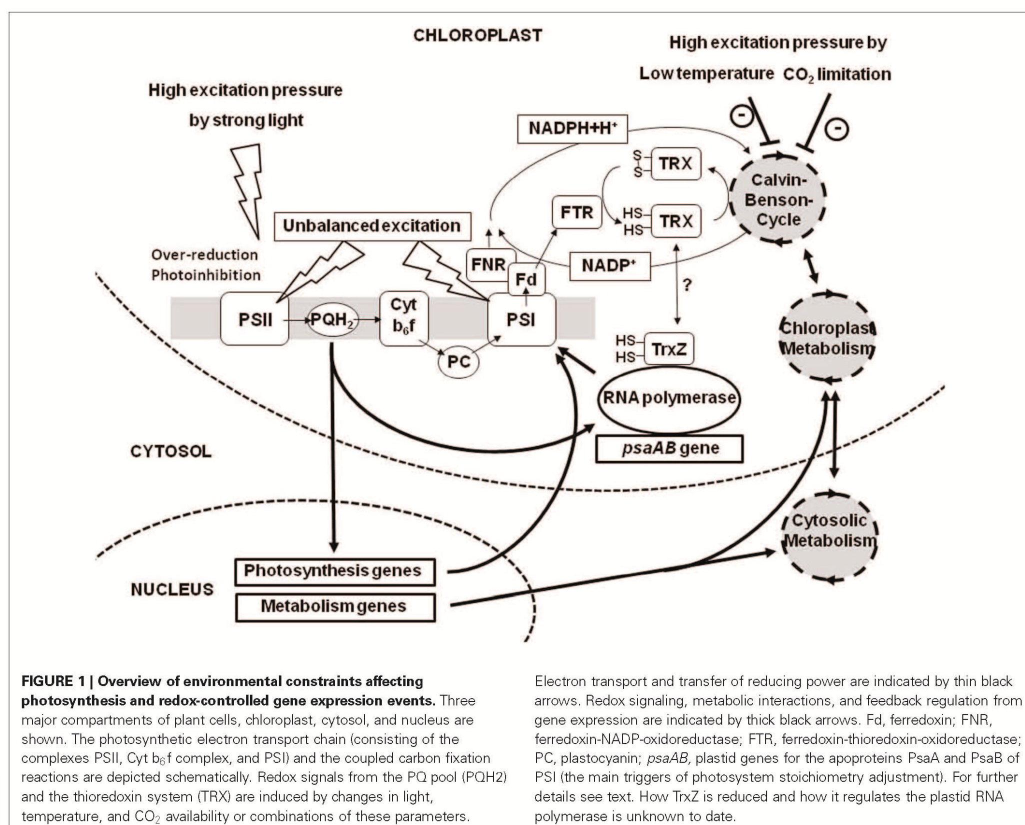

FIGURE 1 | Overview of environmental constraints affecting photosynthesis and redox-controlled gene expression events. Three major compartments of plant cells, chloroplast, cytosol, and nucleus are shown. The photosynthetic electron transport chain (consisting of the complexes PSII, Cyt b_6f complex, and PSI) and the coupled carbon fixation reactions are depicted schematically. Redox signals from the PQ pool (PQH2) and the thioredoxin system (TRX) are induced by changes in light, temperature, and CO_2 availability or combinations of these parameters. Electron transport and transfer of reducing power are indicated by thin black arrows. Redox signaling, metabolic interactions, and feedback regulation from gene expression are indicated by thick black arrows. Fd, ferredoxin; FNR, ferredoxin-NADP-oxidoreductase; FTR, ferredoxin-thioredoxin-oxidoreductase; PC, plastocyanin; *psaAB*, plastid genes for the apoproteins PsaA and PsaB of PSI (the main triggers of photosystem stoichiometry adjustment). For further details see text. How TrxZ is reduced and how it regulates the plastid RNA polymerase is unknown to date.

which are reflected by the reduction state of the electron transport chain, i.e., the PQ pool and the thioredoxin system.

Well known and characterized in this context are the influences of excitation pressure on PSII (**Figure 1**). Under conditions of high excitation pressure absorbed light energy exceeds the demands of the dark reaction leading to a reduction of the electron transport chain while under conditions of low excitation pressure the opposite occurs (Hüner et al., 1998). These two situations are, however, not unambiguous with respect to the inducing environmental condition. High excitation pressure can be induced by sudden and strong increases in light intensity resulting in the absorption of too much photons (Karpinski et al., 1997), but it can be also induced by much weaker light intensities when the efficiency of the dark reaction is strongly restricted by, e.g., a shift to low temperatures or low water availability (Ensminger et al., 2006). All situations will result in a reduced PQ pool and the increased formation of ROS (as well as a number of other signals not clearly defined yet) exacerbating a clear interpretation and understanding of results from experiments under free running conditions. Experimental set-ups, therefore, are usually designed in a way that only one environmental parameter is changed in a distinct manner to understand the respective signaling and the induced acclimation responses. Very common are variations in light intensity at a constant temperature, but also variations of temperature at a constant light intensity are useful to study the effects of excitation pressure (Hüner et al., 1998). Alternatively the redox state of the electron transport chain can be manipulated by growing plants under artificial light sources with varying light quality (Chow et al., 1990; Melis, 1991; Allen, 1992). This set-up uses the fact that the absorption maxima of PSII and PSI differ by 20 nm (680 versus 700 nm). In natural habitats a strong enrichment of far-red wave lengths occurs within a canopy or dense plant population due to selective absorption of blue and red wavelengths in the top leaf layer (Terashima and Hikosaka, 1995; Smith, 2000; Dietzel et al., 2008a). This leads to an overexcitation of PSI relative to PSII and to an imbalance in excitation energy distribution between the PSs (**Figure 1**) which is counteracted by state transitions or PS stoichiometry adjustment. Under laboratory conditions this effect can be mimicked with the use of special light sources which excite preferentially PSII or PSI leading to a reduction or oxidation of the electron transport chain. An advantage of this system is that it works in a weak intensity range (well below 50 μE photons) which largely avoids the formation of ROS and other stress related symptoms allowing to clearly separate low- and high-light effects on the electron transport chain (Mullineaux and Emlyn-Jones, 2005; Piippo et al., 2006; Wagner et al., 2008). It was shown in *Arabidopsis* that separate acclimation strategies to low and high-light conditions exist and that the plant is able to respond in quite different and distinct ways to these environmental signals (Bailey et al., 2001).

Knowledge obtained in such relatively simple experimental systems has been used to understand the much more complex situations in under realistic ecophysiological conditions. To this end recent studies investigated interactions of two or more parameters or the effects of permanently and/or freely fluctuating parameters to study the effects on the redox state of the electron transport chain and the corresponding acclimation responses (Kulheim et al., 2002; Frenkel et al., 2007; Tikkanen et al., 2010). Initial results strongly indicate that such variability in the environment creates a complex situation at the molecular level in the chloroplast which is difficult to interpret and to understand. Systems biology approaches in this field may help (see also below) as these have the power to integrate gene expression and metabolomics data (Bräutigam et al., 2009; Frenkel et al., 2009). However, bioinformatics and modeling needs to be further developed to be useful for future biological applications.

Finally, an important point which must be mentioned is that different species may respond in different ways in one and the same set-up simply because of their differing abilities, e.g., in the dissipation of excess excitation energy *via* non-photochemical quenching (NPQ). This process involves the action of the PsbS protein, a special member of the family of the light-harvesting complex proteins, and the activity of the xanthophyll cycle (Li et al., 2000; Niyogi et al., 2005). These contribute to a conformational change in the light harvesting complex of PSII under high light and the subsequent dissipation of absorbed excess light energy as heat counteracting the generation of ROS. The efficiency of NPQ, however, can be variable depending on the ecological specialization of the species (Demmig-Adams and Adams, 2006). Generalization from one species to other species, thus, is often difficult. However, comparative testing of various species or ecotypes in defined set-ups (now often called phenotyping) opens up the possibility to understand different ecological strategies of plant species and families which might help to engineer more stress-resistant or tolerant crops (Zhu et al., 2010). Development of a basic model with variable input and output parameters which puts redox signals into a framework of environmental acclimation responses in plants thus is highly desirable (Dietz and Pfannschmidt, 2011).

SIGNAL TRANSDUCTION OF CHLOROPLAST REDOX SIGNALS TOWARD THE NUCLEUS

The mechanisms by which redox signals from photosynthesis are transduced to the nucleus are largely not understood. Nevertheless, a number of proteins have been identified which are involved in the mediation of such signals at least within the plastid compartment.

The kinases STN7 and CSK1 are involved in the redox signaling from the PQ pool toward the plastid gene expression machinery which controls the adjustment of PS stoichiometry in response to long-term light quality shifts (Bonardi et al., 2005; Puthiyaveetil et al., 2008). Since PSs are composed also of nuclear encoded components this redox signaling requires a branch toward the nuclear compartment. Mutant analyses strongly suggest that the two signaling branches diverge at or directly after the STN7 kinase (Pesaresi et al., 2009). Further steps in this signaling cascade are unknown, but some experimental evidence exists that mediation of PQ redox signals both to plastid and nuclear gene expression machineries involve phosphorylation-dependent mechanisms (Escoubas et al., 1995; Steiner et al., 2009; Shimizu et al., 2010). As a theoretical possibility it has been discussed that PQ molecules from the thylakoid membrane system may also enter the envelope membrane of chloroplasts and directly signal the redox state to components associated or attached with

it (Pfannschmidt et al., 2003). Experimental data for this are, however, lacking.

The transduction of redox signals from the thioredoxin system is mainly restricted to the understanding of SH-group-mediated control of enzymatic activities in the metabolism while it is not clear how this affects plastid gene expression. Existence of a thiol-dependent signal affecting chloroplast transcription beside the phosphorylation cascade mentioned above could be shown in *in organello* run-on transcription experiments (Steiner et al., 2009) and potential candidates for its transmission are the RNA polymerase subunits PAP6/FLN1 and PAP10/TrxZ (Arsova et al., 2010; Steiner et al., 2011) (**Figure 1**). The molecular connections, however, still require extensive further investigation.

Stress-induced hydrogen peroxide has been discussed to diffuse directly to the cytosol as it is the most stable ROS which can easily pass membranes (Pfannschmidt, 2003; Foyer and Noctor, 2005; Dietzel et al., 2008b). In the cytosol it can interact with a number of potential mediators including MAP kinase cascades or *rimb* (redox imbalanced) components (Kovtun et al., 2000; Heiber et al., 2007) which control down-stream redox regulators such as Rcd1 or Rap2.4a that affect nuclear gene expression (Shaikhali et al., 2008). ROS pattern have been also discussed as long-distance signals transmitted from cell to cell through the complete plant (Mittler et al., 2011). This involves signaling of high-light stress from exposed to unexposed leaves in *Arabidopsis* (Karpinski et al., 1999) as well as induction of cell death by singlet oxygen from PSII (Meskauskiene et al., 2001; Kim et al., 2012). The latter response can be genetically suppressed by inactivation of the chloroplast-located proteins Executer 1 (Ex1) and 2 (Ex2) indicating a role of them in transmission of singlet oxygen signals (Wagner et al., 2004). How this works mechanistically and which functions are exerted by Ex1 and Ex2 is unknown to date. Gun1 (*genomes uncoupled 1*) is a penta-tricopeptide repeat (PPR) protein with still unknown function which is postulated to merge plastidial signals from gene expression and ROS sending it to the nucleus (Cottage et al., 2007; Koussevitzky et al., 2007). This might involve the action of PTM, an envelope-localized PhD transcription factor which has been postulated to be released from plastids upon receiving a plastidial signal (possibly by Gun1) (Sun et al., 2011). After release from the outer membrane by a yet unknown protease it enters the nucleus and controls the expression of the ABI4 (*abscisic acid insensitive 4*) transcription factor. Finally, an indirect way of ROS signaling has been postulated to work *via* the ROS-sensitive glutathione biosynthesis pathway (so far only in *Arabidopsis*) since it appears that the synthesizing enzymes GSH1 and 2 are localized to the chloroplast and the cytosol, respectively. This would require the transport of γ-glutamyl-cysteine out of the chloroplast in order to synthesize the complete glutathione molecule in response to the accumulation of ROS (Mullineaux and Rausch, 2005; Wachter et al., 2005).

In conclusion, the effective transmission of plastidial redox signals both generated within the physiological range and under stress conditions is elusive although a number of interesting working hypotheses exist. How these models relate to each other, therefore, is a matter of future research.

REDOX-INDUCED RESPONSE PATTERNS IN NUCLEAR GENE EXPRESSION

While the transmission of redox signals remained still elusive our understanding of the regulated target genes or gene groups has clearly improved in recent years. Initial evidence for effects of photosynthetic redox signals especially from the PQ pool on nuclear gene expression were obtained in unicellular algae (Escoubas et al., 1995; Maxwell et al., 1995) and, subsequently, also in vascular plants (Petracek et al., 1997; Pfannschmidt et al., 2001; Pursiheimo et al., 2001; Yang et al., 2001). This included changes in the transcript level, translation efficiency, or promoter usage. Typically, these set-ups used light treatments with combinations of electron transport inhibitors to affect the expression of nuclear encoded genes in model organisms like pea (Sullivan and Gray, 1999), tobacco (Pfannschmidt et al., 2001), or *Arabidopsis* (Fey et al., 2005). The favorite target gene in many cases was the Lhcb1 gene [encoding one major protein of the light-harvesting complex of PSII (LHCII)], the "classical" reporter for plastidial signaling since it displays a nicely regulated response pattern under different light treatments. In addition, also other photosynthesis-related genes were tested such as *PetE* (encoding plastocyanin), *Fed1* (encoding ferredoxin 1), or *Nia2* (encoding the cytosolic nitrate reductase) as well as the stress related *Apx2* gene (encoding the ascorbate peroxidase 2) (Karpinski et al., 1997; Petracek et al., 1997; Oswald et al., 2001; Sherameti et al., 2002). Nevertheless, all these reports must be regarded as pilot studies since they were restricted to a very small number of genes. Thus, they allowed the identification of signal origin and some regulation principles but were not representative for the response of specific gene groups such as photosynthesis associated nuclear genes (PhANGs) which are believed to be the primary target of plastidial signals.

With the successful sequencing of the *Arabidopsis* genome array technologies became available which allowed a genome-wide monitoring of nuclear gene expression changes in response to a variety of photosynthetic redox signals. This was first done with *Arabidopsis* plants which were subjected to a PQ reduction signal induced by a light quality shift from PSI- to PSII-light (Fey et al., 2005). In a parallel experiment plants were treated with the same light shift but the redox signal was blocked by the simultaneous application of DCMU. Redox controlled genes were identified by a comparison of the expression patterns in this set-up using a macroarray targeted to genes for proteins with predicted chloroplast location (Kurth et al., 2002). By this means 286 nuclear genes were identified to be under redox control. An additional inhibitor control, however, uncovered some unspecific side effect of the drug indicating that only 54 genes behaved in an "ideal" response pattern as expected if being under PQ redox control (Fey et al., 2005). Nevertheless, the number of regulated genes was unexpectedly high and not restricted to the expected target genes, i.e., PhANGs, but involved genes from all important gene groups such as gene regulation, signal transduction, or various biosynthetic pathways. In a different study *Arabidopsis* plants were subjected to light intensity and light quality shifts and the response pattern was detected with an array with around 8000 randomly selected *Arabidopsis* genes (Piippo et al., 2006). The general response was similar but it was concluded

that the responsible redox signal was initiated in the stroma of chloroplasts not in the PQ pool.

In a more recent study an extensive kinetic approach was chosen to obtain a detailed picture of the dynamics of redox signaling and the corresponding response patterns. To this end *Arabidopsis* plants were subjected to either a reduction or an oxidation signal by using light-quality shifts and samples were taken directly before and 0.5, 2, 8, and 48 h after the shift (Bräutigam et al., 2009). The subsequent gene expression profiling revealed a number of important observations: (1) It demonstrated that the gene expression changes occurred in a very quick and dynamic manner indicating that a single observation time point (as done usually) reveals only a small part of the redox regulated genes. (2) It confirmed that metabolisms genes are a major part of the responsive gene groups and that PhANGs indeed display a unique response pattern among all regulated gene groups (**Figure 1**). (3) It indicated that redox signals from the PQ pool and stromal redox components are simultaneously active and a novel model for cooperative redox signaling was deduced (Bräutigam et al., 2010) which resolved the contradicting conclusion from the earlier studies mentioned above (Fey et al., 2005; Piippo et al., 2006).

