GET IN TUNE:
Chloroplast and Nucleus Harmony

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“We are not to tell nature what she's gotta be. ... She's always got better imagination than we have”.

Richard Feynman
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Abstract:

Photosynthetic eukaryots emerged as a result of several billion years of evolution between proeukaryotic cell and ancestral cyanobacteria that formed modern chloroplasts. The symbiotic relationship led to significant rearrangements in the genomes of the plastid and the nucleus: as many as 90% of all the plastid genes were transferred to the nucleus. The gene transfer has been accompanied by the development of sophisticated regulatory signaling networks originating in the organelle (retrograde) and in the nucleus (anterograde) that coordinate development of the plastid and ensure adequate cell responses to stress signals.

In this thesis I have demonstrated that transcriptional activity of PEP in the chloroplast is essential for proper embryo and seedling development in *Arabidopsis thaliana*. The function of PEP is dependent on the nuclear encoded PEP-associated factor PRIN2 that is able to sense the redox status of the plastid during seedling development and different stress. In response to the plastid status PRIN2 modulates the transcription activity of the PEP enzyme complex. We further established that PRIN2, as an essential component for full PEP activity, is also required to emit the Plastid Gene Expression (PGE) retrograde signal to regulate the Photosynthesis-Associated Nuclear Genes (PhANG) in the nucleus during early seedling growth via GUN1. On the other hand, regulation of PhANG expression during the High Light (HL) conditions requires functional PRIN2 and PEP activity but is GUN1-independent. Another retrograde signal produced by the developing chloroplast is associated with the tetrapyrrole biosynthesis pathway. We have established that accumulation of the chlorophyll intermediate MgProtoIX-ME in the *crd* mutant triggers repression of the PhANG expression, and this negative signal is mediated by a cytoplasmic protein complex containing the PAPP5 phosphatase. The nuclear targets that receive the tetrapyrrole mediated signal are GLK1 and GLK2 transcription factors that control the PhANG expression and the expression of the enzymes involved in the biosynthesis of chlorophyll.

**Keywords:** *Arabidopsis thaliana*, chloroplast, development, gene expression
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Titel: I Samklang: harmoni mellan cellens kloroplaster och kärna

Sammanfattning:


Den här avhandlingen beskriver kloroplastens maskineri för genuttryck (PEP) som en nödvändig komponent för embryo- och växtutvecklingen hos Arabidopsis thaliana. PEP funktionen är beroende av det kärnkodade kloroplastproteinet PRIN2 som är associerat med PEP. PRIN2 mottar redox signaler från plastiden och förändrar genuttrycksaktivitet under kloroplastens utvecklingen eller under olika stressförhållanden. Jag visar dessutom att PRIN2 spelar en viktig roll i överföring av kloroplastens signal som kommunikerar genuttrycksaktivitet (PGE) via GUN1 till kärnan där den styr uttryck av de kärnkodade fotosyntesgenerna (PhANG). Under högljus stressförhållanden styrs dock PhANG-uttrycket av signaler som uppstår från PEP-aktivitet och PRIN2 men som är oberoende av GUN1. Vidare finns det ett annat retrograd signal som har sitt ursprung i biosynthesen av tetrapyrroler. Jag har visat att ackumuleringen av tetrapyrrolen MgProtoIX-ME i crd-mutanten framkallar nedreglering av PhANG-uttryck genom interaktion med ett fosfatas (PAPP5) i cytosolen. GLK1 och GLK2 är två transkriptionsfaktorer som tar emot den tetrapyrrole-medierade signalen i sin tur styr biosyntes av chlorofyll och PhANG uttryck.
List of papers


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Preface

One nice evening in 2013 I gathered with my good old friends and out of the blue received the present: concert film “Celebration Day” by the famous English rock band Led Zeppelin. I immediately suggested to watch it. After band’s drummer (Bonham) died in 1980 Led Zeppelin broke up and thought to be never again together. For many years to see the famous band performing live on stage was just a dream. In 2007 that dream came true, Led Zeppelin played the only show in London O2 Arena with Bonham’s son on the drums. Apparently that was their last performed show. We watched the whole DVD with open-eyed amazement. Such a brilliant performance! Like they have never been apart. All instruments sounded harmonious creating the marvellous, tremendous masterwork. With no obvious leaders in the band, how could four dudes possibly know when to start the note and which tune to play? The answer is simple in theory. They listen to each other and continuously adjust their tunes according to what they hear. That might sound terribly hard, but trust me, it is way easier than trying to play in a band where one of the members’ tune is substituted by the recording. With no chance to adjust the melody or the speed of “non-responding” band member, the whole song turns to be really artificial and static. In fact, most of the time… it simply doesn’t work… at all.

Richard Feynman once mentioned: “In this age of specialization men who thoroughly know one field are often incompetent to discuss another”. I always felt that the more we expand our knowledge in different areas the more chances and possibilities we have to find the clues in order to resolve facing problems. That night in 2013 with the help of the music I found the answer to the question that I faced in plant science during my PhD. The question was “How?” How does the plant cell that has several organelles and the nucleus deal with regulation of their activities? Who is the leader among them? How does it work? Apparently, there is no leader: organelles and nucleus listen to each other and continuously adjust their activities according to what they hear. So make yourself comfortable and let’s see how they actually do it.

Jimmy Kremnev
Umeå, October 2014
INTRODUCTION

ORIGIN OF THE CHLOROPLAST

"Life did not take over the globe by combat, but by networking" (Margulis and Sagan, 1986).

In the late 80’s the basis for life systematics dramatically changed due to the molecular evolution of data based on comparisons of rRNA sequences. Analysis of that data revealed a previously unknown phenomena: prokaryotes are clearly separated into Bacteria and Archaea that show as much difference between each other as between Eukaryota (Balch et al., 1977). Moreover, Eukaryotes appeared to be more closely related to Archaea than to Bacteria. The evidence provided led to the widespread acceptance of the model of universal phylogenetic tree with three domains of life: Bacteria, Archaea, and Eukaryota that was first proposed by Carl Woese (Woese et al., 1990). Increasing amounts of sequence data has also provided new information that strengthened yet another evolutionary theory, first suggested by Konstantin Mereschkowsky in 1905 (Martin and Kowallik, 1999) and further developed by Lynn Margulis: the theory of endosymbiosis (Sagan, 1967).

The endosymbiosis theory states that eukaryots appeared as a result of several billion years’ evolution between the proeukaryotic cell (that shared similarities with some Archaea species) (Horiike et al., 2001; Staub et al., 2004) and the free-living proteobacteria/cyanobacteria ancestors that gave rise to modern organelles: mitochondria/chloroplast. Indeed, several lines of evidence indicate this: mitochondria and plastids divide by binary fission (Nakanishi et al., 2009); some of the lipids of the inner organelle membrane are found exclusively in bacterial cell membrane (Jarvis et al., 2000); organelles have bacteria-like biochemistry and prokaryotic-based
replication, transcriptional, translational machinery (Liere et al., 2011); mitochondria/chloroplasts and bacteria have similar genome organization and high gene homology (Martin et al., 2002). Moreover there are examples of early stages of endosymbiosis that take place nowadays. One of the most intensively studied is Paulinella chromatophora that has acquired a plastid from a different lineage of cyanobacteria than did other plastid-containing organisms (Marin et al., 2005; Yoon et al., 2009). In another example, the recently isolated Hatena flagellate has an algal endosymbiont from the genus Nephroselmis during certain developmental stages. That is followed by the drastic reorganization in morphology and possibly physiology of both partners (Figure 1) (Okamoto and Inouye, 2005; Gould et al., 2008). The giant amoeba Pelomyxa polustris lacks mitochondria but it has Methanobacterium formicicum methanogenic bacterial endosymbionts instead that perform anaerobic respiration (Whatley, 1976; Vanbruggen et al., 1988). Numerous anaerobic ciliates have methanogenic archaea in their cytoplasm and demonstrate different degrees of metabolic interactions (van Hoek et al., 2000). Together with examples of endosymbiotic relations between protists and their eukaryotic endosymbionts (Nowack and Melkonian, 2010) as well as symbionts of multicellular organisms (Dubilier et al., 2008; Moya et al., 2008) it becomes clear that endosymbiotic interactions are widespread in nature and beneficial for organisms. The appearance and evolution of oxygen-producing photosynthetic eukaryotes, including land plants, had global geochemical and ecological impact. However, it is not yet clear how this crucial endosymbiotic relationship between proeukaryote and ancestral oxygenic bacteria has became established.
What might have been the driving forces that led to this tight cooperation? It is possible to imagine the short term benefits that the two organisms could have acquired from their cooperation, such as secreted metabolites and nutrients (Figure 2) (López-Garcia and Moreira, 1999; Huber et al., 2002; Gould et al., 2008). On the other hand, long-term forces that brought the partners to a deeper level of integration may have included shared metabolic pathways (Müller and Martin, 1999; Zomorodipour and Andersson, 1999) and genetic interconnection (Waters et al., 2003; Podar et al., 2013). The chloroplast ancestor possibly was beneficial for the eukaryotic partner not only by providing carbohydrates through photosynthesis, but by also providing O$_2$ to mitochondria that increased ATP production (Kurland and Andersson, 2000).
Photosynthesis is one of the main functions performed in most of the contemporary chloroplasts. Solar light represents a broad spectrum of wavelengths, however only the 400-700nm spectral region is referred to as Photosynthetically Active Radiation (PAR) and is used for photosynthesis (Mccree, 1972). Light absorption occurs by so called Light Harvesting Antenna Complexes (encoded by LHCA and LHCB genes) and energy transfer within them is funnelled towards the two Reaction Centers (RC) where charge separation takes place (Cheng and Fleming, 2009). RCI and RCII together with antenna complexes constitute PSI and PSII photosystems that are specifically excited by Light I (700 nm) and Light II (680 nm) respectively (Allen, 2003). PSII receives electrons from the H₂O molecules.
Chlorophyll is a vital co-factor of light harvesting antenna proteins and a core component of the two photosystems. Chlorophyll belongs to the family of molecules called tetrapyrroles. Apart from chlorophylls, other tetrapyrroles synthesized mainly in plastids are heme, siroheme and phytochromobilin (Tanaka and Tanaka, 2007). Due to the presence of multiple conjugated double bonds, all tetrapyrroles are easily excited by light and are major sources of ROS production. It is therefore not surprising that tetrapyrrole biosynthesis is tightly regulated at several steps (Matsumoto et al., 2004; Tanaka and Tanaka, 2007). The first enzyme of the pathway, GlutRNA reductase, is controlled by heme and Protochlorophyllide (Pchld) through feedback mechanisms (Goslings et al., 2004). The branch point of the pathway where ProtoIX is funnelled either to chlorophyll or the heme biosynthesis is an essential site for regulation: Mg-chelatase is regulated by the ATP/ADP ratio (Cornah et al., 2002), Mg$^{2+}$ ions (Reid and Hunter, 2004), GUN4 protein (Davison et al., 2005) and TRX (Jensen et al, 2000); while Fe-chelatase is under control of the ATP/ADP ratio (Cornah et al., 2002). Moreover, the formation of Chlorophyllide from Protochlorophyllide (Pchld) is driven by the activity of the POR enzyme which is light-dependent (Forreiter and Apel, 1993). Several enzymes of the tetrapyrrole biosynthesis pathway are localized to the plastid membranes; therefore stress factors that affect the integrity of the plastid membrane also inhibit their function (Tanaka and Tanaka, 2007). In addition, enzymes of the pathway are
regulated at the level of their expression and also by the import into the
chloroplast (Matsumoto et al., 2004; Boij et al., 2009), since the respective
proteins are encoded in the nucleus. The chloroplast proteome has
approximately 3000-4000 proteins, compared to the proteome of the modern
closely related bacteria species belonging to the *Synechococcus–*
*Prochlorococcus* clade (Reyes-Prieto et al., 2010), the plastid genomes
contain only 5-10% of the genes present in the respective bacterial species
(Richly and Leister, 2004). This discrepancy is attributed to the fact that
bacterial genes were lost and others were transferred by linear gene transfer
to the nucleus during the course of evolution (Martin et al., 2002; Timmis et
al., 2004; Gould et al., 2008). Another group of genes that contribute to the
plastid proteome represent novel proteins of eukaryotic origin that perform
new functional and regulatory roles in the plastid. All the proteins that
constitute the chloroplast proteome and are encoded in the nucleus have by
necessity acquired a transit peptide sequence that enables them to be
targeted into the chloroplast (Richly and Leister, 2004; Sakamoto et al.,
2008). The genes of bacterial origin that have been retained in the plastid are
involved in either photosynthesis or in housekeeping functions (Martin et al.,
2002; Wakasugi et al., 2001). These must be transcribed and translated in the
plastid within so called nucleoid.

