PLASTID PROTEOSTASIS: RELEVANCE OF TRANSCRIPTION, TRANSLATION AND POST-TRANSLATIONAL MODIFICATIONS

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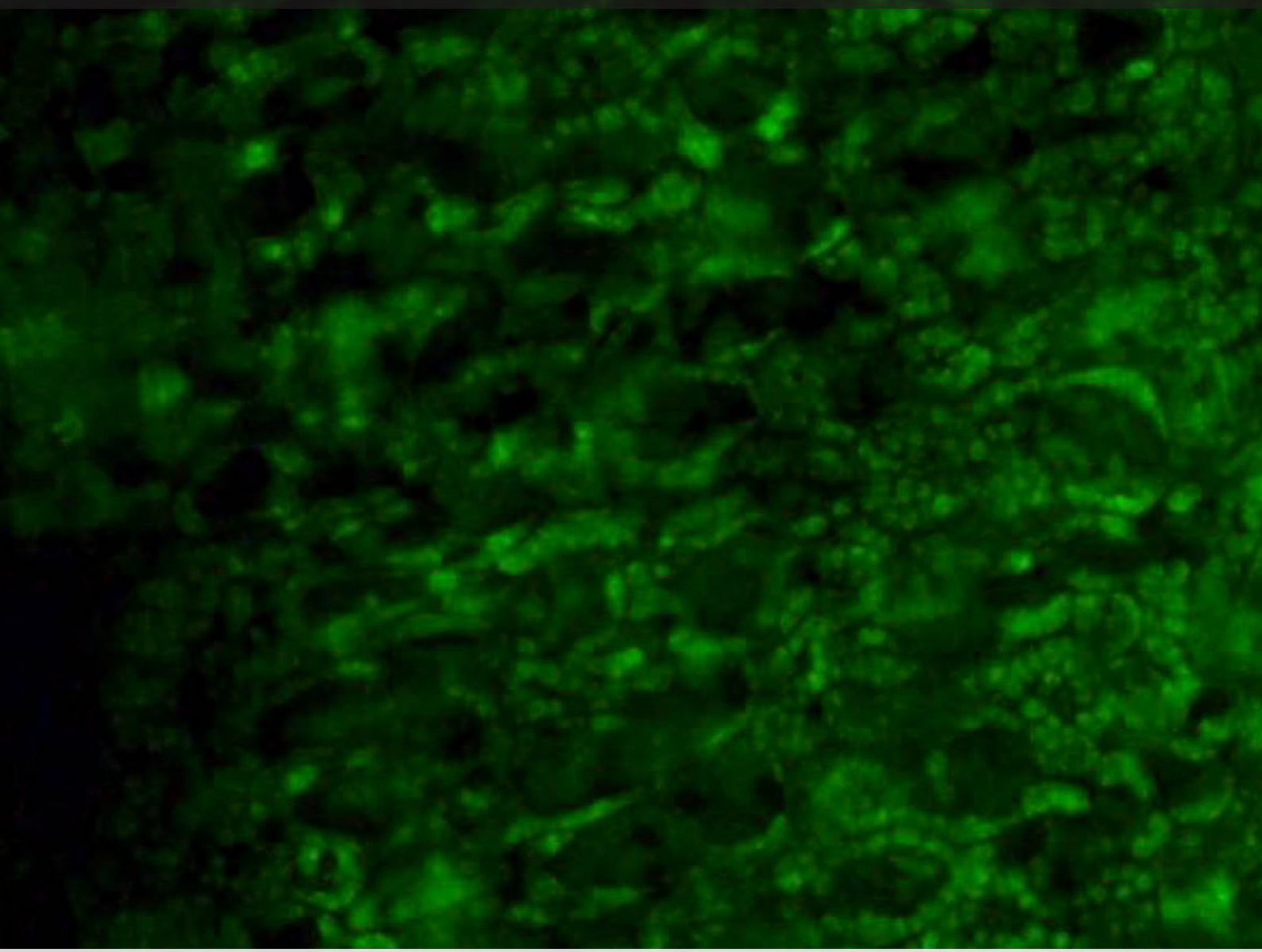

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PLASTID PROTEOSTASIS: RELEVANCE OF TRANSCRIPTION, TRANSLATION AND POST-TRANSLATIONAL MODIFICATIONS

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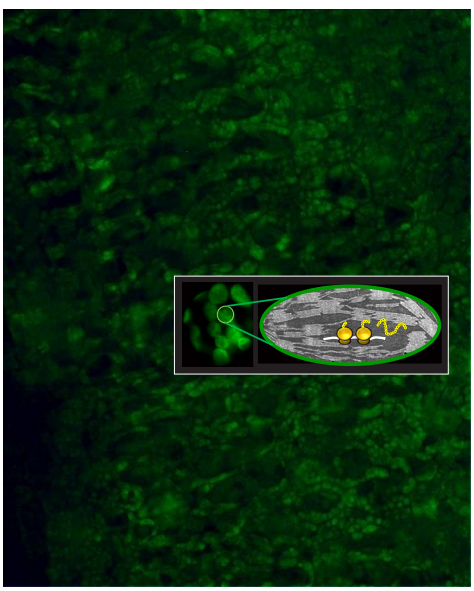

An image of the adaxial side of a sugar beet leaf taken with a conventional fluorescence microscope. The green color is due to autofluorescence of chlorophyll in the chloroplasts. In the box, the optical section of a tobacco protoplast taken with a confocal microscope (at left) and a representation of the chloroplasts structure based on a transmission electron microscopy (TEM) image (at right) is shown. The drawings inside the TEM image represent translation of chloroplast mRNA by 70S ribosomes.

Image: Michele Bellucci.

Due to their bacterial endosymbiotic origin plastids are organelles with both nuclear-encoded and plastid-encoded proteins. Therefore, a highly integrated modulation of gene expression between the nucleus and the plastome is needed in plant cell development. Plastids have retained for the most part a prokaryotic gene expression machinery but, differently from prokaryotes and eukaryotes, they have largely abandoned transcriptional control and switched to predominantly

translational control of their gene expression. Some transcriptional regulation is known to occur, but the coordinate expression between the nucleus and the plastome takes place mainly through translational regulation. However, the regulatory mechanisms of plastid gene expression (PGE) are mediated by intricate plastid-nuclear interactions and are still far from being fully understood. Although, for example, translational autoregulation mechanisms in algae have been described for subunits of heteromeric protein complexes and termed control by epistasy of synthesis (CES), only few autoregulatory proteins have been identified in plant plastids. It should be noted of course that PGE in C. reinhardtii is different from that in plants in many aspects. Another example of investigation in this research area is to understand the interactions that occur during RNA binding between nucleus-encoded RNA-binding proteins and the respective RNA sequences, and how this influences the translation initiation process.

In addition to this, the plastid retains a whole series of mechanisms for the preservation of its protein balance (proteostasis), including specific proteases, as well as molecular chaperones and enzymes useful in protein folding. After synthesis, plastid proteins must rapidly fold into stable three dimensional structures and often undergo co- and posttranslational modifications to perform their biological mission, avoiding aberrant folding, aggregation and targeting with the help of molecular chaperones and proteases.

We believe that this topic is highly interesting for many research areas because the regulation of PGE is not only of wide interest for plant biologists but has also biotechnological implications. Indeed, plastid transformation turns out to be a very promising tool for the production of recombinant proteins in plants, yet some limitations must still be overcome and we believe that this is mainly due to our limited knowledge of the mechanisms in plastids influencing the maintenance of proteostasis.

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EDITORIAL
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Editorial: Plastid Proteostasis: Relevance of Transcription, Translation, and Post-translational Modifications

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Keywords: plastome, gene expression, protein balance, regulation, nuclear-plastid interactions

Editorial on the Research Topic

Plastid Proteostasis: Relevance of Transcription, Translation, and Post-translational Modifications

Plastids are sites of biochemical and biological processes that are fundamental for plant life. The genome of these endosymbiotic organelles encodes for almost one hundred of the three thousand proteins that make up the chloroplast proteome. Genes coding for plastid multi-subunit protein complexes derive from both nuclear and plastid genomes, so it is clear that there is the need of a highly integrated coordination between this two subcellular compartments.

The coordination between the nucleus and the plastome takes place at many different levels, including the modulation of nuclear and plastid transcription, RNA processing and translation, post-translational modifications, and protein targeting. In addition, the plastid retains a whole series of mechanisms for the preservation of its protein balance (proteostasis), including proteases and molecular chaperones (**Figure 1**).

Plastids have largely abandoned transcriptional control switching predominantly to translational and post-translational control of their gene expression, but some transcriptional regulation is known to occur. Transcription of plastid genes is performed by two different types of RNA polymerases: plastid-encoded RNA polymerase (PEP) and a nuclear-encoded RNA polymerases (NEP). Liebers et al. propose that targeted changes in plastid transcription, mostly by controlling the relative activities of NEP and PEP enzymes, impact the establishment of the plastid proteome and these represent key determinants for the transitions between the different plastid types.

The transcriptional regulation mechanisms are still far from being completely elucidated. An unusual light- and stress-responsive promoter (*psbD* LRP), regulated by a AAG-box immediately upstream of the −35 element, has been recently mapped. Shimmura et al. analyzed *psbD* LRP promoter regions in 11 embryophytes, at different evolutionary stages, from liverworts to angiosperms. This analysis identified conserved features of the promoter and facilitated study of its emergence and evolution in plant species.

Among the proteins that regulate plastid gene expression, the nucleus-encoded proteins of the mitochondrial transcription termination factor (mTERF) family have been recently identified. Information on their function is only beginning to emerge. Xu et al. investigate the function of the chloroplast-associated mTERF. They report that these proteins are localized to chloroplast nucleoids and identify two of them involved in the salt stress response.

The import of plastid precursor proteins into plastids is another checkpoint affecting plastid proteostasis that is regulated in response to the fluctuating environmental conditions. This fine regulation ensures the optimal functioning of important biological processes

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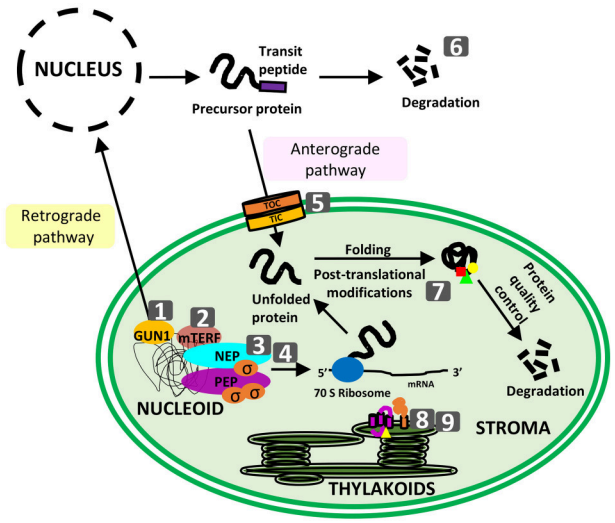

FIGURE 1 | Schematic representation of topics covered in the special issue. Numbers correspond to the following articles: (1) Colombo et al. GUN1, a jack-of-all-trades in chloroplast protein homeostasis and signaling; (2) Xu et al. *Arabidopsis thaliana* mTERF10 and mTERF11, but not mTERF12, are involved in the response to salt stress; (3) Liebers et al. regulatory shifts in plastid transcription play a key role in morphological conversions of plastids during plant development; (4) Shimmura et al. comparative analysis of chloroplast *psbD* promoters in terrestrial plants; (5) Sjuts et al. import of soluble proteins into chloroplasts and potential regulatory mechanisms; (6) Hirosawa et al. ubiquitin-proteasome-dependent regulation of bidirectional communication between plastids and the nucleus; (7) Grabsztunowicz et al. post-translational modifications in regulation of chloroplast function: recent advances; (8) Gabilly and Hamel, maturation of plastid *c*-type cytochromes; (9) Cline et al. CCS2, an octatricopeptide-repeat protein, is required for plastid cytochrome *c* assembly in the green alga *Chlamydomonas reinhardtii*.

taking place in this cellular compartment. The import of plastid precursor proteins is mediated by two distinct translocation complexes called TOC and TIC, located respectively at the outer and at the inner envelope membrane of chloroplasts. The individual steps involved in protein translocation and the corresponding regulation mechanisms used by plants to modulate protein import are reviewed by Sjuts et al.

Upon transition from an endosymbiont to a plant cell organelle, the plastid retains a set of mechanisms that involves enzymes and proteins of prokaryotic origin which are responsible for protein maturation, post-translational modification, correct folding, protein abundance control, and removal of misfolded or damaged components. These processes ensure that plastid proteins are ready to exert their biological mission and require an intricate system of signals from nucleus to plastid and backwards. Plastid-derived signals can regulate availability of nuclear-encoded plastid precursors controlling their *de novo* synthesis, and targeting. Recently, an important player in the chloroplast-to-nucleus retrograde communication has been identified: the protein GENOMES UNCOUPLED1 (GUN1). Recent studies indicate that GUN1 might play a role in the coordination of translation, import, and degradation of plastid proteins. The molecular function of different GUN1 partners has been reviewed by Colombo et al., highlighting its potential role in plastid proteostasis. Another important mechanism during nuclear-plastid interaction is the degradation of multiple components through the ubiquitin–proteasome system. It has become increasingly clear that, together with feedback regulation of nuclear gene expression by plastid-derived signals, this mechanism avoids the accumulation of non-imported proteins in the cytosol. In addition to the anterograde signaling pathway, recent studies in *A. thaliana* demonstrated that also the retrograde signaling pathway can be subjected to ubiquitin–proteasome regulation. Hirosawa et al. review recent advances in understanding how the ubiquitin–proteasome system regulates the nuclear–plastid interaction and plastid biogenesis.

The maturation of plastid proteins is another highly regulated process that in some cases needs complex apparatuses to occur. This is the case of *c*-type cytochromes that require a multicomponent assembly pathway for their maturation, as reviewed by Gabilly and Hamel. The regulation of this pathway has not yet been clarified. Cline et al. describe the functional characterization of the *CCS2* gene of *Chlamydomonas reinhardtii* required for cytochrome *c* assembly. The authors discuss the possible functions of CCS2 in the heme attachment reaction.

A wide range of post-translational modifications (PTMs) contribute to finely regulate the biological processes that take place in plastids. PTMs alter the physicochemical properties of the plastidial proteins thus affecting their function. Grabsztunowicz et al. review the current knowledge on the PTMs regulating important metabolic processes in chloroplasts such as DNA replication and gene expression, photosynthetic carbon assimilation, and starch metabolism and report the known physiological effects of these modifications.

Considering that there is limited knowledge of the combined action among the molecular mechanisms that regulate plastid proteostasis, the goal of this Research Topic is to bring together a set of articles that contribute to our understanding of how transcription, translation, and post-translational modifications (including protein targeting) maintain plastid proteostasis. As a consequence, this Research Topic will help us understand more deeply how plastids function.

AUTHOR CONTRIBUTIONS

MB, DL, and AP prepared the research topic. All the authors edited the manuscripts. FA invited the authors and wrote the editorial. All the authors read and approved the editorial.

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MINI REVIEW
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Regulatory Shifts in Plastid Transcription Play a Key Role in Morphological Conversions of Plastids during Plant Development

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Plastids display a high morphological and functional diversity. Starting from an undifferentiated small proplastid, these plant cell organelles can develop into four major forms: etioplasts in the dark, chloroplasts in green tissues, chromoplasts in colored flowers and fruits and amyloplasts in roots. The various forms are interconvertible into each other depending on tissue context and respective environmental condition. Research of the last two decades uncovered that each plastid type contains its own specific proteome that can be highly different from that of the other types. Composition of these proteomes largely defines the enzymatic functionality of the respective plastid. The vast majority of plastid proteins is encoded in the nucleus and must be imported from the cytosol. However, a subset of proteins of the photosynthetic and gene expression machineries are encoded on the plastid genome and are transcribed by a complex transcriptional apparatus consisting of phage-type nuclear-encoded RNA polymerases and a bacterial-type plastid-encoded RNA polymerase. Both types recognize specific sets of promoters and transcribe partly over-lapping as well as specific sets of genes. Here we summarize the current knowledge about the sequential activity of these plastid RNA polymerases and their relative activities in different types of plastids. Based on published plastid gene expression profiles we hypothesize that each conversion from one plastid type into another is either accompanied or even preceded by significant changes in plastid transcription suggesting that these changes represent important determinants of plastid morphology and protein composition and, hence, the plastid type.

Keywords: plastids, plastid morphology, photomorphogenesis, plant development, transcription, gene regulation, NEP, PEP

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INTRODUCTION

Plastids are cellular organelles that can be found only in plant and algae cells. They are of endosymbiotic origin that traces back to an evolutionary event in which a mitochondriate eukaryote took up a photosynthetically active cyanobacteria-like bacterium and established it as a permanent component of the cell, likely with the help of *Chlamydiae* (Ball et al., 2016). The

most prominent benefit for the eukaryotic cell in this process was the gain of photosynthesis and the concomitant switch from a heterotrophic to an autotrophic lifestyle (Hohmann-Marriott and Blankenship, 2011). The establishment of a stable endosymbiosis was, however, not an immediate evolutionary jump but a long-ongoing adaptation process in which the engulfed cyanobacteria-like ancestor has lost slowly most of its genetic information toward the nucleus of the host cell by horizontal gene transfer (Abdallah et al., 2000; Martin et al., 2002; Reyes-Prieto et al., 2007). Only a small, but highly conserved set of genes finally remained encoded in the plastids' own genome of present plants, the plastome (Bock, 2007; Wicke et al., 2011). The vast majority of the proteome of present-day plant plastids is, therefore, encoded in the nucleus and must be imported from the cytosol (Rolland et al., 2012; Demarsy et al., 2014). Nevertheless, the proper expression of plastid genes is absolutely essential for the build-up of protein complexes involved in plastid gene transcription and translation as well as in metabolic processes such as photosynthesis or fatty acid biosynthesis (Jarvis and Lopez-Juez, 2013; Lyska et al., 2013). All major plastid multi-subunit protein complexes are composed of a patchwork of nuclear and plastid encoded subunits and can be established only by a tight coordination of gene expression between the two genetic compartments (Pogson et al., 2015).

Alongside with these molecular and sub-cellular constraints, the establishment of plastid proteomes is strongly influenced by tissue-dependent and environmental cues. Multicellular, terrestrial plants are comprised of different organs with very divergent tissue organization and function. Plastids in these different tissues display large morphological and functional variations which are tightly connected to the function of the corresponding tissue (Schnepf, 1980; Lopez-Juez and Pyke, 2005). An individual plant, thus, possesses several different plastid types that represent distinct manifestations of the same cell organelle. Interestingly, most of these plastid types can interconvert upon environmentally induced changes in plant and tissue development. These morphological and functional conversions are only possible by corresponding changes in the plastid proteome composition. In this mini-review we focus on the specific changes in plastid gene expression that occur before or during transitions between different plastid types in the course of plant development.

The Different Plastid Types of Plant Cells

Plant cells cannot generate plastids *de novo* but they gain them by inheritance from their progenitor cell. During division of the mother cell plastids are distributed arbitrarily between daughter cells and multiply afterward, by fission using a prokaryotic-type division apparatus (Osteryoung and Pyke, 2014). The final number of plastids within a cell is cell-type specific and depends on regulatory mechanisms that are far from being understood yet (Cole, 2016). In addition, an individual cell does typically contain only one specific plastid type indicating that plastid development and cell development are interlinked. The various developmental lines and possible conversions between plastid types are subsequently discussed using the life cycle of the angiosperm *Arabidopsis* as a model (**Figure 1**). Because of space constraints detailed species-specific differences or special cases will be not considered here.

In *Arabidopsis* (like in most angiosperms) plastids are inherited maternally as a undifferentiated and small precursor form called proplastid (Pyke, 2007). In other species proplastids might be inherited also by paternal or biparental means. Knowledge in this field is poor and active regulation mechanisms remain to be clarified (Greiner et al., 2015). After fertilization of the egg cell *Arabidopsis* embryos undergo a morphological program typical for angiosperms that eventually ends with dry seeds (Le et al., 2010; compare **Figure 1**, outer circle). Like in many other oilseed crops *Arabidopsis* embryogenesis is characterized by an intermediate photosynthetically active period in which proplastids develop into chloroplasts in a stage-specific manner (Tejos et al., 2010). Chloroplast containing cells are already detected at the globular stage, but are most abundant during 6–12 days after fertilization (Allorent et al., 2013). This phase appears to be important for the fitness of the seed (Allorent et al., 2015). In a subsequent desiccation phase these chloroplasts then de-differentiate into non-photosynthetic, colorless leucoplasts, called eoplasts (Mansfield and Briarty, 1991, 1992). After seed imbibition and germination these eoplasts then re-differentiate into various plastid types depending on tissue context and environmental conditions.

In the dark, seedlings follow a developmental program called skotomorphogenesis (Solymosi and Schoefs, 2010). In cotyledons of such seedlings eoplasts develop into etioplasts while those located in hypocotyl and root develop into different types of colorless leucoplasts that are difficult to distinguish at the morphological level. Etioplasts are characteristic for this developmental program and represent an intermediate stand-by state of chloroplast formation. They do not develop a thylakoid membrane system, but a prolamellar body (PLB) that is composed of regular arrangements of NADPH, the enzyme protochlorophyllide-oxido-reductase (POR), the chlorophyll precursor protochlorophyllide and the thylakoid membrane lipids digalactosyl-diacylglycerol (DGDG) and monogalactosyl-diacylglycerol (MGDG; Bastien et al., 2016). Upon illumination another developmental program called photomorphogenesis is initiated by the phytochrome-mediated photoreceptor network that triggers the expression of many nucleus located genes coding for chloroplast proteins (Arsovski et al., 2012). In parallel, thylakoid membranes begin to form and the light-dependent POR induces chlorophyll biosynthesis within the PLB. Chloroplast biogenesis then is usually completed after just 6–24 h. If seeds germinate directly in light the skotomorphogenic program is skipped and the eoplasts within the cotyledons differentiate directly into chloroplasts. Whether or not proplastids and eoplasts represent fully equivalent developmental stages remain to be elucidated. Studies on the transition of de-differentiated desiccoplasts into etio- or chloroplasts after rehydration and illumination in the poikilochlorophyllous plant *Xerophyta humilis* may provide novel clues for the understanding of proplastid/eoplast-to-chloroplast transitions (Solymosi et al., 2013).

During primary leaf formation chloroplasts originate directly from proplastids present in the shoot apical meristem (SAM;

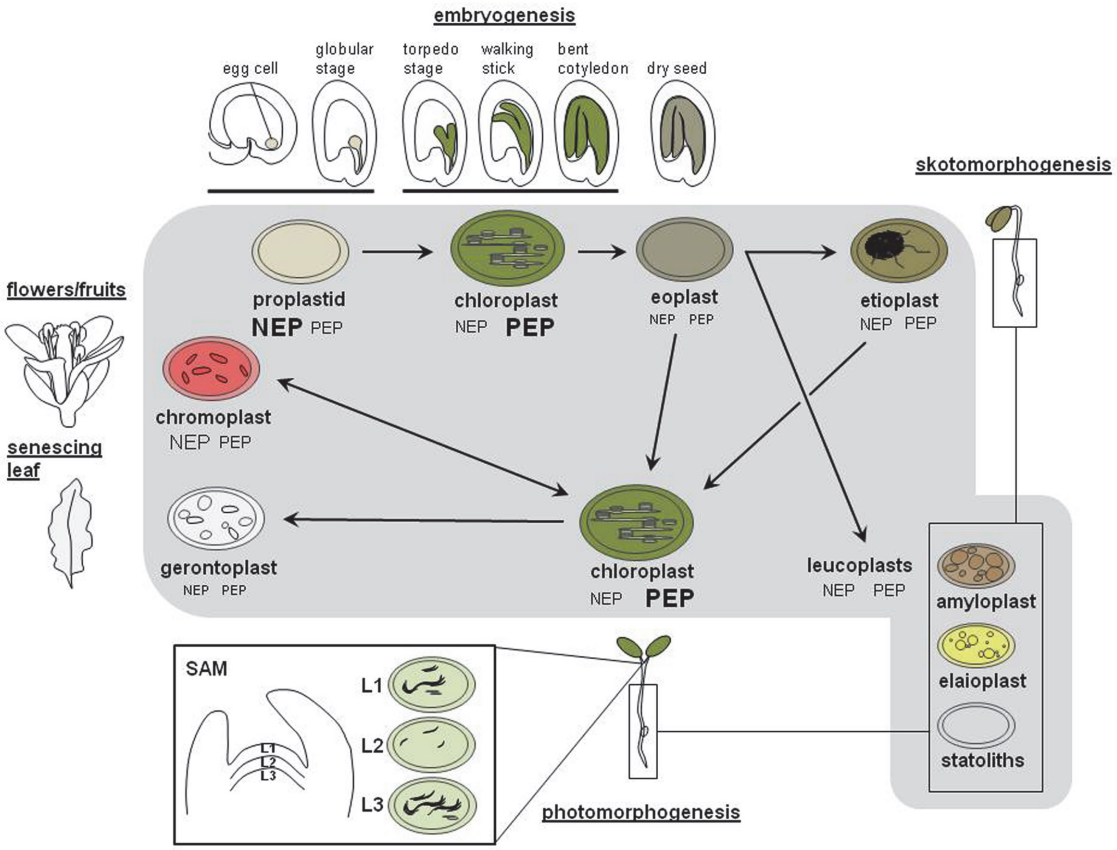

FIGURE 1 | Transitions between the different plastid types during the plant life cycle. Important steps in tissue and body development of an angiosperm from fertilization until flower development are depicted in the outer range of the figure using the well characterized life cycle of *Arabidopsis thaliana*. The inner part (gray background) indicates the major plastid types residing in the tissues of the corresponding developmental stage. Arrows indicate type and direction of transition between these plastid types. The inset depicting a cross-cut through a shoot apical meristem (SAM) with its different stages of chloroplast development has been adapted from (Charuvi et al., 2012). L1 – L3 represent different cell layers of SAM containing chloroplasts with different degree of thylakoid membrane development (indicated by rolling lines). Changes in plastid transcriptional apparatus or activity that occur during these transitions are indicated by symbols NEP and PEP. Size of letters represents the relative activities of the two types of RNA polymerases in the respective plastid type. For details see text.

Charuvi et al., 2012). Fully developed chloroplasts in green parts of plants multiply then by fission until they reach the cell-type specific number. This, however, accounts mainly for the mesophyll tissue while in the epidermis a likely tissue-specific program leads to a differential development of chloroplasts. Guard cells were reported to display high numbers of fully developed chloroplasts while pavement cells contain rather low numbers of relatively small chloroplasts (around half the size of those in mesophyll cells) that may contain reduced levels of chlorophyll (Barton et al., 2016). In reproductive organs such as fruits or flowers chloroplasts usually transform into chromoplasts as part of maturation or developmental programs. In senescing tissues the valuable resources of chloroplasts, notably the nitrogen bound in chlorophylls and photosynthesis proteins such as RubisCO, are reallocated and the plastids turn into gerontoplasts, the aging form of plastids.

In hypocotyls and roots of growing seedlings eoplasts usually develop into a number of colorless plastids commonly summarized under the term leucoplasts. This group comprises amyloplasts, statoliths, and elaioplasts (**Figure 1** and **Table 1**) and, in later stages, may develop also in other parts of the plant. These colorless plastids do develop even if the tissues are exposed to light. This strongly suggests that the transition from proplastids/eoplasts into chloroplasts is actively inhibited in these tissues, likely by internal factors. Recent studies have demonstrated that the developmental block of chloroplast development in *Arabidopsis* roots can be released either genetically or by external hormone treatment (Kobayashi et al., 2012) supporting the view of an active inhibition in chloroplast biogenesis in these tissues. Release of such an inhibition represents not only an artificial effect but does occur also under physiological conditions as some studies reported the presence of fully developed chloroplasts in *Arabidopsis* hypocotyls (Jin et al., 2001; Hermkes et al., 2011). These chloroplasts were found to be involved in phototropic responses suggesting that they play a defined physiological role (Jin et al., 2001). Studying the mechanisms that control this eoplast-chloroplast transition could help to understand principle steps

TABLE 1 | Summary of major plastid types in plant cells.

Plastid type	Tissue appearance	Morphological characteristic	Main function	Remarks	Reference
Proplastids, Eoplasts	Germ cells, embryonic and meristematic tissues	Small with low internal differentiation	Transmission of plastids between cells and generations	Terminological definition in different reports can be ambiguous	Pyke, 2007
Etioplasts	Cotyledons of dark-grown seedlings	Prolamellar body (PLB)	Stand-by state for chloroplast biogenesis		Solymosi and Schoefs, 2010
Chloroplasts	All photo-synthetically active tissues, appearance in hypocotyls and roots under certain conditions possible	Thylakoid membrane system	Photosynthesis, reduction of nitrogen and sulfur, biosyntheses of metabolites	Structural and functional variation depending on photosynthesis type (e.g., C3/C4, CAM)	Jarvis and Lopez-Juez, 2013
Chromoplast	Fruits, flowers, roots, but also formerly green tissues	Strong carotenoid synthesis	Pigment storage, tissue coloration	Internal structures may vary with degree of coloration	Egea et al., 2010
Amyloplasts	Roots and non-green storage tissues	Huge, starch grains for long-term storage	Energy storage	Serve as statoliths in gravi-perception of root columella cells	Pyke, 2007
Elaioplasts	Specialized cells, e.g., tapetal cells of anthers	High amounts of plastoglobuli	Lipid storage for pollen wall		Ting et al., 1998

Typical plastid types found in vascular plants are listed. Leucoplasts are not included as they represent a group of plastids (summarizing all non-green plastids lacking pigments including amyloplasts) rather than defining a specific plastid type. The group of leucoplasts contains also other less prominent plastid types that are not well investigated and not discussed in this review such as root plastids and proteinoplasts. For more information on these specialized plastid forms readers are referred to corresponding reviews (Schnepf, 1980; Pyke, 2007).

of early chloroplast biogenesis and to identify novel regulatory factors of plastid transitions (Chiang et al., 2012).

Shifts in Plastid Transcription during Morphological Transitions of Plastids

The different types of plastids mentioned above perform very different functions that are highly specific for the tissue in which they reside (Pyke, 2007) (**Table 1**). Despite their morphological and functional diversity they all contain the same genome (Bock, 2007). However, their strong functional diversity implies a specific enzymatic configuration for each plastid type. This requires a controlled adjustment in the expression of both plastid and nuclear genes encoding the proteins for each of these specific organelle manifestations. Here, we focus on the adjustment of plastid gene expression.

Molecular and genetic studies uncovered that transcription of plastid genes is performed by two different types of RNA polymerases. One type is comprised by two single-subunit phage-type RNA polymerases encoded by two different nuclear genes (nuclear-encoded RNA polymerases, NEP). These proteins are targeted either only to the plastid (RpoTp) or dually to plastids and mitochondria (RpoTmp). The other type of RNA polymerase is a multi-subunit enzyme of prokaryotic type with four basic subunits encoded in the plastid genome (RpoA, RpoB, RpoC1, and RpoC2; plastid-encoded RNA polymerase, PEP). For promoter recognition this enzyme complex is dependent on the interaction with sigma factors (called Sig1 – Sig6 in *Arabidopsis*) that are encoded in the nucleus (Toyoshima et al., 2005; Schweer et al., 2010; Lerbs-Mache, 2011; Borner et al., 2015; Pfannschmidt et al., 2015). The two types of RNA polymerase activities utilize different promoters and depending on their respective promoter structure the genes on the plastid genome can be categorized into three different classes. Class I comprises genes possessing only PEP promoters (only photosynthesis genes). Class II covers genes that have both NEP and PEP promoters (most other genes including genes for the ATP synthase and many components of the gene expression system). Class III represents genes with NEP promoters only and comprises *ycf2* (encoding a still unknown protein), *accD* (encoding the β-carboxyltransferase subunit of the acetyl CoA carboxylase) and the *rpoBC*$_1$*C*$_2$ operon (Liere et al., 2011). This diversity of promoter structures and the multiplicity of transcriptional components (see also below) represent a prerequisite for efficient transcriptional regulation during plastid conversion where plastid housekeeping genes are preferentially transcribed by NEP and photosynthesis related genes are transcribed by PEP (Allison et al., 1996; Hajdukiewicz et al., 1997).

We propose that targeted changes in plastid transcription, mostly by controlling the relative activities of NEP and PEP enzymes, impact the establishment of the plastid proteome and, therefore, represent key determinants for the transitions between the different plastid types.

Proplastid/Eoplast-Chloroplast Transition

Proplastids can be found only in meristematic cells of plants and in *in vitro* cultured cells. Isolation of proplastids from meristematic cells is technically not feasible. However, as meristematic cells give rise to various plant organs, proplastids might be considered as starting point for differentiation-dependent plastid conversion. Also, plastid gene expression in proplastids and after controlled conversion of proplastids into amyloplasts has been analyzed using *in vitro* cultured cells [(Sakai et al., 1992), see below]. These early experiments already showed that such plastid conversion is accompanied by changes in plastid transcriptional activity.

Proplastid/eoplast-chloroplast conversion-associated changes in plastid gene expression patterns have been characterized

in detail during *Arabidopsis* seed formation and germination (Demarsy et al., 2012; Allorent et al., 2013). Although slight increases of NEP transcribed mRNAs were observed in this transition, the predominant changes concern remarkable increases of mRNAs of photosynthesis related proteins. If proplastid/eoplast-chloroplast conversion is prevented by deletion of plastid *rpo* genes, colorless plastids of 2–5 μm length are formed that might be considered as a genetically induced type of leucoplasts (Allison et al., 1996; De Santis-MacIossek et al., 1999). Thus, establishment of the correct NEP/PEP configuration and their relative activities at a given developmental stage is absolutely essential for successful chloroplast differentiation.

Etioplast-Chloroplast Transition

Etioplasts and their light-induced transition to chloroplasts are well studied in numerous dicotyledonous and monocotyledonous species. Most striking is the very rapid development of thylakoid membranes, increase in chlorophyll content and construction of the photosynthetic apparatus that requires both a massive import of nuclear encoded plastid proteins and high expression of plastid-encoded genes (Lonosky et al., 2004; von Zychlinski et al., 2005; Philippar et al., 2007; Pudelski et al., 2009; Majeran et al., 2010; Ploscher et al., 2011). Etioplasts display just a basic transcriptional activity and accumulate photosynthesis transcripts only to very low levels. Shifting dark-grown seedlings to light, however, rapidly induce a plastome-wide transcript accumulation of photosynthesis genes reaching a maximum level after 10–44 h mRNA levels followed by decrease to approximate pre-illumination levels (Rodermel and Bogorad, 1985). The initial increase in mRNA is followed by subsequent translation of the corresponding proteins (Kanervo et al., 2008). It should be noted that tissue-specific gene expression analyses distinguishing epidermal and mesophyll tissues were never reported and that the results in all studies to date, thus, represent a mixture of both cell types. This is critical with respect to the notion that recent studies suggest a specific sensor function for epidermal chloroplasts (Virdi et al., 2015, 2016). Targeted research on this special type of chloroplasts will be required in order to understand their detailed physiological function.

The light-dependent activation of plastid gene expression during etioplast-chloroplast conversion includes post-translational modifications such as phosphorylation of PEP subunits and sigma factors (Tiller and Link, 1993) and a restructuring of the PEP complex. While in etiolated mustard seedlings PEP was found to exist in its prokaryotic composition (α2, β, β′, β″ subunits), a much larger PEP complex with many additional subunits was purified from fully developed chloroplasts. Studies on intermediate plastids isolated from seedlings illuminated for just 16 h identified both complexes to around equal activities suggesting a light-induced conversion between these two plastid RNA polymerase complexes (Pfannschmidt and Link, 1994). Detailed mass spectrometry analyses identified these subunits and a set of conserved PEP-associated proteins (PAPs) could be defined (Pfannschmidt et al., 2000; Suzuki et al., 2004; Pfalz et al., 2006; Steiner et al., 2011).

PEP-associated proteins are all nuclear encoded and are rapidly light-induced during etioplast-chloroplast transition yielding the observed PEP restructuring (Yagi et al., 2012). Genetic inactivation of any of these PAPs in *Arabidopsis*, maize or rice results in a block of proper chloroplast development and ends up in albinoic phenotypes suggesting that *pap* gene expression and/or subsequent PEP re-structuring represent essential steps in early chloroplast biogenesis. Evolutionary presence of *pap* genes appears to be restricted to terrestrial plants and ferns suggesting that their appearance is connected to the conquest of land (Pfalz and Pfannschmidt, 2013). These genes, thus, likely represent an evolutionary indicator for the development of chloroplast-containing multi-cellular plants (de Vries et al., 2016).

Plastid gene expression changes during etioplast to chloroplast conversion were also analyzed in the monocotyledonous plant maize. In monocotyledons, leaf development is initiated at a basal meristem resulting in a gradient of chloroplast development from the bottom to the tip (Baumgartner et al., 1989, 1993; Hess et al., 1993). This gradient has been used extensively as a model for chloroplast biogenesis. About 51 plastid genes were found to be at least two times higher expressed in tips than in the leaf base (Cahoon et al., 2008). It is, however, still debated how far this plastid developmental gradient reflects the corresponding situation (proplastid-to-chloroplast conversion) in dicotyledonous plants.

Chloroplast-Chromoplast Transition

Chromoplasts mainly develop from chloroplasts in formerly green plant tissues e.g., during fruit ripening or flower development. They can also develop directly from proplastids or amyloplast depending on species and tissue (Egea et al., 2010). Plastid gene expression during conversion of green chloroplasts toward red chromoplasts has been characterized in detail during tomato fruit ripening. In contrast to the rapid etioplast-chloroplast transition in cotyledons, the chloroplast-chromoplast transition in tomato fruits requires several days or even weeks allowing transcript analyses of various intermediary stages. These studies uncovered both systemic and gene-specific effects (Kahlau and Bock, 2008). Most important, green tomato fruits displayed a dramatic reduction in chloroplast transcripts compared to green leaves from the same plant. This indicates that the fruit developmental program provides a dominant repressive impact on plastid transcriptional activities even before ripening effects became visible. This may prevent the unnecessary production of photosynthesis proteins in the tomato fruit already in early stages of ripening.

In contrast, changes in plastid gene expression in the subsequent stages (turning, light red, red) remained relatively subtle suggesting that the chloroplast-chromoplast conversion itself is not accompanied by major changes in plastid gene expression. An exception was observed for the *accD* gene that displayed a targeted accumulation at both, transcript and protein levels. Accumulation of the protein AccD as part of the fatty acid biosynthesis complex may allow the accumulation of lipids necessary for storage of carotenoids produced during fruit ripening. *AccD* gene expression requires at least a low

level of expression of genes involved in transcription/translation. Indeed, the repression by the fruit developmental program was stronger for photosynthesis genes than for genetic system genes (Kahlau and Bock, 2008) suggesting that low levels of plastid gene expression activity may remain. These remaining activities may be directed to the observed targeted *accD* gene expression.

Proplastid-Amyloplast Transition

Amyloplasts are the plastids of storage organ tissues and roots and typically contain high amounts of starch. Systematic gene expression studies in this plastid type were done using potato tubers (Brosch et al., 2007; Valkov et al., 2009). When compared to leaf chloroplasts tuber amyloplasts displayed very low levels of gene expression in terms of transcriptional rate, transcript accumulation and maturation as well as ribosome association of mRNAs and translation. Both, NEP and PEP enzymes are present, but run-on transcription experiments revealed very low transcriptional rates of both enzyme activities. Interestingly, like in chromoplasts *accD* expression appeared to be an exception. It displayed relatively stable transcript levels and ribosome association (Valkov et al., 2009) thus confirming the importance of AccD for the maintenance of plastids regardless of their morphological type. In addition, trans-plastomic inactivation of the plastid *accD* gene in tobacco revealed to be impossible (Kode et al., 2005).

Tissue cultures of tobacco bright-yellow (BY)-2 cells represent another test system to study amyloplasts (Miyazawa et al., 1999; Enami et al., 2011). In presence of cytokinin these cells develop amyloplasts from proplastids. Microarray analysis of the transcriptome did not reveal specific changes between the two plastid types, including the *accD* gene. Interestingly, inhibitors of plastid transcription or translation blocked the hormone-induced differentiation of amyloplasts indicating signaling of plastid gene expression to the hormone-induced plastid developmental pathway. This specific retrograde signaling pathway seems to act *via* intermediates of tetrapyrrole biosynthesis, i.e., haem (Enami et al., 2011).

CONCLUSION AND PERSPECTIVES

In all plastid conversions investigated so far, changes in the plastid transcriptional apparatus and/or transcriptional activity either accompany or even precede the transition. Proper control of plastome transcription, thus, appears to be an important determinant for these developmental steps. Future research will focus on the identification of regulators that may serve as master switches of plastid development in response to internal and external cues (Lopez-Juez, 2007). In addition, more detailed studies on gene expression in proplastids or eoplasts may be highly informative for understanding the molecular regulation of plastid development especially in their initial steps.

AUTHOR CONTRIBUTIONS

ML contributed a figure, contributed to the manuscript text, read, and approved the final version. BG contributed to the manuscript text, read, and approved the final version. FC contributed to the manuscript text, read, and approved the final version. SL-M contributed to the manuscript text, read, and approved the final version. LM contributed to the manuscript text, read, and approved the final version. RB contributed to the manuscript text, read, and approved the final version. TP developed manuscript idea, wrote the manuscript with the help of all co-authors.

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REFERENCES

Abdallah, F., Salamini, F., and Leister, D. (2000). A prediction of the size and evolutionary origin of the proteome of chloroplasts of *Arabidopsis*. *Trends Plant. Sci.* 5, 141–142. doi: 10.1016/S1360-1385(00)01574-0

Allison, L. A., Simon, L. D., and Maliga, P. (1996). Deletion of rpoB reveals a second distinct transcription system in plastids of higher plants. *EMBO J.* 15, 2802–2809.

Allorent, G., Courtois, F., Chevalier, F., and Lerbs-Mache, S. (2013). Plastid gene expression during chloroplast differentiation and dedifferentiation into non-photosynthetic plastids during seed formation. *Plant Mol. Biol.* 82, 59–70. doi: 10.1007/s11103-013-0037-0

Allorent, G., Osorio, S., Vu, J. L., Falconet, D., Jouhet, J., Kuntz, M., et al. (2015). Adjustments of embryonic photosynthetic activity modulate seed fitness in *Arabidopsis thaliana*. *New Phytol.* 205, 707–719. doi: 10.1111/nph.13044

Arsovski, A. A., Galstyan, A., Guseman, J. M., and Nemhauser, J. L. (2012). Photomorphogenesis. *Arabidopsis Book* 10:e0147. doi: 10.1199/tab.0147

Ball, S. G., Bhattacharya, D., and Weber, A. P. (2016). EVOLUTION. Pathogen to powerhouse. *Science* 351, 659–660. doi: 10.1126/science.aad8864

Barton, K. A., Schattat, M. H., Jakob, T., Hause, G., Wilhelm, C., McKenna, J. F., et al. (2016). Epidermal pavement cells of *Arabidopsis* have chloroplasts. *Plant Physiol.* 171, 723–726. doi: 10.1104/pp.16.00608

Bastien, O., Botella, C., Chevalier, F., Block, M. A., Jouhet, J., Breton, C., et al. (2016). New insights on thylakoid biogenesis in plant cells. *Int. Rev. Cell Mol. Biol.* 323, 1–30. doi: 10.1016/bs.ircmb.2015.12.001

Baumgartner, B. J., Rapp, J. C., and Mullet, J. E. (1989). Plastid transcription activity and DNA copy number increase early in barley chloroplast development. *Plant Physiol.* 89, 1011–1018. doi: 10.1104/pp.89.3.1011

Baumgartner, B. J., Rapp, J. C., and Mullet, J. E. (1993). Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley (*Hordeum vulgare*) chloroplast development (evidence for selective stabilization of psbA mRNA). *Plant Physiol.* 101, 781–791. doi: 10.1104/pp.101.3.781

Bock, R. (2007). "Structure, function, and inheritance of plastid genomes," in *Cell and Molecular Biology of Plastids*, ed. R. Bock (Berlin: Springer), 29–63.

Borner, T., Aleynikova, A. Y., Zubo, Y. O., and Kusnetsov, V. V. (2015). Chloroplast RNA polymerases: role in chloroplast biogenesis. *Biochim. Biophys. Acta* 1847, 761–769. doi: 10.1016/j.bbabio.2015.02.004

Brosch, M., Krause, K., Falk, J., and Krupinska, K. (2007). Analysis of gene expression in amyloplasts of potato tubers. *Planta* 227, 91–99. doi: 10.1007/s00425-007-0597-9

Cahoon, A. B., Takacs, E. M., Sharpe, R. M., and Stern, D. B. (2008). Nuclear, chloroplast, and mitochondrial transcript abundance along a maize leaf developmental gradient. *Plant Mol. Biol.* 66, 33–46. doi: 10.1007/s11103-007-9250-z

Charuvi, D., Kiss, V., Nevo, R., Shimoni, E., Adam, Z., and Reich, Z. (2012). Gain and loss of photosynthetic membranes during plastid differentiation in the shoot apex of *Arabidopsis*. *Plant Cell* 24, 1143–1157. doi: 10.1105/tpc.111.094458

Chiang, Y. H., Zubo, Y. O., Tapken, W., Kim, H. J., Lavanway, A. M., Howard, L., et al. (2012). Functional characterization of the GATA transcription factors GNC and CGA1 reveals their key role in chloroplast development, growth, and division in *Arabidopsis*. *Plant Physiol.* 160, 332–348. doi: 10.1104/pp.112.198705

Cole, L. W. (2016). The evolution of per-cell organelle number. *Front. Cell Dev. Biol.* 4:85. doi: 10.3389/fcell.2016.00085

De Santis-MacIossek, G., Kofer, W., Bock, A., Schoch, S., Maier, R. M., Wanner, G., et al. (1999). Targeted disruption of the plastid RNA polymerase genes rpoA, B and C1: molecular biology, biochemistry and ultrastructure. *Plant J.* 18, 477–489. doi: 10.1046/j.1365-313X.1999.00473.x

de Vries, J., Stanton, A., Archibald, J. M., and Gould, S. B. (2016). Streptophyte terrestrialization in light of plastid evolution. *Trends Plant Sci.* 21, 467–476. doi: 10.1016/j.tplants.2016.01.021

Demarsy, E., Buhr, F., Lambert, E., and Lerbs-Mache, S. (2012). Characterization of the plastid-specific germination and seedling establishment transcriptional programme. *J. Exp. Bot.* 63, 925–939. doi: 10.1093/jxb/err322

Demarsy, E., Lakshmanan, A. M., and Kessler, F. (2014). Border control: selectivity of chloroplast protein import and regulation at the TOC-complex. *Front. Plant Sci.* 5:483. doi: 10.3389/fpls.2014.00483

Egea, I., Barsan, C., Bian, W., Purgatto, E., Latche, A., Chervin, C., et al. (2010). Chromoplast differentiation: current status and perspectives. *Plant Cell Physiol.* 51, 1601–1611. doi: 10.1093/pcp/pcq136

Enami, K., Ozawa, T., Motohashi, N., Nakamura, M., Tanaka, K., and Hanaoka, M. (2011). Plastid-to-nucleus retrograde signals are essential for the expression of nuclear starch biosynthesis genes during amyloplast differentiation in tobacco BY-2 cultured cells. *Plant Physiol.* 157, 518–530. doi: 10.1104/pp.111.178897

Greiner, S., Sobanski, J., and Bock, R. (2015). Why are most organelle genomes transmitted maternally? *Bioessays* 37, 80–94. doi: 10.1002/bies.201400110

Hajdukiewicz, P. T., Allison, L. A., and Maliga, P. (1997). The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. *EMBO J.* 16, 4041–4048. doi: 10.1093/emboj/16.13.4041

Hermkes, R., Fu, Y. F., Nurrenberg, K., Budhiraja, R., Schmelzer, E., Elrouby, N., et al. (2011). Distinct roles for *Arabidopsis* SUMO protease ESD4 and its closest homolog ELS1. *Planta* 233, 63–73. doi: 10.1007/s00425-010-1281-z

Hess, W. R., Prombona, A., Fieder, B., Subramanian, A. R., and Borner, T. (1993). Chloroplast rps15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids: evidence for a functioning non-chloroplast-encoded RNA polymerase. *EMBO J.* 12, 563–571.

Hohmann-Marriott, M. F., and Blankenship, R. E. (2011). Evolution of photosynthesis. *Annu. Rev. Plant Biol.* 62, 515–548. doi: 10.1146/annurev-arplant-042110-103811

Jarvis, P., and Lopez-Juez, E. (2013). Biogenesis and homeostasis of chloroplasts and other plastids. *Nat. Rev. Mol. Cell Biol.* 14, 787–802. doi: 10.1038/nrm3702

Jin, X., Zhu, J., and Zeiger, E. (2001). The hypocotyl chloroplast plays a role in phototropic bending of *Arabidopsis* seedlings: developmental and genetic evidence. *J. Exp. Bot.* 52, 91–97. doi: 10.1093/jexbot/52.354.91

Kahlau, S., and Bock, R. (2008). Plastid transcriptomics and translatomics of tomato fruit development and chloroplast-to-chromoplast differentiation: chromoplast gene expression largely serves the production of a single protein. *Plant Cell* 20, 856–874. doi: 10.1105/tpc.107.055202

Kanervo, E., Singh, M., Suorsa, M., Paakarinen, V., Ara, E., Battchikova, N., et al. (2008). Expression of protein complexes and individual proteins upon transition of etioplasts to chloroplasts in Pea (*Pisum sativum*). *Plant Cell Physiol.* 49, 396–410. doi: 10.1093/pcp/pcn016

Kobayashi, K., Baba, S., Obayashi, T., Sato, M., Toyooka, K., Keranen, M., et al. (2012). Regulation of root greening by light and auxin/cytokinin signaling in *Arabidopsis*. *Plant Cell* 24, 1081–1095. doi: 10.1105/tpc.111.092254

Kode, V., Mudd, E. A., Iamtham, S., and Day, A. (2005). The tobacco plastid accD gene is essential and is required for leaf development. *Plant J.* 44, 237–244. doi: 10.1111/j.1365-313X.2005.02533.x

Le, B. H., Cheng, C., Bui, A. Q., Wagmaister, J. A., Henry, K. F., Pelletier, J., et al. (2010). Global analysis of gene activity during *Arabidopsis* seed development and identification of seed-specific transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8063–8070. doi: 10.1073/pnas.1003530107

Lerbs-Mache, S. (2011). Function of plastid sigma factors in higher plants: regulation of plastid gene expression or just preservation of constitutive transcription? *Plant Mol. Biol.* 76, 235–249. doi: 10.1007/s11103-010-9714-4

Liere, K., Weihe, A., and Borner, T. (2011). The transcription machineries of plant mitochondria and chloroplasts: composition, function, and regulation. *J. Plant Physiol.* 168, 1345–1360. doi: 10.1016/j.jplph.2011.01.005

Lonosky, P. M., Zhang, X., Honavar, V. G., Dobbs, D. L., Fu, A., and Rodermel, S. R. (2004). A proteomic analysis of maize chloroplast biogenesis. *Plant Physiol.* 134, 560–574. doi: 10.1104/pp.103.032003

Lopez-Juez, E. (2007). Plastid biogenesis, between light and shadows. *J. Exp. Bot.* 58, 11–26. doi: 10.1093/jxb/erl196

Lopez-Juez, E., and Pyke, K. A. (2005). Plastids unleashed: their development and their integration in plant development. *Int. J. Dev. Biol.* 49, 557–577. doi: 10.1387/ijdb.051997el

Lyska, D., Meierhoff, K., and Westhoff, P. (2013). How to build functional thylakoid membranes: from plastid transcription to protein complex assembly. *Planta* 237, 413–428. doi: 10.1007/s00425-012-1752-5

Majeran, W., Friso, G., Ponnala, L., Connolly, B., Huang, M., Reidel, E., et al. (2010). Structural and metabolic transitions of C4 leaf development and differentiation defined by microscopy and quantitative proteomics in maize. *Plant Cell* 22, 3509–3542. doi: 10.1105/tpc.110.079764

Mansfield, S. G., and Briarty, L. G. (1991). Early embryogenesis in *Arabidopsis thaliana*. II. The developing embryo. *Can. J. Bot.* 69, 461–476. doi: 10.1139/b91-062

Mansfield, S. G., and Briarty, L. G. (1992). Cotyledon cell development in *Arabidopsis thaliana* during reserve deposition. *Can. J. Bot.* 70, 151–164. doi: 10.1139/b92-021

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., et al. (2002). Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 99, 12246–12251. doi: 10.1073/pnas.182432999

Miyazawa, Y., Sakai, A., Miyagishima, S., Takano, H., Kawano, S., and Kuroiwa, T. (1999). Auxin and cytokinin have opposite effects on amyloplast development and the expression of starch synthesis genes in cultured bright yellow-2 tobacco cells. *Plant Physiol.* 121, 461–469. doi: 10.1104/pp.121.2.461

Osteryoung, K. W., and Pyke, K. A. (2014). Division and dynamic morphology of plastids. *Annu. Rev. Plant Biol.* 65, 443–472. doi: 10.1146/annurev-arplant-050213-035748

Pfalz, J., Liere, K., Kandlbinder, A., Dietz, K. J., and Oelmuller, R. (2006). pTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. *Plant Cell* 18, 176–197. doi: 10.1105/tpc.105.036392

Pfalz, J., and Pfannschmidt, T. (2013). Essential nucleoid proteins in early chloroplast development. *Trends Plant Sci.* 18, 186–194. doi: 10.1016/j.tplants.2012.11.003

Pfannschmidt, T., Blanvillain, R., Merendino, L., Courtois, F., Chevalier, F., Liebers, M., et al. (2015). Plastid RNA polymerases: orchestration of enzymes with different evolutionary origins controls chloroplast biogenesis during the plant life cycle. *J. Exp. Bot.* 66, 6957–6973. doi: 10.1093/jxb/erv415

Pfannschmidt, T., and Link, G. (1994). Separation of two classes of plastid DNA-dependent RNA polymerases that are differentially expressed in mustard (*Sinapis alba* L.) seedlings. *Plant Mol. Biol.* 25, 69–81. doi: 10.1007/BF00024199

Pfannschmidt, T., Ogrzewalla, K., Baginsky, S., Sickmann, A., Meyer, H. E., and Link, G. (2000). The multisubunit chloroplast RNA polymerase A from mustard (*Sinapis alba* L.). Integration of a prokaryotic core into a larger complex with organelle-specific functions. *Eur. J. Biochem.* 267, 253–261. doi: 10.1046/j.1432-1327.2000.00991.x

Philippar, K., Geis, T., Ilkavets, I., Oster, U., Schwenkert, S., Meurer, J., et al. (2007). Chloroplast biogenesis: the use of mutants to study the etioplast-chloroplast transition. *Proc. Natl. Acad. Sci. U.S.A.* 104, 678–683. doi: 10.1073/pnas.0610062104

Ploscher, M., Reisinger, V., and Eichacker, L. A. (2011). Proteomic comparison of etioplast and chloroplast protein complexes. *J. Proteom.* 74, 1256–1265. doi: 10.1016/j.jprot.2011.03.020

Pogson, B. J., Ganguly, D., and Albrecht-Borth, V. (2015). Insights into chloroplast biogenesis and development. *Biochim. Biophys. Acta* 1847, 1017–1024. doi: 10.1016/j.bbabio.2015.02.003

Pudelski, B., Soll, J., and Philippar, K. (2009). A search for factors influencing etioplast-chloroplast transition. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12201–12206. doi: 10.1073/pnas.0902145106

Pyke, K. (2007). "Plastid biogenesis and differentiation," in *Cell and Molecular Biology of Plastids*, ed. R. Bock (Berlin: Springer-Verlag), 1–28.

Reyes-Prieto, A., Weber, A. P., and Bhattacharya, D. (2007). The origin and establishment of the plastid in algae and plants. *Annu. Rev. Genet.* 41, 147–168. doi: 10.1146/annurev.genet.41.110306.130134

Rodermel, S. R., and Bogorad, L. (1985). Maize plastid photogenes: mapping and photoregulation of transcript levels during light-induced development. *J. Cell Biol.* 100, 463–476. doi: 10.1083/jcb.100.2.463

Rolland, N., Curien, G., Finazzi, G., Kuntz, M., Marechal, E., Matringe, M., et al. (2012). The biosynthetic capacities of the plastids and integration between cytoplasmic and chloroplast processes. *Annu. Rev. Genet.* 46, 233–264. doi: 10.1146/annurev-genet-110410-132544

Sakai, A., Kawano, S., and Kuroiwa, T. (1992). Conversion of proplastids to amyloplasts in tobacco cultured cells is accompanied by changes in the transcriptional activities of plastid genes. *Plant Physiol.* 100, 1062–1066. doi: 10.1104/pp.100.2.1062

Schnepf, E. (1980). "Types of plastids: their development and interconversions," in *Chloroplasts*, ed. J. Reinert (Berlin: Springer), 4–20.

Schweer, J., Turkeri, H., Kolpack, A., and Link, G. (2010). Role and regulation of plastid sigma factors and their functional interactors during chloroplast transcription - recent lessons from *Arabidopsis* thaliana. *Eur. J. Cell Biol.* 89, 940–946. doi: 10.1016/j.ejcb.2010.06.016

Solymosi, K., and Schoefs, B. (2010). Etioplast and etio-chloroplast formation under natural conditions: the dark side of chlorophyll biosynthesis in angiosperms. *Photosynth. Res.* 105, 143–166. doi: 10.1007/s11120-010-9568-2

Solymosi, K., Tuba, Z., and Boddi, B. (2013). Desiccoplast-etioplast-chloroplast transformation under rehydration of desiccated poikilochlorophyllous *Xerophyta humilis* leaves in the dark and upon subsequent illumination. *J. Plant Physiol.* 170, 583–590. doi: 10.1016/j.jplph.2012.11.022

Steiner, S., Schroter, Y., Pfalz, J., and Pfannschmidt, T. (2011). Identification of essential subunits in the plastid-encoded RNA polymerase complex reveals building blocks for proper plastid development. *Plant Physiol.* 157, 1043–1055. doi: 10.1104/pp.111.184515

Suzuki, J. Y., Ytterberg, A. J., Beardslee, T. A., Allison, L. A., Wijk, K. J., and Maliga, P. (2004). Affinity purification of the tobacco plastid RNA polymerase and in vitro reconstitution of the holoenzyme. *Plant J.* 40, 164–172. doi: 10.1111/j.1365-313X.2004.02195.x

Tejos, R. I., Mercado, A. V., and Meisel, L. A. (2010). Analysis of chlorophyll fluorescence reveals stage specific patterns of chloroplast-containing cells during *Arabidopsis* embryogenesis. *Biol. Res.* 43, 99–111. doi: 10.4067/S0716-97602010000100012

Tiller, K., and Link, G. (1993). Phosphorylation and dephosphorylation affect functional characteristics of chloroplast and etioplast transcription systems from mustard (*Sinapis alba* L.). *EMBO J* 12, 1745–1753.

Ting, J. T., Wu, S. S., Ratnayake, C., and Huang, A. H. (1998). Constituents of the tapetosomes and elaioplasts in *Brassica campestris* tapetum and their degradation and retention during microsporogenesis. *Plant J.* 16, 541–551. doi: 10.1046/j.1365-313x.1998.00325.x

Toyoshima, Y., Onda, Y., Shiina, T., and Nakahira, Y. (2005). Plastid transcription in higher plants. *Crit. Rev. Plant Sci.* 24, 59–81. doi: 10.1080/07352680590910438

Valkov, V. T., Scotti, N., Kahlau, S., Maclean, D., Grillo, S., Gray, J. C., et al. (2009). Genome-wide analysis of plastid gene expression in potato leaf chloroplasts and tuber amyloplasts: transcriptional and posttranscriptional control. *Plant Physiol.* 150, 2030–2044. doi: 10.1104/pp.109.140483

Virdi, K. S., Laurie, J. D., Xu, Y. Z., Yu, J., Shao, M. R., Sanchez, R., et al. (2015). *Arabidopsis* MSH1 mutation alters the epigenome and produces heritable changes in plant growth. *Nat. Commun.* 6:6386. doi: 10.1038/ncomms7386

Virdi, K. S., Wamboldt, Y., Kundariya, H., Laurie, J. D., Keren, I., Kumar, K. R., et al. (2016). MSH1 is a plant organellar DNA binding and thylakoid protein under precise spatial regulation to alter development. *Mol. Plant* 9, 245–260. doi: 10.1016/j.molp.2015.10.011

von Zychlinski, A., Kleffmann, T., Krishnamurthy, N., Sjolander, K., Baginsky, S., and Gruissem, W. (2005). Proteome analysis of the rice etioplast: metabolic and regulatory networks and novel protein functions. *Mol. Cell. Proteom.* 4, 1072–1084. doi: 10.1074/mcp.M500018-MCP200

Wicke, S., Schneeweiss, G. M., dePamphilis, C. W., Muller, K. F., and Quandt, D. (2011). The evolution of the plastid chromosome in land plants: gene content, gene order, gene function. *Plant Mol. Biol.* 76, 273–297. doi: 10.1007/s11103-011-9762-4

Yagi, Y., Ishizaki, Y., Nakahira, Y., Tozawa, Y., and Shiina, T. (2012). Eukaryotic-type plastid nucleoid protein pTAC3 is essential for transcription by the bacterial-type plastid RNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7541–7546. doi: 10.1073/pnas.1119403109

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Comparative Analysis of Chloroplast *psbD* Promoters in Terrestrial Plants

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The transcription of photosynthesis genes encoded by the plastid genome is mainly mediated by a prokaryotic-type RNA polymerase called plastid-encoded plastid RNA polymerase (PEP). Standard PEP-dependent promoters resemble bacterial sigma-70-type promoters containing the so-called −10 and −35 elements. On the other hand, an unusual light- and stress-responsive promoter (*psbD* LRP) that is regulated by a 19-bp AAG-box immediately upstream of the −35 element has been mapped upstream of the *psbD-psbC* operon in some angiosperms. However, the occurrence of the AAG-box containing *psbD* LRP in plant evolution remains elusive. We have mapped the *psbD* promoters in eleven embryophytes at different evolutionary stages from liverworts to angiosperms. The *psbD* promoters were mostly mapped around 500–900 bp upstream of the *psbD* translational start sites, indicating that the *psbD* mRNAs have unusually long 5′-UTR extensions in common. The −10 elements of the *psbD* promoter are well-conserved in all embryophytes, but not the −35 elements. We found that the AAG-box sequences are highly conserved in angiosperms and gymnosperms except for gnetaceae plants. Furthermore, partial AAG-box-like sequences have been identified in the *psbD* promoters of some basal embryophytes such as moss, hornwort, and lycophyte, whereas liverwort has the standard PEP promoter without the AAG-box. These results suggest that the AAG-box sequences of the *psbD* LRP may have evolved from a primitive type of AAG-box of basal embryophytes. On the other hand, monilophytes (ferns) use another type of *psbD* promoter composed of a distinct *cis*-element upstream of the potential −35 element. Furthermore, we found that *psbD* expression is not regulated by light in gymnosperms or basal angiosperms, although they have the well-conserved AAG-box sequences. Thus, it is unlikely that acquisition of the AAG-box containing *psbD* promoter is directly associated with light-induced transcription of the *psbD-psbC* operon. Light- and stress-induced transcription may have evolved independently and multiple times during terrestrial plant evolution.

Keywords: *psbD* LRP, chloroplast, promoter, evolution, stress

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INTRODUCTION

Chloroplasts in plant and algal cells are semiautonomous organelles that have their own genome and gene expression system, reflecting their cyanobacterial origin. Chloroplast transcription is mediated by two distinct RNA polymerase systems, a prokaryotic multi-subunit RNA polymerase (PEP) whose core subunits are encoded by chloroplast genomes and single-subunit

bacteriophage-type RNA polymerases (NEP) that are encoded by the nuclear genome (Hess and Börner, 1999; Liere et al., 2011; Yagi and Shiina, 2012, 2014; Liebers et al., 2017). The PEP core enzyme consists of four major subunits, designated as α, β, β′, and β′′" subunits, which are homologous to bacterial subunits. Another dissociable subunit called a sigma factor allows the core enzyme to initiate transcription from the specific promoters. Multiple sigma factor genes have been identified in embryophytes (Tanaka et al., 1997; Morikawa et al., 1999; Fujiwara et al., 2000; Hara et al., 2001; Kasai et al., 2004; Kubota et al., 2007; Kanazawa et al., 2013), and they play specific roles in transcriptional regulation in response to developmental and/or environmental cues (reviewed by Kanamaru and Tanaka, 2004; Shiina et al., 2005; Schweer et al., 2010; Börner et al., 2015; Chi et al., 2015). Standard PEP-dependent promoters resemble bacterial sigma-70 type promoters containing −10 (TATAAT) and −35 (TTGACA) elements, reflecting their bacterial origin. NEP recognizes distinct types of promoters containing a core YRTA motif (Hess and Börner, 1999; Liere and Maliga, 1999; Liere et al., 2004; Börner et al., 2015). PEP transcribes mainly photosynthesis genes in mature chloroplasts while NEP transcribes housekeeping genes in both chloroplasts and non-photosynthetic plastids (Allison et al., 1996; Hajdukiewicz et al., 1997). The functional coordination of PEP and NEP plays a critical role in plastid differentiation in angiosperms.

In contrast to angiosperms, chloroplasts of the green algae *C. reinhardtii* harbor a simple transcription system, which is dependent on PEP and a single sigma factor SIG1 (Bohne et al., 2006; Yagi and Shiina, 2014). No NEP has been identified in *Chlamydomonas*. It is considered that embryophytes have developed complex transcription systems to adapt to marked environmental stresses. However, the evolutionary process of chloroplast transcription systems in embryophytes remains largely elusive.

Most PEP-dependent genes are actively transcribed in green tissues including the leaves. The chloroplast run-on experiments demonstrated that PEP-dependent transcription is activated by high light compared to normal growth light (Baena-González et al., 2001). However, the accumulation of most PEP-dependent transcripts is not regulated by light/dark transitions or environmental stresses, possibly due to the extraordinary stability of their transcripts (Shiina et al., 1998; Hayes et al., 1999). The only exception is a *psbD* light-responsive promoter designated *psbD* LRP, which is located upstream of a *psbD-psbC* operon encoding D2 (PsbD) and CP47 (PsbC) subunits of the PSII reaction center complex (Christopher et al., 1992; Wada et al., 1994; Allison and Maliga, 1995; To et al., 1996; Hoffer and Christopher, 1997). Transcription from the *psbD* LRP is activated by not only high-irradiance light, but also various abiotic stresses, including salt, high osmolarity and heat (Nagashima et al., 2004) and circadian rhythm (Noordally et al., 2013). The *psbD* LRP contains unique signature sequences named the AAG-box, immediately upstream of the -35 element (Allison and Maliga, 1995; Kim and Mullet, 1995; To et al., 1996; Nakahira et al., 1998; Kim et al., 1999). The AAG-box is composed of two different repeat units (AAGT and GACC/T repeats). *In vitro* transcription assays from the *psbD* LRP revealed that both repeat motifs are important for transcription, but not the −35 element in barley or wheat (Nakahira et al., 1998; Kim et al., 1999). Furthermore, the AAGT repeat interacts with the sequence-specific DNA-binding protein AGF (Kim and Mullet, 1995; Nakahira et al., 1998; Kim et al., 1999). It has also been shown that the stress-responsive plastid sigma factor SIG5 directs the activation of the *psbD* LRP in *Arabidopsis thaliana* (Nagashima et al., 2004; Tsunoyama et al., 2004; Onda et al., 2008).

The *psbD* promoter mapped in *Chlamydomonas* has a well-conserved −10 element, but lacks the AAG-box and standard −35 element (Klein et al., 1992; Klinkert et al., 2005). In addition, nucleotide sequence comparison of the upstream regions of the *psbD* among embryophytes suggests that *A. thaliana* (angiosperm) and *Pinus thunbergii* (gymnosperm) have the *psbD* LRP in their genome, but not the other basal embryophytes *Physcomitrella patens* (moss) and *Marchantia polymorpha* (liverwort) (Kanazawa et al., 2013). These findings suggest that the *psbD* LRP may have emerged during the evolution of embryophytes. However, evolution of the *psbD* promoter remains elusive. In this study, we mapped the promoter region of the *psbD-psbC* operon in eleven embryophytes at different evolutionary stages from liverwort to angiosperm. The results suggest that AAG-box sequences of the *psbD* LRP in angiosperms and gymnosperms may have evolved from the partial AAG-box-like sequences detected in the *psbD* promoters of basic embryophytes such as moss, hornwort, and lycophyte, while monilophytes (ferns) use a distinct type of *psbD* promoter lacking the AAG-box. On the other hand, light-dependent *psbD* expression was not observed in gymnosperms or primitive angiosperms that possess the well-conserved AAG-box, suggesting that the AAG-box containing *psbD* promoter acquisition is unlikely to be associated with the occurrence of light-dependent *psbD* expression.

MATERIALS AND METHODS

Plant Materials and Growth Condition

For primer extension analysis, *A. thaliana*, *Adiantum capillus-veneris*, *P, patens,* and *M. polymorpha* (Tak-1) were grown in the light in growth chambers at 22°C under 16-h-light/8-h-dark (80–100 μmol photons m^{-2} s^{-1}). Other samples (*Laurus nobilis*, *Ginkgo biloba*, *P. thunbergii*, *Equisetum hyemale*, *Psilotum nudum,* and *Lycopodium clavatum* were collected from plants cultivated at Kyoto Botanical Garden. Leaf samples were collected in the daytime, and immediately frozen in liquid N_2. Light-induced gene expression analysis was carried out with plants (*A. capillus-veneris, C. revoluta, P. thunbergii, L. nobilis, A. thaliana*) grown in the growth chambers at 22°C under 16-h-light/8-h-dark (80 μmol photons m^{-2} s^{-1}). Plants were dark-adapted for 72 h, and then exposed to light of 180 μmol photons m^{-2} s^{-1} for 4 h in the growth chamber. Collected leaf samples were immediately frozen in liquid N_2.

Light-induced gene expression analysis (**Supplementary Figures S4–S8**) was also carried out with a wide range of embryophytes at different evolutionary stages from moss to

angiosperms grown in the growth chambers at 22°C under 16-h-light/8-h-dark (80 μmol photons m^{-2} s^{-1}). Plants were dark-adapted for 72 h (D), and then illuminated (275 $\mu molm^{-2}s^{-1}$) for up to 12 h (L). Osmotic stress was achieved by 250 mM mannitol treatment for 6 h to the detached leaves. Collected leaf samples were immediately frozen in liquid N_2.

Arabidopsis thaliana wild-type Columbia ecotype and *AtSIG5*-overexpressing plants were germinated and grown on two layers of filter paper on the one-half Murashige and Skoog (MS) medium containing 0.8% (w/v) agar at 22°C with 16-h light (80 μmol photons m^{-2} s^{-1})/8-h dark cycles for 10 days. For salt and high osmotic stress treatments, the seedlings were transferred to one-half MS medium containing 250 mM NaCl or 250 mM mannitol, and incubated under light (80 μmol photons m^{-2} s^{-1}) for 6–24 h. For low temperature treatment, the seedlings were incubated at 4°C for 6–24 h under light conditions of 50 μmol photons m^{-2} s^{-1}. For light response experiments, the seedlings were dark adapted for 72 h (D) and illuminated for 4 h with white light (80 μmol photons m–2 s–1) or a blue LED light (50 μmol photons m^{-2} s^{-1}) or a red LED light (50 μmol photons m^{-2} s^{-1}).

Transgenic Plants

First strand cDNA of *AtSIG5* was synthesized from total RNA prepared from *Arabidopsis* seedlings using AMV reverse transcriptase (TaKaRa). cDNA was amplified by PCR using KOD-plus-DNA polymerase (TOYOBO) according to the manufacturer's protocols. To obtain an AtSIG5 overexpression construct under the control of CaMV 35S promoter, the GUS gene of the binary vector pBI121 was replaced with the AtSIG5 cDNA. The resulting constructs were introduced into *Agrobacterium tumefaciens* and used to transform the wild-type (Col-0) plants.

Total RNA Isolation, Primer Extension Analysis, and Northern Blot Analysis

Total RNA was extracted from the leaves using the RNeasy Plant Mini kit (Qiagen, United States) or TRIZOL® following the manufacturer's instructions. The primer extension assays were performed on the total RNA using the Primer Extension System (Promega) with the AMV reverse transcriptase following the manufacturer's instructions. Primers used are listed in Supplementary Table S1. Primer extension products were analyzed on a 6% polyacrylamide-7 M urea sequencing gel. For northern blot analysis, total RNA samples (2 μg) were separated by denaturing agarose gelelectrophoresis. After capillary blotting onto Hybond-N nylon membrane, RNA gel blots were hybridized to the randomly primed DNA probes for *psbA* and *psbD* of each plant. The *psbD* UTR probe (−1085 to −726 of the *psbD* translation start codon) was designed to detect specifically the transcripts from the *psbD* LRP in *Arabidopsis*. The *psbD* and *psbA* coding region probes were designed to detect transcripts produced from all multiple promoters in the *psbD-psbC* operon. The *AtSIG5* probe was also prepared using specific primers. The psbD UTR probes specific for each plant were also generated by using PCR primers.

RESULTS

psbD LRP Transcription Is Dependent on SIG5

The *psbD* LRP is a unique PEP-dependent chloroplast promoter, which is responsible for the transcription of the *psbD-psbC* operon. The *psbD-psbC* operon is well-conserved among plants and cyanobacteria. Unlike standard PEP-dependent promoters composed of sigma-70 type −10 (TATAAT) and −35 (TTGACA) elements, it has been shown that *psbD* LRP activity is dependent on the upstream AAG-box in tobacco (Allison and Maliga, 1995), barley (Kim and Mullet, 1995), rice (To et al., 1996), and wheat (Nakahira et al., 1998; **Figure 1A**). On the other hand, the −35 element of the *psbD* LRP is not essential for transcription activity (To et al., 1996; Nakahira et al., 1998; Kim et al., 1999; Thum et al., 2001b). These findings suggest that the upstream AAG-box may take over the role of the pseudo −35 element in the *psbD* LRP.

Multiple promoters have been identified in the *psbD-psbC* operon (Hoffer and Christopher, 1997). The most upstream promoter (∼950 of the psbD translation start site) is a so-called *psbD* LRP that is specifically activated by blue light. To identify specifically mRNAs transcribed from the *psbD* LRP, we used a *psbD* UTR probe (−1085 to −726 of the *psbD* translation start codon) that is designed to be located upstream of the second promoter at −550 (Tsunoyama et al., 2004).