A recent study used a genetic approach for studying redox responsive genes in *Arabidopsis* mutants with defects in the genes *stn7*, *psad1*, or *psae1* (encoding the thylakoid kinase STN7 and the PSI subunits PsaE1 and PsaD1) which are all devoid of state transitions and disturbed in redox signals from the PQ pool (Pesaresi et al., 2009). Comparison of transcript profiles of greenhouse grown plants led to the identification of 56 genes which were regulated in the same manner in all three genotypes representing either potential targets for PQ redox signals or putative representatives for a compensation response. Since in this study Affymetrix full-genome arrays were used also genes for non-chloroplast located proteins could be detected which were 39 out of the identified 56 genes, indicating that plastidial redox signals also affect genes for components in compartments other than plastids (**Figure 1**). This extends the potential influence of plastidial signals to the entire cell, demonstrating that this type of regulation is important not only for the plastid itself but appears to be a general part of the cellular signaling network (see also next chapter).

In an attempt to obtain a more detailed picture of the redox regulation network in *Arabidopsis* a systems biology study performed a meta-analysis of the kinetic and genetic approaches described above supplemented with further data from literature and data bases (Yao et al., 2011). Two transcription factors, ARR10 and ATH1-B, were proposed to be hubs in the redox gene regulatory network while the major photomorphogenesis regulator HY5 was considered to be not specifically affected in its connectivity by light-quality shifts and, thus, being not a specific component of the redox signaling network.

In summary, the recent array approaches revealed a much more complex redox regulation network in plant cells than originally anticipated. Apparently, photosynthetic redox signals do not only adjust photosynthesis genes but also genes coding for metabolic enzymes, signal transduction components, and gene regulation factors. This indicates a major role for redox signals in the cellular signaling networks of plants.

RELATION OF REDOX- AND PHOTORECEPTOR-MEDIATED LIGHT RESPONSES

Light is not only an energy source for plants but also provides important information which regulates major developmental responses such as photomorphogenesis of seedlings, shade avoidance responses, phototropism, circadian rhythms, or flower induction as well as more physiological responses such as chloroplast movement or stomatal opening (Jiao et al., 2007). These responses are regulated by a battery of photoreceptors which detect wavelengths and fluency rates of incident light such as the red/far-red light detecting phytochrome family (Smith, 1995), the blue light detecting cryptochromes and phototropins (Briggs and Christie, 2002; Lin and Shalitin, 2003), or the recently discovered UV-B light receptor (Rizzini et al., 2011). All these light-receptors control specific down-stream regulators which affect nuclear gene expression (Jiao et al., 2007). Since the photosynthetic light reaction can be driven with the same wavelengths it, thus, appears possible that there exists a potential cross-talk between the photoreceptor-mediated signaling networks and light-induced redox signals from photosynthesis. This assumption was initially supported by the observation that light and plastidial signals (induced by norflurazon treatment) act at the same *cis*-elements in the promoters of nuclear photosynthesis genes *Lhcb1* and *RbcS* (Kusnetsov et al., 1996; Strand et al., 2003; Acevedo-Hernandez et al., 2005). However, photoreceptor mutants revealed fully functional photosynthetic acclimation responses (Walters et al., 1999; Fey et al., 2005) indicating that photoreceptors are neither required nor essential for redox-controlled adjustment processes in chloroplasts. On the other hand it could be shown that a number of newly isolated cryptochrome 1 alleles behaved like weak *gun* alleles and it has been hypothesized that Gun1-mediated plastidial signals remodel light-signaling networks by interaction with the basic photomorphogenesis regulators Hy5 (Ruckle et al., 2007). This apparent contradiction can be resolved by identifying the class of plastidial signals being active in this context. Norflurazon treatment and *gun* mutants characterize a distinct class of plastid signals defined as "biogenic control" which represent signaling events essential for the proper build-up of the plastidial compartment (Pogson et al., 2008) and which are decisive especially in the very early phases of chloroplast generation (Pogson and Albrecht, 2011). In contrast, redox signals from photosynthesis become active only after photomorphogenesis was successfully performed, thus representing the most prominent class of signals defined as "operational control" (Pogson et al., 2008). Therefore, it can be hypothesized that photoreceptor-mediated signaling is dominant in morphological programmes which generate new tissues while photosynthetic redox signals become important only in existing tissues which must be functionally adjusted to the environment (**Figure 2**). New observations, however, suggest that this categorization is not as clear as assumed here. Studies in variegation mutants of *Arabidopsis* could demonstrate that the degree of variegation directly and positively correlates with the intensity of excitation pressure on the growing plant (Rosso et al., 2009). Very recent studies of chloroplast

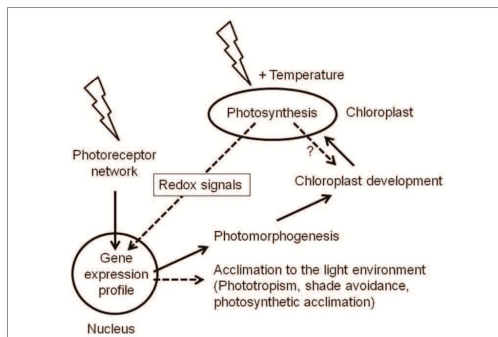

FIGURE 2 | Relation of photoreceptor- and photosynthesis-mediated light signaling. Light (indicated by flash arrows) is sensed by photoreceptors in germinating and growing seedlings. They initiate gene expression profiles in the nucleus which run the photomorphogenic programme. A central part of this programme is the build-up of chloroplasts and the photosynthetic apparatus. As soon as this is functional it serves as additional light and temperature sensing system which affects nuclear gene expression by redox signals. Potential redox signals affecting chloroplast biogenesis are indicated by a question mark. Photoreceptor - and photosynthesis-mediated signals (solid and hatched black arrows, respectively) are integrated in the nucleus and induce modifying programmes which acclimate plant growth and function to the residing environment. The interconnectivity of the respective gene expression profiles is largely unknown; however, it is assumed that photoreceptors are dominant regulators of plant development while photosynthetic redox signals preferentially control acclimation responses.

development in the shoot apex of *Arabidopsis* indicated that the fate of plastid development is determined in a very limited and small cell layer of the shoot apical meristem (Charuvi et al., 2012) providing a morphological indication that supports the likeliness of the excitation pressure model. Furthermore, other results suggest a link between plant resistance responses and plastids which might be light-mediated (Ballare et al., 2012). It appears that plastidial signals can modify plant defence responses although the molecular links are not understood yet (Karpinski et al., 2003; Kangasjarvi et al., 2012). The strong vertical light quality gradients within dense plant populations affect both the phytochrome system *via* the red to far-red ratio as well as the excitation energy distribution between the PSs. A parallel action of both signaling networks thus would be conceivable. Array data obtained in set-ups investigating light-quality regulated genes, however, uncovered only very small overlap between phytochrome- and redox-mediated transcript profiles (Bräutigam et al., 2009). This argues for a parallel rather than an interacting influence on nuclear gene expression; however, to provide final proof for this conclusion array experiments which directly address this specific question needs to be designed and performed.

In conclusion, we need more knowledge to understand how the two fundamental light-dependent signaling networks, controlled by photoreceptors and photosynthetic redox signals, are integrated to regulate nuclear gene expression. As another complication one also needs to consider the action of mitochondria, which are tightly connected to chloroplast function and redox state (Raghavendra and Padmasree, 2003). Future work needs to integrate communication pathways and metabolic interaction of the three different genetic compartments of plant cells in order to obtain a comprehensive view how they respond to environmental constraints in a coordinated manner (Pfannschmidt, 2010).

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10 Manuscript VII

Schröter Y., Steiner S., Weisheit W., Mittag M. and Pfannschmidt T.

A purification strategy for analysis of the nucleotide binding sub-proteome from chloroplasts of mustard cotyledons.

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Title: A purification strategy for analysis of the DNA/RNA-associated sub-proteome from chloroplasts of mustard cotyledons

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ABSTRACT

Plant cotyledons are a tissue that is particularly active in plastid gene expression in order to develop functional chloroplasts from pro-plastids, the plastid precursor stage in plant embryos. Cotyledons, therefore, represent a material being ideal for the study of composition, function and regulation of protein complexes involved in plastid gene expression. Here, we present a pilot study that uses heparin-Sepharose and phospho-cellulose chromatography in combination with isoelectric focussing and denaturing SDS gel electrophoresis (two-dimensional gel electrophoresis) for investigating the nucleotide binding proteome of mustard chloroplasts purified from cotyledons. We describe the technical requirements for a highly resolved biochemical purification of several hundreds of protein spots obtained from such samples. Subsequent mass spectrometry of peptides isolated out of cut spots that had been treated with trypsin identified 58 different proteins within 180 distinct spots. Our analyses indicate a high enrichment of proteins involved in transcription and translation and, in addition, the presence of massive post-translational modification of this plastid protein sub-fraction. The study provides an extended catalogue of plastid proteins from mustard being involved in gene expression and its regulation and describes a suitable purification strategy for further analysis of low abundant gene expression related proteins.

Keywords

Sinapis alba, cotyledon, chloroplast, nucleic acids binding protein, post-translational modification, mass spectrometry

INTRODUCTION

Plant chloroplasts are semiautonomous cell organelles of endosymbiotic origin that emerged from a cyanobacteria-like ancestor (Lopez-Juez and Pyke, 2005). One evolutionary remnant of this origin is their own genome (called plastome) comprising 100-120 genes and a predominantly bacteria-like gene-expression machinery being essential for its proper expression. The plastome gene set in vascular plants is highly conserved and encodes mainly proteins with a function in photosynthesis and the gene expression machinery (Sugiura, 1992). However, for full functionality plastids require the import of many proteins that are encoded by the nuclear compartment since during evolution the endosymbiotic ancestor lost most of its genes to the nucleus of the host cell *via* horizontal gene transfer (Martin et al., 2002;Stoebe and Maier, 2002). These nuclear-encoded plastid proteins are translated in the cytoplasm as precursor molecules that are subsequently imported into plastids with the help of N-terminal transit peptides directing them to their correct sub-compartment (Soll and Schleiff, 2004). After removal of the transit peptide the mature proteins are then assembled into their final configuration together with the plastid-expressed proteins and, therefore, all major multi-subunit complexes (such as photosystems, ribosomes or metabolic enzyme complexes) represent a patchwork of nuclear as well as plastid expressed proteins (Allen et al., 2011).

Based on the prediction of transit peptides and genome-scale proteomics it was estimated that plastids may contain around 1500 - 4000 different proteins (Abdallah et al., 2000;Baerenfaller et al., 2008;Ferro et al., 2010;Van Wijk and Baginsky, 2011). Reference proteomes generated for maize and *Arabidopsis* cover 1564 and 1559 proteins, respectively, so far (Huang et al., 2013) indicating that a large part of the predicted plastid proteome has yet not been detected. This might be caused by the fact that plastids from different tissues (for instance roots, cotyledons, leaves, flowers and fruits) likely contain different protein compositions, but also from the fact that especially regulatory proteins are present in only

trace amounts that are difficult to detect in a matrix of highly abundant proteins, e.g. from photosynthetic apparatus (Huang et al., 2013). Further complexity in the plastid protein complement may derive from the occurrence of multiple post-translational modifications that are essential for regulatory events.

Cotyledons display a high activity in plastid transcription and translation being essential for the light-induced development of chloroplasts out of the embryonic pro-plastids (Baumgartner et al., 1989;Baumgartner et al., 1993). Thus, the proteome of cotyledon plastids comprises a high amount of proteins implicated in gene expression providing a useful source material for the characterisation of the nucleic acids binding proteome. The chloroplast proteome of the dicotyledonous model organism *Arabidopsis thaliana* is well studied in adult leaves, however, an analysis of that of cotyledons is lacking mainly because the small size of the cotyledons is not very suitable for the isolation of chloroplasts and subsequent analyses of their proteins *via* chromatography. In recent investigations, the fast growing cruciferous plant mustard (*Sinapis alba*) demonstrated a high suitability for performing biochemical and physiological analyses of plastid gene expression in cotyledons since the seedlings and their cotyledons are much larger than that of *Arabidopsis* (Oelmuller et al., 1986;Tiller and Link, 1993;Pfannschmidt and Link, 1994;Link, 1996;Baginsky et al., 1997). Isolation of cotyledons in the order of kilograms is easily achieved after just five days of growth and provides enough material even for the biochemical analysis of low-abundant proteins by chromatography followed by mass spectrometry. Since *Sinapis* is a close relative of *Arabidopsis*, peptide data evaluation for the identification of mustard plastid proteins was found to be applicable for well conserved proteins by using the *A. thaliana* or *Brassicales* protein databases (Schröter et al., 2010;Steiner et al., 2011). Thus, the use of mustard as a source for cotyledons combines the advantages of mustard chloroplast preparation with the availability of protein data of well studied organisms like *Arabidopsis thaliana* or some *Brassica* species.

In recent studies, proteins implicated in plastid gene expression in mustard have been isolated by a number of different purification schemes. These include the isolation of the membrane bound insoluble transcriptionally active chromosome (TAC) by ultracentrifugation and gel filtration (Hallick et al., 1976;Bülow et al., 1987;Pfalz et al., 2006) and the isolation of soluble proteins such as RNA polymerases, kinases, RNA binding proteins and sigma factors by various chromatographic steps (Tiller et al., 1991;Nickelsen and Link, 1993;Tiller and Link, 1993;Pfannschmidt and Link, 1994;Liere and Link, 1995;Baginsky et al., 1999). Recently, we applied the purification scheme of plastid isolation followed by protein enrichment *via* heparin-Sepharose (HS) chromatography and visualisation by two-dimensional (2D) blue native (BN)-PAGE to isolate protein complexes such as the RNA polymerase complex as well as a number of gene expression related proteins (Schröter et al. 2010, Steiner et al. 2011). However, these HS purified fractions still included a number of metabolic enzymes which exacerbate the analysis of the nucleic acids binding sub-proteome as they tend to cover low abundant proteins or even hinder their visualisation and identification. Here, we present a pilot characterisation of the nucleic acids binding sub-proteome of chloroplasts from mustard cotyledons. To this end we used HS chromatography followed by a second chromatographic step with phosphocellulose (PC) which was shown to be very effective for isolating nucleic acids binding enzymes like RNA polymerases (Bottomley et al. 1971; Tiller and Link 1993). This was followed by isoelectric focussing (IF) and 2D gel electrophoresis that allowed us to estimate the size of the nucleic acids binding sub-proteome and the ideal IF range for its visualisation and protein determination using mass spectrometry. The use of 2D gel electrophoresis also revealed massive post-translational modifications of the sub-proteome.

RESULTS

Enrichment of nucleic acids binding proteins from mustard chloroplasts

In previous studies we analysed gene expression related protein complexes from isolated mustard chloroplasts using a combination of heparin-Sepharose chromatography followed by a two dimensional blue native (BN)/SDS polyacrylamid gel-electrophoresis (2D BN-PAGE) and electro-spray ionisation-tandem mass spectrometry (ESI-MS/MS). Besides the plastid-encoded RNA polymerase, various CSP41 complexes and translation related proteins, we identified several metabolic enzyme complexes such as GAP-dehydrogenase, ATPases or RubisCO that co-purify in this affinity chromatography. These abundant proteins exacerbated the identification of further low-abundant proteins (Schröter et al., 2010;Steiner et al., 2011). In addition, these studies were focussed on the analysis of large native protein complexes using a BN-PAGE approach. This limited the characterisation of gene expression related proteins that may occur in small complexes or as individual proteins. In this study, we aimed a deeper investigation of the size, composition and complexity of the nucleic acid binding sub-proteome of mustard chloroplasts. To this end, we performed chloroplast isolation and HS chromatography from mustard cotyledons precisely as described before (Schröter et al., 2010). Bound proteins were eluted with a high-salt step, concentrated by dialysis and, for further enrichment of gene expression related proteins, applied to a cation exchange column with PC as matrix as described earlier. Proteins were eluted by a second high-salt step and dialysed against a low-salt storage buffer for analysis and further use (see Materials and Methods). A first comparison of peak fractions with equal protein amounts of both purification steps was done by SDS-PAGE and silver staining (Fig. 1A). The PC fraction exhibited a selective enrichment of many protein bands between 5 and 75 kDa and a strong exclusion of proteins larger than 75-80 kDa. For a more detailed resolution of this protein fraction, we performed 2D gel electrophoresis with an isoelectric focussing (IF) as first dimension followed by a SDS-PAGE (Fig. 1B, C) as second dimension. Using IPG stripes with a non-linear (NL) pH range from 3-11 for the IF and a gradient polyacrylamide gel, we could obtain an overview of the total protein content leading to the identification of around

600 individual spots. We observed two major areas where multiple proteins accumulated on the gel which were located between approx. pH 4.5-7 and pH 9-11. Because of the non-linearity of the IF gradient, proteins at the outer ranges of the IF stripe were poorly resolved which became mainly evident at the basic pH values. Therefore, linear IPG gels were used in addition, overlapping with the first one between pH 3-10 and pH 6-11. The latter gradient resolved the problem with spot accumulation especially observed at the cathode. The higher resolution led to the identification of further proteins leading to a total count of 1079 individual protein spots within the PC fraction which could be distinguished between the different gels. We regard this as the nucleic acids binding sub-proteome of mustard plastids. Our data indicate a significant higher complexity of this specific sub-proteome as it was estimated earlier from the HS fractions (Schröter et al., 2010).