**CHLOROPLAST TRANSCRIPTION**

Chloroplast nucleoids, large protein/DNA complexes, are analogous to the
nucleoids of bacteria. There are as many as 20 nucleoids per chloroplast,
each containing 10-20 copies of plastid DNA (Kuroiwa, 1991) that exhibit
typical beads-on-the-string structure (Figure 3) (Ohniwa et al., 2007).
Proteoimic analysis of purified nucleoids from proplastids and chloroplasts
has identified numerous proteins involved in DNA/RNA/protein metabolism
(Majeran et al., 2012). These are involved in maintaining chloroplast
genome stability, organization, DNA replication, repair as well as proteins involved in RNA metabolism and translation. Contrary to the eukaryotes, bacteria DNA replication, transcription and translation events occur concurrently therefore optimizing all steps of gene expression (Mandal and Breaker, 2004; Merino and Yanofsky, 2005). In addition these processes occur at particular stages of the cell cycle (Srivatsan and Wang, 2008; Merrikh et al., 2012).

Due to their cyanobacterial origin, chloroplasts have inherited a bacterial type of transcriptional machinery, the so called PEP (Plastid Encoded Polymerase). PEP is a multimeric enzyme: the core components are encoded by rpoA, rpoB, rpoC1 and rpoC2 in the chloroplast genome, while six sigma factors (SIG1-SIG6) that recognize promoter sequences are nuclear encoded (Hedtke et al., 1997, Hedtke et al., 2000; Puthiyaveetil et al., 2010).

![Figure 3](image-url). Structure of chloroplast genome from spinach. Each nucleoid contains 30nm beads and 70 nm fiber structures composed of 30 nm beads (Ohniwa et al., 2007). Reprinted with kind permission from Cambridge University Press.

SIG1 targets are psaAB, psbBT, psbEFLJ operons and rbcL and clpP genes (Hanaoka et al., 2012); SIG2 is responsible for transcription of tRNA genes (Kanamaru et al., 2001); SIG3 and SIG4 are required for psbN (Zghidi et al., 2012).
2007) and ndhF (Favory et al., 2005) gene expression, respectively; SIG5 recognizes a specific psbD light responsive promoter (Tsunoyama et al., 2004) and has been shown to be involved in multiple stress responses in Arabidopsis (Nagashima et al., 2004); SIG6 is required for transcription of a wide range of photosynthetic genes and regulates the early stages of chloroplast development (Ishizaki et al., 2005; Lerbs-Mache, 2011).

Apart from the sigma factors that are required for RNA polymerase activity, 40-60 additional subunits (pTACs) encoded in the nucleus have been recruited to PEP during the course of evolution in land plants (Suzuki et al., 2004; Pfannschmidt et al., 2000; Pfalz et al., 2006; Steiner et al., 2011; Melonek et al., 2012). These additional proteins are apparently structural and regulatory subunits (Pfannschmidt and Liere, 2005; Reiss and Link, 1985; Pfannschmidt and Link, 1994; Suck et al., 1996); and the corresponding pTAC mutants are either significantly impaired in development or exhibit a seedling lethal phenotype resembling the ∆rpo mutants of tobacco (Hajdukiewicz et al., 1997). Proteomic studies demonstrated that 10 pTACs are indispensable subunits of PEP while others might be loosely and/or temporarily associated with the complex (Steiner et al., 2011; Pfalz and Pfannschmidt, 2013).

Interestingly, PEP is not the only RNA polymerase present in the plastid. From early studies on ribosome-deficient Hordeum mutants (Hess et al., 1993), iojap mutants of maize (Han et al., 1992) and ∆rpo mutants of tobacco (De Santis-MacIossek et al., 1999) it became clear that there are two additional RNA polymerases (RpoTp and RpoTmp) called NEP (Nuclear Encoded Polymerase) belonging to the same protein family. NEP is a single polypeptide enzyme related to the T7 RNAP of bacteriophages (Hedtke et al., 1997; Weihe and Borner, 1999; Hedtke et al., 2000; Courtois et al., 2007); and inactivation of both RpoT activities leads to abnormal plant
phenotypes and arrested seedling development (Hricova et al., 2006). RpoTp
is present in the chloroplast, while RpoTmp is targeted to both chloroplasts
and mitochondria. Interestingly, though increased expression of RpoTmp
gene has been observed in the sca3 mutant lacking the RpoTp protein, it was
not able to recover the phenotype of the mutant, suggesting that RpoTp and
RpoTmp are only partially redundant (Emanuel et al., 2004; Hricova et al.,
2006). Indeed RpoTmp specifically transcribes the rrn operon, while RpoTp
has a broader range of target genes (Courtois et al., 2007; Azevedo et al.,
2008). Moreover, RpoTp is expressed to similar levels both in cotyledons
and leaf tissue, whereas RpoTmp was mostly expressed only at the early
stages of seedling development (Emanuel et al., 2004, Hricova et al., 2006).
A different sub-plastidial localization of RpoTmp and RpoTp has also been
suggested and while both are membrane bound, RpoTmp is also found in the
stroma of etioplast (Azevedo et al., 2006; Azevedo et al., 2008).

PEP and NEP recognize different promoter sequences in the plastid genome
and vary in transcriptional activity. Indeed, PEP requires the typical bacterial
type -35 (TTGaca); -10 (TAtaaT) promoter elements (Shiina et al., 2005).
On the other hand, four distinct promoters have been characterized for NEP:
Type Ia, Type Ib, Type II (Swiatecka-Hagenbruch et al., 2007) and the
recently identified Pc elements in certain tRNAs and rrn operons (Liere et
al., 2011; Swiatecka-Hagenbruch et al., 2007). Recent genome-wide
mapping of TSSs (Transcriptional Start Site) in barley demonstrated that
most of the plastid genome, including the photosynthesis genes, have
multiple promoters for both PEP and NEP. Many photosynthesis-related
genesis that were previously thought to be solely expressed by PEP appear to
have active NEP promoters and can be transcribed with almost identical 5’
and 3’ ends as from a PEP promoter (Legen et al., 2002; Swiatecka-
Hagenbruch et al., 2007; Zhelyazkova et al., 2012). To date just accD and
rpoB operon are thought to be exclusively expressed by NEP (Hajdukiewicz
et al., 1997). The \textit{rpoB} operon encodes core subunits of PEP, it is therefore feasible that NEP activity is required for establishing the fully functional PEP complex (Emanuel et al., 2004).

Although the majority of the chloroplast genome can be transcribed by either PEP or NEP, in mature chloroplasts PEP activity is predominant. On the other hand, NEP is more active in non-green tissue and during early plastid development (Demarsy et al., 2006; Emanuel et al., 2006; Courtois et al., 2007; Zoschke et al., 2007). These observations indicate that there might be an interplay between PEP- and NEP-dependent transcription during plastid development or in response to environmental cues. A more complex picture emerges as some PEP- and NEP-type promoters appear to be overlapping, suggesting possible competition between PEP and NEP for binding sites (Swiatecka-Hagenbruch et al., 2007). Although impaired PEP-dependent transcription initiates compensatory gene expression driven by NEP (Hanaoka et al., 2005; Zhelyazkova et al., 2012), the complete absence of either PEP or NEP activity results in seedling lethality (Hess et al., 1993; De Santis-MacIossek et al., 1999; Hricova et al., 2006; Pfalz et al., 2006). This might suggest that some chloroplast genes are either solely transcribed by one polymerase and/or transcriptional efficiency of either polymerase is not efficient enough to compensate for the loss of the other (Zhelyazkova et al., 2012).

As it is in prokaryotes, chloroplast genes are organized in operons and RNA transcripts are often polycistronic. Chloroplast transcripts are subjected to several post-transcriptional processes that are performed by nuclear-encoded factors: polycistronic transcript processing, splicing and RNA editing (del Campo et al., 2009). Polycistronic RNA transcribed from the single promoter is cleaved by RNase E and RNase J endoribonucleases associated with putative factors defining sequence-specificity (Li de la Sierra-Gallay et
al., 2008; Schein et al., 2008). Final 3’ ends are generated by PNPase exoribonuclease activity (Yehudai-Resheff et al., 2001) and 5’ end maturation has been attributed to the exoribonuclease activity of RNase J (Mathy et al., 2007), PPR10 (Pfalz et al., 2009) and possibly other 5’ RNA binding proteins (Barkan, 2011). However, in contrast to bacteria, many chloroplast transcripts of higher plants have introns; they therefore must be spliced prior to translation. Proteins belonging to two families have been demonstrated to play roles in splicing: CRM- and PPR-domain proteins (Schmitz-Linneweber and Small, 2008; Stern et al., 2010) but their exact mode of action is largely unknown. PPR family proteins are also involved in the RNA editing process that changes cytidine to uridine and often restores conserved amino acid codons and translation start sites (Chateigner-Boutin and Small, 2007). RNA editing is a complex mechanism and requires both specific PPR proteins and defined cis-binding elements within the editing site (Sasaki et al., 2006; Fujii and Small, 2011). Recent studies have uncovered that PPR proteins have a defined modular amino acid code that allows recognition of specific nucleotide sequences providing extraordinary variability of binding targets and plasticity (Barkan et al., 2012).

Regulation of plastid gene expression occurs at several stages and includes control by light (Finster et al., 2013), redox factors (Pfannschmidt Liere., 2005) and phosphorylation of essential components of the machinery (Steiner et al., 2009) (Figure 4).
Unbalanced excitation of PSI and PSII reaction centers generates a particular redox state of PQ pool that regulates the expression of PSI and PSII genes in order to adjust it to the current light quantity (Pfannschmidt et al., 1999; Li and Sherman, 2000; Tullberg et al., 2000). This signal has been proposed to be mediated via a complex phosphorylation cascade mechanism (Steiner et al., 2009). Phosphorylation of PEP as well as its promoter recognition subunits, SIG1 and SIG6 has been demonstrated (Baginsky et al., 1997; Baena-Gonzalez et al., 2001; Schweer et al., 2010; Shimizu et al., 2010). Kinase activities have been attributed to cpCK2 (PTK) (Ogrzewalla et al., 2002; Schweer et al., 2010) and the prokaryotic-type chloroplast sensor kinase CSK (Puthiyaveetil et al., 2008) that were shown to interact with each other in the yeast-two-hybrid assays (Puthiyaveetil et al., 2010). CSK was also shown to bind SIG1 (Puthiyaveetil et al., 2010) and csk and sig1 mutants exhibit similar expression profile of psaA and psbA genes under Light 1 and Light 2 that excite specifically PSI and PSII, respectively. Several phosphorylation sites were reported to be crucial for the function of SIG1 suggesting that CSK might be phosphorylating SIG1 and therefore
linking redox status of PQ pool and the transcription activity of PEP (Shimizu et al., 2010). Apart from the kinase-dependent signal there might exist a second redox signal dependent on thiol-activities associated with the PSI acceptor site (Steiner et al., 2009). Indeed, cpCK2 function was shown to be controlled by the redox state of glutathione (Ogrzewalla et al., 2002). Redox regulation of PEP is even more prominent since some of the PEP components might be directly involved. Two superoxide dismutases, FSD2 and FSD3 (Myouga et al., 2008), TRXz and its recently identified interacting partner thioredoxin-fold-like protein AtECB1/MRL7 that also binds FSD3, have been implicated in redox regulation of PEP activity (Yu et al., 2014). Absence of either of above-mentioned subunits leads to severe albino phenotype and down-regulation of PEP-dependent gene expression (Pfalz et al., 2006; Steiner et al., 2011). TRXz is unique among the TRXs in the chloroplast: although it displays reducing activity in vitro, it has very specific biochemical properties (Chibani et al., 2011; Bohrer et al., 2012). Mutation of crucial Cys residues in TRXz was shown in vivo to not be essential for PEP activity under normal growth conditions (Wimmelbacher and Börnke, 2014). That might suggest that TRXz is most likely a structural component of PEP or it has a particular function during stress responses and/or plastid development in dark-to-light conditions (Arsova et al., 2010). Identifying redox regulators associated with PEP, revealing their function and ability to receive redox signal from PET will increase our understanding on how photosynthesis is linked to the gene expression machinery of the chloroplast.

PLASTID DEVELOPMENT AND ANTEROGRADE SIGNALLING

As it was mentioned above, establishing the endosymbiosis most likely relied on the development of mutual interaction mechanisms between the partner organisms at biochemical and genetic levels, rather than just the
dependence of the endosymbiont on the eukaryotic host. This interaction is clearly demonstrated by the localization of several metabolic pathways to organelles and cytoplasm, complex import/export transporters (Fischer, 2011), structural/regulatory components of transcriptional apparatus (Liere et al., 2011) and anterograde/retrograde signalling loops that ensure stoichiometric assembly of protein complexes and the ability for their reorganization in response to environmental clues and developmental stage (Pogson et al 2008). Tuning the activities of the chloroplast and the nucleus is especially crucial during plastid development.