As shown in **Figure 1B**, the 4.5- and 3.7-kb transcripts from the *psbD* LRP were specifically detected by the *psbD* LRP UTR probe. As reported by Nagashima et al. (2004), various abiotic stresses including salt, cold, and hyperosmotic stresses induce transcription at the *psbD* LRP in a time-dependent manner (**Figure 1B**). Similarly, SIG5 transcription is activated by abiotic stresses. Previous reports (Nagashima et al., 2004; Tsunoyama et al., 2004) demonstrated that *psbD* LRP activity is abolished in *AtSIG5*-deficient mutants in *Arabidopsis*. In order to further define the role of AtSIG5 in transcription at the *psbD* LRP, we developed SIG5 overexpression lines (SIG5oxA and SIG5oxH) and examined transcription activity from the *psbD* LRP. The accumulation of *psbD* LRP transcripts was significantly increased by the overexpression of *AtSIG5* in illuminated plants irrespective of the presence of white, blue, and red light, but only slightly in the dark (**Figure 1C**). These results clearly demonstrate that SIG5 specifically mediates transcription from the *psbD* LRP in the light. Photoreceptors including CRY1, CRY2, and PhyA have been shown to be involved in the light-induced expression of the *psbD-psbC* operon in *Arabidopsis* (Thum et al., 2001a), while *AtSIG5* overexpression cannot activate transcription from the *psbD* LRP in the dark (**Figure 1C**). Taken together, photoreceptor-mediated signaling may modify SIG5 or SIG5 import in a light-dependent manner and activate transcription at the *psbD* LRP.

psbD Transcripts have Markedly Long 5′-UTRs in Common

Transcription initiation sites of the *psbD-psbC* operon have only been identified in some angiosperm model plants, including barley, wheat, rice, tobacco, and *Arabidopsis* (Yao et al., 1989;

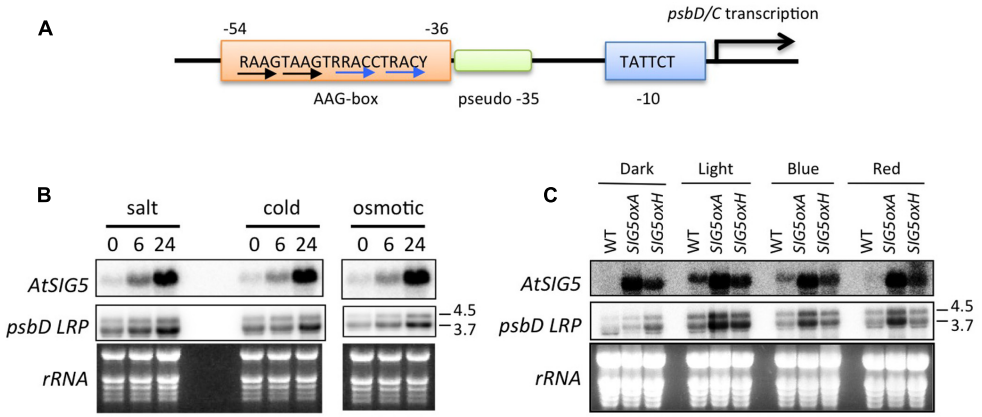

FIGURE 1 | Involvement of AtSIG5 in transcription from the *Arabidopsis psbD* LRP. **(A)** Schematic structure of the *psbD* LRP in *Arabidopsis*. The *psbD* LRP consists of a well-conserved –10 element and an AAG-box upstream of a pseudo –35 element. The conserved 19-bp AAG-box that contains AAGT and GACC/T repeats (black and blue arrows, respectively) is indicated. R, A or G. Y, C or T. **(B)** Northern blot analysis of *psbD* LRP and *AtSIG5* transcripts in *A. thaliana* treated with salt (250 mM NaCl), cold (4°C), and osmotic (250 mM mannitol) stresses for indicated time periods. Total RNAs (2 μg) were electrophoresed in a denatured gel, blotted, and hybridized to ^{32}P-labeled gene-specific probes, *psbD* LRP UTR and *AtSIG5* probes. The psbD probe was used to detect 3.7 and 4.5 kb mRNAs transcribed from the *psbD* LRP. Transcription of *psbD* LRP was significantly induced by abiotic stresses. **(C)** Light-dependent expression of *psbD* LRP transcripts in *AtSIG5* overexpressing plants. The seedlings were dark adapted for 72 h (D) and illuminated for 4 h with white light (80 μmol photons m^{-2} s^{-1}) or a blue LED light (50 μmol photons m^{-2} s^{-1}) or a red LED light (50 μmol photons m^{-2} s^{-1}).

Christopher et al., 1992; Wada et al., 1994; To et al., 1996; Hoffer and Christopher, 1997), and green algae *Chlamydomonas reinhardtii* (Klein et al., 1992). In order to address the evolutionary changes of *psbD* promoter structures, we mapped 5′-ends of *psbD* transcripts of eleven embryophytes at different evolutionary stages from liverworts to angiosperms using primer extension analysis (**Figure 2** and **Supplementary Figure S1**, **S2**). Leaf samples were collected from plants grown in Kyoto Botanical Garden in the daytime, except for *A. thaliana*, *A. capillus-veneris, P. patens,* and *M. polymorpha* that were grown in the light in growth chambers. We estimated the size of primer extension products approximately by comparing their mobility profiles to single-strand DNA ladders. In order to determine the start sites as exactly as possible, we designed appropriate primers that produce primer extension products shorter than 300 bases. Next we searched for sequences similar to the conserved −10 sequences (TATTCT) of the *psbD* LRP in close proximity to the identified transcription initiation sites. Then, we aligned the deduced *psbD* promoter sequences with those of other plants using the −10 element as reference. In this study, we considered the *psbD* transcripts with the most upstream terminus as the primary transcripts, except for *P. thunbergii* and *E. hyemale*, which have another promoter upstream of the potential *psbD* LRP.

Transcription initiation sites from the *psbD* LRP have been mapped at 572, 610, 566, 905, and 948 bp upstream of the *psbD* translation start site of barley (Christopher et al., 1992), wheat (Wada et al., 1994), rice (To et al., 1996), tobacco (Yao et al., 1989), and *Arabidopsis* (Hoffer and Christopher, 1997), respectively. Similarly, 5′-ends of the *psbD* primary transcripts of the *psbD-psbC* operon were mapped at 800–900 bp upstream of the *psbD* gene in the most angiosperms including the basal angiosperm *L. nobilis,* gymnosperm *P. thunbergii*, primitive gymnosperm *G. biloba*, monilophyte (Leptosporangiate fern) *A. capillus-veneris*, monilophyte (Eusporangiate fern) *P. nudum*, monilophyte (Eusporangiate fern) *E. hyemale*, and lycophyte *Huperzia lucidula* (**Figure 3**). Furthermore, we found sequences similar to the *psbD* promoter of *H. lucidula* at the far upstream position (−919) of the *psbD* translation start site in *Anthoceros formosae* (hornwort). On the other hand, 5′-ends of the longest *psbD* transcripts of other bryophytes, *P. patens* (moss), and *M. polymorpha* (liverwort) are located at −246 and −243 of the *psbD* gene, respectively. These results indicate that *psbD* mRNAs have unusually long 5′-UTR extensions in common, except for mosses and liverworts. It is of note that intergenic distances between *psbD* and the upstream *trnT* are much shorter in mosses and liverworts compared with those of other plants.

AAG-Box of the *psbD* LRP Is Highly Conserved among Angiosperms and Gymnosperms, and Partially Conserved in Lycophytes and Mosses

Next, we compared sequences immediately upstream of the *psbD* transcription initiation sites that were mapped in this and previous studies. The typical −10 elements (TATTCT) are well-conserved in all *psbD* promoters (**Figure 4** and Supplementary Table S2). Conversely, the potential −35 elements are less conserved among terrestrial plants and show weak similarity (less than ∼50%) to the consensus sequences (TTGACA). On the other hand, liverworts possess a typical sigma-70 type promoter with conserved −35 and −10 elements with 18-nt spacing. As expected, the AAG-box is well-conserved among angiosperms and gymnosperms. The consensus sequence of the AAG-box is "RAAGTAAGTRRACCTRACYY," which contains an AAGT

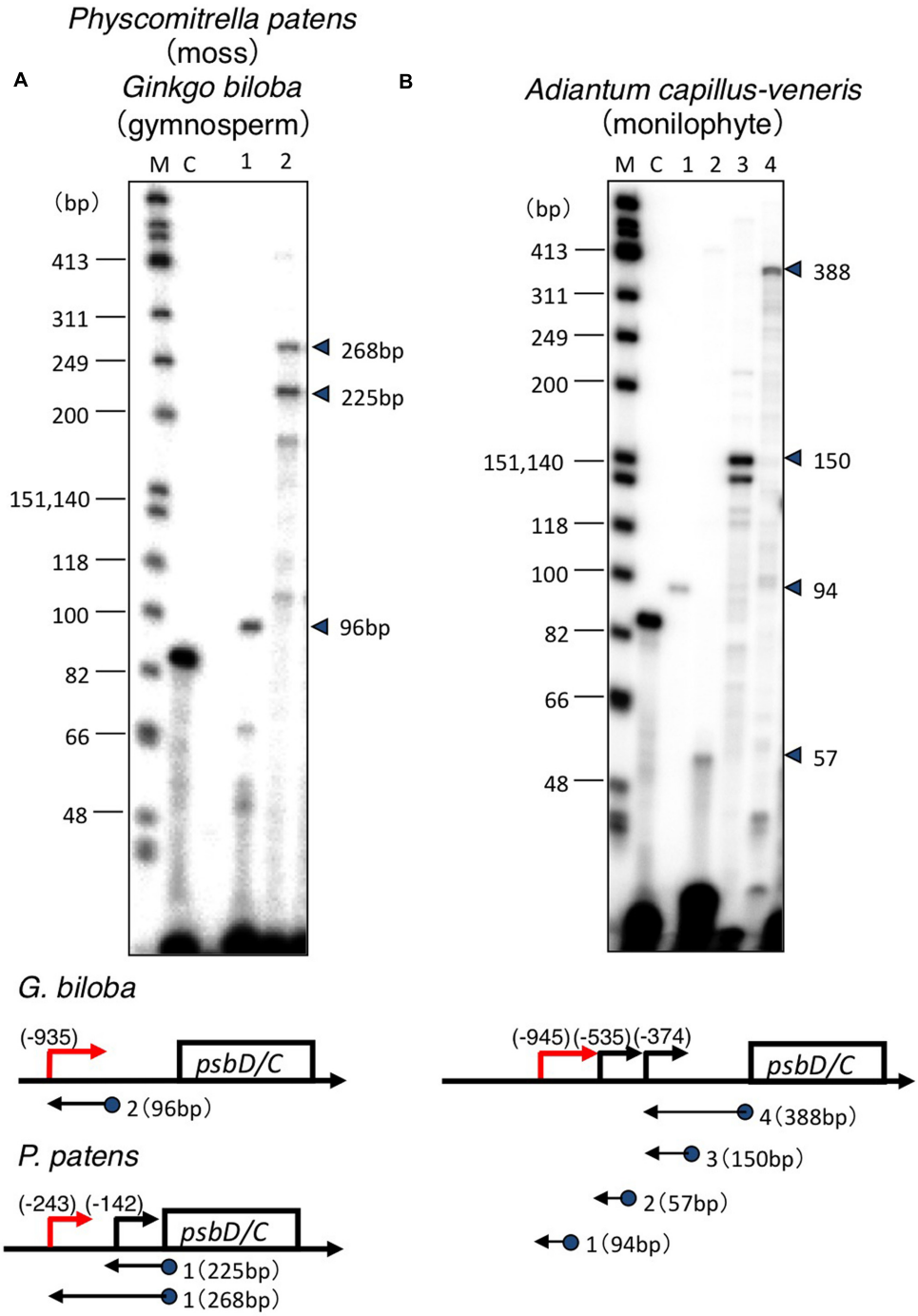

FIGURE 2 | Mapping of the 5′ ends of the *psbD* transcripts. Analysis of the *psbD* transcripts of the moss *P. patens* and primitive gymnosperm *G. biloba* **(A)**, and monilophyte *A. capillus-veneris* **(B)** by primer extension assays. Primers used are indicated by numbers on the top of each lane. The size of the extension product is shown on the right. The position of primers and the size of the extension products are shown on the gene map. The deduced sites of the 5′-end of each transcript are shown as numbers in parentheses. Lane C shows an experiment with the control RNA and primer provided by the manufacturer that produces an 87-base primer extension product.

repeat and a GACC/T repeat. The AAG-box sequences are almost 80% identical in most gymnosperms and angiosperms, including the primitive gymnosperm *G. biloba* and basal angiosperm *L. nobilis* (Supplementary Table S2). We found that a 13-bp core sequence of the AAG-box is also highly conserved (∼85%) in lycophytes and hornworts, and partially conserved

FIGURE 3 | Representative maps of the *psbD* transcripts. The *psbD* transcript 5′-ends identified in **Figure 2** and **Supplementary Figures S1**, **S2** are shown by arrows. The *psbD* LRP-related promoters analyzed in this study are indicated by red shadows.

in mosses (69%), but not in liverworts. The AAG-box of lycophytes and hornworts harbors the GACC/T repeat-like sequences, but lacks the AAGT repeat (**Figure 4B**). On the other hand, neither the AAGT repeat nor GACC/T repeat are conserved in the *psbD* promoter of monilophytes (ferns). Instead, sequences upstream of the −35 element are well-conserved among standard monilophytes and *P. nudum*, but not in the primitive monilophyte *E. hyemale* (**Figure 4B** and **Supplementary Figure S3**). These results suggest that the AAG-box was acquired at a very early stage of embryophyte evolution, and is likely conserved in gymnosperms and angiosperms. On the other hand, monilophytes may have acquired another type of *psbD* promoter with a distinct *cis* element upstream of the −35 element.

The AAG-Box Containing *psbD* Promoter Is not Associated with Light-Induced *psbD* Expression

In order to address whether the *psbD* LRP is responsible for light-induced transcription, we examined the light-induced expression of *psbD* transcripts in some embryophytes, including monilophytes, gymnosperms, and angiosperms. As shown in **Figure 5A**, *psbD* expression is clearly induced by light in *A. thaliana* (angiosperm). However, unexpectedly, no light-induced psbD expression was detected in *L. nobilis* (basal angiosperm), *P. thunbergii* (gymnosperm), or *Cycas revoluta* (primitive gymnosperm), although they all have a well-conserved AAG-box containing *psbD* promoter. Similarly, *psbD* expression

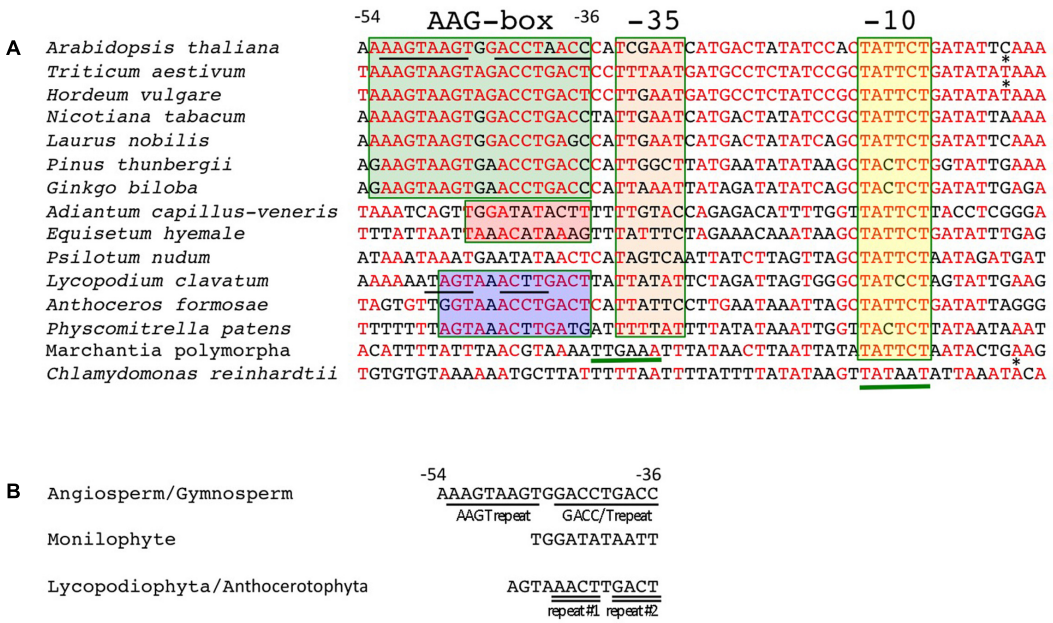

FIGURE 4 | Conserved sequences of the AAG-box containing *psbD* promoters. **(A)** DNA sequences between –54 and +4 of the AAG-box containing *psbD* promoter transcription initiation sites are aligned among the plants analyzed. Transcription initiation sites identified in barley (Kim and Mullet, 1995) and wheat (Nakahira et al., 1998) are indicated by asterisks. Nucleotides that are identical to the wheat sequences are shown in red. The AAG-box in gymnosperms and angiosperms, fern-type upstream sequences, and AAG-box like sequences in basal land plants are indicated by green, red, and blue boxes, respectively. The –35 and –10 elements are indicated by orange and yellow boxes, respectively. The deduced –35 element in *M. polymorpha* sequences and –35 and –10 elements in *C. reinhardtii* are underlined. **(B)** Conserved sequences upstream of the –35 element. Characteristic repeats are underlined.

is also not regulated by light in *A. capillus-veneris* (monilophyte), which does not have the conserved AAG-box in the *psbD* promoter. We further examined the light-mediated regulation of the AAG-box containing *psbD* promoter transcripts by primer extension analysis. As shown in **Figure 5B**, the abundance of the transcripts from the AAG-box containing *psbD* promoter was not regulated by light in *L. nobilis* or *P. thunbergii* (**Figure 5B**). These results suggest that the AAG-box containing *psbD* promoter is not directly associated with light-induced *psbD* expression.

Moreover, we examined light- and osmotic stress-induced *psbD* expression in a wide range of plants. In angiosperms, light-induced *psbD* expression was detected in a number of eudicots (*C. sativus*, *A. thaliana,* and *L. sativa*) and monocots (wheat and maize), whereas *psbD* expression is not regulated by light in basal angiosperms except for *C. glaber* (**Supplementary Figures S4–S6**). It is to be noted that *psbD* expression is activated by long-term illumination (12 h) in *C. glaber* (**Supplementary Figure S6**). In contrast, the osmotic stress-induced expression of *psbD* was detected only in eudicots. Expression of *psbD* was not activated by osmotic stress in monocots and basal angiosperms except for *C. glaber* (**Supplementary Figure S5**, **S6**). Furthermore, neither light nor osmotic stress-induced *psbD* expression was detected in gymnosperms, *Gingko* and *Cycas* (**Supplementary Figure S6**). Although psbD expression was not activated by moderate light (180 $\mu molm^{-2}s^{-1}$) in *Pinus* (**Figure 5**), high light exposure (245 $\mu molm^{-2}s^{-1}$) induced transient expression of *psbD* (**Supplementary Figure S6**). It is of note that gymnosperms and basal angiosperms have a well-conserved AAG-box containing *psbD* promoter. On the other hand, *psbD* expression is induced by light and/or osmotic stress in some monilophytes that lack the typical AAG-box containing *psbD* promoter. In addition, light and salt stress barely affect *psbA* expression in any of the plants examined (**Supplementary Figures S5–S8**). These results indicate that light and salt stress-induced transcription has evolved independently and multiple times during land plant evolution. Furthermore, the AAG-box in the *psbD* promoter is unlikely to be directly associated with the occurrence of light and salt stress-induced *psbD* expression.

Gnetaceae Plants in Gymnosperms Have Lost the AAG-Box Containing *psbD* Promoter

Gnetaceae plants are a unique group of gymnosperms that have evolved a morphological system related to that of the angiosperms. The most upstream transcription initiation sites have been mapped at 317 and 114 bp upstream of the *psbD* translation start site of *Gnetum gnemon* and *Ephedra sinica*, respectively (**Figures 6A,B**). The *psbD* transcripts of gnetaceae have shorter 5′-UTR compared with the standard *psbD* transcripts in other plants. The upstream sequences of the *psbD* transcripts are well-conserved among gnetaceae. No AAG-box-like sequences are found upstream of the *psbD* transcripts in gnetaceae, whereas the gnetaceae *psbD* promoters possess −35- and −10-like sequences (**Figure 6C**). The similar sequences

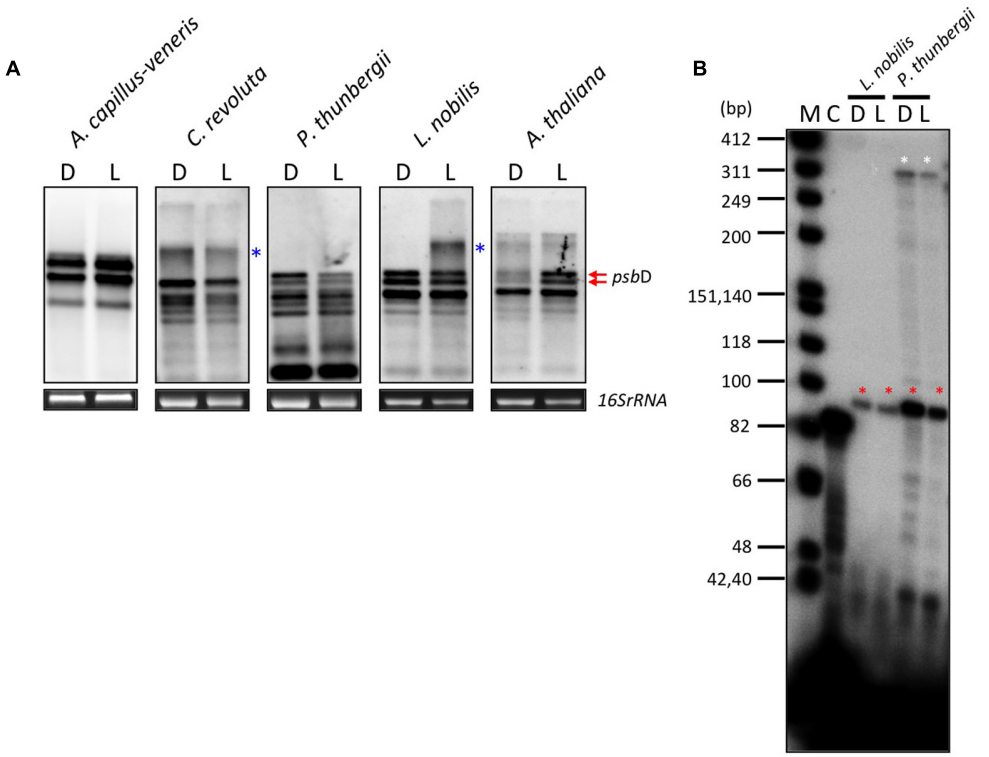

FIGURE 5 | Effects of light on *psbD* transcription. **(A)** DIG-based northern blot analysis of *psbD* transcripts in monilophyte, gymnosperm, and angiosperm. Plants were dark-adapted for 72 h (D) and illuminated for 4 h (180 $\mu molm^{-2}s^{-1}$; L). Previously characterized transcripts from the AAG-box containing *psbD* promoter are indicated by red arrows in *Arabidopsis*. The smear and extremely large bands (indicated by blue asterisks) represent large read-through transcripts of upstream genes. 16S *rRNA* was used as an RNA-loading control for the total RNA sample. **(B)** The AAG-box containing *psbD* promoter transcripts of *L. nobilis* and *P. thunbergii* were analyzed by primer extension assays. Plants were dark-adapted for 72 h (D) and illuminated for 4 h (180 $\mu molm^{-2}s^{-1}$; L). Transcripts from the AAG-box containing *psbD* promoter are indicated by red asterisks. The white asterisks show transcripts from the uncharacterized promoter upstream of the AAG-box containing *psbD* promoter in *P. thunbergii*. Lane C shows an experiment with the control RNA and primer provided by the manufacturer that produces an 87-base primer extension product.

are also found upstream of the psbD-C operon of *Welwitschia mirabilis*. Moreover, AAG-box-like sequences have not been identified in the *trnT-psbD* intergenic regions of gnetaceae. We suggest that the AAG-box containing *psbD* promoter was lost in gnetaceae during their evolution. It would be very interesting to determine whether *psbD* expression is dependent on light in gnetaceaes.

DISCUSSION

The *psbD* and *psbC* genes are organized in a *psbD-psbC* operon, which is well-conserved among plants and cyanobacteria. Transcription from the *psbD-psbC* operon is mediated solely by PEP in green tissues. Multiple transcriptional start sites (TSS) generating mRNAs with heterogeneous 5′ transcript leaders have been mapped on the *psbD-psbC* operon in several angiosperm plants, including tobacco (Yao et al., 1989), barley (Christopher et al., 1992), wheat (Wada et al., 1994), rice (To et al., 1996), and *Arabidopsis* (Hoffer and Christopher, 1997). Light activates the expression of some *psbD-psbC* mRNAs, whereas the accumulation of other mRNAs is not regulated by light. Analysis of the promoter sequences immediately upstream of the light-induced TSS identified an unusual PEP promoter consisting of a core promoter with a weakly conserved −35 element along with an upstream *cis* element termed the AAG-box (**Figure 1A**). This promoter is specifically activated by high-irradiance blue light and UV-A (Christopher and Mullet, 1994), and is designated as the *psbD* light-responsive promoter (*psbD* LRP) or *psbD* blue light-responsive promoter (*psbD* BLRP). As shown in **Figure 1B**, the *psbD* LRP is also activated by various environmental stresses (Nagashima et al., 2004). On the other hand, other light-insensitive promoters mapped on the *psbD-psbC* operon are standard PEP promoters composed of well-conserved −10 and −35 elements.

In order to investigate *psbD LRP* evolution, we mapped promoters responsible for the expression of the *psbD-psbC* operon by primer extension analysis in 11 embryophytes at different evolutionary stages from liverworts to angiosperms. We considered the *psbD* transcripts with the most upstream terminus as the primary transcripts, except for *P. thunbergii* and *E. hyemale,* which have another promoter upstream of the potential AAG-box containing *psbD* promoter. All *psbD* promoters identified at the most upstream mRNA terminus

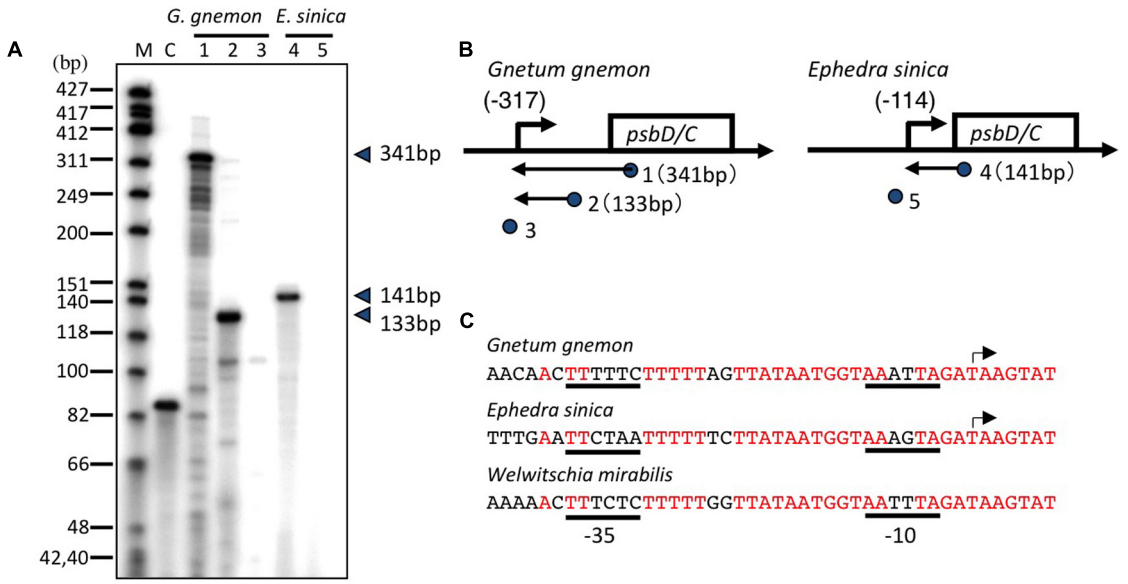

FIGURE 6 | Mapping of the 5′ ends of the *psbD* transcripts in Gnetaceae plants. **(A)** The *psbD LRP* transcripts of the Gnetaceae plants *G. gnemon* (lanes 1–3) and *E. sinica* (lanes 4 and 5) were analyzed by primer extension assays. Primers used are indicated by numbers on the top of each lane. The size of the extension product is shown on the right. Lane C shows an experiment with the control RNA and primer provided by the manufacturer that produces an 87-base primer extension product. **(B)** Representative maps of the *psbD* transcripts. The *psbD* transcript 5′-ends identified by the primer extension assay are shown by arrows. The position of primers and the size of the extension products are shown on the gene map. The deduced sites of the 5′-end of each transcript are shown as numbers in parentheses. No transcript was detected with #3 and #5 primers. **(C)** DNA sequences between –37 and +7 of the AAG-box containing *psbD* promoter transcription initiation sites of *G. gnemon* and *E. sinica* are shown. The corresponding sequences of the *psbD* upstream region of *Welwitschia mirabilis* are also shown. Transcription initiation sites are indicated by arrows. Potential –35 and –10 elements are underlined.

have the well-conserved −10 element (TATTCT) that is similar to the standard −10 element (TATAAT). On the other hand, the potential −35 elements of the *psbD* promoters are less conserved, suggesting the limited role of the −35 element in *psbD* promoter activity. *In vitro* transcription experiments have demonstrated that the −10 element is important for transcription from the AAG-box containing *psbD* promoter, but the −35 element is not essential for transcription in barley, rice, or wheat (To et al., 1996; Nakahira et al., 1998; Kim et al., 1999). It is considered that the −10 element is important for *psbD* promoter activity, but not the poorly conserved −35 element.

We found that the 19 bp AAG-box sequences of the *psbD* promoters are highly conserved among gymnosperms and angiosperms, including the basal angiosperm *L. nobilis* and primitive gymnosperm *G. biloba*. The consensus AAG-box sequence is "RAAGTAAGTRRACCTRACY," which is at least 80% identical in gymnosperms and angiosperms. The AAG-box is composed of two repeat sequences: AAGT and GACC/T repeats. Extensive analyses of the AAG-box containing *psbD* promoter structure using *in vitro* transcription systems have revealed that both the AAGT and GACC/T repeats are important for AAG-box containing *psbD* promoter activity (Kim and Mullet, 1995; To et al., 1996; Nakahira et al., 1998). Moreover, the AAG-box is also partially conserved in basal land plants such as lycophytes, hornworts, and mosses. We identified the shorter conserved AAG-box-like sequences in *H. lucidula* (lycophyte) and *A. formosae* (hornwort). The AAG-box-like sequences in lycophyta and hornworts retain a partially conserved GACC/T repeat, but lack AAGT repeats. Deletion of the AAGT repeat resulted in only a partial reduction of *in vitro* transcription activity of the AAG-box containing *psbD* promoter in wheat, suggesting that the GACC/T repeat is sufficient to mediate AAG-box containing *psbD* promoter activity (Nakahira et al., 1998). Thus, the AAG-box-like sequences may act as a transcription activation element in the *psbD* promoters of basal land plants. Considering the highly conserved −10 element among bryophytes and angiosperms, it is likely that the last common ancestor of bryophytes and spermatophytes likely already possessed an AAG box-containing *psbD* LRP (**Supplementary Figure S9**). The AAG box may have developed to take over the function of the −35 element and support the high-level transcription activity of the AAG-box containing *psbD* promoter.

On the other hand, the AAG-box like sequences have not been identified in the *psbD* promoter of *M. polymorpha* (liverwort). The liverwort *psbD* promoter is composed of the typical −35 element (TTGAAA) and the −10 element (TATTCT) with standard spacing, suggesting that *psbD* is transcribed from a standard PEP promoter in liverworts. It is to be noted that the *psbD* promoter has a well-conserved −10 element, but lacks the AAG-box and standard −35 element in *Chlamydomonas* (Klein et al., 1992; Klinkert et al., 2005).

Monilophytes (ferns) have another type of *psbD* promoter that lacks the conserved AAG-box. Instead, 11-bp sequences upstream of the potential −35 element are well-conserved among standard (*A. capillus-veneris*) and primitive (*P. nudum*) monilophytes. However, it remains elusive whether the conserved sequences upstream of the psbD transcription stat site in monilophytes is required for *psbD* transcription. Interestingly, gnetaceae plants in gymnosperms have lost the AAG-box-containing *psbD* LRP, suggesting that the AAG-box containing *psbD* promoter is not essential for plant development.

It has also been shown that an AAG-box-binding factor (AGF) specifically binds to the AAGT repeat, that it is partially associated with the GACC/T repeats (Kim and Mullet, 1995; Kim et al., 1999), and that it activates transcription from the AAG-box containing *psbD* promoter (Wada et al., 1994; Kim and Mullet, 1995; Allison and Maliga, 1995; To et al., 1996; Nakahira et al., 1998; Kim et al., 1999). PTF1 (plastid transcription factor 1) is a basic helix-loop-helix DNA-binding protein, which binds to the AAG box and is involved in transcription from the *psbD* LRP in *Arabidopsis* (Baba et al., 2001). However, close orthologs of the *Arabidopsis* PTF1 have only been found in angiosperms, suggesting that PTF1 is responsible for AAG-box-dependent transcription at the AAG-box containing *psbD* promoter in angiosperms. However, the role of AGF in the light-dependent transcription remains to be elucidated.

In addition, reverse genetic analysis of sigma factors revealed that the AAG-box containing *psbD* promoter is specifically recognized by SIG5 in *Arabidopsis* (Nagashima et al., 2004; Tsunoyama et al., 2004). Transcription from the AAG-box containing *psbD* promoter is likely to be mediated by SIG5 containing PEP and activated by AGF that binds to the AAG-box. However, overexpression of SIG5 cannot activate transcription from the AAG-box containing *psbD* promoter in the dark (**Figure 1C**). Furthermore, photoreceptors including CRY1, CRY2, and PhyA are involved in the light-induced expression of the *psbD-psbC* operon in *Arabidopsis* (Thum et al., 2001a). Taken together, photoreceptor-mediated signaling may modify SIG5 or SIG5 import into the chloroplasts in a light-dependent manner and activate transcription at the AAG-box containing *psbD* promoter.

SIG5 orthologs have been identified in a number of angiosperms. Moreover, SIG5 has been identified as a gene that was abundant when water availability was low in gymnosperm *Pseudotsuga menziesii* (Hess et al., 2016). Furthermore, SIG5 orthologs have been identified in *P. patens* (Ichikawa et al., 2008) and *Selaginella moellendorffii* (XP_002970534). It has been shown that PpSIG5 is involved in high-intensity light and circadian control of *psbD* expression in the moss *P. patens* (Ichikawa et al., 2004, 2008). Taken together, these results suggest that SIG5 plays a role in transcription from the *psbD* promoter consisting of the AAG-box or AAG-like element in embryophytes including mosses. On the other hand, an *SIG5* ortholog was also identified in the liverwort *M. polymorpha* (Kanazawa et al., 2013). MpSIG5 is not necessary for light-dependent *psbD* expression in *M. polymorpha* (Kanazawa et al., 2013). MpSIG5 may have another role in chloroplast transcription in *M. polymorpha* that lacks the AAG-box containing *psbD* promoter. Furthermore, *SIG5* orthologs have been identified in some charophytes such as *K. flaccidum*, but not in the green alga *C. reinhardtii* or in the primitive red alga *C. merolae*. It is likely that SIG5 was acquired in charophytes before the occurrence of AAG-box containing *psbD* promoter in basal embryophytes.

Transcripts from the AAG-box containing *psbD* promoter are characterized by a markedly long 5′-UTR. All AAG-box containing *psbD* promoter transcripts except for *P. patens* and *M. polymorpha* have a 5′-UTR longer than 500 nucleotides from the translation start site. Detailed mapping of the *psbD* transcripts in angiosperm plants revealed that no intron is present in the 5′-UTR and the AAG-box containing *psbD* promoter transcripts actually have a long 5′-UTR (Wada et al., 1994; Hoffer and Christopher, 1997; Kim et al., 1999). The 5′-UTR of chloroplast transcripts may be involved in the stability of the transcripts and/or efficiency of translation (Shiina et al., 1998). However, no conserved sequences have been identified in the 5′-UTR of embryophytes. Further characterization of the 5′-UTR would shed light on the role of the unusually long 5′-UTR of the AAG-box containing *psbD* promoter in most embryophytes,

This study suggest that the AAG-box containing *psbD* promoter appeared in basal embryophytes more than 450 million years ago, and the common ancestor of bryophytes and spermatophytes likely possessed an AAG box-containing *psbD* promoter. On the other hand, the −35 and −10 elements of the *psbA* and *rbcL* promoters are almost identical among liverworts and angiosperms (**Supplementary Figure S10**). It is suggested that ecological and/or physiological demands may have accelerated the evolution of the AAG-box containing *psbD* promoter in embryophytes. One of the unique characteristics of the AAG-box containing *psbD* promoter is light- and stress-induced transcription. However, extensive expression analysis of *psbD* in a variety of plants revealed that the light and/or stress-induced expression of the *psbD* gene developed independently in several plants. Thus, it is unlikely that that light and/or stress responses of the AAG-box containing *psbD* promoter are directly associated with AAG-box containing *psbD* promoter evolution. Recent studies demonstrated that ABA and the circadian rhythm regulate chloroplast AAG-box containing *psbD* promoter activity via the activation of SIG5 (Noordally et al., 2013; Yamburenko et al., 2015; Belbin et al., 2017) Further analysis of the role of the AAG-box containing *psbD* promoter in chloroplasts may shed light on AAG-box containing *psbD* promoter evolution.

AUTHOR CONTRIBUTIONS

TS, SS, SM, and YN designed research. SS, MN, ShK, SaK, and YI performed research. TS, SS, and MN analyzed data. TS, SS, and MN wrote the paper. SS and MN have contributed equally to this work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01186/full#supplementary-material

FIGURE S1 | Mapping of the 5′ ends of the *psbD* transcripts *psbD* transcripts were isolated from liverwort *M. polymorpha* **(A)**, lycophyte *H. lucidula* **(B)**, and monilophyte *P. nudum* **(C)**. Primers used are indicated by numbers on the top of each lane. The size of the extension product is shown on the right. The position of primers and the size of the extension products are shown on the gene map. The deduced sites of the 5′-end of each transcript are shown as numbers in parentheses. Lane Xs show the results of unrelated samples. Lane C shows an experiment with the control RNA and primer provided by the manufacturer that produces an 87-base primer extension product.

FIGURE S2 | Mapping of the 5' ends of the *psbD* transcripts *psbD* transcripts were isolated from monilophyte *E. hyemale* **(A)**, gymnosperm *P. thunbergii* **(B)**, and angiosperm *L. nobilis* **(C)**. Primers used are indicated by numbers on the top of each lane. The size of the extension product is shown on the left in **(A)** and on the right in **(B,C)**. The position of primers and the size of the extension products are shown on the gene map. The deduced sites of the 5′-end of each transcript are shown as numbers in parentheses. Lane C shows an experiment with the control RNA and primer provided by the manufacturer that produces an 87-base primer extension product.

FIGURE S3 | A comparison of the potential *psbD* promoter sequences in monilophytes DNA sequences upstream of the *psbD* transcription initiation site of *A. capillus-veneris* and corresponding sequences of three other leptosporangiate ferns are compared with the *psbD* promoter sequences of two primitive ferns (*P. nudum* and *E. hyemale*). Transcription initiation sites identified in this study are indicated by asterisks. Nucleotides that are identical to the *Adiantum* sequences are shown in red. The monilophytes-type upstream conserved sequences are indicated by a green box. The −35 and −10 elements are indicated by orange and yellow boxes, respectively.

FIGURE S4 | Northern blot analysis of *psbD* and *psbA* transcripts in eudicots. Plants were dark-adapted for 72 h (D) and illuminated for up to12 h (275 $\mu molm^{-2}s^{-1}$; L). Osmotic stress was applied by 250 mM mannitol for 6 h. Light- or salt stress-induced *psbD* transcripts are indicated by red arrow heads. rRNA stained with EtBr was used as an RNA-loading control for the total RNA sample.

FIGURE S5 | Northern blot analysis of *psbD* and *psbA* transcripts in monocots. Plants were dark-adapted for 72 h (D) and illuminated for up to12 h (275 $\mu molm^{-2}s^{-1}$; L). Osmotic stress was applied by 250 mM mannitol for 6 h. Light- or salt stress-induced *psbD* transcripts are indicated by red arrow heads. rRNA stained with EtBr was used as an RNA-loading control for the total RNA sample.

FIGURE S6 | Northern blot analysis of *psbD* and *psbA* transcripts in basal angiosperms. Plants were dark-adapted for 72 h (D) and illuminated for 12 h (275 $\mu molm^{-2}s^{-1}$; L). Osmotic stress was applied by 250 mM mannitol for 6 h. Light- or salt stress-induced *psbD* transcripts are indicated by red arrow heads. rRNA stained with EtBr was used as an RNA-loading control for the total RNA sample.

FIGURE S7 | Northern blot analysis of *psbD* and *psbA* transcripts in gymnosperms. Plants were dark-adapted for 72 h (D) and illuminated for up to 12 h (275 $\mu molm^{-2}s^{-1}$; L). Osmotic stress was applied by 250 mM mannitol for 6 h. Light- or salt stress-induced *psbD* transcripts are indicated by red arrow heads. rRNA stained with EtBr was used as an RNA-loading control for the total RNA sample.

FIGURE S8 | Northern blot analysis of *psbD* and *psbA* transcripts in monilophytes. Plants were dark-adapted for 72 h (D) and illuminated for up to 12 h (275 $\mu molm^{-2}s^{-1}$; L). Osmotic stress was applied by 250 mM mannitol for 6 h. Light- or salt stress-induced *psbD* transcripts are indicated by red arrow heads. rRNA stained with EtBr was used as an RNA-loading control for the total RNA sample.

FIGURE S9 | Phylogenetic tree of AAG box. Alignments were undertaken with 19 bps sequences (from −54 to −36 of the transcription initiation site) of 18 plants from *C. reinhardtii* to *A. thaliana*.

FIGURE S10 | Comparion of *psbA* and *rbcL* promoters of terrestrial plants.

REFERENCES

Allison, L. A., and Maliga, P. (1995). Light-responsive and transcription-enhancing elements regulate the plastid *psbD* core promoter. *EMBO J.* 14, 3721–3730.

Allison, L. A., Simon, L. D., and Maliga, P. (1996). Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants. *EMBO J.* 15, 2802–2809.

Baba, K., Nakano, T., Yamagishi, K., and Yoshida, S. (2001). Involvement of a nuclear-encoded basic helix-loop-helix protein in transcription of the light-responsive promoter of *psbD*. *Plant Physiol.* 125, 595–603. doi: 10.1104/pp.125.2.595

Baena-González, E., Baginsky, S., Mulo, P., Summer, H., Aro, E. M., and Link, G. (2001). Chloroplast transcription at different light intensities. Glutathione-mediated phosphorylation of the major RNA polymerase involved in redox-regulated organellar gene expression. *Plant Physiol.* 127, 1044–1052. doi: 10.1104/pp.010168

Belbin, F. E., Noordally, Z. B., Wetherill, S. J., Atkins, K. A., Franklin, K. A., and Dodd, A. N. (2017). Integration of light and circadian signals that regulate chloroplast transcription by a nuclear-encoded sigma factor. *New Phytol.* 213, 727–738. doi: 10.1111/nph.14176

Bohne, A. V., Irihimovitch, V., Weihe, A., and Stern, D. B. (2006). *Chlamydomonas reinhardtii* encodes a single sigma70-like factor which likely functions in chloroplast transcription. *Curr. Genet.* 49, 333–340. doi: 10.1007/s00294-006-0060-7

Börner, T., Aleynikova, A. Y., Zubo, Y. O., and Kusnetsov, V. V. (2015). Chloroplast RNA polymerases: Role in chloroplast biogenesis. *Biochim. Biophys. Acta* 1847, 761–769. doi: 10.1016/j.bbabio.2015.02.004

Chi, W., He, B., Mao, J., Jiang, J., and Zhang, L. (2015). Plastid sigma factors: their individual functions and regulation in transcription. *Biochim. Biophys. Acta* 1847, 770–778. doi: 10.1016/j.bbabio.2015.01.001

Christopher, D. A., Kim, M., and Mullet, J. E. (1992). A novel light-regulated promoter is conserved in cereal and dicot chloroplasts. *Plant Cell* 1992, 785–798. doi: 10.1105/tpc.4.7.785

Christopher, D. A., and Mullet, J. E. (1994). Separate photosensory pathways co-regulate blue light/ultraviolet-A-activated *psbD-psbC* transcription and light-induced D2 and CP43 degradation in barley (*Hordeum vulgare*) Chloroplasts. *Plant Physiol.* 104, 1119–1129. doi: 10.1104/pp.104.4.1119

Fujiwara, M., Nagashima, A., Kanamaru, K., Tanaka, K., and Takahashi, H. (2000). Three new nuclear genes, *sigD*, *sigE* and *sigF*, encoding putative plastid RNA polymerase sigma factors in *Arabidopsis thaliana*. *FEBS Lett.* 481, 47–52. doi: 10.1016/S0014-5793(00)01965-7

Hajdukiewicz, P. T., Allison, L. A., and Maliga, P. (1997). The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe

distinct groups of genes in tobacco plastids. *EMBO J.* 16, 4041–4048. doi: 10.1093/emboj/16.13.4041

Hara, K., Morita, M., Takahashi, R., Sugita, M., Kato, S., and Aoki, S. (2001). Characterization of two genes, Sig1 and Sig2, encoding distinct plastid sigma factors(1) in the moss Physcomitrella patens: phylogenetic relationships to plastid sigma factors in higher plants. *FEBS Lett.* 499, 87–91. doi: 10.1016/S0014-5793(01)02530-3

Hayes, R., Kudla, J., and Gruissem, W. (1999). Degrading chloroplast mRNA: the role of polyadenylation. *Trends Biochem. Sci.* 24, 199–202. doi: 10.1016/S0968-0004(99)01388-2

Hess, M., Wildhagen, H., Junker, L. V., and Ensminger, I. (2016). Transcriptome responses to temperature, water availability and photoperiod are conserved among mature trees of two divergent Douglas-fir provenances from a coastal and an interior habitat. *BMC Genomics* 17:682. doi: 10.1186/s12864-016-3022-6

Hess, W. R., and Börner, T. (1999). Organellar RNA polymerases of higher plants. *Int. Rev. Cytol.* 190, 1–59. doi: 10.1016/S0074-7696(08)62145-2

Hoffer, P. H., and Christopher, D. A. (1997). Structure and blue-light-responsive transcription of a chloroplast *psbD* promoter from *Arabidopsis thaliana*. *Plant Physiol.* 115, 213–222. doi: 10.1104/pp.115.1.213

Ichikawa, K., Shimizu, A., Okada, R., Satbhai, S. B., and Aoki, S. (2008). The plastid sigma factor SIG5 is involved in the diurnal regulation of the chloroplast gene *psbD* in the moss Physcomitrella patens. *FEBS Lett.* 582, 405–409. doi: 10.1016/j.febslet.2007.12.034

Ichikawa, K., Sugita, M., Imaizumi, T., Wada, M., and Aoki, S. (2004). Differential expression on a daily basis of plastid sigma factor genes from the moss Physcomitrella patens. Regulatory interactions among PpSig5, the circadian clock, and blue light signaling mediated by cryptochromes. *Plant Physiol.* 136, 4285–4298. doi: 10.1104/pp.104.053033

Kanamaru, K., and Tanaka, K. (2004). Roles of chloroplast RNA polymerase sigma factors in chloroplast development and stress response in higher plants. *Biosci. Biotechnol. Biochem.* 68, 2215–2223. doi: 10.1271/bbb.68.2215

Kanazawa, T., Ishizaki, K., Kohchi, T., Hanaoka, M., and Tanaka, K. (2013). Characterization of four nuclear-encoded plastid RNA polymerase sigma factor genes in the liverwort *Marchantia polymorpha*: blue-light- and multiple stress-responsive SIG5 was acquired early in the emergence of terrestrial plants. *Plant Cell Physiol.* 54, 1736–1748. doi: 10.1093/pcp/pct119

Kasai, K., Kawagishi-Kobayashi, M., Teraishi, M., Ito, Y., Ochi, K., Wakasa, K., et al. (2004). Differential expression of three plastidial sigma factors, OsSIG1, OsSIG2A, and OsSIG2B, during leaf development in rice. *Biosci. Biotechnol. Biochem.* 68, 973–977. doi: 10.1271/bbb.68.973

Kim, M., and Mullet, J. E. (1995). Identification of a sequence-specific DNA binding factor required for transcription of the barley chloroplast blue light-responsive *psbD-psbC* promoter. *Plant Cell* 7, 1445–1457. doi: 10.1105/tpc.7.9.1445

Kim, M., Thum, K. E., Morishige, D. T., and Mullet, J. E. (1999). Detailed architecture of the barley chloroplast *psbD-psbC* blue light-responsive promoter. *J. Biol. Chem.* 274, 4684–4692. doi: 10.1074/jbc.274.8.4684

Klein, U., De Camp, J. D., and Bogorad, L. (1992). Two types of chloroplast gene promoters in *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* 89, 3453–3457. doi: 10.1073/pnas.89.8.3453

Klinkert, B., Schwarz, C., Pohlmann, S., Pierre, Y., Girard-Bascou, J., and Nickelsen, J. (2005). Relationship between mRNA levels and protein accumulation in a chloroplast promoter-mutant of *Chlamydomonas reinhardtii*. *Mol. Genet. Genomics* 274, 637–643. doi: 10.1007/s00438-005-0056-x

Kubota, Y., Miyao, A., Hirochika, H., Tozawa, Y., Yasuda, H., Tsunoyama, Y., et al. (2007). Two novel nuclear genes, *OsSIG5* and *OsSIG6*, encoding potential plastid sigma factors of RNA polymerase in rice: tissue-specific and light-responsive gene expression. *Plant Cell Physiol.* 48, 186–192. doi: 10.1093/pcp/pcl050

Liebers, M., Grübler, B., Chevalier, F., Lerbs-Mache, S., Merendino, L., Blanvillain, R., et al. (2017). Regulatory shifts in plastid transcription play a key role in morphological conversions of plastids during plant development. *Front. Plant Sci.* 8:23. doi: 10.3389/fpls.2017.00023

Liere, K., Kaden, D., Maliga, P., and Börner, T. (2004). Overexpression of phage-type RNA polymerase RpoTp in tobacco demonstrates its role in chloroplast transcription by recognizing a distinct promoter type. *Nucleic Acids Res.* 32, 1159–1165. doi: 10.1093/nar/gkh285

Liere, K., and Maliga, P. (1999). *In vitro* characterization of the tobacco *rpoB* promoter reveals a core sequence motif conserved between phage-type plastid and plant mitochondrial promoters. *EMBO J.* 18, 249–257. doi: 10.1093/emboj/18.1.249

Liere, K., Weihe, A., and Börner, T. (2011). The transcription machineries of plant mitochondria and chloroplasts: Composition, function, and regulation. *J. Plant Physiol.* 168, 1345–1360. doi: 10.1016/j.jplph.2011.01.005

Morikawa, K., Ito, S., Tsunoyama, Y., Nakahira, Y., Shiina, T., and Toyoshima, Y. (1999). Circadian-regulated expression of a nuclear-encoded plastid sigma factor gene (*sigA*) in wheat seedlings. *FEBS Lett.* 451, 275–278. doi: 10.1016/S0014-5793(99)00593-1

Nagashima, A., Hanaoka, M., Shikanai, T., Fujiwara, M., Kanamaru, K., Takahashi, H., et al. (2004). The multiple-stress responsive plastid sigma factor, SIG5, directs activation of the *psbD* blue light-responsive promoter (BLRP) in *Arabidopsis thaliana*. *Plant Cell Physiol.* 45, 357–368. doi: 10.1093/pcp/pch050

Nakahira, Y., Baba, K., Yoneda, A., Shiina, T., and Toyoshima, Y. (1998). Circadian-regulated transcription of the *psbD* light-responsive promoter in wheat chloroplasts. *Plant Physiol.* 118, 1079–1088. doi: 10.1104/pp.118.3.1079

Noordally, Z. B., Ishii, K., Atkins, K. A., Wetherill, S. J., Kusakina, J., Walton, E. J., et al. (2013). Circadian control of chloroplast transcription by a nuclear-encoded timing signal. *Science* 339, 1316–1319. doi: 10.1126/science.1230397

Onda, Y., Yagi, Y., Saito, Y., Takenaka, N., and Toyoshima, Y. (2008). Light induction of Arabidopsis *SIG1* and *SIG5* transcripts in mature leaves: differential roles of cryptochrome 1 and cryptochrome 2 and dual function of SIG5 in the recognition of plastid promoters. *Plant J.* 55, 968–978. doi: 10.1111/j.1365-313X.2008.03567.x

Schweer, J., Türkeri, H., Kolpack, A., and Link, G. (2010). Role and regulation of plastid sigma factors and their functional interactors during chloroplast transcription - recent lessons from *Arabidopsis thaliana*. *Eur. J. Cell Biol.* 89, 940–946. doi: 10.1016/j.ejcb.2010.06.016

Shiina, T., Allison, L., and Maliga, P. (1998). *rbcL* Transcript levels in tobacco plastids are independent of light: reduced dark transcription rate is compensated by increased mRNA stability. *Plant Cell* 10, 1713–1722. doi: 10.1105/tpc.10.10.1713

Shiina, T., Tsunoyama, Y., Nakahira, Y., and Khan, M. S. (2005). Plastid RNA polymerases, promoters and transcription regulators in higher plants. *Int. Rev. Cytol.* 244, 1–68. doi: 10.1016/S0074-7696(05)44001-2

Tanaka, K., Tozawa, Y., Mochizuki, N., Shinozaki, K., Nagatani, A., Wakasa, K., et al. (1997). Characterization of three cDNA species encoding plastid RNA polymerase sigma factors in *Arabidopsis thaliana*: evidence for the sigma factor heterogeneity in higher plant plastids. *FEBS Lett.* 413, 309–313. doi: 10.1016/S0014-5793(97)00906-X

Thum, K. E., Kim, M., Christopher, D. A., and Mullet, J. E. (2001a). Cryptochrome 1, Cryptochrome 2, and Phytochrome A co-activate the chloroplast *psbD* blue light–responsive promoter. *Plant Cell* 13, 2747–2760. doi: 10.1105/tpc.010345

Thum, K. E., Kim, M., Morishige, D. T., Eibl, C., Koop, H.-U., and Mullet, J. E. (2001b). Analysis of barley chloroplast *psbD* light responsive promoter elements in transplastomic tobacco. *Plant Mol. Biol.* 47, 353–366. doi: 10.1023/A:1011616400264

To, K., Cheng, M., Suen, D., Mon, D., Chen, L. O., and Chen, S. G. (1996). Characterization of the light-responsive promoter of rice chloroplast *psbD-C* operon and the sequence-specific DNA binding factor. *Plant Cell Physiol.* 37, 660–666. doi: 10.1093/oxfordjournals.pcp.a028995

Tsunoyama, Y., Ishizaki, Y., Morikawa, K., Kobori, M., Nakahira, Y., Takeba, G., et al. (2004). Blue light-induced transcription of plastid-encoded *psbD* gene is mediated by a nuclear-encoded transcription initiation factor, AtSig5. *Proc. Natl. Acad. Sci. U.S.A.* 101, 3304–3309. doi: 10.1073/pnas.0308362101

Wada, T., Tunoyama, Y., Shiina, T., and Toyoshima, Y. (1994). *In Vitro* analysis of light-induced transcription in the wheat *psbD/C* gene cluster using plastid extracts from dark-grown and short-term-illuminated seedlings. *Plant Physiol.* 104, 1259–1267. doi: 10.1104/pp.104.4.1259

Yagi, Y., and Shiina, T. (2012). Evolutionary aspects of plastid proteins involved in transcription: the transcription of a tiny genome is mediated by a complicated machinery. *Transcription* 3, 290–294. doi: 10.4161/trns.21810

Yagi, Y., and Shiina, T. (2014). Recent advances in the study of chloroplast gene expression and its evolution. *Front. Plant Sci.* 5:61. doi: 10.3389/fpls.2014.00061

Yamburenko, M. V., Zubo, Y. O., and Börner, T. (2015). Abscisic acid affects transcription of chloroplast genes via protein phosphatase 2C-dependent activation of nuclear genes: repression by guanosine-3′-5′-bisdiphosphate and activation by sigma factor 5. *Plant J.* 82, 1030–1041. doi: 10.1111/tpj.12876

Yao, W. B., Meng, B. Y., Tanaka, M., and Sugiura, M. (1989). An additional promoter within the protein-coding region of the *psbD-psbC* gene cluster in tobacco chloroplast DNA. *Nucleic Acids Res.* 17, 9583–9591. doi: 10.1093/nar/17.23.9583

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Arabidopsis thaliana mTERF10 and mTERF11, but Not mTERF12, Are Involved in the Response to Salt Stress

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Plastid gene expression (PGE) is crucial for plant development and acclimation to various environmental stress conditions. Members of the "mitochondrial transcription termination factor" (mTERF) family, which are present in both metazoans and plants, are involved in organellar gene expression. *Arabidopsis thaliana* contains 35 mTERF proteins, of which mTERF10, mTERF11, and mTERF12 were previously assigned to the "chloroplast-associated" group. Here, we show that all three are localized to chloroplast nucleoids, which are associated with PGE. Knock-down of *MTERF10*, *MTERF11*, or *MTERF12* has no overt phenotypic effect under normal growth conditions. However, *in silico* analysis of *MTERF10*, *-11*, and *-12* expression levels points to a possible involvement of mTERF10 and mTERF11 in responses to abiotic stress. Exposing mutant lines for 7 days to moderate heat (30°C) or light stress (400 μmol photons m^{-2} s^{-1}) fails to induce a phenotype in *mterf* mutant lines. However, growth on MS medium supplemented with NaCl reveals that overexpression of *MTERF11* results in higher salt tolerance. Conversely, *mterf10* mutants are hypersensitive to salt stress, while plants that modestly overexpress *MTERF10* are markedly less susceptible. Furthermore, *MTERF10* overexpression leads to enhanced germination and growth on MS medium supplemented with ABA. These findings point to an involvement of mTERF10 in salt tolerance, possibly through an ABA-mediated mechanism. Thus, characterization of an increasing number of plant mTERF proteins reveals their roles in the response, tolerance and acclimation to different abiotic stresses.

Keywords: Arabidopsis, chloroplast, nucleoid, mTERF, acclimation, stress, salt

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INTRODUCTION

Chloroplasts are of cyanobacterial origin (Raven and Allen, 2003) and harbor nowadays a reduced genome that mainly encodes proteins involved in photosynthesis and plastid gene expression (PGE). PGE is crucial for plant development and photosynthesis, but its regulation is only partially understood. This is largely because, although plastids still display characteristics of a prokaryotic-like structure of their genome, their gene expression machinery is much more elaborated compared to that of their cyanobacterial ancestor (reviewed in: Liere et al., 2011). Therefore, PGE requires plenty of proteins encoded in the nucleus that support transcription, splicing, trimming and editing of organellar RNAs, and regulate their translation (Schmitz-Linneweber and Small, 2008; Stern et al., 2010; Hammani et al., 2014; Tiller and Bock, 2014; Börner et al., 2015).

Also the nucleus-encoded proteins of the mitochondrial transcription termination factor (mTERF) family regulate mitochondrial and PGE at diverse levels (Kleine and Leister, 2015). The mTERF proteins have been identified in both plants and metazoans (Linder et al., 2005). Human mTERF1, which is the first characterized mTERF, is one of four mammalian mTERF proteins, and was identified nearly 30 years ago as a factor that acts on transcription termination in mitochondrial extracts (Kruse et al., 1989). Its presumptive function as a transcription terminator (of heavy-strand transcripts) gave the family its name. More recently however, models have been suggested in which mTERF1 acts chiefly as a terminator of antisense transcription (Terzioglu et al., 2013) or in polar replication fork arrest (Shi et al., 2016). The true molecular function of mouse mTERF2 also remains unclear, with some reports suggesting that it binds to the same mitochondrial DNA region as mTERF1 and mTERF3 (Wenz et al., 2009), while another contends that the DNA-binding activity of mTERF2 is not sequence-specific (Pellegrini et al., 2009). Knock-out of *Mterf3* in mice leads to embryonic lethality (Park et al., 2007), and conditional knockout of *Mterf3* in the heart has identified a novel role for its protein product in the biogenesis of metazoan mitochondrial ribosomes (Wredenberg et al., 2013). *Mterf4* knock-out mice are also embryonic lethal (Camara et al., 2011). Interestingly, human mTERF4 forms a complex with NSUN4, which is required for assembly of the small and large ribosomal subunits of the mitochondrial ribosome (Metodiev et al., 2014). Consequently, while the function for mTERF2 remains to be clarified, the remainder of the mammalian mTERFs do not support transcription termination, as it is suggested by their notation, but seem to take part in antisense transcription termination and ribosome biogenesis.

The number of mTERF family members has increased to approximately 30 throughout the evolution of land plants (Kleine, 2012), but information on their functions is only beginning to emerge. Most of the 35 *A. thaliana* mTERF proteins (mTERF1-mTERF35; Kleine, 2012) are localized to chloroplasts and/or mitochondria (Babiychuk et al., 2011), and seven of them (mTERF1, -4, -5, -6, -9, -15, and -18) have been functionally investigated in more detail (reviewed in: Kleine and Leister, 2015; Quesada, 2016). Essential functions of mTERF proteins in plant development are revealed by the effects of complete inactivation of three *MTERF* genes: *A. thaliana* mutants devoid of SOLDAT10 (SINGLET OXYGEN-LINKED DEATH ACTIVATOR10)/mTERF1 (Meskauskiene et al., 2009) or BSM (BELAYA SMERT)/RUG2 (RUGOSA2)/mTERF4 (Babiychuk et al., 2011; Quesada et al., 2011) are arrested in embryo development, and knock-out *mterf6-2* plants are albinotic and stop growing after 2 weeks (Romani et al., 2015). Moreover, the dissection of *mterf* mutants supports an involvement of plant mTERFs in responses to abiotic stress (reviewed in: Kleine and Leister, 2015; Quesada, 2016). Indeed, SOLDAT10 (Meskauskiene et al., 2009) and SUPPRESSOR OF *hot1–4* 1 (SHOT1; Kim et al., 2012) were isolated in forward genetic screens for loci that influence responses to abiotic stress. The *hot1-4* mutant is a dominant-negative allele of the heat-shock protein gene *HSP101*. SHOT1/mTERF18 is a mitochondrial protein and the *shot1-1* missense mutant and the *shot1-2* T-DNA insertion mutant each suppress the heat hypersensitivity of *hot1-4* plants. Moreover, other heat-sensitive mutant phenotypes are also suppressed by *shot1-2*, and *shot1-2* single mutants display a higher heat tolerance (Kim et al., 2012). SOLDAT10 is localized to chloroplasts, and plants homozygous for a weaker *soldat10* allele suffer from mild photo-oxidative stress already in low-light conditions; this results in turn in a stress acclimation response, which appears to confer improved hardiness against a combination of high-light and low-temperature stress (Meskauskiene et al., 2009). Other *mterf* mutants are also linked to stress responses. For example, *mda1* (*mterf5*), and *mterf9* seedlings are less susceptible to osmotic and salt treatments, which might be linked to their decreased sensibility to ABA (Robles et al., 2012, 2015). Furthermore, the *rug2-1* mutant is abnormally sensitive to temperature stress. At 26°C, *rug2-1* homozygotes undergo growth arrest, whereas at 16°C this growth phenotype is not expressed (Quesada et al., 2011).

A co-expression network for all *MTERF* genes (26 out of 35) which were present on the Affymetrix ATH1 genome array has been constructed (Kleine, 2012). The resulting clusters and information related to the subcellular locations of the proteins that are encoded by genes co-expressed with each *MTERF* gene were then used to assign the mTERFs into five groups, referred to as the "chloroplast," "chloroplast-associated," "mitochondrial," "mitochondrion-associated," and the "low expression" clusters.

In the present study, we characterized the members of the "chloroplast-associated" group, which comprises mTERF10 (AT2G34620), mTERF11 (AT3G18870), and mTERF12 (AT4G09620). The sub-chloroplast localization of mTERF10, -11, and -12 was defined by fluorescence microscopy of mTERF-GFP fusions and an RFP fusion protein (as a control for nucleoid localization). Lines with altered *MTERF10*, *MTERF11*, and *MTERF12* levels did not display phenotypes under normal growth conditions. *In silico* analyses with the eFP browser and Genevestigator were conducted, which pointed to an involvement of these three mTERFs in abiotic stress responses. To follow this up, the mutant lines were exposed to moderate heat (30°C), high light (400 μmol photons m^{-2} s^{-1}), or salt (175 mM NaCl) stress, and subjected to ABA treatment. The *mterf10*, *-11*, and *-12* mutant lines responded to heat and high light stress like the wild type (WT). However, lack of mTERF10 or mTERF11 led to enhanced or reduced sensitivity to salt, respectively, while overexpression of *MTERF10* rendered seedlings more tolerant than WT to both salt and ABA.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The mutants *mterf10-1* (SAIL_12A03), *mterf10-2* (SALK_097699), *mterf11-1* (FLAG_357F09), *mterf11-2* (GABI_211D05), and *mterf12-1* (GABI_407E04) were identified in the SIGnAL database (Alonso et al., 2003), the *abi4-1* mutant was ordered from The European Arabidopsis Stock Centre (NASC; ID N8104). All mutants are in the Col-0 background except of *mterf11-1* which is a WS line.

Arabidopsis thaliana plants were grown in long-day conditions (16 h light/8 h dark) on potting soil (Stender). Plants were illuminated with HQI Powerstar 400 W/D lamps and a fluence rate of approximately 100 μmol photons m^{-2} s^{-1}. To accomplish salt and ABA stress experiments, seedlings were grown on plant agar (Sigma-Aldrich) containing half-strength MS medium and 1.5% (w/v) sucrose at 22°C under 100 μmol photons m^{-2} s^{-1} provided by white fluorescent lamps under continuous light or long-day conditions. For salt stress experiments, MS medium was supplemented with 125 mM or 175 mM NaCl as indicated. For ABA experiments, MS medium was supplemented with 1 μM ABA.

Nucleic Acid Extraction

For DNA isolation, leaf tissue was homogenized in extraction buffer containing 200 mM Tris/HCl (pH 7.5), 25 mM NaCl, 25 mM EDTA, and 0.5% (w/v) SDS. After centrifugation, DNA was precipitated by adding isopropyl alcohol. After washing with 70% (v/v) ethanol, the DNA was dissolved in distilled water.

For RNA isolation, frozen tissue was ground in liquid nitrogen. Following the addition of TRIZOL (Invitrogen) and chloroform according to the manufacturer's instructions, RNA was precipitated from the aqueous phase with isopropyl alcohol, then washed with 70% (v/v) ethanol, and dissolved in RNase-free water. Concentration and purity of RNA samples were determined spectroscopically in a GeneQuant pro RNA/DNA Calculator (GE Healthcare Europe GmbH). Isolated RNA was stored at −80°C until further use.

cDNA Synthesis and Real-Time PCR Analysis

cDNA synthesis and real-time PCR analysis were performed as outlined before (Voigt et al., 2010). All reactions were done in triplicate on three biological replicates. The target genes and the respective primers, are listed in **Supplementary Table S1**. The *RCE1* gene was used as an internal reference in other studies (Voigt et al., 2010; Romani et al., 2015). *RCE1* transcript levels are not changed upon diverse conditions, especially not under diverse stress conditions including lincomycin and norflurazon treatment which affect organellar gene expression.

RNAi, Overexpression and Intracellular Protein Localization

To reduce *MTERF12* mRNA levels by RNAi, a 145-bp fragment was amplified from genomic DNA with the primer pair AT4G09620-GST-attB1 and –attB2 (see **Supplementary Table S1**). The gel-purified PCR product was used for BP and LR Clonase reactions (GATEWAY Cloning; Invitrogen) which led to the final construct pB7GWIWG2/MTERF12 (for pB7GWIWG2, see Karimi et al., 2002). For overexpression and localization studies of mTERF10, mTERF11 and mTERF12, cDNAs encompassing the coding regions were amplified by PCR (see **Supplementary Table S1** for primer information). Notably, in our Col-0 strain, *MTERF11* has an additional triplet (CAT; coding for histidine) inserted after nucleotide 27 (relative to the start codon) compared with the coding sequence from The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). *MTERF10*, *MTERF11*, and *MTERF12* were cloned by GATEWAY technology (see above) into pB7FWG2 to generate fusions with enhanced GFP (eGFP), expression of which is controlled by the *Cauliflower mosaic virus* 35S promoter. For RAP-RFP fusions, the pENTR/RAP plasmid (Prof. Jörg Nickelsen, LMU Munich) was introduced into p2GWR7 by GATEWAY cloning. For overexpression of mTERF10, *MTERF10* was introduced by classical cloning with the NcoI restriction enzyme into pCAMBIA1302. For RNAi experiments with *MTERF12* and overexpression of mTERF10 and mTERF11, the plasmids pB7GWIWG2/MTERF12, pCAMBIA1302/MTERF10, and pB7FWG2/MTERF11 were independently transferred into *Agrobacterium tumefaciens*, and *A. thaliana* (ecotype Col-0 for *MTERF10* overexpression and *MTERF12* RNAi; ecotype WS for *MTERF11* overexpression) plants were transformed by the floral-dip method (Clough and Bent, 1998). After seed set, transgenic plants were selected on the basis of their resistance to BASTA (pB7GWIWG2/MTERF12 and pB7FWG2/MTERF11) or hygromycin (pCAMBIA1302/MTERF10), respectively.

For fluorescence visualization, leaves of 3-week-old Col-0 plants grown on MS medium were cut into small pieces and incubated for 16 h at 24°C in the dark in a protoplasting solution (10 mM MES, 20 mM $CaCl_2$, 0.5 M mannitol (pH 5.8), 0.1 g ml^{-1} macerozyme (Duchefa), 0.1 g ml^{-1} cellulase (Duchefa). After isolation and transformation of protoplasts as described (Dovzhenko et al., 2003), preparations were examined with a Fluorescence Axio Imager microscope (Zeiss). Fluorescence was excited with the X-Cite Series 120 fluorescence lamp (EXFO) and images were collected at 500–550 nm (eGFP fluorescence), 570–640 nm (RFP fluorescence) and 670–750 nm (chlorophyll autofluorescence).

Chlorophyll *a* Fluorescence Measurements

In vivo chlorophyll a fluorescence of whole plants was recorded using an imaging chlorophyll fluorometer (ImagingPAM, Walz GmbH, Effeltrich, Germany). Plants were dark adapted for 15 min and then exposed to a pulsed, blue measuring light (1 Hz, intensity 4) and a saturating light flash (intensity 5) to determine the maximum fluorescence F_m and the ratio $(F_m\text{-}F_0)/F_m = F_v/F_m$.

Computational Analyses

Protein sequences were retrieved from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) and The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org). Amino acid sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw; Chenna et al., 2003). The unrooted tree was constructed with the Phylip server Mobyle at the Pasteur Institute (http://mobyle.pasteur.fr/cgi-bin/portal.py#welcome).

RESULTS

All Members of the Chloroplast-Associated mTERF Cluster Are Localized to Nucleoids

The localizations of almost all *A. thaliana* mTERF proteins have been investigated by fluorescence microscopy of mTERF-GFP fusions transiently expressed in isolated protoplasts, and in guard cells of transgenic plants (Babiychuk et al., 2011). These data indicated that mTERF10, -11, and -12 are targeted to chloroplasts. To confirm these results and if possible define the precise locations of the proteins within the chloroplast, the eGFP fluorescence of mTERF10-, mTERF11-, or mTERF12-eGFP fusions, transiently overexpressed in Col-0 protoplasts, was monitored. Localization of all three fusion proteins to chloroplasts was confirmed (**Figure 1A**). However, the fluorescence signals were not uniformly distributed, but

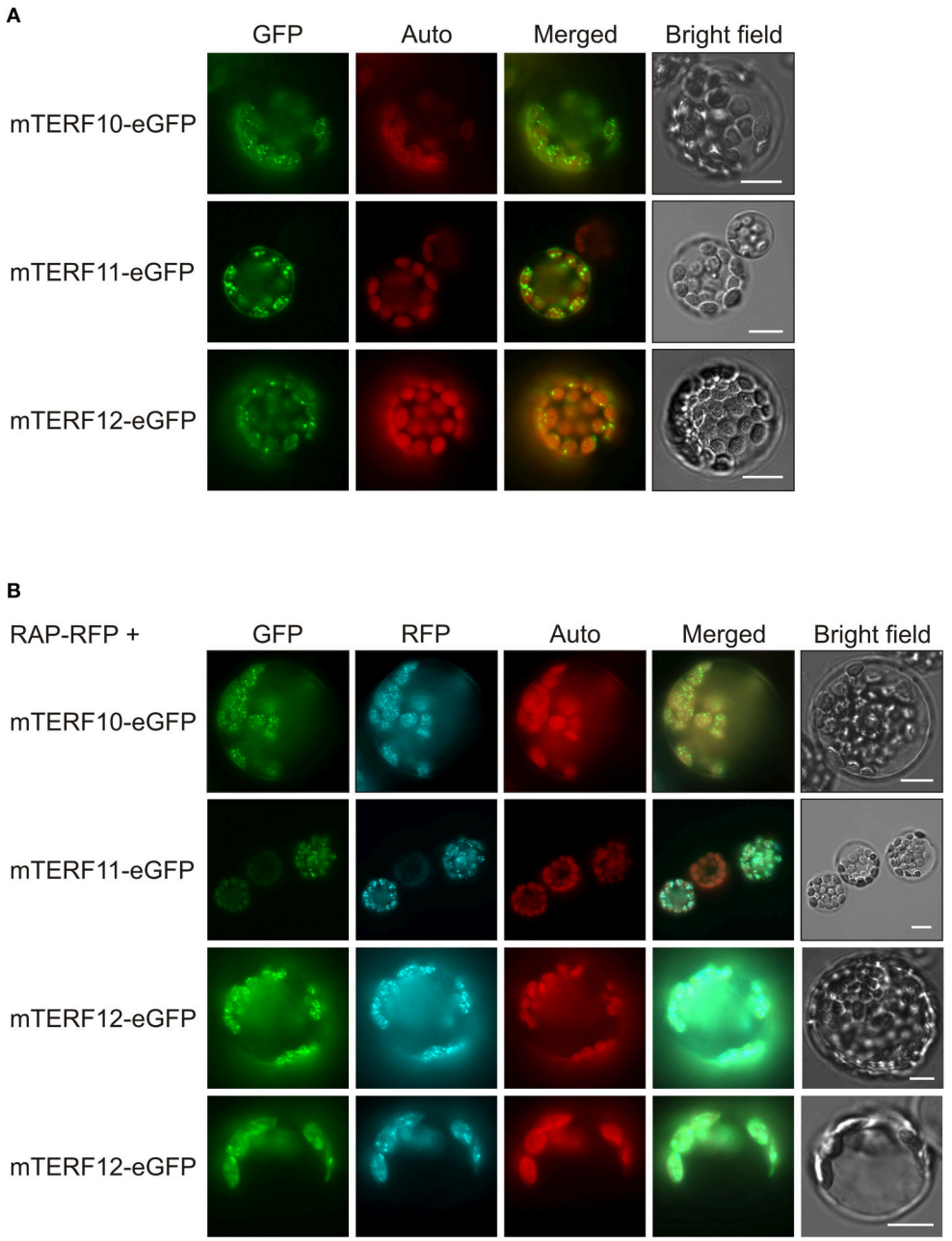

FIGURE 1 | Localization of mTERF10, mTERF11 and mTERF12. **(A)** Fluorescence microscopy of *A. thaliana* protoplasts transiently expressing mTERF10, mTERF11 or mTERF12 fused to eGFP (mTERF10-eGFP, mTERF11-eGFP and mTERF12-eGFP). **(B)** To visualize nucleoids, protoplasts were co-transformed with a RAP-RFP fusion in combination with mTERF10-eGFP, mTERF11-eGFP, or mTERF12-eGFP. The eGFP fluorescence is shown in green (GFP), RFP fluorescence in cyan (RFP), autofluorescence of chloroplasts in red (Auto). The scale bars correspond to 10 μm.

appeared as small dots in the chloroplasts. The size and distribution of these dots were suggestive of nucleoids, which are associated with PGE (Majeran et al., 2012). Localization of the *A. thaliana* RNA-binding protein RAP to nucleoids was previously established using a transiently expressed RAP-eGFP fusion (Kleinknecht et al., 2014). Therefore, Col-0 protoplasts were co-transformed with a RAP-RFP fusion in combination with mTERF10-eGFP, mTERF11-eGFP, or mTERF12-eGFP. Indeed, for each mTERF-eGFP construct, signals were found in dots together with the RFP signal. Merging of both signals confirmed colocalization of the mTERF10, -11, and -12 fusions with RAP, and therefore localization of all three mTERFs to nucleoids (**Figure 1B**). It is noteworthy, that especially mTERF12—and a minor fraction of RAP—tend to be localized in the chloroplast stroma when both mTERF12-eGFP and RAP-RFP are expressed together in protoplasts.