Identification of proteins from the PC fraction by LC-ESI-MS/MS

All 1079 spots were cut out and proteins were subjected to an in-gel tryptic digest. In 153 cases, selected spots were pooled from duplicate gels in order to increase the protein amount for the subsequent measurements. Since a database from *S. alba* is currently not available, protein identification was performed by comparing the determined mass spectrometry data to the *Brassicales* and *Arabidopsis thaliana* databases (compare Materials and Methods). By this means 225 proteins were reliably identified with at least two different peptides in 180 spots indicating that several spots contained more than one protein. In addition, 36 particular proteins were identified in more than one spot (up to 40 different ones) suggesting posttranslational modification of these proteins (Table 1). In total, 58 different proteins were identified. In further analyses, the identified gene models were checked for presence of a plastid transit peptide using TargetP (Emanuelsson et al., 2000). Plastid-directing transit peptides could be predicted for 36 of these proteins, ten of them exhibit an additional luminal transit peptide and four plastid-encoded proteins were identified. Considering a detection

probability of 73% for a transit peptide, we estimated the percentage of true plastid proteins within the PC fraction to be around 94%. Some of the identified proteins were found before in mustard (Pfannschmidt et al., 2000;Pfalz et al., 2006;Schröter et al., 2010), but 36 were identified here for the first time (Table 1).

Based on functional similarities and structural homologies, a categorization of proteins into protein families or subgroups was conducted (Fig. 3). A practical classification mode is given by the modified MapMan bin system (Thimm et al., 2004) of the Plant Proteomics Data Base (PPDB) (Sun et al., 2009). In Table 1, proteins were listed following the PPDB bin grouping as given in column 2. For further comparison, we summarised identified proteins into five major groups. The first group comprises transcription and transcript related proteins, namely subunits of the plastid encoded RNA polymerase (PEPs) and PEP associated proteins (PAPs) as defined in Steiner et al. 2011, other pTACs (pTAC proteins not belonging to the PAPs) and RNA and DNA related proteins (bin 27 and 28, not belonging to PAPs and pTACs). A second large group comprises translation related proteins (bin 29.2 and 29.5). Three further groups cover proteins involved in protein homeostasis (bin 29 and 21 not belonging to PEPs and PAPs), photosynthesis (bin 1) and a miscellaneous group called "others" including various enzymes catalysing metabolic reactions or protein modifications.

PEPs, PAPs and other pTACs

We detected most subunits of the soluble PEP complex including PAP3, PAP4, PAP5, PAP6, PAP8, PAP10, PAP11, PAP12 as well as the PEP core subunit RpoA (Pfalz and Pfannschmidt, 2013). Other PEP core subunits (RpoB, RpoC1, RpoB) and PAP1, PAP2, PAP7 and PAP9 were not identifiable in spots of these gels. Most of the identified proteins of this group became visible as single isolated spots in the acidic range (pH3-6) on the gel (Fig. 4) and at their expected molecular weight. An exception was PAP6 representing the protein fructokinase-like 1 (FLN1) that contains a protein domain of the pfkB-carbohydrate kinase

family (Arsova et al., 2010;Steiner et al., 2011). This protein appeared in a chain of five spots of the same apparent molecular weight but with slightly varying isoelectric points from which the two strongest spots were identified as PAP6 here. This observation suggests post-translational modification of this kinase. In addition, for PAP6 but also for PAP3 and PAP11 one or two spots of lower molecular weight, respectively, were detected suggesting a targeted degradation or proteolytic modification of these two proteins (Fig. 4). For PAP4 and PAP12 only a degradation product was detectable, while a spot of the full length protein was not identified.

Besides PEP and PAP proteins, we identified two proteins described as component of the transcriptionally active chromosome in mustard, PTAC4 and PTAC18 (Pfalz et al., 2006). PTAC4 is the vesicle-inducing protein in plastids 1 (VIPP1) which plays a crucial role in membrane stability (Zhang et al., 2012). The PTAC18 protein belongs to the cupin superfamily that merges proteins with a conserved β-barrel fold, giving this type of protein a strong thermal stability. It represents a family of very diverse members including enzymes and seed storage proteins, but also transcription factors (Dunwell et al., 2001). However, the exact function of pTAC18 is largely unknown. PTAC18 was identified in spot 255 being smaller and more in the acidic range as expected from the predicted protein representing likely a fragment. PTAC4 was identified in spots 243, 278, 280 and 295. 278 and 280 are on the same size but with slightly different IPs suggesting post-translational modification of the protein.

An exceptional constituent of the PC protein fraction represents the protein CSP41 that appears in two forms, CSP41a and CSP41b. Originally described as the chloroplast stem-loop binding protein of 41 kDa (Yang et al., 1996) it has been discussed to be involved in RNA processing and stabilisation as well as in RNA protection (Qi et al., 2012). As described for the HS fractions it represents a dominant protein of the nucleic acids binding proteome of plastids being present in multiple multimeric complexes of highly variable sizes (Schröter et

al., 2010;Qi et al., 2012). In the PC fractions, the two forms of CSP41 appear to be especially enriched as they can be detected in 10 spots of the same apparent molecular weight of around 34 kDa but with different IPs (three for CSP41a and seven for CSP41b). The main accumulation is visible in the middle of the gels between pH 5.5 and 7. The CSP41a spots are by far the strongest spots observed in the whole gel followed by the spots for CSP41b. Roughly estimated they account for 30% – 40% of the total protein content in this fraction making a precise estimate difficult. In addition, the proteins are detectable in 34 less stained and smaller spots of different sizes suggesting massive post-translational modifications as well as multiple degradation or targeted proteolytic events acting on both protein forms. These smaller protein spots of CSP41a/b appear to contain not only random fragments of the proteins but could be observed as reproducible spot pattern in all replicates of nucleic acids binding sub-proteome preparations from mustard.

Translation associated proteins

Numerous proteins identified in this work are directly or indirectly related to translation. In total 12 ribosomal proteins of the large 50S subunit of plastid ribosomes (PRPL) were identified, namely PRPL1, -4, -5, -6, -10, -12, -14, -15, -18, -21,-24, -29. The solely identified protein of the small 30S ribosomal subunit (PRPS) is PRPS5. We also identified two ribosomal subunits that belong to the large subunit of the cytosolic 80S ribosomes (CRPL), CRPL11 and -22-2. *S. alba* proteins of PRPL12-1 and PRPL29 were formerly identified by Pfalz et al. (2006) and PRPL6 by Schröter et al. (2010). The remaining ribosomal proteins listed in Table 1 are identified in mustard plastid protein samples here for the first time.

Beside the ribosomal subunits a number of translation initiation factors were present in the fractions and were detected here for the first time in *S. alba*. Except of eIF1A (a subunit of the cytosolic translation initiation complex) all of them contain a predicted plastid transit peptide. This accounts also to eIF3 which is known as a subunit of a eukaryotic IF. IF2 and

IF3 represent plastid translation initiation factors while elongation factors (EF) EF-Tu and the eukaryotic EF1alpha4 are involved in translation elongation. eIF1A, EF-Tu and EF1-alpha4 appear as single spots while the others were found in several spots suggesting post-translational modifications here, too.

Furthermore, we identified a SpoU methylase that belongs to the class of SPOUT enzymes and introduces a methylation of 2'-OH groups of tRNA or rRNA riboses (Cavaillé et al., 1999;Tkaczuk et al., 2007), and two proteins that are subunits of the nascent polypeptide associated complex (NAC). This dimeric complex is composed of an alpha- and beta-chain and may reversibly bind to ribosomes (Wiedmann et al., 1994). The alpha-NAC-like proteins identified during this work are encoded by different genes in *Arabidopsis* but exhibit a strong similarity within their amino acid sequence. The α-NAC like protein 1 and 3 were determined in the same two spots on the gels representing double spots.

Proteins involved in protein homeostasis, photosynthesis and metabolism

We identified the chloroplast heat shock cognate protein 70-2 (cpHsc70-2) which is the analogue of one of only two stromal Hsp70s in *A. thaliana* plastids (Su and Li, 2008). In addition, we found a TCP-1/cpn60 family chaperonin and a protein disulfide isomerase like 2-1 (PDIL 2-1) belonging to the thioredoxin superfamily and acting as folding catalyst. All proteins are identified in mustard fractions here for the first time and likely function in protein stability or formation. The correct folding of proteins is the last but essential step of gene expression.

The group of photosynthesis related proteins contains four proteins. The alpha and beta subunits of the plastid ATP synthase were formerly identified in *S. alba* (Schröter et al., 2010). Another ATPase, the RubisCO activase and the Rieske cluster of the cytochrome b6/f complex were detected here first by mass spectrometry in the mustard plastid proteome. These proteins are most likely not involved in gene expression but co-purify in the column

chromatography because of their substrate affinities. This is also true for the group of the miscellaneous proteins including the malate dehydrogenases (MDH) and the malate synthase (MLS), both identified in several spots.

Proteins involved in fatty acid metabolism were identified as well. These include acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (CAC3) and FabZ, a beta-hydroxyacyl-acyl carrierprotein (ACP) dehydratase. An earlier study on the purificationof the acetyl-CoA carboxylase multienzyme complex also resulted in the enrichment of nucleoid-associated proteins (Phinney and Thelen, 2005) suggesting a potential physical link between these two larger protein associations.

A third protein found (MFP2) is involved in lipid degradation. It was already identified in the HS-fractions in former experiments (Schröter et al. 2010). We found also a cystein synthase and phosphoserine aminotransferase (PSAT) as well as a pyrroline-5-carboxylate reductase (P5CR) known to be essential for amino acid metabolism and a serine hydroxymethyltransferase (SHMT) being essential for photorespiration. The mustard protein in the PC fractions matches to mitochondrial SHMT1 and 2 peptides of several *Brassicales.* The exact affiliation to one of these SHMTs is unclear since the matching peptides fit to both proteins (Table 1). The PC fractions contain also the myrosinase MB3 (involved in glucosinolate degradation) and a cruciferin fitting best to *A. thaliana* CRU3 (Table 1). Finally, also actin was detected in one spot, although mustard peptides of PC fractions match to different actin types of different *Brassicales*.

DISCUSSION

The plastid nucleic acids binding proteome of mustard

Goal of our study was the establishment of a purification scheme allowing the estimation of size and composition of the plastid nucleic acids binding sub-proteome from mustard. By using heparin-Sepharose and phosphocellulose chromatography coupled to isoelectric

focussing and SDS-PAGE we could reproducibly isolate 1079 protein spots from which we could identify 180 protein spots by mass spectrometry. However, to our surprise these 180 protein spots were found to represent just 58 individual proteins indicating a high degree of post-translational modification of this specific sub-proteome which in part might be caused by differential phosphorylation (Reiland et al., 2009;Reiland et al., 2011). Since we used NaF as phosphatase inhibitor in all preparation steps, the differential phosphorylation states of the analysed proteins should be well conserved. In contrast, different redox states of thiol groups were not maintained during our purification procedure since reducing agents were included in all steps. Detection of a differential redox state in these fractions will require more specific methods such as redox difference gel electrophoresis (redox-DIGE) (Hurd et al., 2007;Hurd et al., 2009). We also observed numerous smaller fragments from several proteins indicating degradation events. These, however, were not random as the spot pattern was reproducible between different preparations suggesting that it is not caused by action of proteases during purification, but by targeted events in the chloroplast. Whether these products represent intermediate steps of protein degradation or whether these fragments perform distinct functions remains to be determined. In summary, this high degree of post-translational modification indicates that the size of the sub-proteome is certainly smaller than the 1079 spots detected. If we assume a similar percentage of individual proteins as within the identified spots (32.2%) for the complete fraction then we estimate 347 proteins for the total nucleic acids binding sub-proteome. Since we identified a number of co-purifying proteins involved in metabolic processes (29.3%), we had to reduce this number to 236 proteins. However, our mass spectrometry determination has a certain bias since we could detect only the fraction of sufficiently abundant proteins which likely is enriched in metabolic enzymes. In addition, a significant part of post-translational modification detected in our fractions is focussed on only two proteins, CSP41a and b which partly compromise our estimate. Without these two proteins, we estimate 314 proteins for the chloroplast nucleic acids binding sub-

proteome. This appears a reasonable number taking into account the proteins that are already known to be involved in the regulation of plastid gene expression such as NEP, PEP, PAPs, pTACs, PPRs, ribosomal proteins and so on. It, however, leaves still some space for the discovery of as yet unidentified regulators that might appear only in trace amounts such as eukaryotic transcription factors (Wagner and Pfannschmidt, 2006).

Specific features of the protein fraction after PC chromatography

PC chromatography is a well established purification step for nucleic acids binding proteins from chloroplasts (Bottomley et al., 1970;Tiller and Link, 1993). Crucial for the quality of these fractions, however, are a thorough chloroplast preparation *via* sucrose gradient centrifugation and a pre-purification step of the chloroplast lysate using HS chromatography. In comparison to results from earlier work using just HS fractions (Schröter et al. 2010) we observed a high enrichment of translation associated proteins and especially of CSP41 proteins. Co-purification of metabolic enzymes as well as components from other cell compartments was clearly reduced. We obtained a good coverage of the subunits for the plastid RNA polymerase complex PEP; however, surprisingly the larger subunits of this complex were not detectable. We observed a significant reduction of proteins above 80 kDa in size within the PC fractions (Fig. 1), however, this might be not the reason for the failure of detection since all other components of the complex were identified in the fractions and especially RpoC2 and RpoB are known to bind DNA/RNA. Since these large subunits are highly conserved and have been successfully detected earlier in HS fractions (Steiner et al., 2011) it is likely that they are not well separated on the IEF. Further analyses using additional enrichment methodologies before the IEF step such as size-exclusion chromatography might help to target this problem in the future.

The largest amount of all identified proteins in the PC fractions is dedicated to translational processes with 43% of all proteins (Fig. 3 C). The 50S subunit of plastid ribosomes contains 33 subunits with 31 orthologues to *Escherichia coli* and the two plastid specific subunits PRPL5 and PRPL6 (Yamaguchi and Subramanian, 2000). The 30S subunit is composed of 21 *E. coli* orthologues and 4 plastid specific proteins with no homologues in other ribosomes (Yamaguchi et al., 2000). Most ribosomal proteins have contact to RNA in various ways, either they are structural components or directly involved in the translational process. Thus, ribosomal proteins contain nucleic acids binding structures which adhere to the used column materials and represent one main component of the nucleic acids binding sub-proteome of plastids. On the 2D-gels most of them accumulate at the higher pH-ranges and the use of the basic IPG-gels of pH 6-11 led to a good resolution of this group of proteins. The identification of 80S ribosomal proteins in plastid fractions is likely caused by the co-purification of particles attached to the outer chloroplast membrane, like known for tonoplast membrane fragments (Schröter et al. 2010). The main regulation of translation occurs at the level of initiation which is performed by initiation factors (IF). In eukaryotes this process is assured *via* 12 eukaryotic IFs (eIF) comprised by 23 polypeptides, whereas in prokaryotes three IFs are sufficient (Kapp and Lorsch, 2004). In plastids orthologues for all bacteria-type translation factors can be found but the translational complex contains additional proteins not present in bacteria (Beligni et al., 2004). Three of the four IFs identified in this study contain a cTP although only IF2 and IF3 are plastid IFs with a prokaryotic origin. The third one, eIF3f, is a subunit of the eIF3 and is important for the basic cell growth and development and influences the expression of about 3000 genes in *A. thaliana* also in interaction with two other eIF3 subunits (Xia et al., 2010). ChloroP predicts a plastid transit peptide of 40 amino acids for eIF3f of *A. thaliana* and it was previously also identified in fractions enriched in plastid nucleoids (Huang et al., 2013). Thus, it seems to be a true plastid protein and not a co-purification of the cytosolic translational apparatus. However, it might be also possible that

this protein possesses a dual localization both in nucleus and plastidscontributingto the coordination of gene expression between the two genetic compartments as proposed for other plant cell proteins (Krause and Krupinska, 2009). The elucidation of the preciserole of eIF3f in plastids and whether it is involved in the regulation of plastid gene expression will be an interesting field of future research.