Chloroplast differentiation, development from proplastid present in meristematic tissues and etioplast in dark grown seedlings to the chloroplast, is a complex process and requires several factors: light and the coordinated action of both retrograde and anterograde signalling pathways (Solymosi and Schoefs, 2010; Pogson and Albrecht, 2011). In the absence of light, the undifferentiated proplastid develops into an etioplast that differs from mature chloroplast by the presence of a characteristic membrane complex (prolamellar body) and distinct pigment composition. Etioplasts have large quantities of Protochlorophyllide (Pchld), a chlorophyll precursor, within the prolamellar body. Pchld is present in two forms, so called non-photoactive and photoactive, and is complexed with the POR enzyme and NADPH (Schoefs and Franck, 2003; Masuda et al., 2003). POR is a light activated enzyme that catalyzes the fast reduction of Protochlorophyllide upon illumination, ensuring that chlorophyll biosynthesis occurs only in light (Lebedev and Timko, 1999). Interestingly, Arabidopsis and other oil seed plants show a particular developmental program, developing chloroplasts during embryogenesis. Functional chloroplasts are detected already at the globular stage of the embryo (Tejos et al., 2010). Indeed, during embryogenesis several sets of genes involved in photosynthesis and carbon fixation are up-regulated, reaching maximum expression levels at the mature
green stage (Spencer et al., 2007). Embryo chloroplasts were shown to fix CO₂ and supply the growing embryo with certain metabolites, energy and O₂ that are needed for various biosynthetic processes, respiration and essential for biomass accumulation (Goffman et al., 2005, Borisjuk et al., 2003; Rolletschek et al., 2003). Functional chloroplasts undergo transition back to the etioplasts during the post-mature embryo stage and remain so until the young seedlings detect light after germination (Rolletschek et al., 2003).

Perception of light in plants is mediated by photoreceptors belonging to phytochrome, cryptochrome and phototropin protein families (Chen et al., 2004). They activate signalling pathways that largely reorganize transcriptional profile in the nucleus and result in developmental changes within a plant cell (Briggs and Olney, 2001; Franklin et al., 2005). Several nuclear factors downstream of photoreceptors are essential for transmitting light signal. COP/DET/FUS proteins act as repressors of photomorphogenesis in darkness (Schwechheimer and Deng, 2000). Several identified cop/det/fus mutants carry mutations within the COP9 signalosome, a multisubunit complex involved in protein degradation (Hardtke and Deng, 2000). Another well-characterized cop/det/fus mutant, cop1, is involved in protein degradation of HY5. HY5 is a nuclear encoded bZIP transcriptional factor and a key positive regulator of light-induced development that binds to the G-box in promoters of light-inducible genes. HY5 acts as a central integrator of multiple photoreceptor signals and directly influences the expression of about 1100 nuclear genes, with 4 of them involved in chloroplast development (Zhang et al., 2011). However, known targets of HY5 also include other transcription factors, and it has been also shown that HY5 is involved in several processes besides photomorphogenesis (Lee et al., 2007b; Cluis et al., 2004). More specific photomorphogenesis related transcription factors downstream of photoreceptors are members of PIF family (Leivar et al., 2012). These transcription factors also bind to the G-
box element but act as repressors of photomorphogenic development in darkness. It has been suggested that HY5 and PIFs may function as an activator/suppressor transcriptional module targeting common promoter G-box cis-element in order to control expression of photosynthesis related genes (Toledo-Ortiz et al., 2014). Another important group of transcription factors involved in regulating photomorphogenesis is GLK1 and GLK2 (Waters et al., 2009). GLK proteins are able to bind to the G-box, to GLK specific cis-elements in promoter sequences, and act as transcriptional activators for more than 100 genes (Fitter et al., 2002; Waters et al., 2009). These include nuclear encoded components of PSI, PSII, their respective antenna proteins, components of the chlorophyll biosynthesis pathway (Kobayashi et al, 2013) and SIG2, which is required for GlutRNA transcription (Waters et al., 2009). GLK1 and GLK2 are indespensible for both etioplal and chloroplast development (Fitter et al., 2002). Indeed, glk1glk2 double mutant demonstrated abnormal etioplast structure and lower amounts of chlorophyll precursors in etioplasts, and in mature chloroplasts mutation in both GLK1 and GLK2 results in mis-regulation of genes encoding components of photosynthetic apparatus and chlorophyll biosynthesis pathway leading to abnormal chloroplast structure and pale-green phenotype of the plants (Waters et al., 2008; Waters et al., 2009).

The photoreceptors and their downstream targets are important for perceiving and transmitting light quality information to regulate the photomorphogenic process. However, photoreceptors have also been shown to register light quantity. For example, a high light signal has been shown to be perceived by the CRY1 photoreceptor and to be transmitted through two separated pathways: HY5-dependent (Kleine et al., 2007) and ZML1/ZML2-dependent (Shaikhali et al., 2012). The resulting activation of photoprotective mechanisms enables the plant to cope with the high light stress, these include expression of proteins essential to prevent
photooxidative damage, such as GPX7, GSTs, PDX and the MYB7 transcription factor (Kleine et al., 2007).

In this section we have discussed that light quality and quantity is perceived by photoreceptors and further transmitted by nuclear encoded factors that largely control the fate of chloroplast development and its response, adjustment to stress signals. These nuclear-to-chloroplast regulatory networks belong to so called anterograde signalling. However, during the recent years it became obvious that in a changing environment (operational control) and during chloroplast biogenesis (biogenic control) numerous chloroplast derived signals are modulating nuclear gene expression to fine tune it to the current status of the plastid (Albrecht et al., 2011).

PLASTID RETROGRADE SIGNALLING

Communication between chloroplast and the nucleus has been first demonstrated in *albostrians* mutants of barley and in Brassica *al* mutant (Bradbeer et al., 1979; Hess et al., 1994); plastid-derived signals were identified as triggers for changes in nuclear gene expression (Fernandez, Strand, 2008; Barajas Lopez et al., 2013) and various other cellular processes including DNA replication, chloroplast and cell development, cell cycle (Sullivan and Gray, 1999; Kobayashi et al., 2009; Burch-Smith et al., 2011; Andriankaja et al., 2012). Numerous different signalling molecules and pathways have been recently associated with chloroplast retrograde signalling (Fernandez and Strand, 2008; Jarvis and Lopez-Juez, 2013; Barajas Lopez et al., 2013). Here I would like to focus particularly on: the tetrapyrrole-mediated signals, photosynthesis related signals (Redox and ROS) and the PGE signalling pathway.
**Plastid gene expression (PGE) signalling pathway**

As it was mentioned earlier, chloroplasts have partially retained prokaryotic gene expression machinery. Due to the fact that chloroplast proteome is encoded in both plastid and nucleus coordinated gene expression must take place (Fernandez and Strand, 2008). Therefore it is feasible that chloroplast must communicate information of its current activity to the nucleus. That can be achieved through retrograde signalling pathway originating from the plastid gene expression machinery. Indeed, inhibition of chloroplast translation by chloramphenicol, erythromycin or lincomycin (Sullivan and Gray, 1999; Koussevitzky et al., 2007) and inhibition of transcriptional activity by tagetitoxin (Mathews and Durbin, 1990) or rifampicin (Woodson et al., 2013) were shown to trigger PGE signalling that in turn inhibits expression of nuclear encoded photosynthesis-associated genes (PhANG). This signal emerges only during early stages of the seedling development, as no effect by the inhibitors was observed in later stages or in mature plants (Sullivan and Gray, 1999; Koussevitzky et al., 2007). Existence of certain time period within which the PGE-based signal is essential indicates that developing chloroplast is particularly sensitive to changing environmental clues and that the plastid gene expression machinery may serve as a sensor that coordinates expression of chloroplast proteome from both the plastids and the nucleus. Interestingly, lincomycin and erythromycin suppress PhANG expression in lip1 and cop1 constitutive morphogenesis mutants in the dark suggesting that light might not directly regulate PGE signalling (Sullivan and Gray, 1999; Ruckle et al., 2007; Woodson et al., 2013) but rather light and PGE signalling may converge at some point (Koussevitzky et al., 2007; Pfannschmidt, 2010; Leister et al., 2011; Leister et al., 2014). It is important to keep in mind though that transcription and translation inhibitors have a serious drawback due to their pleiotropic effects (Mulo et al., 2003), therefore results must be treated with precaution that multiple
signalling pathways might be affected by the inhibitor application (Pfannschmidt, 2010). Direct proof that the gene expression machinery has indeed a role in generating the plastid signal was shown recently by studying functional PEP knockout mutants, sig 2 and sig 6 (Woodson et al., 2013), knockouts of several pTAC components (Pfalz et al., 2006; Steiner et al., 2011) and prolyl-tRNA synthetase RNAi line (Leister et al., 2014). Global analysis of gene expression data revealed that observed changes of PhANG expression in seedlings grown on Lincomycin are partially due to decreased SIG2- and SIG6-dependent PEP activity. Moreover, a crosstalk between PGE and tetrapyrrole-mediated retrograde signalling has been recently suggested as SIG2-dependent PEP transcription of GlutRNA is required for the first steps of tetrapyrrole biosynthesis (Woodson et al., 2013).

One of the candidate proteins involved in PGE mediated signalling is GUN1. The gun1 mutant demonstrates a so called genome uncoupled phenotype during early seedling developmental stages under lincomycin treatment (Koussevitzky et al., 2007); and gun1 mutation introduced in sig2 or sig6 background was able to partially restore PhANG gene expression, even though chloroplast transcription was still compromised (Woodson et al., 2013). Moreover, the gun1 mutant demonstrates delayed chloroplast development in response to light and several developmental abnormalities, including cotyledon opening and hypocotyl elongation (Ruckle and Larkin, 2009). GUN1 encodes a PPR protein, a member of the large family of proteins localized to chloroplast and mitochondria and involved in posttranscriptional events: RNA stability, processing, editing, and translation (Lurin et al., 2004; Schmitz-Linneweber et al., 2006; Delannoy et al., 2007). GUN1 has a characteristic structure with two separate domains, PPR and SMR (Lurin et al., 2004; Moreira and Philippe, 1999); the protein is localized to the sites of active transcription (Koussevitzky et al., 2007). Interestingly, another protein that has similar domain organization as the
GUN1 is PTAC2, essential component of PEP (Steiner et al., 2011). It has been reported that proteins with PPR (Ikeda and Gray, 1999) and SMR motifs (Fukui and Kuramitsu, 2011; Liu et al., 2013) are able to bind DNA, and indeed, DNA binding activity of GUN1 has been demonstrated (Koussevitzky et al., 2007). However, the exact function of GUN1 is still unknown.

**Tetrapyrrole-related signalling**

Apart from *gun1* that was mentioned previously, five other genome uncoupled mutants (*gun2/3/4/5/6*) have been shown to be impaired in the communication between the plastid and the nucleus (Figure 5) (Barajas Lopez et al., 2013). However, GUN2-6 are closely associated with tetrapyrrole metabolism and have been demonstrated to affect flux through the pathway. GUN2 encodes heme oxygenase, GUN3 - phytochromobilin synthase, GUN5 is the H subunit of Mg-chelatase, and GUN4 is a proposed regulator of Mg-chelatase activity (Mochizuki et al., 2001; Larkin et al., 2003). Recently identified GUN6, has a mutation in promoter region of Fe-chelatase 1 and leads to its overexpression (Woodson et al., 2011). The *gun* mutants exhibit impaired communication with the nucleus and maintain nuclear gene expression under photooxidative stress condition, whereas the wild type plants strongly suppress PhANG expression under those conditions (Susek et al., 1993; Woodson et al., 2011). Flux through the tetrapyrrole biosynthesis pathway can be strongly affected by changing environmental conditions and could therefore act as a checkpoint where information about plastid status can be reported to the nucleus (Kindgren et al., 2012).

Studies in animal cells, yeast and *Chlamydomonas reinhardtii* have revealed the role of heme in regulating nuclear gene expression (Zhang and Hach, 1999; Ogawa et al., 2001; von Gromoff et al., 2008).
Figure 5. Suggested components of tetrapyrrole – mediated retrograde signalling. Heme and gun5/MgProtoIX generate two counterbalancing signals that control PhANG expression. The first step of tetrapyrrole biosynthesis among others is dependent upon tRNA expression controlled by PEP.

A recently isolated dominant gun6-1D mutant of Arabidopsis causes overexpression of FC1 gene encoding ferrochelatase 1; and similar to gun2-gun5 mutants it maintains PhANG expression when grown on NF (Woodson et al., 2011), suggesting a positive retrograde signal associated with heme. FC1 gene expression pattern closely resemble that of HEMA2, encoding a protein involved in ALA biosynthesis, and they both show up-regulation upon stress conditions, on contrary to other nuclear genes that are down-regulated (Ujwal et al., 2002; Nagai et al., 2007). It was suggested that there might exist several ALA pools in the chloroplast funneling tetrapyrroles for different pathways and these might dependent on the activity and localization of HEMA1 and HEMA2 enzymes (Czarnecki et al., 2011). Overexpression of both HEMA2 and FC1 result in recovery of nuclear gene expression upon NF treatment suggesting that particular heme biosynthesis pathway/pool is responsible for generating a signal (Woodson et al., 2011).
However, whether it is a distinct heme accumulation or the activity of heme biosynthesis enzymes remains unclear since no satisfactory method for measuring heme content exists (Espinas et al., 2012; Woodson et al., 2011; Voigt et al., 2010).