Identification and Phenotypic Analysis of Mutants for the *MTERF10, MTERF11, MTERF12* Loci

To obtain insight into the physiological functions of mTERFs 10, 11, and 12, T-DNA insertion lines were identified in the SIGnAL database (Alonso et al., 2003). The insertions were confirmed by PCR (**Figure 2A**) and homozygous lines were selected. In the mutants *mterf10-1* (SAIL_12A03) and *mterf10-2* (SALK_097699) the T-DNAs are inserted in the 5′ UTR and the second exon, respectively (**Figure 2B**). The *mterf11-1* (FLAG_357F09) and *mterf11-2* (GABI_211D05) mutants both have their T-DNA insertion in the gene's single exon. For *MTERF12*, only one insertion line could be identified (*mterf12-1*, GABI_407E04), which contains a T-DNA in the promoter region (**Figure 2B**). To repress the *MTERF12* gene by RNAi, Col-0 lines were generated that contained constructs with an inverted repeat of a fragment spanning the first exon and a part of exon 2 of *MTERF12* (**Figure 2A**) which was under control of the constitutive *Cauliflower mosaic virus* 35S promoter.

Figure 2C shows the numbers and distributions of mTERF domains in the mTERF10, -11, and -12 proteins. In mTERF10 and mTERF11, six and five mTERF motifs are predicted by the Simple Modular Architecture Research Tool SMART (http://smart.embl-heidelberg.de/). One mTERF motif has been predicted for mTERF12 (our previous results, and see also Supporting Information of Babiychuk et al., 2011), but this domain is not annotated anymore with confidence by the SMART tool (http://smart.embl-heidelberg.de/smart/show_motifs.pl?ID=Q93ZZ2_ARATH). Thus, the classification of mTERF12 as an mTERF protein must be regarded as uncertain.

All mutants are in the Col-0 background except of *mterf11-1* which is a WS line. Hence, in all following experiments, *mterf11-1* was compared with WS, while Col-0 was used as the WT standard for the other lines. Real-time PCR analysis was employed to determine the extent of repression of *MTERF* transcripts in the different mutant lines (**Figure 3A**). In 3-week-old *mterf10-1* and *mterf10-2* plants, *MTERF10* transcript levels were reduced to 29 and 4% of WT, respectively. To determine *MTERF11* transcript levels, primer pair A was chosen to detect transcripts initiated 5′ of the T-DNA insertions (**Figure 2A**). Using this set-up, *MTERF11* transcript levels were found to be unchanged (*mterf11-1*) and nearly 6-fold induced (*mterf11-2*) relative to their WT (**Figure 3A**). In the *mterf11-1* allele (which is FLAG_357F09), the T-DNA of the pGKB5 vector integrated in the 5′LB–T-DNA–RB3′ direction. It is of note here that the pGKB5 vector used to generate the FLAGdb T-DNA insertion line collection contains the 35S promoter on the LB side (Samson et al., 2002). The 35S promoter drives the expression of *PHOSPHINOTRICIN ACETYL TRANSFERASE* (*PAT*) used to select transgenic plants, and the *PAT* transcripts are terminated by the G7 terminator. It was already shown with two independent FLAG lines as an example that the G7 terminator can be an inefficient terminator in the context of the pGKB5 vector, allowing transcription to continue through and beyond the terminator sequence (Ulker et al., 2008). However, real-time PCR carried out with a primer pair covering the region 3′ of the T-DNA insertion detected greatly reduced *MTERF11* transcript levels in the *mterf11* mutants: 0.09% of WT in *mterf11-1* and 0.01% in *mterf11-2* (**Figure 3A**). *MTERF12* transcript levels were not affected in the *mterf12-1* mutant (**Figure 3A**). Therefore, *MTERF12* RNAi lines were tested for their ability to repress *MTERF12* gene expression. Six independent lines were screened, but the most effectively repressed lines, *mterf12i-1* and *mterf12i-2*, still retained 32% and 59% of WT (Col-0) amounts of *MTERF12* transcripts, respectively (**Figure 3A**). Under normal growth conditions, all identified mutant lines were phenotypically indistinguishable from WT (**Figure 3B**). To look for subtle photosynthetic phenotypes, the maximum quantum yield of photosystem II (F_v/F_m) was measured in Col-0, WS and all *mterf* mutants (**Figure 3B**), but no deviations in this parameter were detected in the mutants.

To summarize, the expression of all mTERF motifs should be strongly reduced in the *mterf10* mutants (particularly *mterf10-2*), while the *mterf11* mutants produce truncated transcripts. Assuming the latter are translated, the protein products would lack the last two mTERF domains (*mterf11-1*) or mTERF domain 5 only (*mterf11-2*) (**Figure 2C**). In the *mterf12i* lines, transcripts including the single putative mTERF domain were—at best—reduced to one-third of Col-0 levels. At all events, none of the *mterf* mutant lines display any obvious phenotype under normal growth conditions.

Phylogenetic Position of the mTERF10, -11, and -12 Proteins

Because the *mterf10*, *-11*, and *-12* mutant lines lacked a clear phenotype under normal growth conditions (**Figure 3**), we asked whether this might be attributable to functional redundancy within the mTERF family. Several *MTERF* genes have undergone tandem duplications (on chromosome 1) and one block duplication (*AT4G19650* and *AT5G45113*; Kleine, 2012). But neither *MTERF12* nor *MTERF10* or *MTERF11* originated from a duplication event, so we can exclude the possibility of

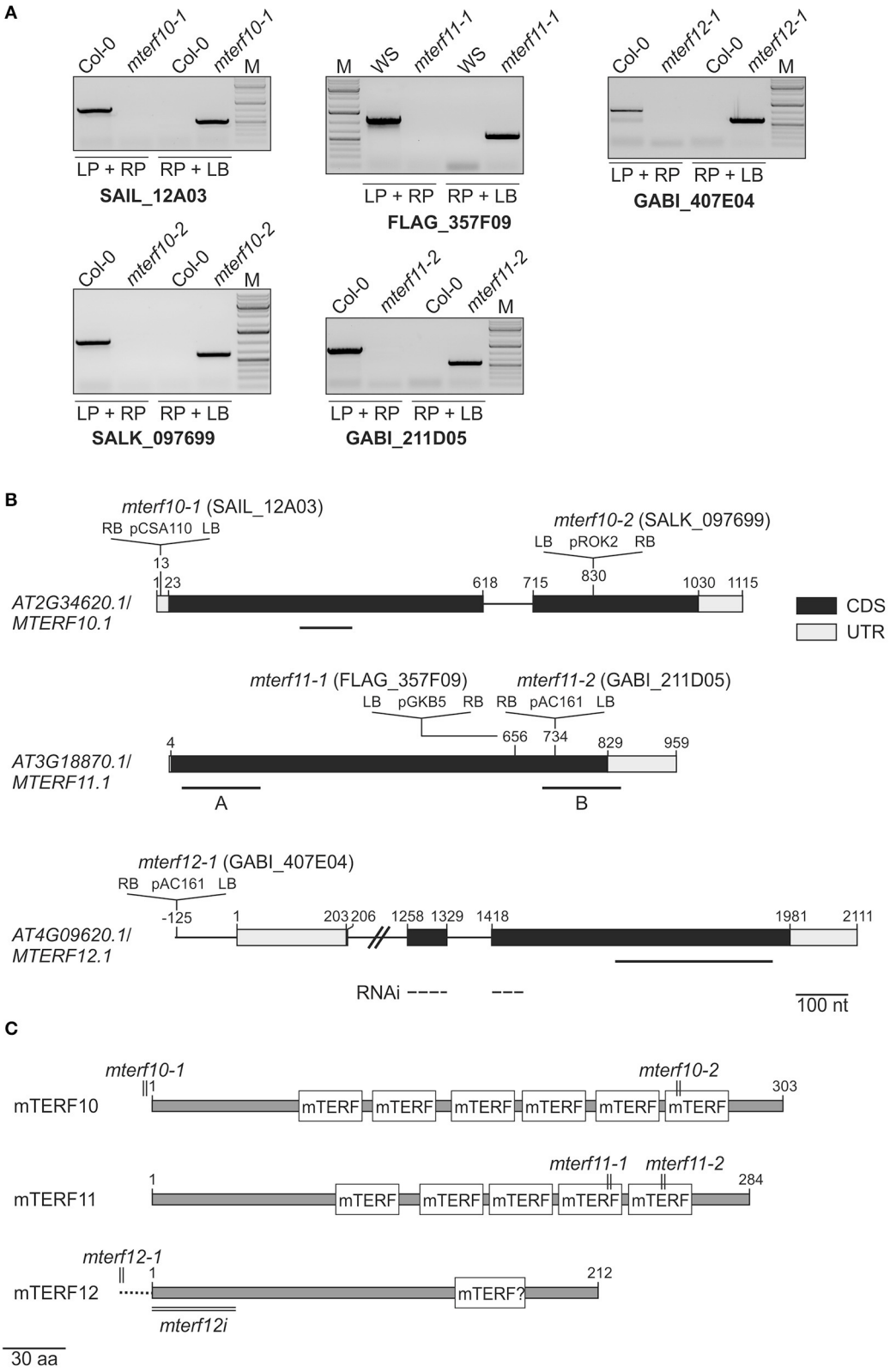

FIGURE 2 | Identification of *mterf10*, *mterf11* and *mterf12* T-DNA insertion mutants, and generation of *MTERF12* RNAi lines. **(A)** Confirmation and identification of homozygous T-DNA insertions in the different *mterf* mutant lines. The combination of the gene-specific left and right primers (LP and RP) was used for amplification of

(Continued)

FIGURE 2 | Continued
sequences around the T-DNA insertion. The combination of RP and T-DNA left border primer (LB) was used for the verification of the T-DNA insertion. **(B)** Schematic representation and T-DNA tagging of the *MTERF10* (*AT2G34620*), *MTERF11* (*AT3G18870*), and *MTERF12* (*AT4G09620*) loci. Exons (black boxes), introns (black lines) and the 5′ and 3′ UTRs (gray boxes) are shown. Numbers are given relative to the transcription start site of the gene loci. Locations and orientation of T-DNA insertions are indicated, as deduced from RP + LB PCR products shown in **(A)** which were subsequently sequenced. Note that the insertions are not drawn to scale. Furthermore, the location of the *MTERF12* RNAi-directed sequence is indicated as a dashed line. **(C)** Schematic representation of mTERF10, mMTERF11, and mTERF12 proteins. The numbers and locations of mTERF domains are shown as white boxes. The relative positions of T-DNA and RNAi tagging are indicated.

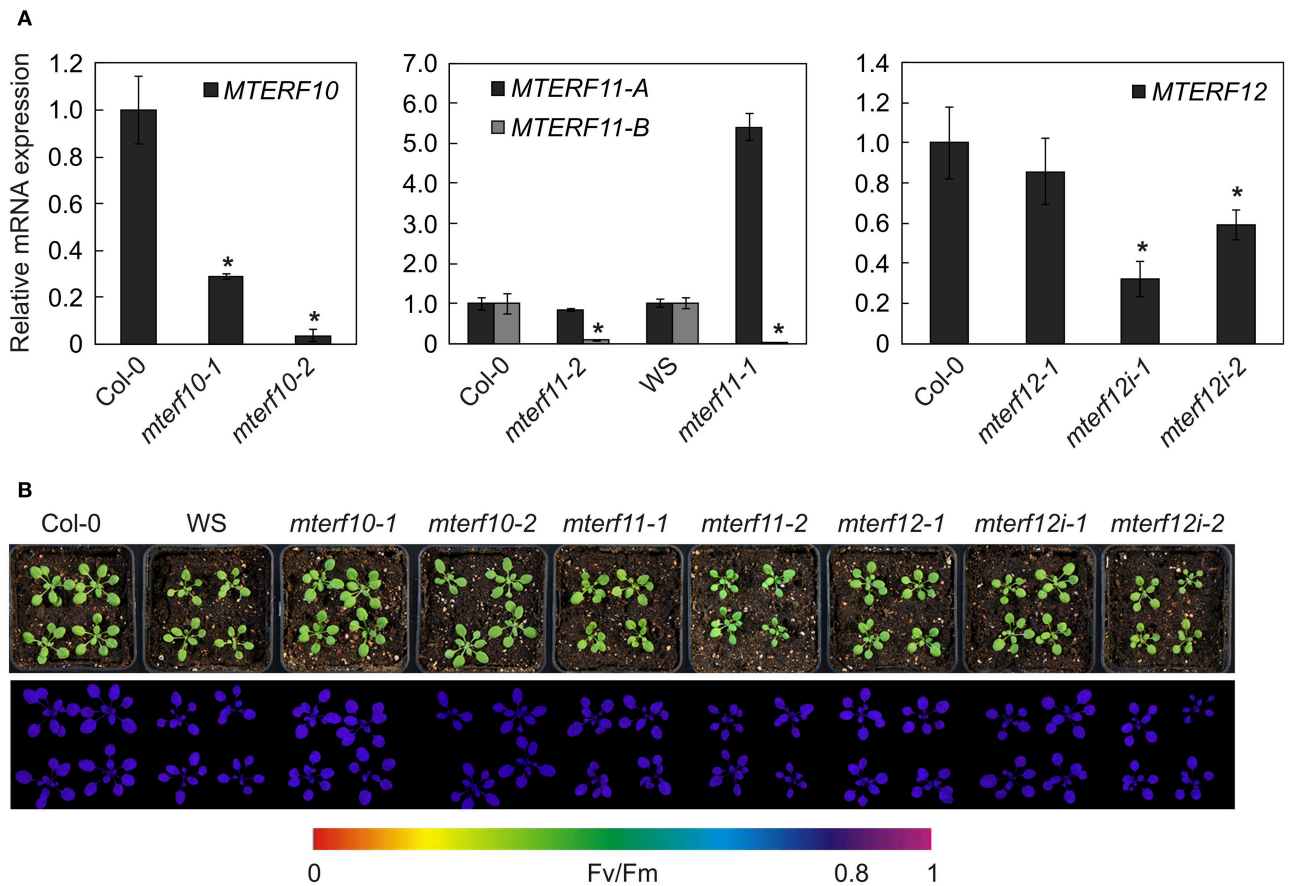

FIGURE 3 | Characterization of *mterf10*, *mterf11*, *mterf12* T-DNA insertion and *MTERF12* RNAi (*mterf12i*) lines. **(A)** Real-time PCR analysis of *MTERF10*, *MTERF11*, *and MTERF12* mRNA levels. Real-time PCR was performed with primers specific for fragments indicated by horizontal black lines below the corresponding gene in **Figure 2B**. Expression values are reported relative to the corresponding transcript levels in Col-0. The results were normalized with respect to the expression level of *At4g36800*, which codes for a RUB1-conjugating enzyme (*RCE1*). Bars indicate standard deviations. Statistically significant differences (*t*-test; $p < 0.05$) between wild-type and mutant samples are indicated by an asterisk. **(B)** Phenotypes of 3-week-old wild-type (WS for *mterf11-1* and Col-0 for the remaining mutant lines) and mutant plants grown under long-day (16/8 h) light conditions. The maximum quantum yield of PSII (F_v/F_m) was measured with an ImagingPAM fluorometer.

protein redundancy arising from gene duplication. To obtain an impression of the overall degree of sequence similarity within the mTERF protein family, we constructed a phylogenetic tree which included in addition to *A. thaliana* mTERFs, mTERF members from the green alga *Chlamydomonas reinhardtii*, *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, and other organisms (**Figure 4**). The tree reveals four main clades. The mTERF members of *C. reinhardtii*, *H. sapiens* and *M. musculus* are all in the same clade. The majority of *A. thaliana* mTERFs form clade I which includes mTERF10 and -11, while mTERF12 along with five other *A. thaliana* mTERFs and one *D. melanogaster* mTERF constitute clade II. The mTERF10, -11, and -12 proteins are most closely related to mTERF1/SOLDAT10 (Meskauskiene et al., 2009), mTERF4/BSM/RUG (Babiychuk et al., 2011; Quesada et al., 2011) and mTERF15 (Hsu et al., 2014) proteins. Mutants for each of these three display phenotypes under normal growth conditions and have been shown to be involved in PGE or mitochondrial gene expression. Moreover, levels of sequence identity/similarity between mTERF10 and mTERF1 (38/68% over a stretch of 240 amino acids), mTERF11 and mTERF4 (26/41% over a stretch of 222 amino acids) and mTERF12 and mTERF15 (29/53% over a stretch of 77 amino acids), respectively, are

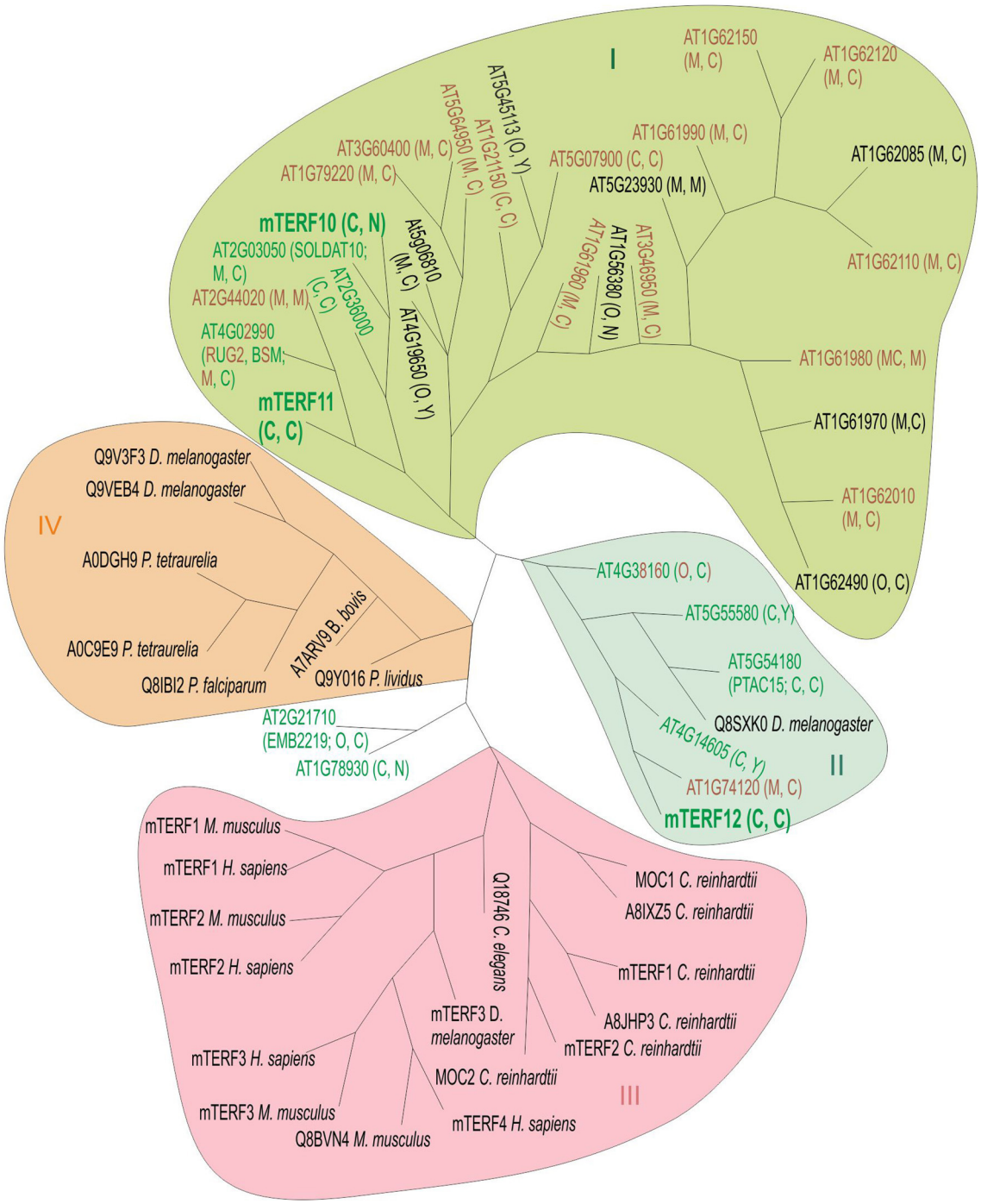

FIGURE 4 | Unrooted phylogenetic tree of mTERF proteins. The tree is based on the amino acid sequences of 35 *A. thaliana* mTERF proteins and 23 other mTERF proteins from *Chlamydomonas reinhardtii* (*C. reinhardtii* MOC1 [Q8LJS6], MOC2 [A8IC10], mTERF1 [E1VD13], mTERF2 [E1VD14], A8IXZ5, A8JHP3), *Drosophila melanogaster* (*D. melanogaster* Q8SXK0, Q9V3F3, Q9VEB4, mTERF3 [Q06YR8]), *Homo sapiens* (*H. sapiens* mTERF1 [Q99551], mTERF2 [Q49AM1], mTERF3[Q96E29], and mTERF [Q7Z6M4]), *Mus musculus* (*M. musculus* mTERF1 [Q8CHZ9], mTERF2 [Q8BKY8], mTERF3 [Q8R3J4], Q8BVN4), *Caenorhabditis elegans* (*C. elegans* Q18746C), *Plasmodium falciparum* (*P. falciparum* Q8IBI2), *Babesia bovis* (*B. bovis* A7ARV9), *Paracentrotus lividus* (*P. lividus* Q9Y016) and *Paramecium tetraurelia* (*P. tetraurelia* A0DGH9). Green, brown and green-brown lettering depicts targeting to chloroplasts, mitochondria or dual targeting to chloroplasts and mitochondria, respectively, as reported elsewhere (Meskauskiene et al., 2009; Babiychuk et al., 2011; Quesada et al., 2011; Romani et al., 2015) and in this article. Letters in parentheses indicate predicted localization by TargetP (http://www.cbs.dtu.dk/services/TargetP) and WoLF PSORT (http://wolfpsort.org). Sequences were aligned by the ClustalW program (see Materials and Methods). The Phylip server Mobyle (see Methods) was used for phylogenetic tree constructions and comparison of distances (model: Jones-Taylor-Thornton matrix), employing a boostrap test with 1,000 replicates. Phylogenetic inference supports the existence

(Continued)

FIGURE 4 | Continued
of four main clades (I–IV). Clade I encompasses proteins encoded by a tandem gene cluster on *A. thaliana* chromosome 1 and several other *A. thaliana* mTERF proteins. In clade III, *C. reinhardtii* mTERFs are grouped together with animal mTERF proteins. Clade IV comprises mTERF proteins from diverse species including paramecium, sea urchin (*P. livides*), parasites (*P. falciparum* and *B. bovis*) together with mTERFs from Drosophila. The mTERF proteins mTERF10 and -11 (highlighted in large, bold letters) form clade I together with 25 other mTERF proteins, while mTERF12 (also highlighted in big, bold letters) is assigned to clade II, together with five other *A. thaliana* mTERFs and one Drosophila mTERF. C, chloroplast; M, mitochondrion; N, nucleus; Y, cytosol; O, other.

noteworthy for the mTERF10/mTERF1 pair, but negligible for the other two.

Changes in *MTERF* Transcript Levels in Response to Abiotic Stresses

To gain deeper insights into the functions of mTERF10, -11, and -12, their mRNA expression patterns were analyzed. Co-expression analysis of 26 *MTERF* genes and their corresponding gene ontology annotations have already been reported (Kleine, 2012). However, that study was designed to provide a global classification. Hence subsequent Genevestigator analyses only dealt with the numbers of conditions/treatments that altered *MTERF* gene expression. In the present study, we extracted *MTERF* transcript levels from the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) with "Abiotic Stress" as a data source (Winter et al., 2007). In these experiments, 18-day-old plants were subjected to different stresses, and samples were taken over a time course of 24 h from stress-treated and control plants. We calculated the relative changes in *MTERF10*, *-11*, and *-12* transcript levels from plants exposed to drought, high salt, heat, or cold compared to control conditions (**Figure 5A**). Because *mda1* (*mterf5*) and *mterf9* mutants are known to exhibit altered stress responses (Robles et al., 2012, 2015), *MTERF5* and *-9* were included for reference. Under drought and heat stress, transcript levels of all investigated *MTERF* genes were only moderately changed (**Figure 5A**). With a 3-fold rise after 1 h of heat stress (MTERF10) and an approximately 0.3-fold change (*MTERF5* and *9*), those transcripts were the most responsive. Under salt and cold treatment, *MTERF* transcript levels tended to be reduced. Under both salt and cold stress, *MTERF10* and *MTERF11* levels were most responsive, and especially after 24 h of cold treatment *MTERF5*, *10*, *11*, and *12* transcript levels were reduced (**Figure 5A**). To confirm these data and to find other conditions under which the *MTERF*s of interest might be regulated, the Genevestigator Perturbations Tool (https://genevestigator.com/gv; Hruz et al., 2008) was employed on all deposited *A. thaliana* ATH1 arrays together with a 2-fold change filter and a p-value of < 0.05. An overview of all changes in *MTERF10*, *MTERF11*, and *MTERF12* mRNA levels in response to perturbations (relative to untreated controls) can be found in **Supplementary Figures S1–S3**. In **Figure 5B** selected conditions are shown which are associated with changes in temperature and light, and with salt and drought stress conditions. Levels of *MTERF10* mRNA were most susceptible to perturbation, being induced by light, raised after germination, and strongly reduced under drought conditions and various cold and high-light regimes, and on exposure to salt or ABA (**Figure 5B**).

Knockdown of *MTERF10* or *MTERF11* Alters Sensitivity to Salt Stress

To experimentally probe the involvement of mTERFs in stress responses, 3-week-old WT and *mterf10*, *-11*, and *-12* mutant plants grown under standard conditions (22°C at 100 μmol photons m^{-2} s^{-1}) were exposed for 7 days to moderate temperature stress (30°C, at a fluence rate of 100 μmol photons m^{-2} s^{-1}) or moderate light stress (400 μmol photons m^{-2} s^{-1} at a temperature of 22°C). After 7 days of moderate temperature stress, the leaf petioles of WT and all *mterf* mutants were shortened, but otherwise all plants looked healthy (**Figure 6A**). After 3 days, F_v/F_m was slightly reduced in all *mterf11* and *mterf12* mutant lines, but was restored to normal levels after 7 days (**Figures 6A,B**). Also after 7 days of moderate light stress, the leaf petioles of WT and all *mterf* mutants were shortened—albeit to a lesser extent. Furthermore, the edges of older leaves in all lines began to show signs of necrosis (**Supplementary Figure S4A**). After 1 h of moderate light stress, F_v/F_m was slightly reduced in all lines (**Supplementary Figure S4B**). This reduction continued in the *mterf11-1* mutant after 2 and 4 h, but all lines recovered to the initial F_v/F_m values after 96 h (**Supplementary Figure S4B**).

After 3 and 24 h of salt stress, *MTERF5* and *MTERF9* transcript levels were reduced to half of those in control conditions (**Figure 5A**), and indeed, *mda1* (*mterf5*) and *mterf9* seedlings are less sensitive to salt and osmotic stresses (Robles et al., 2012, 2015). Because *MTERF10* and *MTERF11* transcripts were reduced to an even larger extent than *MTERF5* and *MTERF9* RNAs following exposure to salt stress for 6 and 24 h (**Figure 5A**), we asked whether inactivation of *MTERF10* or *MTERF11* might enable the mutant plants to better tolerate salt stress. To this end, WT and *mterf* mutant lines were germinated on MS medium (control) and MS medium supplemented with 125 mM or 175 mM NaCl, and germination rates were scored after different time points (**Figure 7A**). All lines germinated to nearly 100% on the control MS medium. Germination rates of all lines with a Col-0 background grown for 48 h on MS medium supplemented with 125 mM NaCl or for 72 h on medium supplemented with 175 mM NaCl were very similar (**Figure 7A**). In the aforementioned conditions, germination rates of Col-0 seeds were approximately 60% (**Figure 7A**). The germination rates of *mterf10-1* and *-2*, *mterf11-2* and all *mterf12* seeds were all lower (ranging from 26 to 47%) than those of Col-0 seeds. However, after 96 h on MS medium supplemented with 175 mM NaCl the germination rates of *mterf12* seeds (76 to 87%) were comparable to that of Col-0 seeds (84%). Interestingly, *mterf10-1*, *mterf10*-2, and *mterf11-2* still displayed enhanced sensitivity to salt inhibition, with germination rates of 54, 55, and 61%, respectively (**Figure 7A**). WS seeds were

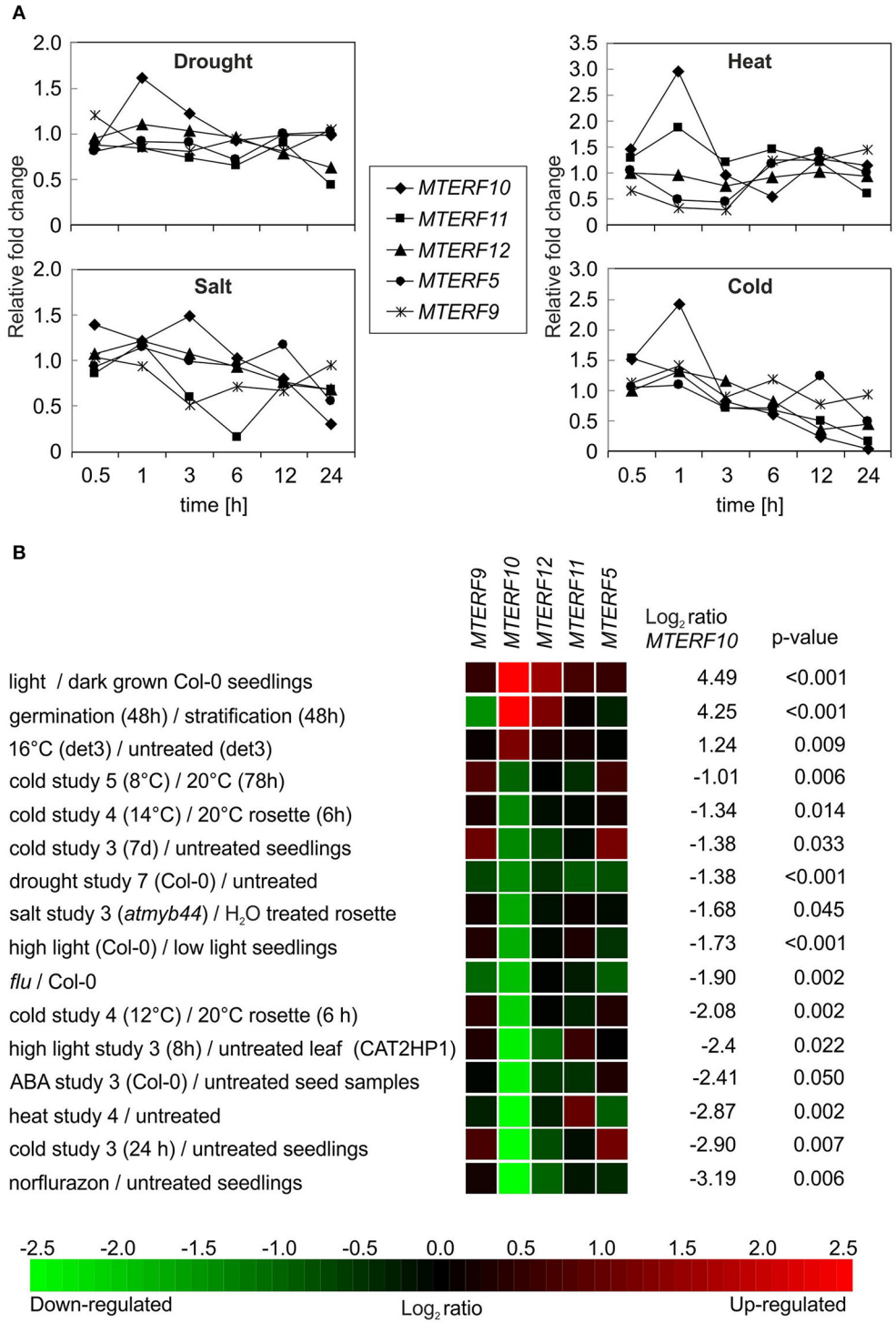

FIGURE 5 | *In silico* analyses of changes in levels of *MTERF* transcripts in response to abiotic stresses. **(A)** *MTERF* transcript levels were extracted from the Arabidopsis eFP Browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) with "Abiotic Stress" as a data source (Winter et al., 2007). Plant material from stress-treated and control plants was analyzed over a time course of 24 h. Here, the expression values are reported relative to the corresponding transcript levels in control conditions. **(B)** The Genevestigator Perturbations Tool (https://genevestigator.com/gv; Hruz et al., 2008) was applied to all available *A.thaliana* microarrays in combination with the 2-fold change filter and a *p*-value of < 0.05. Shown here is a selection of conditions related to abiotic stresses. Conditions were ordered according to the magnitude of the relative change in *MTERF10* mRNA (from high to low). An overview of all transcriptional responses of *MTERF10*, *MTERF11*, and *MTERF12* to perturbations can be found in **Supplementary Figures S1–S3**.

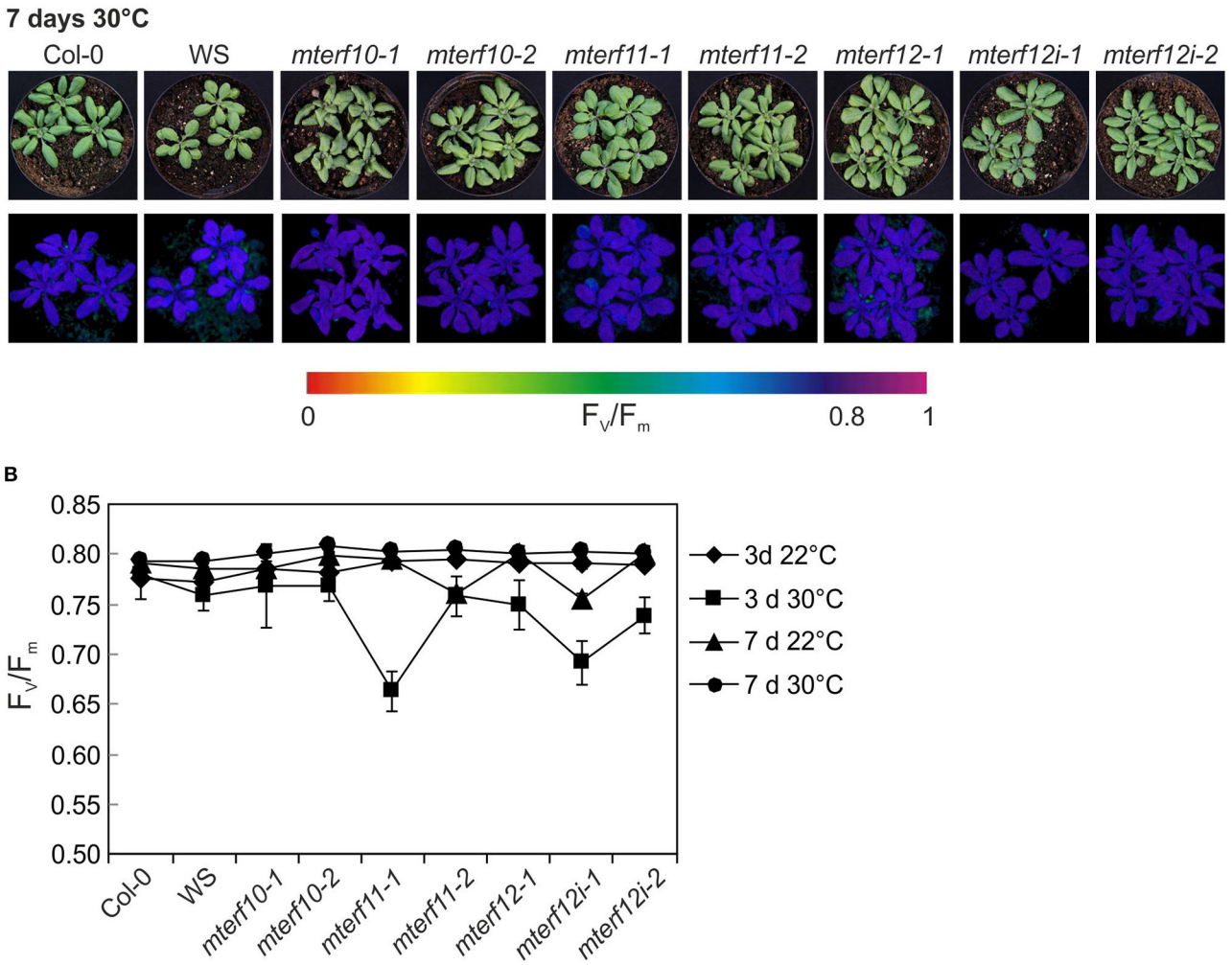

FIGURE 6 | Behavior of wild-type (Col-0) and *mterf10*, *-11* and *-12* mutant plants under moderate heat stress of 30°C. **(A)** To score the phenotypes under moderate heat stress, plants were first grown for 3 weeks under normal growth conditions (100 μmol photons $m^{-2}s^{-1}$, 22°C) and then exposed to 30°C for 7 days. **(B)** The maximum quantum yield of PSII (F_V/F_m) of Col-0 and *mterf* mutant plants was determined after 3 and 7 days (d) in 30°C. The data are shown as mean values ± SD from 8 to 10 different leaves.

very susceptible to salt stress and failed to germinate under the conditions used to investigate the Col-0 descendant lines. For this reason, a milder salt stress treatment was applied to all lines with a WS background. Still, after 72 h growth on MS medium supplemented with 125 mM, the germination rate of WS was only 7% and raised to 48% after 96 h (**Figure 7A**). Although the *mterf11-2* mutant was more sensitive to salt stress compared to Col-0, the germination rates of *mterf11-1* seeds were comparable to their corresponding WT (WS; **Figure 7A**).

To ascertain whether the altered activity of *MTERF10* was indeed responsible for the salt-stress phenotypes and whether overexpression of *MTERF11* might lead to enhanced salt-stress tolerance, *35S:MTERF10:MGFP5* and *35S:MTERF11:EGFP* constructs were introduced into Col-0 and WS, respectively, to generate oe-mTERF10 and oe-mTERF11 lines. Although *MTERF10* mRNA levels were only approximately 2.3-fold higher in oe-mTERF10-1 and oe-mTERF10-2 lines than in Col-0 (**Figure 8A**), these lines—with germination rates of approximately 85 and 95%, respectively, after 48 h on MS medium with 125 mM NaCl and 72 h on MS medium with 175 mM NaCl—were nevertheless resistant to the deleterious effect of salt (**Figure 7A**). This confirms that the salt sensitivity of *mterf10-1* and *mterf10-2* mutants is indeed caused by knockdown of the *MTERF10* gene. Moreover, we identified three oe-mTERF11 lines that displayed a high diversity of *MTERF11* transcript overaccumulation which ranged from 12- to 117-fold (**Figure 8B**). Two of these lines were challenged with salt stress, and actually displayed much higher germination rates than WS and therefore, enhanced salt stress tolerance (**Figure 7A**).

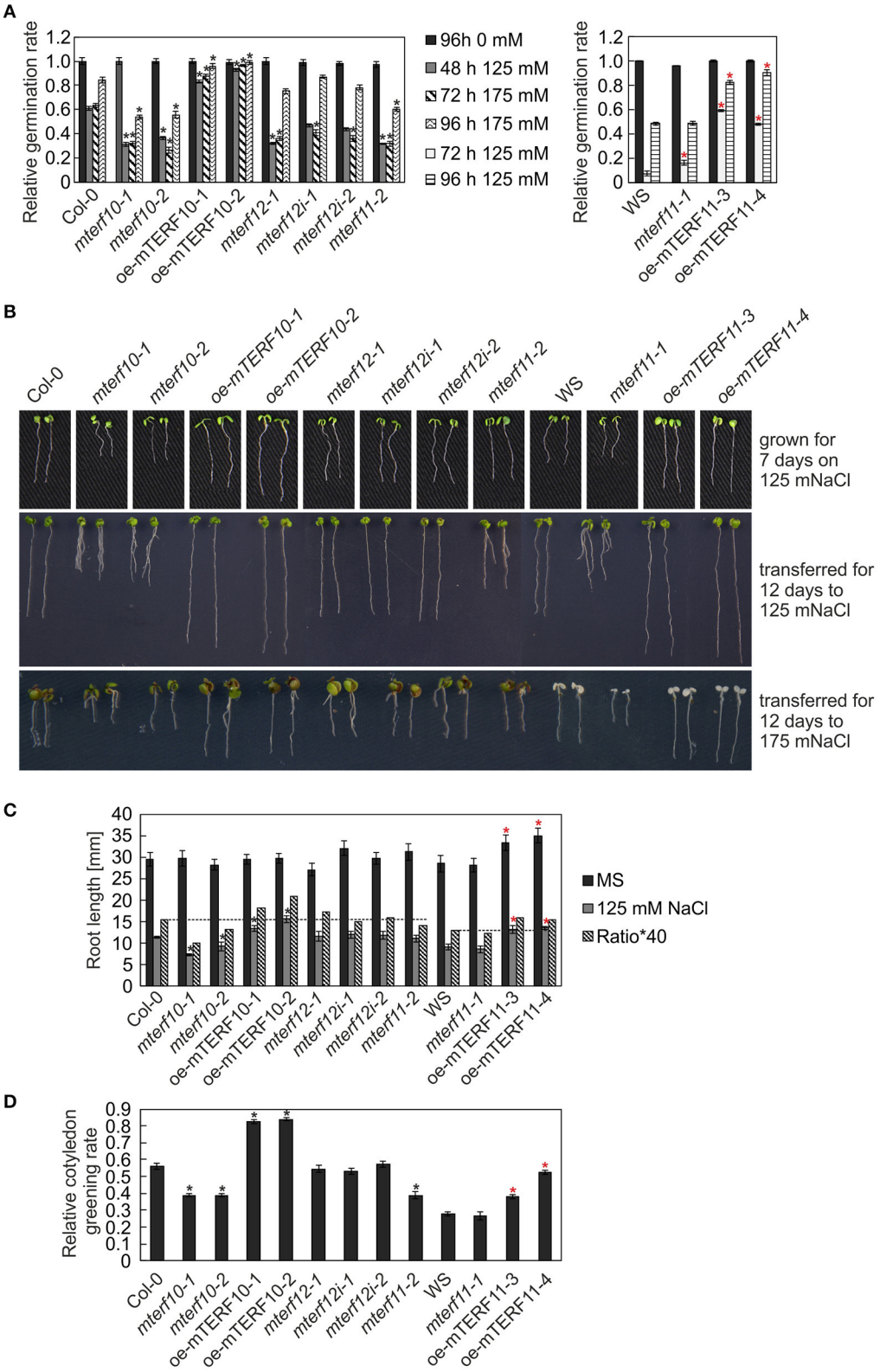

FIGURE 7 | Responses of WT seedlings (WS for *mterf11-1* and oe-mTERF11 lines, and Col-0 for the remaining mutant lines), T-DNA (*mterf10-1* and *-2*, *mterf11-1* and *-2*, and *mterf12-1*), RNAi (*mterf12i-1* and *-2*) and overexpression lines (oe-mTERF10 and oe-mTERF11) to salt stress treatment under long-day conditions. **(A)**

(Continued)

FIGURE 7 | Continued
Seed germination was investigated on MS medium in the absence and presence of 125 and 175 mM NaCl. Radicle emergence was scored after indicated time points. Germination rates were calculated relative to the number of total seeds. **(B)** Phenotypes of 7-day-old WT and mutant seedlings were germinated on either MS medium supplemented with 125 mM NaCl (grown for 7 days on 125 mM NaCl), or on MS medium and transferred directly after radicle emergence for 12 days to MS medium supplemented with 125 mM NaCl (transferred for 12 days to 125 mM Nacl) or 175 mM NaCl (transferred for 12 days to 175 mM NaCl), respectively. The root lengths **(C)** and cotyledon greening rates of seedlings grown on 125 mM NaCl—displayed as the ratio of the number of green cotyledons to the total number of cotyledons **(D)** were determined after 7 and 5 days, respectively. The data in **(A,C,D)** represent mean values ± SD of three independent experiments, each performed with at least 100 seeds per treatment and genotype. Statistically significant differences (*t*-test; $p < 0.05$) between WT (Col-0 or WS) and corresponding mutant lines are indicated by an asterisk (black for Col-0; red for WS).

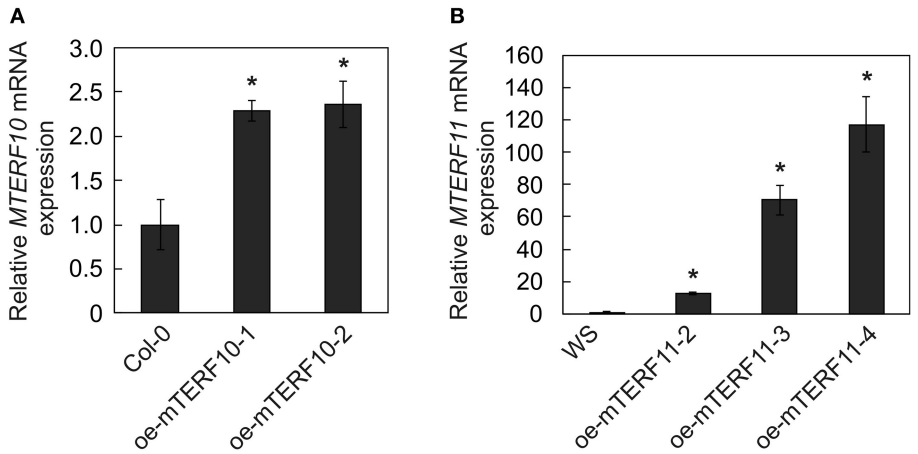

FIGURE 8 | *MTERF10* **(A)** and *MTERF11* **(B)** transcript levels in Col-0 lines overexpressing *MTERF10* (oe-mTERF10) and WS lines overexpressing *MTERF11* (oe-mTERF11), respectively. Transcript levels were determined by real-time PCR analysis. Expression values are reported relative to the corresponding transcript levels in Col-0. The results were normalized with respect to the expression level of *At4g36800* (*RCE1*). Bars indicate standard deviations. Statistically significant differences (*t*-test; $p < 0.05$) between Col-0 and oe-mTERF10 lines and WS and oe-mTERF11 lines, respectively, are indicated by an asterisk.

To investigate this further, the performance of *mterf* mutants and mTERF overexpression lines was investigated during post-germination development. As shown in **Figures 7B,C**, the root lengths of *mterf10-1* and *-2* seedlings challenged with 125 mM NaCl were significantly shorter compared to Col-0, while the root lengths of oe-mTERF10 seedlings were longer (**Figure 7C**). Moreover, compared to WS, overexpression of *MTERF11* results in longer root lengths, reflecting findings of the germination rates (**Figure 7A**). In addition, cotyledon greening—displayed as the ratio of the number of green cotyledons to the total number of cotyledons—of *mterf10-1* and *-2* seedlings was delayed by salt stress, while in contrast, overexpression of *MTERF10* or *MTERF11* enabled seedlings to display higher cotyledon greening rates than their corresponding wild types (**Figure 7D**).

ABA operates as a signal during developmental processes including seed germination, and moreover, in response to abiotic stresses including salt stress (Christmann et al., 2006). Furthermore, *A. thaliana* mutants in which the *ABI4* (*ABSCISIC ACID INSENSITIVE4*) gene has been inactivated are more salt tolerant than WT (Quesada et al., 2000; Shkolnik-Inbar et al., 2013). To investigate whether reduced *MTERF* transcript levels in the *mterf10*, *-11*, and *-12* mutant lines or overexpression of mTERF10 or mTERF11 alter ABA sensitivity, wild-type, *mterf*, oe-mTERF10, oe-mTERF11 and—as a control—*abi4-1* mutant seedlings were grown on MS supplemented with 1 μM ABA, and germination rates were scored after 96 h (lines with a Col-0 background) and 120 h (lines with a WS background). With a 69% germination rate, the control line *abi4-1* germinated better than Col-0 (49%; **Figure 9A**). All *mterf12* lines displayed a slightly, but not significantly, higher germination rate than Col-0, but *mterf10* and *mterf11* lines were as sensitive as Col-0 to ABA stress. Importantly, especially oe-mTERF10 lines were also less susceptible to ABA stress (**Figure 9A**), like they were to salt stress (**Figure 7A**). After 120 h on 1 μM ABA, WS germinated to 32%, and both *mterf11-1* and oe-mTERF11 lines displayed even lower germination rates (**Figure 9A**).

To follow this up, the phenotypes of seedlings grown on 1 μM ABA were examined after 7 days. Col-0 and WS seedlings displayed short roots and cotyledons had only started to emerge (**Figure 9B**). In contrast, *abi4-1* seedlings had longer roots and fully expanded cotyledons. All mutant lines with reduced *MTERF10*, *-11*, or *-12* transcript levels showed the same behavior as the wild types. However, the cotyledon phenotype of the oe-mTERF10 lines was comparable to that of the *abi4-1* mutant (**Figure 9B**). This was also manifested in the higher relative cotyledon greening rate of oe-mTERF10 lines (25 and 30%) compared to Col-0 (**Figure 9C**).

It appears that challenging Arabidopsis seedlings with ABA or salt stress under continuous light reduces germination efficiencies or cotyledon greening of Col-0 to a greater extent

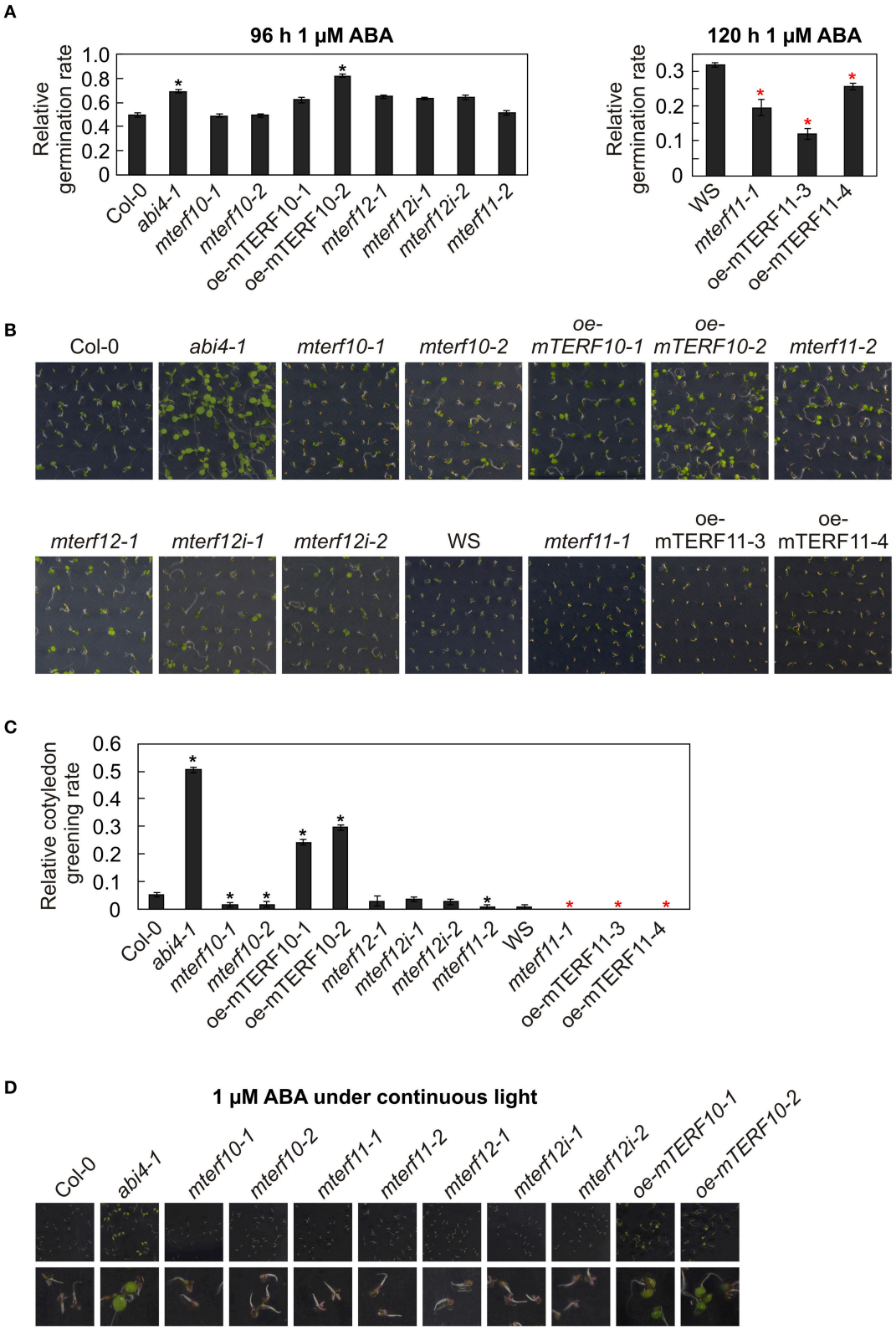

FIGURE 9 | Responses of WT seedlings (WS for *mterf11-1* and oe-mTERF11 lines, and Col-0 for the remaining mutant lines), T-DNA (*mterf10-1* and *-2*, *mterf11-1* and *-2*, and *mterf12-1*), RNAi (*mterf12i-1* and *-2*) and overexpression lines (oe-mTERF10 and oe-mTERF11) and—as control—the *abi4-1* mutant to ABA treatment

(Continued)

FIGURE 9 | Continued
under long-day conditions **(A–C)** and under continuous light **(D)**. **(A)** Seed germination was investigated on MS medium in the absence and presence of 1 μM ABA. Radicle emergence was scored after indicated time points. **(B)** Phenotypes of 7-day-old seedlings grown on MS medium in the presence of 1 μM ABA. **(C)** The ratio of cotyledon greening was determined after 6 days. **(D)** Phenotypes of 7-day-old seedlings grown on MS medium in the presence of 1 μM ABA under continuous light. The pictures of the lower row are magnifications of the pictures above them. The data in **(A,C)** represent mean values ± SD of three independent experiments, each performed with at least 100 seeds per treatment and genotype. Statistically significant differences (*t*-test; $p < 0.05$) between WT (Col-0 or WS) and corresponding mutant lines are indicated by an asterisk (black for Col-0; red for WS).

(Reyes and Chua, 2007; Chen et al., 2008; Hwang et al., 2015) compared to long-day conditions (**Figure 7**; Hu et al., 2013). To tackle the oe-mTERF10 ABA phenotype, we grew Col-0, *mterf* mutant lines and oe-mTERF10 lines in continuous light and a temperature of 20°C on MS medium (control) and MS medium supplemented with 1 μM ABA, and the phenotypes were scored after 7 days (**Figure 9D**). Because lines with a WS background were already very sensitive to ABA in long-day conditions, these lines were omitted. Indeed, Col-0 and *mterf* mutant seedlings displayed even shorter roots compared to long-day conditions and the cotyledons that had started to emerge did not turn green to this time point (**Figure 9D**). In contrast, *abi4-1* seedlings had longer roots and fully expanded green cotyledons. All mutant lines with reduced *MTERF10*, *-11*, or *-12* transcript levels showed the same behavior as Col-0. However, also in continuous light the phenotype of oe-mTERF10 lines was comparable to that of the *abi4-1* mutant, with longer roots and expanded green cotyledons (**Figure 9D**).

In sum, these results indicate that lower or higher *MTERF11* levels result both in increased ABA sensitivity. But, strikingly, higher *MTERF10* levels are associated with decreased sensitivity to ABA, which might in turn be linked to the higher salt tolerance of oe-mTERF10 lines.

DISCUSSION

Arabidopsis thaliana contains 35 mTERF proteins, of which seven have been investigated in more detail (reviewed in: (Kleine and Leister, 2015; Quesada, 2016)). Twenty-six of the 35 mTERFs have been sorted into groups based on their expression profiles and co-expression behavior (Kleine, 2012). The mTERF proteins that have been investigated in more detail are members of the "chloroplast" cluster (mTERF1, -4, -5, -6, and -9; the cluster itself contains 9 members) and the "mitochondrial" cluster (mTERF15 and -18; this cluster contains 7 members). In this study, we added to the inventory of characterized mTERFs and investigated all members of the "chloroplast-associated" cluster (mTERF10, -11, and -12).

In a fluorescence microscopy study of mTERF-GFP fusion proteins, 16 mTERFs were shown to be targeted to mitochondria, 11 to chloroplasts and one to the nucleus/cytosol (Babiychuk et al., 2011). That study revealed localization of mTERF4 to chloroplasts and mTERF6 to mitochondria. Later studies demonstrated that mTERF4 (Quesada et al., 2011) and mTERF6 (Romani et al., 2015) are in fact found in both mitochondria and chloroplasts. The mTERF proteins in the chloroplast-associated group were also assigned to the chloroplasts in the large-scale study cited above (Babiychuk et al., 2011). However, to confirm or extend these data, and also to define the sub-chloroplast localization of mTERF10, -11, and -12, we transiently transformed *A. thaliana* protoplasts with GFP fusion proteins. By co-transformation with a RAP-RFP fusion protein, which is a marker for the chloroplast nucleoid (Kleinknecht et al., 2014), we show that all members of the chloroplast-associated cluster are localized to chloroplast nucleoids (see **Figure 1**). Also the maize homologs of Arabidopsis mTERFs-2, -3, -4, -5, -7, -9, -16, and -27 were identified in enriched maize nucleoids (Majeran et al., 2012) and Arabidopsis mTERF8 was found in preparations of the plastid transcriptionally active chromosome (pTAC; Pfalz et al., 2006) which is related to the nucleoid (Pfalz and Pfannschmidt, 2013). The nucleoid houses proteins that are associated with DNA organization, replication and repair, and furthermore, are involved in transcription, and processing, splicing and editing of transcripts, suggesting that mTERFs participate in PGE (Majeran et al., 2012). In fact, the three plant mTERF proteins whose molecular functions are known do participate in PGE: mTERF4 is involved in chloroplast group II intron splicing (Babiychuk et al., 2011; Hammani and Barkan, 2014), mTERF6 promotes maturation of a chloroplast tRNA (Romani et al., 2015) and in *mterf15* mutants intron splicing of mitochondrial *nad2* transcripts is perturbed (Hsu et al., 2014). Because levels of 16 and 23S rRNAs, and thus chloroplast protein synthesis, are reduced in the *soldat10* mutant (Meskauskiene et al., 2009), it can be assumed that the mTERF1/SOLDAT10 protein is likewise involved in PGE.

Most of the previously characterized *mterf* mutants show phenotypes under normal growth conditions. Inactivation of mTERF1 (Meskauskiene et al., 2009) or mTERF4 (Babiychuk et al., 2011) is embryo lethal, the *mterf6* and *mterf15* knock-out mutants are seedling lethal (Romani et al., 2015) and retarded in growth and development (Hsu et al., 2014), respectively, and *mda1* (*mterf5*) and *mterf9* mutants are small and pale (Robles et al., 2012, 2015). We were unable to discern any phenotypic alterations in *MTERF12* RNAi lines, either under normal or challenging growth conditions. In fact, mTERF12 might not be a bona fide mTERF protein, because an analysis with the SMART tool fails to identify any mTERF domain in mTERF12 (see above). On the other hand, *MTERF12* is expressed at moderate to high levels in many developmental stages and organs (Kleine, 2012), and the mTERF12-eGFP fusion protein is localized to nucleoids (see **Figure 1B**). Therefore, while mTERF12 might not belong to the eponymous family, it may nevertheless be involved in PGE. Moreover, the residual amount of *MTERF12* (32% of WT transcript levels) present in *mterf12i-1* (see **Figure 3A**) may suffice to maintain a WT-like phenotype under all the conditions examined here, or alternatively we may not have hit

upon the conditions required to provoke an abnormal phenotype in *mterf12i* lines. The latter possibility appears to be the more likely. For *MTERF12* mRNA levels are highest in pollen (Wang et al., 2008; Kleine, 2012) and the most pronounced change in *MTERF12* transcript level occurs in response to supplementation with nitrate (see **Supplementary Figure S3**), an intervention to which *mterf12i* lines were not subjected. Moreover, functional redundancy cannot be completely ruled out, although none of the three genes of interest originated from a duplication event (Kleine, 2012) and our phylogenetic tree (see **Figure 4**) and protein sequence comparisons (see above) do not strongly support this idea.

In addition to their pale color and growth-restricted phenotype, the *mda1* (*mterf5*) and *mterf9* mutants are less susceptible to salt and osmotic stresses, perhaps caused by reduced sensitivity to ABA (Robles et al., 2012, 2015). Notably, acclimation outputs are also altered by impairments in several other mTERF proteins (Meskauskiene et al., 2009; Quesada et al., 2011; Kim et al., 2012). Indeed, the emerging role of *A. thaliana* and maize mTERFs in acclimation and stress responses has already been noted and discussed (Zhao et al., 2014; Kleine and Leister, 2015; Quesada, 2016). This notion is especially of importance for crop plants, because plant development and growth is reduced in challenging growth conditions, leading finally to reduced yield. For this reason, several strategies have been tried to produce abiotic stress tolerance crop plants (Sah et al., 2016). With the aim to find a starting point to investigate stress tolerance in cotton, the response of cotton to abiotic stress treatments was studied with a cDNA library derived from samples treated with different stress conditions. Indeed, many transcripts for known stress-related genes, transcription factors and also mTERFs were enriched in this library (Zhou et al., 2016). Moreover, investigation of transcript level changes of six maize *MTERF* genes (maize *MTERF*2, -5, -11, -12, -13, and -28) in response to salt, ABA and NAA treatments showed an upregulation of *MTERF28* transcripts in all tested stress conditions, while *MTERF12* transcript levels were induced nearly 2-fold after salt stress treatment. This suggests that of these tested mTERFs, maize mTERF28 is the strongest candidate participating in all tested stress responses, while mTERF12 might be especially involved in salt stress responses (Zhao et al., 2014). Our results show that in contrast to the strong *mterf* mutant phenotypes which point to essential functions of several mTERFs (Meskauskiene et al., 2009; Babiychuk et al., 2011; Romani et al., 2015), lines with altered *MTERF10* or *MTERF11* levels show only conditional phenotypes, which become manifest under adverse growth conditions (see **Figures 7**, **9**). Strikingly, under continuous light, lower *MTERF10* levels are associated with reductions in salt tolerance, while oe-mTERF10 lines are more tolerant of salt and ABA than wild-type plants. The altered responsiveness to ABA of *oge* and also plastid signaling mutants has been noted before. For example, the "mitochondrial PPR protein PENTATRICOPEPTIDE REPEAT PROTEIN FOR GERMINATION ON NaCl" (PGN; Laluk et al., 2011), the tetrapyrrole biosynthesis proteins GUN4 and GUN5 (Voigt et al., 2010) and the plastid-targeted PPR protein GUN1 (Cottage et al., 2010) alter responses to ABA. Notably, *gun1* mutants show only subtle growth phenotypes, but GUN1 is an important integrator of plastid signals (Koussevitzky et al., 2007). Like mTERF proteins, PPR proteins are typically targeted to chloroplasts or mitochondria, and alter expression of transcripts by influencing editing, turnover, processing or translation (Barkan and Small, 2014). With more than 400 members, the PPR protein family is one of the largest in land plants (Barkan and Small, 2014) and far exceeds the mTERF family in size. The enlargement of the plant PPR family has been linked to the evolution of a complex organellar gene expression system that is characteristic for plant organelles (Barkan and Small, 2014), and this is likely to be true of the mTERF protein family also (Kleine, 2012). Moreover, and in contrast to animals, plants are sessile organisms that are exposed to environmental changes and stresses. During evolution, the expansion and functional diversification of protein families has helped plants to successfully adapt to or tolerate different environmental stresses (Quesada, 2016). The mTERF family is a good case study for this phenomenon. With the characterization of an increasing number of plant mTERF proteins, it is becoming evident that they play a wide range of roles in mediating tolerance and acclimation to different abiotic stresses.

AUTHOR CONTRIBUTORS

Research was designed by TK. Research was performed by DX and TK. The manuscript was prepared by DX, DL, and TK.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01213/full#supplementary-material

Supplementary Figure S1 | Quantification of changes in *MTERF10* mRNA expression in response to perturbations as determined with the Genevestigator Perturbations Tool. The tool was employed on all deposited *A. thaliana* ATH1 arrays together with a 2-fold change filter and a p-value of < 0.05.

Supplementary Figure S2 | Quantification of changes in *MTERF11* mRNA expression in response to perturbations as determined with the Genevestigator Perturbations Tool. The tool was employed on all deposited *A. thaliana* ATH1 arrays together with a 2-fold change filter and a p-value of < 0.05.

Supplementary Figure S3 | Quantification of changes in *MTERF12* mRNA expression in response to perturbations as determined with the Genevestigator Perturbations Tool. The tool was employed on all deposited *A. thaliana* ATH1 arrays together with a 2-fold change filter and a p-value of < 0.05.

Supplementary Figure S4 | Behavior of wild-type (Col-0) and *mterf10*, *-11* and *-12* mutant plants under moderate light stress of 400 μmol photons

m^{-2} s^{-1}. **(A)** To score the phenotypes under moderate heat stress, plants were first grown for 3 weeks under normal growth conditions (100 μmol photons m^{-2} s^{-1}, 22°C) and then exposed to 400 μmol photons m^{-2} s^{-1} for 7 days. **(B)** The maximum quantum yield of PSII (F_v/F_m) of Col-0 and *mterf* mutant plants was determined after the indicated periods of exposure to a fluence of 400 μmol photons m^{-2} s^{-1}. The data are shown as mean values ± SD from 8 to 10 different leaves.

Supplementary Table S1 | Primers used in this study.

REFERENCES

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301, 653–657. doi: 10.1126/science.1086391301/5633/653[pii]

Babiychuk, E., Vandepoele, K., Wissing, J., Garcia-Diaz, M., De Rycke, R., Akbari, H., et al. (2011). Plastid gene expression and plant development require a plastidic protein of the mitochondrial transcription termination factor family. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6674–6679. doi: 10.1073/pnas.1103442108

Barkan, A., and Small, I. (2014). Pentatricopeptide repeat proteins in plants. *Annu. Rev. Plant Biol.* 65, 415–442. doi: 10.1146/annurev-arplant-050213-040159

Börner, T., Aleynikova, A. Y., Zubo, Y. O., and Kusnetsov, V. V. (2015). Chloroplast RNA polymerases: role in chloroplast biogenesis. *Biochim. Biophys. Acta* 1847, 761–769. doi: 10.1016/j.bbabio.2015.02.004

Camara, Y., Asin-Cayuela, J., Park, C. B., Metodiev, M. D., Shi, Y., Ruzzenente, B., et al. (2011). MTERF4 regulates translation by targeting the methyltransferase NSUN4 to the mammalian mitochondrial ribosome. *Cell Metab.* 13, 527–539. doi: 10.1016/j.cmet.2011.04.002

Chen, H., Zhang, J., Neff, M. M., Hong, S. W., Zhang, H., Deng, X. W., et al. (2008). Integration of light and abscisic acid signaling during seed germination and early seedling development. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4495–4500. doi: 10.1073/pnas.0710778105

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., et al. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res.* 31, 3497–3500. doi: 10.1093/nar/gkg500

Christmann, A., Moes, D., Himmelbach, A., Yang, Y., Tang, Y., and Grill, E. (2006). Integration of abscisic acid signalling into plant responses. *Plant Biol. (Stuttg)* 8, 314–325. doi: 10.1055/s-2006-924120

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743. doi: 10.1046/j.1365-313x.1998.00343.x

Cottage, A., Mott, E. K., Kempster, J. A., and Gray, J. C. (2010). The Arabidopsis plastid-signalling mutant gun1 (genomes uncoupled1) shows altered sensitivity to sucrose and abscisic acid and alterations in early seedling development. *J. Exp. Bot.* 61, 3773–3786. doi: 10.1093/jxb/erq186

Dovzhenko, A., Dal Bosco, C., Meurer, J., and Koop, H. U. (2003). Efficient regeneration from cotyledon protoplasts in *Arabidopsis thaliana*. *Protoplasma* 222, 107–111. doi: 10.1007/s00709-003-0011-9

Hammani, K., and Barkan, A. (2014). An mTERF domain protein functions in group II intron splicing in maize chloroplasts. *Nucleic Acids Res.* 42, 5033–5042. doi: 10.1093/nar/gku112

Hammani, K., Bonnard, G., Bouchoucha, A., Gobert, A., Pinker, F., Salinas, T., et al. (2014). Helical repeats modular proteins are major players for organelle gene expression. *Biochimie* 100, 141–150. doi: 10.1016/j.biochi.2013.08.031

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., et al. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics* 2008:420747. doi: 10.1155/2008/420747

Hsu, Y. W., Wang, H. J., Hsieh, M. H., Hsieh, H. L., and Jauh, G. Y. (2014). Arabidopsis mTERF15 is required for mitochondrial nad2 intron 3 splicing and functional complex I activity. *PLoS ONE* 9:e112360. doi: 10.1371/journal.pone.0112360

Hu, Y., Chen, L., Wang, H., Zhang, L., Wang, F., and Yu, D. (2013). Arabidopsis transcription factor WRKY8 functions antagonistically with its interacting partner VQ9 to modulate salinity stress tolerance. *Plant J.* 74, 730–745. doi: 10.1111/tpj.12159

Hwang, J. H., Seo, D. H., Kang, B. G., Kwak, J. M., and Kim, W. T. (2015). Suppression of Arabidopsis AtPUB30 resulted in increased tolerance to salt stress during germination. *Plant Cell Rep.* 34, 277–289. doi: 10.1007/s00299-014-1706-4

Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci.* 7, 193–195. doi: 10.1016/S1360-1385(02)02251-3

Kim, M., Lee, U., Small, I., des Francs-Small, C. C., and Vierling, E. (2012). Mutations in an Arabidopsis mitochondrial transcription termination factor-related protein enhance thermotolerance in the absence of the major molecular chaperone HSP101. *Plant Cell* 24, 3349–3365. doi: 10.1105/tpc.112.101006

Kleine, T. (2012). *Arabidopsis thaliana* mTERF proteins: evolution and functional classification. *Front. Plant Sci.* 3:233. doi: 10.3389/fpls.2012.00233

Kleine, T., and Leister, D. (2015). Emerging functions of mammalian and plant mTERFs. *Biochim. Biophys. Acta* 1847, 786–797. doi: 10.1016/j.bbabio.2014.12.009

Kleinknecht, L., Wang, F., Stube, R., Philippar, K., Nickelsen, J., and Bohne, A. V. (2014). RAP, the sole octotricopeptide repeat protein in Arabidopsis, is required for chloroplast 16S rRNA maturation. *Plant Cell* 26, 777–787. doi: 10.1105/tpc.114.122853

Koussevitzky, S., Nott, A., Mockler, T. C., Hong, F., Sachetto-Martins, G., Surpin, M., et al. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. *Science* 316, 715–719. doi: 10.1126/science. 1140516

Kruse, B., Narasimhan, N., and Attardi, G. (1989). Termination of transcription in human mitochondria: identification and purification of a DNA binding protein factor that promotes termination. *Cell* 58, 391–397. doi: 10.1016/0092-8674(89)90853-2

Laluk, K., Abuqamar, S., and Mengiste, T. (2011). The Arabidopsis mitochondria-localized pentatricopeptide repeat protein PGN functions in defense against necrotrophic fungi and abiotic stress tolerance. *Plant Physiol.* 156, 2053–2068. doi: 10.1104/pp.111.177501

Liere, K., Weihe, A., and Borner, T. (2011). The transcription machineries of plant mitochondria and chloroplasts: composition, function, and regulation. *J. Plant Physiol.* 168, 1345–1360. doi: 10.1016/j.jplph.2011.01.005

Linder, T., Park, C. B., Asin-Cayuela, J., Pellegrini, M., Larsson, N. G., Falkenberg, M., et al. (2005). A family of putative transcription termination factors shared amongst metazoans and plants. *Curr. Genet.* 48, 265–269. doi: 10.1007/s00294-005-0022-5

Majeran, W., Friso, G., Asakura, Y., Qu, X., Huang, M., Ponnala, L., et al. (2012). Nucleoid-enriched proteomes in developing plastids and chloroplasts from maize leaves: a new conceptual framework for nucleoid functions. *Plant Physiol.* 158, 156–189. doi: 10.1104/pp.111.188474

Meskauskiene, R., Wursch, M., Laloi, C., Vidi, P. A., Coll, N. S., Kessler, F., et al. (2009). A mutation in the Arabidopsis mTERF-related plastid protein SOLDAT10 activates retrograde signaling and suppresses $^1O_2$-induced cell death. *Plant J.* 60, 399–410. doi: 10.1111/j.1365-313X.2009.03965.x

Metodiev, M. D., Spahr, H., Loguercio Polosa, P., Meharg, C., Becker, C., Altmueller, J., et al. (2014). NSUN4 is a dual function mitochondrial protein required for both methylation of 12S rRNA and coordination of mitoribosomal assembly. *PLoS Genet.* 10:e1004110. doi: 10.1371/journal.pgen.1004110

Park, C. B., Asin-Cayuela, J., Camara, Y., Shi, Y., Pellegrini, M., Gaspari, M., et al. (2007). MTERF3 is a negative regulator of mammalian mtDNA transcription. *Cell* 130, 273–285. doi: 10.1016/j.cell.2007.05.046

Pellegrini, M., Asin-Cayuela, J., Erdjument-Bromage, H., Tempst, P., Larsson, N. G., and Gustafsson, C. M. (2009). MTERF2 is a nucleoid component in mammalian mitochondria. *Biochim. Biophys. Acta* 1787, 296–302. doi: 10.1016/j.bbabio.2009.01.018

Pfalz, J., Liere, K., Kandlbinder, A., Dietz, K. J., and Oelmuller, R. (2006). pTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. *Plant Cell* 18, 176–197. doi: 10.1105/tpc.105.036392

Pfalz, J., and Pfannschmidt, T. (2013). Essential nucleoid proteins in early chloroplast development. *Trends Plant Sci.* 18, 186–194. doi: 10.1016/j.tplants.2012.11.003

Quesada, V. (2016). The roles of mitochondrial transcription termination factors (MTERFs) in plants. *Physiol. Plant.* 157, 389–399. doi: 10.1111/ppl.12416

Quesada, V., Ponce, M. R., and Micol, J. L. (2000). Genetic analysis of salt-tolerant mutants in *Arabidopsis thaliana*. *Genetics* 154, 421–436.