The dominant proteins in the PC fractions are the two proteins named CSP41a and CSP41b (Yang et al., 1996;Yang and Stern, 1997). CSP41a and b were also detected in isolates of the PEP-complex as one of the most abundant component (Pfannschmidt et al. 2000; Suzuki et al. 2004; Schröter et al. 2010) but they appear not to belong to the PAPs but co-purify with these fractions because of the enormous size of their largest conglomerates (Peltier et al., 2006;Schröter et al., 2010;Qi et al., 2012). Here, we identified CSP41a in 8 and CSP41b in 40 spots of diverse sizes and isoelectric points. Thereby, both form a defined spot pattern which was congruent in most replicates of the 2D-gels prepared for this work. This suggests that not only a multimerisation of CSP41a/b occurs but maybe also an integration of defined fragment species of the proteins that might be important for specific functions. In addition to targeted fragmentation, the spot pattern after 2D SDS-PAGE suggests also a strong post-translational modification of the two proteins. Indeed, phosphorylation and lysine acetylation have been reported for the corresponding *Arabidopsis* proteins (Reiland et al., 2009;Finkemeier et al., 2011;Reiland et al., 2011). The spot pattern as well as the positions of the two proteins in the 2d-gels is highly reminiscent to those recently reported for *Arabidopsis* (Qi et al., 2012). The only difference occurs in the number of identified spots which were 6 Csp41a and 5 Csp41b in *Arabidopsis* while in mustard we observed 3 Csp41a and 7 Csp41b variants (besides the fragmented versions) (Fig. 5). This suggests the action of at least some species-specific modifications of the proteins.

Conclusion

Here, we describe the technical requirements for a highly resolved biochemical purification of several hundreds of protein spots representing the nucleic acids binding sub-proteome of plastids. Our analyses indicate a high enrichment of proteins involved in transcription and translation and, in addition, the presence of massive post-translational modification of this plastid protein sub-fraction. Furthermore, our study provides an extended catalogue of plastid proteins from mustard being involved in gene expression and its regulation and describes a suitable purification strategy for further analysis of low abundant gene expression related proteins.

MATERIALS AND METHODS

Plant growth and Isolation of plastids

Mustard seedlings (*Sinapis alba* L., var. Albatros) were cultivated under permanent white light illumination at 20°C and 60% humidity. Cotyledons were harvested under the respective light and stored on ice before homogenisation in ice-cold isolation buffer in a Waring Blender and filtering through muslin and nylon. Chloroplast isolation by differential centrifugation and sucrose gradient centrifugation in a gradient between 30 to 55% sucrose was conducted as described earlier (Schröter et al. 2010)

Isolation of nucleic acids binding proteins by HS- and PC-chromatography

Lysis of plastids and the chromatography at HS CL-6B was performed according to Tiller and Link 1993; Steiner et al. 2009). Proteins were washed, eluted with 1.2 M $(NH_4)_2SO_4$ and the peak fractions detected via protein quantification assays (RC DC™, Bio-Rad Laboratories, Inc., Hercules, CA, USA) (Schröter et al. 2010). For PC chromatography pooled HS peak fractions were diluted to 10 % (v/v) glycerol with dilution buffer (50 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 0.1 % (v/v) TritonX-100, 10 mM sodium fluoride, 62.5 mM $(NH_4)_2SO_4$, 6

mM 2-mercaptoethanol). Activation and equilibration of PC (cellulose phosphate ion-exchanger P11, Whatman™ GE healthcare UK Limited, Little Chalfont, UK) to pH 7.6 occurred following the distributor´s instructions. Diluted HS proteins were applied to disposable PD-10 columns (Amersham™ GE healthcare UK Limited, Little Chalfont, UK) filled with activated PC, closed carefully and rotated gently for 60 min at 4°C. After fixing the column on a stand and washing with washing buffer (50 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 0.1 % (v/v) TritonX-100, 10 mM sodium fluoride, 50 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 10 % (v/v) glycerol) proteins were eluted in 3 ml fractions with elution buffer (50 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 0.1 % (v/v) TritonX-100, 10 mM sodium fluoride, 1.2 M $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 10 % (v/v) glycerol) and dialyzed against storage buffer (50 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 0.1 % (v/v) TritonX-100, 10 mM sodium fluoride, 50 mM $(NH_4)_2SO_4$, 5 mM 2-mercaptoethanol, 50 % (v/v) glycerol). Peak fractions were determined by a protein quantification assay (RC DC™, Bio-Rad Laboratories, Inc., Hercules, CA, USA), pooled and stored at -20°C

2D gel electrophoresis

For 2D gel electrophoresis an acetone precipitation of dialyzed proteins from PC chromatography was used to remove the storage buffer following manuals instruction (2-D Electrophoresis principles and methods, 2004, GE healthcare UK Limited, Little Chalfont, UK). For first dimension 18 cm IPG-stripes pH 3-11NL, pH 6-11 and pH3-10 were used (GE Healthcare UK Limited, Little Chalfont, UK). IPG-stripes were rehydrated in rehydration buffer (8 M urea, 0.5 % (w/v) chaps, 0.2 % (w/v) DTT, 0.5 % (v/v) IPG-Buffer, 0.002% (w/v) bromophenol blue) for about 14- 16 hours. An amount of 400 µg precipitated and dried protein per stripe was resolved in rehydration buffer and applicated on the IPG-stripes as cup-loading procedure following manufacturer´s instructions (2-D Electrophoresis principles and methods, 2004, GE Healthcare UK Limited, Little Chalfont, UK). For focussing of proteins

the following protocol was used on IPGphor (Amersham™ GE Healthcare UK Limited, Buckinghamshire, UK) 6h step and hold 150V, 3h step and hold 300V, 6h gradient 1200V, 3h gradient 8000V, 3h step and hold 8000V. After isoelectric focussing IPG-strips were equilibrated twice in equilibration solution (50 mM Tris-HCl, ph 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, 0.002% (w/v) bromophenol blue) first with addition of 2 % (w/v) DTT for 15 min under gentle agitation and after removing the first solution second with addition of 2.5 % (w/v) iodacetamide (IAA, for alkylation of thiol groups) again for 15 min and gently agitated as described (2-D Electrophoresis principles and methods, 2004, GE Healthcare UK Limited, Little Chalfont, UK). As second dimension a SDS PAGE in gradient gels of 7.5-20% acrylamide with Rhinohide™ gel strengthener (Molecular Probes, Inc., Eugene, OR, USA) was used following manual instructions. Afterwards gels were stained with silver according to manufacturer´s instruction (Amersham™ GE Healthcare UK Limited, Buckinghamshire, UK).

Tryptic digest, LC/ESI-MS/MS and data analysis

The spot pattern of the different gels was compared. Matching low abundant spots were pooled (as indicated in Supp. Table 1) to increase the detectable protein amount. Tryptic digest of protein spots was conducted after destaining as referred (Mørtz et al., 1994;Stauber et al., 2003). Mass spectrometry was carried out at LCQ™-DecaXP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) using a data-dependent scan procedure with four cyclic scan events as described in Schröter et al. 2010. The first cycle, a full MS scan of the mass range m/z 450–1200, was followed by three dependent MS/MS scans of the three most abundant ions. Sample run and data acquisition was performed using the Xcalibur™ software (Version1.3 © Thermo Finnigan 1998–2001). 76 of the low abundant spots were measured at a Finnigan LTQ linear ion trap mass spectrometer (Thermo Finnigan, Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled online after a nano HPLC

Ultimate 3000 (Dionex, Thermo Fisher Scientific Inc., Waltham, MA, USA) (Schmidt et al., 2006). After one full MS the instrument was set to measure the collision induced dissociation pattern of the four most abundant ions and exclude the measured once for 10 sec from newly measuring.

The resulting spectra were analyzed using the Proteome Discoverer vs. 1.0 (Thermo Fisher Scientific Inc., Waltham, MA, USA) with the implemented Sequest algorithm (Link et al., 1999). Therefore, a database of all RefSeq (reference sequence) sequences of *Arabidopsis thaliana* and *Arabidopsis lyrata* as well as the complete *Brassica napus* and *Capsella rubella* and the remaining *Brassicales* proteins of NCBI was created (NCBI 2012.03.19 109146 sequences: *Arabidopsis* RefSeq 67924 sequences (35375 *A. thaliana*, 32549 *A. lyrata*) + *Brassica napus* 10622 sequences + *Capsella rubella* 4246 sequences + other *brassicales* 26354 sequences). The Proteome Discoverer Software was set to adjust the Xcorr to reach a false discovery rate of ≤ 1% (Veith et al., 2009). All proteins with at least two unique peptides were taken for further analysis.

For transit peptide prediction the web-based tools TargetP (http://www.cbs.dtu.dk/services/TargetP/) (Emanuelsson et al., 2000) was used and for prediction of the transit peptide length the web-tool ChloroP (http://www.cbs.dtu.dk/ services/ ChloroP/) (Emanuelsson et al., 1999). For further analyses identified proteins were grouped into bins according to the modified MapMan system of the plant proteome database (ppdb) ((http://ppdb.tc.cornell.edu/dbsearch/mapman.aspx) (Sun et al., 2009) based on the MapManBins of (Thimm et al., 2004).

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TABLES

Table 1. Functional categorization and characterization of proteins from the phosphocellulose fraction identified by LC-ESI-MS/MS

Identified proteins are named in the first column according to the annotation of the respective gene at NCBI. They are grouped into different classes written bold at the beginning of each group as defined in results. Proteins within each group are sorted alphabetically. Spots: number of the spots containing the respective protein. MapMan bin: classification groups for proteins according to the modified MapMan system of the plant proteome database (ppdb) ((http://ppdb.tc.cornell.edu/dbsearch/mapman.aspx) ©Klaas J. van Wijk Lab, Cornell University; Sun et al. 2009) based on the MapManBins of Thimm et al. (2004); Accession: gi identification number and At gene accession number; cTP: possibility of a plastid transit peptide and the respective reliability class (RC); Reference: first identification of the protein in mustard by mass spectrometry.

Protein	spots	MapMan bin	Accession	ATG	cTP	Reference
PEP, PAPs, pTACs						
Chloroplast RNA steem-loop binding protein 41a (CSP41a)	8	27.3.99	15229384	AT3G63140	cTP 0.983 RC 1	Pfannschmidt et al. 2000
Chloroplast RNA steem-loop binding protein 41b (CSP41b)	40	27	15217485	AT1G09340	—	Schröter et al. 2010
Fructokinase-like 1 (FLN1)	3	29.4.1	15232415	AT3G54090	cTP 0.624 RC 3	Pfalz et al. 2006
Iron superoxide dismutase 3 (FSD3)	1	21.6	15237281	AT5G23310	cTP 0.945 RC 2	Pfannschmidt et al. 2000
Plastid encoded RNA polymerase alpha (RpoA)	2	27.2	7525065	AtCg00740	—	Pfannschmidt et al. 2000
Plastid transcriptionally active chromosome 4 (pTAC4)	4	29.3.3	18408237	AT1G65260	cTP 0.965 RC 1	Pfalz et al. 2006
Plastid transcriptionally active chromosome 6 (pTAC6)	1	28.3	79318316	AT1G21600	cTP 0.802 RC 2	Pfalz et al. 2006
Plastid transcriptionally active chromosome 7 (pTAC7)	1	35.2	334187898	AT5G24314	cTP 0.899 RC 2	Pfalz et al. 2006
Plastid transcriptionally active chromosome 10 (pTAC10)	3	28.3	297816052	—	cTP 0.791 RC 3	Pfalz et al. 2006
Plastid transcriptionally active chromosome 12 (pTAC12)	1	28.3	30686151	AT2G34640	cTP 0.563 RC 4	Pfalz et al. 2006
Plastid transcriptionally active chromosome 18 (pTAC18)	1	35.2	15225202	AT2G32180	cTP 0.712 RC 4	Pfalz et al. 2006
Thioredoxin z (TRX z)	1	21.1	15230779	AT3G06730	cTP 0.858 RC 3	Pfalz et al. 2006

UDP-N-acetylmuamoylalanyl-d-glutamate-2,6-diaminopimelate ligase (MurE)	3	28.3	240254313	AT1G63680	cTP 0.695 RC 3	Pfalz et al. 2006
Translation						
Alpha-nascent polypeptide associated complex like protein 1 (Alpha-NAC-like protein 1)	1	29.2.4	15230476	AT3G12390	—	this work
Alpha-nascent polypeptide associated complex like protein 3 (Alpha-NAC-like protein 3)	1	29.2.4	240256288	AT5G13850	—	this work
Cytosolic ribosomal protein L11 (CRPL11)	1	29.2.1.2.2.11	79595462	AT2G42740	—	this work
Cytosolic ribosomal protein L22-2 (CRPL22-2)	2	29.2.1.2.2.22	145331980	AT3G05560	—	this work
Elongation factor 1-alpha4 (EF1-alpha4)	1	29.2.4	186532608	AT5G60390	—	this work
Elongation factor tu (EFtu)	1	29.2.4	15237059	AT4G20360	cTP 0.975 RC 1	Pfalz et al. 2006
Eukaryotic translation initiation factor 1A (eIF1A)	1	29.2.3	334188030	AT5G35680	—	this work
Eukaryotic translation initiation factor 3 (eIF3)	2	29.5.11.20	15225611	AT2G39990	cTP 0.797 RC 2	this work
Plastid ribosomal protein L1 (PRPL1)	21	29.2.1.1.1.2.1	15229443	AT3G63490	cTP 0.937 RC 1	this work
Plastid ribosomal protein L4 (PRPL4)	12	29.2.1.1.1.2.4	79317147	AT1G07320	cTP 0.826 RC 2	this work
Plastid ribosomal protein L5 (PRPL5)	1	29.2.1.1.1.2.5	15234136	AT4G01310	cTP 0.828 RC 3	this work
Plastid ribosomal protein L6 (PRPL6)	17	29.2.1.1.1.2.6	15220443	AT1G05190	cTP 0.495 RC 5	Schröter et al. 2010
Plastid ribosomal protein L10 (PRPL10)	6	29.2.1.1.1.2.10	15240644	AT5G13510	cTP 0.887 RC 2	this work
Plastid ribosomal protein L12-1 (PRPL12-1)	2	29.2.1.1.1.2.12	15232274	AT3G27830	cTP 0.941 RC 1	Pfalz et al. 2006
Plastid ribosomal protein L12-3 (PRPL12-3)	2	29.2.1.1.1.2.12	15232276	AT3G27850	cTP 0.955 RC 1	this work
Plastid ribosomal protein L14 (PRPL14)	1	29.2.1.1.1.2	297848252	—	—	this work
Plastid ribosomal protein L15 (PRPL15)	2	29.2.1.1.1.2.15	15230931	AT3G25920	cTP 0.866 RC 2	this work
Plastid ribosomal protein L18 (PRPL18)	1	29.2.1.1.1.2.18	15221153	AT1G48350	cTP 0.863 RC 2	this work
Plastid ribosomal protein L21 (PRPL21)	2	29.2.1.1.1.2.21	15219695	AT1G35680	cTP 0.981 RC 1	this work
Plastid ribosomal protein L24 (PRPL24)	2	29.2.1.1.1.2.24	30696487	AT5G54600	cTP 0.920 RC 2	this work
Plastid ribosomal protein L29 (PRPL29)	1	29.2.1.1.1.2.29	257717595	—	cTP 0.924 RC 2	Pfalz et al. 2006