Opposite to the putative positive heme-related pathway there is evidence of counterbalancing repressing pathway associated with the Mg-ProtoIX/Mg-protoIX-ME (Kindgren et al., 2011; Barajas Lopez et al., 2013). Accumulation of Mg-ProtoIX/Mg-protoIX-ME has been originally thought to initiate the changes in nuclear gene expression and was further supported by the characterization of gun5 mutant (Johanningmeier and Howell., 1984; Kropat et al., 1997; Strand et al., 2003). This model has been recently updated and it was proposed that changes in the flux of the biosynthesis pathway (Moulin et al., 2008; Mochizuki et al., 2008) and/or rapid, transient MgProtoIX/ME accumulation of the intermediates (Zhang et al., 2011; Kindgren et al., 2012) might be the sources of the signal. Indeed, molecules belonging to the porphyrin family, including chlorophyll precursors, catabolites and heme can be exported from the chloroplast to cytoplasm (Thomas and Weinstein, 1990; Jacobs and Jacobs, 1993; Ankele et al., 2007). Further evidence on the possible role of MgProtoIX/ MgProtoIX-ME pool itself as a signalling factor has been achieved by identifying putative MgProtoIX interacting proteins (Kindgren et al., 2011). In recent proteomic study a large number of cytoplasmic proteins were found to be associated with MgProtoIX/MgProtoIX-ME (Kindgren et al., 2011). HSP90 was one of the proteins identified, and a regulatory complex of MgProtoIX/ MgProtoIX-ME and HSP90 was demonstrated to be crucial during oxidative stress to transmit negative signal to the nucleus through the HY5 transcription factor. The very similar regulatory pathway for heme signalling that includes HSP70/HSP90/HAP1 has been described previously in yeast (Zhang and Hack, 1999).
The presence of several signalling molecules, multiple signalling pathways and regulatory mechanisms associated with tetrapyrrole biosynthesis means first, that it serves as an indicator for changes in environment and developmental status of the chloroplast; and second, that these signals might need to be converged to master regulators that control nuclear gene expression (Richly et al., 2003; Biehl et al., 2005).

**ROS and redox signals**

It is not only the flux through the tetrapyrrole biosynthesis pathway that responds to changes in plastid homeostasis, the photosynthesis apparatus itself can provide direct information about current status of the chloroplast. Indeed, processing information during the deetiolation process, acclimation to various stress conditions that affects photosynthetic activity is crucial for adjusting both chloroplast and nuclear gene expression (Brautigam et al., 2010). The signals that are linked to the photosynthetic electron chain activity are associated with redox status of particular components of PET and the accumulation of different ROS species (Figure 6) (Barajas Lopez et al., 2013). ROS are produced and scavenged continuously during photosynthesis, however under stress conditions a rapid burst of ROS can occur that causes oxidation of lipids, proteins and therefore affect cell integrity and function (Foyer and Allen, 2003). ROS produced in the chloroplast are singlet oxygen (\( ^1\text{O}_2 \)) generated by PSII and superoxide ion (\( \text{O}_2^- \)) formed by PSI that rapidly is converted to \( \text{H}_2\text{O}_2 \) (Apel and Hirt, 2004). Information on one aspect of the ROS signal transduction pathway was obtained by studying the flu (Meskauskiene et al., 2001) and the npq1lut2 (Alboresi et al., 2011) mutants that overaccumulate \( ^1\text{O}_2 \). Singlet oxygen was shown to be mainly activating cellular death responses rather than plastid proteome adjustment (Danon et al., 2005). The signal transduction pathway includes EX1/EX2 proteins localized to the plastid membrane (Lee et al.,
2007) and possibly the oxidation product of β-carotene (Ramel et al., 2013), though the exact function of the putative signal carriers and signal transduction pathway is yet unknown.

![Figure 6. Retrograde redox signals generated by photosynthetic electron transport activity of the chloroplast.](image)

On the other hand, H$_2$O$_2$ has been shown to activate a separate signalling pathway that acts antagonistically to the one generated by $^1$O$_2$ (Gadjev et al., 2006; Vanderauwera et al., 2005). The H$_2$O$_2$ signal generated in the chloroplast can be further transmitted through a yet unknown cascades to certain redox regulators, such as RCD1 or RAP2.4a in the nucleus (Shaikhali et al., 2008; Hiltscher et al., 2014). Genes that are induced by H$_2$O$_2$ include ascorbate peroxidise APX2 and large number of other genes involved in plant stress responses (Vanderauwera et al., 2005). Although it is clear that different ROS signals initiate separate pathways and cell response mechanisms, it is also important to mention that ROS species are produced in other cellular compartments than the plastids, therefore the origin and strength of ROS signal must be differentiated. Mechanisms for such selectivity are currently unknown (Moller and Sweetlove, 2010).
Another type of signal is directly generated by the activity of Photosynthetic Electron Transport chain (PET) (Brautigam et al., 2009). Redox signal can be partially triggered by changes of the redox status of PQ. Indeed, oxidized by DCMU or reduced by DBMIB, the PQ pool has been shown in several organisms to affect nuclear gene expression (Escoubas et al., 1995; Pfanschmidt, 2003). However, further analysis of redox-triggered regulation of nuclear gene expression demonstrated that only a small amount of genes are sensitive to the changes in redox status of the PQ pool (Hihara et al., 2003), suggesting that there might be other signalling factors (Fey et al., 2005a and 2005b). Redox state of PSI acceptor site and the redox state of thioredoxin have been proposed as a second site of the PET signal (Scheibe et al., 2005). Identification of the recently isolated redox imbalanced mutants (rimb) that couple the redox state of the PSI acceptor site with the expression of 2-Cys-peroxiredoxin (2-CPA) can provide a better understanding of redox generated retrograde signalling pathway (Heiber et al., 2007; Hilscher et al., 2014). PET imbalance and subsequent change in the redox state of the chloroplast can be also induced by HL stress. Overreduction of PET after HL treatment affects activity of PGE machinery that further serves as a retrograde signal. The ysl mutant defective in editing of rpoB transcript and mutants with compromised PEP activity show severe misregulation of PhANG expression, suggesting that chloroplast expression is important to send a HL-induced redox signal (Zhou et al., 2009; Kindgren et al., 2012).

During recent years it became obvious that anterograde signals and chloroplast retrograde signatures are both important for plant development. However, one must keep in mind that these two regulatory networks do not exist separately but rather represent a regulatory loop, a commonly used regulatory mechanism in biological systems. The term “regulatory loop” is used in relation to the connected systems, where each of them control the activity of the other. It is very difficult to determine the original cause of
regulation in the case of feedback systems or regulatory loops since one component regulates the activity of the second and the second in turn influences the first. Therefore, feedback regulatory networks should rather be studied as the whole phenomena (Åström and Murray, 2008). Despite the fact that retrograde and anterograde signalling pathways are studied intensively separately, the activity of both systems in parallel in response to changing condition, is poorly understood. As it was demonstrated above, light quality and quantity information is perceived by both chloroplast and the nucleus; and these two components complement each other in order to proceed with a plant developmental program and to respond adequately to environmental changes. The main challenge for the photosynthetic endosymbiont that was retained within a cell was to create a regulatory network to coordinate its gene expression machinery and photosynthetic activity with the host metabolism and transcription/translation activity (Barajas Lopez et al., 2013; Petrillo et al., 2014). One of the sites for that regulatory network in the chloroplast is the plastid expression machinery. It integrates signals that occur within the plastid (such as changes in PET, ROS pools and tetrapyrrole biosynthesis pathway) (Brautigam et al., 2010; Link, 2003; Woodson et al., 2012) and those that emerge from the nucleus in the form of the structural/regulatory proteins (Fernandez and Strand, 2007; Barajas Lopez et al., 2013). The site for the reciprocal control in the nucleus includes the transcription factors HY5 (Ruckle et al., 2007; Kindgren et al., 2012), GLK (Waters et al., 2009) and ABI4 (Koussevitzky et al., 2007). Moreover, cis-elements for light and retrograde signals are often common or overlapping (Nott et al., 2006; Koussevitzky et al., 2007); and it is hard to separate them (Martinez-Hernandez et al., 2002; Ruckle et al., 2007) which further suggests that both signalling pathways are in fact integrated together in a regulatory loop, rather than acting separately.
Aim

The aim of this thesis work was to reveal new components of the retrograde signalling pathways and to understand their function during development and in response to stress signals in the model plant *Arabidopsis thaliana*. 
RESULTS AND DISCUSSION

REDOX REGULATED PROTEINS ARE INVOLVED IN
CHLOROPLAST TRANSCRIPTION

Isolation of the prin2.1 mutant and subcellular localization of
the PRIN2 protein

The screen for redox insensitive mutants impaired in down regulation of
LHC gene expression after exposure to High Light (HL) (1000 µmol photons
m\(^{-2}\)s\(^{-1}\), 3 h) resulted in the identification of prin2.1 mutant (Paper I, Figure 1A). The EMS mutant prin2.1 and the T-DNA insertion mutant prin2.2
(GABI_772D02) displayed striking phenotype with pale/albino cotyledons,
pale-green leaves and reduced growth compared to the wild type (Paper I,
Figure 2A and 2B). Analysis of chloroplast structure using TEM revealed
that both prin2.1 and prin2.2 seedlings grown at 150 µmol photons m\(^{-2}\)s\(^{-1}\)
displayed abnormal chloroplasts with reduced number of thylakoid
membranes and grana stacks (Paper I, Figure S3). Map-based cloning and
consequent SOLiD System Sequencing identified a mutation in the
At1g10522 gene that results in a protein lacking 6 amino acids at the C-
terminial (Paper I, Figure 2A). In silico prediction of PRIN2 localization
(Emanuelsson et al., 2007) suggested the presence of chloroplast transite
peptide. To confirm localization in vivo we expressed PRIN2::YFP fusion
protein in Arabidopsis protoplasts. Interestingly, the signal from the protein
was detected in the chloroplast and co-localized with the signal from the
PEND::CFP construct expressing bZIP domain of the PEND protein (Paper
I, Figure 3). PEND has been reported to bind DNA both in chloroplast and
in the nucleus (Arsova et al., 2010), therefore we assumed that PRIN2 might
be localized to the chloroplast nucleoids. This finding was further supported
by the co-localization of PRIN2::YFP with PTAC12::CFP (Paper I, Figure S5), known component of pTAC (Pfalz et al., 2006) and identification of maize PRIN2 homolog in nucleoid fraction of the chloroplasts (Majeran et al., 2012). In silico analysis of PRIN2 promoter sequence revealed several light-responsive cis-elements suggesting light regulation of gene expression (Table 1). Indeed, PRIN2 is mostly expressed in seedlings and leaves of rosette plants, while the expression is low in root tissue (Paper I, Figure S2A). Moreover, its expression increases at the beginning of the light period, reaching maximum at the end of the day and further decreasing during the night (Paper I, Figure S2B).

**PRIN2 is a redox regulated protein**

PRIN2 is a conserved protein among land plants and is absent in *Chlamydomonas spp.* or cyanobacteria, suggesting that the protein appeared late during evolution and therefore might perform a putative regulatory function (Pfannschmidt and Liere, 2005). Gene sequence analysis revealed two conserved cysteine residues within the mature protein: C68 and C115, suggesting possibility for redox regulation (Paper V, Figure 2A). C68 is located at the N-terminus, while C115 is in the middle of the mature PRIN2 protein sequence. Since prin2.1 was isolated in a screen for redox insensitive mutants it was tempting to investigate possible redox activity of C68 and C115. We analyzed the in vitro response of the wild type and mutant proteins where one or two of the respective cysteine residues were substituted with Ser (termed S68, S115 and S68/S115 respectively). In non-reducing SDS-PAGE two bands were observed in wild type PRIN2, corresponding to the monomer (≈14 kDa) and the dimer (≈34 kDa). Increasing concentrations of the H$_2$O$_2$ induced formation of the dimer; on
contrary, increasing the concentrations of DTT results in reduction of the dimer (Paper V, Figure 2C).

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Table 1. cis-elements in PRIN2 promoter sequence predicted in silico by PlantCARE
The S68 mutant protein was present only in monomeric form irrespective of DTT or \( \text{H}_2\text{O}_2 \) treatment, suggesting that C115 is not a redox active cysteine and that C68 is responsible for the dimer formation of PRIN2 (Paper V, Figure 2C). In addition, the C68-C68 bond reduction in S115 mutant required higher concentrations of DTT compared to what is required for the reduction of the wild type protein suggesting that mutation of the C115 cysteine probably affects the redox activity and/or availability of C68 to redox regulation (Paper V, Figure 2D). These data indicate that PRIN2 responds to the changes in the redox environment and the C68 cysteine is required for dimer formation.