Quesada, V., Sarmiento-Manus, R., Gonzalez-Bayon, R., Hricova, A., Perez-Marcos, R., Gracia-Martinez, E., et al. (2011). Arabidopsis RUGOSA2 encodes an mTERF family member required for mitochondrion, chloroplast and leaf development. *Plant J.* 68, 738–753. doi: 10.1111/j.1365-313X.2011.04726.x

Raven, J. A., and Allen, J. F. (2003). Genomics and chloroplast evolution: what did cyanobacteria do for plants? *Genome Biol.* 4:209.

Reyes, J. L., and Chua, N. H. (2007). ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. *Plant J.* 49, 592–606. doi: 10.1111/j.1365-313X.2006.02980.x

Robles, P., Micol, J. L., and Quesada, V. (2012). Arabidopsis MDA1, a nuclear-encoded protein, functions in chloroplast development and abiotic stress responses. *PLoS ONE* 7:e42924. doi: 10.1371/journal.pone.0042924

Robles, P., Micol, J. L., and Quesada, V. (2015). Mutations in the plant-conserved MTERF9 alter chloroplast gene expression, development and tolerance to abiotic stress in *Arabidopsis thaliana*. *Physiol. Plant.* 154, 297–313. doi: 10.1111/ppl.12307

Romani, I., Manavski, N., Morosetti, A., Tadini, L., Maier, S., Kuhn, K., et al. (2015). A member of the arabidopsis mitochondrial transcription termination factor family is required for maturation of chloroplast transfer RNAIle(GAU). *Plant Physiol.* 169, 627–646. doi: 10.1104/pp.15.00964

Sah, S. K., Reddy, K. R., and Li, J. (2016). Abscisic acid and abiotic stress tolerance in crop plants. *Front. Plant Sci.* 7:571. doi: 10.3389/fpls.2016.00571

Samson, F., Brunaud, V., Balzergue, S., Dubreucq, B., Lepiniec, L., Pelletier, G., et al. (2002). FLAGdb/FST: a database of mapped flanking insertion sites (FSTs) of *Arabidopsis thaliana* T-DNA transformants. *Nucleic Acids Res.* 30, 94–97. doi: 10.1093/nar/30.1.94

Schmitz-Linneweber, C., and Small, I. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci.* 13, 663–670. doi: 10.1016/j.tplants.2008.10.001

Shi, Y., Posse, V., Zhu, X., Hyvarinen, A. K., Jacobs, H. T., Falkenberg, M., et al. (2016). Mitochondrial transcription termination factor 1 directs polar replication fork pausing. *Nucleic Acids Res.* 44, 5732–5742. doi: 10.1093/nar/gkw302

Shkolnik-Inbar, D., Adler, G., and Bar-Zvi, D. (2013). ABI4 downregulates expression of the sodium transporter HKT1;1 in Arabidopsis roots and affects salt tolerance. *Plant J.* 73, 993–1005. doi: 10.1111/tpj.12091

Stern, D. B., Goldschmidt-Clermont, M., and Hanson, M. R. (2010). Chloroplast RNA metabolism. *Annu. Rev. Plant Biol.* 61, 125–155. doi: 10.1146/annurev-arplant-042809-112242

Terzioglu, M., Ruzzenente, B., Harmel, J., Mourier, A., Jemt, E., Lopez, M. D., et al. (2013). MTERF1 binds mtDNA to prevent transcriptional interference at the light-strand promoter but is dispensable for rRNA gene transcription regulation. *Cell Metab.* 17, 618–626. doi: 10.1016/j.cmet.2013.03.006

Tiller, N., and Bock, R. (2014). The translational apparatus of plastids and its role in plant development. *Mol. Plant* 7, 1105–1120. doi: 10.1093/mp/ssu022

Ulker, B., Peiter, E., Dixon, D. P., Moffat, C., Capper, R., Bouche, N., et al. (2008). Getting the most out of publicly available T-DNA insertion lines. *Plant J.* 56, 665–677. doi: 10.1111/j.1365-313X.2008.03608.x

Voigt, C., Oster, U., Bornke, F., Jahns, P., Dietz, K. J., Leister, D., et al. (2010). In-depth analysis of the distinctive effects of norflurazon implies that tetrapyrrole biosynthesis, organellar gene expression and ABA cooperate in the GUN-type of plastid signalling. *Physiol. Plant.* 138, 503–519. doi: 10.1111/j.1399-3054.2009.01343.x

Wang, Y., Zhang, W. Z., Song, L. F., Zou, J. J., Su, Z., and Wu, W. H. (2008). Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in Arabidopsis. *Plant Physiol.* 148, 1201–1211. doi: 10.1104/pp.108.126375

Wenz, T., Luca, C., Torraco, A., and Moraes, C. T. (2009). mTERF2 regulates oxidative phosphorylation by modulating mtDNA transcription. *Cell Metab.* 9, 499–511. doi: 10.1016/j.cmet.2009.04.010

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., and Provart, N. J. (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLoS ONE* 2:e718. doi: 10.1371/journal.pone.0000718

Wredenberg, A., Lagouge, M., Bratic, A., Metodiev, M. D., Spahr, H., Mourier, A., et al. (2013). MTERF3 regulates mitochondrial ribosome biogenesis in invertebrates and mammals. *PLoS Genet.* 9:e1003178. doi: 10.1371/journal.pgen.1003178

Zhao, Y., Cai, M., Zhang, X., Li, Y., Zhang, J., Zhao, H., et al. (2014). Genome-wide identification, evolution and expression analysis of mTERF gene family in maize. *PLoS ONE* 9:e94126. doi: 10.1371/journal.pone.0094126

Zhou, B., Zhang, L., Ullah, A., Jin, X., Yang, X., and Zhang, X. (2016). Identification of multiple stress responsive genes by sequencing a normalized cDNA library from sea-land cotton (*Gossypium barbadense* L.). *PLoS ONE* 11:e0152927. doi: 10.1371/journal.pone.0152927

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REVIEW
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Import of Soluble Proteins into Chloroplasts and Potential Regulatory Mechanisms

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Chloroplasts originated from an endosymbiotic event in which a free-living cyanobacterium was engulfed by an ancestral eukaryotic host. During evolution the majority of the chloroplast genetic information was transferred to the host cell nucleus. As a consequence, proteins formerly encoded by the chloroplast genome are now translated in the cytosol and must be subsequently imported into the chloroplast. This process involves three steps: (i) cytosolic sorting procedures, (ii) binding to the designated receptor-equipped target organelle and (iii) the consecutive translocation process. During import, proteins have to overcome the two barriers of the chloroplast envelope, namely the outer envelope membrane (OEM) and the inner envelope membrane (IEM). In the majority of cases, this is facilitated by two distinct multiprotein complexes, located in the OEM and IEM, respectively, designated TOC and TIC. Plants are constantly exposed to fluctuating environmental conditions such as temperature and light and must therefore regulate protein composition within the chloroplast to ensure optimal functioning of elementary processes such as photosynthesis. In this review we will discuss the recent models of each individual import stage with regard to short-term strategies that plants might use to potentially acclimate to changes in their environmental conditions and preserve the chloroplast protein homeostasis.

Keywords: chloroplast, protein import, TOC, TIC, plastid proteostasis, acclimation

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INTRODUCTION

Chloroplasts are unique photosynthetic organelles that evolved through an endosymbiotic event ~1.5 billion years ago. A formerly free-living cyanobacterium was engulfed via phagocytosis by an ancestral eukaryotic host that already contained mitochondria (Gould et al., 2008). During evolution, a dramatic reduction in the bacterial endosymbiont genome size occurred, since 95% of the genes encoding the ~3000 proteins acting in the chloroplasts were transferred to the host nucleus that attained control over its new organelle. The plastid genome encodes the residual ~100 genes (Sugiura, 1989; Martin et al., 2002; Timmis et al., 2004). As a consequence, nuclear-encoded chloroplast proteins that were originally encoded on the endosymbiont genome are now translated in the cytosol and are post-translationally translocated into the allocated organelle (Leister, 2003). This translocation process requires a first-sorting event of the so-called preproteins. According

to their chloroplast-specific targeting peptide, which is called chloroplast transit peptide (cTP), the preproteins are targeted to the receptor-equipped destination organelle. Previously, this term has been used to define both the (1) N-terminal peptide which is cleaved off in the stroma upon import and (2) the sequence which is necessary and sufficient for import of a cargo protein into the chloroplast. However, these peptides differ from each other, as the sequence of (1) is determined by the processing site and does not contain parts of the mature protein, whereas (2) could also include domains from the mature protein. In order to avoid confusion, Rolland et al tried to find a suitable nomenclature for this issue. The term cTP refers to the sequence of the preprotein which is required for chloroplast targeting and cleaved off upon import. The cTP is determined by the processing site and is not part of the mature protein. In contrast, the sequence which is necessary and sufficient for import of a cargo protein into the chloroplast is called transit peptide. This transit peptide includes the cTP and possibly part of the mature protein (Comai et al., 1988; Bionda et al., 2010; Rolland et al., 2016). During targeting, preproteins can interact with different cytosolic chaperones that enable the cell to keep the preproteins in an unfolded, and hence import-competent, state. After recruiting the chaperoned complexes to the chloroplast outer envelope membrane, translocation is initiated. In the majority of cases, import across the outer and inner envelope membrane of chloroplasts is facilitated by two distinct translocation complexes, called TOC (translocon on the outer chloroplast membrane) and TIC (translocon on the inner chloroplast membrane), respectively. Once inside the stroma, a stromal processing peptidase (SPP) cleaves off the cTP, and the remaining mature protein undergoes folding and insertion or further direction to intraorganeller targets, again with the guidance of stromal chaperones (Richter and Lamppa, 1998; Richter et al., 2005).

It has been long known that import activity correlates with protein demand during plastid development of a plant life (Dahlin and Cline, 1991). In young and fast dividing tissues, the protein demand is especially high, in comparison to adult and non-dividing cell parts. It has been shown that protein import into plastids is developmentally regulated (Li and Teng, 2013). However, since plants are sessile organisms, even mature tissues of a plant are constantly exposed to fluctuating environmental conditions such as temperature and light, and plants must therefore regulate their protein content within the chloroplast to ensure optimal functioning of processes such as photosynthesis. Specifically, the photosynthesis rate depends on different intensities of light and temperature, hence all subunits of the involved complexes have to be produced, imported and assembled according to the current demand.

Several studies noticed that the actual chloroplast proteome is indeed influenced by short-term applications of varying temperature or light conditions, meaning the plant is effectively acclimating upon external stimuli (Dutta et al., 2009; Grimaud et al., 2013). In contrast to adaptation, during which a trait evolves over a longer period of time by means of natural selection, in our understanding acclimation refers to an environmentally inducible and mostly even reversible event which occurs within the organism's lifetime. Several upstream mechanisms exist, such as changes in the transcription rate of preproteins or involved import receptors upon stress applications. One mentionable example is the upregulation of the TOC GTPase genes upon salt stress in tomato seedlings (Yan et al., 2014). However, these transcriptional mechanisms will not be part of this review; instead, the chloroplast protein import process itself is one advisable target to be highly regulated at different stages, thus leading to a dynamic acclimation of import activity. This acclimation can be achieved by means of post-translational mechanisms such as reversible phosphorylation or oxidation/reduction of both to-be imported and import-related proteins.

Here, we review the individual steps involved in protein translocation into chloroplasts and touch on regulation mechanisms that plants might use to modulate protein import. It is worth mentioning that our understanding of import regulation is still developing. Therefore, we have tried to summarize what is known so far and what the available data from different research groups might mean concerning regulatory mechanisms. These overall speculations might contribute to our current understanding of how plants potentially acclimate to external stimuli by fine-tuning their organellar protein import.

CYTOSOLIC SORTING OF PREPROTEINS AND TARGETING TO THE ORGANELLE – THE ROLE OF REVERSIBLE PHOSPHORYLATION

After completion of translation on cytoplasmic ribosomes, the initial step of protein import is the accurate targeting of these newly synthesized preproteins. To avoid mistargeting, chloroplast-destined preproteins harbor an N-terminal cTP that specifically targets them to the chloroplast outer membrane (Bruce, 2001). Unexpectedly, conserved characteristics specific to chloroplast proteins across plant species are missing and the sequences of cTPs are highly heterogeneous in their length and properties. They merely display an overall positive net charge, resulting from the lack of acidic amino acids (Bruce, 2001). Regarding the fact that mitochondrial proteins have specific and conserved features within their N-terminal targeting sequence across plant species, the lack of such a consensus sequence for chloroplast-targeted proteins is striking, thus rendering the question of how specificity for the chloroplast is achieved and mistargeting between these organelles is avoided. One potential hypothesis for the heterogeneity could be different preferences of the preproteins for plastid types, which is determined by distinct cTP features (Li and Teng, 2013).

To sustain import competency by keeping preproteins in an unfolded structure, cytosolic chaperones are involved. Up to now, the most prominent chaperone thought to facilitate appropriate

recruiting of preproteins is Hsp70. Both cTPs and the mature part of preproteins have been shown to interact directly with this chaperone, and import activity is clearly stimulated in the presence of Hsp70 (Rial et al., 2000).

Apart from Hsp70, another component has been identified in cytosolic preprotein targeting: a 14-3-3 protein preferentially binds to phosphorylated serines or threonines within the cTP, which in association with the chaperone Hsp70 leads to increased import efficiency of preproteins. This assembly has been designated the cytosolic guidance complex (May and Soll, 2000) (**Figure 1**). Phosphorylation is mediated by the recently identified STY kinases 7, 18, and 46; a knockout of two and concurrent knockdown of the third kinase led to severe phenotypes in chloroplast biogenesis during greening (Lamberti et al., 2011). However, it seemed that dephosphorylation plays a more crucial role in the actual import process than phosphorylation. It could be shown that under the applied conditions – removal of the phosphorylation site within the binding motif of the cTP for 14-3-3 proteins – the kinetics, rather than the fidelity, of targeting to chloroplasts was impaired (May and Soll, 2000; Nakrieko et al., 2004). In contrast, phosphorylated precursors, or those containing a glutamic acid residue instead to mimic phosphorylation, are only imported very slowly (Waegemann and Soll, 1996). *In vivo* studies showed that an *Arabidopsis* mutant which mimicked the phosphorylated serine in the cTP of the photosynthetic precursor pHcf136 resulted in reduced import activity, and hence impaired photosystem II assembly, most prominent in cotyledons (Nickel et al., 2015). This is probably due to the impossibility of dephosphorylation occurring within the cTP and clearly demonstrates that import and assembly of photosynthetic proteins is highly dependent on a proper phosphorylation/dephosphorylation cycle prior to translocation. Once this process cannot be completed, the chloroplast protein homeostasis is misbalanced.

Like Hsp70, the chaperone Hsp90 is able to bind to both the cTP and mature region of a different subset of preproteins. Its presence alone stimulates protein import into isolated chloroplasts (Qbadou et al., 2006; Fellerer et al., 2011). In contrast to the Hsp70/14-3-3 guidance complex, Hsp90-bound preprotein favors a distinct docking station at the OEM, which will be defined below.

As neither the guidance complex nor the phosphorylation event is essential for successful import, it is highly tempting to speculate that under specific conditions phosphorylation has a regulatory function rather than an essential role in protein import. As phosphorylation is generally a fast response, one can assume that different external stimuli trigger the phosphorylation to regulate protein import. Independent from protein import, this has been shown not only for light-dependent phosphorylation in photosynthetic reactions, but also as a general response to different stress stimuli (Grieco et al., 2016).

It would be interesting to know if the *in vivo* phosphorylation/dephosphorylation circuit of preproteins is enhanced or reduced under stress conditions such as high light treatment, and to define the influence of this regulation mechanism on protein import. Furthermore, whether this effect would be due to either an enhanced activation of the mentioned STY kinases or due to the inhibition of the yet unknown phosphatase remains an interesting question to address.

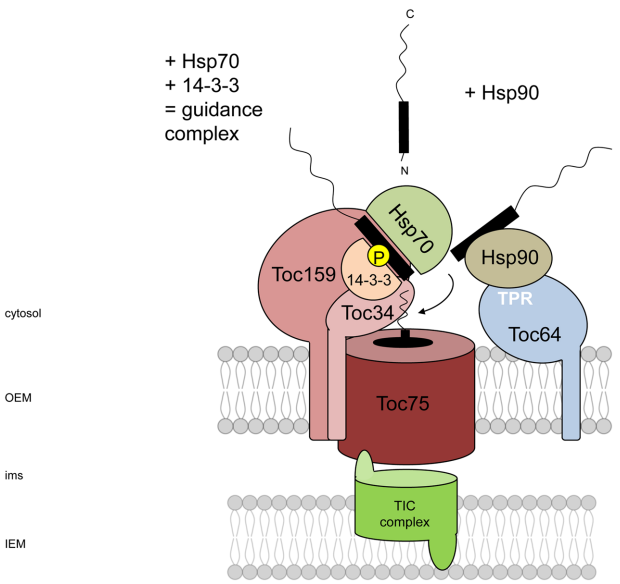

FIGURE 1 | Chaperone involvement in cytosolic targeting and recognition of preproteins at the outer envelope membrane of chloroplasts. Preproteins could be chaperoned by the guidance complex or by Hsp90 alone. The guidance complex is represented by Hsp70 that binds to both mature region and cTP of the preprotein and 14-3-3 proteins which bind to the phosphorylated cTP. Hsp70-chaperoned preproteins are recognized by the GTP-dependent receptor proteins Toc159 and Toc34, followed by delivery to the import channel Toc75, whereas precursor proteins bound to Hsp90 are docked to the third receptor Toc64 via its TPR domain and are then handed over to Toc34.

CROSSING THE OUTER ENVELOPE MEMBRANE VIA THE TOC COMPLEX

After synthesis and sorting in the cytosol, the preproteins are recognized at the OEM. This is mainly mediated by the two GTP-dependent receptor proteins Toc34 and Toc159 (Kessler and Schnell, 2009). Both proteins are anchored C-terminally in the OEM and expose their GTP-binding domains toward the cytosol, in consistency with their role as preprotein receptors. Together with a third protein, Toc75, which is deeply embedded in the lipid bilayer and forms the protein conducting channel (Hinnah et al., 1997), they build up a stable complex, resulting in a heterotrimeric TOC core complex (**Figure 1**). Determination of the apparent mass of 500 kDa of the pea multiprotein complex leads to a stoichiometry of 1:4:4 of Toc159/Toc34/Toc75 (Schleiff et al., 2003). Both receptors belong to a plant-specific family of eukaryotic-originated GTPases, sharing some general features. Toc159 is a tripartite protein consisting of three functional domains: an intrinsically disordered acidic domain (A-domain), the GTPase domain (G-domain) and the membrane anchor domain (M-Domain with a mass of ∼54 kDa) (Bölter et al.,

1998a; Chen et al., 2000; Richardson et al., 2009). Toc34 contains a cytosolic GTPase domain and is anchored into the OEM by a single transmembrane domain. Both proteins Toc34 and Toc159 bind to distinct regions of the N-terminal cTP, hence they could act simultaneously in receiving preproteins (Becker et al., 2004).

The GTPase activity plays a central role in preprotein recognition and delivery, as non-hydrolyzable GTP analogs inhibit preprotein binding and translocation (Young et al., 1999). Interestingly each individual GTPase domain is dispensable for the plant (Agne et al., 2009; Aronsson et al., 2010), however, a viable plant lacking both domains from both receptors could not yet be isolated. The minimal structure required for sufficient assembly of the TOC complex and to support protein import is the M-domain of Toc159, which can partially complement the loss of Toc159 in *ppi2* mutant plants (Lee et al., 2003).

Toc34 is believed to exist as a homodimer in its GDP-bound state, which exhibits a preprotein-binding site in its GTPase domain (Sun et al., 2002). Upon preprotein delivery, GTPase activity is stimulated and exchanges GDP to GTP. Toc34 in its GTP-bound state binds preproteins with high affinity, which triggers not only the disruption of the Toc34-dimer but also promotes heterodimerization of Toc34 and Toc159. This GTP-heterodimer-complex is now referred to as the active TOC complex (Becker et al., 2004). GTP hydrolysis results in reduced affinity toward the preprotein, the subsequent transfer of the preprotein into the Toc75 channel and the initiation of membrane translocation (Oreb et al., 2007). Taken together, the hypothesized model clearly demonstrates that the receptors are working as GTP/GDP-regulated switches to control preprotein binding and delivery. However, there are still missing factors, such as the GTPase-activating protein or GTP-exchange factor, although it could be shown that peptides from cTPs can stimulate GTPase activity (Jelic et al., 2003).

In *Arabidopsis*, different homologs of the TOC receptors exist, which enhances complexity and specificity toward binding proteins. The Toc159 family consist of four genes, each of them differentially participating in chloroplast biogenesis. These are atToc159, atToc132, atToc120 and atToc90, which show high similarity in their G and M domains, but a high variation in sequence and length of the dynamic A-domain (Bauer et al., 2000; Kubis et al., 2004).

The most abundant isoform is atToc159, consequently the knockout *toc159* (*ppi2*) mutant shows an albino phenotype and is seedling lethal, but can grow hetero-autotrophically (Bauer et al., 2000; Bischof et al., 2011). The latter, and the fact that atToc159 exhibits high expression levels in juvenile developmental stages, led to the suggestion that atToc159 constitutes the primary receptor for photosynthetic precursor proteins, which will be discussed below (Bauer et al., 2000). AtToc90 can complement the albino phenotype of *ppi2* and restores photoautotrophic growth, indicating that atToc90 has a similar function to atToc159 (Infanger et al., 2011). Based on expression pattern and the ability to rescue the *toc159* mutant phenotype, the different TOC receptors are classified in two groups: the above-mentioned group of atToc159 and atToc90, and a second group consisting of atToc132 and atToc120. AtToc132 and atToc120 are expressed at similar levels throughout all tissues and are functionally exchangeable (Ivanova et al., 2004; Kubis et al., 2004). However, atToc120 cannot rescue the phenotype of *toc159*, clearly emphasizing a distinct specificity toward preproteins.

The *Arabidopsis* Toc34 family comprises atToc34 and atToc33, which likewise display differential developmental expression profiles. AtToc33 is highly expressed in juvenile, photosynthetic-active tissues, whereas atToc34 is expressed at low levels throughout all developmental stages and all organs. In line with this expression profile, the *toc33 (ppi1)* mutant showed a pale phenotype during early development, but reached near-WT appearance after 2 weeks of growth. AtToc33 and atToc34 functionally overlap. Different observations led to this conclusion. First, both proteins show 65% sequence similarity; secondly, a small fraction of atToc33 co-immunoprecipitated with atToc120/atToc132, which was originally shown only for atToc34; thirdly, the double knockout of atToc33 and atToc34 is embryo lethal; and last and most critically, atToc34 can complement the *ppi1* phenotype (Jarvis et al., 1998; Ivanova et al., 2004; Kubis et al., 2004).

Different studies led to the overall assumption that various isoforms of the GTPases associate with distinct TOC complexes and may prefer a particular set of precursors. It was suggested that atToc159/atToc90 bind to atToc33, whereas atToc120 and/or atToc132 form a complex together with atToc34 (Ivanova et al., 2004). Originally the idea was favored that the various TOC complexes represent distinct pathways for incoming preproteins. It was stated that the complex consisting of the most abundant isoforms atToc159 and atToc33 preferentially imports highly demanded photosynthetic preproteins, whereas the other TOC isoforms form a translocation complex with specificity toward housekeeping proteins (Ivanova et al., 2004; Kubis et al., 2004; Smith et al., 2004). However, this over-simplified model has been rejected due to a large-scale proteomic and transcriptomic approach by Bischof et al. (2011), in which they identified an import defect for different functional subsets of preproteins in *ppi2* protoplasts. Similar to this observation, equal numbers of photosynthetic and non-photosynthetic preproteins were identified to interact with both atToc159 and atToc132 (Dutta et al., 2014). Nevertheless, the distinct preferential import pathways could be a subtle hint for selectivity of target preproteins, possibly in different developmental stages or under diverse external environmental conditions. As the Toc34 isoforms are functionally interchangeable, preprotein selectivity could be mediated by the Toc159 family. Recent hints are pointing toward a specificity-conferring role of the variable A-domains of the Toc159 receptor family (Inoue et al., 2010).

A third component was identified to assist in receiving preproteins, named Toc64. Its potential role in protein import has been concluded from its ability to bind a precursor protein and the transient association with the other TOC components (Sohrt and Soll, 2000). In contrast to the above-mentioned receptor proteins, Toc64 serves as an initial docking station for Hsp90-bound preproteins und subsequently delivers these

preproteins to Toc34 (Qbadou et al., 2006). Toc64 harbors three cytosolic tetratricopeptide repeat (TPR) domains, mediating the interaction with Hsp90 (**Figure 1**). This is a typical feature of proteins interacting with Hsp70/90-associated proteins (Young et al., 2003). The same holds true for a plant ER receptor TPR7 (Schweiger et al., 2012) and interestingly, a Toc64 homolog, namely OM64, was found in plant mitochondria, replacing the mitochondrial TOM70 present in mammals and fungi but absent in plants. Instead, the protein OM64 with a C-terminal TPR domain serves as a receptor for mitochondrial-destined proteins (Chew et al., 2004). Although *in vitro* a strong interaction between Hsp90 and Toc64 could be measured with a K_D of 2.4–15.5 μm (Schweiger et al., 2012) the essentiality of these TPR proteins *in vivo* is still under debate. Since chloroplasts lacking Toc64 sustain their import capacity, it is feasible that this docking protein rather constitutes more an additional regulatory component to the general TOC receptor complex than being an essential constituent. However, it could be shown that atToc33 and Toc64 cooperate in preprotein import, hence it is reasonable to say that atToc33 can overcome the loss of Toc64 function as preproteins are still recognized (Sommer et al., 2013), while only chaperone binding is lost.

After the preprotein has been delivered to the receptor proteins, it has to be translocated through the membrane. The preprotein-conducting channel in the OEM is represented by the beta barrel protein Toc75 (Schnell et al., 1994). The essential nature of Toc75 is demonstrated by its gene being a single copy conserved throughout all plant lineages and the embryo lethality of knockout lines (Jackson-Constan and Keegstra, 2001). The protein belongs to the Omp85 superfamily, which is exclusively found in gram-negative bacteria, mitochondria and plastids (Bölter et al., 1998b). Typically for this family, the structure of Toc75 exhibits two features: 16-18 arranged beta strands forming the C-terminal beta domain, and several POTRA domains at its N-terminus (Clantin et al., 2007). Irrespective of the fact that POTRA domains are required for Toc75 function (Paila et al., 2016), the orientation and thus exact molecular function of these POTRA domains remain a matter of debate. On the one hand, it is assumed that these domains are facing the cytosolic side of the OEM, assisting in preprotein interaction (Sommer et al., 2011). However, a recent study proposed a localization of the POTRA domains in the intermembrane space (Chen et al., 2016).

In vitro analyses showed preprotein binding during import and the import process itself being inhibited with Toc75 antibodies (Tranel et al., 1995). Electrophysiological analyses revealed that reconstituted Toc75 in lipid bilayers forms a voltage-gated channel with a pore size of 14Å at its narrowest part (Hinnah et al., 2002). In contrast to the other TOC components, Toc75 harbors an N-terminal bipartite transit peptide. One part directs the protein into the stroma where the SPP cleaves off this portion once the extreme N-terminus reaches the stroma, whereas the second cleavage site is processed by a plastidic type I signal peptidase (Plsp1), which is localized to the IEM (Inoue et al., 2005).

CROSSING THE INTERMEMBRANE SPACE AND INNER ENVELOPE MEMBRANE VIA THE TIC COMPLEX

Successful import requires not only the interaction between preproteins and outer membrane receptors, but also the formation of super complexes between the translocons of both OEM and IEM via contact sites that enable the preprotein to pass through both membranes simultaneously (Schnell and Blobel, 1993). Both complexes are facing the intermembrane space, thus some proteins localized in this compartment have to be involved in the import process. However, only limited knowledge about import-related factors of the intermembrane space is available. Presently, the only member identified in this compartment to be involved in protein translocation is the soluble protein Tic22. Tic22 has been shown to interact with preproteins during protein import (Kouranov et al., 1998). Structural and functional studies led to the hypothesis that Tic22 is working as a molecular chaperone, as *Arabidopsis* mutants lacking Tic22 showed growth and biogenesis defect and a decreased import activity (Kasmati et al., 2013; Rudolf et al., 2013). One potential role for Tic22 would be, like the cytosolic counterparts, to ensure proper targeting and prevent misfolding during the transfer between TOC and TIC. However, this role has not been confirmed yet.

The TIC counterpart of the TOC core channel Toc75 is Tic110. Tic110 was the first TIC component described Schnell et al. (1994) and is the second most abundant protein in the IEM (Lübeck et al., 1996). It was found in a supercomplex associated with TOC components and incoming preproteins, suggesting a functional role as the central part of the IEM translocon (Lübeck et al., 1996).

Reconstitution of a Tic110-protein lacking its two N-terminal hydrophobic transmembrane stretches (pea sequence: aa91-966, ΔN-110) resulted in a cation-selective channel with a diameter of 1.7 nm, which is similar to the diameter of the channel of Toc75 and hence sufficient for preprotein threading (Heins et al., 2002; Balsera et al., 2009) (**Figure 2**). However, two controversial models concerning the topology and function of Tic110 still persist. Undoubtedly and universally accepted is the fact that the 110-kDa protein is anchored into the membrane by its two N-terminal, highly hydrophobic helices (Inaba et al., 2003; Balsera et al., 2009). In our current topological model, we can combine the essential functions of Tic110, which has been under discussion for a long time. On the one hand, Tic110 assembles into its channel-like structure via its four amphipathic helices, substantiating its function as the main translocation pore. The four membrane-spanning helices consequently lead to the formation of two loops that are extended into the intermembrane space, which could be confirmed by limited proteolysis experiments (Lübeck et al., 1996; Balsera et al., 2009). On the other hand, a large part of the C-terminus is protruding into the stroma and thus could fulfill the additional function of Tic110 acting as a scaffold for chaperones and co-chaperones (Inaba et al., 2005). The crystal structure of a *Cyanidioschyzon merolae* Tic110 version, which consists of the C-terminus including only the last amphipathic helix, is proposed

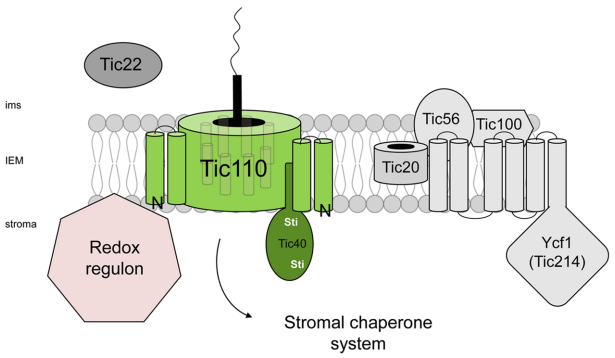

FIGURE 2 | Crossing the inner envelope membrane of chloroplasts via the TIC complex. The counterpart of the outer channel protein is the IEM protein Tic110 which is a functional dimer. Two hydrophobic domains anchor the protein into the IEM whereas further eight amphipathic helices are involved in the channel formation. Tic40 is supposed to interact with Tic110 with its Sti1 domain and acts further as a scaffold for stromal chaperones. Controversial, the 1MDa-complex depicted on the right side comprises atTic20 as the channel protein, atTic56 embedded in the complex, atTic100 located at the IMS and the plastid encoded Ycf1 (atTic214) with its six transmembrane domains and a large stromal C-terminus.

to be too flattened and elongated to form a channel protein (Tsai et al., 2013). However, as it is unlikely that such a shortened protein can fold into its native conformational structure, it is still reasonable to assume that the full-length Tic110 protein is able to build the channel protein via its amphipathic, membrane-spanning helices.

Like Toc75, Tic110 is encoded by a single gene and constitutively expressed in all tissues. Homozygous T-DNA insertion lines are embryolethal, and heterozygous plants already exhibit a clear growth and greening defect, clearly emphasizing the necessity of Tic110 in chloroplast biogenesis and overall plant viability (Kovacheva et al., 2005). Import of Tic110 is achieved by targeting the protein into the stroma and after cleavage of the cTP, Tic110 is re-inserted into the lipid bilayer of the IEM (Vojta et al., 2007).

Using a cross-linking strategy, another TIC component could be directly associated to Tic110, named Tic40. Tic40 consists of a single transmembrane helix which anchors the protein at the IEM, resulting in a large stroma-facing, soluble domain. This C-terminal part harbors two Hip/Hop/Sti domains, building binding sites for Tic110 and the stromal Hsp70/93 chaperones. The main function of Tic40 is to co-chaperone the translocation process of incoming preproteins by coordinating Hsp93 activity (Chou et al., 2006) (**Figure 2**).

A further TIC component, named Tic20, was identified by its ability to covalently cross-link with a precursor protein en route to the chloroplast (Kouranov and Schnell, 1997; Kouranov et al., 1998). Structural prediction indicated three or four hydrophobic transmembrane domains (Kouranov et al., 1998). Tic20 is essential in *Arabidopsis*. Chloroplasts isolated from Tic20 antisense lines are impaired in preprotein import (Chen et al., 2002). In addition, early phylogenetic analysis indicated a relation of Tic20 with bacterial amino acid transporter and cyanobacterial proteins of unknown function suggesting a role as a translocation channel (Reumann and Keegstra, 1999). However, a latter study including many more genomes was unable to reproduce these claims (Gross and Bhattacharya, 2009). Nonetheless, the important role of Tic20 in chloroplast biogenesis is evident and it was proposed early on by Reumann et al. (2005) that Tic20 and Tic110 form independent preprotein translocation channels. Besides this circumstantial evidence for the notion, direct support comes from electrophysiological studies using either heterologously expressed and purified Tic20 (Kovács-Bogdán et al., 2011) or a 1MDa-complex from *Arabidopsis*, of which Tic20 is one constituent (Kikuchi et al., 2009, see below), which both showed the channel-forming capacity of the applied material. Using a cleavable proteinA-tagged variant of Tic20 expressed in transgenic *Arabidopsis* plants, the authors were able to purify the 1MDa complex via affinity purification. The obtained complex contained three other proteins in addition to Tic20: atTic56, atTic100 and atTic214 (Ycf1) (Kikuchi et al., 2013) (**Figure 2**).

Interestingly, Ycf1 is one of the last enigmatic open-reading frames of the chloroplast genome without an assigned function (Drescher et al., 2000). It is predicted to contain at least six transmembrane helices at its N-terminus (de Vries et al., 2015). AtTic56 and atTic100 are nuclear-encoded proteins, the first deeply embedded in the holo-complex without any predicted transmembrane domain, whereas the latter is supposed to associate with the complex on the intermembrane space site (Kikuchi et al., 2013). However, major questions came up concerning the exact physiological roles of the involved proteins. So far, for the potential involvement of Tic100, no data are available. However, for atTic56, a proteomic analysis showed that most of the chloroplast proteins are still imported into the organelle in *atTic56* mutant plants, pointing toward a still functioning import machinery (Köhler et al., 2015). Furthermore, an alternative role independent from protein import for atTic56 was suggested, since Köhler et al. established a link between processing of plastid rRNA and the assembly of plastid ribosomes. They stated that a defect in plastid ribosome construction is responsible for the albino phenotype of *atTic56-1* mutant plants, thus leading to a potential role of atTic56 in ribosome assembly and establishment of a functional plastid translation machinery (Köhler et al., 2016). Even more importantly, since Ycf1 is missing not only in all grasses but also in a variety of dicotyledonous plants, one can speculate about its overall significance in protein import. The critical question is: how do plants that are completely lacking this gene manage to retain their functional import machinery (de Vries et al., 2015)? Since Ycf1 is an essential protein in *Arabidopsis*, it is difficult to study protein import in knockout plants. Nonetheless, ecotypes of *Arabidopsis* can be grown on media containing spectinomycin, which is a specific inhibitor of plastid translation (Wirmer and Westhof, 2006). Under these conditions it could be shown that Ycf1 is truly absent in *Arabidopsis* plants, thus enabling to study its role in protein import (Bölter and Soll, 2016; Köhler et al., 2016). Presumably, the seed contains sufficient Ycf1 protein for the plants to germinate, and spectinomycin-induced signaling leads to compensatory mechanisms that ensure

survival on the antibiotic. Interestingly, as these two studies show, precursor proteins that depend on the general protein import machinery are still efficiently imported into the plastids, thus excluding the role of Yfc1 as a constituent of the main protein channel. Furthermore, the nuclear-encoded Tic20 is also not detectable under spectinomycin treatment, implying a feedback mechanism between plastid and nucleus concerning the assembly of the 1MDa complex (Bölter and Soll, 2016). Instead of being a main translocation factor, Ycf1 could be involved in the assembly of a plastid fatty acid synthase (ACCase). Under spectinomycin, plants are also lacking the plastid-encoded subunit AccD but are able to complement for that loss by upregulating the expression and import of a nuclear-encoded and plastid-targeted protein (Acc2). This upregulation only appears if Ycf1 is strongly diminished, suggesting a functional role of Ycf1 in assembling the ACCase holoenzyme (Bölter and Soll, 2016). Recently, Ycf1 was shown to be a target of a nuclear-encoded translational activator named PBR1, which is important for thylakoid biogenesis, suggesting it could play a role in this process (Yang et al., 2016). Although a potential role of Ycf1 in protein import cannot entirely be excluded, more research is needed to clarify its functional role(s).

Besides the discrepancies concerning the main translocation machinery, additional TIC components have been identified which are called the redox regulon. This regulon includes the proteins Tic55, Tic62, and Tic32 (Stengel et al., 2009). Tic55 is a Rieske protein, while both Tic62 and Tic32 are dehydrogenases. All proteins have been found in complexes containing Tic110; specifically, Tic32 shows a direct interaction with the N-terminus of Tic110 (Hörmann et al., 2004; Stengel et al., 2009). The role of these redox regulon members will be discussed in detail below.

COMPLETION OF THE TRANSLOCATION PROCESS: THE STROMAL CHAPERONE SYSTEM

Upon reaching the stroma, the preprotein translocation proceeds by removing the cTP and subsequently folding into an active structure. Four distinct destinations for the imported proteins are possible: stroma, IEM, thylakoids and thylakoid lumen. The mature protein is either re-inserted into the IEM or, due to a bipartite transit peptide, directed to the thylakoids using different sorting mechanisms for further processing and assembly (Schünemann, 2007). The removal of the cTP is carried out by a soluble SPP which is essential for plants (Richter and Lamppa, 1998; Trösch and Jarvis, 2011). Import is an energy-consuming process resulting from nucleotide-hydrolysis. Although the TOC members are able to hydrolyze GTP, this provides only the minimal energy required for the irreversible initiation of protein import and is not the driving force for sufficient and complete import, so the energy must originate from a different source. It has been shown that the energy is provided in the form of ATP, which is hydrolyzed by stromal chaperones, leading to a sufficient motor activity for preprotein crossing of the OEM and IEM of the chloroplast (Pain and Blobel, 1987). Various chaperones have been determined as being involved in the folding of proteins and/or consuming the required energy via ATP hydrolysis, mainly the chloroplast Hsp70, Hsp90, Hsp93 and Cpn60 (Kessler and Blobel, 1996; Akita et al., 1997; Nielsen et al., 1997; Inoue et al., 2013). However, Cpn60, the homolog of bacterial GroEL, is most likely exclusively involved in protein folding and assembly of the newly imported mature proteins, especially Rubisco (Goloubinoff et al., 1989).

Hsp93 (bacterial ClpC) is a member of the Hsp100 family, which itself belongs to the broader AAA+ family (ATPases associated with various cellular activities) (Moore and Keegstra, 1993). Hsp100 proteins contain one or two AAA+ domains, and are typically arranged into a hexameric structure with a central pore which is sufficient for protein threading (Rosano et al., 2011). *Arabidopsis* features two genes encoding for the isoforms Hsp93-V and Hsp93-III. Beside the putative function of providing energy coming from ATP hydrolysis, Hsp93 has been shown to be a regulatory chaperone for the Clp protease system, thus functioning in quality control and potential degradation of the incoming preproteins (Kovacheva et al., 2005).

Originally, three chloroplast Hsp70 isoforms in pea were reported. Two of them are located in the stroma whereas one is supposed to reside in the intermembrane space (Ratnayake et al., 2008). However, in *Arabidopsis* the gene coding for the latter has not yet been identified, leaving doubts about the existence or identity of such an intermembrane-space chaperone. *Arabidopsis* double null mutants of the stromal Hsp70 isoforms are embryo lethal and single mutants already exhibit biogenesis and import defects (Su and Li, 2010).

CpHsp90 was identified in complexes containing import intermediates at late import stages that also contain Tic110 and Hsp93 (Inoue et al., 2013). A specific and reversible Hsp90 ATPase inhibitor arrests protein import in chloroplasts, whereas initial binding to the TOC complex is not impaired, clearly emphasizing a role of cpHsp90 in late import stages (Nakamoto et al., 2014).

Due to the complexity of the chaperone system in chloroplasts, there is an ongoing discussion about the specificity and import-related function of each individual chaperone, resulting in different models. It is still not completely clear which protein is the potential candidate to constitute the main motor protein for providing the import energy. In mitochondria and ER, the responsible driving force is believed to come from ATP hydrolysis performed by Hsp70 chaperones which are located in the matrix and lumen, respectively (Park and Rapoport, 2012; Dudek et al., 2013). Thus, it was long thought that cpHsp70s are likewise the main motor in chloroplasts. In that context, it seems logic that the responsible ATPase interacts directly with the incoming preproteins, or at least associates with the TIC translocon and for a long time, this scenario could not be shown for stromal Hsp70, hence it seemed unlikely that Hsp70 alone provides the required power. However, it could be shown in 2010 for the moss *P. patens* that Hsp70 is indeed involved in protein import into chloroplasts as a stromal Hsp70 co-immunoprecipitated with early-import intermediates, as well as with Tic40 and Hsp93 (Shi and Theg, 2010). In agreement with this, *Arabidopsis* mutants lacking the chloroplast isoforms of Hsp70 showed a reduced import level of preproteins, which could also be demonstrated in

the moss *Physcomitrella patens* (Su and Li, 2010; Shi and Theg, 2010). Furthermore, it was suggested that the ATP requirements correlate with the activity of moss Hsp70, emphasizing the idea that cpHsp70 is the only energy-providing motor, at least in moss (Shi and Theg, 2010). Interestingly, *Arabidopsis* double mutants of Hsp93 and Hsp70 showed an additive effect in decreased import capacity compared to the single knockout mutants, leading to the theory that both proteins are acting at least partially in parallel as independent import players (Su and Li, 2010). This idea was somewhat supported later on: it was hypothesized that Hsp70 is the motor protein whereas Hsp93 is stably associated with the Clp protease complex at the IEM, suggesting a permanent role in quality control and degradation of preproteins and not a role in powering protein translocation (**Figure 3A**). In this study, the authors used a transgenic line in which the interaction of Hsp93 with the protease ClpP was disrupted, but the protein itself was still localized to the IEM and interaction with Tic110 was also ensured (Flores-Pérez et al., 2016). This enabled the study of the role of Hsp93 in protein import independent from its role in proteolysis. However, the truncated version could not complement the *hsp93* import defective phenotype, thus excluding the possibility of Hsp93 being the main motor functioning in protein import (Flores-Pérez et al., 2016).

In remarkable contrast to the above-mentioned observations, a recent study on that topic could show that Hsp93 directly binds to both the N-terminal cTP and the mature part of incoming preproteins, thus clearly indicating a role in early import stages and challenging the above-mentioned theory (Huang et al., 2015). These authors favor the hypothesis that both chaperones could prefer different regions of the preprotein and thus provide different modes of translocation force, which would result in additive import defects in the double mutants. This would also hold true if Hsp93 was to be the primary motor for the cTP and Hsp70 for the mature region (**Figure 3B**). Preprotein processing takes place during binding to Hsp93 and thus, binding to the mature protein is also detected. In their model, Hsp70 is entirely responsible for interacting with the mature protein, acting in parallel and one defined step after the action of Hsp93 (**Figure 3B**).

Taken together, and taking the described discrepancies into account, it remains unclear why the chloroplast evolved such a complex and divergent chaperone system in comparison to other subcellular compartments such as mitochondria or the ER. However, as the cytosolic chaperones display distinct preprotein affinities, it is still reasonable to say that the stromal counterparts do the same, while keeping the opportunity to react efficiently toward different import conditions resulting from potential environmental stimuli.

POTENTIAL INVOLVEMENT OF IMPORT REGULATION IN PLANT ACCLIMATION

Translocation efficiency of chloroplast proteins is highly dependent on post-translational modifications, enabling the plant to react quickly and efficiently toward external stimuli. The above-mentioned import steps can be influenced by various regulation mechanisms, including redox-mediated circuits of both cytosolic and stromal pathways, and phosphorylation-dependent activities.

Redox-Sensing at the Outer Envelope Membrane

Redox-mediated communication and regulation within cellular processes had already been present in the prokaryotic ancestor, thus leading to a range of reduction- and oxidation-driven regulation in the organelle. One of the best-studied mechanisms in the bacterial ancestor is the bacterial disulfide bond (Dsb) that ensures accurate folding of periplastic proteins (Guddat et al., 1998). The central component is DsbA, which contains a highly redox-active CXXC motif and can bind to its substrate during protein import. Similar to this, a thiol-dependent oxidation mechanism has been addressed to the thylakoid lumen (Gopalan et al., 2004).

Since even mitochondrial intermembrane-destined proteins are imported in an oxidation-driven reaction by the mitochondrial disulfide relay (Herrmann et al., 2009), this might also be the case for chloroplast import. However, this field has only recently gained more attention. Redox-mediated regulation could be observed at different stages of the import process. Both in the OEM as well as in the IEM, import-involved proteins exhibit redox-active properties.

Import activity is highly stimulated *in vitro* upon the addition of reducing agents like DTT or TCEP, and impaired by oxidizing substrates, suggesting a potential role of cysteines and disulfide bridges (Stengel et al., 2009). Interestingly, all TOC components contain several conserved cysteines. Seven are found in Toc75, present in both vascular and non-vascular plants. The POTRA domain contains four of them. In the case of cytosolic-facing POTRA domains, these cysteines could be involved in redox-mediated regulation. Toc159 displays five cysteines; two of them are conserved and are located within the GTPase domain. One of these two is also present in the GTPase domain of Toc34 and is fairly exposed (Sun et al., 2002), suggesting a suitable target for redox reactions. In the last identified TOC component, Toc64, ten cysteines could be found, of which six are conserved through vascular and non-vascular plants.

In their reduced state, Toc159, Toc34 and Toc75 are loosely attached, harboring different reduced thiols and thus, forming the so-called 'active' TOC complex, prepared for preprotein recognition and binding (**Figure 4A**). Upon oxidation, intra- and intermolecular disulfide bridges are generated, commonly between Toc159, Toc34 and Toc75, resulting in a heteromeric TOC complex (Seedorf et al., 1995). Different hypotheses concerning the mode of action have been suggested. Oxidized on the one hand, this bulky complex could inhibit the import rate by simply blocking the channel and thus preventing the entrance of incoming proteins (**Figure 4B**). However, another mechanism suggests that not only does channel blocking occur, but also the preprotein-binding capacity of the receptor proteins is already altered as the cysteines are located within the preprotein binding, the GTPase, domain (Stengel et al., 2010). Up to now,

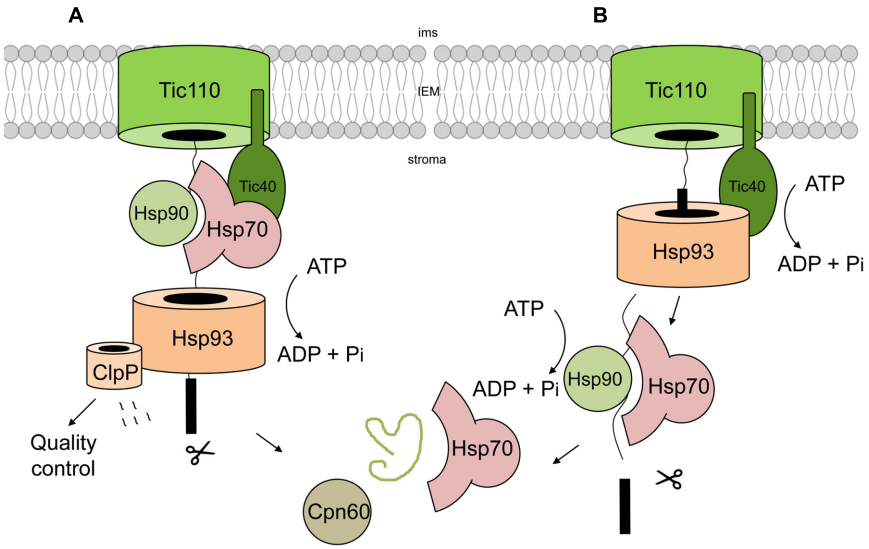

FIGURE 3 | The stromal chaperone system. Two different models have been hypothesized concerning the main import motor of the chaperones. One model **(A)** involves a secondary function of Hsp93, assuming that this protein acts mainly in the quality control pathway by degrading mistargeted or wrongly folded proteins. In this model the main energy is consumed by Hsp70 and not by Hsp93 (Flores-Pérez et al., 2016). A recent study suggest that Hsp93 interacts subsequently with incoming preprotein at the N-terminal cTP, whereas Hsp70 binds to the mature parts of the protein (Huang et al., 2015). This enable the two chaperone systems to interact at least partially in parallel with the preproteins. After completing of the import by processing the cTP, proteins are folded with the help of various chaperones like Cpn60 and Hsp70 **(B)**.

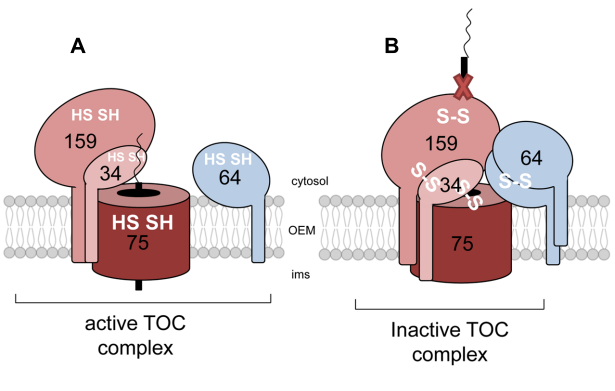

FIGURE 4 | Redox regulation at the outer envelope membrane. Disulfide bridges between conserved cysteine residues of the TOC constituents are involved in the redox modulation of the constituents of the OEM. Under reducing conditions, the TOC receptors are loosely attached, thus forming the open and active TOC complex **(A)**. Upon oxidation due to various external stimuli the generated intra- and intermolecular disulfide bridges lead to a blocked TOC complex which inhibits import of precursor proteins either by blocking the channel or altering the binding capacity of the receptor toward the preproteins **(B)**.

all these experiments have been carried out by adding, reducing or oxidizing agents *in vitro*, and so far, a discrete physiological role is still missing. However, it is still reasonable to assume that changing environmental conditions led to different redox states in the cytosol, due to the production of reactive oxygen species for example, and hence affecting the redox modulation of the translocation apparatus.

Redox Sensing at the Inner Envelope Membrane

Regarding the fact that the TOC complex could be regulated in a thiol-dependent mechanism, it can be supposed that this regulation is also effective for the translocase of the IEM.

Indeed, a thiol-dependent interaction between Tic110 and Tic40 has been observed, but its *in vivo* role has to be clarified (Stahl et al., 1999). Tic110 itself has been found to contain one or two regulatory disulfide bridges (Balsera et al., 2009). These intramolecular bridges could have a critical influence on the structure and function of the central TIC component. Switches between reduction and oxidation of these disulfide bridges could either lead to an open or closed formation of Tic110, respectively, and thereby limit the amount of incoming preproteins (**Figure 5**). The stromal thioredoxin family has been demonstrated to operate on disulfide bonds of Tic110 (Balsera et al., 2009). The redox state of thioredoxins is directly linked to both photosynthetic activity and other redox-dependent mechanisms in the organelle, thus it might act as a transport signal that eventually reaches the import machinery to regulate the chloroplast import rate. The intermembrane space protein Tic22 contains a conserved cysteine (Glaser et al., 2012), which could be involved in intramolecular disulfide bridges leading to dimerization of Tic22. Furthermore, since Tic110 exposes one cysteine into the IMS, a possible disulfide bond between the soluble Tic22 and the pore protein Tic110 during preprotein is also a hypothesis. However, no redox-mediated modulation has been reported so far and this hypothesis has to be addressed experimentally.

A direct read-out for the stromal redox state is the ratio between NADPH and NADP+. These reduction equivalents

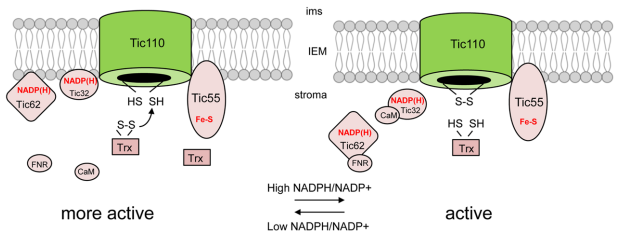

FIGURE 5 | Import regulation of the TIC complex from the stromal site. Similar to the redox regulation at the OEM import of precursor proteins is accelatered under reducing conditions, suggestively due to an open conformation of the main channel, Tic110. A second regulation mechanism involves the stromal redox state, which is reflected by the NADPH/NADP$^+$ ratio. A low NADPH/NADP$^+$ ratio could be shown to enhance the import rate compared to a higher NADPH/NADP$^+$ ratio.

deliver electrons which are required for enzymatic activities of many biosynthetic pathways within the organelle. All the required enzymes for a subset of different pathways have to be imported at a specific rate depending on the actual need within the organelles. Therefore, protein import activity must be regulated according to these requirements, which could be mediated by the stromal redox state. Independent studies have shown that the stromal redox state influences the import activity of a subset of preproteins (Stengel et al., 2009; Zhang et al., 2016). Interestingly, several components, namely Tic62, Tic55 and Tic32, have been shown to associate dynamically with the core complex, leading to the assumption that these transient TIC components act in a regulatory mechanism in response to the stromal redox state (Stengel et al., 2008). One prominent candidate was Tic62, which showed a triple-localization pattern, shuttling from a membrane associated state at the IEM as well as the thylakoids to the stroma in response to changing NADP+/NADPH ratios, and thus being able to mediate signals from the photosynthetically active thylakoids to the import machinery (Stengel et al., 2008). Since one important function of Tic62 in vascular plants is the binding of the chloroplast-targeted ferredoxin-NADP(+) oxidoreductase (FNR) to these membranes via specific C-terminal motifs (Alte et al., 2010), its shuttling could significantly influence electron-transfer processes from this photosynthetic enzyme to different acceptor proteins which could display a signal transduction chain. As Tic62 possesses a NADPH binding site and acts as a dehydrogenase *in vitro*, it might also be involved in a direct electron transfer onto yet unknown acceptor proteins (Stengel et al., 2008). Furthermore, another binding partner of FNR, named Trol, has been characterized. This thylakoid-localized protein harbors a similar single C-terminal extension as is found repeatedly in Tic62 and was demonstrated to interact with FNR (Jurić et al., 2009; Lintala et al., 2014). It could be shown that Trol also associates with the IEM, thus it might also participate in the signal transduction chain involving Tic62/FNR (Jurić et al., 2009). Interestingly, the FNR binding C-terminal motif is exclusively found in vascular plants, leading to the assumption that this regulatory mechanism has evolved later in evolution. This might suggest that, for all other plants, the ecological pressure was not high enough to evolve a system that regulates their protein import activity in response to changing stromal redox conditions, which is in contrast to the old evolutionary regulation mechanism of thiol oxidation. A second protein possibly involved in redox regulation is Tic32, which is another member of a dehydrogenase family capable of transferring electrons. Like Tic62, the affinity toward the TIC complex is lower under reduced conditions. Interestingly, Tic32 is also subject to calmodulin/Ca2+ dependent regulation. It could be shown that calmodulin (CaM) directly binds to Tic32, which promotes import, and that specific inhibition of this interaction decreased import efficiency (Chigri et al., 2006). Thus, two very different modes of action can regulate the TIC translocon (**Figure 5**). The third member of the redox regulon is Tic55, a Rieske protein found in close proximity of Tic110. It is anchored to the IEM by two alpha helices and exposes its C-terminal region into the stroma. Recently, Tic55 was identified as a potential thioredoxin target by affinity chromatography on a Trx-column (Bartsch et al., 2008), which is supported by the presence of a CXXC motif. The molecular function of Tic55 is still unclear, but recently, a study was published in which a hydroxylation activity during leaf-senescence-dependent chlorophyll breakdown was demonstrated for Tic55 (Hauenstein et al., 2016). This potential function of Tic55 connects chlorophyll metabolism to the chloroplast import demand and could function as a coordinator of the chloroplast homeostasis, similar to GUN1, which is a mediator of retrograde signaling. Under stress conditions, when chlorophyll is degraded, Tic55 could relay the required information which would eventually reach the nucleus in order to respond efficiently toward external stimuli. All the presented import regulation mechanisms are clearly involved in fine-tuning of the process rather than representing a molecular on/off switch, since single knockout mutants of the redox regulon components have, so far, no reported defects in protein import (Bölter et al., 2015).

Phosphorylation of the TOC Complex

The number of import sites per chloroplast was estimated, leading to different results: counting the number of radioactive mature proteins inside the organelle led to an estimated number of 3,500 (Friedman and Keegstra, 1989), whereas the approach using immunogold labeling of ultrathin sections with antibodies against main import components resulted in a higher number of 35,000 import sites (Morin and Soll, 1997). The discrepancy between these numbers can be explained by the fact that the immunogold labeling informs us about the total number of import complexes in the envelope, while the radioactive experiment gives us a measure of the fraction of these complexes that are actively importing. The switch between activity and non-activity of import complexes is likely to be modulated by the number of preproteins in need of being imported, amongst other signals.

It has been suggested that heterodimerization of the receptors, as well as their preprotein-binding capacity, is regulated by phosphorylation. PsToc34 and atToc33 are phosphorylated, whereas atToc34 is not, giving the opportunity to hypothesize that this represents specificity toward a different subset of

preproteins (Jelic et al., 2003). The phosphorylation might negatively affect GTP and preprotein binding of the respective receptor, and the whole TOC integrity is negatively influenced by phosphorylation *in vitro* (Oreb et al., 2007).

Signals triggering phosphorylation are, however, still not well defined. Data from transgenic *Arabidopsis* mutant lines showed that a phosphomimicking mutant of atToc33 is indeed affected in import capacity, whereas a non-phosphorylatable version of atToc33 exhibited a WT-like phenotype (Aronsson et al., 2006; Oreb et al., 2007). The latter observation in particular clearly indicates that phosphorylation mediated regulation is not a common or permanent regulation mechanism during plant development but rather an on/off switch in response to either a short period of a developmental change or to different, yet undefined, external stimuli. This could be the case, for example, upon cold or high light stress where the protein demand in the chloroplast is changed, or a specific subset of proteins is required. Under these conditions, fast post-translational modification machinery is required and phosphorylation of the TOC receptors might represent a relevant and efficient target for such an event. Regulation could occur in two ways. On the one hand, the overall import rate is affected (reduced, if phosphorylated) by downregulating the affinity to preproteins. On the other hand, phosphorylation could change the TOC complex stability, which would lead to the import of a distinct subset of client proteins.

Under this aspect, it would make sense if the responsible kinase was located in close proximity to avoid long shuttling pathways and to ensure specificity. In pea chloroplasts a 98-kDa, ATP-dependent outer membrane-attached kinase was identified as the responsible kinase (Sveshnikova et al., 2000). However, an *Arabidopsis* homolog is still missing, thus the identity of the responsible kinase remains elusive. Besides atToc33 (psToc34), the Toc159 family is also a target for phosphorylation, which is putatively mediated by a 70-kDa, ATP-dependent kinase (Fulgosi and Soll, 2002). All members are highly phosphorylated in their variable A-domain, consequently leading to a distinct phosphorylation pattern, ranging from many phosphorylation sites (atToc159) to few (all others) (Agne et al., 2010). As the A-domain between the members already displays a heterogeneous profile in sequence characteristics, the phosphorylation event could either be irrelevant or, contrarily, even enhance specificity toward preproteins.

Besides having a direct effect, phosphorylation could also act as part of a signaling cascade or promote indirectly another post-translational mechanism, like ubiquitination. It has been shown that phosphorylation can indeed have a negative or positive effect on ubiquitination (Hunter, 2007). This would provide a link to a recently made observation. Ling and Jarvis (2016) identified an OEM E3 ubiquitin ligase (SP1), which upon abiotic stress marks TOC components for degradation. It must be clarified if phosphorylation enhances this effect, which would provide new insights into the regulation made by phosphorylation.

DEDICATION

We dedicate this review article to the memory of Kentaro Inoue and his significant contributions to the chloroplast import field.

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AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication

REFERENCES

Agne, B., Andrès, C., Montandon, C., Christ, B., Ertan, A., Jung, F., et al. (2010). The acidic A-domain of *Arabidopsis* Toc159 occurs as a hyperphosphorylated protein. *Plant Physiol.* 153, 1016–1030. doi: 10.1104/pp.110.158048

Agne, B., Infanger, S., Wang, F., Hofstetter, V., Rahim, G., Martin, M., et al. (2009). A toc159 import receptor mutant, defective in hydrolysis of GTP, supports preprotein import into chloroplasts. *J. Biol. Chem.* 284, 8670–8679. doi: 10.1074/jbc.M804235200

Akita, M., Nielsen, E., and Keegstra, K. (1997). Identification of protein transport complexes in the chloroplastic envelope membranes via chemical cross-linking. *J. Cell Biol.* 136, 983–994. doi: 10.1083/jcb.136.5.983

Alte, F., Stengel, A., Benz, J. P., Petersen, E., Soll, J., Groll, M., et al. (2010). Ferredoxin:NADPH oxidoreductase is recruited to thylakoids by binding to a polyproline type II helix in a pH-dependent manner. *Proc. Natl. Acad. Sci. U.S.A.* 107, 19260–19265. doi: 10.1073/pnas.1009124107

Aronsson, H., Combe, J., Patel, R., and Jarvis, P. (2006). In vivo assessment of the significance of phosphorylation of the *Arabidopsis* chloroplast protein import receptor, atToc33. *FEBS Lett.* 580, 649–655. doi: 10.1016/j.febslet.2005.12.055

Aronsson, H., Combe, J., Patel, R., Agne, B., Martin, M., Kessler, F., et al. (2010). Nucleotide binding and dimerization at the chloroplast pre-protein import receptor, atToc33, are not essential in vivo but do increase import efficiency. *Plant J. Cell Mol. Biol.* 63, 297–311. doi: 10.1111/j.1365-313X.2010.04242.x

Balsera, M., Goetze, T. A., Kovács-Bogdán, E., Schürmann, P., Wagner, R., Buchanan, B. B., et al. (2009). Characterization of Tic110, a channel-forming protein at the inner envelope membrane of chloroplasts, unveils a response to Ca(2+) and a stromal regulatory disulfide bridge. *J. Biol. Chem.* 284, 2603–2616. doi: 10.1074/jbc.M807134200

Bartsch, S., Monnet, J., Selbach, K., Quigley, F., Gray, J., von Wettstein, D., et al. (2008). Three thioredoxin targets in the inner envelope membrane of chloroplasts function in protein import and chlorophyll metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4933–4938. doi: 10.1073/pnas.0800378105

Bauer, J., Chen, K., Hiltbunner, A., Wehrli, E., Eugster, M., Schnell, D., et al. (2000). The major protein import receptor of plastids is essential for chloroplast biogenesis. *Nature* 403, 203–207. doi: 10.1038/35003214

Becker, T., Jelic, M., Vojta, A., Radunz, A., Soll, J., and Schleiff, E. (2004). Preprotein recognition by the Toc complex. *EMBO J.* 23, 520–530. doi: 10.1038/sj.emboj.7600089

Bionda, T., Tillmann, B., Simm, S., Beilstein, K., Ruprecht, M., and Schleiff, E. (2010). Chloroplast import signals: the length requirement for translocation in vitro and in vivo. *J. Mol. Biol.* 402, 510–523. doi: 10.1016/j.jmb.2010.07.052

Bischof, S., Baerenfaller, K., Wildhaber, T., Troesch, R., Vidi, P.-A., Roschitzki, B., et al. (2011). Plastid proteome assembly without Toc159: photosynthetic protein import and accumulation of N-acetylated plastid precursor proteins. *Plant Cell* 23, 3911–3928. doi: 10.1105/tpc.111.092882

Bölter, B., May, T., and Soll, J. (1998a). A protein import receptor in pea chloroplasts, Toc86, is only a proteolytic fragment of a larger polypeptide. *FEBS Lett.* 441, 59–62. doi: 10.1016/S0014-5793(98)01525-7

Bölter, B., Soll, J., Schulz, A., Hinnah, S., and Wagner, R. (1998b). Origin of a chloroplast protein importer. *Proc. Natl. Acad. Sci. U.S.A.* 95, 15831–15836. doi: 10.1073/pnas.95.26.15831

Bölter, B., and Soll, J. (2016). Ycf1/Tic214 is not essential for the accumulation of plastid proteins. *Mol. Plant* 10, 219–221. doi: 10.1016/j.molp.2016.10.012

Bölter, B., Soll, J., and Schwenkert, S. (2015). Redox meets protein trafficking. *Biochim. Biophys. Acta* 1847, 949–956. doi: 10.1016/j.bbabio.2015.01.010

Bruce, B. D. (2001). The paradox of plastid transit peptides: conservation of function despite divergence in primary structure. *Biochim. Biophys. Acta* 1541, 2–21. doi: 10.1016/S0167-4889(01)00149-5

Chen, K., Chen, X., and Schnell, D. J. (2000). Initial binding of preproteins involving the Toc159 receptor can be bypassed during protein import into chloroplasts. *Plant Physiol.* 122, 813–822. doi: 10.1104/pp.122.3.813

Chen, X., Smith, M. D., Fitzpatrick, L., and Schnell, D. J. (2002). In vivo analysis of the role of atTic20 in protein import into chloroplasts. *Plant Cell* 14, 641–654. doi: 10.1105/tpc.010336

Chen, Y.-L., Chen, L.-J., and Li, H.-M. (2016). Polypeptide transport-associated domains of the Toc75 channel protein are located in the intermembrane space of chloroplasts. *Plant Physiol.* 172, 235–243. doi: 10.1104/pp.16.00919

Chew, O., Lister, R., Qbadou, S., Heazlewood, J. L., Soll, J., Schleiff, E., et al. (2004). A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor. *FEBS Lett.* 557, 109–114. doi: 10.1016/S0014-5793(03)01457-1

Chigri, F., Hörmann, F., Stamp, A., Stammers, D. K., Bölter, B., Soll, J., et al. (2006). Calcium regulation of chloroplast protein translocation is mediated by calmodulin binding to Tic32. *Proc. Natl. Acad. Sci. U.S.A.* 103, 16051–16056. doi: 10.1073/pnas.0607150103

Chou, M.-L., Chu, C.-C., Chen, L.-J., Akita, M., and Li, H. (2006). Stimulation of transit-peptide release and ATP hydrolysis by a cochaperone during protein import into chloroplasts. *J. Cell Biol.* 175, 893–900. doi: 10.1083/jcb.200609172

Clantin, B., Delattre, A.-S., Rucktooa, P., Saint, N., Méli, A. C., Locht, C., et al. (2007). Structure of the membrane protein FhaC: a member of the Omp85-TpsB transporter superfamily. *Science* 317, 957–961. doi: 10.1126/science.1143860

Comai, L., Larson-Kelly, N., Kiser, J., Mau, C. J., Pokalsky, A. R., Shewmaker, C. K., et al. (1988). Chloroplast transport of a ribulose bisphosphate carboxylase small subunit-5-enolpyruvyl 3-phosphoshikimate synthase chimeric protein requires part of the mature small subunit in addition to the transit peptide. *J. Biol. Chem.* 263, 15104–15109.

Dahlin, C., and Cline, K. (1991). Developmental regulation of the plastid protein import apparatus. *Plant Cell* 3, 1131–1140. doi: 10.1105/tpc.3.10.1131

de Vries, J., Sousa, F. L., Bölter, B., Soll, J., and Gould, S. B. (2015). YCF1: a green TIC? *Plant Cell* 27, 1827–1833. doi: 10.1105/tpc.114.135541

Drescher, A., Ruf, S., Calsa, T., Carrer, H., and Bock, R. (2000). The two largest chloroplast genome-encoded open reading frames of higher plants are essential genes. *Plant J.* 22, 97–104. doi: 10.1046/j.1365-313x.2000.00722.x

Dudek, J., Rehling, P., and van der Laan, M. (2013). Mitochondrial protein import: common principles and physiological networks. *Biochim. Biophys. Acta* 1833, 274–285. doi: 10.1016/j.bbamcr.2012.05.028

Dutta, S., Mohanty, S., and Tripathy, B. C. (2009). Role of temperature stress on chloroplast biogenesis and protein import in pea. *Plant Physiol.* 150, 1050–1061. doi: 10.1104/pp.109.137265

Dutta, S., Teresinski, H. J., and Smith, M. D. (2014). A split-ubiquitin yeast two-hybrid screen to examine the substrate specificity of atToc159 and atToc132, two *Arabidopsis* chloroplast preprotein import receptors. *PLoS ONE* 9:e95026. doi: 10.1371/journal.pone.0095026

Fellerer, C., Schweiger, R., Schöngruber, K., Soll, J., and Schwenkert, S. (2011). Cytosolic HSP90 cochaperones HOP and FKBP interact with freshly synthesized chloroplast preproteins of *Arabidopsis*. *Mol. Plant* 4, 1133–1145. doi: 10.1093/mp/ssr037

Flores-Pérez, Ú., Bédard, J., Tanabe, N., Lymperopoulos, P., Clarke, A. K., and Jarvis, P. (2016). Functional analysis of the Hsp93/ClpC chaperone at the chloroplast envelope. *Plant Physiol.* 170, 147–162. doi: 10.1104/pp.15.01538

Friedman, A. L., and Keegstra, K. (1989). Chloroplast protein import?: quantitative analysis of precursor binding. *Plant Physiol.* 89, 993–999. doi: 10.1104/pp.89.3.993

Fulgosi, H., and Soll, J. (2002). The chloroplast protein import receptors Toc34 and Toc159 are phosphorylated by distinct protein kinases. *J. Biol. Chem.* 277, 8934–8940. doi: 10.1074/jbc.M110679200

Glaser, S., van Dooren, G. G., Agrawal, S., Brooks, C. F., McFadden, G. I., Striepen, B., et al. (2012). Tic22 is an essential chaperone required for protein import into the apicoplast. *J. Biol. Chem.* 287, 39505–39512. doi: 10.1074/jbc.M112.405100

Goloubinoff, P., Christeller, J. T., Gatenby, A. A., and Lorimer, G. H. (1989). Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature* 342, 884–889. doi: 10.1038/342884a0

Gopalan, G., He, Z., Balmer, Y., Romano, P., Gupta, R., Héroux, A., et al. (2004). Structural analysis uncovers a role for redox in regulating FKBP13, an immunophilin of the chloroplast thylakoid lumen. *Proc. Natl. Acad. Sci. U.S.A.* 101, 13945–13950. doi: 10.1073/pnas.0405240101

Gould, S. B., Waller, R. F., and McFadden, G. I. (2008). Plastid evolution. *Annu. Rev. Plant Biol.* 59, 491–517. doi: 10.1146/annurev.arplant.59.032607.092915

Grieco, M., Jain, A., Ebersberger, I., and Teige, M. (2016). An evolutionary view on thylakoid protein phosphorylation uncovers novel phosphorylation hotspots with potential functional implications. *J. Exp. Bot.* 67, 3883–3896. doi: 10.1093/jxb/erw164

Grimaud, F., Renaut, J., Dumont, E., Sergeant, K., Lucau-Danila, A., Blervacq, A.-S., et al. (2013). Exploring chloroplastic changes related to chilling and freezing tolerance during cold acclimation of pea (*Pisum sativum* L.). *J. Proteomics* 80, 145–159. doi: 10.1016/j.jprot.2012.12.030

Gross, J., and Bhattacharya, D. (2009). Revaluating the evolution of the Toc and Tic protein translocons. *Trends Plant Sci.* 14, 13–20. doi: 10.1016/j.tplants.2008.10.003

Guddat, L. W., Bardwell, J. C., and Martin, J. L. (1998). Crystal structures of reduced and oxidized DsbA: investigation of domain motion and thiolate stabilization. *Struct. Lond. Engl.* 1993, 757–767.