Plastid ribosomal protein S5 (PRPS5)	14	29.2.1.1.1.1.5	15226167	AT2G33800	cTP 0.929 RC 1	this work
Translation initiation factor 2 (IF2)	7	29.2.3	15220055	AT1G17220	cTP 0.537 RC 3	this work
Translation initiation factor 3 (IF3)	2	29.2.3	18417644	AT4G30690	cTP 0.782 RC 2	this work
tRNA/rRNA methyltransferase (SpoU)	3	29.2.7	30680811	AT2G19870	—	this work
Protein homeostasis						
Chloroplast heat shock cognate protein 70-2 (cpHSC70-2)	1	29.6	15240578	AT5G49910	cTP 0.993 RC 1	this work
Protein disulfide isomerase like 2-1 (PDI-like 2-1)	2	21.1	145331431	AT2G47470	—	this work
T-complex protein 1/ chaperonin60 family protein (TCP1/cpn60)	4	29.6	15242093	AT5G20890	—	this work
Photosynthesis						
ATPsynthase alpha	1	1.1.4	7525018	AtCg00120	—	Schröter et al. 2010
ATPsynthase beta	3	1.1.4	7525040	AtCg00480	—	Schröter et al. 2010
Rieske Cluster	2	1.1.3	30679426	AT4G03280	cTP 0.652 RC 3	this work
RubisCO activase	2	1.3.13	30687999	AT2G39730	cTP 0.888 RC 1	this work
Others						
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (CAC3)	1	11.1.1	30687368	AT2G38040	cTP 0.927 RC 2	this work
Actin	1	31.1	79324605	AT2G37620	—	this work
Cruciferin 3 (CRU3)	2	33.1	15235321	AT4G28520	—	this work
cystein synthase	1	13.1.5.3.1	334184908	AT2G43750	cTP 0.938 RC 1	this work
Fatty acid biosynthesis z (FabZ)	2	11.1.5	72255615	—	cTP 0.872 RC 2	this work
Malate dehydrogenase (MDH)	3	8.2.99	15232820	AT3G47520	cTP 0.911 RC 1	this work
Malate synthase (MLS)	4	6.2	334187411	AT5G03860	—	this work
Multi functional protein 2 (MFP2)	5	11.9.4.9	15231317	AT3G06860	—	Schröter et al. 2010
Myrosinase	1	16.5.1	127734		—	this work
Phosphoserine aminotransferase (PSAT)	4	13.1.5.1.2	15237069	AT4G35630	cTP 0.938 RC 1	this work

Pyrroline-5-carboxylate reductase (P5CR)	3	13.2.2.3	145334418	AT5G14800	—	this work
Serine hydroxymethyltransferase 1 (SHMT 1)	8	25.1	15235745	AT4G37930	—	Schröter et al. 2010
Serine hydroxymethyltransferase 2 (SHMT 2)	8	25.1	30690404	AT5G26780	—	Schröter et al. 2010

FIGURE LEGENDS

Figure 1: Overview of purification procedure and protein visualisation by 2D PAGE. A) Protein peak fractions after HS and PC chromatography in a silver stained 7-20% SDS polyacrylamide gel. 20 µg of protein per lane was separated. Sizes of marker proteins are given in the right margin. **B)** Flow chart of the complete protein isolation and identification procedure. Arrows indicate the purification stages separated by 1D (Fig. 1A) or 2D PAGE (Fig. 1C). **C)** Protein pattern of PC peak fractions in silver stained 7.5-20% SDS acrylamide gels using three different pH gradients in the first dimension. The pH gradient used is indicated in the upper left corner of each gel and the pH range is given in detail below each gel. Marker sizes are given in the right margin. Dotted lines indicate the overlapping pH areas. 400 µg of total protein separated in each gel. **D)** Numbering of protein spots visualised in the 2D gel with pH 3-11NL for first dimension as shown in Fig.1C. Marker sizes and pH range are given in the margin or below the gel, respectively.

Figure 2: Numbers of total, analyzed and identified spots of the PC peak fractions. Proteins separated in the 2D gels shown in Fig. 1 C are given in yellow boxes at the top. Protein groups corresponding to Table 1 are displayed below in coloured boxes. At the left side of each box the number of putative plastid proteins per bin being either plastid encoded or for which a plastid transit peptide was predicted is given. At the right side proteins without these properties are given.

Figure 3. Overview and comparison of the protein content in HS and PC fractions. A) Distribution of the identified proteins of the HS fractions analysed in Schröter et al. 2010 and classification into groups in correlation to the recent work. **B)** Percentage of identified proteins of PC peak fractions analysed in this work and classified into groups as shown in Table 1. **C)** Distribution of solely the plastid proteins of the recent PC fractions to functional groups according to table 1 but with an aggregation of "PEPs and PAPs" with "Other pTACs" and a part of "DNA and RNA" to one bin "Transcription".

Figure 4. Essential polymerase-associated proteins (PAPs) of the soluble PEP complex. Positions of PAPs in the 2D gel after isoelectric focussing of the PC fraction on a pH 3-11NL and pH 6-11 gradient. Spot identity is given at the right margin. Fragments are additionally indicated by an asterisk. Marker sizes and pH range are given at right margin and above or below the gel, respectively. The gel is silver-stained.

Figure 5. Distribution of CSP41a and b spots in the pH 3-11 NL 2D-gel. CSP41a is drawn in yellow and CSP41b in orange. Marker sizes and pH range are given right beside and above the silver stained gel respectively.

Supplemental Figure S1. Silver stained 2D-gels of the PC fractions with isoelectric focusing for the first dimension in pH gradients between 6-11, 3-11 NL and 3-10 indicated in the upper left corner of each gel. The second dimension is performed in a 7.5-20% SDS polyacrylamide gel. Spots are marked and numbered in yellow. Marker sizes and pH range are given right beside and below the gel respectively.

Supplemental Table 1. Identified peptides from the PC fraction

Spots are listed in numerical order. Accession numbers of proteins belonging to the same spot are listed in an order starting with the highest peptide coverage. Spot nr., identification number of the protein containing spot on the 2D-gels (see Suppl. Fig 1). Descriptions of depicted proteins are given as stated in the databases (see Methods). Coverage, coverage of the depicted proteins by the identified peptides; calc. pI, calculated pI of the depicted proteins based on the protein sequences in the database; MW, calculated molecular weight based on the protein sequences in the databases; z, peptide ion charge; lower case "m" in the peptide sequence, oxidized form of methionine; lower case "w" oxidized form of tryptophane; lower case "c" cystein with carbamidomethylation; lower case "k" acetylation of lysine

Supplemental Table 2. Detailed characterization of proteins from the PC fraction identified by LC-ESI-MS/MS

Identified proteins are given in the first column according to the annotation of the respective gene at NCBI. Proteins were sorted according to the MapMan bin numbering in the second column representing classification groups for proteins according to the modified MapMan system of the plant proteome database (ppdb) ((http://ppdb.tc.cornell.edu/dbsearch/mapman.aspx) ©Klaas J. van Wijk Lab, Cornell University (Sun et al. 2009) based on the MapManBins of Thimm et al. (2004). Spots: number of spots containing the respective protein. Spot nr: identification number of the protein containing spot on the 2D-gels. NCBI accession: gi identification number at NCBI (The National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/); ATG: gene accession of the first matching *Arabidopsis thaliana* hit or (if no *A. thaliana* protein was matching) the best matching other organism and the respective *A. thaliana* gene accession determined by a protein-protein blast at NCBI in brackets; cTP: probability of a plastid transit peptide; MW [kDa]: calculated theoretical molecular weight in kilodalton; MW(-cTP) [kDa]: calculated theoretical molecular weight without chloroplast transit peptide (cTP) in

kilodalton; MW(-lTP) [kDa]: calculated theoretical molecular weight without cTP and luminal transit peptide (lTP) in kilodalton; PI: calculated isoelectric point; PI (-cTP): calculated isoelectric point without cTP; PI (-lTP): calculated isoelectric point without cTP and lTP. The last 6 parameter were provided at the related accession entry by ppdb (http://ppdb.tc.cornell.edu/dbsearch/searchacc.aspx).