We further wanted to see which forms of the protein are present in vivo. Recombinant full-length PRIN2 was expressed in E.coli, purified on Ni\(^{2+}\)-NTI agarose column and used for injection into rabbits to raise up antibodies. Unfortunately, we were unable to detect PRIN2 protein in the wild type plants, suggesting that protein levels are quite low despite relatively high gene expression (Paper I, Figure S2B). It is worth mentioning that many bacterial proteins performing regulatory function or required during specific cell cycle period are often present at extremely low levels due to the strict proteolytic control making their detection difficult (Kanemori et al., 1999). Indeed, proteolysis appears to be an essential regulatory mechanism that allows rapid degradation of the target, resulting in immediate response (Gur et al., 2011). Some of the well-studied examples include E. coli heat shock \( \sigma^{32} \) sigma factor that is present at extremely low concentrations with a half-life of only 1 minute due to the proteolysis by FtsH protease (Herman et al., 1995); and SOS response regulator LexA that is degraded by ClpXP (Neher et al., 2003). It is apparent that despite major transcription and post-transcription regulation of gene expression, post-translation control by proteolysis is essential for fine-tuning of regulatory networks (Gur et al., 2011).
Since we were unable to detect PRIN2 in the wild type plant using the recombinant PRIN2 antibody, we decided to use a PRIN2 over-expressor line for further studies. Two bands corresponding to PRIN2 monomer and dimer were identified in non-reducing SDS-PAGE (Paper V, Figure 2B). To further complement our studies we used protein extract from 12h dark adapted and 3 h HL treated Arabidopsis plants to induce changes in redox state of the chloroplast stroma (Nikkanen et al., 2014). HL treatment increased, while dark adjustment decreased the amounts of the monomer (Paper V, Figure 3A), suggesting that PRIN2 undergoes dimer/monomer transitions when redox state of the stroma was modulated, confirming behavior of the protein in vitro (Paper V, Figure 2).

During the course of evolution electron transport chain of photosystem (PET) has also been used to detect different light quantity and quality. Dark adapted photosystem generates more oxidized stroma and PQ pool. On contrary, afternoon sun represents the “ideal” conditions for photosynthesis, where both longer and shorter wavelengths are present and therefore both reaction centers act in such an orchestrated way that PQ pool and chloroplast stroma are mainly reduced (Brautigam et al., 2010). Reducing power is used in anabolic metabolism such as biosynthesis of carbohydrates in Calvin-Benson cycle (Brautigam et al., 2009); reduction of antioxidants such as APX and GPX (Gill and Tuteja, 2010); and also used in several redox regulatory systems: NADPH-, ferredoxin-, thioredoxin-, and glutaredoxin-based (Zaffagnini et al., 2012; Nikkanen et al., 2014) that control enzyme activity and function. Thioredoxins (TRX) are specifically reduced by Ferredoxin-Thioredoxin Reductase (FTR) associated with PSI; and the activated dithiol group of TRX is used to reduce target proteins (Nikkanen et al., 2014). As many as 11 members of plastid TRX are known in Arabidopsis (Meyer et al., 2005) and recently identified TRXz is specifically localized in chloroplast nucleoids (Arsova et al., 2010). Since we have
observed that the PRIN2 protein was able to undergo transitions between monomeric/dimeric forms in response to redox both \textit{in vitro} (\textbf{Paper V, Figure 2C}) and possibly also \textit{in vivo} (\textbf{Paper V, Figure 2B}), we decided to test if PRIN2 can be an \textit{in vitro} target for chloroplast TRX. Indeed, PRIN2 dimer was efficiently reduced by 2µM TRXf1 (\textbf{Paper V, Figure 3B}), while almost no reduction was observed when using 2µM TRXz (data not shown). TRXz has distinct biochemical properties compared with other plastid TRX: while FTR efficiently reduces TRXx, it is unable to reduce TRXz. Moreover, less negative $E_m$ potential of TRXz (-311 mV at pH 7.9) (Chibani et al., 2011) than $E_m$ of other plastid TRX (-365 to -335 mV) (Collin et al., 2003) suggests that TRXz can be reduced by them. Indeed, both TRXf, TRXm and to less extent TRXx and TRXy increased the amount of TRXz monomer \textit{in vitro} (Bohrer et al., 2012). Distinct biochemical properties of TRXz indicate that it might have acquired a different function. Indeed, TRXz has been also identified as a core component of PEP (Arsova et al., 2010) and it has been recently suggested that cystein residues of TRXz are not essential for its function within an RNA polymerase during normal growth conditions (Wimmelbacher and Börnke, 2014). That implies that TRXz can be rather a structural subunit of PEP.

The ability of TRXf1 to be an efficient reductant for PRIN2, on the other hand (\textbf{Paper V, Figure 3B}), suggests that chloroplast TRX may serve as one of the potential reducing factors for modulating the monomer/dimer forms of PRIN2 \textit{in vivo} (\textbf{Paper V, Figure 2B}). Pea IGS-TRX-F/TRX-M plants with reduced expression of both TRXf and TRXm proteins due to the gene silencing have been recently described (Luo et al., 2012). These mutants might be very useful to study transitions of PRIN2 forms in the future.
PRIN2 is involved in PEP dependent transcription

The localization of PRIN2 to plastid nucleoids (Paper I, Figure 3) (Majeran et al., 2012) and co-localization with PTAC12 (Paper I, Figure S5), known component of PEP suggested that PRIN2 might be involved in DNA/RNA metabolism in the chloroplast. We have analyzed chloroplast transcriptome profile of *prin2.1* and *prin2.2* mutant seedlings under 150 μmol photons m⁻²s⁻¹ by Real-Time PCR. Photosynthesis-associated genes that are predominantly transcribed from PEP promoters showed lower transcript levels the in *prin2.1* and *prin2.2* seedlings compared to wild type (Paper I, Figure 4). Similarly, *psaA* and *psbA* transcript levels in mature rosette plants was decreased in both mutants compared to the wild type, suggesting that PEP-dependent activity is impaired in *prin2* mutants throughout life cycle (Paper I, Figure S6). On the other hand, house-keeping genes, predominantly transcribed by NEP in seedlings and rosette plants showed significantly higher transcript levels in the mutant alleles compared to wild type (Paper I, Figure 4 and S6). Increased transcript levels of the NEP genes has previously been demonstrated in several mutants deficient in PEP activity: *Arpo* (Hess et al., 1993; Silhavy and Maliga, 1998; De Santis-Maciossek, et al 1999; Krause te al., 2000), PTAC (Pfalz et al., 2006), *ysl* (Zhou et al., 2009), *sig2* (Kanamaru et al., 2001), *sig6* (Ishizaki et al., 2005), *trxZ* (Arsova et al., 2010). The mechanism for that up-regulation is yet unknown; however it is assumed that it might be a compensatory effect for absence of functional PEP machinery. It was originally proposed that tRNA_{Glu}, required for translation and also a precursor in tetrapyrrole biosynthesis pathway might be a putative repressor of NEP activity (Hanaoka et al., 2005). However, subsequent studies demonstrated non-specific binding of chloroplast tRNA to NEP, suggesting that a different regulatory mechanism might be involved (Bohne et al., 2009; Zoschke et al.,
The similar phenotype and plastid gene expression profile observed in prin2 mutants (Paper I, Figure 2B) and mutants deficient in either core components of PEP or additional proteins constituting TAC (Pfalz et al., 2006; Arsova et al., 2010) suggested that PRIN2 might indeed be a component of PEP-dependent transcription machinery. To prove that decreased steady-state levels of PEP-dependent transcripts are indeed due to decreased transcription efficiency and not due to post-transcriptional effects such as decreased RNA stability, we have performed run-on transcription assay for psaA, psbA, 16S rDNA in wild type and prin2.2 mutant chloroplasts. Indeed, transcription elongation efficiency was significantly lower for all studied transcripts (Paper V, Figure 1D), proving that PRIN2 is indeed essential for PEP-dependent transcription activity. We further tested the possibility that PRIN2 is able to directly bind DNA in vitro. Recombinant PRIN2 protein was used in Electrophoretic Mobility Shift Assay (EMSA) with several different DNA sequences carrying PEP promoter for psaA, psbA, psbD; and NEP promoter: rpoB, ysf1, clpP. DNA/PRIN2 complexes were observed for all of the promoters (Paper V, Figure 1A, B). We further used psaA, psbA, psbD, rpoB, ysf1 non-labeled DNA as competitors for ysf1/PRIN2 binding, and all of the probes were competing for interaction with DNA (Paper V, Figure 1C). Sequence analysis of selected DNA probes did not reveal any common elements that could have resulted in similar protein/DNA shift in EMSA, indicating that PRIN2 is sequence non-specific DNA binding protein.

Transcription regulation in bacteria involves different classes of DNA binding proteins. Promoter recognition is initiated within 35-10 elements by sigma factors (Feklistov and Darst, 2011); α subunit of RNAP itself is binding to so-called Upstream element (UP) that is important for initiation of transcription (Gaal et al., 1996). Furthermore, various Transcription Factors (TFs) are required for RNAP activity. Transcription repressors often bind to
promoter region and therefore hinder RNAP activity. On the other hand, activators tend to bind upstream cis-elements and recruit RNAP complex. Specificity of Transcription factor to certain cis-element and position within a promoter defines its repressor/activator function (Madan Babu and Teichmann, 2003). Interestingly, organization of the bacterial chromosome by histone-like proteins also affects transcription. Bacterial DNA binding protein H-NS is a repressor of gene expression (Schröder and Wagner, 2002); however, another histone-like protein HU has been recently demonstrated to act antagonistically to H-NS and activate transcription (Dame and Goosen, 2002). On the contrary to sequence-specific TF, histone-like proteins bind DNA sequence non-specifically, and therefore have the global effect on gene expression (Dorman and Deighan, 2003).

As it was mentioned previously, the subunits of the chloroplast localized PEP core are homologous to bacterial RNAP (Pfannschmidt et al., 2000; Steiner et al., 2011). Similarly to bacteria, CTD domain of the α-subunit and σ-factors can recognize plastid promoter regions (Ishizaki et al., 2005; Yagi et al., 2012). One of additional, eukaryotic subunits of PEP polymerase, PTAC3, was recently shown to recognize promoter elements specific to photosynthesis-related genes (Yagi et al., 2012). To date little is known about site-specific chloroplast TFs involved in the PEP-dependent transcription. The CDF1 transcription factor has been characterized to be important for transcription specifically from rbcL upstream element in maize (Lam et al., 1988); the RLBP transcription factor was shown to bind to the promoter region of rbcL in tobacco and to be required for efficient transcription (Kim et al., 2002); the bHLH transcription factor PTF1 has been recently demonstrated to bind to AAG element in the psbD promoter (Baba et al., 2001); and CDF2, is a specific repressor of PEP-dependent rRNA transcription (Bligny et al., 2000). Similarly to bacteria, nucleoid organizing proteins have been also described in chloroplasts (Majeran et al.,
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2012). One of nucleoid-compacting proteins, bifunctional sulphide reductase (SiR) has been shown to negatively regulate global plastid gene expression, similarly to bacterial N-HS (Sekine et al., 2002).

PRIN2 is a DNA-binding protein, (Paper V, Figure 1), that is essential for transcription of photosynthesis-related genes. We have demonstrated that mutations of PRIN2 most likely decreases the activity of the PEP polymerase (Paper V, Figure 1D). That suggests that PRIN2 is neither a general transcription activator responding to specific stimuli, nor is it a transcription factor specific for a particular gene. On contrary, it is essential for transcription of specific set of photosynthesis-related genes, transcribed by PEP in seedlings and mature plants (Paper I, Figure 4 and Figure S6).

A few explanations are feasible; first, PRIN2 might acquire sequence specificity to certain cis-element within promoters common for all photosynthesis-related genes through interaction with sequence-specific DNA binding protein. It is possible to test this hypothesis by analyzing DNA binding regions by Chromatin immunoprecipitation (ChIP) assay. On the other hand, PRIN2 might be a part of the DNA binding domain of PEP that can stabilize/regulate PEP/DNA association during transcription. The fact that prin2.2 mutants are still able to transcribe PEP-dependent genes though with less efficiency according to the Run-On transcription assay (Paper V, Figure 1D) makes the second assumption plausible.

PRIN2 regulates PEP activity in a redox-dependent manner

To test if PRIN2 cysteine residues are essential for the regulation of chloro plast gene expression in planta, we transformed prin2.2 mutant plants with constructs carrying wild type, S68, S115 or S68/S115 cDNA under the control of 35S promoter. Plants were transformed with wild type copy, S68 and S68/S115 cDNA all recovered the wild type growth phenotype (Paper
V, Figure 4A) and psaA, psbA gene expression in 7 days old seedlings (Paper V, Figure 5A). On contrary, the S115 mutant cDNA was not able to restore neither wild type growth phenotype (Paper V, Figure 4A), nor the chloroplast gene expression (Paper V, Figure 5A). This data could be explained by the particular in vitro behaviour of S115 protein that requires higher levels of DTT to reduce the C68-C68 bond compared to the wild type protein (Paper V, Figure 2D). Therefore, in planta data confirms in vitro behavior of the proteins and suggests that the monomer is the active form of PRIN2; and effective transition from dimer to the monomer in response to light is the redox controlled mechanism for regulation of PEP activity.