Hauenstein, M., Christ, B., Das, A., Aubry, S., and Hörtensteiner, S. (2016). A role for TIC55 as a hydroxylase of phyllobilins, the products of chlorophyll breakdown during plant senescence. *Plant Cell* 28, 2510–2527. doi: 10.1105/tpc.16.00630

Heins, L., Mehrle, A., Hemmler, R., Wagner, R., Küchler, M., Hörmann, F., et al. (2002). The preprotein conducting channel at the inner envelope membrane of plastids. *EMBO J.* 21, 2616–2625. doi: 10.1093/emboj/21.11.2616

Herrmann, J. M., Kauff, F., and Neuhaus, H. E. (2009). Thiol oxidation in bacteria, mitochondria and chloroplasts: common principles but three unrelated machineries? *Biochim. Biophys. Acta* 1793, 71–77. doi: 10.1016/j.bbamcr.2008.05.001

Hinnah, S. C., Hill, K., Wagner, R., Schlicher, T., and Soll, J. (1997). Reconstitution of a chloroplast protein import channel. *EMBO J.* 16, 7351–7360. doi: 10.1093/emboj/16.24.7351

Hinnah, S. C., Wagner, R., Sveshnikova, N., Harrer, R., and Soll, J. (2002). The chloroplast protein import channel Toc75: pore properties and interaction with transit peptides. *Biophys. J.* 83, 899–911. doi: 10.1016/S0006-3495(02)75216-8

Hörmann, F., Küchler, M., Sveshnikov, D., Oppermann, U., Li, Y., and Soll, J. (2004). Tic32, an essential component in chloroplast biogenesis. *J. Biol. Chem.* 279, 34756–34762. doi: 10.1074/jbc.M402817200

Huang, P.-K., Chan, P.-T., Su, P.-H., Chen, L.-J., and Li, H. (2015). Chloroplast Hsp93 directly binds to transit peptides at an early stage of the preprotein import process. *Plant Physiol.* 170, 857–866. doi: 10.1104/pp.15.01830

Hunter, T. (2007). The age of crosstalk: phosphorylation, ubiquitination, and beyond. *Mol. Cell* 28, 730–738. doi: 10.1016/j.molcel.2007.11.019

Inaba, T., Alvarez-Huerta, M., Li, M., Bauer, J., Ewers, C., Kessler, F., et al. (2005). *Arabidopsis* tic110 is essential for the assembly and function of the protein

import machinery of plastids. *Plant Cell* 17, 1482–1496. doi: 10.1105/tpc.105.030700

Inaba, T., Li, M., Alvarez-Huerta, M., Kessler, F., and Schnell, D. J. (2003). atTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts. *J. Biol. Chem.* 278, 38617–38627. doi: 10.1074/jbc.M306367200

Infanger, S., Bischof, S., Hiltbrunner, A., Agne, B., Baginsky, S., and Kessler, F. (2011). The chloroplast import receptor Toc90 partially restores the accumulation of Toc159 client proteins in the *Arabidopsis thaliana* ppi2 mutant. *Mol. Plant* 4, 252–263. doi: 10.1093/mp/ssq071

Inoue, H., Li, M., and Schnell, D. J. (2013). An essential role for chloroplast heat shock protein 90 (Hsp90C) in protein import into chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3173–3178. doi: 10.1073/pnas.1219229110

Inoue, H., Rounds, C., and Schnell, D. J. (2010). The molecular basis for distinct pathways for protein import into *Arabidopsis* chloroplasts. *Plant Cell* 22, 1947–1960. doi: 10.1105/tpc.110.074328

Inoue, K., Baldwin, A. J., Shipman, R. L., Matsui, K., Theg, S. M., and Ohme-Takagi, M. (2005). Complete maturation of the plastid protein translocation channel requires a type I signal peptidase. *J. Cell Biol.* 171, 425–430. doi: 10.1083/jcb.200506171

Ivanova, Y., Smith, M. D., Chen, K., and Schnell, D. J. (2004). Members of the Toc159 import receptor family represent distinct pathways for protein targeting to plastids. *Mol. Biol. Cell* 15, 3379–3392. doi: 10.1091/mbc.E03-12-0923

Jackson-Constan, D., and Keegstra, K. (2001). *Arabidopsis* genes encoding components of the chloroplastic protein import apparatus. *Plant Physiol.* 125, 1567–1576. doi: 10.1104/pp.125.4.1567

Jarvis, P., Chen, L. J., Li, H., Peto, C. A., Fankhauser, C., and Chory, J. (1998). An *Arabidopsis* mutant defective in the plastid general protein import apparatus. *Science* 282, 100–103. doi: 10.1126/science.282.5386.100

Jelic, M., Soll, J., and Schleiff, E. (2003). Two Toc34 homologues with different properties. *Biochemistry (Mosc.)* 42, 5906–5916. doi: 10.1021/bi034001q

Jurić, S., Hazler-Pilepić, K., Tomašić, A., Lepedus, H., Jelicić, B., Puthiyaveetil, S., et al. (2009). Tethering of ferredoxin:NADP+ oxidoreductase to thylakoid membranes is mediated by novel chloroplast protein TROL. *Plant J. Cell Mol. Biol.* 60, 783–794. doi: 10.1111/j.1365-313X.2009.03999.x

Kasmati, A. R., Töpel, M., Khan, N. Z., Patel, R., Ling, Q., Karim, S., et al. (2013). Evolutionary, molecular and genetic analyses of Tic22 homologues in *Arabidopsis thaliana* chloroplasts. *PLoS ONE* 8:e63863. doi: 10.1371/journal.pone.0063863

Kessler, F., and Blobel, G. (1996). Interaction of the protein import and folding machineries of the chloroplast. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7684–7689. doi: 10.1073/pnas.93.15.7684

Kessler, F., and Schnell, D. (2009). Chloroplast biogenesis: diversity and regulation of the protein import apparatus. *Curr. Opin. Cell Biol.* 21, 494–500. doi: 10.1016/j.ceb.2009.03.004

Kikuchi, S., Bédard, J., Hirano, M., Hirabayashi, Y., Oishi, M., Imai, M., et al. (2013). Uncovering the protein translocon at the chloroplast inner envelope membrane. *Science* 339, 571–574. doi: 10.1126/science.1229262

Kikuchi, S., Oishi, M., Hirabayashi, Y., Lee, D. W., Hwang, I., and Nakai, M. (2009). A 1-megadalton translocation complex containing Tic20 and Tic21 mediates chloroplast protein import at the inner envelope membrane. *Plant Cell* 21, 1781–1797. doi: 10.1105/tpc.108.063552

Köhler, D., Helm, S., Agne, B., and Baginsky, S. (2016). Importance of translocon subunit Tic56 for rRNA processing and chloroplast ribosome assembly. *Plant Physiol.* 172, 2429–2444. doi: 10.1104/pp.16.01393

Köhler, D., Montandon, C., Hause, G., Majovsky, P., Kessler, F., Baginsky, S., et al. (2015). Characterization of chloroplast protein import without Tic56, a component of the 1-megadalton translocon at the inner envelope membrane of chloroplasts. *Plant Physiol.* 167, 972–990. doi: 10.1104/pp.114.255562

Kouranov, A., Chen, X., Fuks, B., and Schnell, D. J. (1998). Tic20 and Tic22 are Nnw components of the protein import apparatus at the chloroplast inner envelope membrane. *J. Cell Biol.* 143, 991–1002. doi: 10.1083/jcb.143.4.991

Kouranov, A., and Schnell, D. J. (1997). Analysis of the interactions of preproteins with the import machinery over the course of protein import into chloroplasts. *J. Cell Biol.* 139, 1677–1685. doi: 10.1083/jcb.139.7.1677

Kovacheva, S., Bédard, J., Patel, R., Dudley, P., Twell, D., Ríos, G., et al. (2005). In vivo studies on the roles of Tic110, Tic40 and Hsp93 during chloroplast protein import. *Plant J. Cell Mol. Biol.* 41, 412–428. doi: 10.1111/j.1365-313X.2004.02307.x

Kovács-Bogdán, E., Benz, J. P., Soll, J., and Bölter, B. (2011). Tic20 forms a channel independent of Tic110 in chloroplasts. *BMC Plant Biol.* 11:133. doi: 10.1186/1471-2229-11-133

Kubis, S., Patel, R., Combe, J., Bédard, J., Kovacheva, S., Lilley, K., et al. (2004). Functional specialization amongst the *Arabidopsis* Toc159 family of chloroplast protein import receptors. *Plant Cell* 16, 2059–2077. doi: 10.1105/tpc.104.023309

Lamberti, G., Gügel, I. L., Meurer, J., Soll, J., and Schwenkert, S. (2011). The cytosolic kinases STY8, STY17, and STY46 are involved in chloroplast differentiation in *Arabidopsis*. *Plant Physiol.* 157, 70–85. doi: 10.1104/pp.111.182774

Lee, K. H., Kim, S. J., Lee, Y. J., Jin, J. B., and Hwang, I. (2003). The M domain of atToc159 plays an essential role in the import of proteins into chloroplasts and chloroplast biogenesis. *J. Biol. Chem.* 278, 36794–36805. doi: 10.1074/jbc.M304457200

Leister, D. (2003). Chloroplast research in the genomic age. *Trends Genet.* 19, 47–56. doi: 10.1016/S0168-9525(02)00003-3

Li, H., and Teng, Y.-S. (2013). Transit peptide design and plastid import regulation. *Trends Plant Sci.* 18, 360–366. doi: 10.1016/j.tplants.2013.04.003

Ling, Q., and Jarvis, P. (2016). Plant signaling: ubiquitin pulls the trigger on chloroplast degradation. *Curr. Biol.* 26, R38–R40. doi: 10.1016/j.cub.2015.11.022

Lintala, M., Schuck, N., Thormählen, I., Jungfer, A., Weber, K. L., Weber, A. P. M., et al. (2014). *Arabidopsis* tic62 trol mutant lacking thylakoid-bound ferredoxin-NADP+ oxidoreductase shows distinct metabolic phenotype. *Mol. Plant* 7, 45–57. doi: 10.1093/mp/sst129

Lübeck, J., Soll, J., Akita, M., Nielsen, E., and Keegstra, K. (1996). Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. *EMBO J.* 15, 4230–4238.

Martin, W., Rujan, T., Richly, E., Hansen, A., Cornelsen, S., Lins, T., et al. (2002). Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 99, 12246–12251. doi: 10.1073/pnas.182432999

May, T., and Soll, J. (2000). 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. *Plant Cell* 12, 53–64. doi: 10.2307/3871029

Moore, T., and Keegstra, K. (1993). Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. *Plant Mol. Biol.* 21, 525–537. doi: 10.1007/BF00028809

Morin, X. K., and Soll, J. (1997). Immunogold labelling of cryosectioned pea chloroplasts and initial localization of the proteins associated with the protein import machinery. *Planta* 201, 119–127. doi: 10.1007/BF01007696

Nakamoto, H., Fujita, K., Ohtaki, A., Watanabe, S., Narumi, S., Maruyama, T., et al. (2014). Physical interaction between bacterial heat shock protein (Hsp) 90 and Hsp70 chaperones mediates their cooperative action to refold denatured proteins. *J. Biol. Chem.* 289, 6110–6119. doi: 10.1074/jbc.M113.524801

Nakrieko, K.-A., Mould, R. M., and Smith, A. G. (2004). Fidelity of targeting to chloroplasts is not affected by removal of the phosphorylation site from the transit peptide. *Eur. J. Biochem.* 271, 509–516. doi: 10.1046/j.1432-1033.2003.03950.x

Nickel, C., Soll, J., and Schwenkert, S. (2015). Phosphomimicking within the transit peptide of pHCF136 leads to reduced photosystem II accumulation in vivo. *FEBS Lett.* 589, 1301–1307. doi: 10.1016/j.febslet.2015.04.033

Nielsen, E., Akita, M., Davila-Aponte, J., and Keegstra, K. (1997). Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. *EMBO J.* 16, 935–946. doi: 10.1093/emboj/16.5.935

Oreb, M., Zoryan, M., Vojta, A., Maier, U. G., Eichacker, L. A., and Schleiff, E. (2007). Phospho-mimicry mutant of atToc33 affects early development of *Arabidopsis thaliana*. *FEBS Lett.* 581, 5945–5951. doi: 10.1016/j.febslet.2007.11.071

Paila, Y. D., Richardson, L. G., Inoue, H., Parks, E. S., McMahon, J., Inoue, K., et al. (2016). Multi-functional roles for the polypeptide transport associated domains

of Toc75 in chloroplast protein import. *eLife* 5:e12631. doi: 10.7554/eLife.12631

Pain, D., and Blobel, G. (1987). Protein import into chloroplasts requires a chloroplast ATPase. *Proc. Natl. Acad. Sci. U.S.A.* 84, 3288–3292. doi: 10.1073/pnas.84.10.3288

Park, E., and Rapoport, T. A. (2012). Mechanisms of Sec61/SecY-mediated protein translocation across membranes. *Annu. Rev. Biophys.* 41, 21–40. doi: 10.1146/annurev-biophys-050511-102312

Qbadou, S., Becker, T., Mirus, O., Tews, I., Soll, J., and Schleiff, E. (2006). The molecular chaperone Hsp90 delivers precursor proteins to the chloroplast import receptor Toc64. *EMBO J.* 25, 1836–1847. doi: 10.1038/sj.emboj.7601091

Ratnayake, R. M. U., Inoue, H., Nonami, H., and Akita, M. (2008). Alternative processing of *Arabidopsis* Hsp70 precursors during protein import into chloroplasts. *Biosci. Biotechnol. Biochem.* 72, 2926–2935. doi: 10.1271/bbb.80408

Reumann, S., Inoue, K., and Keegstra, K. (2005). Evolution of the general protein import pathway of plastids (Review). *Mol. Membr. Biol.* 22, 73–86. doi: 10.1080/09687860500041916

Reumann, S., and Keegstra, K. (1999). The endosymbiotic origin of the protein import machinery of chloroplastic envelope membranes. *Trends Plant Sci.* 4, 302–307. doi: 10.1016/S1360-1385(99)01449-1

Rial, D. V., Arakaki, A. K., and Ceccarelli, E. A. (2000). Interaction of the targeting sequence of chloroplast precursors with Hsp70 molecular chaperones. *Eur. J. Biochem.* 267, 6239–6248. doi: 10.1046/j.1432-1327.2000.01707.x

Richardson, L. G., Jelokhani-Niaraki, M., and Smith, M. D. (2009). The acidic domains of the Toc159 chloroplast preprotein receptor family are intrinsically disordered protein domains. *BMC Biochem.* 10:35. doi: 10.1186/1471-2091-10-35

Richter, S., and Lamppa, G. K. (1998). A chloroplast processing enzyme functions as the general stromal processing peptidase. *Proc. Natl. Acad. Sci. U.S.A.* 95, 7463–7468. doi: 10.1073/pnas.95.13.7463

Richter, S., Zhong, R., and Lamppa, G. (2005). Function of the stromal processing peptidase in the chloroplast import pathway. *Physiol. Plant.* 123, 362–368. doi: 10.1111/j.1399-3054.2005.00476.x

Rolland, V., Badger, M. R., and Price, G. D. (2016). Redirecting the cyanobacterial bicarbonate transporters BicA and SbtA to the chloroplast envelope: soluble and membrane cargos need different chloroplast targeting signals in plants. *Front. Plant Sci.* 7:185. doi: 10.3389/fpls.2016.00185

Rosano, G. L., Bruch, E. M., and Ceccarelli, E. A. (2011). Insights into the Clp/HSP100 chaperone system from chloroplasts of *Arabidopsis thaliana*. *J. Biol. Chem.* 286, 29671–29680. doi: 10.1074/jbc.M110.211946

Rudolf, M., Machettira, A. B., Groß, L. E., Weber, K. L., Bolte, K., Bionda, T., et al. (2013). In vivo function of Tic22, a protein import component of the intermembrane space of chloroplasts. *Mol. Plant* 6, 817–829. doi: 10.1093/mp/sss114

Schleiff, E., Soll, J., Küchler, M., Kühlbrandt, W., and Harrer, R. (2003). Characterization of the translocon of the outer envelope of chloroplasts. *J. Cell Biol.* 160, 541–551. doi: 10.1083/jcb.200210060

Schnell, D. J., and Blobel, G. (1993). Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. *J. Cell Biol.* 120, 103–115. doi: 10.1083/jcb.120.1.103

Schnell, D. J., Kessler, F., and Blobel, G. (1994). Isolation of components of the chloroplast protein import machinery. *Science* 266, 1007–1012. doi: 10.1126/science.7973649

Schünemann, D. (2007). Mechanisms of protein import into thylakoids of chloroplasts. *Biol. Chem.* 388, 907–915.

Schweiger, R., Müller, N. C., Schmitt, M. J., Soll, J., and Schwenkert, S. (2012). AtTPR7 is a chaperone-docking protein of the Sec translocon in *Arabidopsis*. *J. Cell Sci.* 125, 5196–5207. doi: 10.1242/jcs.111054

Seedorf, M., Waegemann, K., and Soll, J. (1995). A constituent of the chloroplast import complex represents a new type of GTP-binding protein. *Plant J.* 7, 401–411. doi: 10.1046/j.1365-313X.1995.7030401.x

Shi, L.-X., and Theg, S. M. (2010). A stromal heat shock protein 70 system functions in protein import into chloroplasts in the moss *Physcomitrella* patens. *Plant Cell* 22, 205–220. doi: 10.1105/tpc.109.071464

Smith, M. D., Rounds, C. M., Wang, F., Chen, K., Afitlhile, M., and Schnell, D. J. (2004). atToc159 is a selective transit peptide receptor for the import of nucleus-encoded chloroplast proteins. *J. Cell Biol.* 165, 323–334. doi: 10.1083/jcb.200311074

Sohrt, K., and Soll, J. (2000). Toc64, a new component of the protein translocon of chloroplasts. *J. Cell Biol.* 148, 1213–1221. doi: 10.1083/jcb.148.6.1213

Sommer, M., Rudolf, M., Tillmann, B., Tripp, J., Sommer, M. S., and Schleiff, E. (2013). Toc33 and Toc64-III cooperate in precursor protein import into the chloroplasts of *Arabidopsis thaliana*. *Plant Cell Environ.* 36, 970–983. doi: 10.1111/pce.12030

Sommer, M. S., Daum, B., Gross, L. E., Weis, B. L. M., Mirus, O., Abram, L., et al. (2011). Chloroplast Omp85 proteins change orientation during evolution. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13841–13846. doi: 10.1073/pnas.1108626108

Stahl, T., Glockmann, C., Soll, J., and Heins, L. (1999). Tic40, a new "old" subunit of the chloroplast protein import translocon. *J. Biol. Chem.* 274, 37467–37472. doi: 10.1074/jbc.274.52.37467

Stengel, A., Benz, J. P., Buchanan, B. B., Soll, J., and Bölter, B. (2009). Preprotein import into chloroplasts via the Toc and Tic complexes is regulated by redox signals in *Pisum sativum*. *Mol. Plant* 2, 1181–1197. doi: 10.1093/mp/ssp043

Stengel, A., Benz, J. P., Soll, J., and Bölter, B. (2010). Redox-regulation of protein import into chloroplasts and mitochondria: similarities and differences. *Plant Signal. Behav.* 5, 105–109. doi: 10.4161/psb.5.2.10525

Stengel, A., Benz, P., Balsera, M., Soll, J., and Bölter, B. (2008). TIC62 redox-regulated translocon composition and dynamics. *J. Biol. Chem.* 283, 6656–6667. doi: 10.1074/jbc.M706719200

Su, P.-H., and Li, H. (2010). Stromal Hsp70 is important for protein translocation into pea and *Arabidopsis* chloroplasts. *Plant Cell* 22, 1516–1531. doi: 10.1105/tpc.109.071415

Sugiura, M. (1989). The chloroplast chromosomes in land plants. *Annu. Rev. Cell Biol.* 5, 51–70. doi: 10.1146/annurev.cb.05.110189.000411

Sun, Y.-J., Forouhar, F., Li Hm, H., Tu, S.-L., Yeh, Y.-H., Kao, S., et al. (2002). Crystal structure of pea Toc34, a novel GTPase of the chloroplast protein translocon. *Nat. Struct. Biol.* 9, 95–100. doi: 10.1038/nsb744

Sveshnikova, N., Soll, J., and Schleiff, E. (2000). Toc34 is a preprotein receptor regulated by GTP and phosphorylation. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4973–4978. doi: 10.1073/pnas.080491597

Timmis, J. N., Ayliffe, M. A., Huang, C. Y., and Martin, W. (2004). Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nat. Rev. Genet.* 5, 123–135. doi: 10.1038/nrg1271

Tranel, P. J., Froehlich, J., Goyal, A., and Keegstra, K. (1995). A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO J.* 14, 2436–2446.

Trösch, R., and Jarvis, P. (2011). The stromal processing peptidase of chloroplasts is essential in *Arabidopsis*, with knockout mutations causing embryo arrest after the 16-cell stage. *PLoS ONE* 6:e23039. doi: 10.1371/journal.pone.0023039

Tsai, J.-Y., Chu, C.-C., Yeh, Y.-H., Chen, L.-J., Li, H.-M., and Hsiao, C.-D. (2013). Structural characterizations of the chloroplast translocon protein Tic110. *Plant J. Cell Mol. Biol.* 75, 847–857. doi: 10.1111/tpj.12249

Vojta, L., Soll, J., and Bölter, B. (2007). Protein transport in chloroplasts - targeting to the intermembrane space. *FEBS J.* 274, 5043–5054. doi: 10.1111/j.1742-4658.2007.06023.x

Waegemann, K., and Soll, J. (1996). Phosphorylation of the transit sequence of chloroplast precursor proteins. *J. Biol. Chem.* 271, 6545–6554. doi: 10.1074/jbc.271.11.6545

Wirmer, J., and Westhof, E. (2006). Molecular contacts between antibiotics and the 30S ribosomal particle. *Methods Enzymol.* 415, 180–202. doi: 10.1016/S0076-6879(06)15012-0

Yan, J., Smith, M. D., Glick, B. R., and Liang, Y. (2014). Effects of ACC deaminase containing rhizobacteria on plant growth and expression of Toc GTPases in tomato (*Solanum lycopersicum*) under salt stress. *Botany* 92, 775–781. doi: 10.1139/cjb-2014-0038

Yang, X.-F., Wang, Y.-T., Chen, S.-T., Li, J.-K., Shen, H.-T., and Guo, F.-Q. (2016). PBR1 selectively controls biogenesis of photosynthetic complexes by modulating translation of the large chloroplast gene Ycf1 in *Arabidopsis*. *Cell Discov.* 2:16003. doi: 10.1038/celldisc.2016.3

Young, J. C., Hoogenraad, N. J., and Hartl, F. U. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial

import receptor Tom70. *Cell* 112, 41–50. doi: 10.1016/S0092-8674(02)01250-3

Young, M. E., Keegstra, K., and Froehlich, J. E. (1999). GTP promotes the formation of early-import intermediates but is not required during the translocation step of protein import into chloroplasts. *Plant Physiol.* 121, 237–244. doi: 10.1104/pp.121.1.237

Zhang, D.-W., Yuan, S., Xu, F., Zhu, F., Yuan, M., Ye, H.-X., et al. (2016). Light intensity affects chlorophyll synthesis during greening process by metabolite signal from mitochondrial alternative oxidase in *Arabidopsis*. *Plant Cell Environ.* 39, 12–25. doi: 10.1111/pce.12438

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REVIEW
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Post-translational Modifications in Regulation of Chloroplast Function: Recent Advances

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Post-translational modifications (PTMs) of proteins enable fast modulation of protein function in response to metabolic and environmental changes. Phosphorylation is known to play a major role in regulating distribution of light energy between the Photosystems (PS) I and II (state transitions) and in PSII repair cycle. In addition, thioredoxin-mediated redox regulation of Calvin cycle enzymes has been shown to determine the efficiency of carbon assimilation. Besides these well characterized modifications, recent methodological progress has enabled identification of numerous other types of PTMs in various plant compartments, including chloroplasts. To date, at least N-terminal and Lys acetylation, Lys methylation, Tyr nitration and S-nitrosylation, glutathionylation, sumoylation and glycosylation of chloroplast proteins have been described. These modifications impact DNA replication, control transcriptional efficiency, regulate translational machinery and affect metabolic activities within the chloroplast. Moreover, light reactions of photosynthesis as well as carbon assimilation are regulated at multiple levels by a number of PTMs. It is likely that future studies will reveal new metabolic pathways to be regulated by PTMs as well as detailed molecular mechanisms of PTM-mediated regulation.

Keywords: chloroplast, phosphorylation, photosynthesis, post-translational modification, redox regulation

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INTRODUCTION

Chloroplasts are sites of versatile metabolism. In addition to photosynthetic reactions, chloroplasts host a number of other processes, such as nitrogen and sulfur assimilation, amino acid and fatty acid biosynthesis as well as accumulation of pigments, photoreceptors, and hormones. Chloroplasts are surrounded by the envelope membrane, and the majority of nuclear-encoded chloroplast proteins are imported through the envelope into the plastid via the Toc/Tic machinery. The subchloroplastic destination of a specific protein is determined by the information buried within the primary amino acid sequence, either in the form of cleavable transit peptide or as an internal targeting signal. Due to their endosymbiotic origin, biosynthesis and function of chloroplasts is not only dependent on nuclear control, but also on the expression of approximately 120 plastome encoded genes, mostly involved in photosynthesis and plastid gene expression (Sugiura, 1992; Green, 2011). Obviously, coordination of gene expression between these compartments as well

Abbreviations: PS, photosystem; *psa*, genes encoding subunits of Photosystem II; *psb*, genes encoding subunits of Photosystem II; PTM, post-translational modification; RA, Rubisco activase; RB, RNA binding protein; *rps*, genes encoding ribosome subunits; *trn*, genes encoding chloroplast transferRNAs.

as integration of plastid metabolism with the rest of the cell are required to induce appropriate physiological responses to various environmental stimuli, thereby enabling successful growth and reproduction of the plants. This coordination takes place at many different levels, including the control of nuclear and plastid transcription, RNA processing and translation, protein translocation and assembly of protein complexes as well as functional adjustments of specific enzymes and/or pathways.

Recent interest and methodological progress on PTMs of non-histone proteins has revealed that also a great number of chloroplast proteins are post-translationally modified, which denotes for covalent processing of a mature protein. The most well-studied chloroplast protein modifications: (de)phosphorylation, conveyed by kinases and phosphatases, and oxidation-reduction (including disulfide-thiol exchange of Cys residues, regulated via thioredoxins) have been extensively reviewed (e.g., Buchanan and Balmer, 2005; Tikkanen and Aro, 2012; Michelet et al., 2013; Rochaix, 2013) and thus are not described in detail in the present article. Other PTM types, such as acetylation, methylation, glycosylation, nitration and nitrosylation, sumoylation, and glutathionylation have been identified in chloroplast proteins much later. As only limited information is available for these PTMs, it is currently not possible to conclude whether a given PTM is found in the chloroplasts of all plant and algal species, or whether it is specific for a certain group of organisms. In addition to the PTMs modifying a given amino acid, recent studies have shown that a number of chloroplast proteins are prone to N-terminal trimming resulting in different N-termini or N-terminal acetylation (Lehtimäki et al., 2015; Rowland et al., 2015). It is intriguing that both nuclear- and chloroplast-encoded proteins may be subjected to these modifications (Lehtimäki et al., 2015). In most cases the site of the PTM (cytosol or plastid) and/or the responsible enzymes have remained obscure. PTMs alter the physicochemical properties and thus the function of proteins in different ways depending on the modification and the molecular environment. The molecular structures of the different chloroplast PTM are presented in **Figure 1**. Here, we will draw together the current understanding of the PTMs regulating distinct metabolic processes in chloroplasts, and review the known physiological effects of these modifications.

CHLOROPLAST MACHINERY FOR DNA REPLICATION AND GENE EXPRESSION

Organellar genomes are organized as nucleoids (also called as transcriptionally active chromosomes or TACs), DNA-protein complexes, which have been identified as the sites for both DNA replication and transcription (Melonek et al., 2016). Recently, proteomic analyses have suggested that also mRNA processing, splicing, editing, and ribosome assembly occur in association with the nucleoid, which supports the idea of co-transcriptional translation of plastid-encoded genes (Majeran et al., 2012). Although only few examples are thoroughly studied, PTMs of various types have been shown to regulate chloroplast genome replication and gene expression at multiple levels (**Figure 2**).

Even if a glycosylation machinery has been identified only in endoplasmic reticulum, some chloroplast proteins have been found to be glycosylated suggesting an existence of vesicular Toc/Tic independent chloroplast protein import route (Villarejo et al., 2005). One of the glycosylated proteins is the pea chloroplast protein p43, which associates with and activates the chloroplast DNA polymerase (Chen et al., 1996). Specifically, the N-terminal domain of p43 is highly O-arabinosylated (Gaikwad et al., 1999). Glycosylation of the protein is required for the induction of polymerization activity, although DNA binding is retained even if the protein is deglycosylated (Gaikwad et al., 1999, 2000). In addition to DNA replication, transcriptional activity of chloroplast genes is (partly) regulated by PTMs. Two different types of RNA polymerases, the plastid-encoded polymerase PEP, and the nuclear-encoded polymerase NEP, are responsible for the transcription of plastid-encoded genes (Shiina et al., 2005). The core subunits of PEP polymerase are associated with nuclear-encoded sigma factors, which are regulated by (de)phosphorylation (Link, 2003; Shimizu et al., 2010). Ser phosphorylation of the sigma factors (SIG6 being the most well studied one) is at least partly conveyed by the plastid transcription kinase (PTK), which is a chloroplast Ser/Thr protein kinase (Baginsky et al., 1997; Baena-González et al., 2001; Ogrzewalla et al., 2002; Salinas et al., 2006; Schweer et al., 2010). The kinase itself is regulated via autophosphorylation and glutathione-dependent redox regulation (Baginsky et al., 1997, 1999; Ogrzewalla et al., 2002). Effect of sigma factor phosphorylation on transcription depends on the sigma factor and the transcribed gene in question: for instance phosphorylation of the Thr_{170} in SIG1 inhibits transcription of the *psaA* gene (Shimizu et al., 2010), while phosphorylation of $Ser_{94/95}$ and/or Ser_{174} in SIG6 enhances transcription of the *atpB* and *trnK* genes with no apparent effect on the transcription of the *psbA* gene (Schweer et al., 2010).

Processing of the chloroplast transcripts is also affected by phosphorylation and redox regulation of RNA binding proteins. For instance phosphorylation of endoribonuclease p54, which is responsible for the 3′ processing of the plastid *trnK* and *rps16*, affects the RNA processing activity but not the cleavage specificity (Nickelsen and Link, 1993; Liere and Link, 1994). Additionally, the processing activity of p54 was modulated by glutathione (Liere and Link, 1994). Phosphorylation of 24 kDa (24RNP) and 28 kDa (28RNP) RNA-binding proteins, associated with a complex regulating the maturation of the 3′ end of chloroplast transcripts (Hayes et al., 1996), has been shown to affect the affinity of the proteins to RNA. Specifically, phosphorylation of 24RNP increased its binding capacity to *petD* and *psbA* 3′ UTR (Loza-Tavera et al., 2006), whereas phosphorylation of 28RNP resulted in decreased affinity to RNA (Lisitsky and Schuster, 1995). Recently, it was shown that phosphorylation status of the 24RNP and 28RNP (and apparently other unidentified RNA binding proteins) mediates the interplay between the *petD* mRNA stability and processing (Vargas-Suarez et al., 2013).

The translational machinery of the chloroplast is composed of prokaryotic-type 70S ribosomes organized in small (Yamaguchi et al., 2000, 2003) and large (Yamaguchi and Subramanian, 2000) subunits. Chloroplast ribosomes contain rRNA and proteins,

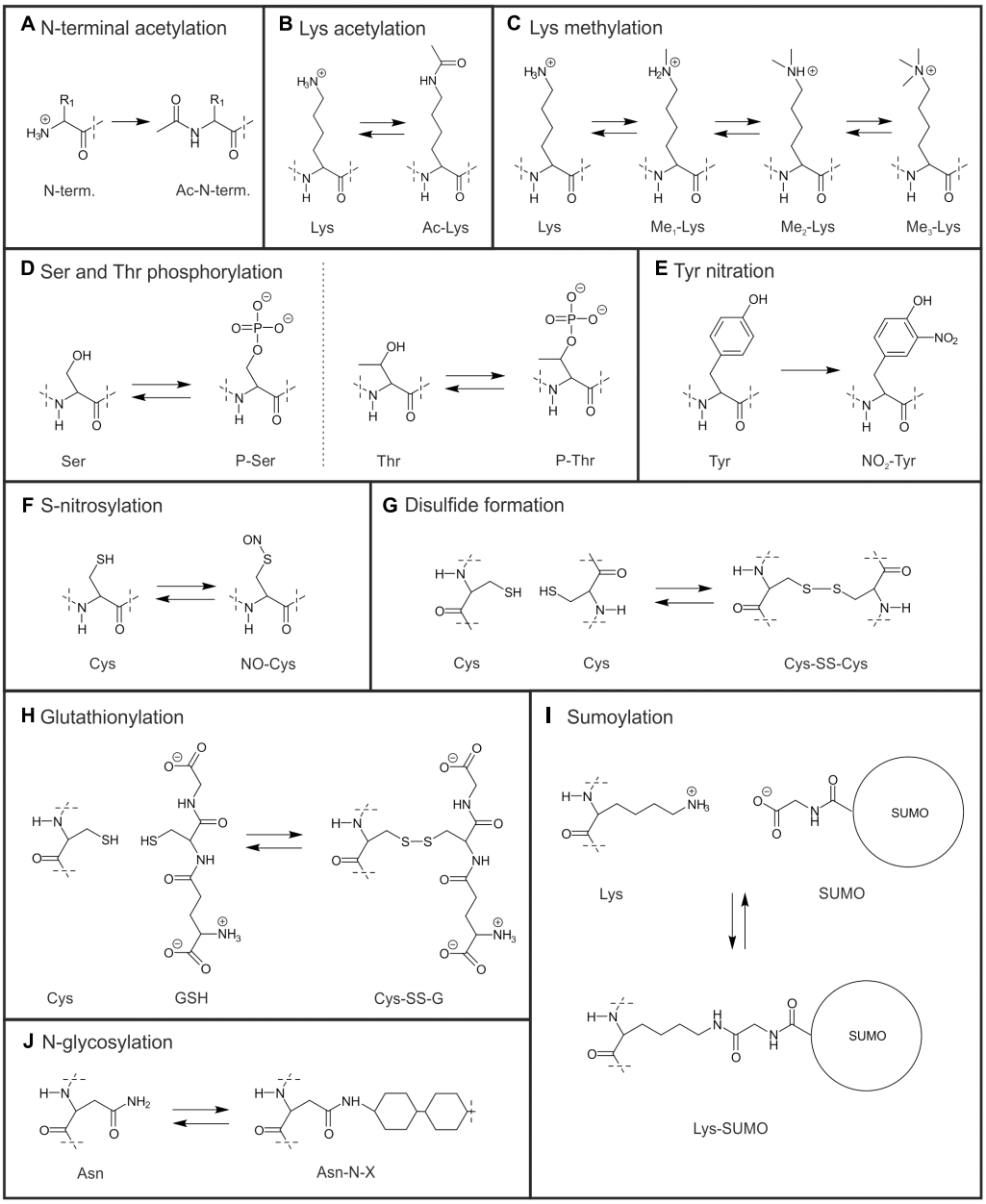

FIGURE 1 | Molecular structures of chloroplast Post-translational modifications (PTMs). (A) N-terminal acetylation (Ac denotes acetyl group and N-term. the N-terminal amino acid of a protein). **(B)** Lys acetylation. **(C)** Lys mono-, di- and trimethylation (Me denotes methyl group). **(D)** Ser and Thr phosphorylation (P denotes phospho group). **(E)** Tyr nitration. **(F)** S-nitrosylation. **(G)** Disulfide formation (-SS- denotes disulfide bridge). **(H)** Glutathionylation of Cys (GSH denotes reduced and G oxidized glutathione). **(I)** Lys sumoylation. **(J)** N-glycosylation of Asn (-N-X denotes N-linked glycosyl group). Dash line indicates where structures have been cut off.

which are encoded both by the nuclear and chloroplast genomes (Carroll, 2013). Several ribosomal proteins in chloroplasts are targets of extensive PTMs, including formyl group or formyl methionine removal, N- and C-terminal processing, acetylation and monomethylation of N-terminal amino acids, trimethylation of Lys (Kamp et al., 1987; Schmidt et al., 1992; Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000; Alban et al., 2014) as well as phosphorylation (Guitton et al., 1984; Posno et al., 1984; Wagner et al., 2006). Recently, the enzyme responsible for the trimethylation of the internal Lys in Arabidopsis plastid ribosomal protein L11 (RPL11) has been identified as PrmA-like (Protein Arg methyltransferase-like) protein (Alban et al., 2014; Mazzoleni et al., 2015). Although depletion of Arabidopsis *PRMA-like* gene did not result in any phenotypic effects, mapping of the trimethylated Lys on the surface of the RPL11 protein allows hypothesizing that methylation might influence the stalk

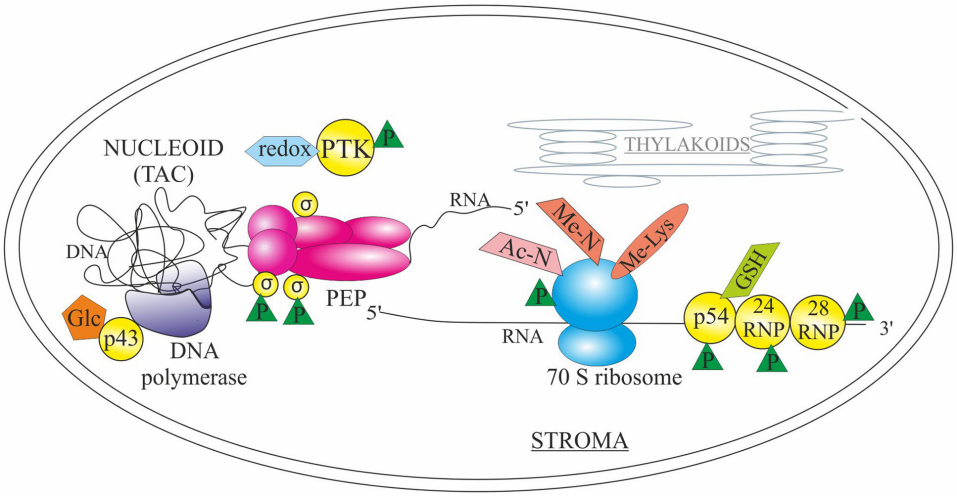

FIGURE 2 | Post-translational modifications in the regulation of plastid machinery for DNA replication and gene expression. Glycosylation (Glc) of chloroplast protein p43 induces the activity of DNA polymerase. The sigma factors (σ) associated with the plastid encoded RNA polymerase PEP are regulated by (de)phosphorylation (P) conveyed by the plastid transcription kinase (PTK), which is autophosphorylated and redox-regulated. Glutathione-mediated redox regulation (GSH) and phosphorylation of endoribonuclease p54 affects the processing activity of *trnK* and *rps16* transcripts. Phosphorylation of RNA-binding proteins 24RNP and 28RNP affect the binding capacity to the 3′ end of chloroplast transcripts. RPL11 subunit of the 70S ribosome is trimethylated (Me-Lys), and ribosomes are also subjected to acetylation and monomethylation of N-terminal amino acid (Ac-N and Me-N, respectively), as well as phosphorylation. See text for details.

region, which is responsible for the recruitment of initiation, elongation and release factors (Mazzoleni et al., 2015).

A special case in the chloroplast gene expression processes is the regulation of *psbA* gene expression, which has been under intense study for decades. The *psbA* gene encodes the light-sensitive PSII core subunit D1, which is constantly degraded and resynthesized in a light-responsive PSII repair cycle (Aro et al., 1993; Mulo et al., 2008). It has been shown that in chloroplasts of green algae and higher plants *psbA* gene expression is mainly controlled at post-transcriptional levels (Mulo et al., 2012). In *Chlamydomonas reinhardtii*, ADP-dependent phosphorylation of the cPDI (chloroplast protein disulfide isomerase or RB60) protein in darkness leads to release of the protein from the 5′ UTR of *psbA* mRNA and cessation of translation (Danon and Mayfield, 1994). Additionally, binding of RB47 to the *psbA* mRNA is controlled via redox regulation of disulfide groups in RB60 (Danon and Mayfield, 1994; Alergand et al., 2006). It has also been hypothesized that phosphorylation of the spinach 28RNP (in addition to participating in 3′ UTR processing, see above) and ribosomal protein(s) might provide a light-dependent translation control mechanism for the chloroplast, especially during the repair cycle of PSII (Lisitsky and Schuster, 1995; Trebitsh et al., 2000; Yamaguchi and Subramanian, 2003).

LIGHT REACTIONS OF PHOTOSYNTHESIS

Light reactions of photosynthesis, i.e., capture of light energy by the light harvesting complex (LHC) for the production of reducing power (NADPH) occur at the thylakoid membrane via the thylakoid-embedded pigment-protein complexes, namely PSII, Cyt b_6f, and PSI. Concomitantly, protons are pumped into the thylakoid lumen, and ADP is photophosphorylated to ATP upon release of the generated proton gradient via the ATP synthase (**Figure 3**). NADPH and ATP, in turn, are used for numerous reactions, carbon assimilation being the major process. PSII functions as an oxygen-plastoquinone oxidoreductase, which is prone to light-induced photoinhibition (Aro et al., 1993; Tyystjärvi, 2013). The PSII core proteins D1 and D2 as well as the inner antenna protein CP43 and a minor PSII subunit PsbH are targets for light-dependent Thr phosphorylation (**Figure 3**) catalyzed mainly by the STN8 kinase (Bellafiore et al., 2005; Bonardi et al., 2005; Fristedt and Vener, 2011), while the PSII CORE PHOSPHATASE is responsible for the reverse reaction (i.e., dephosphorylation; Samol et al., 2012). PSII protein phosphorylation is involved in the folding of the thylakoid membrane, which affects the lateral migration of damaged D1 protein from grana stacks to stroma lamellae for degradation and resynthesis (Tikkanen et al., 2008; Fristedt et al., 2009). Another well-studied phosphorylation process is involved in the balancing electron transfer between PSII and PSI according to ambient environmental cues (i.e., light quality and quantity). Phosphorylation of the light harvesting proteins Lhcb1, Lhcb2 and Lhcb4 is catalyzed by the STN7 kinase (Depege et al., 2003; Bellafiore et al., 2005), and instead of PSII, the phosphorylated LHC trimers deliver excitation energy to PSI (so called state transitions) to adjust the absorption cross sections of the two PSs (Rochaix, 2014). Dephosphorylation of LHC by the PPH1/TAP38 (chloroplast protein phosphatase/thylakoid associated phosphatase of 38 kDa) protein phosphatase, in turn, results in redistribution of excitation energy toward PSII (Pribil et al., 2010; Shapiguzov et al., 2010). The STN7 kinase is activated by the binding of plastoquinol to the Q_o site of Cyt b_6f complex

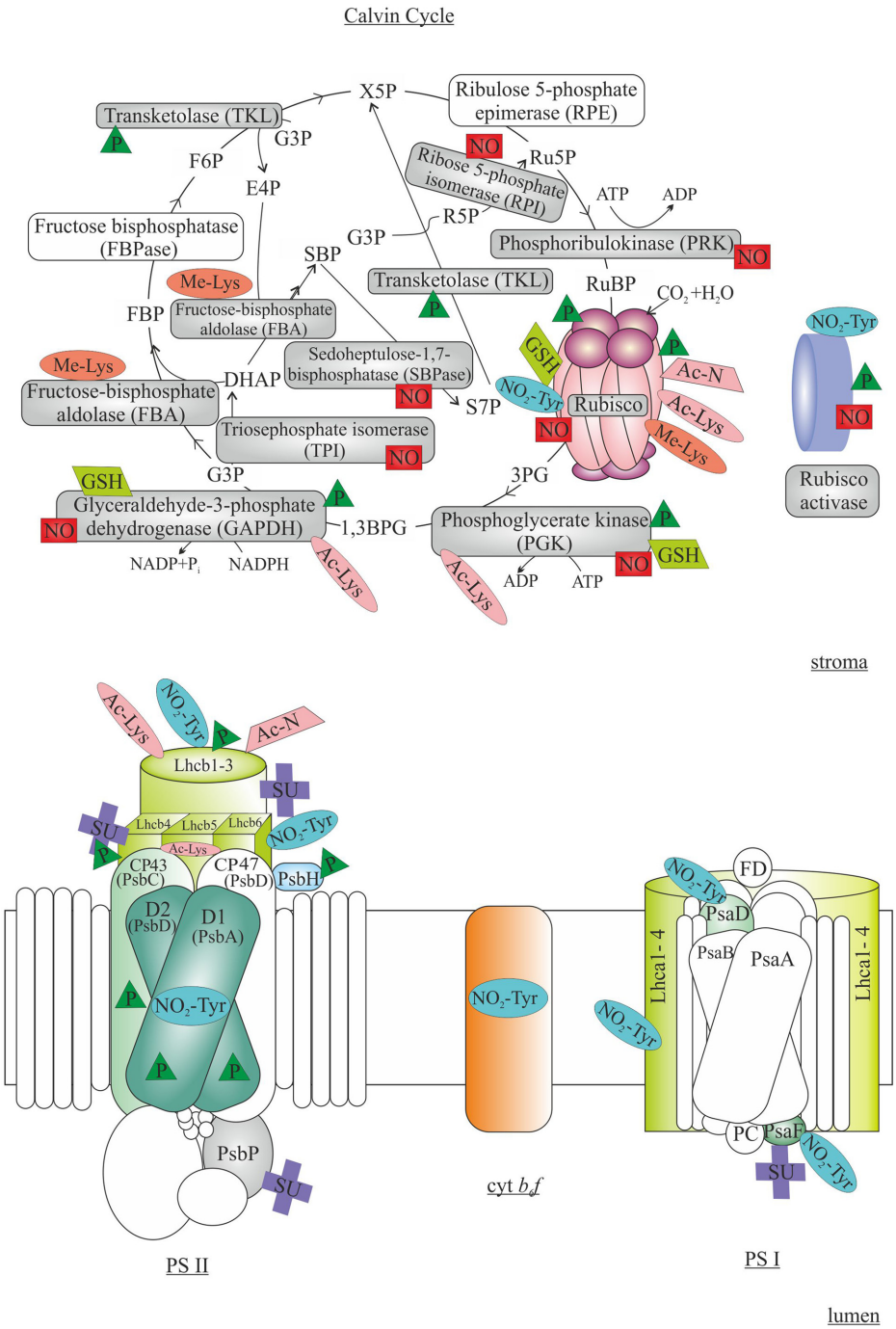

FIGURE 3 | Post-translational modifications in regulation of photosynthetic light reactions (Lower) and Calvin cycle (Upper). In light reactions, phosphorylation (P) of Photosystem (PS) II subunits D1, D2, CP43 and PsbH are involved in PSII repair cycle, while phosphorylation of light harvesting proteins (Lhcb) is required for state transitions. Tyr nitration (NO_2-Tyr), N-terminal and Lys acetylation (Ac-N and Ac-Lys, respectively) and sumoylation (SU) of various PSII, Cytochrome b_6f (Cyt b_6f) and PSI subunits have been detected. In Calvin cycle, the function of Rubisco is controlled by a multitude of PTMs, including phosphorylation, Tyr-nitration, acetylation, Lys methylation (Me-Lys), nitrosylation (NO) and glutathionylation (GSH). Additionally, several other enzymes functioning in the Calvin cycle and activation of Rubisco are targets of various PTMs. The subchloroplastic sites of the PTMs are not indicated in the figure. 3PG, 3-phosphoglycerate; 1,3BPG, 1,3-bisphosphoglycerate; G3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; F6P, fructose 6-phosphate; X5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate; S7P, sedoheptulose 7-phosphate; SBP, sedoheptulose 1,7-bisphosphate; E4P, erythrose 4-phosphate. The modified proteins are indicated with colors, while the non-modified proteins are shown as transparent. See text for details.

(Vener et al., 1997; Lemeille et al., 2009) and inhibited by stromal reductants (Rintamäki et al., 2000).

In addition to phosphorylation, LHC proteins are prone to various other PTMs (**Figure 3**), such as N-terminal acetylation (Michel et al., 1991; Wu et al., 2011; Rowland et al., 2015), Lys-acetylation (Finkemeier et al., 2011; Wu et al., 2011), Tyr-nitration (Galetskiy et al., 2011b) and sumoylation (Elrouby and Coupland, 2010; López-Torrejón et al., 2013). As acetylation neutralizes the positive charge either on the protein N-terminus or on Lys residue, it has numerous implications in biologic processes including determination of enzyme activity, protein stability and mediation of protein–protein interactions (Hwang et al., 2010; Bienvenut et al., 2011; Scott et al., 2011; Hoshiyasu et al., 2013). Accordingly, acetylation of the Lhcb1 and Lhcb2 proteins appear to be involved in the regulation of LHC attachment to the PSII complexes: the peripheral LHC antenna loosely bound to PSII showed higher level of Lys acetylation than the PSII-LHCII supercomplexes (Wu et al., 2011). In contrast to phosphorylation, acetylation status did not respond to changes in illumination (Wu et al., 2011). It is also worth noting that only the N-terminally trimmed form of Lhcb5 starting with Leu_{38} (other forms starting with Phe_{39} or Ser_{40}) were reported to be Lys acetylated, indicating a cross-talk between N-terminal processing and acetylation of chloroplast proteins (Wu et al., 2011). Neither the chloroplast acetylation machinery (Dinh et al., 2015) nor the enzymes responsible for N-terminal processing (Rowland et al., 2015) have been thoroughly characterized yet. Also Tyr nitration of proteins representing PSII (including D1), Cyt b_6f, PSI as well as LHC has been detected (Galetskiy et al., 2011a,b). Protein Tyr nitration is a marker of nitrosative stress, and it can irreversibly modify the conformation of proteins thus affecting the catalytic activity and susceptibility to proteolysis (Corpas et al., 2007). Indeed, changes in light conditions resulted in variation in nitration levels in different PSII-LHCII complexes, suggesting that nitration might be involved in photodamage, disassembly of complexes and subsequent degradation of proteins (Galetskiy et al., 2011a). It has also been found that LHC may be post-translationally modified by sumoylation (Elrouby and Coupland, 2010; López-Torrejón et al., 2013), which refers to covalent binding of the small ubiquitin-like modifier (SUMO) protein (Miura et al., 2007). Sumoylation has been implicated in the regulation of protein localization, interactions and catalytic activity (Vierstra, 2012). Obviously, the exact effects of these PTMs on the function of LHC require further studies.

CARBON ASSIMILATION AND STARCH METABOLISM

The photosynthetic carbon reduction cycle, i.e., the Calvin cycle, is a multistep pathway in which redox equivalents and chemical energy (NADPH and ATP) originating from the light reactions is utilized for the reduction of atmospheric carbon dioxide into organic compounds. Calvin cycle involves 11 stromal enzymes, which catalyze 13 distinct reactions. In the first step, inorganic CO_2 is fixed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), producing 3-phosphoglycerate (3PG), which is first phosphorylated and then reduced into glyceraldehyde-3-phosphate (G3P). G3P then exits from the Calvin cycle and is further used for the synthesis of more complex sugars, including starch that is the most abundant storage polyglucan in nature (Tetlow and Emes, 2014). Several Calvin cycle enzymes have been reported to be activated in light upon reduction of specific disulfide bonds by thioredoxin (Pedersen et al., 1966; Jensen and Bassham, 1968; Buchanan and Wolosiuk, 1976; Wolosiuk and Buchanan, 1976; Buchanan, 1980). In addition to redox regulation, all steps of CO_2 fixation and starch metabolism are carefully controlled by multiple (PTM-dependent) mechanisms which balance the rate of starch synthesis with the availability of energy and carbon in different plant tissues and under various environmental conditions (**Figure 3**).

Rubisco

In terrestrial plants and green alga, Rubisco exists as a holocomplex composed of eight nuclear-encoded small subunits (RBCS) and eight plastid-encoded large subunits (RBCL). Among the other enzymes involved in Calvin cycle Rubisco has been reported as a target of reversible phosphorylation in many plant species (**Figure 3**) (Reiland et al., 2009, 2011; Facette et al., 2013; Wang et al., 2014; Roitinger et al., 2015), RBCL being phosphorylated in response to light (Budde and Randall, 1990; Wang et al., 2014). The RBCL and RBCS subunits of Rubisco have been shown to contain multiple phosphorylation sites (Cao et al., 2011; Wang et al., 2014). Phosphorylation of the highly conserved RBCL residues Ser_{208}, Thr_{246}, Tyr_{239} and Thr_{330}, located in the close proximity to RuBP binding site, might affect the catalytic activity of the enzyme (Lohrig et al., 2009; Hodges et al., 2013). Indeed, dephosphorylation of RBCL has been shown to result in decreased activity of the enzyme (Chen et al., 2011), perhaps via affecting the interaction between Rubisco and RA (Guitton and Mache, 1987; Aggarwal et al., 1993; Hodges et al., 2013). Moreover, it has been suggested that dephosphorylation of RBCL and/or RBCS may lead to dissociation of Rubisco holocomplex (Guitton and Mache, 1987; Aggarwal et al., 1993; Hodges et al., 2013).

Rubisco has also been found as a target of both N-terminal acetylation and Lys acetylation (**Figure 3**). In spinach, RBCL is post-translationally processed by removal of Met_1 and Ser_2 followed by the acetylation of the penultimate amino acid (Mulligan et al., 1988). Although N-acetylation of proteins in general is known to modify their activity and stability, the detailed significance and mechanism of RBCL N-termini modification remains unknown (Mulligan et al., 1988; Houtz et al., 1992; Zybailov et al., 2008). Lys acetylation of the Rubisco subunits has been identified only recently, and it has been reported as a dynamic modification in response to the changes in the energy status in plants under different light conditions (Gao et al., 2016). The Rubisco holocomplex contains multiple Lys acetylation sites (e.g., nine in Arabidopsis and thirteen in wheat; Finkemeier et al., 2011), which are localized either in the catalytic center of Rubisco (Cleland et al., 1998; Finkemeier et al., 2011), at the interface between the two RBCL subunits (Knight et al., 1990; Finkemeier et al., 2011) or at the site crucial for

the formation of tertiary structure of Rubisco (Knight et al., 1990). Therefore, Lys-acetylation has been suspected to affect Rubisco activity and interactions between the subunits and with other molecules, and indeed recent studies have shown negative regulation of Rubisco activity by Lys acetylation (Finkemeier et al., 2011; Gao et al., 2016). Thus, acetylation of Rubisco might provide a mechanism to coordinate the function of light reactions and carbon assimilation with the carbon status of the cell.

In addition to acetylation, Lys residues of RBCL may be methylated (**Figure 3**). In many organisms (e.g., pea and tobacco), RBCL is considered as the main stromal methylprotein (Alban et al., 2014). Trimethylation of RBCL at Lys_{14} has been found in several plant species (Alban et al., 2014; Ma et al., 2016) as a modification catalyzed by the large subunit Rubisco methyltransferase (LSMT), a highly conserved SET-domain protein lysine methyltransferase found in all plant species (Dirk et al., 2006). Despite numerous studies, the role of Lys_{14} trimethylation of RBCL (as well as the role of methylation for other chloroplastic methylproteins) has not been identified (Clarke, 2013; Ma et al., 2016). Interestingly, in Arabidopsis, spinach, and wheat plants RBCL is not methylated at Lys_{14} indicating species-specific differences in regulatory mechanisms (Houtz et al., 1992; Mininno et al., 2012; Ma et al., 2016). The methylation of chloroplast proteins seems to be biologically important, as a mutant impaired in PTAC14 (plastid-located SET-domain methyltransferase) exhibits defects in chloroplast differentiation and shows an albino phenotype (Steiner et al., 2011). On the other hand, the LSMT knockdown plants do not show any decrease in CO_2 assimilation and growth (Mininno et al., 2012).

Intriguingly, some Calvin cycle enzymes, including Rubisco, have been reported to be modified by peroxynitrite (**Figure 3**) (Cecconi et al., 2009; Lozano-Juste et al., 2011; Barroso et al., 2013). It has been suggested that Tyr-nitration of RBCL (and RA) might act as a modulator of plant defense-related responses including hypersensitive responses (Cecconi et al., 2009). On the other hand, Tyr-nitration of abundant proteins such as those involved in carbon metabolism might function as a non-specific scavenging system for reactive nitrogen forms under stress conditions. Reversibility of Tyr-nitration is still discussed, thus additionally raising new questions about a potential function as a specific signaling event (Souza et al., 2008; Baudouin, 2011).

The reversible S-nitrosylation of Rubisco Cys residues has been reported both *in vitro* and *in vivo* for several plant species in response to nitric oxide (NO) -releasing compounds or to abiotic stresses (**Figure 3**) (Abat et al., 2008; Abat and Deswal, 2009; Fares et al., 2011; Vanzo et al., 2016). As the redox-active thiols in Cys residues can be modified by the covalent binding of NO resulting in the formation of S-nitrosothiol (Lindermayr et al., 2005), it is plausible that S-nitrosylation of Cys residues adjacent to the Rubisco active site in Arabidopsis might regulate the activity of the enzyme and degradation of the protein (Takahashi and Yamasaki, 2002; Marcus et al., 2003; Romero-Puertas et al., 2008). Indeed, recent enzymatic activity assays have revealed that Rubisco inactivation in response to S-nitrosylation is probably the main cause of reduction in carbon fixation upon various stress conditions (Clark et al., 2000; Abat et al., 2008; Abat and Deswal, 2009).

Another modification of Cys residues is protein S-glutathionylation, a well-described mechanism of signal transduction and protein regulation in mammals (Chrestensen et al., 2000). S-glutathionylation is a reversible post-translational formation of a mixed disulfide between the Cys residue of protein and glutathione. Previously, three Cys residues in RBCL and one in RBCS have been identified as targets of S-glutathionylation in plants (Rouhier et al., 2005), green alga (Zaffagnini et al., 2012a) and cyanobacteria (Sakr et al., 2013; Chardonnet et al., 2015). Protein S-glutathionylation probably protects specific Cys residues against irreversible oxidation under stress conditions (Ito et al., 2003; Zaffagnini et al., 2012b), but this PTM can also result in modulation of protein activity (Klatt and Lamas, 2000; Fratelli et al., 2004) and localization (Chardonnet et al., 2015). Nevertheless, the functional significance of Rubisco S-glutathionylation is not known yet.

Activation and Function of the Calvin Cycle

Although PTMs of Rubisco have been extensively studied, also numerous other enzymes involved in carbon assimilation have been shown to possess multiple PTMs (**Figure 3**). As RA is responsible for removing inhibitors from Rubisco active center and thus contributes to initiation of carbon fixation, the stimuli affecting the RA activity is reflected in the yield of the entire carbon assimilation cycle. In green alga *C. reinhardtii,* RA is phosphorylated at Ser_{53} by the thylakoid-localized Stn7 ortholog Stt7 kinase (see above; Lemeille et al., 2010). RA is mainly localized in the stroma, but a smaller portion of the enzyme has been found in association with the thylakoid membrane (Jin et al., 2006). It has been suggested that phosphorylation of RA increases the attachment of RA to the membrane, protecting Stt7 against proteolysis (Lemeille et al., 2009, 2010). The relocation could also be a mechanism reducing the activity of Rubisco under specific environmental conditions (Lemeille et al., 2010). In Arabidopsis plants, RA is phosphorylated at two sites, Thr_{78} and Ser_{172} (Boex-Fontvieille et al., 2014). In the dark, the phosphorylation percentage of Thr_{78} increases (Reiland et al., 2009; Kim et al., 2016). As Thr_{78} is located in the region crucial for Rubisco interaction (Zhang and Portis, 1999; Kim et al., 2016), it has been suggested that Thr_{78} phosphorylation inhibits Rubisco activation (van de Loo and Salvucci, 1996; Stotz et al., 2011; Boex-Fontvieille et al., 2014). However, the importance of Thr_{78} phosphorylation for the Rubisco activation requires further investigation as the Thr_{78} is not conserved and replaced by Ile in maize and rice (Baginsky, 2016).

In addition to Rubisco, three other enzymes involved in Calvin cycle have been reported as phosphoproteins. Phosphoglycerate kinase (PGK) enzyme catalyzing the transfer of phosphate group from ATP to 3PG is phosphorylated in Arabidopsis, rice, and maize plants (Reiland et al., 2009; Facette et al., 2013; Roitinger et al., 2015; Baginsky, 2016). The two latter species

share the identical phosphorylation site VGAVSpSPK whereas in Arabidopsis PGK is phosphorylated in a domain much closer to the N-terminus. The kinase responsible for phosphorylation is unknown, but the phosphorylation motif suggests proline-directed kinase as a possible candidate (Baginsky, 2016). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) possesses several phosphorylation sites, but as the sites differ significantly between different organisms, it is plausible that phosphorylation is not a major determinant of GAPDH activity in chloroplasts (Baginsky, 2016). Moreover, the main transketolase isoform in Arabidopsis (TKL1) is phosphorylated in a Ca^{2+} dependent manner at Ser_{428}, and phosphorylation affects enzyme activity (Rocha et al., 2014). Although Ser_{428} is conserved in higher plants, Ser_{428} has been found phosphorylated only in Arabidopsis plants (Hou et al., 2015; Baginsky, 2016). It is intriguing that PGK and GAPDH are also targets of Lys acetylation and S-glutathionylation (Finkemeier et al., 2011; Zaffagnini et al., 2012a; Chardonnet et al., 2015; Shen et al., 2015). The enzymatic activity of GAPDH and PGK is increased upon deacetylation, but the functional importance of S-glutathionylation of GAPDH and PGK remains to be elucidated (Finkemeier et al., 2011; Shen et al., 2015). These examples indicate that further studies are urgently needed in order to fully understand the dynamic regulation of Calvin cycle enzymes and to pinpoint the responsible enzymes involved (Friso and van Wijk, 2015; Baginsky, 2016).

Furthermore, a number of other enzymes involved in carbon assimilation have been shown to be post-translationally modified. For instance, fructose 1,6-bisphosphate aldolase (FBA) is trimethylated at a conserved Lys residue close to the C-terminus of the protein, however, without any effect on catalytic activity or the oligomeric state of the enzyme (Mininno et al., 2012; Ma et al., 2016). In poplar trees sedoheptulose-bisphosphatase (SBPase), RA, ribose-5-phosphate isomerase (RPI), phosphoribulokinase (PRK), GAPDH, triosephosphate isomerase (TPI), and PGK were S-nitrosylated during short-term oxidative stress induced by NO treatment (Vanzo et al., 2014, 2016), but the functional importance has not been described yet (Lindermayr et al., 2005; Abat et al., 2008; Romero-Puertas et al., 2008; Abat and Deswal, 2009).

Starch Metabolism

Starch synthesis and degradation occur in a coordinated manner on a diurnal basis. In leaves, starch is synthesized during the day and degraded in darkness (Kötting et al., 2010). Reversible protein phosphorylation plays an important role also in the regulation of starch metabolism (Tetlow et al., 2004a, 2008; Grimaud et al., 2008; Reiland et al., 2009), and five different phosphoproteins (phosphoglucose isomerase, phosphoglucomutase, starch synthase and two subunits of ADP-glucose pyrophosphorylase) involved in starch biosynthesis have been identified in Arabidopsis leaves (Geigenberger, 2011). Interestingly, starch synthase has been reported to be phosphorylated in a light dependent manner, i.e., exclusively at the end of the dark period (Reiland et al., 2009). Analyses of amyloplasts and chloroplasts from *Triticum aestivum* (wheat) have shown that some isoforms of starch-branching enzymes (SBE) are catalytically activated by phosphorylation and deactivated by dephosphorylation of one or more of their Ser residues (Tetlow et al., 2004b). Additionally, phosphorylation is apparently involved in the formation of protein complexes composed of starch synthase, SBE isoforms as well as other enzymes with undefined role(s) (Tetlow et al., 2004b; Kötting et al., 2010). It has been speculated that the physical association of the enzymes could alter their activities thus improving the efficiency of starch polymer construction (Kötting et al., 2010; Geigenberger, 2011). Moreover, numerous enzymes involved in starch metabolism, such as glucan water dikinase (GWD, also termed SEX1), starch excess4 (SEX4), β-amylase 1 (BAM1), ADP-glucose pyrophosphorylase, ADP-Glc transporter and class II SBE (Mikkelsen et al., 2005; Balmer et al., 2006; Sokolov et al., 2006; Valerio et al., 2011; Tuncel et al., 2014) are redox activated by thioredoxin. However, it is worth noting that redox modification of starch biosynthesis enzymes in response to light (and other environmental stimuli; reviewed in Kötting et al., 2010; Geigenberger, 2011) is not the only determinant of starch accumulation in plants, but most probably other (PTM-dependent) regulatory mechanisms will be identified in the future (Li et al., 2012).

CONCLUSION

Recently developed new experimental tools, i.e., PTM-specific antibodies and stains as well as enrichment techniques and high quality equipment for mass spectrometry have enabled identification of a range of PTMs in chloroplast proteins. Detailed knowledge about the effects of protein phosphorylation and redox regulation on the photosynthetic reactions already exists, but the regulation of most metabolic pathways in the chloroplast is poorly understood. Because a specific amino acid residue may be targeted by different PTM types (e.g., Lys methylation or Lys acetylation), and because different PTMs may have either antagonistic or cooperative effects, it will be important to reveal the entire PTM code of a protein(s) in order to understand the physiological significance of PTM-mediated regulation in a given metabolic pathway. Future studies are likely to reveal novel modification types as well as molecular mechanisms of PTM-dependent regulation of various metabolic pathways in chloroplasts.

AUTHOR CONTRIBUTIONS

PM, MG, and MK have made substantial intellectual contribution to the work, participated in writing and revised the paper. MK and MG have drawn the figures. All authors have approved the paper for publication.

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REFERENCES

Abat, J. K., and Deswal, R. (2009). Differential modulation of S-nitrosoproteome of *Brassica juncea* by low temperature: change in S-nitrosylation of rubisco is responsible for the inactivation of its carboxylase activity. *Proteomics* 9, 4368–4380. doi: 10.1002/pmic.200800985

Abat, J. K., Mattoo, A. K., and Deswal, R. (2008). S-nitrosylated proteins of a medicinal CAM plant *Kalanchoe pinnata*- ribulose-1,5-bisphosphate carboxylase/oxygenase activity targeted for inhibition. *FEBS J.* 275, 2862–2872. doi: 10.1111/j.1742-4658.2008.06425.x

Aggarwal, K. K., Saluja, D., and Sachar, R. C. (1993). Phosphorylation of rubisco in *Cicer arietinum*: non-phosphoprotein nature of rubisco in *Nicotiana tabacum*. *Phytochemistry* 34, 329–335. doi: 10.1016/0031-9422(93)80004-C

Alban, C., Tardif, M., Mininno, M., Brugiere, S., Gilgen, A., Ma, S., et al. (2014). Uncovering the protein lysine and arginine methylation network in *Arabidopsis* chloroplasts. *PLoS ONE* 9:e95512. doi: 10.1371/journal.pone.0095512

Alergand, T., Peled-Zehavi, H., Katz, Y., and Danon, A. (2006). The chloroplast protein disulfide isomerase RB60 reacts with a regulatory disulfide of the RNA-binding protein RB47. *Plant Cell Physiol.* 47, 540–548.

Aro, E. M., Virgin, I., and Andersson, B. (1993). Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochim. Biophys. Acta* 1143, 113–134.

Baena-González, E., Baginsky, S., Mulo, P., Summer, H., Aro, E.-M., and Link, G. (2001). Chloroplast transcription at different light intensities. Glutathione-mediated phosphorylation of the major RNA polymerase involved in redox-regulated organellar gene expression. *Plant Physiol.* 127, 1044–1052. doi: 10.1104/pp.010168

Baginsky, S. (2016). Protein phosphorylation in chloroplasts - a survey of phosphorylation targets. *J. Exp. Bot.* 67, 3873–3882. doi: 10.1093/jxb/erw098

Baginsky, S., Tiller, K., and Link, G. (1997). Transcription factor phosphorylation by a protein kinase associated with chloroplast RNA polymerase from mustard (*Sinapis alba*). *Plant Mol. Biol.* 34, 181–189. doi: 10.1023/A:1005802909902

Baginsky, S., Tiller, K., Pfannschmidt, T., and Link, G. (1999). PTK, the chloroplast RNA polymerase-associated protein kinase from mustard (*Sinapis alba*), mediates redox control of plastid in vitro transcription. *Plant Mol. Biol.* 39, 1013–1023. doi: 10.1023/A:1006177807844

Balmer, Y., Vensel, W. H., Hurkman, W. J., and Buchanan, B. B. (2006). Thioredoxin target proteins in chloroplast thylakoid membranes. *Antioxid. Redox Signal.* 8, 1829–1834. doi: 10.1089/ars.2006.8.1829

Barroso, J. B., Valderrama, R., and Corpas, F. J. (2013). Immunolocalization of S-nitrosoglutathione, S-nitrosoglutathione reductase and tyrosine nitration in pea leaf organelles. *Acta Physiol. Plant* 35, 2635–2640. doi: 10.1007/s11738-013-1291-0

Baudouin, E. (2011). The language of nitric oxide signalling. *Plant Biol.* 13, 233–242. doi: 10.1111/j.1438-8677.2010.00403.x

Bellafiore, S., Barneche, F., Peltier, G., and Rochaix, J. D. (2005). State transitions and light adaptation require chloroplast thylakoid protein kinase STN7. *Nature* 433, 892–895.

Bienvenut, W. V., Espagne, C., Martinez, A., Majeran, W., Valot, B., Zivy, M., et al. (2011). Dynamics of post-translational modifications and protein stability in the stroma of *Chlamydomonas reinhardtii* chloroplasts. *Proteomics* 11, 1734–1750. doi: 10.1002/pmic.201000634

Boex-Fontvieille, E., Daventure, M., Jossier, M., Hodges, M., Zivy, M., and Tcherkez, G. (2014). Phosphorylation pattern of rubisco activase in *Arabidopsis* leaves. *Plant Biol.* 16, 550–557. doi: 10.1111/plb.12100

Bonardi, V., Pesaresi, P., Becker, T., Schleiff, E., Wagner, R., Pfannschmidt, T., et al. (2005). Photosystem II core phosphorylation and photosynthetic acclimation require two different protein kinases. *Nature* 437, 1179–1182.

Buchanan, B. B. (1980). Role of light in the regulation of chloroplast enzymes. *Ann. Rev. Plant Physiol.* 31, 341–371. doi: 10.1146/annurev.pp.31.060180.002013

Buchanan, B. B., and Balmer, Y. (2005). Redox regulation: a broadening horizon. *Annu. Rev. Plant Biol.* 56, 187–220. doi: 10.1146/annurev.arplant.56.032604.144246

Buchanan, B. B., and Wolosiuk, R. A. (1976). Photosynthetic regulatory protein found in animal and bacterial cells. *Nature* 264, 669–670. doi: 10.1038/264669a0

Budde, R. J., and Randall, D. D. (1990). Light as a signal influencing the phosphorylation status of plant proteins. *Plant Physiol.* 94, 1501–1504.

Cao, X., Gao, Y., Wang, Y., Li, C. M., Zhao, Y. B., Han, Z. H., et al. (2011). Differential expression and modification of proteins during ontogenesis in *Malus domestica*. *Proteomics* 11, 4688–4701. doi: 10.1002/pmic.201100132

Carroll, A. J. (2013). The *Arabidopsis* cytosolic ribosomal proteome: from form to function. *Front. Plant Sci.* 4:32. doi: 10.3389/fpls.2013.00032

Cecconi, D., Orzetti, S., Vandelle, E., Rinalducci, S., Zolla, L., and Delledonne, M. (2009). Protein nitration during defense response in *Arabidopsis thaliana*. *Electrophoresis* 30, 2460–2468. doi: 10.1002/elps.200800826

Chardonnet, S., Sakr, S., Cassier-Chauvat, C., Le Marechal, P., Chauvat, F., Lemaire, S. D., et al. (2015). First proteomic study of S-glutathionylation in cyanobacteria. *J. Proteome Res.* 14, 59–71. doi: 10.1021/pr500625a

Chen, W., Gaikwad, A., Mukherjee, S. K., Choudhary, N. R., Kumar, D., and Tewari, K. K. (1996). A 43 kDa DNA binding protein from the pea chloroplast interacts with and stimulates the cognate DNA polymerase. *Nucleic Acids Res.* 24, 3953–3961. doi: 10.1093/nar/24.20.3953

Chen, X., Zhang, W., Zhang, B., Zhou, J., Wang, Y., Yang, Q., et al. (2011). Phosphoproteins regulated by heat stress in rice leaves. *Proteome Sci.* 9:37. doi: 10.1186/1477-5956-9-37

Chrestensen, C. A., Starke, D. W., and Mieyal, J. J. (2000). Acute cadmium exposure inactivates thioltransferase (glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. *J. Biol. Chem.* 275, 26556–26565. doi: 10.1074/jbc.M004097200

Clark, D., Durner, J., Navarre, D. A., and Klessig, D. F. (2000). Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. *Mol. Plant Microbe Interact.* 13, 1380–1384. doi: 10.1094/MPMI.2000.13.12.1380

Clarke, S. G. (2013). Protein methylation at the surface and buried deep: thinking outside the histone box. *Trends Biochem. Sci.* 38, 243–252. doi: 10.1016/j.tibs.2013.02.004

Cleland, W. W., Andrews, T. J., Gutteridge, S., Hartman, F. C., and Lorimer, G. H. (1998). Mechanism of rubisco: the carbamate as general base. *Chem. Rev.* 98, 549–562.

Corpas, F. J., del Rio, L. A., and Barroso, J. B. (2007). Need of biomarkers of nitrosative stress in plants. *Trends Plant Sci.* 12, 436–438.

Danon, A., and Mayfield, S. P. (1994). ADP-dependent phosphorylation regulates RNA-binding in vitro: implications in light-modulated translation. *EMBO J.* 13, 2227–2235.

Depege, N., Bellafiore, S., and Rochaix, J. D. (2003). Role of chloroplast protein kinase Stt7 in LHCII phosphorylation and state transition in *Chlamydomonas*. *Science* 299, 1572–1575. doi: 10.1126/science.1081397

Dinh, T. V., Bienvenut, W. V., Linster, E., Feldman-Salit, A., Jung, V. A., Meinnel, T., et al. (2015). Molecular identification and functional characterization of the first Nα-acetyltransferase in plastids by global acetylome profiling. *Proteomics* 15, 2426–2435. doi: 10.1002/pmic.201500025

Dirk, L. M., Trievel, R. C., and Houtz, R. L. (2006). 7 non-histone protein lysine methyltransferases: structure and catalytic roles. *Enzymes* 24, 179–228. doi: 10.1016/S1874-6047(06)80009-0

Elrouby, N., and Coupland, G. (2010). Proteome-wide screens for small ubiquitin-like modifier (SUMO) substrates identify *Arabidopsis* proteins implicated in diverse biological processes. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17415–17420. doi: 10.1073/pnas.1005452107

Facette, M. R., Shen, Z., Bjornsdottir, F. R., Briggs, S. P., and Smith, L. G. (2013). Parallel proteomic and phosphoproteomic analyses of successive stages of maize leaf development. *Plant Cell* 25, 2798–2812. doi: 10.1105/tpc.113.112227

Fares, A., Rossignol, M., and Peltier, J. B. (2011). Proteomics investigation of endogenous S-nitrosylation in *Arabidopsis*. *Biochem. Biophys. Res. Commun.* 416, 331–336. doi: 10.1016/j.bbrc.2011.11.036

Finkemeier, I., Laxa, M., Miguet, L., Howden, A. J., and Sweetlove, L. J. (2011). Proteins of diverse function and subcellular location are lysine acetylated in *Arabidopsis*. *Plant Physiol.* 155, 1779–1790. doi: 10.1104/pp.110.171595

Fratelli, M., Gianazza, E., and Ghezzi, P. (2004). Redox proteomics: identification and functional role of glutathionylated proteins. *Expert Rev. Proteomics* 1, 365–376. doi: 10.1586/14789450.1.3.365

Friso, G., and van Wijk, K. J. (2015). Posttranslational protein modifications in plant metabolism. *Plant Physiol.* 169, 1469–1487. doi: 10.1104/pp.15.01378

Fristedt, R., and Vener, A. V. (2011). High light induced disassembly of photosystem II supercomplexes in *Arabidopsis* requires STN7-dependent phosphorylation of CP29. *PLoS ONE* 6:e24565. doi: 10.1371/journal.pone.0024565

Fristedt, R., Willig, A., Granath, P., Crevecoeur, M., Rochaix, J. D., and Vener, A. V. (2009). Phosphorylation of photosystem II controls functional macroscopic folding of photosynthetic membranes in *Arabidopsis*. *Plant Cell* 21, 3950–3964. doi: 10.1105/tpc.109.069435

Gaikwad, A., Tewari, K. K., Kumar, D., Chen, W., and Mukherjee, S. K. (1999). Isolation and characterisation of the cDNA encoding a glycosylated accessory protein of pea chloroplast DNA polymerase. *Nucleic Acids Res.* 27, 3120–3129. doi: 10.1093/nar/27.15.3120

Gaikwad, A., Van Hop, D., and Mukherjee, S. K. (2000). Carboxy terminal region of a chloroplast DNA polymerase accessory factor stimulates DNA polymerase activity. *Indian J. Biochem. Biophys.* 37, 424–432.