Figure 1.JPEG

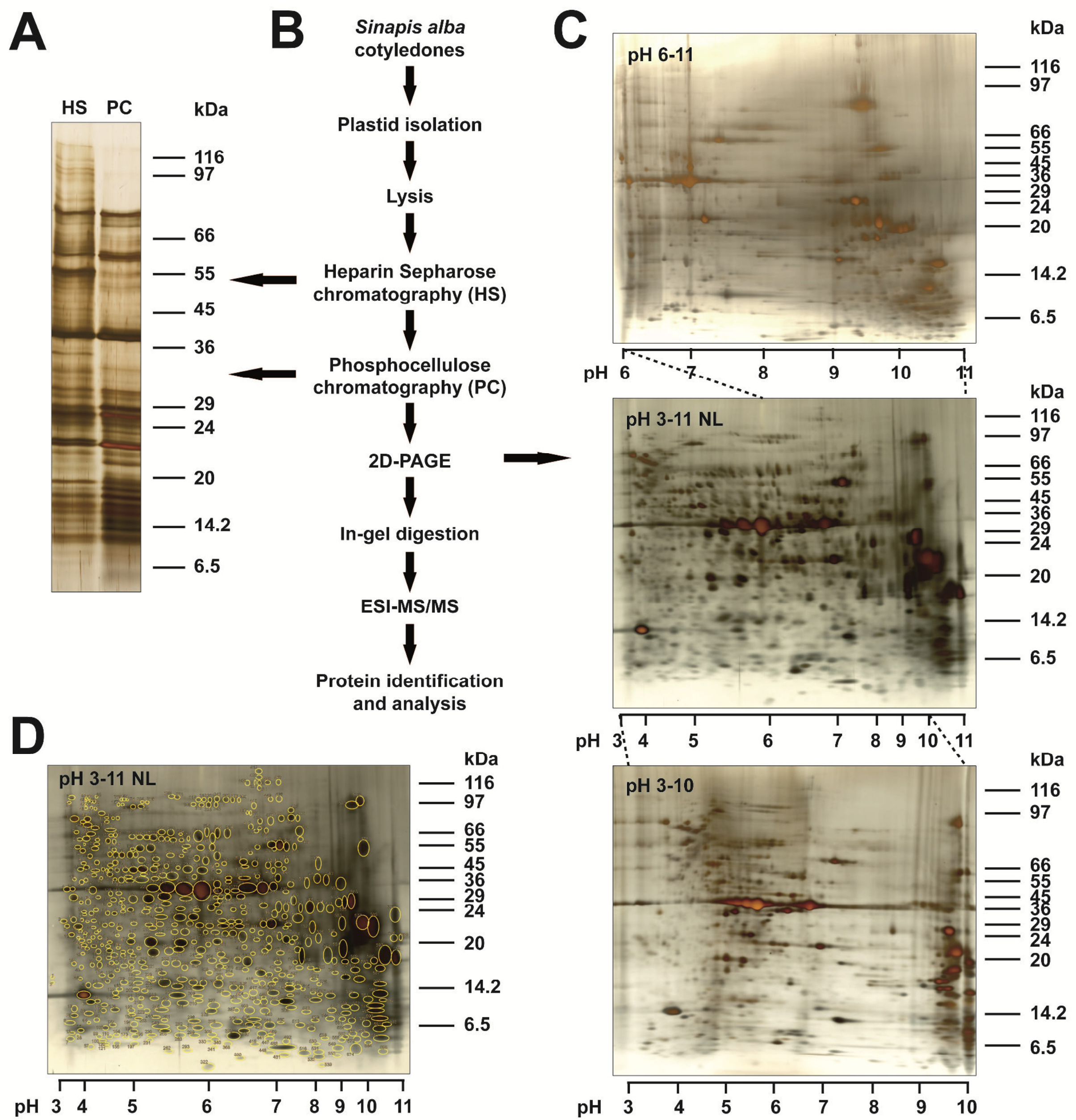


Figure 2.JPEG

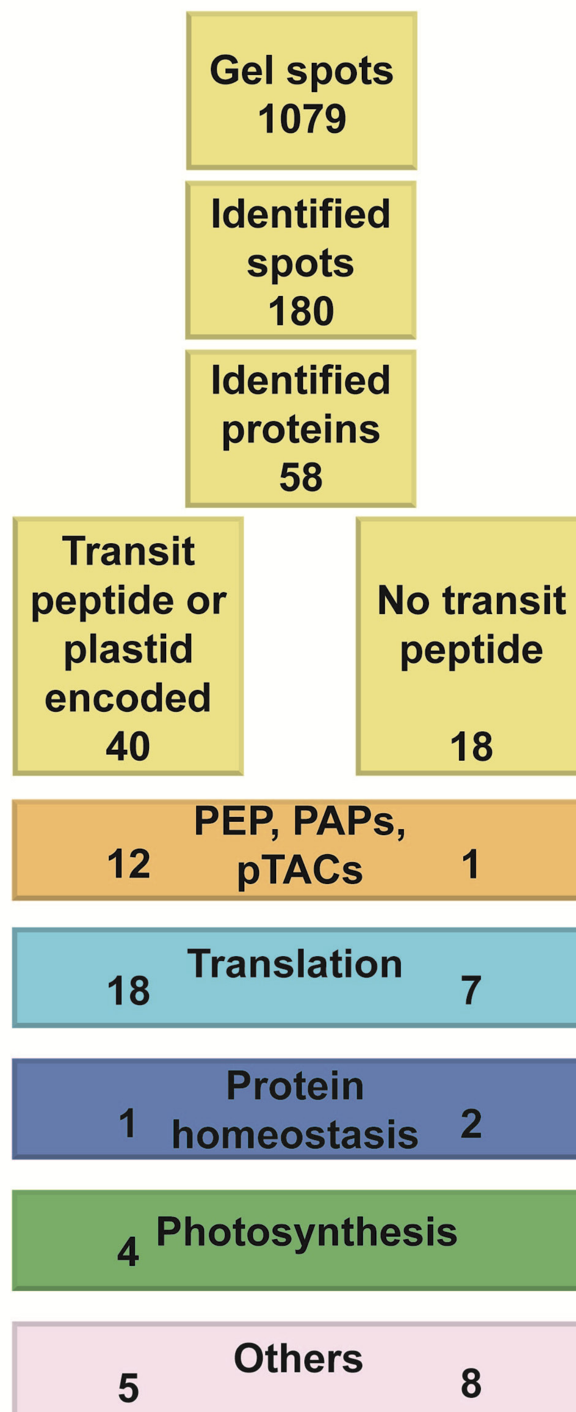


Figure
3.JPEG

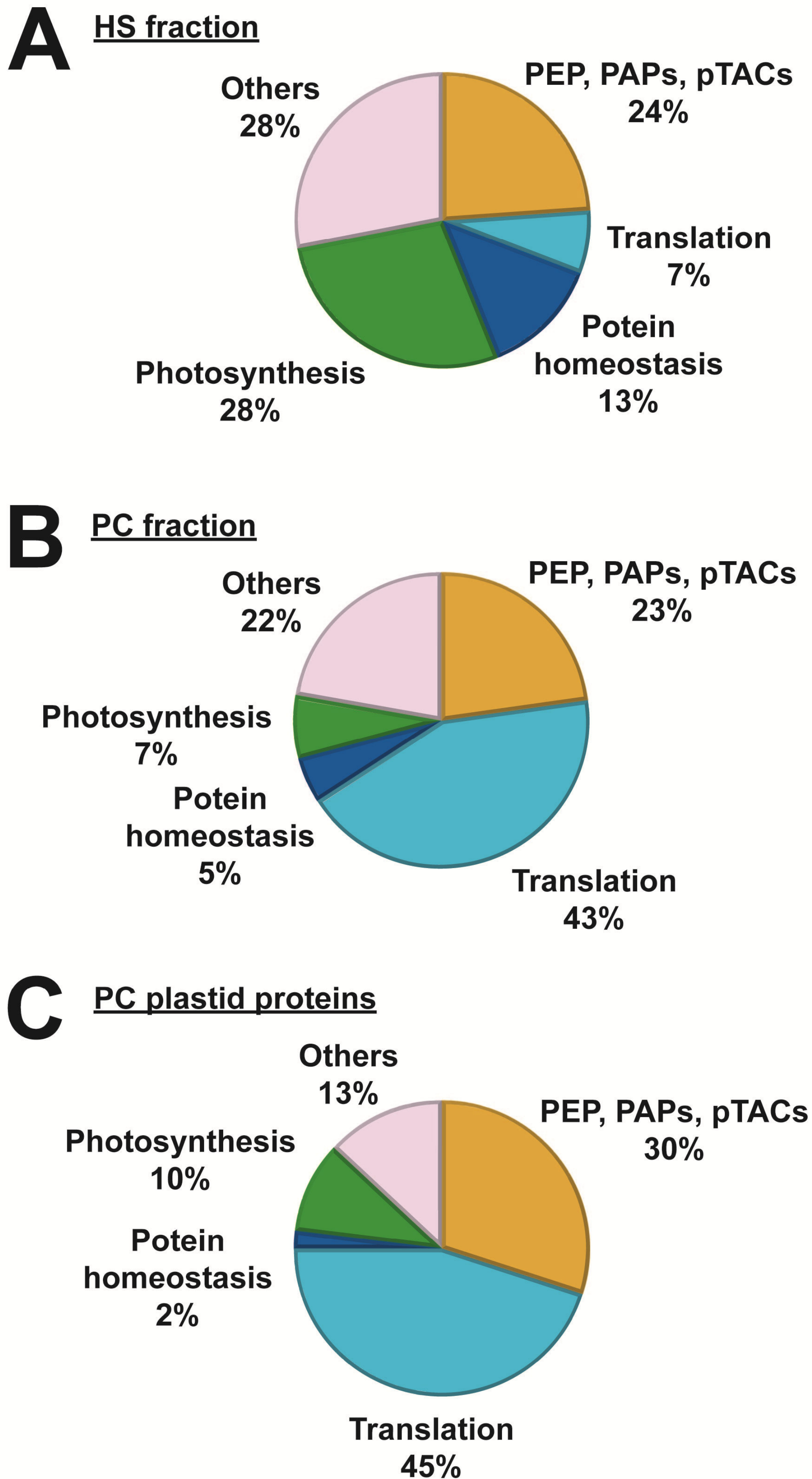


Figure
4.JPEG

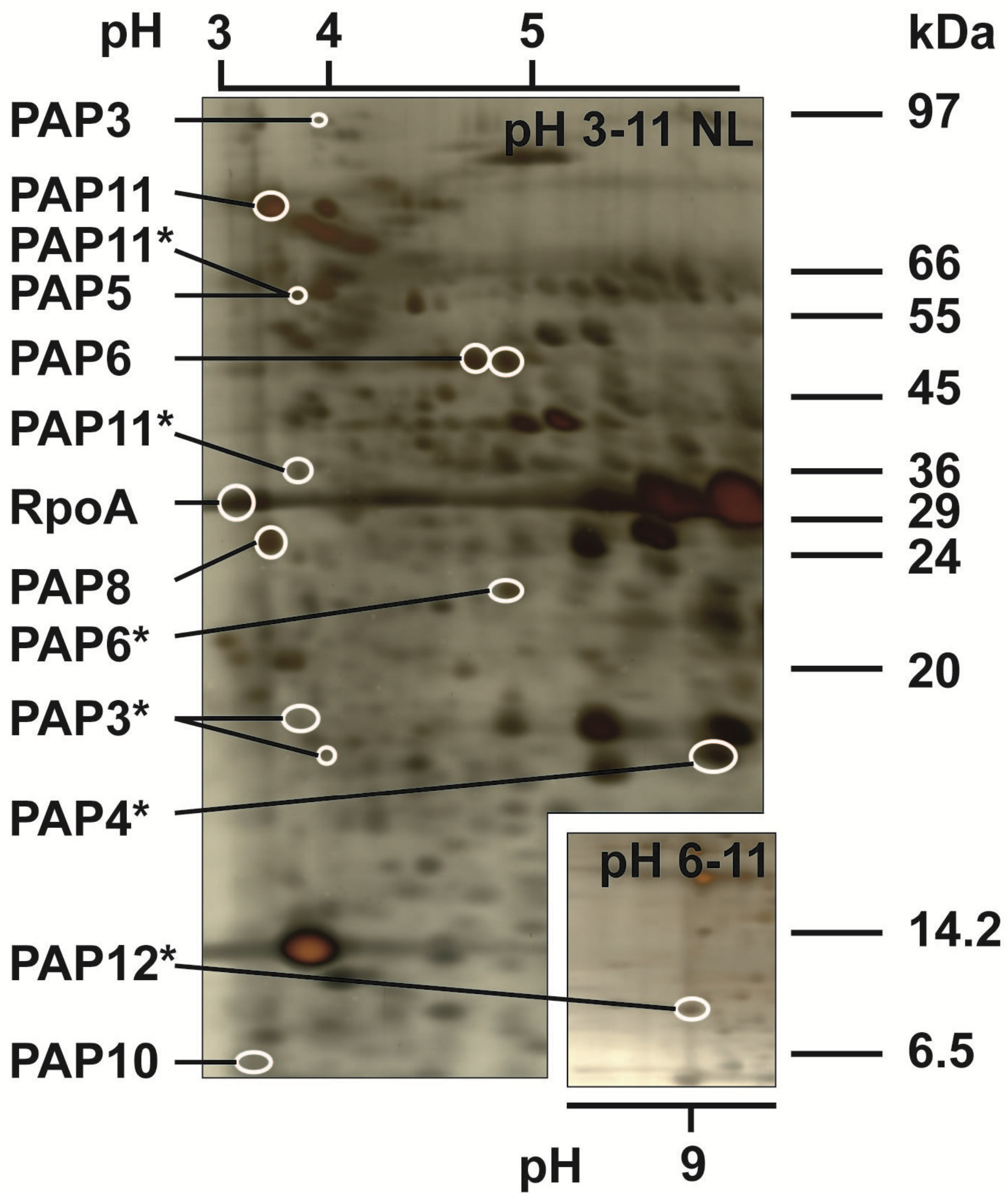
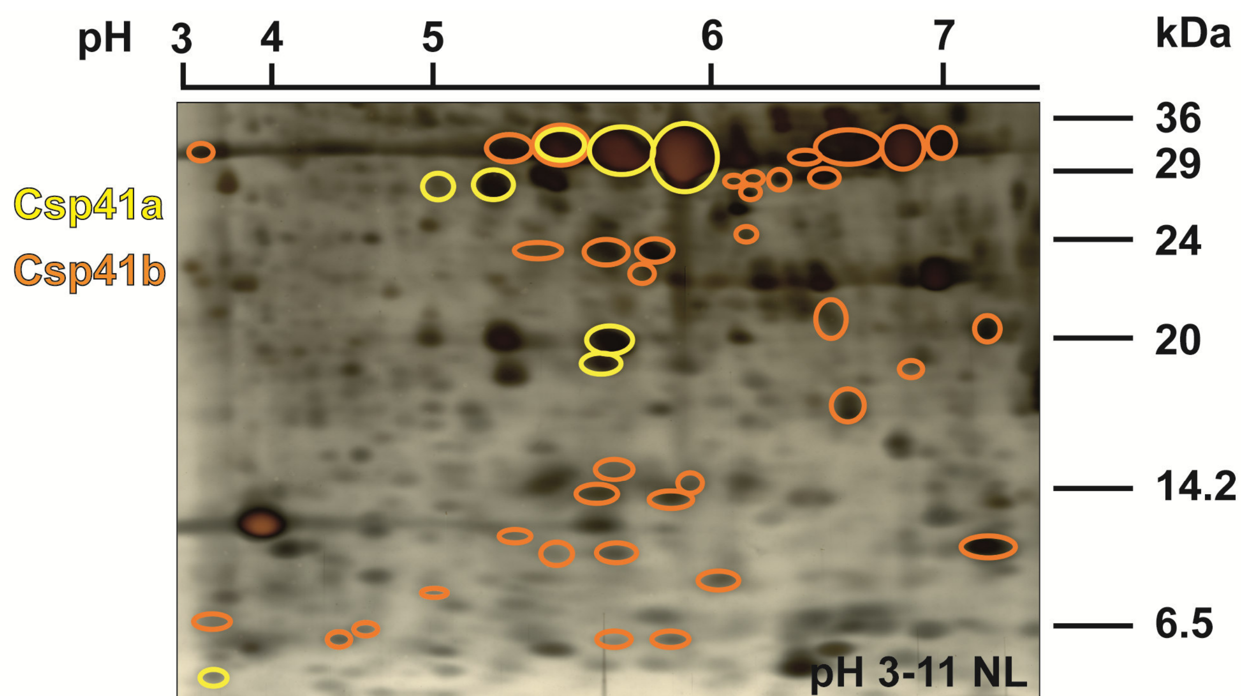


Figure 5.JPEG



11 General discussion

For a detailed understanding of processes during redox signal induced plastid gene expression the analysis of the nucleotide binding plastid sub-proteome is of particular interest. This work combines the analysis of the knowledge about redox signal transmission and its influence on plastid gene expression as well as classical biochemical methods with modern analytical tools for a fundamental study of the plastid gene expression machinery.

11.1 Redox signal triggered gene expression

The review article Pfannschmidt *et al*. 2008, Dietzel *et al*. 2008 and Pfalz *et al*. 2012 highlight the actual ken about chloroplast to nucleus communication and *vice versa* to date as well as the influence on nuclear and plastid gene expression. As discussed there the knowledge of these complex processes are marginal and need to be investigated in detail. Pfannschmidt *et al*. 2008 describes the generation of a redox state caused by imbalances of the PET and acclimation responses in terms of STR and LTR. Furthermore the role of the PQ as signal giver and the STN7 kinase as signal sensor in redox signal transmission is discussed. Putative phosphorylation cascades and an involvement of thioredoxins in redox signal mediation to the plastid gene expression machinery are postulated and the redox signal transduction to the nucleus is discussed (Fig. 2, Pfannschmidt *et al*. 2008). An influence of redox signals on primary nuclear and plastid target genes are part of this review as well because of their importance for understanding cellular processes and transduction pathways. The communication within plastids and plant cells highlighted here is complex and represents a network of different signals of various origins, signal transducing cascades and responding elements. A model for the integration of various signals in plant cells is given in this review too (Fig. 3, Pfannschmidt *et al*. 2008). Experimental approaches for the generation of a redox state of the PQ pool by inhibitor treatment, the use of knock out mutants or defined illumination conditions are discussed in detail and base for further studies within this work as referred in the introduction.

Dietzel *et al*. 2008 represents a review contributing to the book “The Chloroplast”. It treats of the retrograde signaling between plastids and nucleus covering the examined fields to date. This comprises the plastid signaling depending on plastid gene expression and on pigment biosynthesis and the crosstalk of both signals. Furthermore the interaction of photo-morphogenesis related signals induced by whether developing plastids or light and its interaction. Most important for the context of this work is the disquisition of signals depending on photosynthesis and reactive oxygen species and in special these evolving from the PET (Dietzel *et al*. 2008).

Pfalz *et al*. 2012 includes recent advances and findings concerning the redox signal generation and mediation. It also considers the publications Steiner *et al*. 2009, Schröter *et al*. 2010 and Steiner *et al*. 2011 and comprehends the new investigations into existing models. It focuses on the relation of physiological redox signals to the environment and signal transduction pathways to nucleus gene expression.

As discussed in these reviews the redox signal mediation to the adjusting gene expression machineries in plastids and nucleus is complex and most likely represents a network of several signal cascades interacting with each other. The knowledge about components of redox signal transmission as well as elements directly influencing the expression of target genes is marginal.

11.2 LTR in mustard cotyledons

For a better understanding of these adaption processes the plastid gene expression in dependency of a defined redox state was examined within this work. As prefatory described an experimental approach was established inducing a defined PQ redox state in mustard (Steiner *et al*. 2009; Schröter *et al*. 2010). Mustard cotyledons were shown to be ideal objects for this purpose. They deliver that high amount of biomass what is needed for the complex biochemical purification procedures used here. The harvest of 7 days old mustard cotyledons of 1 culture soil plate of 30x50 cm delivers between 150 and 250 g plant material. After plastid isolation and HS-Chromatography about 4 mg protein remain and about 1.6 mg protein after PC-chromatography. For one 2D BN-PAGE 140 µg of HS protein fraction is necessary and for one 2D gel

with isoelectric focusing (IEF) as first dimension 400 µg PC protein fraction were used. With *Arabidopsis* a vast multiple of culture soil plates and growing time has to be invested for getting similar amounts of cotyledon biomass because of the small size and slower growing of this species. On the other hand there is only a limited knowledge about *S. alba* genome, proteome and metabolic data available. But the close relationship of *S. alba* to *A. thaliana* is a big advantage for comparing new analytical data of mustard with known facts of *Arabidopsis* and other *Brassicales*. That enables the identification of mustard proteins and the attribution and inclusion into established biological systems and pattern.
In Steiner *et al*. 2009 the responses of mustard cotyledons to a PS-light and light switch induced redox state were analyzed *via* Chlorophyll fluorescence measurement and compared to *A. thaliana*. Mustard displays a typical and similar acclimation pattern as *A. thaliana* and is therefore comparable with the photosynthetic performance of former studies of *A. thaliana* (Pfannschmidt *et al*. 1999a; Piipo *et al*. 2006; Wagner *et al*. 2008; Fig. 1 and Table 1, Steiner *et al*. 2009). The redox control at the *psaA* promoter and the usage of the promoter was studied in a primer extension assay (Fig. 3 Steiner *et al*. 2009). In contrast to *A. thaliana*, with two 5'-transcript ends for the *psaA* mRNA, *S. alba* contains only one end (Summer *et al*. 2000; Fey *et al*. 2005; Fig. 3, Steiner *et al*. 2009). The transcript accumulation in dependency to the used light regime is consistent with earlier studies and displays the redox regulation of the *psaA* gen in mustard at this single transcript initiation site (Fig. 3, Steiner *et al*. 2009).

11.3 Characterization of the HS protein fractions

The present publications, Steiner *et al*. 2009, Schröter *et al*. 2010, Steiner *et al*. 2011 and Schröter *et al*. 2014, describe the methodology for the preparation of the nucleotide binding sub-proteome of *S. alba* cotyledons and its analysis and identification of the single proteins. For Steiner *et al*. 2009 and Schröter *et al*. 2010 mustard cotyledons were subjected to a PSI-II or PSII-I light-regime during growth the other studies were performed with white light plants. Isolated intact mustard plastids were lysed and subjected to HS chromatography. The resulting protein fraction may contain impurities caused by contaminations of other cell compartments. To define the kind and level of contamination several

protein fractions at different purification steps within the used procedure were tested for their composition of cellular compartments (Schröter *et al*. 2010). The tested aliquots of total cell extract (TL), lysate of intact chloroplasts (CL), peak fraction after HS chromatography (HS) and a cell nuclei fraction (N) were subjected to western immuno-analysis. The used antisera were directed against marker proteins for plastids and other cell compartments. The isolated chloroplast fractions were shown to be free of cytosolic and nuclear proteins but contain a small amount of mitochondria and tonoplast contaminations caused by co-purification with chloroplasts *via* sucrose gradient centrifugation. Also obviously was a disappearance of RubisCO subunits and LhcII by comparing the CL with the HS fraction on the Ponceau S stained blotting membrane and only traces of RbcS which remain detectable *via* immuno-assay. This and the substantial differences in the protein band pattern between CL and HS fraction on stained blotting membrane point to a selective enrichment of nucleic acid-binding proteins (Fig.3, Schröter *et al*. 2010). The excellent quality of prepared fractions was fundamental for further examinations including further chromatography steps in Schröter *et al*. 2014.