Redox – regulated transcription regulators have been identified in several organisms. One of the best studied examples in bacteria is OxyR that senses levels of hydrogen peroxide and activates expression of antioxidant genes including catalase, GST, glutathione reductase (Rosner and Storz, 1997). OxyR exists as a tetramer in solution and oxidation with H$_2$O$_2$ results in formation of an intramolecular disulfide bond between two conserved cystein residues resulting in an active form of the protein that induces transcription (Storz et al., 1990). In mammals AP-1 activator (Fos and Jun), p53, NF-$k$B transcription factors turn into the active form when critical cystein residues are reduced (Fritz et al., 2003). Plant transcription factors MYB domain R2R3 protein (Heine et al., 2004), HAHR1 and HAHB10 proteins containing homeodomain (Tron et al., 2002), bZIP16, bZIP68 and GBF1 (Shaikhali et al., 2012) are regulated via the reduction/oxidation of cystein residues. An AP-2 domain Rap2.4a protein shows an even more complex redox regulation. The protein forms intermolecular disulfide bond that stabilize the active form, the homodimer. Further oxidation of the homodimer or its complete reduction decreases its transcription activity (Shaikhali et al., 2008).
Our data describes a new chloroplastic DNA binding factor, PRIN2, which activates PEP dependent transcription most likely in its monomeric form (Paper V, Figure 6). We have also demonstrated that the chloroplastic TRXf serves as a reducing power for PRIN2 monomerization in vitro (Paper V, Figure 3B) and therefore can be one of the possible regulators of dimer-to-monomer transition in response to light. As it was previously described for sigma factors, increased binding of the protein to the DNA might repress transcription initiation (Tiller and Link, 1993a and 1993b). It would be interesting to investigate if the reason for the reduced transcription of the chloroplast genes may be the enhanced DNA binding of the PRIN2 dimer in the C115 PRIN2 mutant and also in wild type PRIN2 under oxidized stromal conditions. Indeed, that can provide a missing link between redox regulation of PRIN2, DNA binding properties of the protein and functional activity of PEP.

The redox state of the chloroplast changes in response to the environmental conditions. The idea that the photosynthesis apparatus act as a chloroplast sensor has been developed by Anderson et al., (1995). Indeed, photosystem itself serves as a perfect indicator of light quality and quantity. The two connected redox systems in PET, PQ- and TRX- dependent have been described to regulate various processes in the chloroplast (Brautigam et al., 2009). Signal transduction from TRX is mediated by thiol-mediated reactions; on the other hand, the redox state of the PQ pool initiates a phosphorylation cascade. Predominant excitation of PSII generates oxidized TRX pool and PQ pool that is mainly reduced. The opposite situation happens under PSI light conditions. Dark adapted plants have therefore both the TRX and PQ pools oxidized, while under moderate light growth conditions, both are reduced and represent the ideal conditions for chloroplast activities (Pfannschmidt and Liere 2005; Brautigam et al., 2009).

An elegant hypothesis was proposed by Brautigam and colleagues...
(Brautigam et al., 2010): instead of treating these two redox signals independently, authors suggested that they should rather act in parallel defining a particular “state” condition and driving adaption of photosystems to changing light conditions. Indeed, two redox signals are regulating LhcbII kinase activity involved in state transition (Zer et al., 2009; Lemeille and Rochaix, 2010); initiation of translation of psbA in Chlamydomonas reinhardtii requires priming signal from PQ (Trebitsh and Danon, 2001) and activating signal from reduced TRX (Danon and Mayfield, 1994; Kim and Mayfield, 1997). The same transcription regulation might also happen in plants (Shen et al., 2001): chloroplast gene expression in mustard has been shown to depend both on kinase activity and a redox signal (Steiner et al., 2009). Moreover, analysis of phosphoproteome of DNA binding protein fraction from chloroplast identified as many as 40 phosphorylated proteins with relatively small molecular mass, that show differential phosphorylation and promoter recognition under PSI and PSII light (Steiner et al., 2009). cpCK2A kinase has been shown recently to be associated with PEP (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002), and together with another kinase, CSK, is implicated in phosphorylation of several PEP subunits and SIG1, SIG6 factors upon changes in PET activity and redox state of the chloroplast (Baginsky et al., 1997; Ogrzewalla et al., 2002; Schweer et al., 2010; Shimizu et al., 2010). Phosphorylation of PEP components results in changes of PEP activity and promoter preferences. Interestingly, in silico analysis of the PRIN2 protein sequence revealed a conserved Thr residue, a putative target for CK2 phosphorylation (Figure 7). Surprisingly, this Thr residue is within the stretch of 6 amino acids absent in the prin2.1 EMS mutant (Paper I, Figure 2A). We have demonstrated that gene expression in prin2.1 is insensitive to the PET derived signal modulated by DCMU and DBMIB treatment (Paper I, Figure 8A and B). It is therefore tempting to speculate that the observed prin2.1 mutant
phenotype and chloroplast gene expression pattern might be due to the absence of proper phosphorylation of the protein.

Here we would like to propose following model of PRIN2 regulation of chloroplast gene expression (Paper V, Figure 6). PET chain generates signals dependent on current light quality. In darkness, redox state of the chloroplast is oxidized (Brautigam et al., 2010).

![Prediction of CK2 phosphorylation site in PRIN2 protein sequence by GPS2.1 software (Xue et al., 2011).](image)

Oxidized stroma of the chloroplast shifts PRIN2 towards formation of the inactive dimeric form (Paper V, Figure 2C, 3A and 6). Under light conditions where the balance between PSI and PSII is equilibrated, redox state of the stroma is mostly reduced, resulting in a shift towards the PRIN2 monomer possibly through reduction by TRX and activation of PEP-dependent transcription. An additional redox signal might be involved during light limitation at photosystems through kinase activities of CSK and/or PTK that might phosphorylate PRIN2 on Thr residue and affect its activity and/or DNA binding.
PRIN2 interacts with the CSP41b protein which is associated with the PEP complex

To understand the mechanism(s) by which PRIN2 regulates PEP activity we have searched for PRIN2 interacting partners using Co-IP approaches. Arabidopsis thaliana plants overexpressing PRIN2-MYC under the control of the 35S promoter were used to isolate chloroplast and further co-immunoprecipitate PRIN2 containing complex (Paper IV, Figure 1A). In two independent experiments we have identified the CSP41b protein (Chloroplast Stem Loop Binding Protein 41 kDa), previously found in nucleoid preparations (Majeran et al., 2012) and in association with PEP (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002; Bollenbach et al., 2009). We further confirmed the specificity of interaction between PRIN2 and CSP41b by direct Co-IP in Arabidopsis protoplasts (Paper IV, Figure 1C). CSP41b protein and its ortholog CSP41a are of cyanobacterial origin and are well conserved in the plant kingdom. Both CSP41b and CSP41a have been identified among proteins with redox active cysteins (Ströher and Dietz, 2008) and shown to be organized in High Molecular weight (HMW) complexes upon changing redox state of stroma (Qi et al., 2012).

Various chloroplast functions have been assigned to the CSP41 proteins, pointing to their multifunctionality. CSP41a and CSP41b have been described as endoribonucleases found in association with 70S ribosomes of Chlamydomonas reinhardtii (Yamaguchi et al., 2003) and Arabidopsis (Beligni and Mayfield, 2008); CSP41b has been identified in PEP preparations and implicated in transcription regulation (Pfannschmidt 2000; Bollenbach et al., 2009), and post-transcriptional stabilization of RNA (Qi et al., 2012). Identification of CSP41b in association with PEP polymerase and its effect on PEP-dependent gene expression suggests that it might be one of the additional nuclear subunits of PEP required for its function. Several
different HMW complexes containing CSP41 proteins were identified on BN-PAGE: 0.8-2 MDa; 950 kDa; 224 kDa; 106-126 kDa (Peltier et al., 2006; Olinares et al., 2010) indicating that different attributed functions of the protein might depend on the exact protein complex and interacting partners.

Indeed, we have confirmed that recombinant CSP41b protein was present in HMW complexes ranging from 40-700 kDa on the Native-PAGE (Paper IV, Figure 1B) in accordance with results obtained by (Qi et al., 2012). Similarly, recombinant PRIN2 migrated as a continuous protein complex ranging from 20-66 kDa. Interestingly, incubation of PRIN2 and CSP41b together resulted in rearrangement of protein complexes and formation of one protein complex of ≈60 kDa that contained both PRIN2 and CSP41b suggesting protein interaction in vitro (Paper IV, Figure 1B). The presence of redox active cystein residue(s) in both CSP41b and PRIN2 and capacity to oligomerize (Qi et al., 2012)/dimerize (Paper V, Figure 3A) upon oxidized stroma condition indicates that CSP41b and PRIN2 might be orchestrated together by the redox signals originated in plastid. Whether interaction of PRIN2 with CSP41b and protein complex rearrangement is also redox mediated remains at this point an open question.

The csp41b-2 T-DNA insertion line and the prin2.2 mutant show very similar phenotypes, chloroplast structure revealed by TEM and thylakoid complex organization shown by Native-PAGE (Paper IV, Figure 2A, B and C) (Hassidim et al., 2007; Bollenbach et al., 2009; Qi et al., 2012). We further studied expression of several chloroplast genes in both prin2.2 and csp41b-2 mutants and similarly to previously reported results (Bollenbach et al., 2009; Qi et al., 2012), csp41b-2 and prin2-2 seedlings have decreased levels of genes that are predominantly transcribed from PEP-dependent promoter (psaA, psbA, psbD, rbcL). On the other hand, NEP dependent genes (accD, rpoB, ycf2) showed higher expression compared to wild type
Run-On transcription assay performed for \textit{csp41b-2} (Bollenbach et al., 2009) and \textit{prin2.2} (\textbf{Paper V, Figure 1D}) clearly demonstrated similar impairment in transcription elongation suggesting that the two proteins might be associated in a protein complex that directly affects function of PEP.

Previous studies have demonstrated that CSP41b is an RNA binding protein with specificity towards photosynthesis-related mRNA (Qi et al., 2012). We further wondered if CSP41b protein can affect PRIN2 DNA binding activity in EMSA assay. Interestingly, CSP41b alone was also able to bind psaA197 promoter fragment (\textbf{Paper IV, Figure 1D}). The difference in sizes observed for PRIN2/DNA and CSP41b/DNA complexes suggests that either PRIN2 forms higher molecular weight complexes upon DNA binding compared to CSP41b or it binds to several regions on the psaA197 probe. Incubation of PRIN2 and CSP41b together with PsA197 probe resulted in formation of an intermediate sized band that most likely represented psaA197-PRIN2/CSP41b heteromeric protein complex, further confirming that the two proteins are indeed interacting with each other and also upon DNA binding \textit{in vitro} (\textbf{Paper IV, Figure 1D}). Though earlier reports have demonstrated that CSP41b is an RNA binding protein (Qi et al., 2012), our results indicate that CSP41b also has a DNA binding activity. Indeed, there are several proteins that have both RNA and DNA binding properties. These include $\beta/\beta'$ subunits of RNA polymerase, $\sigma70$ factor, p53 protein, STAT1 (Cassiday and Maher, 2002; Suswam et al., 2005), GUN1 (Koussevitzky et al., 2007). Particularly interesting function has been assigned to the transcription factor TFIIIA. TFIIIA is required for 5S RNA transcription by RNAP III and binds to certain region within its gene (Churchill et al., 1990). However, it also serves as a storage protein for 5S RNA transcript (Honda and Roeder, 1980). Different sets of Zinc-finger domains within TFIIIA protein are responsible for binding to DNA at specific sequences within 5S
gene, and interaction with distinct secondary structures of 5S RNA (Clemens et al., 1993). This example of interaction with both DNA and RNA and therefore distinct function within transcription and post-transcriptional regulation might also be the case for the CSP41b protein.