Galetskiy, D., Lohscheider, J. N., Kononikhin, A. S., Popov, I. A., Nikolaev, E. N., and Adamska, I. (2011a). Mass spectrometric characterization of photooxidative protein modifications in *Arabidopsis thaliana* thylakoid membranes. *Rapid Commun. Mass Spectrom.* 25, 184–190. doi: 10.1002/rcm.4855

Galetskiy, D., Lohscheider, J. N., Kononikhin, A. S., Popov, I. A., Nikolaev, E. N., and Adamska, I. (2011b). Phosphorylation and nitration levels of photosynthetic proteins are conversely regulated by light stress. *Plant Mol. Biol.* 77, 461–473. doi: 10.1007/s11103-011-9824-7

Gao, X., Hong, H., Li, W. C., Yang, L., Huang, J., Xiao, Y. L., et al. (2016). Downregulation of Rubisco activity by non-enzymatic acetylation of RbcL. *Mol. Plant.* 9, 1018–1027. doi: 10.1016/j.molp.2016.03.012

Geigenberger, P. (2011). Regulation of starch biosynthesis in response to a fluctuating environment. *Plant Physiol.* 155, 1566–1577. doi: 10.1104/pp.110.170399

Green, B. R. (2011). Chloroplast genomes of photosynthetic eukaryotes. *Plant J.* 66, 34–44. doi: 10.1111/j.1365-313X.2011.04541.x

Grimaud, F., Rogniaux, H., James, M. G., Myers, A. M., and Planchot, V. (2008). Proteome and phosphoproteome analysis of starch granule-associated proteins from normal maize and mutants affected in starch biosynthesis. *J. Exp. Bot.* 59, 3395–3406. doi: 10.1093/jxb/ern198

Guitton, C., Dorne, A. M., and Mache, R. (1984). In organello and in vitro phosphorylation of chloroplast ribosomal proteins. *Biochem. Biophys. Res. Commun.* 121, 297–303.

Guitton, C., and Mache, R. (1987). Phosphorylation in vitro of the large subunit of the ribulose-1,5-bisphosphate carboxylase and of the glyceraldehyde-3-phosphate dehydrogenase. *Eur. J. Biochem.* 166, 249–254. doi: 10.1111/j.1432-1033.1987.tb13509.x

Hayes, R., Kudla, J., Schuster, G., Gabay, L., Maliga, P., and Gruissem, W. (1996). Chloroplast mRNA 3′-end processing by a high molecular weight protein complex is regulated by nuclear encoded RNA binding proteins. *EMBO J.* 15, 1132–1141.

Hodges, M., Jossier, M., Boex-Fontvieille, E., and Tcherkez, G. (2013). Protein phosphorylation and photorespiration. *Plant Biol.* 15, 694–706. doi: 10.1111/j.1438-8677.2012.00719.x

Hoshiyasu, S., Kohzuma, K., Yoshida, K., Fujiwara, M., Fukao, Y., Yokota, A., et al. (2013). Potential involvement of N-terminal acetylation in the quantitative regulation of the epsilon subunit of chloroplast ATP synthase under drought stress. *Biosci. Biotechnol. Biochem.* 77, 998–1007.

Hou, Y., Qiu, J., Tong, X., Wei, X., Nallamilli, B. R., Wu, W., et al. (2015). A comprehensive quantitative phosphoproteome analysis of rice in response to bacterial blight. *BMC Plant Biol.* 15:163. doi: 10.1186/s12870-015-0541-2

Houtz, R. L., Poneleit, L., Jones, S. B., Royer, M., and Stults, J. T. (1992). Posttranslational modifications in the amino- terminal region of the large subunit of ribulose- 1,5-bisphosphate carboxylase/oxygenase from several plant species. *Plant Physiol.* 98, 1170–1174.

Hwang, C. S., Shemorry, A., and Varshavsky, A. (2010). N-terminal acetylation of cellular proteins creates specific degradation signals. *Science* 327, 973–977. doi: 10.1126/science.1183147

Ito, H., Iwabuchi, M., and Ogawa, K. (2003). The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in *Arabidopsis thaliana*: detection using biotinylated glutathione. *Plant Cell Physiol.* 44, 655–660. doi: 10.1093/pcp/pcg098

Jensen, R. G., and Bassham, J. A. (1968). Photosynthesis by isolated chloroplasts III. Light activation of the carboxylation reaction. *Biochim. Biophys. Acta* 153, 227–234. doi: 10.1016/0005-2728(68)90164-3

Jin, S. H., Hong, J., Li, X. Q., and Jiang, D. A. (2006). Antisense inhibition of Rubisco activase increases Rubisco content and alters the proportion of Rubisco activase in stroma and thylakoids in chloroplasts of rice leaves. *Ann. Bot.* 97, 739–744.

Kamp, R. M., Srinivasa, B. R., von Knoblauch, K., and Subramanian, A. R. (1987). Occurrence of a methylated protein in chloroplast ribosomes. *Biochemistry* 26, 5866–5870. doi: 10.1021/bi00392a043

Kim, S. Y., Bender, K. W., Walker, B. J., Zielinski, R. E., Spalding, M. H., Ort, D. R., et al. (2016). The plastid casein kinase 2 phosphorylates Rubisco activase at the Thr-78 site but is not essential for regulation of Rubisco activation state. *Front. Plant Sci.* 7:404. doi: 10.3389/fpls.2016.00404

Klatt, P., and Lamas, S. (2000). Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. *Eur. J. Biochem.* 267, 4928–4944.

Knight, S., Andersson, I., and Branden, C. I. (1990). Crystallographic analysis of ribulose 1,5-bisphosphate carboxylase from spinach at 2.4 A resolution. Subunit interactions and active site. *J. Mol. Biol.* 215, 113–160.

Kötting, O., Kossmann, J., Zeeman, S. C., and Lloyd, J. R. (2010). Regulation of starch metabolism: the age of enlightenment? *Curr. Opin. Plant Biol.* 13, 321–329. doi: 10.1016/j.pbi.2010.01.003

Lehtimäki, N., Koskela, M. M., and Mulo, P. (2015). Posttranslational modifications of chloroplast proteins: an emerging field. *Plant Physiol.* 168, 768–775. doi: 10.1104/pp.15.00117

Lemeille, S., Turkina, M. V., Vener, A. V., and Rochaix, J. D. (2010). Stt7-dependent phosphorylation during state transitions in the green alga *Chlamydomonas reinhardtii*. *Mol. Cell. Proteomics* 9, 1281–1295. doi: 10.1074/mcp.M000020-MCP201

Lemeille, S., Willig, A., Depege-Fargeix, N., Delessert, C., Bassi, R., and Rochaix, J. D. (2009). Analysis of the chloroplast protein kinase Stt7 during state transitions. *PLoS Biol.* 7:e45. doi: 10.1371/journal.pbio.1000045

Li, J., Almagro, G., Munoz, F. J., Baroja-Fernandez, E., Bahaji, A., Montero, M., et al. (2012). Post-translational redox modification of ADP-glucose pyrophosphorylase in response to light is not a major determinant of fine regulation of transitory starch accumulation in *Arabidopsis* leaves. *Plant Cell Physio.* 53, 433–444. doi: 10.1093/pcp/pcr193

Liere, K., and Link, G. (1994). Structure and expression characteristics of the chloroplast DNA region containing the split gene for tRNA(gly) (UCC) from mustard (*Sinapis alba* L.). *Curr. Genet.* 26, 557–563. doi: 10.1007/BF00309950

Lindermayr, C., Saalbach, G., and Durner, J. (2005). Proteomic identification of S-nitrosylated proteins in *Arabidopsis*. *Plant Physiol.* 137, 921–930.

Link, G. (2003). Redox regulation of chloroplast transcription. *Antioxid. Redox. Signal.* 5, 79–87. doi: 10.1089/152308603321223568

Lisitsky, I., and Schuster, G. (1995). Phosphorylation of a chloroplast RNA-binding protein changes its affinity to RNA. *Nucleic Acids Res.* 23, 2506–2511.

Lohrig, K., Muller, B., Davydova, J., Leister, D., and Wolters, D. A. (2009). Phosphorylation site mapping of soluble proteins: Bioinformatical filtering reveals potential plastidic phosphoproteins in *Arabidopsis thaliana*. *Planta* 229, 1123–1134. doi: 10.1007/s00425-009-0901-y

López-Torrejón, G., Guerra, D., Catala, R., Salinas, J., and del Pozo, J. C. (2013). Identification of SUMO targets by a novel proteomic approach in plants. *J. Integr. Plant Biol.* 55, 96–107. doi: 10.1111/jipb.12012

Lozano-Juste, J., Colom-Moreno, R., and Leon, J. (2011). In vivo protein tyrosine nitration in *Arabidopsis thaliana*. *J. Exp. Bot.* 62, 3501–3517. doi: 10.1093/jxb/err042

Loza-Tavera, H., Vargas-Suarez, M., Diaz-Mireles, E., Torres-Marquez, M. E., Gonzalez de la Vara, L. E., Moreno-Sanchez, R., et al. (2006). Phosphorylation of the spinach chloroplast 24 kDa RNA-binding protein (24RNP) increases its binding to petD and psbA 3′ untranslated regions. *Biochimie* 88, 1217–1228.

Ma, S., Martin-Laffon, J., Mininno, M., Gigarel, O., Brugiere, S., Bastien, O., et al. (2016). Molecular evolution of the substrate specificity of chloroplastic aldolases/Rubisco lysine methyltransferases in plants. *Mol. Plant* 9, 569–581. doi: 10.1016/j.molp.2016.01.003

Majeran, W., Friso, G., Asakura, Y., Qu, X., Huang, M., Ponnala, L., et al. (2012). Nucleoid-enriched proteomes in developing plastids and chloroplasts from maize leaves: a new conceptual framework for nucleoid functions. *Plant Physiol.* 158, 156–189. doi: 10.1104/pp.111.188474

Marcus, Y., Altman-Gueta, H., Finkler, A., and Gurevitz, M. (2003). Dual role of cysteine 172 in redox regulation of ribulose 1,5-bisphosphate

carboxylase/oxygenase activity and degradation. *J. Bacteriol.* 185, 1509–1517. doi: 10.1128/JB.185.5.1509-1517.2003

Mazzoleni, M., Figuet, S., Martin-Laffon, J., Mininno, M., Gilgen, A., Leroux, M., et al. (2015). Dual targeting of the protein methyltransferase PrmA contributes to both chloroplastic and mitochondrial ribosomal protein L11 methylation in *Arabidopsis*. *Plant Cell Physiol.* 56, 1697–1710. doi: 10.1093/pcp/pcv098

Melonek, J., Oetke, S., and Krupinska, K. (2016). Multifunctionality of plastid nucleoids as revealed by proteome analyses. *Biochim. Biophys. Acta* 1864, 1016–1038. doi: 10.1016/j.bbapap.2016.03.009

Michel, H., Griffin, P. R., Shabanowitz, J., Hunt, D. F., and Bennett, J. (1991). Tandem mass spectrometry identifies sites of three post-translational modifications of spinach light-harvesting chlorophyll protein II. Proteolytic cleavage, acetylation, and phosphorylation. *J. Biol. Chem.* 266, 17584–17591.

Michelet, L., Zaffagnini, M., Morisse, S., Sparla, F., Perez-Perez, M. E., Francia, F., et al. (2013). Redox regulation of the Calvin-Benson cycle: something old, something new. *Front. Plant Sci.* 4:470. doi: 10.3389/fpls.2013.00470

Mikkelsen, R., Mutenda, K. E., Mant, A., Schurmann, P., and Blennow, A. (2005). Alpha-glucan, water dikinase (GWD): a plastidic enzyme with redox-regulated and coordinated catalytic activity and binding affinity. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1785–1790.

Mininno, M., Brugiere, S., Pautre, V., Gilgen, A., Ma, S., Ferro, M., et al. (2012). Characterization of chloroplastic fructose 1,6-bisphosphate aldolases as lysine-methylated proteins in plants. *J. Biol. Chem.* 287, 21034–21044. doi: 10.1074/jbc.M112.359976

Miura, K., Jin, J. B., and Hasegawa, P. M. (2007). Sumoylation, a post-translational regulatory process in plants. *Curr. Opin. Plant Biol.* 10, 495–502.

Mulligan, R. M., Houtz, R. L., and Tolbert, N. E. (1988). Reaction-intermediate analogue binding by ribulose bisphosphate carboxylase/oxygenase causes specific changes in proteolytic sensitivity: the amino-terminal residue of the large subunit is acetylated proline. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1513–1517.

Mulo, P., Sakurai, I., and Aro, E. M. (2012). Strategies for psbA gene expression in cyanobacteria, green algae and higher plants: from transcription to PSII repair. *Biochim. Biophys. Acta* 1817, 247–257. doi: 10.1016/j.bbabio.2011.04.011

Mulo, P., Sirpio, S., Suorsa, M., and Aro, E. M. (2008). Auxiliary proteins involved in the assembly and sustenance of photosystem II. *Photosynth. Res.* 98, 489–501. doi: 10.1007/s11120-008-9320-3

Nickelsen, J., and Link, G. (1993). The 54 kDa RNA-binding protein from mustard chloroplasts mediates endonucleolytic transcript 3′ end formation in vitro. *Plant J.* 3, 537–544. doi: 10.1046/j.1365-313X.1993.03040537.x

Ogrzewalla, K., Piotrowski, M., Reinbothe, S., and Link, G. (2002). The plastid transcription kinase from mustard (*Sinapis alba* L.). A nuclear-encoded CK2-type chloroplast enzyme with redox-sensitive function. *Eur. J. Biochem.* 269, 3329–3337. doi: 10.1046/j.1432-1033.2002.03017_269_13.x

Pedersen, T. A., Kirk, M., and Bassham, J. A. (1966). Inhibition of photophos phorylation and photosynthetic carbon cycle reactions by fatty acids and esters. *Biochim. Biophys. Acta* 112, 189–203. doi: 10.1016/0926-6585(66)90320-7

Posno, M., van Noort, M., Debise, R., and Groot, G. S. (1984). Isolation, characterization, phosphorylation and site of synthesis of Spinacia chloroplast ribosomal proteins. *Curr. Genet.* 8, 147–154. doi: 10.1007/BF00420227

Pribil, M., Pesaresi, P., Hertle, A., Barbato, R., and Leister, D. (2010). Role of plastid protein phosphatase TAP38 in LHCII dephosphorylation and thylakoid electron flow. *PLoS Biol.* 8:e1000288. doi: 10.1371/journal.pbio.1000288

Reiland, S., Finazzi, G., Endler, A., Willig, A., Baerenfaller, K., Grossmann, J., et al. (2011). Comparative phosphoproteome profiling reveals a function of the STN8 kinase in fine-tuning of cyclic electron flow (CEF). *Proc. Natl. Acad. Sci. U.S.A.* 108, 12955–12960. doi: 10.1073/pnas.1104734108

Reiland, S., Messerli, G., Baerenfaller, K., Gerrits, B., Endler, A., Grossmann, J., et al. (2009). Large-scale *Arabidopsis* phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. *Plant Physiol.* 150, 889–903. doi: 10.1104/pp.109.138677

Rintamäki, E., Martinsuo, P., Pursiheimo, S., and Aro, E. M. (2000). Cooperative regulation of light-harvesting complex II phosphorylation via the plastoquinol and ferredoxin-thioredoxin system in chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 97, 11644–11649. doi: 10.1073/pnas.180054297

Rocha, A. G., Mehlmer, N., Stael, S., Mair, A., Parvin, N., Chigri, F., et al. (2014). Phosphorylation of *Arabidopsis* transketolase at Ser428 provides a potential paradigm for the metabolic control of chloroplast carbon metabolism. *Biochem. J.* 458, 313–322. doi: 10.1042/BJ20130631

Rochaix, J. D. (2013). Redox regulation of thylakoid protein kinases and photosynthetic gene expression. *Antioxid. Redox Signal.* 18, 2184–2201. doi: 10.1089/ars.2012.5110

Rochaix, J. D. (2014). Regulation and dynamics of the light-harvesting system. *Annu. Rev. Plant Biol.* 65, 287–309. doi: 10.1146/annurev-arplant-050213-040226

Roitinger, E., Hofer, M., Kocher, T., Pichler, P., Novatchkova, M., Yang, J., et al. (2015). Quantitative phosphoproteomics of the ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia-mutated and rad3-related (ATR) dependent DNA damage response in *Arabidopsis thaliana*. *Mol. Cell. Proteomics* 14, 556–571. doi: 10.1074/mcp.M114.040352

Romero-Puertas, M. C., Campostrini, N., Matte, A., Righetti, P. G., Perazzolli, M., Zolla, L., et al. (2008). Proteomic analysis of S-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response. *Proteomics* 8, 1459–1469. doi: 10.1002/pmic.200700536

Rouhier, N., Villarejo, A., Srivastava, M., Gelhaye, E., Keech, O., Droux, M., et al. (2005). Identification of plant glutaredoxin targets. *Antioxid. Redox Signal.* 7, 919–929. doi: 10.1089/ars.2005.7.919

Rowland, E., Kim, J., Bhuiyan, N. H., and van Wijk, K. J. (2015). The *Arabidopsis* chloroplast stromal N-terminome: complexities of amino-terminal protein maturation and stability. *Plant Physiol.* 169, 1881–1896. doi: 10.1104/pp.15.01214

Sakr, S., Dutheil, J., Saenkham, P., Bottin, H., Leplat, C., Ortega-Ramos, M., et al. (2013). The activity of the *Synechocystis* PCC6803 AbrB2 regulator of hydrogen production can be post-translationally controlled through glutathionylation. *Int. J. Hydrogen Energy* 38, 13547–13555.

Salinas, P., Fuentes, D., Vidal, E., Jordana, X., Echeverria, M., and Holuigue, L. (2006). An extensive survey of CK2 alpha and beta subunits in *Arabidopsis*: multiple isoforms exhibit differential subcellular localization. *Plant Cell Physiol.* 47, 1295–1308. doi: 10.1093/pcp/pcj100

Samol, I., Shapiguzov, A., Ingelsson, B., Fucile, G., Crevecoeur, M., Vener, A. V., et al. (2012). Identification of a photosystem II phosphatase involved in light acclimation in *Arabidopsis*. *Plant Cell* 24, 2596–2609. doi: 10.1105/tpc.112.095703

Schmidt, J., Herfurth, E., and Subramanian, A. R. (1992). Purification and characterization of seven chloroplast ribosomal proteins: evidence that organelle ribosomal protein genes are functional and that NH2-terminal processing occurs via multiple pathways in chloroplasts. *Plant Mol. Biol.* 20, 459–465. doi: 10.1007/BF00040605

Schweer, J., Turkeri, H., Link, B., and Link, G. (2010). AtSIG6, a plastid sigma factor from *Arabidopsis*, reveals functional impact of cpCK2 phosphorylation. *Plant J.* 62, 192–202. doi: 10.1111/j.1365-313X.2010.04138.x

Scott, D. C., Monda, J. K., Bennett, E. J., Harper, J. W., and Schulman, B. A. (2011). N-terminal acetylation acts as an avidity enhancer within an interconnected multiprotein complex. *Science* 334, 674–678. doi: 10.1126/science.1209307

Shapiguzov, A., Ingelsson, B., Samol, I., Andres, C., Kessler, F., Rochaix, J. D., et al. (2010). The PPH1 phosphatase is specifically involved in LHCII dephosphorylation and state transitions in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 4782–4787. doi: 10.1073/pnas.0913810107

Shen, Y., Wei, W., and Zhou, D. X. (2015). Histone acetylation enzymes coordinate metabolism and gene expression. *Trends Plant Sci.* 20, 614–621. doi: 10.1016/j.tplants.2015.07.005

Shiina, T., Tsunoyama, Y., Nakahira, Y., and Khan, M. S. (2005). Plastid RNA polymerases, promoters, and transcription regulators in higher plants. *Int. Rev. Cytol.* 244, 1–68.

Shimizu, M., Kato, H., Ogawa, T., Kurachi, A., Nakagawa, Y., and Kobayashi, H. (2010). Sigma factor phosphorylation in the photosynthetic control of photosystem stoichiometry. *Proc. Natl. Acad. Sci. U.S.A.* 107, 10760–10764. doi: 10.1073/pnas.0911692107

Sokolov, L. N., Dominguez-Solis, J. R., Allary, A. L., Buchanan, B. B., and Luan, S. (2006). A redox-regulated chloroplast protein phosphatase binds to starch diurnally and functions in its accumulation. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9732–9737.

Souza, J. M., Peluffo, G., and Radi, R. (2008). Protein tyrosine nitration- functional alteration or just a biomarker? *Free Radic. Biol. Med.* 45, 357–366. doi: 10.1016/j.freeradbiomed.2008.04.010

Steiner, S., Schroter, Y., Pfalz, J., and Pfannschmidt, T. (2011). Identification of essential subunits in the plastid-encoded RNA polymerase complex reveals building blocks for proper plastid development. *Plant Physiol.* 157, 1043–1055. doi: 10.1104/pp.111.184515

Stotz, M., Mueller-Cajar, O., Ciniawsky, S., Wendler, P., Hartl, F. U., Bracher, A., et al. (2011). Structure of green-type Rubisco activase from tobacco. *Nat. Struct. Mol. Biol.* 6, 1366–1370. doi: 10.1038/nsmb.2171

Sugiura, M. (1992). "The chloroplast genome," in *10 Years Plant Molecular Biology*, eds R. A. Schilperoort and L. Dure (Berlin: Springer), 149–168. doi: 10.1007/978-94-011-2656-4_10

Takahashi, S., and Yamasaki, H. (2002). Reversible inhibition of photophosphorylation in chloroplasts by nitric oxide. *FEBS Lett.* 512, 145–148.

Tetlow, I. J., Beisel, K. G., Cameron, S., Makhmoudova, A., Liu, F., Bresolin, N. S., et al. (2008). Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes. *Plant Physiol.* 146, 1878–1891. doi: 10.1104/pp.108.116244

Tetlow, I. J., and Emes, M. J. (2014). A review of starch-branching enzymes and their role in amylopectin biosynthesis. *IUBMB Life* 66, 546–558. doi: 10.1002/iub.1297

Tetlow, I. J., Morell, M. K., and Emes, M. J. (2004a). Recent developments in understanding the regulation of starch metabolism in higher plants. *J. Exp. Bot.* 55, 2131–2145. doi: 10.1093/jxb/erh248

Tetlow, I. J., Wait, R., Lu, Z., Akkasaeng, R., Bowsher, C. G., Esposito, S., et al. (2004b). Protein phosphorylation in amyloplasts regulates starch branching enzyme activity and protein-protein interactions. *Plant Cell* 16, 694–708. doi: 10.1105/tpc.017400

Tikkanen, M., and Aro, E. M. (2012). Thylakoid protein phosphorylation in dynamic regulation of photosystem II in higher plants. *Biochim. Biophys. Acta* 1817, 232–238. doi: 10.1016/j.bbabio.2011.05.005

Tikkanen, M., Nurmi, M., Kangasjärvi, S., and Aro, E. M. (2008). Core protein phosphorylation facilitates the repair of photodamaged photosystem II at high light. *Biochim. Biophys. Acta* 1777, 1432–1437. doi: 10.1016/j.bbabio.2008.08.004

Trebitsh, T., Levitan, A., Sofer, A., and Danon, A. (2000). Translation of chloroplast psbA mRNA is modulated in the light by counteracting oxidizing and reducing activities. *Mol. Cell. Biol.* 20, 1116–1123. doi: 10.1128/MCB.20.4.1116-1123.2000

Tuncel, A., Cakir, B., Hwang, S. K., and Okita, T. W. (2014). The role of the large subunit in redox regulation of the rice endosperm ADP-glucose pyrophosphorylase. *FEBS J.* 281, 4951–4963. doi: 10.1111/febs.13041

Tyystjärvi, E. (2013). Photoinhibition of photosystem II. *Int. Rev. Cell. Mol. Biol.* 300, 243–303. doi: 10.1016/B978-0-12-405210-9.00007-2

Valerio, C., Costa, A., Marri, L., Issakidis-Bourguet, E., Pupillo, P., Trost, P., et al. (2011). Thioredoxin-regulated beta-amylase (BAM1) triggers diurnal starch degradation in guard cells, and in mesophyll cells under osmotic stress. *J. Exp. Bot.* 62, 545–555. doi: 10.1093/jxb/erq288

van de Loo, F. J., and Salvucci, M. E. (1996). Activation of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) involves Rubisco activase Trp16. *Biochemistry* 35, 8143–8148. doi: 10.1021/bi9604901

Vanzo, E., Ghirardo, A., Merl-Pham, J., Lindermayr, C., Heller, W., Hauck, S. M., et al. (2014). S-nitroso-proteome in poplar leaves in response to acute ozone stress. *PLoS ONE* 9:e106886. doi: 10.1371/journal.pone.0106886

Vanzo, E., Merl-Pham, J., Velikova, V., Ghirardo, A., Lindermayr, C., Hauck, S. M., et al. (2016). Modulation of protein S-nitrosylation by isoprene emission in poplar. *Plant Physiol.* 170, 1945–1961. doi: 10.1104/pp.15.01842

Vargas-Suarez, M., Castro-Sanchez, A., Toledo-Ortiz, G., Gonzalez de la Vara, L. E., Garcia, E., and Loza-Tavera, H. (2013). Protein phosphorylation regulates in vitro spinach chloroplast petD mRNA 3′-untranslated region stability, processing, and degradation. *Biochimie* 95, 400–409. doi: 10.1016/j.biochi.2012.10.012

Vener, A. V., van Kan, P. J., Rich, P. R., Ohad, I., and Andersson, B. (1997). Plastoquinol at the quinol oxidation site of reduced cytochrome bf mediates signal transduction between light and protein phosphorylation: thylakoid protein kinase deactivation by a single-turnover flash. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1585–1590.

Vierstra, R. D. (2012). The expanding universe of ubiquitin and ubiquitin-like modifiers. *Plant Physiol.* 160, 2–14. doi: 10.1104/pp.112.200667

Villarejo, A., Buren, S., Larsson, S., Dejardin, A., Monne, M., Rudhe, C., et al. (2005). Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast. *Nat. Cell Biol.* 7, 1224–1231. doi: 10.1038/ncb1330

Wagner, L. E. II, Betzenhauser, M. J., and Yule, D. I. (2006). ATP binding to a unique site in the type-1 S2- inositol 1,4,5-trisphosphate receptor defines susceptibility to phosphorylation by protein kinase A. *J. Biol. Chem.* 281, 17410–17419.

Wang, Y., Wang, Y., Zhao, Y. B., Chen, D. M., Han, Z. H., and Zhang, X. Z. (2014). Protein phosphorylation differs significantly among ontogenetic phases in malus seedlings. *Proteome Sci.* 12, 31. doi: 10.1186/1477-5956-12-31

Wolosiuk, R. A., and Buchanan, B. B. (1976). Studies on the regulation of chloroplast NADP-linked glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.* 251, 6456–6461.

Wu, X., Oh, M. H., Schwarz, E. M., Larue, C. T., Sivaguru, M., Imai, B. S., et al. (2011). Lysine acetylation is a widespread protein modification for diverse proteins in *Arabidopsis*. *Plant Physiol.* 155, 1769–1778. doi: 10.1104/pp.110.165852

Yamaguchi, K., Beligni, M. V., Prieto, S., Haynes, P. A., McDonald, W. H., Yates, J. R. III, et al. (2003). Proteomic characterization of the *Chlamydomonas reinhardtii* chloroplast ribosome. Identification of proteins unique to the 70 S ribosome. *J. Biol. Chem.* 278, 33774–33785. doi: 10.1074/jbc.M301934200

Yamaguchi, K., and Subramanian, A. R. (2000). The plastid ribosomal proteins. Identification of all the proteins in the 50 S subunit of an organelle ribosome (chloroplast). *J. Biol. Chem.* 275, 28466–28482. doi: 10.1074/jbc.M005012200

Yamaguchi, K., and Subramanian, A. R. (2003). Proteomic identification of all plastid-specific ribosomal proteins in higher plant chloroplast 30 S ribosomal subunit. *Eur. J. Biochem.* 270, 190–205. doi: 10.1046/j.1432-1033.2003.03359.x

Yamaguchi, K., von Knoblauch, K., and Subramanian, A. R. (2000). The plastid ribosomal proteins. Identification of all the proteins in the 30 S subunit of an organelle ribosome (chloroplast). *J. Biol. Chem.* 275, 28455–28465. doi: 10.1074/jbc.M004350200

Zaffagnini, M., Bedhomme, M., Groni, H., Marchand, C. H., Puppo, C., Gontero, B., et al. (2012a). Glutathionylation in the photosynthetic model organism *Chlamydomonas reinhardtii*: a proteomic survey. *Mol. Cell. Proteomics* 11, M111.014142. doi: 10.1074/mcp.M111.014142

Zaffagnini, M., Bedhomme, M., Marchand, C. H., Morisse, S., Trost, P., and Lemaire, S. D. (2012b). Redox regulation in photosynthetic organisms: focus on glutathionylation. *Antioxid. Redox Signal.* 16, 567–586. doi: 10.1089/ars.2011.4255

Zhang, N., and Portis, A. R. (1999). Mechanism of light regulation of Rubisco: a specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9438–9443. doi: 10.1073/pnas.96.16.9438

Zybailov, B., Rutschow, H., Friso, G., Rudella, A., Emanuelsson, O., Sun, Q., et al. (2008). Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. *PLoS ONE* 3:e1994. doi: 10.1371/journal.pone.0001994

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Maturation of Plastid *c*-type Cytochromes

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Cytochromes *c* are hemoproteins, with the prosthetic group covalently linked to the apoprotein, which function as electron carriers. A class of cytochromes *c* is defined by a C*XX*CH heme-binding motif where the cysteines form thioether bonds with the vinyl groups of heme. Plastids are known to contain up to three cytochromes *c*. The membrane-bound cytochrome *f* and soluble cytochrome c_6 operate in photosynthesis while the activity of soluble cytochrome c_{6A} remains unknown. Conversion of apo- to holocytochrome *c* occurs in the thylakoid lumen and requires the independent transport of apocytochrome and heme across the thylakoid membrane followed by the stereospecific attachment of ferroheme via thioether linkages. Attachment of heme to apoforms of plastid cytochromes *c* is dependent upon the products of the *CCS* (for **c**ytochrome ***c*** **s**ynthesis) genes, first uncovered via genetic analysis of photosynthetic deficient mutants in the green alga *Chlamydomonas reinhardtii*. The CCS pathway also occurs in cyanobacteria and several bacteria. CcsA and CCS1, the signature components of the CCS pathway are polytopic membrane proteins proposed to operate in the delivery of heme from the stroma to the lumen, and also in the catalysis of the heme ligation reaction. CCDA, CCS4, and CCS5 are components of trans-thylakoid pathways that deliver reducing equivalents in order to maintain the heme-binding cysteines in a reduced form prior to thioether bond formation. While only four CCS components are needed in bacteria, at least eight components are required for plastid cytochrome *c* assembly, suggesting the biochemistry of thioether formation is more nuanced in the plastid system.

Keywords: thylakoid lumen, photosynthesis, cytochrome *c*, heme, thioether, cytochrome b_6f

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CYTOCHROMES OF THE *c*-TYPE

Cytochromes of the *c*-type, often generically referred to as cytochromes *c,* are membrane-bound or soluble metalloproteins occurring in energy-transducing membranes in archaea, bacteria, mitochondria, and plastids, where they function as electron carriers in respiration and photosynthesis (Thony-Meyer, 1997; Bonnard et al., 2010; Kletzin et al., 2015). Cytochromes *c*, on the positive side (or *p*-side)[1] of energy-transducing membranes, carry one or several hemes (ferroprotoporphyrin IX) as a prosthetic group covalently attached via thioether bonds to a heme

[1]The *p*-side corresponds to the bacterial or archaeal periplasm, the thylakoid lumen and the mitochondria intermembrane space.

binding site in the apoprotein. The most common heme binding site consists of a C*XX*CH motif where the first and second cysteines are, respectively, linked to the vinyl-2 and vinyl-4 groups of heme and the intervening residue *X* can be any amino-acid except cysteine in naturally occurring cytochromes *c* (Allen et al., 2004; Bowman and Bren, 2008). The histidine residue serves as the proximal axial ligand of the iron atom. A distant histidine, methionine, or, less commonly, other residues in the apocytochrome provide distal axial ligation of the heme group (Bowman and Bren, 2008).

Variations of the heme binding site are rare and one example is the A/FXXCH motif of mitochondrial cytochromes *c* in Euglenozoa that bind the vinyl-4 group of heme via a single thioether bond (Priest and Hajduk, 1992; Fülöp et al., 2009). Other non-canonical heme binding sites occur in bacterial cytochromes *c* and contain three, four, or fifteen intervening residues between the cysteines instead of two (Herbaud et al., 2000; Aragão et al., 2003; Hartshorne et al., 2007) or a lysine instead of a histidine as the proximal heme ligand (Einsle et al., 1999). Another atypical cytochrome *c* is also the only known example of a cytochrome *c* on the negative side (*n*-side)[2] of the membrane. This is cytochrome b_6 of the b_6f complex in plastids and cyanobacteria and cytochrome *b* of the *bc* complex in firmicutes, which contain a heme covalently attached via a single thioether bond (de Vitry, 2011). The heme binding cysteine faces the *n*-side of the membrane and is not found in a motif, unlike other *p*-side localized cytochromes *c*. Moreover, there are no amino-acid residues serving as proximal and distal ligands of heme, differentiating this cytochrome *c* from all other *c*-type cytochromes occurring on the *p*-side (de Vitry, 2011).

CYTOCHROME *c* MATURATION SYSTEMS

All *p*-side localized holocytochromes *c* are assembled on the *p*-side of the membrane. This requires the apoform and the heme moiety, both of which are transported independently across at least one biological membrane. Conversion of apocytochrome to its holoform requires free sulfhydryls at the C*XX*CH motif, provision of heme under the reduced form (ferroheme), and stereospecific attachment of the prosthetic group via catalysis of the thioether bond linkage (Mavridou et al., 2013; Travaglini-Allocatelli, 2013). Extensive genetic and biochemical analyses in bacteria, plants, and fungi revealed that the operation of three distinct assembly pathways called Systems I, II, and III is required for cytochrome *c* maturation, depending on the location (Kranz et al., 2009; Mavridou et al., 2013; Travaglini-Allocatelli, 2013; Verissimo and Daldal, 2014; Babbitt et al., 2015). The diversity of maturation systems is surprising, considering the biochemical requirements for heme attachment to apocytochrome *c* are believed to be universal and thioether bond formation appears, *a priori*, a simple chemical reaction (Bowman and Bren, 2008). Each System can be recognized by prototypical assembly factors but the number of such assembly factors and their features differ considerably among the different Systems (**Table 1**). An additional layer of complexity is the apparent "mosaic" distribution of Systems I, II, and III among organisms and the different energy transducing membranes (Mavridou et al., 2013; Travaglini-Allocatelli, 2013). Several evolutionary scenarios accounting for the origin and distribution of the different maturation systems have been proposed but the complexity of cytochrome *c* maturation as a biochemical process still remains mysterious (Bertini et al., 2007; Allen et al., 2008; Giegé et al., 2008; Kranz et al., 2009; Allen, 2011).

PLASTID *c*-TYPE CYTOCHROMES

Three *c*-type cytochromes, have been identified within the thylakoid lumen of various plastids: the membrane-bound cytochrome *f* and the soluble cytochromes c_6 and c_{6A}. While cytochrome *f* and c_6 are known to function as electron carriers in photosynthesis, cytochrome c_{6A} function remains enigmatic despite having been discovered 15 years ago (Howe et al., 2006). All plastid cytochromes *c* contain a single heme attached to a C*XX*CH motif on the apoprotein. Cytochrome *f*, a catalytic subunit of the cytochrome b_6f complex, is universal in all photosynthetic eukaryotes (and cyanobacteria) and is essential for photosynthesis (Martinez et al., 1994). Cytochrome c_6 is found in cyanobacteria and the plastid of eukaryotic algae, where it is widely distributed among green, red and brown algal lineages (Sandmann et al., 1983; Kerfeld and Krogmann, 1998). Cytochrome c_6 is involved in the transfer of electrons from cytochrome *f* of the cytochrome b_6f complex to Photosystem I (Merchant and Dreyfuss, 1998). In green algae and cyanobacteria, cytochrome c_6 acts as a substitute for plastocyanin in Cu-deficient conditions (Merchant and Bogorad, 1987a,b). Cytochrome c_{6A} occurs in the thylakoid lumen of land plants and green algae but appears absent in red algae and diatoms (Wastl et al., 2004).

Cytochrome c_{6A} was discovered in *Arabidopsis* as a protein interacting with the lumen-localized immunophilin FKBP13 in a yeast two-hybrid screen (Gupta et al., 2002a,b; Buchanan and Luan, 2005). It was initially postulated that cytochrome c_{6A} acts as a substitute for plastocyanin (Gupta et al., 2002a), as in green algae and cyanobacteria where cytochrome c_6 can replace plastocyanin (Merchant and Bogorad, 1987a,b). However, loss of cytochrome c_{6A} in *Arabidopsis* has no visible phenotype even under Cu deficient conditions (Gupta et al., 2002a). Moreover, an *Arabidopsis* plastocyanin-deficient mutant is unable to grow photoautotrophically even when cytochrome c_{6A} is overexpressed (Weigel et al., 2003). *In vitro*, cytochrome c_{6A} is unable to provide electrons to Photosystem I (Molina-Heredia et al., 2003). This observation accounts for the fact that cytochrome c_{6A} cannot act as a functional substitute for plastocyanin *in vivo*. Hence, cytochrome c_{6A} does not appear to function in the known electron transfer reactions of photosynthesis, which is consistent with its extremely low abundance in the thylakoid. The presence of a disulfide bond in holocytochrome c_{6A} led to the proposal that the molecule acts as an oxidant of luminal proteins dithiols with heme providing

[2]The *n*-side corresponds to the bacterial or archaeal cytoplasm, the plastid stroma and the mitochondrial matrix.

TABLE 1 | Prototypical components of cytochrome *c* maturation pathways.

Function/Activity	System I	System II		System III
		Bacteria	Plastids	
Transmembrane heme transport	?	CcsA, CcsB[4]	CCS1, CcsA	?
Heme handling	CcmABCDE[1]	CcsA, CcsB	CCS1, CcsA	HCCS
Heme reduction	CcmF[2]	?	?	?
Apocytochrome c chaperoning	CcmH[3]	CcsB	CCS1	HCCS
Maintenance of reduced CXXCH sulfhydryls	DsbD CcmG	DsbD CcsX	CCDA CCS5 CCS4	?
Thioether bond formation	CcmFH	CcsA, CcsB	CCS1, CcsA	HCCS
Unknown			CCS2, CCS3, CCS6	

The prototypical components of Systems I, II, and III are indicated according to their proposed function in the maturation process. Bacterial cytochromes c are assembled via System I or II. Plastid cytochrome c assembly relies on System II and mitochondrial cytochromes c are matured via System I or III. System III is restricted to mitochondria and is defined by a single component, holocytochrome c synthase (HCCS). The nomenclature for Escherichia coli (System I), Bordetella pertussis (System II bacteria), and Chlamydomonas reinhardtii (System II plastids) is used here. With the exception of System II, for which a heme transport across the membrane is supported by experimental evidence, there is no description of transmembrane heme delivery routes in Systems I and III. '?' indicates that there is no component identified for this activity in the maturation process. System I: CcmABCDE[1] is a periplasmic heme handling route defined by an ABC transporter (CcmAB), a member of the Heme Handling Protein (HHP) family (CcmC), and a small transmembrane protein (CcmD) that are required to load heme onto a heme chaperone (CcmE) (Kranz et al., 2009). CcmF[2] and thiol-disulfide oxidoreductase CcmH[3] are forming a complex postulated to carry the holocytochrome c synthase activity (Kranz et al., 2009). System II: CcsB[4] is the bacterial ortholog of plastid CcsA.

electrons for re-oxidation of the cysteine pair (Marcaida et al., 2006). Additional experimental exploration is required to test this hypothesis.

SYSTEM II, A MULTICOMPONENT ASSEMBLY PATHWAY REQUIRED FOR MATURATION OF PLASTID CYTOCHROMES *c*

Plastid cytochromes *c* are matured via System II, also referred to as the CCS pathway, a multicomponent assembly machinery (Hamel et al., 2009; Bonnard et al., 2010; Simon and Hederstedt, 2011). System II first emerged through genetic screens for photosynthesis-impaired *ccs* mutants (*ccs* for *c*ytochrome *c* *s*ynthesis) in the green alga *Chlamydomonas reinhardtii* (Hamel et al., 2009; Simon and Hederstedt, 2011). The *Chlamydomonas ccs* mutants were isolated on the basis of a dual deficiency in the holoforms of both cytochrome *f* and cytochrome c_6. All *ccs* mutants are photosynthetic deficient because loss of cytochrome *f* assembly results in a b_6f-minus phenotype (Howe and Merchant, 1992; Inoue et al., 1997; Xie et al., 1998; Dreyfuss and Merchant, 1999; Page et al., 2004). Pulse-chase experiments revealed that both plastid apocytochromes *c* are synthesized, imported in the thylakoid lumen, and processed by lumen-resident signal peptidase, but they fail to be converted to their respective holoforms (Howe and Merchant, 1993, 1994; Xie et al., 1998). Based on these experiments, it was concluded the *ccs* mutants exhibit a defect in the heme attachment to apoforms of cytochrome *c* in the thylakoid lumen (Howe and Merchant, 1993, 1994; Xie et al., 1998). The *ccs* mutants are also expected to display a defect in cytochrome c_{6A}. However, this could not be tested because holocytochrome c_{6A} could not be detected in a wild-type strain. The defect in the *ccs* mutants is specific to plastid *c*-type cytochromes since plastocyanin, another lumen-resident metalloprotein, is normally assembled (Howe and Merchant, 1993, 1994; Xie et al., 1998). The *ccs* mutants are not affected for the covalent attachment of heme to the single *n*-side facing cysteine in cytochrome b_6, a structural subunit of the b_6f complex (Kuras et al., 2007). Catalysis of this thioether bond in cytochrome b_6, occurs on the stromal side of the thylakoid membrane and is dependent upon the *CCB* gene products (de Vitry, 2011).

CCSA AND CCS1, A HEME DELIVERY COMPLEX WITH HOLOCYTOCHROME *c* SYNTHASE ACTIVITY

The first CCS component to be identified is plastid-encoded CcsA (Xie and Merchant, 1996; Hamel et al., 2003), a thylakoid membrane protein belonging to the HHP (Heme Handling Protein) superfamily (Lee et al., 2007), which is defined by the highly conserved tryptophan-rich WWD motif and conserved histidine residues (**Figure 1**). This feature is also shared by CcmC and CcmF, two HHPs in System I shown to relay heme on the bacterial periplasmic space (Richard-Fogal et al., 2009; Richard-Fogal and Kranz, 2010) (**Table 1**). The other prototypical component is CCS1, a thylakoid membrane protein with little sequence conservation and lacking domains or structural features speaking to a specific chemical function, with the exception of an invariant histidine (Inoue et al., 1997). Because all photosynthetic plastid genomes (with a few exceptions) encode a CcsA-like protein, the CCS pathway is believed to operate in the plastids of all photosynthetic eukaryotes. System II also occurs in cyanobacteria, a majority of the Gram-positive bacteria, proteobacteria of the β-, δ-, and ε groups, and aquificales (Hamel et al., 2009; Bonnard et al., 2010; Simon and Hederstedt, 2011).

Detailed studies, including topological studies of algal CcsA and cyanobacterial Ccs1, site-directed mutagenesis of conserved residues, and molecular analysis of existing *ccs1* alleles, established that CcsA and CCS1 are polytopic membrane proteins with functional domains exposed to the lumen and four

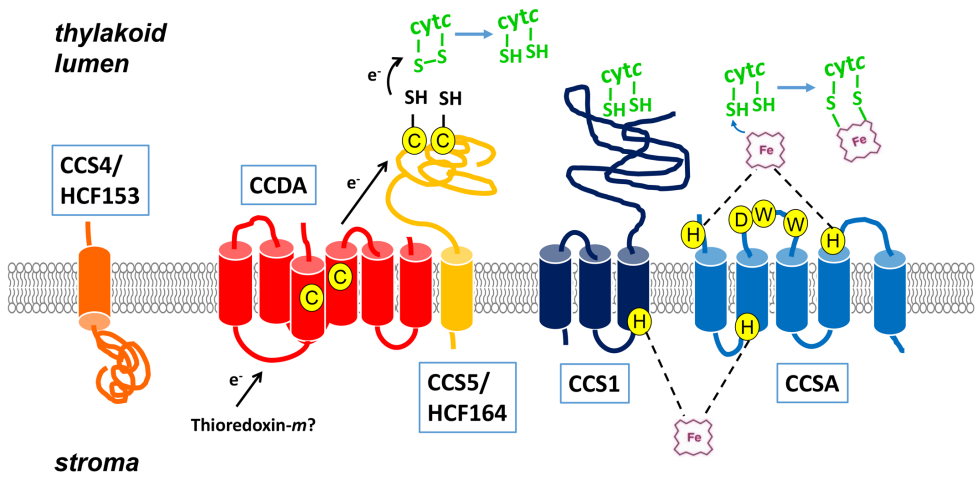

FIGURE 1 | A Model for plastid cytochrome *c* maturation. Plastid cytochrome *c* maturation involves two different pathways: (1) a *trans*-thylakoid disulfide reducing route with CCDA (red), CCS5/HCF164 (light orange), and CCS4/HCF153 (orange). The CCDA topology was deduced using PhoA/LacZ topological reporters (Page et al., 2004). CCS5/HCF164 is a membrane anchored, lumen-facing, thioredoxin-like protein (Lennartz et al., 2001). Cysteines (C) are circled and highlighted in yellow. Electron (e^-) transfer is indicated by black arrows. Stromal thioredoxin-*m* is the possible reductant for the CCDA-CCS5/HCF164 pathway, which conveys electrons to reduce disulfide-bonded *CXXCH* motif in apocytochrome *c*. CCS4/HCF153 is bound to the thylakoid membrane (Lennartz et al., 2006) and the soluble part of the protein is predicted to be facing the stroma based on the positive-inside rule (Gabilly et al., 2011). The role of CCS4/HCF153 in cytochrome *c* biogenesis is unclear, but it may be involved in the transport of reductant across the membrane. (2) A heme delivery/cytochrome *c* synthase pathway composed of CCS1 (dark blue) and CcsA (light blue). CcsA and CCS1 topologies were deduced using PhoA/LacZ topological reporters (Hamel et al., 2003; Dreyfuss et al., 2003). The CCS1/CcsA complex binds and channels the reduced heme from the stroma to the thylakoid lumen and catalyzes thioether bond formation. Two histidines, one from CCS1 and one from CcsA, on the stromal side of the thylakoid membrane could function in relaying the heme across the membrane. Two histidines in CcsA on the luminal side could act by coordinating heme that interacts with the WWD domain. Strictly conserved histidines (H), tryptophans (W), and asparatic acids (D) are circled and highlighted in yellow. The thylakoid membrane is drawn in gray.

strictly conserved essential histidine residues on both the lumen and stromal sides (Dreyfuss et al., 2003; Hamel et al., 2003) (**Figure 1**). Functional domains include the WWD signature motif for CcsA and a large hydrophilic C-terminal extension for CCS1. The C-terminal domain was postulated to chaperone apocytochrome *c* from studies of cyanobacterial Ccs1 (Tichy and Vermaas, 1999). In *Chlamydomonas,* a 200 kDa CCS1-containing complex in wild-type no longer accumulated in a *ccsA* mutant, suggesting that CcsA and CCS1 occur in a complex. The reduced abundance of CCS1 in some *ccs* mutants suggests the 200 kDa complex may contain other CCS components besides CcsA and CCS1 (Hamel et al., 2003). This led to the proposal that these two proteins act together to relay heme via histidinyl coordination from its site of synthesis, the stroma, to the lumen. In the lumen, heme is relayed to the WWD domain and coordinated by two histidine residues in CcsA (Hamel et al., 2003; **Figure 1**).

Experimental proof that CcsA and Ccs1 catalyze the heme attachment reaction was provided with the finding that Ccs1-CcsA fusion proteins, naturally occurring in several ε-proteobacteria, could assemble reporter cytochrome(s) *c* in an *Escherichia coli* strain lacking its endogenous cytochrome *c* assembly machinery (Feissner et al., 2006; Frawley and Kranz, 2009; Goddard et al., 2010; Kern et al., 2010; Richard-Fogal et al., 2012). Biochemical evidence supporting a possible role of CcsA and Ccs1 in heme transport from the cytoplasm to the periplasm came from studies of the Ccs1-CcsA fusion from *Helicobacter hepaticus*. Spectroscopic analysis of the recombinant fusion protein identified the presence of heme. Mutagenesis of the two periplasm-facing histidines highlighted the importance of these residues for the binding of heme and its maintenance in a reduced state (Frawley and Kranz, 2009). This led to the hypothesis that CcsA carries a heme binding site on the periplasmic space, presumably required for the cytochrome *c* synthase activity. To test the function of the two transmembrane cytoplasm-facing histidines in Ccs1 and CcsA, these residues were mutated in the recombinant protein. Because heme is synthesized in the cytoplasm and was no longer detected in the mutated form of the protein, it was concluded that these histidines provide an entry site for heme through the lipid bilayer on the cytoplasmic side of the membrane.

This implied that Ccs1-CcsA functions in channeling heme from the cytoplasm to the periplasm, but a direct heme transport activity remains to be demonstrated. By analogy, it is plausible that plastid CcsA and CCS1 also function in a heme relay pathway from stroma to lumen and carry the cytochrome *c* synthase activity but this has not been tested. While candidate components for the chemical reduction of heme were identified in System I, it is unknown how this process is achieved in System II (**Table 1**).

OPERATION OF *TRANS*-THYLAKOID DISULFIDE REDUCING PATHWAYS

The operation of a thylakoid transmembrane thiol-disulfide relay in plastid cytochrome *c* maturation emerged with the description of two thiol-disulfide oxidoreductases at the

thylakoid membrane, namely CCDA, a member of the DsbD family, and HCF164, a membrane-anchored, lumen-facing protein that displays similarity to thioredoxin-like CcmG and CcsX (**Table 1**) (Lennartz et al., 2001; Page et al., 2004; Motohashi and Hisabori, 2006; Motohashi and Hisabori, 2010). In bacteria using Systems I and II, cytochrome *c* maturation requires the provision of reductants via sequential thiol-disulfide exchanges involving a cytoplasmic thioredoxin, a thiol-disulfide reductase of the DsbD family, and a periplasmic thioredoxin-like protein (CcmG in System I or CcsX in System II) (**Table 1**) (Mavridou et al., 2013; Travaglini-Allocatelli, 2013). The working model is that the apocytochrome *c* C*XX*CH motif is first disulfide bonded by the disulfide bond forming enzymes residing in the periplasm and subsequently reduced by a thioredoxin-like protein (CcmG or CcsX) dedicated to the heme attachment reaction (Mavridou et al., 2013; Travaglini-Allocatelli, 2013). Reverse-genetic analysis in *Arabidopsis* indicates a function for CCDA and HCF164 in holocytochrome *f* accumulation, but a possible defect in the heme attachment reaction was not investigated (Lennartz et al., 2001; Page et al., 2004).

The biochemical requirement for thiol-disulfide chemistry in plastid cytochrome *c* biogenesis was demonstrated with the identification of CCS5, the *Chlamydomonas* ortholog of thioredoxin-like HCF164 (Gabilly et al., 2010). CCS5 physically interacts with plastid apocytochromes *c* and a recombinant form of the CCS5 molecule is active as a reductase when apocytochrome *c* with a disulfide-bonded C*XX*CH motif is provided as a substrate in an *in vitro* reaction (Gabilly et al., 2010). Application of exogenous thiols to the *ccs5*-null mutant rescues the photosynthetic deficiency and holocytochrome *f* assembly, an indication that CCS5 acts as a disulfide reductase *in vivo* (Gabilly et al., 2010). By analogy to the bacterial pathway, CCS5/HCF164 is likely to be maintained reduced by the activity of CCDA but this remains to be experimentally tested (**Figure 1**). The source of reducing equivalents on the stromal side was attributed to thioredoxin-*m* (Trx-*m*) (**Figure 1**) based on the observation that the redox active cysteines in CCDA and HCF164 undergo reduction in isolated thylakoid membranes when Trx-*m* is added exogenously (Motohashi and Hisabori, 2010). Complete loss of function of CCDA or HCF164 in *Arabidopsis* and CCS5 in *Chlamydomonas* does not abolish plastid cytochrome *c* maturation, an indication that another mechanism for delivery of reductant must exist (Lennartz et al., 2001; Page et al., 2004; Gabilly et al., 2010).

Evidence of an additional pathway for the supply of reducing power was provided with the finding that the *ccs4* mutant is restored for cytochrome *c* assembly by application of exogenous thiols (Gabilly et al., 2011). CCS4 is a small protein with an N-terminal membrane anchor and a C-terminal domain predicted to be exposed to the stromal side of the thylakoid membrane but does not display any motif or residue (such as cysteines) suggesting a role in thiol-based redox chemistry (Gabilly et al., 2011). CCS4 exhibits similarity to *Arabidopsis* HCF153, a thylakoid membrane anchored protein with a stromal facing C-terminal domain required for cytochrome b_6f accumulation (Lennartz et al., 2006). In addition to the thiol-dependent photosynthetic rescue of the *ccs4* mutant, the placement of CCS4 in a disulfide-reducing pathway for cytochrome *c* assembly is further substantiated by the fact that ectopic expression of CCDA, a thiol/disulfide oxidoreductase of the DsbD family, at the thylakoid membrane suppresses the *ccs4* mutant (Gabilly et al., 2011). As none of the *CCS* loci correspond to *CCDA* (Page et al., 2004), the CCDA-dependent suppression of the *ccs4* mutant provides indirect evidence for the function of CCDA in plastid cytochrome *c* maturation. The suppression can be explained by a compensatory effect due to enhanced expression of the thiol-disulfide oxidoreductase CCDA. The activity of CCS4 in the heme attachment reaction so far remains unclear but one attractive scenario is that it controls the delivery of reducing power through the membrane via transport of a reductant. There is precedence for this in bacterial periplasm where reducing power, in the form of cysteine or glutathione, is transferred from the cytoplasm to the periplasm via specific transporters (Pittman et al., 2005; Ohtsu et al., 2010).

OTHER CCS COMPONENTS UNIQUE TO PLASTID CYTOCHROME *C* MATURATION

In bacteria using the CCS pathway, CcsA, Ccs1, a thiol-disulfide reductase of the DsbD family, and a thioredoxin-like protein are the only components required to complete holocytochrome *c* assembly (Beckett et al., 2000; Le Brun et al., 2000). From the genetic analysis of the *Chlamydomonas ccs* mutants, it appears that cytochrome *c* maturation in plastids is a more complicated process than in bacteria. This seems counter-intuitive considering that bacteria can assemble numerous mono and multiheme cytochromes *c* via the CCS pathway, while plastids only need to mature up to three monoheme cytochromes. In addition to CcsA, CCS1, CCDA, and HCF164/CCS5, plastid cytochrome *c* maturation also requires CCS4 and the products of the *CCS2*, *CCS3*, and *CCS6* genes (Xie et al., 1998; Page et al., 2004), which remain uncharacterized. The fact that single alleles map to the *Chlamydomonas CCS3, CCS4, CCS5,* and *CCS6* loci suggests that mutant screens for plastid cytochrome *c* deficient mutants are not saturated and additional CCS loci could still be uncovered (Howe and Merchant, 1992; Xie et al., 1998; Dreyfuss and Merchant, 1999; Page et al., 2004).

AUTHOR CONTRIBUTIONS

SG and PH wrote the manuscript jointly. SG designed the figure.

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REFERENCES

Allen, J. W., Ginger, M. L., and Ferguson, S. J. (2004). Maturation of the unusual single-cysteine (XXXCH) mitochondrial c-type cytochromes found in trypanosomatids must occur through a novel biogenesis pathway. *Biochem. J.* 383(Pt 3), 537–542.

Allen, J. W., Jackson, A. P., Rigden, D. J., Willis, A. C., Ferguson, S. J., and Ginger, M. L. (2008). Order within a mosaic distribution of mitochondrial *c*-type cytochrome biogenesis systems? *FEBS J.* 275, 2385–2402. doi: 10.1111/j.1742-4658.2008.06380.x

Allen, J. W. A. (2011). Cytochrome *c* biogenesis in mitochondria – Systems III and V. *FEBS J.* 278, 4198–4216. doi: 10.1111/j.1742-4658.2011.08231.x

Aragão, D., Frazão, C., Sieker, L., Sheldrick, G. M., LeGall, J., and Carrondo, M. A. (2003). Structure of dimeric cytochrome *c*3 from *Desulfovibrio gigas* at 1.2 A resolution. *Acta Crystallogr. D Biol. Crystallogr.* 59(Pt 4), 644–653. doi: 10.1107/S0907444490300194X

Babbitt, S. E., Sutherland, M. C., Francisco, B. S., Mendez, D. L., and Kranz, R. G. (2015). Mitochondrial cytochrome *c* biogenesis: no longer an enigma. *Trends Biochem. Sci.* 40, 446–455. doi: 10.1016/j.tibs.2015.05.006

Beckett, C. S., Loughman, J. A., Karberg, K. A., Donato, G. M., Goldman, W. E., and Kranz, R. G. (2000). Four genes are required for the system II cytochrome *c* biogenesis pathway in *Bordetella pertussis*, a unique bacterial model. *Mol. Microbiol.* 38, 465–481. doi: 10.1046/j.1365-2958.2000.02174.x

Bertini, I., Cavallaro, G., and Rosato, A. (2007). Evolution of mitochondrial-type cytochrome *c* domains and of the protein machinery for their assembly. *J. Inorg. Biochem.* 101, 1798–1811. doi: 10.1016/j.jinorgbio.2007.02.001

Bonnard, G., Corvest, V., Meyer, E. H., and Hamel, P. P. (2010). Redox processes controlling the biogenesis of *c*-type cytochromes. *Antioxid. Redox Signal.* 13, 1385–1401. doi: 10.1089/ars.2010.3161

Bowman, S. E. J., and Bren, K. L. (2008). The chemistry and biochemistry of heme c: functional bases for covalent attachment. *Nat. Prod. Rep.* 25, 1118–1130. doi: 10.1039/b717196j

Buchanan, B. B., and Luan, S. (2005). Redox regulation in the chloroplast thylakoid lumen: a new frontier in photosynthesis research. *J. Exp. Bot.* 56, 1439–1447. doi: 10.1093/jxb/eri158

de Vitry, C. (2011). Cytochrome *c* maturation system on the negative side of bioenergetic membranes: CCB or System IV. *FEBS J.* 278, 4189–4197. doi: 10.1111/j.1742-4658.2011.08373.x

Dreyfuss, B. W., Hamel, P. P., Nakamoto, S. S., and Merchant, S. (2003). Functional analysis of a divergent system II protein, Ccs1, involved in *c*-type cytochrome biogenesis. *J. Biol. Chem.* 278, 2604–2613. doi: 10.1074/jbc.M208652200

Dreyfuss, B. W., and Merchant, S. (1999). "*CCS5*, a new locus required for chloroplast *c*-type synthesis," in *Proceedings of the XIth International Congress on Photosynthesis*, eds J. Pusztai and G. Garab (Dordrecht: Kluwer Academic Publishers), 3139–3142.

Einsle, O., Messerschmidt, A., Stach, P., Bourenkov, G. P., Bartunik, H. D., Huber, R., et al. (1999). Structure of cytochrome *c* nitrite reductase. *Nature* 400, 476–480. doi: 10.1038/22802

Feissner, R. E., Richard-Fogal, C. L., Frawley, E. R., Loughman, J. A., Earley, K. W., and Kranz, R. G. (2006). Recombinant cytochromes *c* biogenesis systems I and II and analysis of haem delivery pathways in *Escherichia coli*. *Mol. Microbiol.* 60, 563–577. doi: 10.1111/j.1365-2958.2006.05132.x

Frawley, E. R., and Kranz, R. G. (2009). CcsBA is a cytochrome *c* synthetase that also functions in heme transport. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10201–10206. doi: 10.1073/pnas.0903132106

Fülöp, V., Sam, K. A., Ferguson, S. J., Ginger, M. L., and Allen, J. W. A. (2009). Structure of a trypanosomatid mitochondrial cytochrome *c* with heme attached via only one thioether bond and implications for the substrate recognition requirements of heme lyase. *FEBS J.* 276, 2822–2832. doi: 10.1111/j.1742-4658.2009.07005.x

Gabilly, S. T., Dreyfuss, B. W., Karamoko, M., Corvest, V., Kropat, J., Page, M. D., et al. (2010). CCS5, a thioredoxin-like protein involved in the assembly of plastid *c*-type cytochromes. *J. Biol. Chem.* 285, 29738–29749. doi: 10.1074/jbc.M109.099069

Gabilly, S. T., Kropat, J., Karamoko, M., Page, M. D., Nakamoto, S. S., Merchant, S. S., et al. (2011). A novel component of the disulfide-reducing pathway required for cytochrome *c* assembly in plastids. *Genetics* 187, 793–802. doi: 10.1534/genetics.110.125369

Giegé, P., Grienenberger, J. M., and Bonnard, G. (2008). Cytochrome *c* biogenesis in mitochondria. *Mitochondrion* 8, 61–73. doi: 10.1016/j.mito.2007.10.001

Goddard, A. D., Stevens, J. M., Rondelet, A., Nomerotskaia, E., Allen, J. W., and Ferguson, S. J. (2010). Comparing the substrate specificities of cytochrome *c* biogenesis Systems I and II: bioenergetics. *FEBS J.* 277, 726–737. doi: 10.1111/j.1742-4658.2009.07517.x

Gupta, R., He, Z., and Luan, S. (2002a). Functional relationship of cytochrome c_6 and plastocyanin in *Arabidopsis*. *Nature* 417, 567–571.

Gupta, R., Mould, R. M., He, Z., and Luan, S. (2002b). A chloroplast FKBP interacts with and affects the accumulation of Rieske subunit of cytochrome *bf* complex. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15806–15811. doi: 10.1073/pnas.222550399

Hamel, P., Corvest, V., Giege, P., and Bonnard, G. (2009). Biochemical requirements for the maturation of mitochondrial *c*-type cytochromes. *Biochim. Biophys. Acta* 1793, 125–138. doi: 10.1016/j.bbamcr.2008.06.017

Hamel, P. P., Dreyfuss, B. W., Xie, Z., Gabilly, S. T., and Merchant, S. (2003). Essential histidine and tryptophan residues in CcsA, a system II polytopic cytochrome *c* biogenesis protein. *J. Biol. Chem.* 278, 2593–2603. doi: 10.1074/jbc.M208651200

Hartshorne, R. S., Kern, M., Meyer, B., Clarke, T. A., Karas, M., Richardson, D. J., et al. (2007). A dedicated haem lyase is required for the maturation of a novel bacterial cytochrome *c* with unconventional covalent haem binding. *Mol. Microbiol.* 64, 1049–1060. doi: 10.1111/j.1365-2958.2007.05712.x

Herbaud, M. L., Aubert, C., Durand, M. C., Guerlesquin, F., Thöny-Meyer, L., and Dolla, A. (2000). *Escherichia coli* is able to produce heterologous tetraheme cytochrome c_3 when the *ccm* genes are co-expressed. *Biochim. Biophys. Acta* 1481, 18–24. doi: 10.1016/S0167-4838(00)00117-5

Howe, C. J., Schlarb-Ridley, B. G., Wastl, J., Purton, S., and Bendall, D. S. (2006). The novel cytochrome c6 of chloroplasts: a case of evolutionary bricolage? *J. Exp. Bot.* 57, 13–22. doi: 10.1093/jxb/erj023

Howe, G., and Merchant, S. (1992). The biosynthesis of membrane and soluble plastidic *c*-type cytochromes of *Chlamydomonas reinhardtii* is dependent on multiple common gene products. *EMBO J.* 11, 2789–2801.

Howe, G., and Merchant, S. (1993). Maturation of thylakoid lumen proteins proceeds post-translationally through an intermediate *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 90, 1862–1866. doi: 10.1073/pnas.90.5.1862

Howe, G., and Merchant, S. (1994). Role of heme in the biosynthesis of cytochrome c_6. *J. Biol. Chem.* 269, 5824–5832.

Inoue, K., Dreyfuss, B. W., Kindle, K. L., Stern, D. B., Merchant, S., and Sodeinde, O. A. (1997). *CCS1*, a nuclear gene required for the post-translational assembly of chloroplast *c*-type cytochromes. *J. Biol. Chem.* 272, 31747–31754. doi: 10.1074/jbc.272.50.31747

Kerfeld, C. A., and Krogmann, D. W. (1998). PHOTOSYNTHETIC CYTOCHROMES c IN CYANOBACTERIA, ALGAE, AND PLANTS. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 397–425. doi: 10.1146/annurev.arplant.49.1.397

Kern, M., Eisel, F., Scheithauer, J., Kranz, R. G., and Simon, J. (2010). Substrate specificity of three cytochrome *c* haem lyase isoenzymes from *Wolinella succinogenes*: unconventional haem *c* binding motifs are not sufficient for haem *c* attachment by NrfI and CcsA1. *Mol. Microbiol.* 75, 122–137. doi: 10.1111/j.1365-2958.2009.06965.x

Kletzin, A., Heimerl, T., Flechsler, J., van Niftrik, L., Rachel, R., and Klingl, A. (2015). Cytochromes *c* in Archaea: distribution, maturation, cell architecture, and the special case of *Ignicoccus hospitalis*. *Front. Microbiol.* 6:439. doi: 10.3389/fmicb.2015.00439

Kranz, R. G., Richard-Fogal, C., Taylor, J.-S., and Frawley, E. R. (2009). Cytochrome *c* biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. *Microbiol. Mol. Biol. Rev.* 73, 510–528. doi: 10.1128/mmbr.00001-09

Kuras, R., Saint-Marcoux, D., Wollman, F. A., and de Vitry, C. (2007). A specific *c*-type cytochrome maturation system is required for oxygenic photosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9906–9910. doi: 10.1073/pnas.0702340104

Le Brun, N. E., Bengtsson, J., and Hederstedt, L. (2000). Genes required for cytochrome *c* synthesis in *Bacillus subtilis*. *Mol. Microbiol.* 36, 638–650. doi: 10.1046/j.1365-2958.2000.01883.x

Lee, J. H., Harvat, E. M., Stevens, J. M., Ferguson, S. J., and Saier, M. H. Jr. (2007). Evolutionary origins of members of a superfamily of integral membrane cytochrome *c* biogenesis proteins. *Biochim. Biophys. Acta* 1768, 2164–2181. doi: 10.1016/j.bbamem.2007.04.022

Lennartz, K., Bossmann, S., Westhoff, P., Bechtold, N., and Meierhoff, K. (2006). HCF153, a novel nuclear-encoded factor necessary during a post-translational step in biogenesis of the cytochrome *bf* complex. *Plant J.* 45, 101–112. doi: 10.1111/j.1365-313X.2005.02605.x

Lennartz, K., Plücken, H., Seidler, A., Westhoff, P., Bechtold, N., and Meierhoff, K. (2001). *HCF164* encodes a thioredoxin-like protein involved in the biogenesis of the cytochrome b_6f complex in *Arabidopsis*. *Plant Cell* 13, 2539–2552. doi: 10.1105/tpc.13.11.2539

Marcaida, M. J., Schlarb-Ridley, B. G., Worrall, J. A., Wastl, J., Evans, T. J., Bendall, D. S., et al. (2006). Structure of cytochrome c_{6A}, a novel dithio-cytochrome of *Arabidopsis thaliana*, and its reactivity with plastocyanin: implications for function. *J. Mol. Biol.* 360, 968–977. doi: 10.1016/j.jmb.2006.05.065

Martinez, S. E., Huang, D., Szczepaniak, A., Cramer, W. A., and Smith, J. L. (1994). Crystal structure of chloroplast cytochrome *f* reveals a novel cytochrome fold and unexpected heme ligation. *Structure* 2, 95–105. doi: 10.1016/S0969-2126(00)00012-5

Mavridou, D. A. I., Ferguson, S. J., and Stevens, J. M. (2013). Cytochrome *c* assembly. *IUBMB Life* 65, 209–216. doi: 10.1002/iub.1123

Merchant, S., and Bogorad, L. (1987a). The Cu(II)-repressible plastidic cytochrome *c*. Cloning and sequence of a complementary DNA for the pre-apoprotein. *J. Biol. Chem.* 262, 9062–9067.

Merchant, S., and Bogorad, L. (1987b). Metal ion regulated gene expression: use of a plastocyanin-less mutant of *Chlamydomonas reinhardtii* to study the Cu(II)-dependent expression of cytochrome *c*-552. *EMBO J.* 6, 2531–2535.

Merchant, S., and Dreyfuss, B. W. (1998). Posttranslational assembly of photosynthetic metalloproteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 25–51. doi: 10.1146/annurev.arplant.49.1.25

Molina-Heredia, F. P., Wastl, J., Navarro, J. A., Bendall, D. S., Hervas, M., Howe, C. J., et al. (2003). Photosynthesis: a new function for an old cytochrome? *Nature* 424, 33–34. doi: 10.1038/424033b

Motohashi, K., and Hisabori, T. (2006). HCF164 receives reducing equivalents from stromal thioredoxin across the thylakoid membrane and mediates reduction of target proteins in the thylakoid lumen. *J. Biol. Chem.* 281, 35039–35047. doi: 10.1074/jbc.M605938200

Motohashi, K., and Hisabori, T. (2010). CcdA is a thylakoid membrane protein required for the transfer of reducing equivalents from stroma to thylakoid lumen in the higher plant chloroplast. *Antioxid. Redox Signal.* 13, 1169–1176. doi: 10.1089/ars.2010.3138

Ohtsu, I., Wiriyathanawudhiwong, N., Morigasaki, S., Nakatani, T., Kadokura, H., and Takagi, H. (2010). The L-cysteine/L-cystine shuttle system provides reducing equivalents to the periplasm in *Escherichia coli*. *J. Biol. Chem.* 285, 17479–17487. doi: 10.1074/jbc.M109.081356

Page, M. L. D., Hamel, P. P., Gabilly, S. T., Zegzouti, H., Perea, J. V., Alonso, J. M., et al. (2004). A homolog of prokaryotic thiol disulfide transporter CcdA is required for the assembly of the cytochrome b_6f complex in *Arabidopsis* chloroplasts. *J. Biol. Chem.* 279, 32474–32482. doi: 10.1074/jbc.M404285200

Pittman, M. S., Robinson, H. C., and Poole, R. K. (2005). A bacterial glutathione transporter (*Escherichia coli* CydDC) exports reductant to the periplasm. *J. Biol. Chem.* 280, 32254–32261. doi: 10.1074/jbc.M503075200

Priest, J. W., and Hajduk, S. L. (1992). Cytochrome *c* reductase purified from *Crithidia fasciculata* contains an atypical cytochrome c_1. *J. Biol. Chem.* 267, 20188–20195.

Richard-Fogal, C., and Kranz, R. G. (2010). The CcmC:heme:CcmE complex in heme trafficking and cytochrome *c* biosynthesis. *J. Mol. Biol.* 401, 350–362. doi: 10.1016/j.jmb.2010.06.041

Richard-Fogal, C. L., Frawley, E. R., Bonner, E. R., Zhu, H., San Francisco, B., and Kranz, R. G. (2009). A conserved haem redox and trafficking pathway for cofactor attachment. *EMBO J.* 28, 2349–2359. doi: 10.1038/emboj.2009.189

Richard-Fogal, C. L., San Francisco, B., Frawley, E. R., and Kranz, R. G. (2012). Thiol redox requirements and substrate specificities of recombinant cytochrome *c* assembly systems II and III. *Biochim. Biophys. Acta* 1817, 911–919. doi: 10.1016/j.bbabio.2011.09.008

Sandmann, G., Reck, H., Kessler, E., and Boger, P. (1983). Distribution of plastocyanin and soluble plastidic cytochrome *c* in various classes of algae. *Arch. Microbiol.* 134, 23–27. doi: 10.1007/BF00429401

Simon, J., and Hederstedt, L. (2011). Composition and function of cytochrome *c* biogenesis System II. *FEBS J.* 278, 4179–4188. doi: 10.1111/j.1742-4658.2011.08374.x

Thony-Meyer, L. (1997). Biogenesis of respiratory cytochromes in bacteria. *Microbiol. Mol. Biol. Rev.* 61, 337–376.

Tichy, M., and Vermaas, W. (1999). Accumulation of pre-apocytochrome *f* in a *Synechocystis* sp. PCC 6803 mutant impaired in cytochrome *c* maturation. *J. Biol. Chem.* 274, 32396–32401. doi: 10.1074/jbc.274.45.32396

Travaglini-Allocatelli, C. (2013). Protein machineries involved in the attachment of heme to cytochrome *c*: protein structures and molecular mechanisms. *Scientifica* 2013:505714. doi: 10.1155/2013/505714

Verissimo, A. F., and Daldal, F. (2014). Cytochrome *c* biogenesis System I: an intricate process catalyzed by a maturase supercomplex? *Biochim. Biophys. Acta* 1837, 989–998. doi: 10.1016/j.bbabio.2014.03.003

Wastl, J., Purton, S., Bendall, D. S., and Howe, C. J. (2004). Two forms of cytochrome c6 in a single eukaryote. *Trends Plant Sci.* 9, 474–476. doi: 10.1016/j.tplants.2004.08.005

Weigel, M., Varotto, C., Pesaresi, P., Finazzi, G., Rappaport, F., Salamini, F., et al. (2003). Plastocyanin is indispensable for photosynthetic electron flow in *Arabidopsis thaliana*. *J. Biol. Chem.* 278, 31286–31289. doi: 10.1074/jbc.M302876200

Xie, Z., Culler, D., Dreyfuss, B. W., Kuras, R., Wollman, F. A., Girard-Bascou, J., et al. (1998). Genetic analysis of chloroplast *c*-type cytochrome assembly in *Chlamydomonas reinhardtii*: one chloroplast locus and at least four nuclear loci are required for heme attachment. *Genetics* 148, 681–692.