The protein pattern of the isolated HS fractions is visible on silver stained SDS PAGE gels (Fig. 2, Steiner *et al*. 2009; Fig. 1, Schröter *et al*. 2010; Fig. 1, Schröter *et al*. 2014). A comparison of protein pattern between PSI-II and PSII-I light fractions in these gels is hindered by the high quantity of different proteins within. Light–quality effects on single proteins may be masked by other proteins of the same size (Steiner *et al*. 2009). The characterization of the *psaA* and *psbA* promoter binding efficiency of the HS fractions in an EMSA approach offers any differences between PSI-II and PSII-I acclimated plastids (Fig. 4, Steiner *et al*. 2009). Complemented by Southwestern experiments with the same fractions 20-30 different proteins were shown to interact with the promoter in a Light-quality dependent way (Fig. 5, Steiner *et al*. 2009). Hence an influence of the redox state on promoter binding capacity was demonstrated there. The effects of differential phosphorylation of proteins on the promoter binding behavior between PSI-II and PSII-I fractions was tested as well here (Steiner *et al*. 2009). As incipient described an involvement of kinases in redox signal transmission was already shown for STN7 and CSK (Bonardi *et al*. 2005; Puthiyaveetil *et al*. 2008). Western-immuno-assays with regards to short and

long term effects on the phosphorylation pattern offered Light-quality dependent differences on around 40 proteins also in a time dependent manner here (Fig. 6 and Fig. 7, Steiner *et al*. 2009). This high number of influenced proteins confirms the involvement of phosphorylation cascades on plastid gene expression possibly as widespread mechanism (Steiner *et al*. 2009). Obviously the general phosphorylation pattern between the PSI-II and PSII-I fractions is similar concerning all three amino acids tested, thus representing the genuine phosphorylation state of both fractions. The majority of the phosphorylation sites detected seem not to be involved in LTR regulation. Nevertheless three candidates for redox responsive factors were found, showing differences in phosphorylation pattern and promoter binding activity (Steiner *et al*. 2009). The endogenous kinase activity is quite different between PSI-II and PSII-I HS fractions and display differential phosphorylated proteins in both fractions, pointing to varying kinase activities and accessibilities of phosphorylation sites (Fig. 8, Steiner *et al*. 2009). In run-on transcription assays was shown that kinase inhibition and thiol reduction together have a strong influence on *psaA* and *psbA* transcription (Fig. 9, Steiner *et al*. 2009). Thus phosphorylation and dithiol sites were necessary for plastid transcriptional regulation (Steiner *et al*. 2009).

Taken together in Steiner *et al*. 2009 was shown that the isolated HS fractions contain numerous small DNA binding proteins or even plastid *psaA* and *psbA* promoter binding proteins. Furthermore any proteins within these fractions are influenced or controlled by phosphorylation also in a Light-quality dependent manner. These findings confirm the existence of a complex network of interacting proteins for signal transmission to plastid gene expression. This might include the action of several kinases, like STN7 and CSK, and post-translational modifications, like dithiol reduction (Steiner *et al*. 2009).

11.4 2D BN-PAGE

The more detailed analysis of the protein content within the HS fractions was performed using 2D BN-PAGE (Schröter *et al*. 2010; Steiner *et al*. 2011). The visualization of the isolated HS-protein fractions on 2D BN-gels displayed ten oligomeric protein complexes between 1000-250kDa and eleven smaller protein

complexes or even spots below 200kDa (Fig.2 and Table1, Schröter *et al*. 2010). By using different polyacrylic gradients for first dimension the whole fraction content was obviously (Fig.2, Schröter *et al*. 2010). For Steiner *et al*. 2011 only the second oligomeric complex was of interest containing the PEP complex and its associated proteins as discussed later.

For protein identification of the single spots a tryptic digestion and ESI-MS/MS measurement was conducted. The identification of measured peptides succeeded using a *Brassicales* Database. The sequence homology between *S. alba* and *Arabidopsis* as well as other *Brassicales* was shown to be sufficient for the identification of mustard proteins. Thus mustard could be established as additional model plant to *Arabidopsis* especially for the application of complex biochemical analysis and purification schemes and MS studies (Schröter *et al*. 2010; Steiner *et al*. 2011; Schröter *et al*. 2014).

The investigation of effects of photosynthetic light acclimation on the protein composition of plastids was of special interest in Schröter et al. 2010. Any differences in the protein pattern generated by the illumination systems PSI-II and PSII-I should be obviously on 2D BN-gels as well. An effect was visible for the proteins of spot 11 of the 2D BN-gels as an enrichment in PSII-I fractions (Fig. 2, Schröter *et al*. 2010). It contains the fructose-1,6-bisphosphatase and the translation elongation factor EF-tu (Table 1, Schröter *et al*. 2010). It was demonstrated before that redox control has a strong effect on plastid translation and possibly EF-tu is involved in conversion of this signal (Trebitsh *et al*. 2000; Trebitsh and Danon 2001; Schröter *et al*. 2010).

Further differences are not visible within these gels confirming redox signal mediation by phosphorylation or other posttranslational modifications instead of up or down regulation of the abundance of a single protein. Therefore preparations of further studies are performed with white light cotyledons (Steiner *et al*. 2011; Schröter *et al*. 2014).

11.5 PC chromatography

Plenty of contaminating proteins in the HS fractions exacerbate the analysis of gene expression related proteins as discussed later here in detail. Many photosynthetic and metabolic proteins were enriched by HS chromatography

which tends to cover low abundant proteins and have to be removed (Table 1, Schröter *et al*. 2010). The use of PC for isolating the nucleotide binding sub-proteome of mustard plastids in addition to HS chromatography provides a potent protein fraction, containing the essential elements of plastid gene expression (Schröter *et al*. 2014). PC chromatography is a well-tried method for separating nucleotide binding enzymes like RNA polymerases (Burgess *et al*. 1969; Bottomley *et al*. 1971; Tiller and Link 1993). Thus it is not a new tool in protein analysis but in combination with 2D-gelelectrophoresis and modern mass spectrometrical analysis the ancient method gets a new position.

The comparison of HS fractions and PC fractions in a silver stained polyacrylamide gel offered a different protein pattern with an evident reduction as well as enrichment of proteins (Fig.1 A, Schröter *et al*. 2014). An overview of the whole PC Sample in 2D-gels was achieved by using a pH range from 3-11NL (non linear) for the isoelectric focussing of proteins in the first dimension and a gradient polyacrylamide gel as second dimension (Fig.1 C, Schröter *et al*. 2014). The non linear pH gradient of the gel tends to an accumulation of proteins at the outer ranges and poorly resolved spots in these areas. Overlapping pH gradients for the first dimension between pH 3-10 and pH 6-11 solved this problem and the whole protein content of the PC fractions was displayed (Fig. 1 C, Schröter *et al*. 2014). Drawing the overlapping 2D-gels together a pattern of 1079 spots is visible, representing the nucleotide binding sub-proteome of mustard plastids (Fig. 1 C and D and supplemental Fig. 1, Schröter *et al*. 2014). Many spots are doubled representing the same protein on different gels, cognoscible on the detailed spot pattern of overlapping gel areas (Supplemental fig. 1, Schröter *et al*. 2014). Altogether 284 spots can be selected visually as analysable with a sufficient protein amount for the identification *via* the used mass spectrometry method. Altogether 58 different proteins were identified in 180 of the strongest spots by using again a *Brassicales* database (Table 1 and Supplemental table 1, Schröter *et al*. 2014).

About 62% of all proteins of this work were first identified in *S. alba* by a mass spectrometry approach. Totally 32 protein were first detected in the HS fractions of Schröter *et al*. 2010 and 33 more in the PC fractions of Schröter *et al*. 2014.

The identified proteins may be categorized as defined in Schröter *et al*. 2014. This enables the comparison of the results of both publications, Schröter *et al*.

2010 and Schröter *et al*. 2014. The classification is based on the modified MapMan bin system (Thimm *et al*. 2004) of the Plant Proteomics Data Base (PPDB) (Sun *et al*. 2009). Proteins of Schröter *et al*. 2014 were sorted according to these bins and further on dedicated to 5 functional groups. The first group contains the components of the soluble PEP complex as defined in Steiner *et al*. 2011 and described following. Two more proteins were unified with the existing PAPs because of their obviously importance for transcription in 2013 (Pfalz and Pfannschmidt 2013). A number of pTACs with additional functions in gene expression but not directly associated to the soluble PEP complex as well as the CSP41a and b protein remain and were dedicated to this group as well. This first group comprises transcription and transcript related proteins as well as additional RNA and DNA related proteins and is called “PEP, PAPs and pTACs”. The other proteins may be grouped into translation associated proteins (Translation), proteins involved in protein homeostasis, photosynthesis or belong to the miscellaneous group “others”. The results of Schröter *et al*. 2010 were already grouped in Schröter *et al*. 2014 for comparison of HS and PC fractions (Fig. 3, Schröter *et al*. 2014).

11.6 PEP, PAPs and pTACs

The main focus of this work was on gene expression related proteins. As mentioned earlier the transcriptional PEP complex and its associated proteins is depicted in complex 2 of the 2D BN-gels without contaminations of other complexes migrating in this gel area during first dimension (Schröter *et al*. 2010; Steiner *et al*. 2011). About 24% of the HS fraction comprised PEP associated and transcription related proteins grouped into “PEP, PAPs and pTACs” (Fig. 2 and Table 1, Schröter *et al*. 2010; Fig. 3, Schröter *et al*. 2014). Most of the PEP components identified in Schröter *et al*. 2010 were already known but one new PAP was identified within this work (Schröter *et al*. 2010). The protein of spot 2.7 was already described as compound of the TAC-fraction and the PEP complex but first identified here as Thioredoxin z (TRXz) and later on established as PAP10 (Pfalz *et al*. 2006; Arsova *et al*. 2010; Fig. 2 and Table 1, Schröter *et al*. 2010; Steiner *et al*. 2011). Arsova *et al*. (2010) ascertains the interaction of TRXz with, PAP6, another PEP associated protein. This pfkB-

carbohydrate kinase family protein, fructokinase-like 1 (FLN1), is present in the HS- and PC-fractions too and a well known member of the PEP complex since Suzuki *et al*. (2004) identified in *A. thaliana*. Recently it was shown that PAP4/FSD3 and the TRXz of PAP10 interact with a novel thioredoxin in higher plants, AtECB1, for regulation of plastid gene expression and development (Yua *et al*. 2014).

In Steiner *et al*. 2011 independent HS preparations of this complex were compared to highly purified PEP preparations after glycerol gradient centrifugation. The protein bands which were consistent on SDS-PAGE gels of both preparations were regarded to be permanent PEP subunits (Fig.1, Steiner *et al*. 2011). These 15 proteins comprise the basic PEP composition of the PEP subunits $RpoC_2$, RpoB, $RpoC_1$ and RpoA and essential PEP associated proteins (PAPs), namely PAP1 (pTAC3), PAP2 (pTAC2), PAP3 (pTAC10), PAP4 (FSD3), PAP5 (pTAC12/HEMERA), PAP6 (FLN1), PAP7 (pTAC14), PAP8 (pTAC6), PAP9 (FSD2) and PAP10 (TRXz) (Table 1, Steiner *et al*. 2011). The indispensability of PAPs for PEP activity was confirmed by phenotypic characterisation of *A. thaliana* knock-out mutants of the respective gene (Schröter *et al*. 2010; Steiner *et al*. 2011). All PAP knock-out plants show an albino or pale green phenotype caused by severe defects in chloroplast development (Steiner *et al*. 2011). Nevertheless they were able to survive on sugar supplemented medium and the gene knock out is not lethal in general. As mentioned before the PAPs were supplemented by two more subunits PAP11 (MurE-ligase) and PAP12 (pTAC7), displaying an albino phenotype in the respective knock-out mutants too (Pfalz and Pfannschmidt 2013).

The PEP subunit content of the PC fractions of Schröter *et al*. 2014 reflects the different isolation spectra of the chromatographic methods. Clearly cognizable is the massive reduction of proteins above approximately 80 kDa in the PC fractions which tends to a reduction of almost all of the higher molecular weight PEP subunits and PAPs to unidentifiable amounts (Fig. 4 and Table 1, Schröter *et al*. 2014). Only RpoA and PAPs from PAP12 to PAP3 remain identifiable in this work whereas the HS fractions contain the whole PEP complex in sufficient amounts (Fig. 2 and Table 1, Schröter *et al*. 2010; Fig 1. Steiner *et al*. 2011; Fig. 4 and Table 1, Schröter *et al*. 2014). An improvement of the biochemical

purification procedure is planned to solve the size cut-off problem through chromatography.

The CSP41a and b proteins were handled separate here because of their vast amount in HS and PC fractions and their variability. Detected first as eponymous chloroplast RNA stem-loop binding protein of 41 kDa in spinach, it was shown to be RNA-binding and ribosome associated in *Chlamydomonas reinhardtii* as RAP38 and RAP41 as well as in *A. thaliana* named CSP41a and CSP41b (Yang *et al*. 1996; Baker *et al*. 1998; Bollenbach and Stern 2003a and b; Yamaguchi *et al*. 2003; Bollenbach *et al*. 2004; Beligni and Mayfield 2008). CSP41a and b were also detected in isolates of the PEP-complex as one of the most abundant component (Pfannschmidt *et al*. 2000). They are involved in the gene expression process in general and are thus important for transcriptional and translational processes (Bollenbach *et al*. 2009; Qi *et al*. 2012). Here we dedicate both proteins to the group of PEP, PAPs and pTACs. In former investigations and the HS fractions of Schröter *et al*. 2010 CSP41a and b showed a pattern of broad protein bands on acrylamide gels pointing to the formation of diverse complexes of different sizes (Peltier *et al*. 2006; Fig. 2, Schröter *et al*. 2010; Qi *et al*. 2012). In the PC fractions of Schröter *et al*. 2014 we identified CSP41a in 8 and CSP41b in 40 spots of diverse sizes and isoelectric points (Fig. 5, Schröter *et al*. 2014). Thereby both form a defined spot pattern which is congruent in the replicates of the 2D-gels prepared for this work. This indicates massive post-translational modifications and degradations to CSP41a and b.

11.7 Translation associated proteins in HS and PC fractions

The largest difference between HS and PC fractions is obviously within the translation associated proteins. The biggest amount of all identified proteins in the PC fractions is dedicated to the translational processes with 43% of all proteins (Fig. 3 and Table 1, Schröter *et al*. 2014). Compared to only 7% after HS chromatography a massive enrichment of translation related proteins occurred (Table 1, Schröter *et al*. 2010; Fig. 3, Schröter *et al*. 2014). Thereby ribosomal subunits contribute most with about 60% of all proteins of this group in PC fractions (Table 1, Schröter *et al*. 2014). The central element of the

organellar gene expression machinery is the 70S ribosome. Ribosomal proteins contain nucleotide binding structures for their contact to the different structural or gene expression relevant RNA types. Hence they are ideal adhesion partner for the used column materials. The identification of eukaryotic 80S ribosomal proteins in plastid fractions is formerly documented during examination of stroma protein fractions, recorded in the ppdb (http://ppdb.tc.cornell.edu/) as part of a study of Olinares *et al*. 2010. Thus the identification of such cytosolic compounds was not surprising. The plastid isolation procedure usually excludes the cytosole but plastid membrane attached particles, like the tonoplast membrane, proteins involved in cytosolic gene expression close to the organelle or assistants of protein translocation across the chloroplast membrane, remove only hardly during plastid isolation and remain in the fractions (Schröter *et al*. 2010). Compared to only two identified ribosomal proteins with one of them cytosolic in Schröter *et al*. 2010 the 14 plastid and 2 cytosolic ribosomal subunits of the PC fractions display the immense enrichment of nucleotide binding proteins through the combination of both chromatographic steps (Table 1, Schröter *et al*. 2010; Table 1, Schröter *et al*. 2014).

Further translation associated proteins were identified here too. Divers plastid and cytosolic translation initiation factors (IF) were found in the PC fractions (Table 1, Schröter *et al*. 2014). The plastid translation elongation factor Tu (EF-Tu) was found in the HS and the PC fractions (Table 1, Schröter *et al*. 2010; Table 1, Schröter *et al*. 2014). Additionally the cytosolic EF1-alpha4 was found among the PC proteins (Table 1, Schröter *et al*. 2014). The cytosolic translation factors as well as the SpoU methylase and the two Alpha-NAC-like proteins identified here represent most likely cytosolic contaminations, which are easy to enrich because of their nucleotide binding nature (Schröter *et al*. 2014). An exception is the eukaryotic translation initiation factor 3f (eIF3f) found in PC fractions (Table 1, Schröter *et a*l. 2014). This original cytosolic subunit of the eIF3 is involved in plenty regulatory events of the basic cell development which exceed its classic function as translation factor (Xia *et al*. 2010). Moreover this cytosolic factor contains a cTP and is most probably a real plastid protein beside it was previously found in plastid nucleoids (Huang *et al*. 2013; Schröter *et al*. 2014).