**PEP activity is essential during embryogenesis**

To study the genetic interaction between PRIN2 and CSP41b-2, we attempted to generate a csp41b-2prin2.2 double mutant. However the double mutant was embryo lethal (Paper IV, Figure 3A). Analysis of the heterozygous CSP41b-2prin2.2/csp41b-2prin2.2 mutant siliques demonstrated that development of embryos that most likely correspond to the double mutant was aborted at a broad range of developmental stages. Opaque ovules did not develop further and turned into dark, shrunken seeds that were unable to germinate on MS media (Paper IV, Figure 3A). Embryogenesis is an intricate process that requires tight coordination of gene and metabolic activities of both the sporophyte and the developing embryo. Interestingly, about one-third of all known embryo-defective Arabidopsis mutants were described to have mutations in plastid localized proteins that are involved in energy production (Allen et al., 2009), biosynthesis of essential metabolites (Xiang et al. 2011), protein import or gene expression (Apuya et al., 2002; Bryant et al., 2011; McElver et al., 2001; Tzafrir et al., 2004) suggesting a crucial role of the chloroplast during embryo development. In Arabidosis and other oil-seed plants functional chloroplasts appear already at the globular stage of embryogenesis and their number according to chlorophyll autofluorescence increases and reaches its maximum at the mature green stage (Tejos et al., 2010) (Paper IV, Figure 3B and C). In addition to the embryo lethality of the csp41b-2prin2.2 double mutant, normal embryo development was also affected in prin2.2 and
csp41b-2 single mutants. Chlorophyll autofluorescence data clearly demonstrated that from LC to MG stage, csp41b-2 embryos were distinctively not as uniformly green as was the wild type embryo, with chloroplast containing tissue being localized to the epidermal layers of the embryo (Paper IV, Figure 3C). The prin2.2 mutant embryos showed an even more severe phenotype: they were significantly paler than the wild type embryos throughout embryogenesis (Paper IV, Figure 3B). A visible phenotype of the embryo was supported by the TEM pictures of chloroplasts from respective genotypes at MG stage (Paper IV, Figure 4). As it has been shown in mature rosette plants (Paper IV, Figure 2B) (Hassidim et al., 2007), chloroplasts of csp41b-2 mutant embryos developed chloroplasts with large areas devoid of membrane structures, with less thylakoid membranes and grana stacks. Even more severe chloroplast structure was observed in the prin2.2 embryo that not only had less thylakoid membranes, they were also often mis-oriented from the chloroplast axis but also had numerous vesicles (Paper IV, Figure 4). Similarly to gene expression in mature rosette plants (Paper IV, Figure 1D), the embryos of prin2.2 and csp41b-2 single mutants showed impaired expression of psaA, psbA, psbD genes (Paper IV, Figure 5C), suggesting that the previously demonstrated defect in PEP-mediated gene expression in mature csp41b-2 and prin2.2 plants is maintained also during the embryo development and could account for the observed impairment in embryogenesis.

Transcription of the genes encoding components of photosynthesis and components involved in energy production have been shown to be gradually up-regulated from the preglobular to the mature green stage during embryo development in Arabidopsis (Le et al., 2010) (Paper IV, Figure 5A). However, these transcription changes were preceded by an increased expression levels of RPO encoding the plastid NEP polymerase (Paper IV, Figure 5B). To date, the rpoBC operon encoding core subunits of PEP is the
only operon in chloroplast transcribed exclusively by NEP (Swiatecka-Hagenbruch et al., 2007; Zhelyazkova et al., 2012). It is therefore feasible that expression of \textit{RPOT} should be early during development to transcribe subunits of PEP: \textit{rpoA}, \textit{rpoB} and \textit{rpoC1/C2}. Moreover, transcription of PEP core subunits in the plastid was accompanied by transcription of additional components required for PEP function such as: \textit{PTAC3}, \textit{PTAC10}, \textit{PTAC12}, \textit{FLN1}, \textit{PTAC6}, \textit{PRIN2} and \textit{CSP41B} (Pfannschmidt et al., 2000; Pfalz et al., 2006) (\textbf{Paper IV, Figure 5B}). This data indicates that formation of functional PEP during embryogenesis is dependent upon expression of all additional PEP components and assembling them with core PEP to produce fully active holoenzyme that efficiently transcribes photosynthesis-related gens, such as \textit{psbA}, \textit{B}, \textit{C}, \textit{D} and \textit{psaA}, \textit{B} (\textbf{Paper IV, Figure 5A}). Observed embryo lethality of the \textit{csp41b-2prin2.2} double mutant suggests first, that transition from NEP- to efficient PEP-dependent transcription of photosynthesis-related genes is essential for embryo development; and PRIN2-CSP41b complex is required for PEP activity in the chloroplast during embryo development.

\textbf{PEP TRANSCRIPTION AND THE STATE OF THE CHLOROPLAST ARE LINKED TO THE NUCLEUS}

As it was mentioned earlier, chloroplast proteome is encoded in both plastid and the nucleus (Fernandez and Strand, 2008) and activities of them both must be coordinated for proper plant development (\textbf{Paper IV}). This coordination occurs partially through Plastid Gene Expression (PGE) mediated signaling. The exact source of the signal is unknown; however, decreased activity of gene expression in the chloroplast triggers changes in the nucleus (Koussevitzky et al., 2007). Genes that are regulated by this signaling are related to photosynthesis and therefore termed Photosynthesis-Associated Nuclear Genes (PhANG). Identification of the PRIN2 protein as
an important regulator of PEP activity led us to study the PhANG expression in the prin2 mutants. Indeed, both prin2.1 and prin2.2 have decreased levels of LHCB expression in seedlings (Paper I, Figure 5B), similar to the results obtained in lincomycin inhibitor studies (Sullivan and Gray, 1999). The PGE triggered signal is active only during early seedling development (Koussevitzky et al., 2007) suggesting that during these stages chloroplast is particularly sensitive to changing environmental conditions. Indeed, PhANG expression was restored to the wild type level in the rosette plants of the prin2 mutants (Paper I, Figure S6).

One of the known components of PGE signalling is GUN1, a PPR protein localized to plastid nucleoids (Koussevitzky et al., 2007). It shows a genome uncoupled phenotype when treated with inhibitors of plastid gene expression (Paper I, Figure 6) and has been proposed to act as a master regulator downstream of several signalling pathways (Koussevitzky et al., 2007). Indeed, the prin2.1gun1-1 and prin2.2gun1-1 double mutants demonstrated wild type expression of LHCB1.1, LHCB2.4 and RBCS in seedlings despite that PEP dependent transcription was halted due to PRIN2 mutation (Paper I, Figure 5). That suggests, that gun1 is indeed downstream of the PGE-generated retrograde signal and that GUN1 protein is not involved in the PEP-dependent transcription in light-grown seedlings. We have further demonstrated that PRIN2 and GUN1 interact in Arabidopsis protoplasts according to CoIP assay suggesting that GUN1 might obtain direct information from PEP machinery through its interaction with PRIN2 (data not shown). Localization of GUN1 in plastid nucleoids, its co-localization with another PPR protein within TAC (Koussevitzky et al., 2007), genetic (Paper I, Figure 5) and physical interaction with PEP-associated redox protein PRIN2 might suggest that GUN1 can sense compromised activity of gene expression machinery and send this information further to the nucleus.
COMPONENTS OF HIGH LIGHT SIGNAL PERCEPTION IN THE NUCLEUS AND THE CHLOROPLAST

As we and others have shown earlier that the plastid gene expression machinery is a target for redox regulation that originates within PET and plays an important role under low and moderate light growth conditions (Pfannschmidt and Liere, 2005; Brautigam et al., 2010). However, when the photon fluency exceeds the capacity of the PET, reaction centers become photoinhibited followed by overreduction of PSI and production of ROS that is not counterbalanced by the activity of scavenging enzymes (Karpinski et al., 2003; Foyer and Allen, 2003). That leads to irreversible damage of cell structures, lipids, proteins and affects cellular activities. Sensing the High Light signal and initiation of regulatory pathways that coordinate both chloroplast and nuclear gene expression is therefore crucial (Rossel et al., 2002).

It has been recently shown that one component of the High light signal is perceived by the nucleus through the activity of the CRY1 photoreceptor (Kleine et al., 2007). Exposure of cry1 mutant plants to high light results in misregulation of 77 genes associated with stress response mechanisms: such as GPX7, PDX2 involved in vitamin B6 metabolism, MYB transcription factor, CHS enzyme of phenylpropanoid pathway and GST ERD9 (Kleine et al., 2007). Under the same conditions 26 out of those 77 genes were also misregulated in HY5, nuclear encoded bZIP transcriptional factor involved in plant photomorphogenesis. Analysis of microarray data revealed that promoters of the HY5 – mediated HL induced genes are enriched in G-box and G-variant box elements, responsible for HY5 binding (Kleine et al., 2007).

Analysis of HY5-independent CRY1 mediated HL responsive genes revealed two new cis-elements, CryR1 and CryR2 that were enriched in promoter regions of respective genes (Kleine et al., 2007). A novel
biochemical approach was designed to identify proteins binding to CryR1 cis-element, resulted in the identification of the ZML2 transcription factor belonging to GATA type zinc finger domain protein family (Shaikhali et al., 2012). ZML2 has two close homologs in Arabidopsis, ZML1 and ZIM; and all three of them belong to a distinct group within GATA type transcription factors based on their protein sequence (Teakle et al., 2002). We have demonstrated that ZML1 and ZML2 are interacting with each other in yeast and in Arabidopsis protoplasts using a Co-IP approach in vivo (Paper III, Figure 8C); and act as transcriptional activators for CRY1 mediated HY5-independent activation of ELIP2, GPX7, ERD9, and MYB7 gene expression in response to high light (Shaikhali et al., 2012). We have also demonstrated that there is no direct interaction between ZML1 or ZML2 and CRY1 (Paper III, Figure 8C) suggesting that photoreceptor and two transcription factors are linked through yet unknown component to regulate expression of the genes involved in photoprotective mechanisms towards High light stress (Shaikhali et al., 2012).

High Light induces retrograde signalling pathways also in the chloroplasts, and these are mediated by the Redox state PET (Escoubas et al., 1995; Karpinski et al., 1999), activity of intrinsic kinases that phosphorylate photosystem membrane proteins (Rintamaki et al., 1997; Vener et al., 1998) and signalling initiated by ROS (Apel and Hirt, 2004). These multiple signals regulate PhANG expression in the nucleus (Escoubas et al., 1995; Fey et al 2005). Both prin2.1 and prin2.2 seedlings and rosette plants were unable to repress LHCB1.1 after HL exposure (Paper I, Figure 2). The mutants showed wild type expression of ELIP1 under these conditions, suggesting that HL induced PRIN2-mediated signalling is also independent from CRY1 mediated pathway (Paper I, Figure S4). We further tested the possibility that functional PEP is required for HL induced retrograde
signalling and have complemented our studies with ys-I mutant affected in the editing of rpoB transcript (Zhou et al., 2009), a component of plastid import machinery, tic-40 (Chou et al., 2003; Chiu and Li, 2008), gun1 and gun5 mutants (Koussevitzky et al., 2007; Strand et al., 2003). While wild type, gun1, gun5 and tic-40 mutants showed strong repression of LHCBI.1 and LHCBI.2 genes upon HL exposure, neither prin2 mutants, nor ys1 repressed PhANG genes suggesting that PEP activity is required for HL – induced retrograde signaling (Paper I, Figure 7). Moreover, prin2.1gun1 and prin2.2gun1 double mutants showed the same mis-regulation as single prin2 mutants, suggesting that gun1 is not involved in HL-mediated signalling (Paper I, Figure 7).

Since HL induces multiple effects in the chloroplasts including changes in redox of the stroma, PET components and ROS pool (Fernandez and Strand, 2008). We tested if PRIN2 is mediating the PET-derived retrograde signaling. Consistent with previous reports (Escoubas et al., 1995 and Brautigam et al., 2009), application of DCMU induced, while treatment with DBMIB reduced LHCBI.1 and LHCBI.2 gene expression in wild type seedlings, respectively. However, similarly to the HL treatment, prin2.1 and prin2.2 were insensitive to DCMU/DBMIB treatment (Paper I, Figure 8). The fact that prin2 mutants were also unable to regulate psbA expression under DCMU/DBMIB might suggest that changes in PET might be perceived by fully functional PEP and its altered transcription activity is further reported to the nucleus. Application of DCMU/DBMIB as well as the growth under Light 1/Light 2 results in simultaneous changes in PQ pool state and the redox state of the stroma (Brautigam et al., 2010). Whether one of those signals or the combination of both are perceived by PEP polymerase and further transmitted to the nucleus remains to be established.
We have previously discussed that chloroplast and particularly its transcription is essential during embryo development of Arabidopsis (Paper IV). Next we have also demonstrated that chloroplast gene expression machinery is crucial for regulating PhANG expression during early seedling growth (Paper V). In this section we would like to take a final step further and demonstrate that there is the complex interplay between retrograde and light-initiated anterograde signalling that creates a regulatory feedback loop adjusting plastid development.