Xie, Z., and Merchant, S. (1996). The plastid-encoded *ccsA* gene is required for heme attachment to chloroplast *c*-type cytochromes. *J. Biol. Chem.* 271, 4632–4639. doi: 10.1074/jbc.271.9.4632

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CCS2, an Octatricopeptide-Repeat Protein, Is Required for Plastid Cytochrome *c* Assembly in the Green Alga *Chlamydomonas reinhardtii*

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In bacteria and energy generating organelles, *c*-type cytochromes are a class of universal electron carriers with a heme cofactor covalently linked via one or two thioether bonds to a heme binding site. The covalent attachment of heme to apocytochromes is a catalyzed process, taking place via three evolutionarily distinct assembly pathways (Systems I, II, III). System II was discovered in the green alga *Chlamydomonas reinhardtii* through the genetic analysis of the *ccs* mutants (**c**ytochrome ***c*** **s**ynthesis), which display a block in the apo- to holo- form conversion of cytochrome *f* and c_6, the thylakoid lumen resident *c*-type cytochromes functioning in photosynthesis. Here we show that the gene corresponding to the *CCS2* locus encodes a 1,719 amino acid polypeptide and identify the molecular lesions in the *ccs2-1* to *ccs2-5* alleles. The CCS2 protein displays seven degenerate amino acid repeats, which are variations of the **o**ctatrico**p**eptide-**r**epeat motif (OPR) recently recognized in several nuclear-encoded proteins controlling the maturation, stability, or translation of chloroplast transcripts. A plastid site of action for CCS2 is inferred from the finding that GFP fused to the first 100 amino acids of the algal protein localizes to chloroplasts in *Nicotiana benthamiana*. We discuss the possible functions of CCS2 in the heme attachment reaction.

Keywords: plastid, photosynthesis, cytochrome *c*, heme, assembly factor, OPR

INTRODUCTION

Energy-transducing membranes are specialized membranes in archaea, bacteria, mitochondria, and chloroplasts, which rely on electron carriers to generate the proton gradient necessary for ATP synthesis. In energy-transducing membranes, the *c*-type cytochromes, also generically referred to as cytochromes *c*, are a class of structurally diverse metalloproteins with one or more covalently linked heme(s) (ferro-protoporphyrin IX) that participate in electron transfer reactions (Thony-Meyer, 1997; Bonnard et al., 2010; Verissimo and Daldal, 2014). On the positive side (*p*-side) of the energy-transducing membrane (i.e., bacterial or archaeal periplasm, mitochondrial intermembrane space, thylakoid lumen), a group of cytochromes *c* occur as both soluble and membrane proteins with the heme moiety typically attached *via* a thioether linkage to two cysteine sulfhydryl(s) in a characteristic motif in the apocytochrome *c*. The consensus motif, also referred to as the heme-binding site, is CX_nCH where n is usually equal to two and X can be any residue except

cysteine in naturally occurring cytochromes *c*. The histidine in this motif acts as a ligand of ferroheme (Bowman and Bren, 2008; Allen et al., 2009). Heme attachment to the heme-binding site is a catalyzed reaction *in vivo* and requires heme transport across at least one biological membrane and covalent linkage of ferroheme to free sulfhydryls of the heme binding cysteines in the CX_nCH motif (Bonnard et al., 2010; Mavridou et al., 2013).

To date, three Systems (I, II, and III) have been identified as being necessary for the post-translational assembly of cytochromes *c* located on the *p*-side of the membrane, where these molecules function. These systems have been identified through extensive investigation of both bacterial and eukaryotic models (Kranz et al., 2009; Allen, 2011; de Vitry, 2011; Mavridou et al., 2013; Verissimo and Daldal, 2014). In plastids, the heme attachment reaction occurs on the luminal side of the thylakoid membrane and is under the control of System II. This system is composed of multiple pathways and occurs in plastids of all photosynthetic eukaryotes, cyanobacteria, and most proteobacteria of the β-, δ-, and ε-group (Hamel et al., 2009; Bonnard et al., 2010; Simon and Hederstedt, 2011).

System II first emerged through genetic screens for photosynthesis-impaired *ccs* mutants (*ccs* for **c**ytochrome **c** **s**ynthesis) in the green alga *Chlamydomonas reinhardtii* (Hamel et al., 2009; Simon and Hederstedt, 2011; Gabilly and Hamel, 2017). The *ccs* mutants were isolated on the basis of photosynthetic deficiency due to loss of plastid *c*-type cytochromes, namely membrane-bound cytochrome *f* and soluble cytochrome c_6 (Howe and Merchant, 1992). These two plastid cytochromes *c* reside in the thylakoid lumen and function in photosynthesis. The defect is specific to plastid cytochromes *c,* as abundance of mitochondrial cytochromes *c* was unaffected in the *ccs* mutants (Howe and Merchant, 1992). Pulse-chase analyses revealed that precursor forms of apocytochrome *f* and c_6 are synthesized, translocated to the lumen, and therein cleaved by the thylakoid peptidase. However, they are not converted to their respective holoforms. This indicates that the *CCS* loci control the heme attachment reaction in the lumen (Howe and Merchant, 1992, 1993, 1994; Xie et al., 1998; Gabilly et al., 2010). At least seven loci, plastid *ccsA* and nuclear *CCS1* to *CCS6*, were uncovered through genetic analysis of the *ccs* mutants isolated via several UV and insertional mutageneses (Xie et al., 1998; Page et al., 2004; Gabilly et al., 2010).

Functional analysis of plastid CcsA and Ccs1 led to the proposal that these two proteins act together to relay heme from its site of synthesis, the stroma, to its site of function, the lumen, possibly in an assembly complex alongside other CCS factors (Xie and Merchant, 1996; Inoue et al., 1997; Dreyfuss et al., 2003; Hamel et al., 2003). Biochemical studies supported this model with evidence that bacterial CcsA and Ccs1 operate as a functional unit with a dual activity in heme transport across the membrane and heme ligation to apocytochromes *c* in the periplasmic space (Frawley and Kranz, 2009).

In addition to heme delivery and attachment, the maintenance of the cysteine sulfhydryls (reduced vs. oxidized) within the heme binding site is a prerequisite for covalent linkage of heme (Bonnard et al., 2010; Sanders et al., 2010; Verissimo and Daldal, 2014). The proposed model is that the cysteines are first oxidized by disulfide bond forming enzymes on the *p*-side of the membrane and must subsequently be reduced to provide free sulfhydryls for the heme ligation reaction. In plastids and bacteria, this requirement is fulfilled by the operation of a disulfide reducing pathway, which conveys electrons across the energy-transducing membrane via thiol-disulfide exchanges in order to reduce the disulfide bond formed between the heme-linking cysteines in the heme binding motif of apocytochromes *c* (Karamoko et al., 2013; Verissimo and Daldal, 2014; Gabilly and Hamel, 2017).

In bacteria, the CcsA/Ccs1-dependent heme delivery/attachment pathway and the disulfide-reducing pathway are all that is required for cytochrome *c* assembly (Beckett et al., 2000; Le Brun et al., 2000; Feissner et al., 2005). However, screening of *ccs* mutants in *Chlamydomonas reinhardtii* has revealed four additional *CCS* loci (*CCS2*, *CCS3*, *CCS4,* and *CCS6*), an indication that the assembly of cytochromes *c* in the plastid is a more complicated process (Xie et al., 1998; Page et al., 2004). While we have proposed that CCS4 regulates the disulfide reducing pathway in the plastid (Gabilly et al., 2011), the gene products for *CCS2*, *CCS3,* and *CCS6* remain unknown.

In this article, we report the molecular identification of the *CCS2* locus by functional complementation of the *ccs2* mutant. The *CCS2* gene encodes a plastid-localized, 170 kDa protein that was previously identified by bioinformatics to be a member of the **o**ctatrico**p**eptide **r**epeat (OPR) family (Eberhard et al., 2011).

MATERIALS AND METHODS

Strains and Culture Condition

The *ccs2-1* through *ccs2-5* strains were used in complementation experiments (Xie et al., 1998). Mutants *ccs2-1* and *ccs2-2* were crossed to wild-type strain $3A^+$ (*mt*$^+$ *arg7-8*) and $4C^-$ (*mt*$^-$ *arg7-8*), respectively, to generate the *ccs2-1 arg7-8* and *ccs2-2 arg7-8* recipient strains. The *ccs2-1 arg7-8* mutant was then crossed to wild-type strain CC425 (*arg7-8 cw15*) to generate the *ccs2-1 arg7-8 cw15* mutant used for detection of the HA-tagged CCS2 protein. The MCA1-HA expressing strain is described in Raynaud et al. (2007). Strains were maintained at 25°C on Tris-acetate phosphate (TAP) liquid or solid medium supplemented with 400 mg/mL arginine (Harris, 1989) at 0.6 $\mu E/m^2/s$. Complemented *ccs2* strains were assessed for restoration of photoautotrophic growth on minimal medium (Min) (Harris, 1989) or acetate containing (1.7 mM) minimal medium. For protein extraction, wild-type and complemented strains were grown in liquid TAP supplemented with arginine under 50 $\mu E/m^2/s$ illumination while *ccs* mutants were grown under 0.6 $\mu E/m^2/s$ illumination. Cell wall-less mutants (*cw15*) were cultured in liquid and on solid media supplemented with 50 mM sorbitol. Copper-free media for induction of cytochrome c_6 was prepared as described previously (Howe and Merchant, 1992; Quinn and Merchant, 1998).

Molecular Cloning of the *CCS2* Gene

An indexed cosmid library of *Chlamydomonas* genomic DNA was used for transformation by electroporation as described

by Shimogawara et al. (1998) with the following exceptions: the vector backbone was the cosmid pCB412 containing the *ARG7* marker and the transformation required a 30 min incubation in autolysin (to digest the cell wall) followed by electroporation of 5 μg of DNA per cosmid pool on a Biorad Micropulser at 1300 V. Transformants were selected under 50 $\mu E/m^2/s$ light on minimal media supplemented with 1.7 mM acetate. Plasmid pMOL+8.2 kb was generated by cloning the 8.2 kb *Bam*HI/*Hind*III *CCS2* genomic fragment isolated from complementing cosmid (c8G6), into *Bam*HI/*Hind*III digested pMOLUC (Cha et al., 2002).

Assembly of the *CCS2* cDNA

Chlamydomonas RNAs were extracted and retro-transcribed using a bacterial reverse transcriptase from the Roche Transcriptor High Fidelity cDNA Synthesis Kit and the *CCS2* specific primers CCS2.30 and CCS2.66STP. Overlapping fragments were amplified with the following primer pairs: CCS2.69 and CCS2.66STP, CCS2.19 and CCS2.54, CCS2.21 and CCS2.18, CCS2.79 and CCS2.02, CCS2.27 and CCS2.28, CCS2.51 and CCS2.26, and, finally, CCS2.81ATG and CCS2.70 using DV Ready Mix (Sigma) and the total retro-transcribed RNAs as a template. Amplified fragments were isolated after electrophoresis in agarose gel, purified, re-amplified and AT-cloned into pGEM-T Easy for sequencing. All primer sequences are listed in Supplementary Table S1.

Construction of HA-Tagged CCS2 and CCS2-GFP Expressing Constructs

Versions of the *CCS2* gene carrying an internal HA tag were created by cutting pMOL+8.2 kb by *Bsi*WI or *Bsp*EI and inserting the HA-tag sequence via In-Fusion® (CloneTech). The HA-tag sequence was generated using PCR based fill-in of primers CCS2-HA_BsiW1-F and CCS2-HA_BsiW1-R or CCS2-HA_BspEI-F and CCS2-HA_BspEI-R. Introduction of an internal HA tag sequence at the *Bsp*EI site (residue 298) abolished CCS2 function while the tag at *Bsi*WI (residue 1672) had no impact (not shown).

To generate the series of "p8" plasmids used in **Figure 5**, a 606 bp sequence was synthesized (Genscript) and cloned into pUC57 (pUC57+ccs2bit). This sequence contained two distinct fragments that were modified from the *Chlamydomonas* genomic DNA. The first was a 207 bp fragment corresponding to the 3′ end of *CCS2* genomic DNA that both changed the stop codon in the *CCS2* ORF into an alanine and added the restriction sites *Xba*I, *Swa*I, and *Spe*I downstream of the stop codon. The second was a 394 bp fragment encompassing the 5′ end of *CCS2* genomic DNA designed to remove the native *CCS2* promoter and introduce restriction sites *Bgl*II and *Xho*I upstream of the ATG.

Carboxy-terminus 3xHA tags were generated by cutting pMOL+8.2 kb by *Bsi*WI (146 bp upstream the stop codon) and *PshA*1 (570 bp downstream of the stop codon in the 3′ UTR) and cloning the 207 bp fragment, which was amplified by primers CCS2-BsiWI-F and CCS2-PshAI-R using pUC57+ccs2bits as a template. The 207 bp fragment was cloned using In-Fusion® and the resulting plasmid is p8-noS. The p8-noS plasmid was then digested by *Xba*I and *Spe*I and a sequence corresponding to a 3xHA tag (and including a stop), created through PCR fill-in of primers 8.3xHA-f and 8.3xHA-r, was inserted via In-Fusion®. This created vector p8-3xHA expressing CCS2-HA from its native promotor. The plasmids expressing CCS2 or CCS2-HA under the *PSAD* promoter were generated in a similar manner. The 394 bp fragment was amplified from pUC57+ccs2bits using primers CCS2-XcmI.R and CCS2-XcmI.F and inserted via In-Fusion® after digestion of pMOL+8.2 kb and p8-3xHA by *Xcm*I (322 bp from the initiation codon). This created plasmids p8-noP and p8-3xHAnoP. These constructs were then used to generate plasmids p8-PROM and p8-3xHA+P. To create these vectors, the *PSAD* promoter and 5′ UTR were amplified from plasmid pSL18 (Pollock et al., 2004), using primers 8.PROM-f and 8.PROM-r, and inserted via In-Fusion® at the introduced restriction sites *Bgl*II and *Xho*I.

The plasmid pGWB5/ccs2target, expressing the CCS2-GFP fusion protein, was constructed from pUC57+ccs2targeting, which contains the first 300 bp from *CCS2* ORF, corresponding to the first 100 amino acids of CCS2, cloned into pUC57. This 300 bp sequence was codon optimized for expression in tobacco and synthesized by GenScript. Using primers CCS2.t1 and CCS2.t2, the sequence was amplified from the pUC57+ccs2targeting template and then inserted in frame with the GFP reporter in the expression vector pGWB5 (Nakagawa et al., 2007) using entry vector pENTR/SD/TOPO (Invitrogen) via TOPO cloning. The GFP-NLS/GFP-NES expressing construct in pK7WGF2 (Karimi et al., 2002) is a gift from Dr. I. Meier (Ohio State University). The NLS (nuclear localization sequence) is from the SV40 large T-antigen. The NES (nuclear export signal) is from the HIV-1 Rev response element. Plasmids were transferred to *Agrobacterium tumefaciens* GV3101 by electroporation.

Protein Preparation and Analysis

Cytochrome *f* and c_6 detection was performed as in Howe and Merchant (1992). Soluble fractions for cytochrome c_6 detection were obtained by freeze-thaw fractionation of cells grown in copper deficient conditions. Fractions were electrophoretically separated and cytochromes *c* revealed by immunodetection or a heme-staining procedure (Howe and Merchant, 1992). Anti-HA immunoblotting analysis was performed on whole cells extracts, prepared as follows: cells were grown to early logarithmic phase under 0.6 $\mu E/m^2/s$ illumination and moved into 30 $\mu E/m^2/s$ light for 5 h. Cells were then pelleted and re-suspended in 10 mM $NaPO_4$ buffer with protease inhibitors (4 mM benzamine, 0.4 mM 6-amino-*n*-hexanoic acid, 2 mM PMSF, 10 μM leupeptin, 1 μM pepstatin, 1 mM ortho-phenanthroline, 40 μg/mL chymostatin, 10 μM E-64) (Kurvari et al., 1998) to a final concentration of 3×10^8 cells. Laemmli Buffer was then added to a final concentration of 1.2×10^8 cells and 0.1 M DTT. Cells were placed in a sonication bath for 120 s and the solubilized protein sample was denatured for 20 min at 70°C before electrophoresis. Polyclonal antisera raised against *Chlamydomonas* cytochrome c_6, cytochrome *f* GST-fusion protein, and CF_1 were used for immunodetection by alkaline phosphatase-conjugated secondary antibodies. The rat

monoclonal anti-HA antibody (clone 3F10) (Roche) was used for immunodetection by peroxidase-conjugated secondary antibody.

Fluorescence Rise and Decay Kinetics

Fluorescence rise and decay kinetics were taken as described in Gabilly et al. (2010) except that strains were grown in liquid medium overnight and measurements were recorded on 20 μL culture aliquots against a white background. Fluorescence transients were measured using Handy Fluorcam from Photon System Instruments. The fluorescence is in arbitrary units (A.U.) and recorded over a 3-s illumination period.

GFP Fluorescence and Imaging

Nicotiana benthamiana was transformed with *Agrobacterium* carrying the GFP-CCS2 or NLS-GFP/NES-GFP expressing construct *via* infiltration after a 1 h incubation in Induction Medium (10 mM $MgCl_2$, 10 mM 2-[*N*-Morpholino]ethanesulfonic acid, 100 μM Acetosyringone). After 3 days, protoplasts were extracted from infiltrated leaves by a 30-min incubation in a Digestion Buffer (1.5% cellulose, 0.4% macerozyme, 0.4 M mannitol, 20 mM KCl, 20 mM 2-[*N*-Morpholino]ethanesulfonic acid -KOH pH 5.5, 10 mM $CaCl_2$, 0.1% Bovine serum albumin) and concentrated with a 1 s spin at 100 rpm. Supernatant was removed and protoplasts were re-suspended in 100 μL Digestion Buffer before imaging. GFP-dependent fluorescence was taken at 515 nm and chlorophyll auto-fluorescence at 650 nm on a Nikon Cl confocal microscope (Eclipse C90i) using a medium aperture. Images were processed using the NIS-Elements software.

Analysis of OPR Motifs in Proteins

Information presented in **Figure 4** was generated by the MEME algorithm (Bailey and Elkan, 1994). Individual protein sequences (Supplementary Figure S1) were entered into the program and base settings were altered to search for any number of repetitions, one motif, between 70 and 600 sites, and between 28 and 50 amino acids wide. The OPR motifs identified by the MEME program (Supplementary Figure S2) were fed into Weblogo 3.3 (Crooks et al., 2004).

RESULTS

Cloning of the *CCS2* Gene by Functional Complementation of the *ccs2-2* Mutant

To gain further insights into plastid cytochrome *c* assembly, we sought to clone the gene corresponding to the *CCS2* locus, defined by the *ccs2-1* to *-5* alleles (Xie et al., 1998). All *ccs* mutants display a b_6f-deficient phenotype due to loss of cytochrome *f* assembly and hence are unable to grow photoautotrophically (Howe and Merchant, 1992; Xie et al., 1998). Using the photosynthetic deficient phenotype of the *ccs* mutants, we cloned the *CCS2* gene via transformation of the *ccs2-2 arg7-8* double mutant with an indexed cosmid library (Purton and Rochaix, 1994; Zhang et al., 1994). Transformants were selected for restored growth on acetate-containing minimal medium under standard illumination (50 $\mu E/m^2/s$). Two cosmids (c8G6 and c5D9) were isolated based on their ability to restore photosynthetic growth to the *ccs2-2* mutant. Sequence analysis revealed that c8G6 contains a 30.2 kb region from chromosome 19 while c5D9 appears to have rearranged. The complementing activity in c8G6 could be narrowed down to an 8.2 kb *Bam*HI-*Hind*III fragment. Both c8G6 and the cloned 8.2 kb *Bam*HI-*Hind*III fragment complemented strains *ccs2-1* through *ccs2-5*, restoring photosynthetic growth (**Figure 1A**).

To test if the cytochrome *f* assembly was restored in the complemented transformants, we performed analyses of fluorescence rise and decay kinetics (**Figure 1B**). In such experiments, the emitted fluorescence of excited chlorophyll in photosystem II is taken as an indication of the functionality of the b_6f complex, which receives electrons from photosystem II. As seen in **Figure 1B**, the rise and plateau curve for *ccs2* is characteristic of a specific block in electron transfer at the level of the cytochrome b_6f complex because of its impaired assembly in the absence of membrane-bound holocytochrome

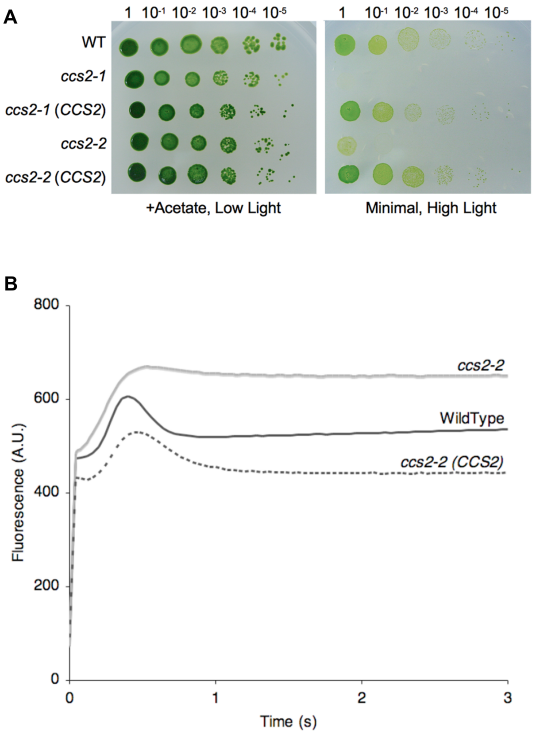

FIGURE 1 | The *CCS2* gene restores photoautotropic growth to the *ccs2* mutants. For **(A,B)**, wild-type CC124 (WT), *ccs2-1* and *ccs2-2* strains, and *CCS2* complemented mutants were used. **(A)** Ten-fold dilution series of algal cultures were plated on solid medium, with or without acetate, and incubated under low light (0.6 $\mu E/m^2/s$) or high light (50 $\mu E/m^2/s$), respectively, for 1 week at 25°C. The *ccs2-3*, *ccs2-4*, and *ccs2-5* mutants are also restored for photoautotrophic growth upon transformation with the *CCS2* gene (not shown). **(B)** Representative fluorescence rise and decay kinetics indicate restoration of the cytochrome b_6f complex in the complemented *ccs2* mutant. While all complemented mutants were restored, only the *ccs2-2* strain is shown. The fluorescence is in arbitrary units (A.U.) and recorded over a 3-s illumination period after a dark adaption period.

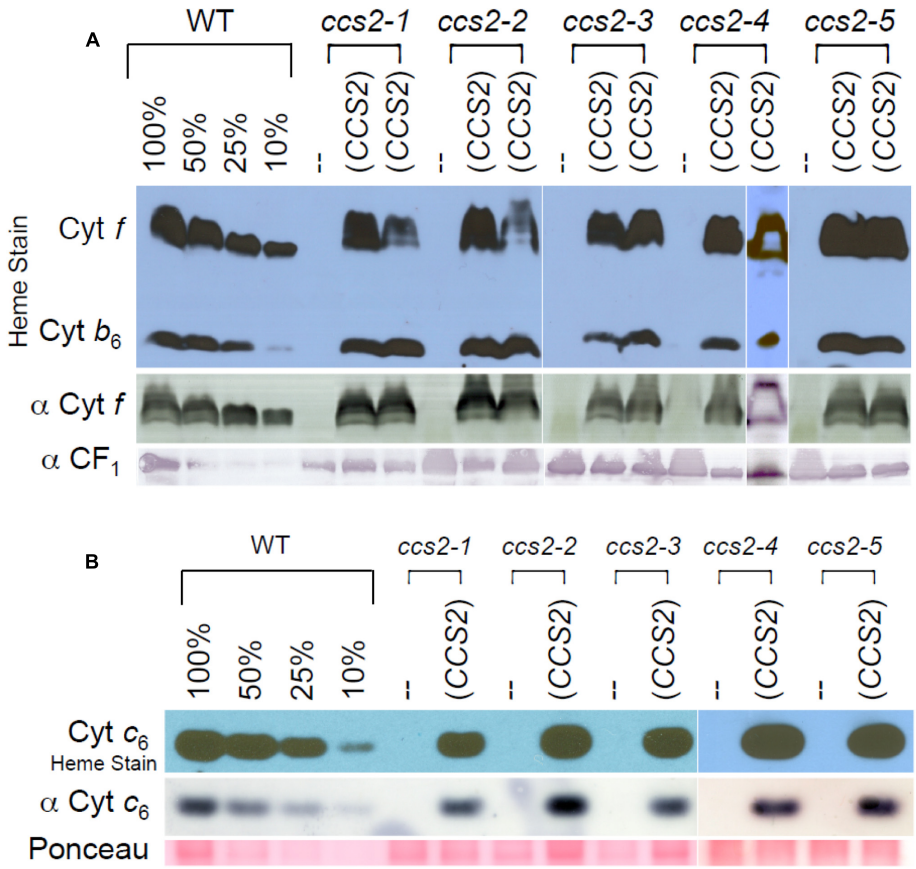

FIGURE 2 | *Restoration of plastid cytochrome c* assembly in the *CCS2* complemented strains. For **(A,B)**, the *ccs2-1* to *ccs2-5* mutants were complemented with the 8.2 kb genomic fragment containing the *CCS2* gene. Dilutions of the wild-type sample serve to estimate the cytochrome *f* **(A)** and cytochrome c_6 **(B)** abundance. **(A)** The wild-type CC124 strain (WT), *ccs2* mutants, and two independently complemented *ccs2* transformants for each *ccs2* allele (*CCS2*) were analyzed for cytochrome *f* accumulation via heme stain and immunoblotting. Detection of the CF_1 of the ATPase is shown as a loading control. Note that heme staining also reveals the presence of covalently attached heme c_i in holocytochrome b_6. As evidenced from the heme stain, holocytochrome b_6 accumulates to a lower level due to loss of cytochrome *f* assembly in the *ccs2* mutants. Different levels of holocytochrome *f* accumulation in the transformants might reflect differential expression of the introduced *CCS2* gene due to position effect of non-homologous integration of the construct in the chromosome. **(B)** Same as in **(A)**, except only one transformant was analyzed for cytochrome c_6 accumulation by heme staining and immunoblotting. Ponceau staining is shown as a loading control. The white lines in **(A,B)** indicate assembly from independent immunoblots.

f. When the energy absorbed by the chlorophyll cannot be utilized, in this case as a result of a block in photosynthetic transfer through cytochrome b_6f, an increase in the chlorophyll fluorescence is observed. In wild-type and complemented strains, the decay phase corresponds to the re-oxidation of the quinone pool, the primary electron acceptor of the photosystem II, by the cytochrome b_6f complex. This indicates holocytochrome *f* assembly and cytochrome b_6f functionality is restored in the complemented strains. In accord with this result, we also showed that both holocytochrome *f* and cytochrome c_6 accumulation is restored to wild-type levels in the complemented strains (**Figures 2A,B**).

The *CCS2* Gene Encodes a Protein of the OPR Family

Because of the large size, low abundance, and high GC content (74%) of the *CCS2* mRNA, the corresponding full-length cDNA proved difficult to amplify. Instead, overlapping cDNA fragments, approximately 2 kb in length, were amplified and the full-length transcript was extrapolated by aligning the sequenced fragments with the *CCS2* genomic DNA. Sequence comparison of the assembled cDNA with the current gene model *Cre03.g213201* extended the 5′ end of exon 1 and identified four introns within the *CCS2* gene (**Figure 3A**). The *Chlamydomonas* transcriptome from the Joint Genome Institute, University of California (JGI-UCLA) and Genoscope[1] corroborates this transcript sequence under the previous unique gene ID Cre19.g757200. We assigned the start codon in exon 1 based on the fact that this is the 5′ most ATG preceded by stop codons in all three reading frames.

Sequencing of the genomic DNA in the *ccs2-1* to *ccs2-5* mutants revealed molecular lesions in the *CCS2* coding sequence (**Figure 3A**). All lesions introduced nonsense (*ccs2-1* and *ccs2-3*)

[1]http://genomes.mcdb.ucla.edu/Cre454/

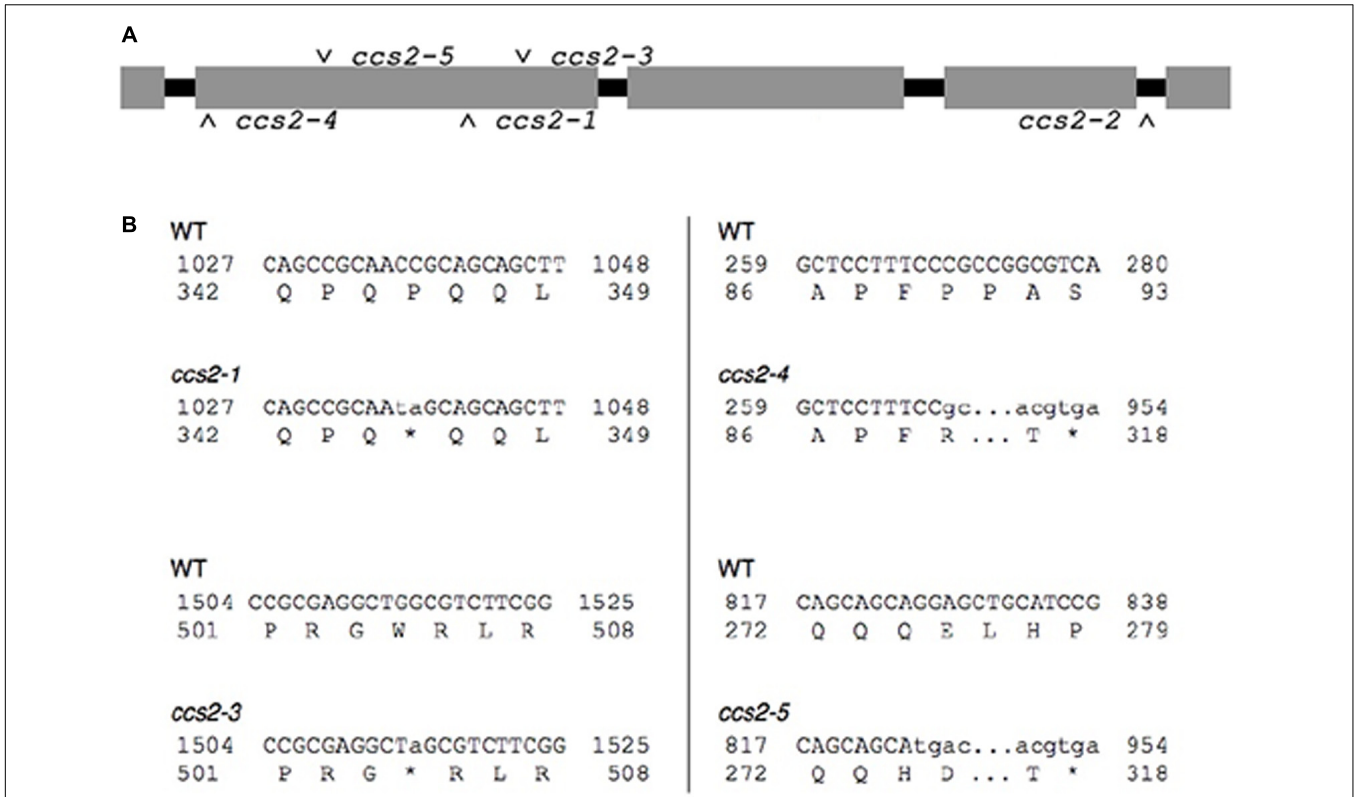

FIGURE 3 | *Molecular identification of the ccs2-1* to *ccs2-5* mutations. **(A)** Schematic representation of the *CCS2* immature transcript. Introns are shown in black and exons in gray. The position of the molecular lesions in the *ccs2* alleles is indicated. **(B)** Identified mutations in the *ccs2* alleles. The top line corresponds to the nucleotide sequence of the *CCS2* ORF and numbers displayed to either side refer to position of the nucleotides within the ORF. The lower-case letters indicate the nucleotide sequence due to change(s) induced by the UV mutagenesis. The lower line shows the corresponding amino acid sequence and the change within the protein sequence resulting from the molecular lesion(s). The asterisk indicates a stop in the protein sequence. The *ccs2-1* allele is a CC to TA mutation at position 1036-1037 and the *ccs2-3* allele is a G to A change at position 1514. In the *ccs2-4*, deletion of a single C occurred at position 269. The *ccs2-5* mutation combines a G to T mutation at position 826 and deletion of a single G at position 829. The GenBank accession number for the *CCS2* nucleotide sequence is KC292647.

or frameshift (*ccs2-4* and *ccs2-5*) mutations, which result in truncations within the first 500 amino acids of the protein except for the *ccs2-2* allele (**Figure 3B**). In the *ccs2-2* mutant, a single guanine has been deleted from a stretch of 11 guanines in intron 4, 10 bp downstream of the 3′ end of exon 4. It is possible that this change impairs the splicing of intron 4 in the *CCS2* transcript. The identification of the molecular lesions confirms that the complementing sequence we isolated corresponds to the wild-type *CCS2* gene rather than an extragenic suppressor of the *ccs2* mutation.

The *CCS2* gene encodes a 1,719 amino acid protein with a predicted molecular weight of 171,753 Da. The most striking feature of the CCS2 protein is the presence of several 38–40 amino acid repeats occurring between residues 720 and 1610 (**Figure 4AB**). Such motifs, named OPR for **o**ctatrico**p**eptide **r**epeats were first defined in TBC2 (translation factor for chloroplast *psbC* mRNA), a nuclear encoded protein required for the translation of *psbC* RNA in the chloroplast (Auchincloss et al., 2002). OPRs are also recognized in other factors controlling translation (TDA1, TAB1), maturation (RAT2, RAA1, RAA8, RAP), or stability (MCG1,MBI1) of chloroplast transcripts (Balczun et al., 2005; Merendino et al., 2006; Eberhard et al., 2011; Rahire et al., 2012; Kleinknecht et al., 2014; Marx et al., 2015; Wang et al., 2015).

The OPR family is further characterized by the presence of low complexity regions, which are regions containing little diversity in their amino acid composition (Balczun et al., 2005; Merendino et al., 2006; Eberhard et al., 2011; Rahire et al., 2012; Marx et al., 2015; Wang et al., 2015). Indeed, we noted the occurrence of several stretches of three or more alanine, glycine, serine, or glutamine repeats in CCS2 (**Figure 4A**). CCS2 has a high content of alanine (24.2%), glycine (10.7%), proline (8.7%), and leucine (8.7%), a feature shared by other OPR proteins (Auchincloss et al., 2002; Balczun et al., 2005; Merendino et al., 2006; Eberhard et al., 2011; Rahire et al., 2012; Marx et al., 2015; Wang et al., 2015). OPR motifs found in CCS2 are highlighted in **Figure 4B** and a consensus OPR motif from all OPR containing proteins that have been functionally identified to date, with the exception of NCC1 and NCC2 (Boulouis et al., 2015), can be seen in **Figure 4C**. The relative locations of the motifs in the proteins used to generate the consensus motif in **Figure 4C** can be seen in **Figure 4B**.

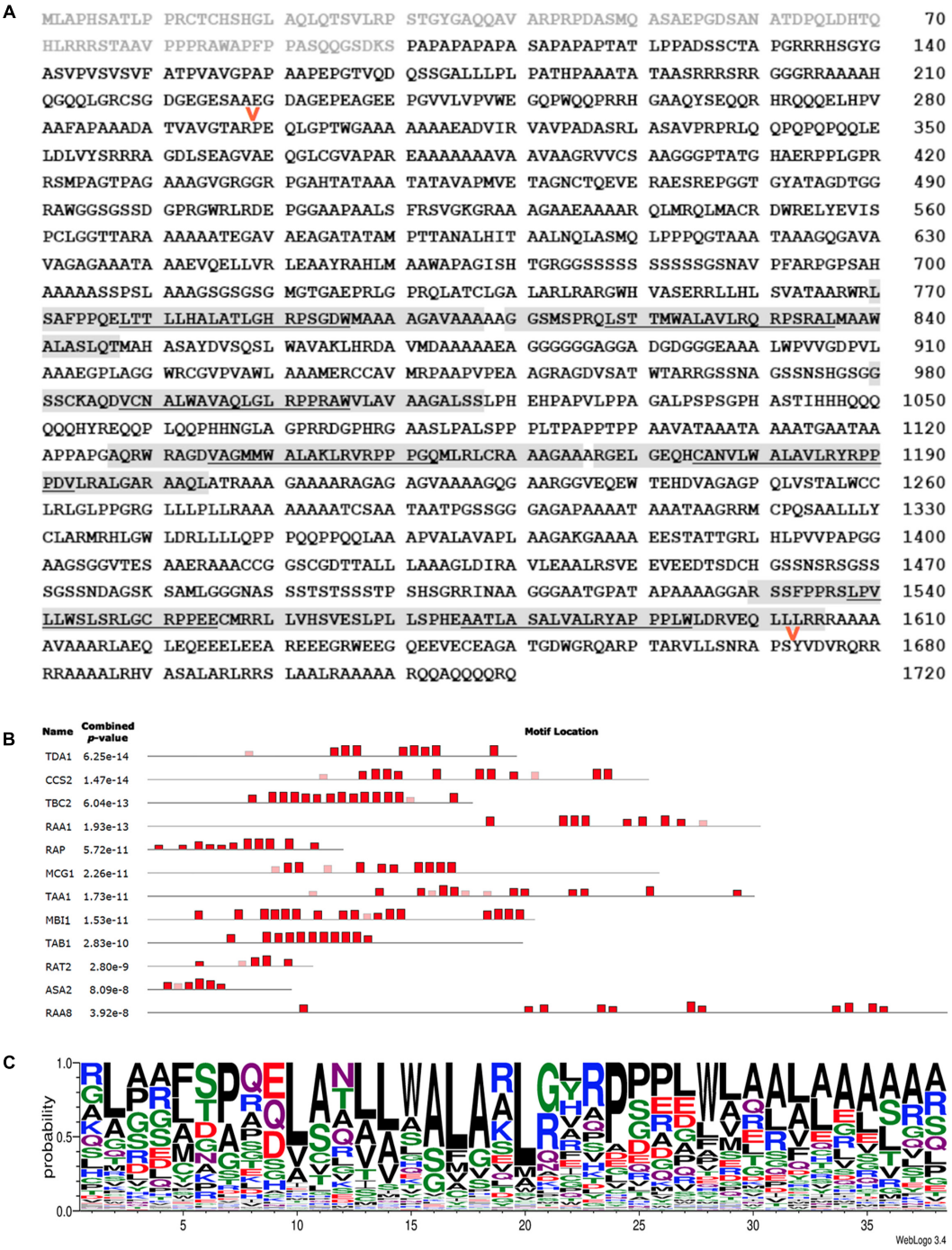

FIGURE 4 | CCS2 is an OPR protein. **(A)** Amino acid sequence of the deduced CCS2 protein. Light gray text indicates the protein sequence that was fused to GFP for targeting experiments (**Figure 6**), light gray highlighting indicates OPRs recognized with *p*-values of at least $1e^{-10}$, as shown in **(B)**. The underlined portion indicates the location of the previously recognized "PPPEW" motif. The red arrows indicate the positions of internal HA tags that were introduced to test neutrality of the tag with respect to CCS2 function (see Materials and Methods). **(B)** MEME-generated distribution of OPR motifs within the *Chlamydomonas* OPR proteins that have been functionally characterized. Heights of blocks indicate relative proportion of the *p*-value $1e^{-10}$. Red boxes represent motifs found by the MEME program while faded boxes represent motifs found by other programs within the MEME Suite. Sequences of *Chlamydomonas* OPR proteins CCS2 (KC292647), ASA2 (EDP00850.1), TBC2 (CAD20887.1), TDA1 (CCA62914.20), RAA1 (CAE53330.1), TAB1 (ADY68544.1), RAT2 (EDP02536.1), TAA1 (Cre06.g262650), RAA8 (Cre10.g440000), MCG1 (Cre10.g429400), MBI1 (Cre06.g272450), and Arabidopsis RAP (OAP08625.1) were used (see Supplementary Figure S1). **(C)** WebLogo consensus sequence of OPRs identified by the MEME program depicted in red in **Figure 4B** (Bailey and Elkan, 1994; Crooks et al., 2004). Letter height indicates the probability of a particular amino acid (y-axis) at a given position within the 38 amino acid repeat (x-axis). OPR sequences used to calculate this consensus sequence are found in Supplementary Figure S2.

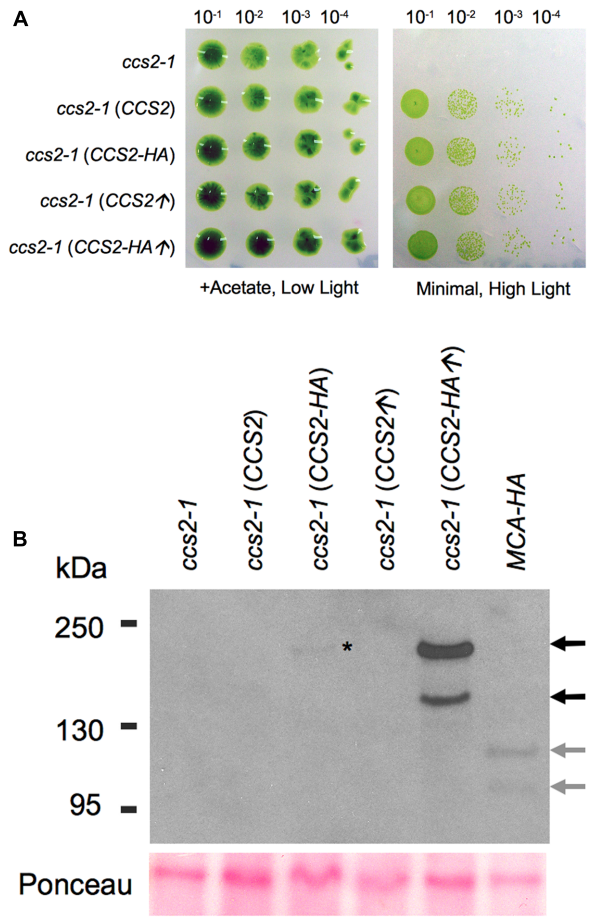

FIGURE 5 | Immunodetection of CCS2-HA in whole cells. For **(A,B)**, a cell wall minus *ccs2-1* strain was transformed with plasmids carrying the 8.2 kb genomic fragment (*CCS2*), the 8.2 kb genomic fragment expressing HA tagged CCS2 (*CCS2-HA*), the *CCS2* coding sequence under the *PSAD* promoter (*CCS2*↑), and HA tagged *CCS2* coding sequence under the *PSAD* promoter (*CCS2-HA*↑), respectively. One representative transformant, selected on minimal medium, was used for the analysis. **(A)** Ten-fold dilution series of algal cells on acetate containing (0.6 $\mu E/m^2/s$) or minimal media (50 $\mu E/m^2/s$), incubated for 1 week at 25°C. **(B)** Anti-HA immunodetection using whole cell extracts. The HA-tagged MCA1 strain is used as a control (Raynaud et al., 2007). MCA1 is a plastid-localized PPR protein (PPR for pentatricopeptide repeat). Black arrows indicate the two CCS2 species, gray arrows indicate the two MCA1 species. The lower bands correspond to the expected molecular mass for CCS2 (~176 kDa) and MCA1 (~105 kDa). The asterisk indicates the CCS2 species of high molecular weight that is detected in the *ccs2* strain expressing CCS2-HA under its native promoter. Ponceau staining serves as a loading control.

Immunodetection of the CCS2 Protein

In order to detect CCS2 and assess its subcellular localization, a series of constructs expressing HA tagged proteins were engineered. The tagged proteins were either expressed under the control of the native *CCS2* promoter or the *PSAD* promoter, which allows increased expression of *Chlamydomonas* genes (Fischer and Rochaix, 2001). We saw no difference in the ability of the two HA-tagged *CCS2* constructs to complement the *ccs2-1* mutation as compared to the WT gene (**Figure 5A**).

Immunoblotting analysis against whole cell extracts from transformants over-expressing CCS2-HA revealed that the protein occurs as two species, one of which appears to migrate at the expected size (≃176 kDa) (**Figure 5B**). A similar pattern was also seen in cells expressing HA-tagged MCA1, a PPR (PPR for **p**entatrico**p**eptide **r**epeat) protein with plastid localization (Raynaud et al., 2007; Boulouis et al., 2011). This suggests that the immunoreactive species with higher mobility may be a result of the extraction conditions and/or the denaturation step needed to immunodetect CCS2 (see Materials and Methods). Detection of CCS2-HA was only possible from freshly grown cultures when a cocktail of protease inhibitors was used immediately followed by denaturing at 70°C (instead of 100°C), an indication that the protein is likely highly sensitive to proteolysis. Interestingly, Rahire et al. (2012) noted that the OPR protein TDA1 was also very susceptible to proteolysis.

The CCS2 Protein Localizes to the Plastid

A chloroplast targeting sequence is assigned by both ChloroP (Emanuelsson et al., 1999) and WoLF PSORT (Horton et al., 2007) at the N-terminus of CCS2, an indication that CCS2 might reside in the plastid. However, the extraction methods necessary for detection of CCS2-HA precluded the use of subcellular fractionation to determine protein localization. Hence, we tested the ability of the CCS2 N-terminus to direct GFP to the plastid in a heterologous system such as *N. benthamiana*. This is justified, as targeting sequences from *Chlamydomonas* nuclear-encoded proteins retain their function as transit peptides for import into chloroplasts in several species of land plants including *Nicotiana* (Nakazato et al., 2003; Falciatore et al., 2005; Levitan et al., 2005; Li et al., 2016; Yamaoka et al., 2016). To this end, we constructed CCS2-GFP, which encoded a protein consisting of the first 100 amino acids of CCS2, including the putative targeting sequence, translationally fused to the amino-terminus of GFP. This CCS2-GFP expressing construct was introduced into *N. benthamiana* leaves. Fluorescence microscopy shows clear overlay of chlorophyll auto-fluorescence, which is a feature of the chloroplast, and GFP fluorescence (**Figure 6**). These results indicate that the first 100 amino acids of CCS2 are sufficient to target GFP to the chloroplast of *N. benthamiana* and hence the CCS2 protein is likely localized to the plastid of *Chlamydomonas*.

DISCUSSION

Identification of CCS2 as an OPR Protein Controlling Cytochrome *c* Assembly

Here, we have identified CCS2, a chloroplast localized cytochrome *c* assembly factor that, based on primary sequence similarity, appears to be unique to specific branches of the chlorophycean algae. This protein has been recognized as a member of the recently designated OPR family, which contains 43 members in *Chlamydomonas* (Eberhard et al., 2011). OPRs are defined by loosely conserved repeats of 38–40 amino acids

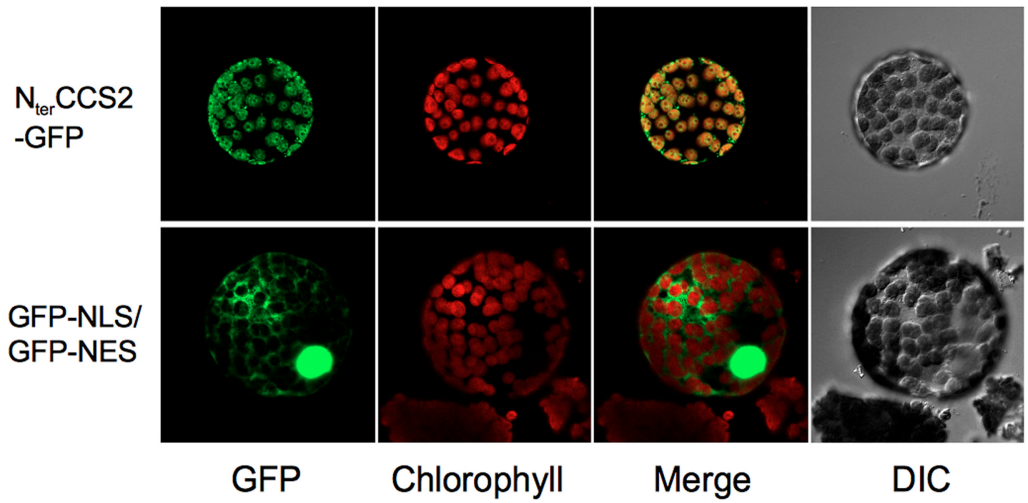

FIGURE 6 | Localization of GFP to chloroplast via CCS2 N-terminal sequence. Top row (N$_{ter}$CCS2-GFP): imaging of a protoplast from *Nicotiana benthamiana* leaves transformed with the pGWB5/ccs2target plasmid expressing the first 100 amino acids of CCS2 fused to GFP. Bottom row (GFP-NLS/GFP-NES): imaging of a protoplast from *N. benthamiana* leaves transformed with the construct expressing a NLS-GFP/NES-GFP fusion protein shuttling between the nucleus and the cytosol via a nuclear localization signal (NLS) and a nuclear export signal (NES). DIC, differential interference contrast microscopy.

(Auchincloss et al., 2002; Eberhard et al., 2011; Rahire et al., 2012).

Via bioinformatics analysis, OPR proteins have been assigned to the 'α-solenoid' superfamily, which contains proteins defined by similar tandem repeats, such as TPRs (tetratricopeptide repeat, 34 amino acids) and PPRs (pentatricopeptide repeat, 35 amino acids) (Eberhard et al., 2011; Rahire et al., 2012). Three dimensional structure analyses of TPRs (Das et al., 1998; D'Andrea and Regan, 2003; Zeytuni and Zarivach, 2012) and PPRs (Ringel et al., 2011; Nakamura et al., 2012; Shen et al., 2016) indicate that these motifs result in a series of anti-parallel α-helices. Protein predictions using I-TASSER (Zhang, 2008; Roy et al., 2010) suggest that the OPR proteins are organized in a similar manner (Auchincloss et al., 2002; Eberhard et al., 2011; Rahire et al., 2012). I-TASSER also predicts regions of anti-parallel α-helices in CCS2 (not shown). TPR proteins act as scaffolds to mediate protein–protein interactions and control a wide range of cellular functions such as the cell cycle, transcription in the nucleus, or protein import into mitochondria and peroxisomes (D'Andrea and Regan, 2003). On the other hand, all the PPR proteins described so far were shown to be control maturation, stability, or translation of organellar RNAs, presumably via direct interaction with their target transcript(s) (Barkan and Small, 2014; Manna, 2015).

The PPR family has expanded greatly in the plant lineage, with 450 representatives in *Arabidopsis* alone, while the typical non-plant, eukaryotic genome encodes for fewer than 40 representatives (Barkan and Small, 2014; Manna, 2015). Similar to the PPR protein family expansion seen in land plants, chlorophycean algae have an expansion in the number of OPR containing proteins (Eberhard et al., 2011; Rahire et al., 2012). The majority of functionally characterized OPR proteins are involved in translation, maturation, or stability of transcripts in the chloroplast of *Chlamydomonas*. For instance, TBC2, TDA1, and TAB1 are factors involved in the translation of the *psbC*, *atpA*, or *psaB* transcripts, respectively (Auchincloss et al., 2002; Eberhard et al., 2011; Rahire et al., 2012), while RAA1/RAA8 and RAT2 are involved in the maturation of *psaA* and *tscA* RNAs (Balczun et al., 2005; Merendino et al., 2006; Marx et al., 2015). In *Arabidopsis*, RAP is the sole OPR containing protein and loss of RAP function yields a defect in the maturation of the chloroplast 16S ribosomal RNA (Kleinknecht et al., 2014). Recent studies have uncovered the role of MCG1 and MBI1 in stabilizing the *petG* mRNA, encoding a small subunit of the cytochrome b_6f complex and the *psbI* mRNA, coding for a small subunit of photosystem II, respectively (Wang et al., 2015).

If the role of most OPR containing proteins as transcript-interacting factors was inferred from the phenotypic analysis of loss-of-function mutations, the demonstration that OPR motifs interact directly with their relevant target RNAs was only provided for TAB1 (Rahire et al., 2012). A recent study revealed that gain of function mutations in the OPR motifs of NCC1 and NCC2 confer an ability to recognize chloroplast transcripts as novel targets of action (Boulouis et al., 2015). This suggests that specific amino acids within these OPR motifs must govern nucleotide recognition, but the basis of this specificity still remains to be deciphered.

The molecular mass range of the functionally identified OPR proteins extends from 45 to 269 kDa with an average length of 1428 amino acids and 5–17 OPR motifs. These repeats are found primarily in the central regions of proteins while the carboxyl- and amino-termini are typically characterized by stretches of single amino acid repeats (Auchincloss et al., 2002; Merendino et al., 2006; Rahire et al., 2012). The OPR motifs themselves can be highly divergent, and so the repeat count per protein fluctuates depending on how stringently one defines a single OPR motif

(**Figure 4B**). This divergence is reflected in the fact that OPR repeats in CCS2 are characterized by a LWALAR consensus motif (**Figure 4A**) while repeats in TBC2, TDA1, and TAB1 contain a PPPEW sequence (Auchincloss et al., 2002; Eberhard et al., 2011; Rahire et al., 2012). While in some instances OPR motifs are serially repeated, the motifs can also be separated by gaps (**Figure 4B**), often by stretches of amino acids such as alanine. We noted the frequent occurrence of a poly-glutamine stretch upstream of the OPR motif in CCS2. Interestingly, glutamine-rich regions are known to form polar zippers involved in protein interaction but they have also been recognized as motifs for transcriptional activation (Courey and Tjian, 1988; Stott et al., 1995).

That CCS2 functions directly in the heme attachment reaction in the thylakoid lumen is very unlikely, considering its large size and the absence of a typical bipartite targeting sequence for luminal localization. Considering its inclusion in the OPR family, one possibility is that CCS2 controls the maturation, stability, or translation of a chloroplast transcript in the stroma involved in cytochrome *c* assembly. In the chloroplast genome, the *ccsA* gene encodes a heme delivery factor required for cytochrome *c* maturation (Xie and Merchant, 1996). While the level of *ccsA* transcript in the *ccs2* mutants was previously shown to be unaltered, indicating that CCS2 is not required to stabilize the *ccsA* mRNA (Xie et al., 1998), the hypothesis that CCS2 is involved in the translation of *ccsA* remains plausible. Unfortunately, this was not tested due to the lack of an anti-CcsA antibody. Another likely scenario is that CCS2 functions like a TPR containing protein and mediates the assembly of protein complexes containing other CCS factors. The OPR family also includes ASA2 (ATP Synthase Associated protein), a subunit of the unusual mitochondrial ATP synthase, found only in chlorophycean algae (van Lis et al., 2007; Cano-Estrada et al., 2010) (**Figure 4B**). While a 200 kDa CCS complex containing *ccsA* (40 kDa) and Ccs1 (60 kDa) has been identified at the thylakoid membrane (Hamel et al., 2003), it is unlikely that the 170 kDa CCS2 is a component of this complex because of its large size. However, it is conceivable that CCS2 could stabilize the CCS complex via protein-protein interactions or facilitate the recruitment of the components of this complex on the stromal side of the thylakoid membrane.

AUTHOR CONTRIBUTIONS

SC executed the experiments, interpreted the data and wrote the manuscript; PH designed the experiments, interpreted the data and wrote the manuscript; IL executed experiments.

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SUPPLEMENTARY MATERIAL

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REFERENCES

Allen, J. W., Sawyer, E. B., Ginger, M. L., Barker, P. D., and Ferguson, S. J. (2009). Variant *c*-type cytochromes as probes of the substrate specificity of the *E. coli* cytochrome *c* maturation (Ccm) apparatus. *Biochem. J.* 419, 177–184. doi: 10.1042/BJ20081999

Allen, J. W. A. (2011). Cytochrome c biogenesis in mitochondria – systems III and V. *FEBS J.* 278, 4198–4216. doi: 10.1111/j.1742-4658.2011.08231.x

Auchincloss, A. H., Zerges, W., Perron, K., Girard-Bascou, J., and Rochaix, J. D. (2002). Characterization of Tbc2, a nucleus-encoded factor specifically required for translation of the chloroplast *psbC* mRNA in *Chlamydomonas reinhardtii*. *J. Cell Biol.* 157, 953–962.

Bailey, T. L., and Elkan, C. (1994). Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 2, 28–36.

Balczun, C., Bunse, A., Hahn, D., Bennoun, P., Nickelsen, J., and Kuck, U. (2005). Two adjacent nuclear genes are required for functional complementation of a chloroplast trans-splicing mutant from *Chlamydomonas reinhardtii*. *Plant J.* 43, 636–648.

Barkan, A., and Small, I. (2014). Pentatricopeptide repeat proteins in plants. *Annu. Rev. Plant Biol.* 65, 415–442. doi: 10.1146/annurev-arplant-050213-040159

Beckett, C. S., Loughman, J. A., Karberg, K. A., Donato, G. M., Goldman, W. E., and Kranz, R. G. (2000). Four genes are required for the System II cytochrome *c* biogenesis pathway in *Bordetella pertussis*, a unique bacterial model. *Mol. Microbiol.* 38, 465–481.

Bonnard, G., Corvest, V., Meyer, E. H., and Hamel, P. P. (2010). Redox processes controlling the biogenesis of *c*-type cytochromes. *Antioxid. Redox Signal.* 13, 1385–1401. doi: 10.1089/ars.2010.3161

Boulouis, A., Drapier, D., Razafimanantsoa, H., Wostrikoff, K., Tourasse, N. J., Pascal, K., et al. (2015). Spontaneous dominant mutations in chlamydomonas highlight ongoing evolution by gene diversification. *Plant Cell* 27, 984–1001. doi: 10.1105/tpc.15.00010

Boulouis, A., Raynaud, C., Bujaldon, S., Aznar, A., Wollman, F. A., and Choquet, Y. (2011). The nucleus-encoded trans-acting factor MCA1 plays a critical role in the regulation of cytochrome *f* synthesis in *Chlamydomonas* chloroplasts. *Plant Cell* 23, 333–349. doi: 10.1105/tpc.110.078170

Bowman, S. E. J., and Bren, K. L. (2008). The chemistry and biochemistry of heme c: functional bases for covalent attachment. *Nat. Prod. Rep.* 25, 1118–1130. doi: 10.1039/b717196j

Cano-Estrada, A., Vázquez-Acevedo, M., Villavicencio-Queijeiro, A., Figueroa-Martínez, F., Miranda-Astudillo, H., Cordeiro, Y., et al. (2010). Subunit-subunit interactions and overall topology of the dimeric mitochondrial ATP synthase of

Polytomella sp. *Biochim. Biophys. Acta* 1797, 1439–1448. doi: 10.1016/j.bbabio.2010.02.024

Cha, H. J., Dalal, N. G., Pham, M.-Q., Kramer, S. F., Vakharia, V. N., and Bentley, W. E. (2002). Increased efficiency of cloning large DNA fragments using a lower copy number plasmid. *Biotechniques* 32, 992–998.

Courey, A. J., and Tjian, R. (1988). Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 55, 887–898.

Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome Res.* 14, 1188–1190.

D'Andrea, L. D., and Regan, L. (2003). TPR proteins: the versatile helix. *Trends Biochem. Sci.* 28, 655–662.

Das, A. K., Cohen, P. W., and Barford, D. (1998). The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. *EMBO J.* 17, 1192–1199.

de Vitry, C. (2011). Cytochrome *c* maturation system on the negative side of bioenergetic membranes: CCB or System IV. *FEBS J.* 278, 4189–4197. doi: 10.1111/j.1742-4658.2011.08373.x

Dreyfuss, B. W., Hamel, P. P., Nakamoto, S. S., and Merchant, S. (2003). Functional analysis of a divergent System II protein, Ccs1, involved in *c*-type cytochrome biogenesis. *J. Biol. Chem.* 278, 2604–2613.

Eberhard, S., Loiselay, C., Drapier, D., Bujaldon, S., Girard-Bascou, J., Kuras, R., et al. (2011). Dual functions of the nucleus-encoded factor TDA1 in trapping and translation activation of *atpA* transcripts in *Chlamydomonas reinhardtii* chloroplasts. *Plant J.* 67, 1055–1066. doi: 10.1111/j.1365-313X.2011.04657.x

Emanuelsson, O., Nielsen, H., and von Heijne, G. (1999). ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci.* 8, 978–984.

Falciatore, A., Merendino, L., Barneche, F., Ceol, M., Meskauskiene, R., Apel, K., et al. (2005). The FLP proteins act as regulators of chlorophyll synthesis in response to light and plastid signals in *Chlamydomonas*. *Genes Dev.* 19, 176–187. doi: 10.1101/gad.321305

Feissner, R. E., Beckett, C. S., Loughman, J. A., and Kranz, R. G. (2005). Mutations in cytochrome assembly and periplasmic redox pathways in *Bordetella pertussis*. *J. Bacteriol.* 187, 3941–3949.

Fischer, N., and Rochaix, J. D. (2001). The flanking regions of *PsaD* drive efficient gene expression in the nucleus of the green alga *Chlamydomonas reinhardtii*. *Mol. Genet. Genomics* 265, 888–894.

Frawley, E. R., and Kranz, R. G. (2009). CcsBA is a cytochrome *c* synthetase that also functions in heme transport. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10201–10206. doi: 10.1073/pnas.0903132106

Gabilly, S. T., and Hamel, P. P. (2017). Maturation of plastid *c*-type cytochromes. *Front. Plant Sci.* 8:1313. doi: 10.3389/fpls.2017.01313

Gabilly, S. T., Dreyfuss, B. W., Karamoko, M., Corvest, V., Kropat, J., Page, M. D., et al. (2010). CCS5, a thioredoxin-like protein involved in the assembly of plastid *c*-type cytochromes. *J. Biol. Chem.* 285, 29738–29749. doi: 10.1074/jbc.M109.099069

Gabilly, S. T., Kropat, J., Karamoko, M., Page, M. D., Nakamoto, S. S., Merchant, S. S., et al. (2011). A novel component of the disulfide-reducing pathway required for cytochrome *c* assembly in plastids. *Genetics* 187, 793–802. doi: 10.1534/genetics.110.125369

Hamel, P., Corvest, V., Giege, P., and Bonnard, G. (2009). Biochemical requirements for the maturation of mitochondrial c-type cytochromes. *Biochim. Biophys. Acta* 1793, 125–138. doi: 10.1016/j.bbamcr.2008.06.017

Hamel, P. P., Dreyfuss, B. W., Xie, Z., Gabilly, S. T., and Merchant, S. (2003). Essential histidine and tryptophan residues in CcsA, a System II polytopic cytochrome *c* biogenesis protein. *J. Biol. Chem.* 278, 2593–2603.

Harris, E. H. (1989). *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*. San Diego, CA: Academic Press.

Horton, P., Park, K. J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C. J., et al. (2007). WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* 35, W585–W587.

Howe, G., and Merchant, S. (1992). The biosynthesis of membrane and soluble plastidic *c*-type cytochromes of *Chlamydomonas reinhardtii* is dependent on multiple common gene products. *EMBO J.* 11, 2789–2801.

Howe, G., and Merchant, S. (1993). Maturation of thylakoid lumen proteins proceeds post-translationally through an intermediate *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 90, 1862–1866.

Howe, G., and Merchant, S. (1994). Role of heme in the biosynthesis of cytochrome c_6. *J. Biol. Chem.* 269, 5824–5832.

Inoue, K., Dreyfuss, B. W., Kindle, K. L., Stern, D. B., Merchant, S., and Sodeinde, O. A. (1997). *CCS1*, a nuclear gene required for the post-translational assembly of chloroplast *c*-type cytochromes. *J. Biol. Chem.* 272, 31747–31754.

Karamoko, M., Gabilly, S. T., and Hamel, P. P. (2013). Operation of trans-thylakoid thiol-metabolizing pathways in photosynthesis. *Front. Plant Sci.* 4:476. doi: 10.3389/fpls.2013.00476

Karimi, M., Inze, D., and Depicker, A. (2002). GATEWAY vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* 7, 193–195.

Kleinknecht, L., Wang, F., Stube, R., Philippar, K., Nickelsen, J., and Bohne, A. V. (2014). RAP, the sole octotricopeptide repeat protein in *Arabidopsis*, is required for chloroplast *16S* rRNA maturation. *Plant Cell* 26, 777–787. doi: 10.1105/tpc.114.122853

Kranz, R. G., Richard-Fogal, C., Taylor, J.-S., and Frawley, E. R. (2009). Cytochrome *c* biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. *Microbiol. Mol. Biol. Rev.* 73, 510–528. doi: 10.1128/mmbr.00001-09

Kurvari, V., Grishin, N. V., and Snell, W. J. (1998). A gamete-specific, sex-limited homeodomain protein in *Chlamydomonas*. *J. Cell Biol.* 143, 1971–1980.

Le Brun, N. E., Bengtsson, J., and Hederstedt, L. (2000). Genes required for cytochrome *c* synthesis in *Bacillus subtilis*. *Mol. Microbiol.* 36, 638–650.

Levitan, A., Trebitsh, T., Kiss, V., Pereg, Y., Dangoor, I., and Danon, A. (2005). Dual targeting of the protein disulfide isomerase RB60 to the chloroplast and the endoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6225–6230. doi: 10.1073/pnas.0500676102

Li, Z., Peers, G., Dent, R. M., Bai, Y., Yang, S. Y., Apel, W., et al. (2016). Evolution of an atypical de-epoxidase for photoprotection in the green lineage. *Nat. Plants* 2:16140. doi: 10.1038/nplants.2016.140

Manna, S. (2015). An overview of pentatricopeptide repeat proteins and their applications. *Biochimie* 113, 93–99. doi: 10.1016/j.biochi.2015.04.004

Marx, C., Wünsch, C., and Kück, U. (2015). The octatricopeptide repeat protein Raa8 is required for chloroplast trans splicing. *Eukaryot. Cell* 14, 998–1005. doi: 10.1128/ec.00096-15

Mavridou, D. A. I., Ferguson, S. J., and Stevens, J. M. (2013). Cytochrome *c* assembly. *IUBMB Life* 65, 209–216. doi: 10.1002/iub.1123

Merendino, L., Perron, K., Rahire, M., Howald, I., Rochaix, J. D., and Goldschmidt-Clermont, M. (2006). A novel multifunctional factor involved in trans-splicing of chloroplast introns in *Chlamydomonas*. *Nucleic Acids Res.* 34, 262–274.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., et al. (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J. Biosci. Bioeng.* 104, 34–41.

Nakamura, T., Yagi, Y., and Kobayashi, K. (2012). Mechanistic insight into pentatricopeptide repeat proteins as sequence-specific RNA-binding proteins for organellar RNAs in plants. *Plant Cell Physiol.* 53, 1171–1179. doi: 10.1093/pcp/pcs069

Nakazato, E., Fukuzawa, H., Tabata, S., Takahashi, H., and Tanaka, K. (2003). Identification and expression analysis of cDNA encoding a chloroplast recombination protein REC1, the chloroplast RecA homologue in *Chlamydomonas reinhardtii*. *Biosci. Biotechnol. Biochem.* 67, 2608–2613. doi: 10.1271/bbb.67.2608

Page, M. L. D., Hamel, P. P., Gabilly, S. T., Zegzouti, H., Perea, J. V., Alonso, J. M., et al. (2004). A homolog of prokaryotic thiol disulfide transporter CcdA is required for the assembly of the cytochrome b_6f complex in *Arabidopsis* chloroplasts. *J. Biol. Chem.* 279, 32474–32482. doi: 10.1074/jbc.M404285200

Pollock, S. V., Prout, D. L., Godfrey, A. C., Lemaire, S. D., and Moroney, J. V. (2004). The *Chlamydomonas reinhardtii* proteins Ccp1 and Ccp2 are required for long-term growth, but are not necessary for efficient photosynthesis, in a low-CO_2 environment. *Plant Mol. Biol.* 56, 125–132.

Purton, S., and Rochaix, J. D. (1994). Complementation of a *Chlamydomonas reinhardtii* mutant using a genomic cosmid library. *Plant Mol. Biol.* 24, 533–537.

Quinn, J. M., and Merchant, S. (1998). Copper-responsive gene expression during adaptation to copper deficiency. *Methods Enzymol.* 297, 263–279.

Rahire, M., Laroche, F., Cerutti, L., and Rochaix, J. D. (2012). Identification of an OPR protein involved in the translation initiation of the PsaB subunit of photosystem I. *Plant J.* 72, 652–661. doi: 10.1111/j.1365-313X.2012.05111.x

Raynaud, C., Loiselay, C., Wostrikoff, K., Kuras, R., Girard-Bascou, J., Wollman, F. A., et al. (2007). Evidence for regulatory function of nucleus-encoded factors on mRNA stabilization and translation in the chloroplast. *Proc. Natl. Acad. Sci. U.S.A.* 104, 9093–9098.

Ringel, R., Sologub, M., Morozov, Y. I., Litonin, D., Cramer, P., and Temiakov, D. (2011). Structure of human mitochondrial RNA polymerase. *Nature* 478, 269–273. doi: 10.1038/nature10435

Roy, A., Kucukural, A., and Zhang, Y. (2010). I-TASSER: a unified platform for automated protein structure and function prediction. *Nat. Protoc.* 5, 725–738. doi: 10.1038/nprot.2010.5

Sanders, C., Turkarslan, S., Lee, D.-W., and Daldal, F. (2010). Cytochrome *c* biogenesis: the Ccm system. *Trends Microbiol.* 18, 266–274. doi: 10.1016/j.tim.2010.03.006

Shen, C., Zhang, D., Guan, Z., Liu, Y., Yang, Z., Yang, Y., et al. (2016). Structural basis for specific single-stranded RNA recognition by designer pentatricopeptide repeat proteins. *Nat. Commun.* 7:11285. doi: 10.1038/ncomms11285

Shimogawara, K., Fujiwara, S., Grossman, A., and Usuda, H. (1998). High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. *Genetics* 148, 1821–1828.

Simon, J., and Hederstedt, L. (2011). Composition and function of cytochrome *c* biogenesis System II. *FEBS J.* 278, 4179–4188. doi: 10.1111/j.1742-4658.2011.08374.x

Stott, K., Blackburn, J. M., Butler, P. J., and Perutz, M. (1995). Incorporation of glutamine repeats makes protein oligomerize: implications for neurodegenerative diseases. *Proc. Natl. Acad. Sci. U.S.A.* 92, 6509–6513.

Thony-Meyer, L. (1997). Biogenesis of respiratory cytochromes in bacteria. *Microbiol. Mol. Biol. Rev.* 61, 337–376.

van Lis, R., Mendoza-Hernandez, G., Groth, G., and Atteia, A. (2007). New insights into the unique structure of the F0F1-ATP synthase from the chlamydomonad algae *Polytomella* sp. and *Chlamydomonas reinhardtii*. *Plant Physiol.* 144, 1190–1199.

Verissimo, A. F., and Daldal, F. (2014). Cytochrome *c* biogenesis System I: an intricate process catalyzed by a maturase supercomplex? *Biochim. Biophys. Acta* 1837, 989–998. doi: 10.1016/j.bbabio.2014.03.003

Wang, F., Johnson, X., Cavaiuolo, M., Bohne, A. V., Nickelsen, J., and Vallon, O. (2015). Two *Chlamydomonas* OPR proteins stabilize chloroplast mRNAs encoding small subunits of photosystem II and cytochrome b_6f. *Plant J.* 82, 861–873. doi: 10.1111/tpj.12858

Xie, Z., Culler, D., Dreyfuss, B. W., Kuras, R., Wollman, F. A., Girard-Bascou, J., et al. (1998). Genetic analysis of chloroplast c-type cytochrome assembly in *Chlamydomonas reinhardtii*: one chloroplast locus and at least four nuclear loci are required for heme attachment. *Genetics* 148, 681–692.

Xie, Z., and Merchant, S. (1996). The plastid-encoded *ccsA* gene is required for heme attachment to chloroplast *c*-type cytochromes. *J. Biol. Chem.* 271, 4632–4639.

Yamaoka, Y., Achard, D., Jang, S., Legéret, B., Kamisuki, S., Ko, D., et al. (2016). Identification of a *Chlamydomonas* plastidial 2-lysophosphatidic acid acyltransferase and its use to engineer microalgae with increased oil content. *Plant Biotechnol. J.* 14, 2158–2167. doi: 10.1111/pbi.12572

Zeytuni, N., and Zarivach, R. (2012). Structural and functional discussion of the tetra-trico-peptide repeat, a protein interaction module. *Structure* 20, 397–405. doi: 10.1016/j.str.2012.01.006

Zhang, H., Herman, P. L., and Weeks, D. P. (1994). Gene isolation through genomic complementation using an indexed library of *Chlamydomonas reinhardtii* DNA. *Plant Mol. Biol.* 24, 663–672.

Zhang, Y. (2008). I-TASSER server for protein 3D structure prediction. *BMC Bioinformatics* 9:40. doi: 10.1186/1471-2105-9-40

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frontiers in Plant Science

REVIEW
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GUN1, a Jack-Of-All-Trades in Chloroplast Protein Homeostasis and Signaling

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The *GENOMES UNCOUPLED 1 (GUN1)* gene has been reported to encode a chloroplast-localized pentatricopeptide-repeat protein, which acts to integrate multiple indicators of plastid developmental stage and altered plastid function, as part of chloroplast-to-nucleus retrograde communication. However, the molecular mechanisms underlying signal integration by GUN1 have remained elusive, up until the recent identification of a set of GUN1-interacting proteins, by co-immunoprecipitation and mass-spectrometric analyses, as well as protein–protein interaction assays. Here, we review the molecular functions of the different GUN1 partners and propose a major role for GUN1 as coordinator of chloroplast translation, protein import, and protein degradation. This regulatory role is implemented through proteins that, in most cases, are part of multimeric protein complexes and whose precise functions vary depending on their association states. Within this framework, GUN1 may act as a platform to promote specific functions by bringing the interacting enzymes into close proximity with their substrates, or may inhibit processes by sequestering particular pools of specific interactors. Furthermore, the interactions of GUN1 with enzymes of the tetrapyrrole biosynthesis (TPB) pathway support the involvement of tetrapyrroles as signaling molecules in retrograde communication.

Keywords: nucleoid, GUN1, protein homeostasis, retrograde signaling, biogenic control

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INTRODUCTION

Upon illumination, proplastids differentiate into functional chloroplasts in developing photosynthetic tissues of cotyledons, leaves, and stems (Jarvis and López-Juez, 2013). Chloroplast biogenesis also occurs during the growth of young green tissues, as cells expand and mature chloroplasts undergo division by binary fission (Okazaki et al., 2010). This process is characterized macroscopically by rapid greening of the young chloroplast and microscopically by the concomitant formation of thylakoid membranes and the reorganization of nucleoids, i.e., DNA-containing structures without defined boundaries, which differ in number, size, and distribution within plastids at different developmental stages, and harbor the plastid gene expression (PGE) machinery (Pfalz and Pfannschmidt, 2013; Melonek et al., 2016).