11.8 Protein homeostasis related proteins

Closely connected to translation is the generation of the right protein folding. Thereby molecular chaperons like heat shock proteins play a crucial role but mostly comprising additional functions as well. Also protein degrading proteins belong to the group "protein homeostasis" as found in the HS fractions (Schröter *et al*. 2010). An amount of 13% proteins were identified dedicated to protein homeostasis among the HS proteins and only 5% remain after PC chromatography (Table 1, Schröter *et al*. 2010; Table 1 and Fig. 3 Schröter *et al*. 2014). This reduction displays the enforced selective purification of nucleotide binding proteins *via* the second chromatographic step. Any of these proteins represent clear cytosolic compounds which are presumably co-purified with the cytosolic ribosomes attached to the nascent polypeptide chains as they emerge from the ribosome. There are several cytosolic chaperones binding in that manner during translation proceeds like Hsp70, Hsp40 and TCP1 (Frydman *et al*. 1994; McCallum *et al*. 2000; Etchells *et al*. 2005).

11.9 Proteins involved in photosynthesis

A number of photosynthesis related proteins were identified in Schröter *et al*. 2010 (Table 1). Totally 28% of all proteins of the HS fractions may be dedicated to this group (Table 1, Schröter *et al*. 2010; Fig. 3, Schröter *et al*. 2014). These proteins are constitutional compounds of plastid samples and represent contaminants which co-purify because of their large amount in the starting material and/or affinity to the negatively charged column material. The amount of photosynthesis related proteins could be reduced by PC chromatography to only 7% (Fig. 3, Schröter *et al*. 2014). The subunits of the chloroplast ATP synthase are observed after HS and PC protein purification (Schröter *et al*. 2010; Schröter *et al*. 2014). They are good to enrich with the used chromatographic methods because of their ATP binding nature. But additionally they are also components of the nucleoids of *A. thaliana* leafs prepared by Huang *et al*. (2013). Same is applicable for the RubisCO activase with an ATPase function and linked nucleotide binding domain. The RubisCO activase and also the Rieske protein was detectable in nucleoid preparations of *A.*

thaliana before (Huang *et al*. 2013). The Lhc proteins, the RubisCO and some other calvin cycle proteins of the HS fractions were removed by PC chromatography (Schröter *et al*. 2010; Schröter *et al*. 2014).

11.10 Other identified proteins in HS and PC fractions

The remaining proteins have miscellaneous functions in plants and were dedicated to one group called "others". Also within these proteins a strong reduction is noticeably between 28% HS and 22% PC proteins (Table 1, Schröter *et al*. 2010; Table 1 and Fig. 3, Schröter *et al*. 2014). Most of the proteins of this group in Schröter *et al*. 2010 belong to the vacuolar ATP synthase which has an apparent affinity to the HS column material like the chloroplast ATP synthase too. Nevertheless all of these vacuolar ATP synthase subunits were removed by PC chromatography (Table 1, Schröter *et al*. 2010; Table 1, Schröter *et al*. 2014). Also removed by PC chromatography are the chloroplastic phospho-glucose-isomerase, the general regulatory factors (GF14) and the 2-Cys peroxiredoxins found in Schröter *et al*. 2010 (Table 1, Schröter *et al*. 2010; Table 1, Schröter *et al*. 2014).

Five of the 13 "other" proteins of Schröter *et al*. 2014 contain a predicted cTP and thus represent true plastid proteins. Namely these are MDH, CAC3, FabZ, cysteine synthase and PSAT (Table 1, Schröter *et al*. 2014). They seem not to be directly involved in gene expression but all five were formerly identified in nucleoid fractions of *A. thaliana* chloroplasts (Huang *et al*. 2013). MDH, CAC3, PSAT and the cysteine synthase are somehow involved in processes which require ATP, NAD+ or PLP for their reactions and thus whether inoculate a domain for binding well to the phosphate groups of the used column material or even show an affinity to nucleotides and acidic compounds caused by their whole nature.

The nine remaining proteins with different functions seem to be contaminations of other compartments since they lack a cTP. However five of them where identified in plastid fractions before. The MFP2 was previously found in the *S. alba* plastids, *A. thaliana* Stroma and even isolated nucleoids (Schröter *et al*. 2010, Huang *et al*. 2013, Nishimura *et al*. 2013). The Myrosinase was identified in *Arabidopsis* plastoglobules, thylakoids, stroma and nucleoids (Giacomelli *et*

al. 2006; Zybailov *et al.* 2008; Zybailov *et al.* 2009; Lundquist et al. 2012, Huang *et al.* 2013, Nishimura *et al.* 2013). SHMT 1 and 2 are previously found in plastids and plastid stroma fractions and SHMT 1 additionally in nucleoids and plastoglobules (Gulas *et al.* 2006, Huang *et al.* 2013; Lundquist *et al.* 2013; Nishimura *et al.* 2013). Finally CRU3 was shown to be compound of *Arabidopsis* plastoglobules (Lundquist *et al.* 2013). Thus a plastid localisation of these five proteins can't be excluded as well as an involvement of some of them in gene expression caused by their identification in plastid nucleoids. Disregarding the potential affiliation to nucleoids MFP2, SHMT 1 and 2 and CRU3 are possibly nucleotide binding because of the phosphate residue containing energy carrier they use.

Only three identified proteins of Schröter *et al.* 2014 remain as real contaminants without cTP and any hint for chloroplast localisation. P5CR with a NAD(P) binding domain, MLS and Actin as structure element with high affinity to ATP. These proteins as part of HS fractions in unidentifiable amount were enriched through PC chromatography to a sufficient quantity.

11.11 Proteins with plastid affiliation

The transit peptide (TP) as attribute of proteins imported into plant cell organelles and essential for the passage across organelle membranes gives us a hint for the protein localisation. It is detectable with prediction programs like TargetP (Emanuelsson *et al.* 2000). A plastid TP was predicted for 25 of the 47 identified proteins of the HS fraction and 36 of the 58 identified proteins of the PC fractions contain a TP as well. Further on 7 of the HS and 4 of the PC proteins were plastid encoded (Schröter *et al.* 2010; Schröter *et al.* 2014). This denotes a high degree of plastid proteins in the isolated fractions. The exclusion of all probably non plastidic proteins as contaminations will turn the picture further on. For the results in Fig. 3 C of Schröter *et al.* 2014 only proteins with plastid transit peptide (cTP) or plastid origin were used. At the end 30% of the identified proteins of PC fractions with clear plastid affiliation are involved in transcription. As implicated in translational processes 45% were identified, 2% are involved in protein homeostasis, 10% are photosynthetic and 13% other proteins. Unfortunately this target sequences are not that similar, varying in

length and amino acid composition. Thus some potentially plastid proteins were predicted to belong to mitochondria or even as not-targeted to any organelle. Problems occur also concerning dual targeting proteins with transit peptides for both, mitochondria and plastids. Therefore the number of real plastid proteins is most probably higher than calculated.

11.12 Conclusion

This work participates on the elucidation of the plastid redox signal transmission to plastid gene expression as fundamental process in plant acclimation to environmental changes. An effect of plastid redox signals on the expression of essential plastid genes was examined as well as an involvement of phosphorylation and thioredoxin mediated redox signal transduction. Within this work a fundamental contribution to the investigation of the plastid gene expression machinery and its constituents was achieved by establishing mustard as additional model organism with huge advantages for complex biochemical approaches.

Abstract

The actual keen about redox signal development at the plastid photosynthetic apparatus, transmission and the reply to the signal was highlighted by the contribution of three review article to this work. Pfannschmidt *et al*. 2008 summarizes short and long term acclimation responses (STR and LTR respectively) to redox signals of the Plastoquinone (PQ) pool and the involvement of putative phosphorylation cascades and thioredoxins as well as the influence of the redox state on primary target genes in plastids and nucleus. Further on experimental approaches for the generation of a defined redox state at the photosynthetic electron transport (PET) chain was discussed. Dietzel *et al*. 2008 reviews the different types of retrograde signals between plastids and nucleus as well as the complexity and interaction of the signaling cascades and networks and in Pfalz *et al*. 2012 the environmental influences on gene expression and recent findings within plastid redox signaling were discussed.

For a detailed investigation of the adaption of plastid gene expression responding to plastid redox signals the gene expression machinery of chloroplasts itself was studied. An experimental approach was used for the generation of a defined redox signal in mustard cotyledons, the following isolation of its chloroplasts and further on the nucleotide binding sub-proteome using heparin-Sepharose (HS) (Steiner *et al*. 2009; Schröter *et al*. 2010). The characterization and comparison of mustard cotyledons acclimated to redox signal inducing Light-qualities with *Arabidopsis thaliana* cotyledons was important for the integration of new findings within *Sinapis alba* into established models (Steiner *et al*. 2009). An effect on the transcriptional regulation of the two plastome-encoded genes *psaAB* and *psbA* was studied here concerning promoter recognition and specificity (Steiner *et al*. 2009). The impact of phosphorylation events on gene expression was surveyed and confirmed by determination of the phosphorylation state of the HS fractions, the endogenous kinase activity and the cooperative influence of kinase activity and thiol redox state on Chloroplast transcription (Steiner *et al*. 2009).

HS proteins fractions contain a high degree of DNA and especially *psaA* and *psbA* binding proteins which were identified using mass spectrometry and *Brassicales* databases (Steiner *et al*. 2009; Schröter *et al*. 2010; Steiner *et al*.

2011). Special emphasis was on the analysis of the essential subunits of the plastid-encoded plastid RNA-polymerase (PEP) which was well to prepare by 2 dimensional (2D) blue native (BN) gel electrophoresis (Schröter *et al*. 2010; Steiner *et al*. 2011). The degree of proteins involved in gene expression was strongly increased by the use of a second chromatographic step with Phosphocellulose (PC) additional to HS (Schröter *et al*. 2014). Visualization and identification of this nucleotide binding sub-proteome was the aim of the last publication included into this work giving access to a precise view on the gene expression related proteome of mustard plastids (Schröter *et al*. 2014).

Zusammenfassung

Drei *review* Artikel beleuchten das aktuelle Wissen über die Redoxsignalentwicklung im plastidären Photosyntheseapparat, die Signalübermittlung und –beantwotung. Pfannschmidt *et al.* 2008 fasst Kurzzeit- und Langzeitantworten auf Redoxsignale des Plastochinonpools zusammen und darüberhinaus die Einbeziehung von Phosphorylierungskaskaden und Thioredoxinen sowie den Einfluss des Redoxstatus auf primäre Zielgene in Plastiden und dem Zellkern. Desweiteren wurden experimentelle Ansätze für die Erzeugung eines definierten Redoxstatus in der photosynthetischen Elektronentransportkette diskutiert. Dietzel *et al.* 2008 fasst die verschiedenen Typen retrograder Signale zwischen Plastiden und Zellkern zusammen sowie die Komplexität und Interaktion der Signalkaskaden und –netzwerke und in Pfalz *et al.* 2012 werden die Umwelteinflüsse auf die Genexpression und aktuelle Erkenntnisse über Redoxsignale diskutiert.

Für eine detailierte Untersuchung der Genexpressionsadaption als Antwort auf plastidäre Redoxsignale wurde die Genexpressionsmaschinerie der Chloroplasten direkt studiert. Ein definiertes Redoxsignal wurde in Senfkeimlingen generiert, anschließend die Chloroplasten und schließlich die nukleotidbindenden Proteine mittels Chromatographie über Heparinsepharose (HS) isoliert (Steiner *et al.* 2009; Schröter *et al.* 2010). Der Vergleich und die Charakterisierung der Senfkeimlinge, die an das Redoxsignal induzierende Licht akklimatisiert waren, mit *Arabidopsis thaliana* Keimlingen war wichtig für die Integration neuer Erkenntnisse über *Sinapis alba* in etablierte Modelle (Steiner *et al.* 2009). Der Effekt auf die transkriptionale Regulierung der zwei plastomkodierten Gene psaAB und psbA wurde hinsichtlich Promotererkennung und –spezifität untersucht (Steiner *et al.* 2009). Die Auswirkung von Phosphorylierungen auf die Genexpression wurde, durch die Bestimmung des Phosphorylierungsgrades der HS Fraktionen, der endogene Kinaseaktivität und des kooperativen Einflusses der Kinaseaktivität und des Thiolredoxstatus auf die Chloroplastentranskription, untersucht (Steiner *et al.* 2009).

HS Fraktionen besitzen einen hohen Grad an DNA- und speziell *psaA*- und *psbA*-bindenden Proteinen, die durch Massenspektrometrie und Analyse mit *Brassicales*-Datenbanken identifiziert werden können (Steiner *et al.* 2009;

Schröter *et al*. 2010; Steiner *et al*. 2011). Der Schwerpunkt lag bei der Analyse der essentiellen Untereinheiten der plastidenkodierten plastidären RNA-Polymerase (PEP), die gut durch 2 dimensionale (2D) *blue native* (BN) Gelelektrophorese präpariert werden konnte (Schröter *et al*. 2010; Steiner *et al*. 2011).

Der Anteil an Proteinen der Genexpression konnte durch eine zweite Chromatographie über Phosphocellulose (PC) zusätzlich zur HS-Chromatographie erzielt werden (Schröter *et al*. 2014). In der letzten Publikation dieser Arbeit geht es vorrangig um die Visualisierung und Identifizierung dieses nukleotidbindenden Teilproteoms, wodurch ein Zugang zu einem detaillierteren Einblick in das genexpressionsrelevante Proteom der Senfplastiden erzielt wurde (Schröter *et al*. 2014).

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Eigenständigkeitserklärung

Mir ist die geltende Promotionsordnung der Biologisch Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena bekannt. Ich erkläre, dass ich die vorliegende Arbeit selbständig verfasst und nur unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe. Bei der Bearbeitung des Manuskriptes wurde ich von Prof. Dr. Thomas Pfannschmidt unterstützt, die zugrundeliegenden Publikationen basieren auf der Arbeit der angegebenen Autoren. Es wurden weder Promotionsberater in Anspruch genommen noch geldwerte Leistungen für Arbeiten im Zusammenhang mit den Inhalten dieser Dissertation vergeben. Diese Dissertation wird erstmalig an einer Hochschule eingereicht.

Jena, den 28.08.2014

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Yvonne Schattschneider

Scientific publications and presentations

Articles

Pfalz J, Liebers M, Hirth M, Grübler B, Holtzegel U, Schröter Y, Dietzel L, Pfannschmidt T. Environmental control of plant nuclear gene expression by chloroplast redox signals. Front Plant Sci. 2012 Nov 19;3:257.

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Talks

23.01.2009: 7. Mitteldeutsche Pflanzenphysiologie-Tagung in Halle
“Analysis of the nucleotide binding sub-proteome of plastids by mass spectrometry “

08.10.2008: 12. Jahrestagung der Deutschen Sektion der Internationalen Gesellschaft für Endocytobiologie in München
“Identifikation plastidärer Proteine durch Massenspektrometrie”

23.2.2008: 6. Mitteldeutsche Pflanzenphysiologie-Tagung Jena
“Identification of plastid proteins by mass spectrometry”

Posters

06.09.-10.09.2009 Botanikertagung 2009 in Leipzig
“Analysis and identification of the nucleotide binding sub-proteome of plastids by mass spectrometry”
Yvonne Schröter, Sebastian Steiner, Lars Dietzel and Thomas Pfannschmidt

17.02.-20.02.2009 Pflanzenmolekularbiologie-Tagung 2009 in Dabringhausen
„Analysis of the nucleotide binding sub-proteome of plastids by mass spectrometry”
Yvonne Schröter, Sebastian Steiner, Lars Dietzel and Thomas Pfannschmidt

25.06.-27.06.2007 Tagung: "Redox signal integration: From stimulus to networks and genes" in Bielefeld
„Identification of redox-regulated DNA-binding proteins in chloroplasts of higher plants by mass spectrometry“
Yvonne Schröter, Sebastian Steiner and Thomas Pfannschmidt

03.09-07.09.2007 Botanikertagung 2007 in Hamburg
“Identification of redox regulated DNA-binding plastid proteins through mass spectrometry”
Yvonne Schröter, Sebastian Steiner and Thomas Pfannschmidt