Light exposure of etiolated seedling changes the expression of nearly one-third of all nuclear encoded genes including those encoding chloroplast localized proteins. Light perception and signal transduction is mediated by PHY/CRY/PHOT photoreceptors (Gyula et al., 2003) and downstream targets, such as the bZIP HY5 that regulates various photomorphogenesis responses including the expression of photosynthesis-related genes and downstream TF, such as GLK1 and GLK2 (Oyama et al., 1997). Indeed, plastid undergoes severe developmental changes upon exposure to light: transition from etioplast to photosynthetically active chloroplast. Etioplast has a characteristic paracrystalline membrane structure, prolamellar body, that is rich in lipids, precursor of the chlorophyll, protochlorophyllide and POR/NADPH enzyme complex (Schoefs and Franck, 2003). One of the last steps of chlorophyll biosynthesis, conversion of Pchld to chlorophyllide catalyzed by POR is a strictly light-dependent reaction that ensures that a massive production of chlorophyll molecules will happen immediately after light exposure. It is feasible that the trigger for etioplast-to-chloroplast
transition is therefore dependent upon initial light signalling. Moreover, chlorophyll biosynthesis is strictly regulated, since all of the tetrapyrroles are photoreactive molecules and can produce ROS upon light exposure if they are not properly quenched or sequestered (Tanaka and Tanaka, 2007). All of the enzymes involved in the tetrapyrrole biosynthesis and its regulatory factors are nuclear encoded suggesting further anterograde regulation mechanism. However, development of the chloroplast requires additional signals from the plastid itself that can adjust expression of nuclear encoded factors required for its function (Fernandez and Strand, 2008). Moreover, it has been demonstrated that both chloroplast- and light-derived signals utilize common/adjacent promoter elements suggesting reciprocal regulation of the pathways (Strand et al., 2003; Koussevitzky et al., 2007).

One of chloroplast retrograde signals is linked to tetrapyrrole biosynthesis and accumulation of MgProtoIX/ME (Strand et al., 2003; Zhang et al., 2011; Kindgren et al., 2012). MgProtoIX-ME is a substrate for the aerobic cyclase enzyme which is very sensitive to oxidative stress (Stenbaek et al., 2008). Therefore changes in environmental conditions and activity of PET can disturb the flux through tetrapyrrole biosynthesis pathway and affect the levels of intermediates that in turn result in changes of PhANG expression.

In our studies we used a T-DNA insertion mutant chl27/crd that over-accumulates MgProtoIX/ME due to a less functional cyclase enzyme complex (Tottey et al., 2003; Bang et al., 2008). The mutation leads to reduction of chlorophyll b content and a pale phenotype of the crd plants (Paper II, Figure 1A and 1B). Furthermore, the crd mutant displays impaired chloroplast structure with less grana stacks and thylakoid membranes (Paper II, Figure 1C). In accordance with that is structural rearrangement of thylakoid membrane complexes observed in the mutant compared with those of the wild type (Paper II, Figure 1A and 1D).
It has demonstrated that MgProtoIX/ME can be exported or released from the chloroplast upon oxidative stress conditions *in vivo* and therefore tetrapyrroles themselves may serve a signalling molecules in the tetrapyrrole mediated retrograde signalling pathway (Ankele et al., 2007). To further test the hypothesis that cytosolic accumulation of MgProtoIX is required for signal transduction Kindgren et al (2011) performed a biochemical approach to identify proteins interacting with MgProtoIX/ME. Among the proteins isolated a type 5 2A Ser/Thr Phytochrome-associated protein Phosphatase 5 (PAPP5) were found, previously described to be involved in de-phosphorylating of Pfr form and enhancing the phytochrome-mediated light response (Ryu et al., 2005). When using spectrofluorometry approach we were unable to show direct binding of MgProtoIX to recombinant PAPP5 *in vitro* (Paper II, Figure S5A). However, while using a native protein complex immunoprecipitated from tobacco plants in our binding assay instead of recombinant PAPP5 we were able to detect interaction with MgProtoIX (Paper II, Figure 6). There are several possible explanations for this: post-translational modification of PAPP5 might be essential for binding; a native protein complex with PAPP5 subunit is required for interaction; or PAPP5 is associated with yet another tetrapyrrole binding protein.

To further understand the nature and functional interaction between MgProtoIX and PAPP5 we have crossed *papp5*, a T-DNA insertion mutant, and *crd*. On contrary to *crd, papp5* single mutant and papp5*crd* double mutant show wild type levels of chlorophyll, normal growth rates, wild type structure of the plastid and proper organization of thylakoid membrane complexes in mature plants (Paper II, Figure 1). Similarly to the wild type, *papp5* and *papp5*crd developed normally during de-etiolation process and recovered wild type levels of the chlorophyll; while, *crd* mutant showed less thylakoid membranes and grana stacks in the chloroplast and was
significantly delayed in greening process (Paper II, Figure 2 and 3). That suggests that recovery of the wild type phenotype in papp5crd occurs both in mature grown plants and during de-etiolation process. Interestingly, papp5 mutant showed higher chlorophyll levels compared to wild type after light exposure suggesting that PAPP5 might act as a repressor factor of chlorophyll biosynthesis under those conditions (Paper II, Figure 3). We further studied nuclear gene expression during de-etiolation process. In accordance with the previous phenotypic observations, papp5crd show recovered induction of LHCB2.4 and GLK1, GLK2 expression compared to the crd single mutant. Moreover, similarly to the increased chlorophyll levels, papp5 mutant demonstrated higher expression levels of LHCB2.4, GLK1 and GLK2 compared to wild type, indicating that PAPP5 might indeed be a negative regulator of nuclear genes associated with chloroplast function (Paper II, Figure 4B and C).

Due to the decreased activity of MgProtoIX-ME cyclase in the crd mutant, it accumulates large quantities of MgProtoIX/ME that has been demonstrated to be behind the repression of PhANG expression (Bang et al., 2008). To test the possibility that the recovery of papp5crd mutant phenotype and gene expression might happen due to a rescue of cyclase activity and therefore wild type levels of MgProtoIX/ME are restored, we analyzed the tetrapyrrole content in the mutants during the de-etiolation process. Interestingly, both papp5crd and crd show higher levels of MgProtoIX/ME compared to wild type. In addition, the CRD protein content has not be recovered either in the papp5crd double mutant, suggesting that the papp5 mutation does not restore the normal flux through the tetrapyrrole pathway (Paper II, Figure 5A). These results indicate that PAPP5 is rather a signalling component downstream of MgProtoIX/ME accumulation. The signalling role of MgProtoIX/ME has been recently challenged and it was suggested that rather a change of the flux through the tetrapyrrole pathway, a specific pool
of Mg-ProtoIX/ME or the activity of Mg-chelatase are actual sources of the signal (Mochizuki et al., 2008; Moulin et al., 2008). In order to test if cytosolic accumulation of MgProtoIX/Me could be the signal perceived by PAPP5, we analyzed PhANG expression in papp5 mutant following MgProtoIX treatment. Indeed, it resulted in accumulation of large quantities of MgProtoIX in the cytosol of wild type and papp5 mutant. In agreement with previous results (Bang et al., 2008; Kindgren et al., 2012), an increase of MgProtoIX content through feeding resulted in a suppression of LHCB2.4, GLK1, GLK2 expression in the wild type. Interestingly, the feeding had no effect in the papp5 mutant (Paper II, Figure 7) therefore phenocopying the papp5crd expression profile (Paper II, Figure 4).

The PAPP5 protein belongs to the type 52A serine/threonine protein phosphatase family (Chinkers, 2001). To test the possibility that PAPP5 phosphatase activity is required for transmission of the MgProtoIX/ME-mediated signal to the nucleus, we treated seedlings with okadaic acid that specifically blocks activity of PAPP5 (de la Fuente van Bentem et al., 2005) and other 2A phosphatases (Bialojan and Takai, 1988) in vitro. Indeed, LHCB2.4, GLK1 and GLK2 gene expression was restored in the crd mutant treated with okadaic acid compared to the untreated control, suggesting that phosphatase activity of PAPP5 is crucial to mediate the signal. The observed in vivo binding of the PAPP5 containing protein complex to MgProtoIX (Paper II, Figure 6) suggests that it is rather the PAPP5 interacting partner that directly interacts with MgProtoIX; and PAPP5 might be involved in regulation and/or interaction/transmission of the signal through protein de-phosphorylation (Paper II, Figure 9). PAPP5 has been shown to act as a co-chaperone with HSP90 for correct folding and functioning of disease resistance proteins in tomato (de la Fuente van Bentem et al., 2005). Interestingly, HSP90 was recently identified in the same proteomics approach for MgProtoIX/ME - binding proteins as was the PAPP5
(Kindgren et al., 2011); and direct binding of MgProtoIX/ME to HSP90 has been demonstrated (Kindgren et al., 2012). HSP90 RNAi lines have reduced levels of HSP90 family proteins and were unable to transmit the MgProtoIX/ME signal to the nucleus in order to regulate PhANG expression (Kindgren et al., 2011) HSP90/MgProtoIX complex and its downstream component, transcription factor HY5, resembles yeast regulatory complex HSP70/HSP90/HAP1 involved in heme signaling (Mense and Zhang, 2006). Whether PAPP5 is a component of HSP90/HY5 regulon that transmits the negative MgProtoIX-mediated signal to the nucleus remains to be established.

Primary light signal transmitted from photoreceptors to HY5 regulates numerous genes involved in photomorphogenesis (Gyula et al., 2003). However, the majority of HY5-regulated genes are photosynthesis-specific transcription factors including GLK1 and GLK2 (Lee et al., 2007). Higher levels of GLK1 and GLK2 expression in papp5 and recovered wild type levels expression in papp5crd suggests that these transcription factors may receive the tetrapyrrole mediated retrograde signal. GLK1 and GLK2 have been shown to control expression of the genes involved in chlorophyll biosynthesis and photosynthesis and therefore to be essential during the establishment of the chloroplast (Waters et al., 2008; Waters et al., 2009). Indeed, glk1glk2 double mutant shows very pale phenotype, decreased chlorophyll content, reduced growth and reduced expression levels of PhANGs (Fitter et al., 2002) therefore resembling very much the crd mutant. Recent studies have demonstrated that GLK1 and GLK2 expression respond differentially to chloroplast signals generated by NF and lincomycin (Waters et al., 2009), that might suggest a feedback regulatory loop. Developing chloroplast emits a tetrapyrrole-mediated retrograde signal which is transmitted through PAPP5 to GLK1 and GLK2 transcription factors that in turn regulates expression of PhANG and chlorophyll biosynthesis genes.
(Paper II, Figure 9). Moreover, this model can explain the phenotype of the $crd$ mutant by reduced expression of $GLK1$ and $GLK2$ and recovery of $papp5crd$ mutant that occurs through increased expression of $PhANGs$ and increased chloroplast performance.
Conclusions and future perspectives

The work in this thesis reveals new aspects of the communication network between plastid and nucleus. We tried to demonstrate this mutual interaction under physiologically relevant conditions: during de-etiolation, embryogenesis and high-light stress.

Many new components of redox retrograde signalling have been recently identified, however their biochemical functions remain elusive in most cases. Even less information is available about factors involved in PGE signalling. We have identified a new protein, PRIN2, that is associated with the PEP transcription machinery of the chloroplast and is involved in redox- and PGE signalling from the plastid to the nucleus. PEP is a unique multisubunit enzyme which contains core subunits homologous to bacterial RNAP and 50 additional proteins of eukaryotic origin. Despite recent progress in identification and characterisation of mutants lacking respective subunits, almost no information is available about biochemical function of these proteins associated with the PEP complex. We have demonstrated that PRIN2 receives redox signals during plastid development from PET and regulates the activity of PEP through changes in its dimer/monomeric state. Moreover, PRIN2 and its interacting partner CSP41b are essential for PEP activity during embryogenesis in Arabidopsis. Bacterial RNAP has been thoroughly studied and its protein subunit interaction and stoichiometry is well known. Creating a similar “interaction map” of all identified PEP subunits and their stoichiometry within the complex will help uncovering their function and regulation.

Another retrograde signalling pathway that remains a very competitive area for studies is associated with the tetrapyrrole biosynthesis. There are still many unresolved questions regarding controversies in the nature of the
signal and its transmission to the nucleus. The presence of several candidate signalling molecules and different experimental conditions where they appear to be active might indicate that tetrapyrroline biosynthesis is essentially the site for numerous retrograde signals that report the state of the chloroplast to the nucleus. In this thesis we have demonstrated that MgProtoIX binds to a yet unknown cytoplasmic protein complex containing PAPP5 phosphatase. We have also shown that tetrapyrrole signalling is essential during de-etiolation in order to coordinate the activities of the chloroplast and the nucleus. The nuclear components that receive this tetrapyrrole-mediated retrograde signal are the GLK1/GLK2 transcription factors that in turn control chlorophyll biosynthesis, expression of PhANG. The tetrapyrrole-GLK signalling pathway represents a regulatory loop controlling chloroplast development. GLK1/GLK2 and other transcription factors that are involved in retrograde signalling bind to common cis-elements that also integrate light signals. Therefore, attempts to separate anterograde and retrograde signalling might be futile. Instead, a systems biology approach might be useful to describe the nature and the activity of these complex networks.

In this work we have revealed new components of the chloroplast and nucleus communication network and have demonstrated that tuned activities of both compartments are essential for the development of the plant. Several billion years’ evolution of proeukaryot and cyanobacterial ancestor resulted not in the creation of hegemony of one compartment over the other, but rather blended them and created totally new life form – the photosynthetic eukaryote. Yes, individual instruments might sound beautiful in solo, but the pure harmony and power uncovers when they all sound together. In tune.
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