At the molecular level, this rather complex biogenic transition is achieved by cytosolic synthesis of chloroplast-targeted proteins, followed by import, assembly, folding, and degradation of unfolded/misfolded proteins (Jarvis and López-Juez, 2013). Indeed, the plastid genome itself (the

plastome) comprises fewer than 100 protein-coding genes, and the vast majority of the 2000–3000 proteins that make up the chloroplast proteome are encoded in the nucleus (Richly and Leister, 2004). In particular, precursor proteins carrying N-terminal transit peptides initially interact with two multiprotein complexes termed Translocon at the outer envelope membrane of chloroplasts (TOC) and Translocon at the inner envelope membrane of chloroplasts (TIC), which facilitate their active transport through the chloroplast envelope, powered by an ATP import motor, consisting of the stromal heat-shock protein 93 (Hsp93), heat-shock protein 70 (Hsp70), and heat-shock protein 90 (Hsp90; Flores-Perez and Jarvis, 2013; Inoue et al., 2013; Shi and Theg, 2013a,b). Upon translocation, proteins are exposed to different proteolytic systems of prokaryotic origin, which are responsible for protein maturation, control of protein abundance, and removal of either misfolded or damaged components. Among these, the stromal protease Clp is a multimeric complex made of chaperones and serine protease subunits, which serve general housekeeping functions. In contrast, the thylakoid-associated FtsH (Filamentous temperature sensitive H) proteases are zinc-containing metalloendopeptidases that have both chaperone and proteolytic functions, and participate in the Photosystem II repair cycle, together with the DEG serine proteases (Kato and Sakamoto, 2010; Van Wijk, 2015).

Besides translation and post-translational processes, chloroplast biogenesis also requires transcriptional coordination of thousands of nuclear genes with the expression of the comparatively few plastid genes in order to meet the needs of the developing chloroplast (Chan et al., 2016; Kleine and Leister, 2016). This is achieved through extensive exchange of information between plastids and the nucleus, for instance, via biogenic retrograde signaling—a system in which developmentally relevant stimuli in plastids induce the accumulation of specific signaling molecules that relay information to the nucleus, and in turn adjust the expression of nuclear genes to the needs of the plastids (Pogson et al., 2008; Woodson and Chory, 2008; Chan et al., 2016).

During the last 30 years, experiments with the carotenoid biosynthesis inhibitor norfluorazon (NF) and the inhibitor of plastid translation lincomycin (LIN), each of which arrests chloroplast development at the proplastid stage and represses the expression of photosynthesis-associated nuclear genes (PhANGs), have provided insights into the plastid's biogenic retrograde pathways (Oelmüller and Mohr, 1986; Oelmüller et al., 1986).

Six *genome uncoupled* (*gun*) mutants have been characterized in *Arabidopsis thaliana* that fail to repress transcription of the nuclear gene *Lhcb1.2* after NF treatment, and are thus impaired in retrograde signaling (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003; Koussevitzky et al., 2007; Adhikari et al., 2011; Woodson et al., 2011). Five of these genes, *GUN2-6*, were found to be involved in tetrapyrrole biosynthesis (TPB), whereas *GUN1*, which encodes a nucleoid-localized pentatricopeptide repeat protein (PPR), has been shown to have a role in PGE, and to act as an integrator of multiple retrograde signals, since *gun1* mutants are unique in exhibiting a *gun* phenotype in response to both norfluorazon and lincomycin (Gray et al., 2003; Koussevitzky et al., 2007). However, the exact molecular role of GUN1 remained enigmatic until the new insights provided by the recent identification of a set of GUN1-interacting proteins (Tadini et al., 2016; **Table 1**).

Based on the functions of these partners, GUN1 appears to take part in multiple processes essential for chloroplast biogenesis and maintenance of the chloroplast proteome. GUN1-mediated control of plastid ribosomal protein S1 (PRPS1) accumulation, together with co-immunoprecipitation (CoIP) of proteins involved in different steps of plastid translation, support the involvement of GUN1 in the regulation of plastid protein synthesis. Furthermore, the presence of several chaperones in the CoIP mixture suggests a role for GUN1 in the coordination of chloroplast protein import and protein degradation.

Intriguingly, several GUN1 interactors appear to accumulate to higher levels upon induction of the unfolded protein response (UPR) in *Chlamydomonas reinhardtii* chloroplasts, which is triggered upon conditional repression of the catalytic subunit of Clp protease (ClpP1; Ramundo et al., 2013; Ramundo and Rochaix, 2014; Rochaix and Ramundo, 2015). This finding suggests the possible involvement of GUN1 in the UPR signaling pathway.

In this review, we describe the functional roles of the different GUN1 protein partners and propose some testable hypotheses that should clarify the molecular role of GUN1 in chloroplast biogenesis and chloroplast protein homeostasis.

GUN1 IS FOUND IN PLASTID NUCLEOIDS AND INTERACTS WITH THE TRANSCRIPTIONAL MACHINERY

GUN1 encodes a member of PPR-containing protein family, which has a small MutS-related (SMR) domain at the C-terminal end and a plastid targeting signal sequence at its N terminus. PPR motifs have been shown to mediate interactions with nucleic acids, and the SMR domain is found in proteins that act in DNA repair and recombination. However, *in vivo* RNA and DNA immunoprecipitation on chip (NIP-chip), as well as one-hybrid assays, have failed to detect any stable interaction of GUN1 with nucleic acids (Tadini et al., 2016), in contrast to a previous report, in which a GUN1 fragment containing both the PPR and SMR domains was shown to bind DNA *in vitro* (Koussevitzky et al., 2007). Nevertheless, GUN1 appears to be associated with nucleoids in the chloroplast, and more specifically with the domain of active plastid transcription, as shown by the relatively large and distinct foci of a fluorescent GUN1-YFP (Yellow Fluorescence Protein) chimera that co-localize with a Plastid Transcriptionally Active Chromosome 2-Cyan Fluorescence Protein (pTAC2-CFP) fusion in chloroplasts of mesophyll cells (Koussevitzky et al., 2007). However, although the repertoire of nucleoid-associated proteins so far identified is quite extensive, the GUN1 protein is not listed in any of the chloroplast or nucleoid/pTAC proteomes published to date (for a review see Melonek et al., 2016), most probably because it accumulates in very small amounts at specific developmental stages or under

TABLE 1 | GUN1 interactors together with their functions and impacts on plant development.

Designation	AGI code	Mutant phenotype[a]	Molecular function/Defect	Nucleoid subunit[b]	Identification assay[c]	References
TRANSCRIPTION AND RNA METABOLISM						
pTAC6/PAP8	AT1G21600	Albino	Low PEP activity	+	CoIP-MS	Pfalz et al., 2006; Steiner et al., 2011; Pfalz and Pfannschmidt, 2013
RH3/EMB1138	AT5G26742	Embryo lethal	RNA splicing of group II introns, assembly of the 50S ribosomal particle	+	CoIP-MS	Asakura et al., 2012; Majeran et al., 2012
AtPPR_3g49240/ EMB1796	AT3G49240	Embryo lethal	n.d.	+	CoIP-MS	Cushing et al., 2005; Majeran et al., 2012
TRANSLATION						
rpl2	ATCG00830	n.d.	Promotes translation initiation	+	CoIP-MS	Manuell et al., 2007; Melonek et al., 2016
rps3	ATCG00800	Essential for cell survival in tobacco	Promotes translation initiation	+	CoIP-MS	Manuell et al., 2007; Fleischmann et al., 2011; Melonek et al., 2016
rps4	ATCG00380	Essential for cell survival in tobacco	Involved in the assembly of the 30S ribosomal particle; binds to16S rRNA	+	CoIP-MS	Rogalski et al., 2008; Shoji et al., 2011; Melonek et al., 2016
PRPL10/ EMB3136	AT5G13510	Embryo lethal	Part of the L12 stalk and required for translation, since it recruits auxiliary translation factors such as cpIF2	–	CoIP-MS	Baba et al., 2006; Bryant et al., 2011; Shoji et al., 2011; Pfalz and Pfannschmidt, 2013
PRPS1	AT5G30510	n.d.	Promotes translation initiation	–	Y2H; BiFC	Manuell et al., 2007; Shoji et al., 2011; Tadini et al., 2016
cpIF2/FUG1	AT1G17220	Embryo lethal	Promotes translation initiation; leaky mutant alleles suppress leaf variegation in *var* mutants	–	CoIP-MS	Miura et al., 2007
PROTEIN IMPORT, PROTEIN FOLDING, AND PROTEIN UNFOLDING/DEGRADATION						
Hsp93-III/ClpC2	AT3G48870	Single mutant identical to WT; *hsp93-III hsp93-V* double mutant is embryo lethal	Cooperates with Tic110 and Tic40 in chloroplast protein import; chaperone in the Clp protease complex	–	CoIP-MS	Inaba et al., 2003; Kovacheva et al., 2005; Chou et al., 2006; Sakamoto, 2006; Kovacheva et al., 2007; Van Wijk, 2015
Hsp93-V/ClpC1	At5g50920	Single mutant exhibits a chlorotic phenotype; *hsp93-III hsp93-V* double mutant is embryo lethal	Cooperates with Tic110 and Tic40 in chloroplast protein import; chaperone in the Clp protease complex	+	CoIP-MS	Inaba et al., 2003; Kovacheva et al., 2005; Chou et al., 2006; Sakamoto, 2006; Kovacheva et al., 2007; Van Wijk, 2015; Melonek et al., 2016
Hsp70-1	AT4G24280	Single mutant exhibits variegated cotyledons, malformed leaves, growth retardation and impaired root growth; *hsp70-1 hsp70-2* double mutant is lethal	Involved in chloroplast protein import, folding and onward guidance of newly imported polypeptide chains	+	CoIP-MS	Su and Li, 2008; Shi and Theg, 2010; Su and Li, 2010; Liu et al., 2014; Melonek et al., 2016
Hsp70-2	AT5G49910	Single mutant identical to WT; *hsp70-1 hsp70-2* double mutant is lethal	Involved in chloroplast protein import, folding and onward guidance of newly imported polypeptide chains	–	CoIP-MS	Su and Li, 2008; Shi and Theg, 2010; Liu et al., 2014; Su and Li, 2010
ptCpn60α1	AT2G28000	Albino	Involved in folding and onward guidance of newly imported polypeptide chains; essential for plastid division in *A. thaliana*; involved in Rubisco and NdhH assembly	+	CoIP-MS	Gutteridge and Gatenby, 1995; Apuya et al., 2001; Suzuki et al., 2009; Peng et al., 2011; Flores-Perez and Jarvis, 2013; Melonek et al., 2016

(Continued)

TABLE 1 | Continued

Designation	AGI code	Mutant phenotype[a]	Molecular function/Defect	Nucleoid subunit[b]	Identification assay[c]	References
ptCpn60β1	AT1G55490	Leaves of the *len1* mutant have wrinkled and irregular surfaces and display lesions due to spontaneous cell death	Involved in folding and onward guidance of newly imported polypeptide chains; essential for plastid division in *A. thaliana*; involved in Rubisco and NdhH assembly	–	CoIP-MS	Gutteridge and Gatenby, 1995; Boston et al., 1996; Kessler and Blobel, 1996; Jackson-Constan et al., 2001; Ishikawa et al., 2003; Ishikawa, 2005; Suzuki et al., 2009; Flores-Perez and Jarvis, 2013
TPB ENZYMES						
CHLD	AT1G08520	Albino	Encodes the D subunit of the Mg-chelatase enzyme, involved in chlorophyll biosynthesis	–	Y2H; BiFC	Strand et al., 2003; Tanaka et al., 2011
PBGD	AT5G08280	n.d.	Porphobilinogen deaminase activity. Enzyme in the tetrapyrrole biosynthesis pathway	–	Y2H; BiFC	Tanaka et al., 2011
UROD2	AT2G40490	n.d.	Uroporphyrinogen decarboxylase activity; Enzyme in the tetrapyrrole biosynthesis pathway	–	Y2H; BiFC	Tanaka et al., 2011
FC1	AT5G26030	No visible phenotype; overexpression of the *FC1* gene is responsible for the *gun6* phenotype	Encodes ferrochelatase I, involved in heme biosynthesis	–	Y2H; BiFC	Tanaka et al., 2011; Woodson et al., 2011
DIVERSE FUNCTIONS						
rbcL	ATCG00490	Essential for photoautotrophy	Large subunit of Rubisco	+	CoIP-MS	Phinney and Thelen, 2005; Majeran et al., 2012; Huang et al., 2013
ATP-synthase β subunit	ATCG00480	Essential for photoautotrophy	Beta subunit of the thylakoid ATP synthase complex	+	CoIP-MS	Phinney and Thelen, 2005; Pfalz et al., 2006; Majeran et al., 2012; Melonek et al., 2012; Huang et al., 2013
RER4	AT5G12470	Mutant exhibits stunted growth, weak leaf reticulation and smaller mesophyll cells	Integral component of chloroplast outer and inner envelope membranes; possibly involved in retrograde signaling, supply of metabolites, control of ROS	–	CoIP-MS	Perez-Perez et al., 2013
2-Cys PrxA	AT3G11630	Mutant exhibits increased tolerance to photo-oxidative stress	Involved in peroxide detoxification in the chloroplast; functions as a redox sensor and chaperone; controls the conversion of Mg-protoporphyrin monomethyl ester into protochlorophyllide	–	CoIP-MS	Stenbaek et al., 2008; Rey et al., 2007; Pulido et al., 2010; König et al., 2013; Dietz, 2016

Note that proteins Q9SIP7 (AT2G31610) and Q42112 (AT3G09200) reported to be identified in coimmunoprecipitates of GUN1-GFP (Tadini et al., 2016) are not listed in this Table, since they have been described as subunits of cytosolic ribosomes. Furthermore, the protein Q9C5C2 (AT5G25980) has not been included, since it localizes to the tonoplast (Agee et al., 2010).

n.d., not determined.

[a]Phenotype of knock-out mutants is described.

[b]Protein already identified as part of chloroplast nucleoid by proteomic approaches.

[c]Assays used to identify the corresponding protein as a GUN1 interactor: coimmunoprecipitation followed by mass spectrometry (CoIP-MS), yeast two-hybrid (Y2H) analysis, and Bimolecular Fluorescence Complementation (BiFC).

particular physiological conditions. This inference is supported by CoIP experiments with a Green Fluorescence Protein (GUN1-GFP) fusion and subsequent mass spectrometry (MS), which identified several nucleoid subunits as interactors with GUN1 (Tadini et al., 2016; **Table 1**).

pTAC6 is among the GUN1 interactors, and it has been reported to interact directly with the plastid-encoded RNA polymerase (PEP), building together with pTAC2 and other polymerase-associated proteins (PAPs) the soluble RNA polymerase (sRNPase) complex (Pfalz et al., 2006), a central

component of nucleoids (Steiner et al., 2011; **Figure 1**). Intriguingly, pTAC6 (also known as PAP8) contains no known domain and exhibits no homologies that could provide hints as to its function in PGE (Steiner et al., 2011). However, functional genomics analyses have indicated that homozygous *pap* knockout lines develop white cotyledons, fail to accumulate chlorophyll even under low light intensities, and do not produce primary leaves unless they are cultivated on MS medium supplemented with sucrose (for a review, see Pfalz and Pfannschmidt, 2013). Furthermore, analyses of PGE in *pap* mutants revealed strong repression of the accumulation of PEP-dependent transcripts, whereas levels of nucleus-encoded RNA polymerase (NEP)-dependent transcripts were not depleted, while some were enhanced, indicating that pTAC6/PAP8 and the other PAP proteins are essential for the activity of PEP (see **Table 1**).

GUN1 CONTROLS PLASTID TRANSLATION AND RIBOSOME BIOGENESIS

GUN1 also interacts with several ribosomal subunits, such as the plastid-encoded ribosomal proteins L2, S3, and S4 (rpl2, rps3, and rps4) and the nucleus-encoded plastid ribosomal protein L10 (PRPL10; **Figure 1**). Furthermore, yeast two-hybrid and Bimolecular Fluorescence Complementation (BiFC) assays revealed a physical interaction between GUN1 and PRPS1 (Tadini et al., 2016). Ribosomal proteins have been reproducibly detected in nucleoid and pTAC proteomes (Melonek et al., 2016), further supporting the existence of a translational subdomain within the nucleoids, as proposed by Pfalz and Pfannschmidt (2013). The homologs of PRPL10, rpl2, PRPS1, rps3, and rps4 are essential components of the protein biosynthetic machinery in *Escherichia coli* (Baba et al., 2006; Shoji et al., 2011) and the indispensability of rps3 and rps4 has been also proven in tobacco plastids (Rogalski et al., 2008; Fleischmann et al., 2011). Furthermore, PRPL10 is annotated as EMBryo defective 3136 (EMB3136) in the SeedGenes Project database (http://www.seedgenes.org/), and in its absence Arabidopsis embryo development arrests at the globular stage (Bryant et al., 2011). Mutants devoid of PRPS1 have not been described. However, given the conservation of PRPS1 function in prokaryotes and chloroplasts, it can be confidently assumed that complete lack of PRPS1 is lethal in Arabidopsis.

Taking into consideration the function of these ribosomal proteins, it can be argued that their interaction with GUN1 has a dual purpose. On the one hand, GUN1 modulates protein synthesis by controlling the abundance of PRPS1, which, together with rps3 and rps2, has been reported to form the domain responsible for the interaction of the 30S ribosomal subunit with mRNA, promoting translation initiation (Manuell et al., 2007; Tadini et al., 2016). This role is supported further by the stable interaction of GUN1 with the chloroplast translation initiation

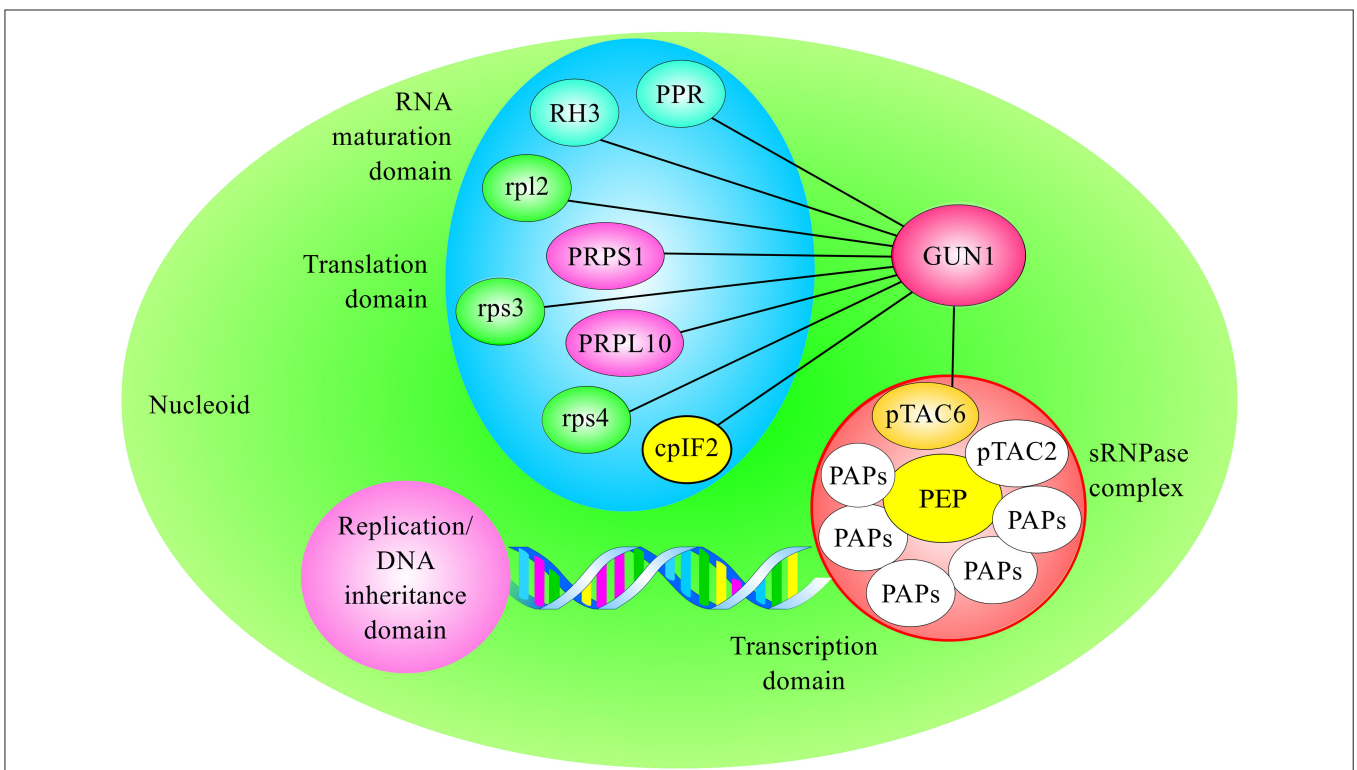

FIGURE 1 | Schematic overview of GUN1 protein interactors involved in gene transcription, ribosome biogenesis and plastid translation. The scheme takes into account the partition of nucleoids into functional subdomains proposed by Pfalz and Pfannschmidt (2013). PPR refers to AtPPR_3g49240, also known as EMB1796, as reported in **Table 1**.

factor 2 (cpIF2; Tadini et al., 2016), also known as FUG1, and reported to be essential for chloroplast biogenesis (Miura et al., 2007).

On the other hand, GUN1 seems to be involved in the process of ribosome biogenesis too, since nucleoid-associated ribosomes are thought to be in various stages of assembly, with several rRNA maturation steps occurring in a co-transcriptional and assembly-assisted manner, as in prokaryotic systems (Bohne, 2014). For instance, the DEAD-box-containing, ATP-dependent RNA helicase 3 (RH3), which has been functionally linked to the chloroplast nucleoid (Majeran et al., 2012), is among the proteins that interact with GUN1 (Tadini et al., 2016; see also **Figure 1** and **Table 1**). RH3 is directly involved in the splicing of group II introns in the *rpl2*, *trnA*, *trnI*, and *rps12* transcripts and could be coimmunoprecipitated with immature and mature 23S rRNA (Asakura et al., 2012). Furthermore, the PPR protein At3g49240 also known as AtPPR_3g49240, according to the PPR protein database (http://www.plantenergy.uwa.edu.au/applications/ppr/ppr.php), is also part of GUN1's interactors, and its maize ortholog, GRMZM2G074599_P01, has been identified in the chloroplast nucleoid (Majeran et al., 2012). The gene is annotated as embryo defective 1796 (EMB1796) in the SeedGenes database, since the complete lack of AtPPR_3g49240 leads to the arrest of embryonic development at the globular stage (Cushing et al., 2005), further supporting the essential role of GUN1 interactors in chloroplast biogenesis.

GUN1 AND THE IMPORT OF CHLOROPLAST PROTEINS

Almost a quarter of the GUN1 interactors identified by CoIP-MS are chaperones (see **Table 1**), a relatively high proportion when compared with the extensive repertoire of protein functions found within the nucleoid (Melonek et al., 2016). The stromal Hsp93 and Hsp70 chaperones mediate different steps in protein import into the chloroplast stroma, whereas the 60 KD chaperonin Cpn60 is thought to be involved in the folding of newly imported mature proteins and to function downstream of Hsp93 and Hsp70 (Kessler and Blobel, 1996; Jackson-Constan et al., 2001; Flores-Perez and Jarvis, 2013). Furthermore, the two genes most highly co-regulated with *GUN1* encode the proteins TIC110 and TOC159 (Tadini et al., 2016), which are part of the outer and inner chloroplast translocons, respectively, suggesting a role of GUN1 in chloroplast protein import (**Figure 2**).

The Hsp93 Chaperones

In Arabidopsis, there are two nearly identical isoforms of Hsp93, termed Hsp93-V and Hsp93-III (or ClpC1 and ClpC2, respectively) and both interact with GUN1. The two proteins are highly homologous, but Hsp93-V is expressed at much higher levels than Hsp93-III (Kovacheva et al., 2005, 2007), and only Hsp93-V has been reported as a component of the nucleoid proteome (Phinney and Thelen, 2005; Majeran et al., 2012; Melonek et al., 2012; Huang et al., 2013). Furthermore, both *hsp93* single mutants are viable whereas *hsp93-III hsp93-V* double mutant is embryo-lethal, indicating that the two proteins have redundant functions in Arabidopsis chloroplasts (Constan et al., 2004; Sjögren et al., 2004; Kovacheva et al., 2007).

The current model for chloroplast protein import assumes that the preprotein transit peptide interacts with the TOC, and is subsequently transported through the TIC in an energy-dependent process (Shi and Theg, 2013b). In particular, the Tic110–Tic40 interaction is proposed to trigger the release of the transit peptide from Tic110 and enable the association of the preprotein with Hsp93 (Inaba et al., 2003). Tic40 then stimulates ATP hydrolysis by Hsp93, which harnesses the energy released to draw the preprotein into the stroma (Chou et al., 2006).

The Hsp70 Chaperones

Recent work has also demonstrated the involvement of Hsp70 in protein translocation into chloroplasts, as part of the translocon energy-dependent engine together with Hsp93 and Hsp90 (Inoue et al., 2013; Liu et al., 2014). Like Hsp93, Hsp70 proteins occur in two isoforms, Hsp70-1 and Hsp70-2, in the chloroplasts of Arabidopsis (Su and Li, 2008) and only Hsp70-1 was found in the proteomes of pTAC and crude nucleoids (for a review see Melonek et al., 2016). However, both Hsp70 proteins have been identified as GUN1 interactors (Tadini et al., 2016). Protein import assays using chloroplasts isolated from the Arabidopsis Hsp70 knockout mutants *hsp70-1* and *hsp70-2* showed that stromal Hsp70s are important for the import of both photosynthetic and non-photosynthetic precursor proteins, especially in early developmental stages (Su and Li, 2010). Furthermore, no *hsp70-1 hsp70-2* double mutant has ever been isolated. Thus, the two Hsp70s are likely to have redundant functions that are essential for plant development and chloroplast biogenesis.

The Cpn60 Chaperonins

After preproteins delivered to the stroma have been processed, they may require accessory factors to enable them to fold into their functional conformation, or to reach their final intra-organellar destination. The stromal molecular chaperones Hsp70, Cpn60, and Cpn10 are all believed to mediate the folding or onward guidance of newly imported polypeptide chains (Boston et al., 1996; Jackson-Constan et al., 2001). In particular, immunoprecipitation experiments have revealed that Cpn60 operates in close proximity with Tic110 (Kessler and Blobel, 1996), while import experiments have shown a transient association of mature, newly imported proteins with the Cpn60-Tic110 complex, suggesting that Tic110 can recruit Cpn60 in an ATP-dependent manner for the folding of proteins upon their arrival in the stroma. It has also been suggested that stromal Hsp70 and Cpn60 act sequentially to facilitate the maturation of imported proteins, particularly those destined for the thylakoid membranes (Madueno et al., 1993; Tsugeki and Nishimura, 1993; Peng et al., 2011). The Arabidopsis genome encodes two members of the Cpn60α family, denoted ptCpn60α1 and ptCpn60α2, and four members of Cpn60β, known as ptCpn60β1–β4 (Suzuki et al., 2009). Two of them, ptCpn60α1 and ptCpn60β2, have been linked to the nucleoid proteome (Melonek et al., 2016), and ptCpn60α1 and ptCpn60β1 are among the GUN1 interactors identified via the CoIP-MS strategy (see **Table 1**). The complete loss of ptCpn60α1, in the

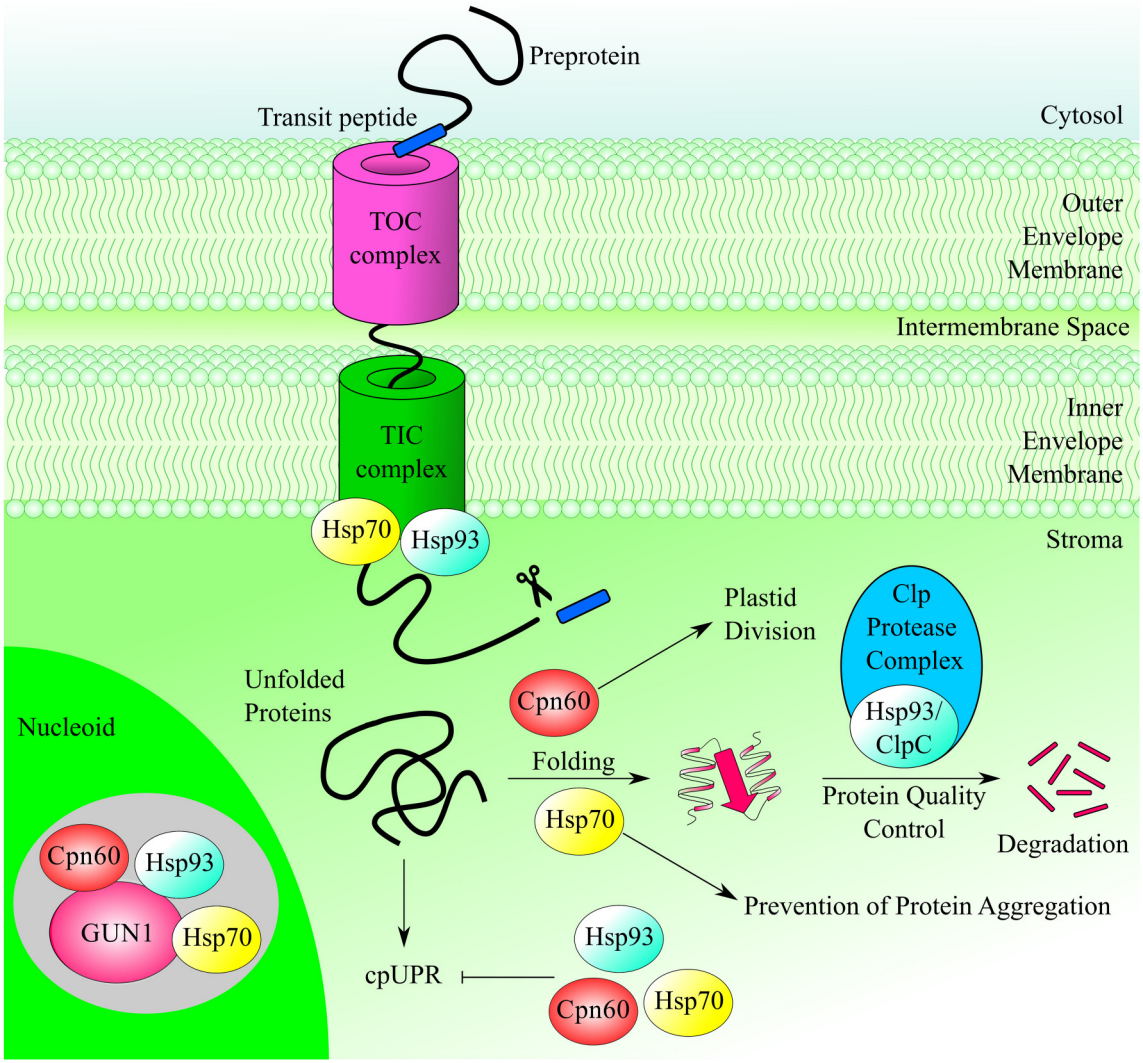

FIGURE 2 | GUN1 interacts with different plastid chaperones. The chaperones Hsp93, Hsp70, and Cpn60 participate in different processes within the chloroplast, such as protein import, protein folding/unfolding, prevention of protein aggregation, and regulation of plastid division, and they might play a key role in the chloroplast Unfolded Protein Response (cpUPR). CoIP-MS analysis has shown that they are also part of GUN1-containing protein complexes.

mutant termed *schlepperless* (*slp*), causes retardation of embryo development before the heart stage and an albino seedling phenotype, indicating that ptCpn60α1 is essential for chloroplast biogenesis (Apuya et al., 2001). Conversely, plants devoid of ptCpn60β1, also known as *lesion initiation 1* (*len1*), have leaves with wrinkled and irregular surfaces and undergo localized, spontaneous cell death in the absence of pathogen attack, i.e., lesion formation, under short-day conditions (Ishikawa et al., 2003).

OTHER FUNCTIONS OF PLASTID CHAPERONES

Besides their roles in plastid protein import, all GUN1-interacting chaperones are present in the stroma at significant amounts relative to their association with the chloroplast import apparatus and perform various other functions together with different protein complexes (**Figure 2**). For instance Hsp93, also termed ClpC, acts as a regulatory chaperone in the Clp protease complex, the most abundant stromal protease with general household functions (Sakamoto, 2006; Van Wijk, 2015). Clp substrates are selected through various signals intrinsic to amino acid sequences and the ATP-dependent ClpC chaperone activity helps to progressively unfold selected substrates that are delivered to the ClpPR core for degradation into small peptides (~8–10 amino acids long; Olinares et al., 2011).

Similarly, Cpn60 forms a large oligomeric protein complex (>600 KDa) that promotes the assembly of Rubisco (Gutteridge and Gatenby, 1995). In particular, it has been observed that the large subunit of Rubisco (RbcL) is specifically associated with

Cpn60 before assembly into the holoenzyme and that the Cpn60-RbcL complex is an obligatory intermediate. Furthermore, Cpn60 proteins have been shown to be essential for plastid division in *A. thaliana* (Suzuki et al., 2009). Thus, mesophyll cells in *ptcpn60α1-1* (a missense mutant) and *ptcpn60β1-1* (a protein null) plants, contain fewer and larger chloroplasts, indicating that normal levels of plastid Cpn60 are required for the correct folding of the stromal plastid division proteins and/or regulation of FtsZ (Filamentous temperature-sensitive Z) polymer dynamics (Suzuki et al., 2009).

The same holds true for the Hsp70 proteins, which are also involved in modulation of protein activity, regulation of protein degradation and prevention of irreversible protein aggregation when they are free in the stroma (Su and Li, 2008). Potentially GUN1 can be involved in a multitude of activities, besides plastid protein import, thus further investigations are needed to clarify the functional significance of GUN1–chaperone interactions.

GUN1 AND THE CHLOROPLAST UNFOLDED PROTEIN RESPONSE (cpUPR)

Chaperones, together with enzymes that process and degrade proteins, are also necessary to maintain protein folding homeostasis in the various compartments of eukaryotic cells. Distinct signal transduction pathways, known as unfolded protein responses (UPRs), have evolved to couple the unfolded/misfolded protein load to the expression of specific chaperones and enzymes that promote folding and the disposal of misfolded proteins in each compartment.

The unfolded protein response was first discovered in the endoplasmic reticulum (ER) in yeast, where inhibition of protein folding leads to the transcriptional up-regulation of several chaperones (Cox et al., 1993), and subsequently in mitochondria, where accumulation of unfolded proteins in the mitochondrial matrix stimulates the expression of nuclear gene transcripts coding for mitochondrial chaperones (Aldridge et al., 2007; Lin and Haynes, 2016). Compared to yeast and metazoans, studies of plant UPRs are less advanced, and molecular details are known mainly for the ER-dependent UPR, which shows certain similarities with the process in multicellular eukaryotes, as well as plant-specific features (Ruberti et al., 2015). Recently, the possible existence of a chloroplast UPR (cpUPR) has been investigated in the green alga *Chlamydomonas reinhardtii*. Taking advantage of a repressible chloroplast gene expression system (Rochaix et al., 2014), Ramundo et al. (2014) induced the selective gradual depletion of the essential stromal Clp protease, in order to follow the early and late events caused by the decrease in its abundance. Temporal profiles of gene expression and protein accumulation revealed a marked increase in levels of chaperones, including Hsp70B, upon Clp depletion. Similar data have also been reported for Arabidopsis, where up-regulation of chloroplast chaperones and protein-sorting components occurred upon constitutive repression of Clp (Rudella et al., 2006; Zybailov et al., 2009). In particular, characterization of total leaf proteomes of WT and *clpr2-1* highlighted differential expression of 768 proteins. The largest functional category quantified (with 205 proteins) comprised proteins involved in translation, folding and degradation. Strikingly, all the chaperones interacting with GUN1, including Hsp93, Hsp70, Cpn60, as well as the DEAD box RNA helicase RH3, are among those up-regulated (by between 1.6- and 8.5-fold) in *clpr2-1* leaves, whereas no significant change in the chloroplast ribosomal protein population was observed (Zybailov et al., 2009).

Taken together, these findings suggest that disruption of protein homeostasis in organelles can be sensed and transduced to the nucleus to induce the expression of a specific set of factors responsible for promoting folding and monitoring protein quality control (Ramundo and Rochaix, 2014; Rochaix and Ramundo, 2015). After entering the higher plant chloroplast, these factors are able to interact with the nucleoid-associated GUN1 protein (**Figure 2**), which might therefore play a role in the cpUPR process.

GUN1 AND CHLOROPLAST METABOLISM

The large subunit of ribulose bisphosphate carboxylase (RbcL) and the β subunit of the ATP synthase are also among the interactors of GUN1 identified by CoIP-MS analysis (Tadini et al., 2016). Because of their relatively high abundance in the chloroplast proteome, it is tempting to assume that these proteins are simply contaminants. However, RbcL and subunits of the ATP synthase have been repeatedly identified in the pTAC/nucleoid proteomes, even though different procedures were employed for isolation of crude nucleoid fractions and highly purified pTAC complexes (for a review see Melonek et al., 2016), thus suggesting these proteins might have a dual localization to the chloroplast stroma and nucleoids. The nucleoid association of RbcL and ATP synthase, i.e., of proteins that are not directly involved in core nucleoid functions, might also indicate that nucleoids also monitor photosynthesis and energy metabolism and respond appropriately to any perturbations (**Figure 3**).

Unlike RbcL and the ATP synthase β subunit, RETICULATA-RELATED 4 (RER4), an integral component of the chloroplast envelope membranes with three transmembrane α-helices, has never been identified in the pTAC/nucleoid proteome, although it appears to be an interactor of GUN1 (**Table 1**). The mutant *rer4-1* exhibits leaf reticulation, having green veins that stand out against paler intervein tissue, with fewer and smaller mesophyll cells than those of the wild type leaves (Perez-Perez et al., 2013). The molecular function of RER4 remains to be established. However, some hints as to its role in the chloroplast can be derived from features of the *rer4-1* mutant phenotype. A possible involvement of RER4 in retrograde signaling is suggested by the altered growth and development of mesophyll cells. Alternatively, the absence of RER4 might deplete the supply of essential metabolites during early stages of leaf development, which could explain the aberrant mesophyll structure. Furthermore, RER4 has been suggested to be involved in the control of reactive oxygen species (ROS), since the reticulated pigmentation of the *rer4-1* mutant grown under long-day conditions can be rescued

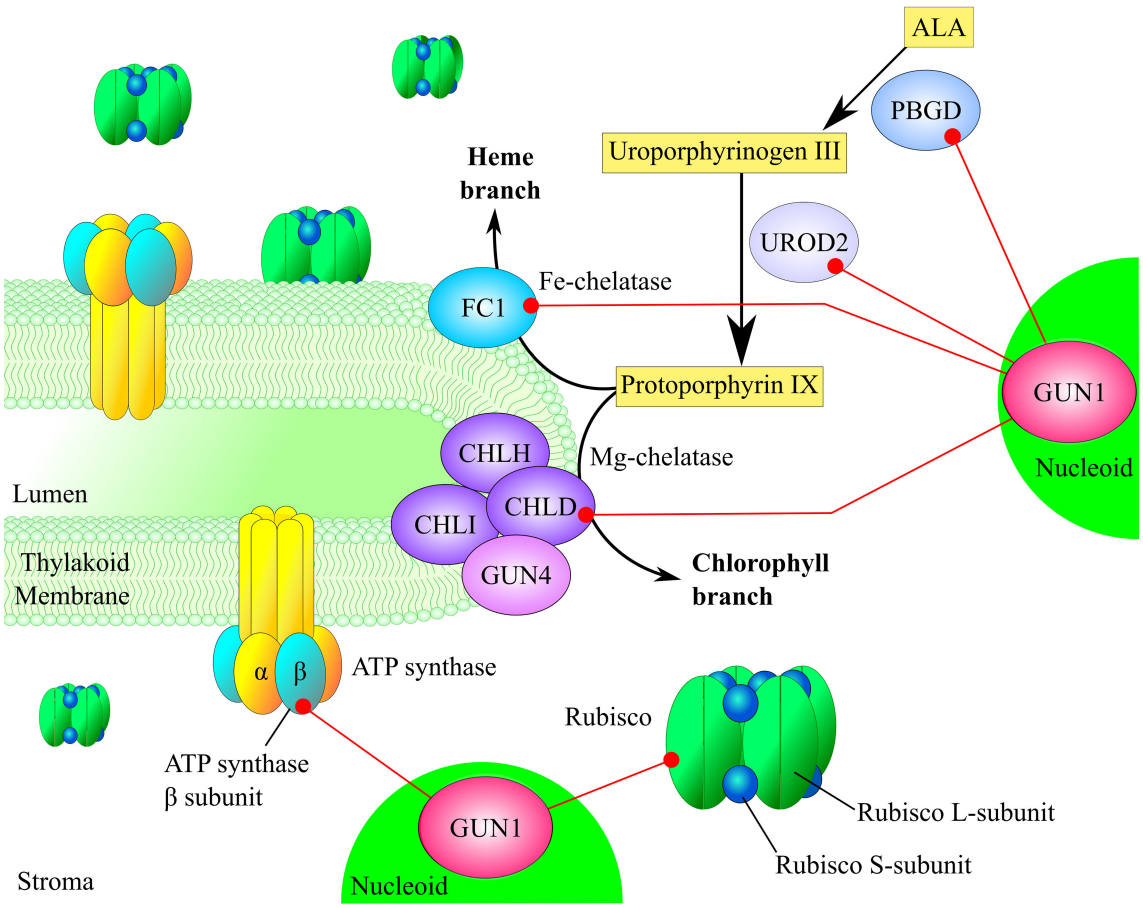

FIGURE 3 | GUN1 is involved in photosynthesis and tetrapyrrole biosynthesis. The large subunit of Rubisco and the β-subunit of the thylakoid ATP synthase have been coimmunoprecipitated with GUN1, supporting a role for GUN1 in coordinating nucleoid activities with chloroplast metabolism. GUN1 also interacts with four enzymes of the tetrapyrrole biosynthesis pathway, i.e., the D subunit of Mg chelatase (CHLD), porphobilinogen deaminase (PBGD), uroporphyrinogen III decarboxylase (UROD2), and ferrochelatase I (FC1), as shown by yeast two-hybrid and Bimolecular Fluorescence Complementation. Note that the proteins RER4 and 2-Cys PrxA have not been included in this scheme for reasons of clarity.

by a short-day photoperiod, which markedly dampens ROS accumulation.

The 2-Cys peroxiredoxin A (2-Cys Prx A; see also **Table 1**), another interactor with GUN1, appears also to have a role in ROS scavenging (Rey et al., 2007; Pulido et al., 2010; Dietz, 2016) and, like RER4, it has never been reported to be part of the pTAC/nucleoid proteome (Pfalz et al., 2006; Majeran et al., 2012; Huang et al., 2013). 2-Cys Prx A and the highly homologous 2-Cys Prx B function as redox sensors and chaperones, thanks to the flexibility of their protein structure (König et al., 2013), and they have been shown to control the conversion of Mg-protoporphyrin monomethyl ester into protochlorophyllide (Stenbaek et al., 2008).

The involvement of GUN1 in TPB is further supported by its interaction with four TPB enzymes, namely subunit D of Mg chelatase (CHLD), porphobilinogen deaminase (PBGD), uroporphyrinogen III decarboxylase (UROD2), and ferrochelatase I (FC1), as demonstrated by both yeast two-hybrid and BiFC assays (Tadini et al., 2016; **Figure 3**). Interestingly, mutants defective in three of these GUN1 interactors—CHLD, PBGD, and FC1—have themselves been described as *gun* mutants (Strand et al., 2003; Huang and Li, 2009; Woodson et al., 2011), but have never been identified in crude nucleoid preparations, unlike subunit I of Mg chelatase (CHLI; Melonek et al., 2012; Huang et al., 2013).

GUN1 AND PLASTID PROTEIN HOMEOSTASIS: SOME TESTABLE HYPOTHESES

The recent identification of the GUN1 protein's partners in chloroplasts of Arabidopsis by means of CoIP-MS studies as well as in yeast two-hybrid and BiFC assays (Tadini et al., 2016) strongly suggests a major role for GUN1 in plastid protein homeostasis (**Figure 4**). This regulatory role involves proteins that are, in most cases, members of multimeric protein complexes and whose functions are often context-dependent. Furthermore, most GUN1 interactors appear to participate in four major processes:

Chloroplast Protein Synthesis

A wealth of evidence accumulated during the last two decades supports the primacy of plastid protein synthesis in the control of chloroplast gene expression (Choquet and Wollman, 2002; Manuell et al., 2007; Tiller and Bock, 2014; Sun and Zerges, 2015). In this context, GUN1 has been suggested to regulate translation in plastids by modulating the abundance and binding affinity of PRPS1 (Tadini et al., 2016). In particular, PRPS1 is the only ribosomal protein that shuttles between ribosome-bound and ribosome-free forms (Merendino et al., 2003; Delvillani et al., 2011), the latter being more abundant in plants that lack GUN1. Based on observations in *E. coli*, where the unbound form is thought to inhibit translation by competing with ribosomes for mRNAs (Delvillani et al., 2011), it can be argued that the GUN1-dependent equilibrium between the two PRPS1 states has an important role in controlling polysome assembly and protein synthesis in chloroplasts (**Figure 4A**). However, further investigations are needed to clarify this issue. For instance, lines characterized by the ectopic expression of *PRPS1* or carrying *PRPS1* constructs under the control of inducible promoters, coupled with assays aimed to measure the translation rate in plastids, should allow us to verify the role of PRPS1 in modulating protein synthesis. Furthermore, GUN1 controls the abundance of PRPS1 at the post-transcriptional level. This suggests the involvement of an as yet unidentified plastid protease in this aspect of GUN1 function. In addition, the significance of the interaction of GUN1 with other ribosomal proteins, factors involved in ribosome biogenesis and regulators of plastid protein synthesis remains to be elucidated.

Chloroplast Protein Import and Degradation

Based on the observations reported above, it appears that GUN1 may well control the interactions of a sub-set of chaperones, promoting plastid protein import when their association with the TIC complex is favored, and stimulating protein degradation, folding/unfolding when they interact with proteases or other protein complexes in the stroma or in the thylakoid membranes (**Figure 4B**). Such a regulatory mechanism would enable GUN1 to coordinate protein translocation across the chloroplast envelope with protein degradation in the stroma,

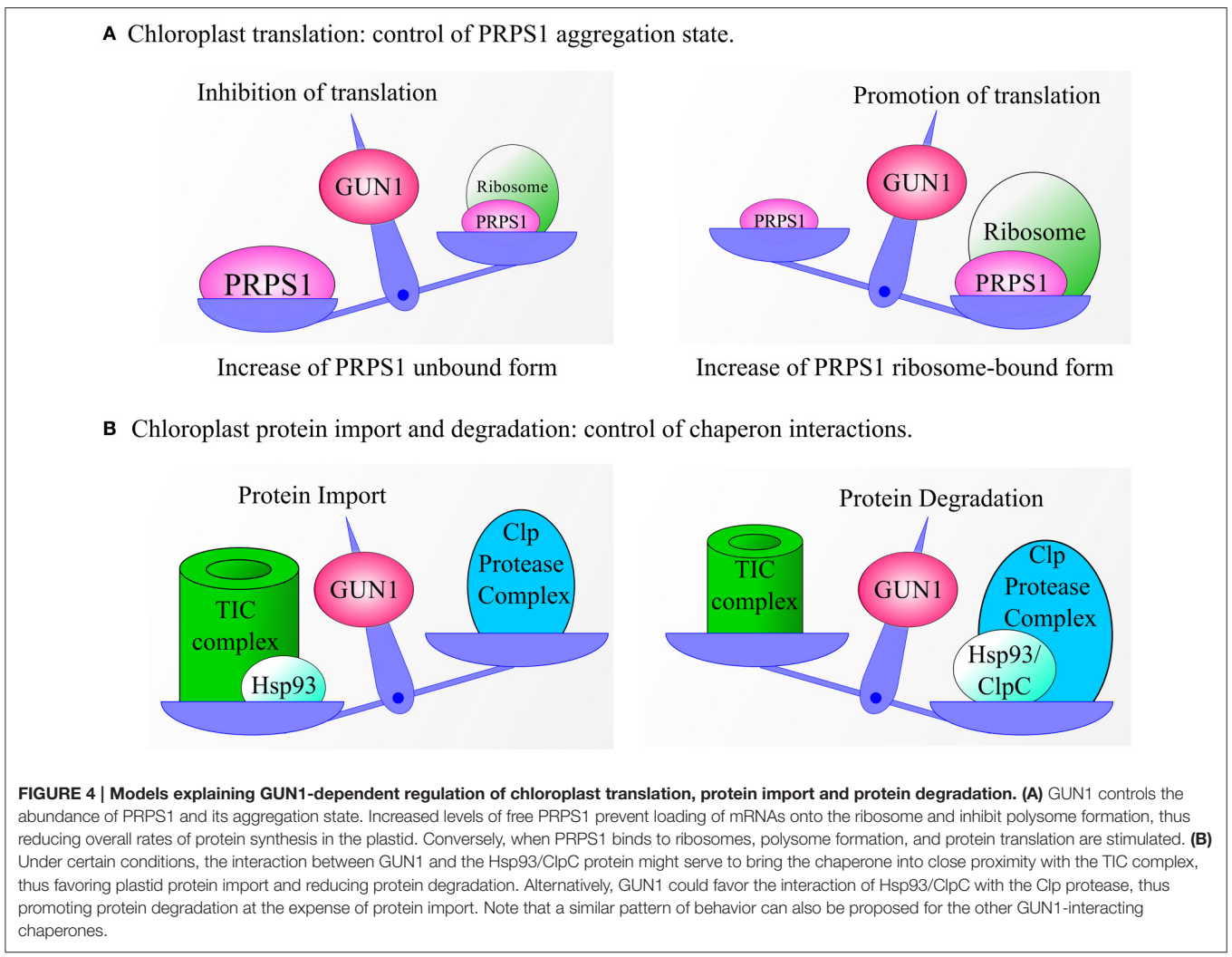

FIGURE 4 | Models explaining GUN1-dependent regulation of chloroplast translation, protein import and protein degradation. (A) GUN1 controls the abundance of PRPS1 and its aggregation state. Increased levels of free PRPS1 prevent loading of mRNAs onto the ribosome and inhibit polysome formation, thus reducing overall rates of protein synthesis in the plastid. Conversely, when PRPS1 binds to ribosomes, polysome formation, and protein translation are stimulated. **(B)** Under certain conditions, the interaction between GUN1 and the Hsp93/ClpC protein might serve to bring the chaperone into close proximity with the TIC complex, thus favoring plastid protein import and reducing protein degradation. Alternatively, GUN1 could favor the interaction of Hsp93/ClpC with the Clp protease, thus promoting protein degradation at the expense of protein import. Note that a similar pattern of behavior can also be proposed for the other GUN1-interacting chaperones.

as well as with plastid division, thus modulating the protein content of the chloroplast in accordance with physiological requirements.

Relatively simple biochemical analyses can be used to verify the importance of GUN1 in influencing the interactions of the stromal chaperones, such as protein complex fractionation via sucrose-gradient ultracentrifugation and/or Blue-Native PAGE coupled with two-dimensional (2D) SDS-PAGE, and immunoblot analyses. Furthermore, the interactions of GUN1 with chaperones should be shown to occur at the plastid envelope and protein import efficiency should be tested in chloroplasts isolated from *gun1* and WT seedlings in order to implicate GUN1 in regulating plastid protein import.

Retrograde Signal Induction

GUN1 may well be a master regulator of plastid-to-nucleus communication in *A. thaliana*, as it appears to integrate signals derived from perturbations in PGE, TPB, and redox state, in order to modulate nuclear gene expression. Indeed, components of all three pathways have been shown to interact with GUN1, suggesting that signal integration might take place through physical interaction.

Due to the limited abundance of GUN1, as indicated by the fact that the protein has yet to be detected in plastid proteome studies, it is tempting to disregard the idea that its physical interaction with PGE-, TPB-, and redox-related proteins could lead to protein sequestration and directly to differences in protein translation, TPB, and redox balance (Koussevitzky et al., 2007; Pogson et al., 2008; Woodson and Chory, 2008; Kleine and Leister, 2016). Nevertheless, a direct association with GUN1 could control protein abundance through post-transcriptional mechanisms, as in the case of PRPS1 and CHLD (Tadini et al., 2016). Thus, control of CHLD and possibly of FC1 levels could alter the tetrapyrrole flux and influence the abundance of the tetrapyrrole intermediate Mg-protoporphyrin IX (Mg-ProtoIX), or the tetrapyrrole product Fe-protoporphyrin IX (heme), which have been reported to act as negative and positive retrograde signals, respectively (for a review, see Chan et al., 2016). Alternatively, the interaction of GUN1 with the near-identical paralogs ClpC1 and ClpC2 could contribute to the coordination of plastid protein content with tetrapyrrole biosynthesis. Indeed, the activity of the stromal Clp protease has been shown to modulate tetrapyrrole flux by controlling (i) the accumulation of chlorophyll a oxygenase, which converts chlorophyll a into chlorophyll b (Nakagawara et al., 2007), and (ii) the level of glutamyl-tRNA reductase (GluTR), thus regulating the rate-limiting reaction in tetrapyrrole synthesis—the conversion of glutamate-1-semialdehyde into 5-aminolevulinic acid (Apitz et al., 2016).

Therefore, accurate determination of tetrapyrrole intermediates should be performed in *gun1* mutant and WT backgrounds. The analyses should be restricted to young seedlings or even to different developmental stages of the chloroplast, in line with the roles of tetrapyrrole and GUN1-mediated signaling in chloroplast development.

CONCLUDING REMARKS

In the past decade, substantial progress has been made in elucidating retrograde signaling, with the identification of multiple retrograde pathways and more than 40 components involved at different levels in chloroplast-to-nucleus communication. Nevertheless, the molecular function of GUN1 has remained unclear until the recent identification of the GUN1 protein's partners. Based on the functional roles of GUN1 interactors and the embryo lethal or albino phenotypes of most of the corresponding knock-out mutants, we have learned that GUN1 plays a role in chloroplast biogenesis, possibly by controlling protein turnover and protein import, and through the coordination of plastid and nuclear gene expression. Furthermore, GUN1 could have a role in the cpUPR process. Nonetheless, the involvement of GUN1 in plastid biogenesis and protein homeostasis is only just beginning to be understood. For instance, other approaches will be needed to validate the GUN1's protein partners identified by CoIP-MS. The use of a GUN1-GFP protein chimera, expressed under the control of a strong constitutive promoter such as the Cauliflower Mosaic Virus 35S (35S-CaMV), is indeed prone to the identification of false interactors. CoIP-MS studies using a GUN1 specific antibody appears to be the ideal strategy to identify protein partners. Alternatively, the use of GUN1 chimeras under the control of GUN1 native promoter is also practicable. Moreover, we do not know whether all these activities take place within one GUN1-containing nucleoid or if there are different nucleoids/locations for each GUN1-dependent function. The developmental stages of the chloroplast itself may even show distinct patterns of compartmentalization of the different functions. In addition, GUN1's interactions with its diverse partners might have quite different functional consequences: (i) promote specific functions, by bringing enzymes into close proximity with their own substrates and, ultimately, controlling the enzyme abundance, (ii) inhibit processes by sequestering sub-pools of specific proteins and, also in this case, controlling their abundance.

We are confident that future work, based on the exciting breakthroughs discussed in this Review, will shed new light on the molecular functions of GUN1 and its involvement in chloroplast biogenesis and protein homeostasis.

AUTHOR CONTRIBUTIONS

MC, LT, CP, RF, and PP participated to the organization of the manuscript. MC and PP designed and conceived the pictures. PP wrote the manuscript.

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REFERENCES

Adhikari, N. D., Froehlich, J. E., Strand, D. D., Buck, S. M., Kramer, D. M., and Larkin, R. M. (2011). GUN4-porphyrin complexes bind the ChlH/GUN5 subunit of Mg-Chelatase and promote chlorophyll biosynthesis in Arabidopsis. *Plant Cell* 23, 1449–1467. doi: 10.1105/tpc.110.082503

Agee, A. E., Surpin, M., Sohn, E. J., Girke, T., Rosado, A., Kram, B. W., et al. (2010). MODIFIED VACUOLE PHENOTYPE1 is an Arabidopsis myrosinase-associated protein involved in endomembrane protein trafficking. *Plant Physiol.* 152, 120–132. doi: 10.1104/pp.109.145078

Aldridge, J. E., Horibe, T., and Hoogenraad, N. J. (2007). Discovery of genes activated by the mitochondrial unfolded protein response (mtUPR) and cognate promoter elements. *PLoS ONE* 2:e874. doi: 10.1371/journal.pone.0000874

Apitz, J., Nishimura, K., Schmied, J., Wolf, A., Hedtke, B., Van Wijk, K. J., et al. (2016). Posttranslational control of ALA synthesis includes GluTR degradation by Clp protease and stabilization by GluTR-binding protein. *Plant Physiol.* 170, 2040–2051. doi: 10.1104/pp.15.01945

Apuya, N. R., Yadegari, R., Fischer, R. L., Harada, J. J., Zimmerman, J. L., and Goldberg, R. B. (2001). The Arabidopsis embryo mutant schlepperless has a defect in the chaperonin-60alpha gene. *Plant Physiol.* 126, 717–730. doi: 10.1104/pp.126.2.717

Asakura, Y., Galarneau, E., Watkins, K. P., Barkan, A., and Van Wijk, K. J. (2012). Chloroplast RH3 DEAD box RNA helicases in maize and Arabidopsis function in splicing of specific group II introns and affect chloroplast ribosome biogenesis. *Plant Physiol.* 159, 961–974. doi: 10.1104/pp.112.197525

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., et al. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2, 2006.0008. doi: 10.1038/msb4100050

Bohne, A. V. (2014). The nucleoid as a site of rRNA processing and ribosome assembly. *Front. Plant Sci.* 5:257. doi: 10.3389/fpls.2014.00257

Boston, R. S., Viitanen, P. V., and Vierling, E. (1996). Molecular chaperones and protein folding in plants. *Plant Mol. Biol.* 32, 191–222. doi: 10.1007/BF00039383

Bryant, N., Lloyd, J., Sweeney, C., Myouga, F., and Meinke, D. (2011). Identification of nuclear genes encoding chloroplast-localized proteins required for embryo development in Arabidopsis. *Plant Physiol.* 155, 1678–1689. doi: 10.1104/pp.110.168120

Chan, K. X., Phua, S. Y., Crisp, P., McQuinn, R., and Pogson, B. J. (2016). Learning the languages of the chloroplast: retrograde signaling and beyond. *Annu. Rev. Plant Biol.* 67, 25–53. doi: 10.1146/annurev-arplant-043015-111854

Choquet, Y., and Wollman, F. A. (2002). Translational regulations as specific traits of chloroplast gene expression. *FEBS Lett.* 529, 39–42. doi: 10.1016/S0014-5793(02)03260-X

Chou, M. L., Chu, C. C., Chen, L. J., Akita, M., and Li, H. M. (2006). Stimulation of transit-peptide release and ATP hydrolysis by a cochaperone during protein import into chloroplasts. *J. Cell Biol.* 175, 893–900. doi: 10.1083/jcb.200609172

Constan, D., Froehlich, J. E., Rangarajan, S., and Keegstra, K. (2004). A stromal Hsp100 protein is required for normal chloroplast development and function in Arabidopsis. *Plant Physiol.* 136, 3605–3615. doi: 10.1104/pp.104.052928

Cox, J. S., Shamu, C. E., and Walter, P. (1993). Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell* 73, 1197–1206. doi: 10.1016/0092-8674(93)90648-A

Cushing, D. A., Forsthoefel, N. R., Gestaut, D. R., and Vernon, D. M. (2005). Arabidopsis emb175 and other ppr knockout mutants reveal essential roles for pentatricopeptide repeat (PPR) proteins in plant embryogenesis. *Planta* 221, 424–436. doi: 10.1007/s00425-004-1452-x

Delvillani, F., Papiani, G., Dehò, G., and Briani, F. (2011). S1 ribosomal protein and the interplay between translation and mRNA decay. *Nucleic Acids Res.* 39, 7702–7715. doi: 10.1093/nar/gkr417

Dietz, K. J. (2016). Thiol-based peroxidases and ascorbate peroxidases: why plants rely on multiple peroxidase systems in the photosynthesizing chloroplast? *Mol. Cells* 39, 20–25. doi: 10.14348/molcells.2016.2324

Fleischmann, T. T., Scharff, L. B., Alkatib, S., Hasdorf, S., Schöttler, M. A., and Bock, R. (2011). Nonessential plastid-encoded ribosomal proteins in tobacco: a developmental role for plastid translation and implications for reductive genome evolution. *Plant Cell* 23, 3137–3155. doi: 10.1105/tpc.111.088906

Flores-Pérez, U., and Jarvis, P. (2013). Molecular chaperone involvement in chloroplast protein import. *Biochim. Biophys. Acta* 1833, 332–340. doi: 10.1016/j.bbamcr.2012.03.019

Gray, J. C., Sullivan, J. A., Wang, J. H., Jerome, C. A., and Maclean, D. (2003). Coordination of plastid and nuclear gene expression. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358, 135–144. discussion 144–145. doi: 10.1098/rstb.2002.1180

Gutteridge, S., and Gatenby, A. A. (1995). Rubisco synthesis, assembly, mechanism, and regulation. *Plant Cell* 7, 809–819. doi: 10.1105/tpc.7.7.809

Huang, M., Friso, G., Nishimura, K., Qu, X., Olinares, P. D., Majeran, W., et al. (2013). Construction of plastid reference proteomes for maize and Arabidopsis and evaluation of their orthologous relationships; the concept of orthoproteomics. *J. Proteome Res.* 12, 491–504. doi: 10.1021/pr300952g

Huang, Y. S., and Li, H. M. (2009). Arabidopsis CHLI2 can substitute for CHLI1. *Plant Physiol.* 150, 636–645. doi: 10.1104/pp.109.135368

Inaba, T., Li, M., Alvarez-Huerta, M., Kessler, F., and Schnell, D. J. (2003). atTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts. *J. Biol. Chem.* 278, 38617–38627. doi: 10.1074/jbc.M306367200

Inoue, H., Li, M., and Schnell, D. J. (2013). An essential role for chloroplast heat shock protein 90 (Hsp90C) in protein import into chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3173–3178. doi: 10.1073/pnas.1219229110

Ishikawa, A. (2005). Tetrapyrrole metabolism is involved in lesion formation, cell death, in the Arabidopsis lesion initiation 1 mutant. *Biosci. Biotechnol. Biochem.* 69, 1929–1934. doi: 10.1271/bbb.69.1929

Ishikawa, A., Tanaka, H., Nakai, M., and Asahi, T. (2003). Deletion of a chaperonin 60 beta gene leads to cell death in the Arabidopsis lesion initiation 1 mutant. *Plant Cell Physiol.* 44, 255–261. doi: 10.1093/pcp/pcg031

Jackson-Constan, D., Akita, M., and Keegstra, K. (2001). Molecular chaperones involved in chloroplast protein import. *Biochim. Biophys. Acta* 1541, 102–113. doi: 10.1016/S0167-4889(01)00148-3

Jarvis, P., and López-Juez, E. (2013). Biogenesis and homeostasis of chloroplasts and other plastids. *Nat. Rev. Mol. Cell Biol.* 14, 787–802. doi: 10.1038/nrm3702

Kato, Y., and Sakamoto, W. (2010). New insights into the types and function of proteases in plastids. *Int. Rev. Cell Mol. Biol.* 280, 185–218. doi: 10.1016/S1937-6448(10)80004-8

Kessler, F., and Blobel, G. (1996). Interaction of the protein import and folding machineries of the chloroplast. *Proc. Natl. Acad. Sci. U.S.A.* 93, 7684–7689. doi: 10.1073/pnas.93.15.7684

Kleine, T., and Leister, D. (2016). Retrograde signaling: organelles go networking. *Biochim. Biophys. Acta.* 1857, 1313–1325. doi: 10.1016/j.bbabio.2016.03.017

König, J., Galliardt, H., Jütte, P., Schäper, S., Dittmann, L., and Dietz, K. J. (2013). The conformational bases for the two functionalities of 2-cysteine peroxiredoxins as peroxidase and chaperone. *J. Exp. Bot.* 64, 3483–3497. doi: 10.1093/jxb/ert184

Koussevitzky, S., Nott, A., Mockler, T. C., Hong, F., Sachetto-Martins, G., Surpin, M., et al. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. *Science* 316, 715–719. doi: 10.1126/science. 1140516

Kovacheva, S., Bédard, J., Patel, R., Dudley, P., Twell, D., Ríos, G., et al. (2005). *In vivo* studies on the roles of Tic110, Tic40 and Hsp93 during chloroplast protein import. *Plant J.* 41, 412–428. doi: 10.1111/j.1365-313X.2004.02307.x

Kovacheva, S., Bédard, J., Wardle, A., Patel, R., and Jarvis, P. (2007). Further *in vivo* studies on the role of the molecular chaperone, Hsp93, in plastid protein import. *Plant J.* 50, 364–379. doi: 10.1111/j.1365-313X.2007.03060.x

Larkin, R. M., Alonso, J. M., Ecker, J. R., and Chory, J. (2003). GUN4, a regulator of chlorophyll synthesis and intracellular signaling. *Science* 299, 902–906. doi: 10.1126/science.1079978

Lin, Y. F., and Haynes, C. M. (2016). Metabolism and the UPR(mt). *Mol. Cell* 61, 677–682. doi: 10.1016/j.molcel.2016.02.004

Liu, L., McNeilage, R. T., Shi, L. X., and Theg, S. M. (2014). ATP requirement for chloroplast protein import is set by the Km for ATP hydrolysis of stromal Hsp70 in *Physcomitrella patens*. *Plant Cell* 26, 1246–1255. doi: 10.1105/tpc.113.121822

Madueno, F., Napier, J. A., and Gray, J. C. (1993). Newly imported rieske iron-sulfur protein associates with Both Cpn60 and Hsp70 in the chloroplast stroma. *Plant Cell* 5, 1865–1876. doi: 10.1105/tpc.5.12.1865

Majeran, W., Friso, G., Asakura, Y., Qu, X., Huang, M., Ponnala, L., et al. (2012). Nucleoid-enriched proteomes in developing plastids and chloroplasts from maize leaves: a new conceptual framework for nucleoid functions. *Plant Physiol.* 158, 156–189. doi: 10.1104/pp.111.188474

Manuell, A. L., Quispe, J., and Mayfield, S. P. (2007). Structure of the chloroplast ribosome: novel domains for translation regulation. *PLoS Biol.* 5:e209. doi: 10.1371/journal.pbio.0050209

Melonek, J., Matros, A., Trösch, M., Mock, H. P., and Krupinska, K. (2012). The core of chloroplast nucleoids contains architectural SWIB domain proteins. *Plant Cell* 24, 3060–3073. doi: 10.1105/tpc.112.099721

Melonek, J., Oetke, S., and Krupinska, K. (2016). Multifunctionality of plastid nucleoids as revealed by proteome analyses. *Biochim. Biophys. Acta.* 1864, 1016–1038. doi: 10.1016/j.bbapap.2016.03.009

Merendino, L., Falciatore, A., and Rochaix, J. D. (2003). Expression and RNA binding properties of the chloroplast ribosomal protein S1 from *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* 53, 371–382. doi: 10.1023/B:PLAN.0000006941.56233.42

Miura, E., Kato, Y., Matsushima, R., Albrecht, V., Laalami, S., and Sakamoto, W. (2007). The balance between protein synthesis and degradation in chloroplasts determines leaf variegation in Arabidopsis yellow variegated mutants. *Plant Cell* 19, 1313–1328. doi: 10.1105/tpc.106.049270

Mochizuki, N., Brusslan, J. A., Larkin, R., Nagatani, A., and Chory, J. (2001). Arabidopsis genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* 98, 2053–2058. doi: 10.1073/pnas.98.4.2053

Nakagawara, E., Sakuraba, Y., Yamasato, A., Tanaka, R., and Tanaka, A. (2007). Clp protease controls chlorophyll b synthesis by regulating the level of chlorophyllide a oxygenase. *Plant J.* 49, 800–809. doi: 10.1111/j.1365-313X.2006.02996.x

Oelmüller, R., Levitan, I., Bergfeld, R., Rajasekhar, V. K., and Mohr, H. (1986). Expression of nuclear genes as affected by treatments acting on the plastids. *Planta* 168, 482–492. doi: 10.1007/BF00392267

Oelmüller, R., and Mohr, H. (1986). Photooxidative destruction of chloroplasts and its consequences for expression of nuclear genes. *Planta* 167, 106–113. doi: 10.1007/BF00446376

Okazaki, K., Kabeya, Y., and Miyagishima, S. Y. (2010). The evolution of the regulatory mechanism of chloroplast division. *Plant Signal. Behav.* 5, 164–167. doi: 10.4161/psb.5.2.10461

Olinares, P. D., Kim, J., and van Wijk, K. J. (2011). The Clp protease system; a central component of the chloroplast protease network. *Biochim. Biophys. Acta* 1807, 999–1011. doi: 10.1016/j.bbabio.2010.12.003

Peng, L., Fukao, Y., Myouga, F., Motohashi, R., Shinozaki, K., and Shikanai, T. (2011). A chaperonin subunit with unique structures is essential for folding of a specific substrate. *PLoS Biol.* 9:e1001040. doi: 10.1371/journal.pbio.1001040

Pérez-Pérez, J. M., Esteve-Bruna, D., González-Bayón, R., Kangasjarvi, S., Caldana, C., Hannah, M. A., et al. (2013). Functional redundancy and divergence within the Arabidopsis RETICULATA-RELATED gene family. *Plant Physiol.* 162, 589–603. doi: 10.1104/pp.113.217323

Pfalz, J., Liere, K., Kandlbinder, A., Dietz, K. J., and Oelmüller, R. (2006). pTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. *Plant Cell* 18, 176–197. doi: 10.1105/tpc.105.036392

Pfalz, J., and Pfannschmidt, T. (2013). Essential nucleoid proteins in early chloroplast development. *Trends Plant Sci.* 18, 186–194. doi: 10.1016/j.tplants.2012.11.003

Phinney, B. S., and Thelen, J. J. (2005). Proteomic characterization of a triton-insoluble fraction from chloroplasts defines a novel group of proteins associated with macromolecular structures. *J. Proteome Res.* 4, 497–506. doi: 10.1021/pr049791k

Pogson, B. J., Woo, N. S., Förster, B., and Small, I. D. (2008). Plastid signalling to the nucleus and beyond. *Trends Plant Sci.* 13, 602–609. doi: 10.1016/j.tplants.2008.08.008

Pulido, P., Spínola, M. C., Kirchsteiger, K., Guinea, M., Pascual, M. B., Sahrawy, M., et al. (2010). Functional analysis of the pathways for 2-Cys peroxiredoxin reduction in *Arabidopsis thaliana* chloroplasts. *J. Exp. Bot.* 61, 4043–4054. doi: 10.1093/jxb/erq218

Ramundo, S., Casero, D., Mühlhaus, T., Hemme, D., Sommer, F., Crèvecoeur, M., et al. (2014). Conditional depletion of the chlamydomonas chloroplast ClpP protease activates nuclear genes involved in autophagy and plastid protein quality control. *Plant Cell* 26, 2201–2222. doi: 10.1105/tpc.114.124842

Ramundo, S., Rahire, M., Schaad, O., and Rochaix, J. D. (2013). Repression of essential chloroplast genes reveals new signaling pathways and regulatory feedback loops in chlamydomonas. *Plant Cell* 25, 167–186. doi: 10.1105/tpc.112.103051

Ramundo, S., and Rochaix, J. D. (2014). Chloroplast unfolded protein response, a new plastid stress signaling pathway? *Plant Signal. Behav.* 9:e972874. doi: 10.4161/15592316.2014.972874

Rey, P., Becuwe, N., Barrault, M. B., Rumeau, D., Havaux, M., Biteau, B., et al. (2007). The *Arabidopsis thaliana* sulfiredoxin is a plastidic cysteine-sulfinic acid reductase involved in the photooxidative stress response. *Plant J.* 49, 505–514. doi: 10.1111/j.1365-313X.2006.02969.x

Richly, E., and Leister, D. (2004). An improved prediction of chloroplast proteins reveals diversities and commonalities in the chloroplast proteomes of Arabidopsis and rice. *Gene* 329, 11–16. doi: 10.1016/j.gene.2004.01.008

Rochaix, J. D., and Ramundo, S. (2015). Conditional repression of essential chloroplast genes: evidence for new plastid signaling pathways. *Biochim. Biophys. Acta* 1847, 986–992. doi: 10.1016/j.bbabio.2014.11.011

Rochaix, J. D., Surzycki, R., and Ramundo, S. (2014). Tools for regulated gene expression in the chloroplast of Chlamydomonas. *Methods Mol. Biol.* 1132, 413–424. doi: 10.1007/978-1-62703-995-6_28

Rogalski, M., Schöttler, M. A., Thiele, W., Schulze, W. X., and Bock, R. (2008). Rpl33, a nonessential plastid-encoded ribosomal protein in tobacco, is required under cold stress conditions. *Plant Cell* 20, 2221–2237. doi: 10.1105/tpc.108.060392

Ruberti, C., Kim, S. J., Stefano, G., and Brandizzi, F. (2015). Unfolded protein response in plants: one master, many questions. *Curr. Opin. Plant Biol.* 27, 59–66. doi: 10.1016/j.pbi.2015.05.016

Rudella, A., Friso, G., Alonso, J. M., Ecker, J. R., and Van Wijk, K. J. (2006). Downregulation of ClpR2 leads to reduced accumulation of the ClpPRS protease complex and defects in chloroplast biogenesis in Arabidopsis. *Plant Cell* 18, 1704–1721. doi: 10.1105/tpc.106.042861

Sakamoto, W. (2006). Protein degradation machineries in plastids. *Annu. Rev. Plant Biol.* 57, 599–621. doi: 10.1146/annurev.arplant.57.032905.105401

Shi, L. X., and Theg, S. M. (2010). A stromal heat shock protein 70 system functions in protein import into chloroplasts in the moss *Physcomitrella patens*. *Plant Cell* 22, 205–220. doi: 10.1105/tpc.109.071464

Shi, L. X., and Theg, S. M. (2013a). The chloroplast protein import system: from algae to trees. *Biochim. Biophys. Acta* 1833, 314–331. doi: 10.1016/j.bbamcr.2012.10.002

Shi, L. X., and Theg, S. M. (2013b). Energetic cost of protein import across the envelope membranes of chloroplasts. *Proc. Natl. Acad. Sci. U.S.A.* 110, 930–935. doi: 10.1073/pnas.1115886110

Shoji, S., Dambacher, C. M., Shajani, Z., Williamson, J. R., and Schultz, P. G. (2011). Systematic chromosomal deletion of bacterial ribosomal protein genes. *J. Mol. Biol.* 413, 751–761. doi: 10.1016/j.jmb.2011.09.004

Sjögren, L. L., Macdonald, T. M., Sutinen, S., and Clarke, A. K. (2004). Inactivation of the clpC1 gene encoding a chloroplast Hsp100 molecular chaperone causes growth retardation, leaf chlorosis, lower photosynthetic activity, and a specific reduction in photosystem content. *Plant Physiol.* 136, 4114–4126. doi: 10.1104/pp.104.053835

Steiner, S., Schröter, Y., Pfalz, J., and Pfannschmidt, T. (2011). Identification of essential subunits in the plastid-encoded RNA polymerase complex reveals building blocks for proper plastid development. *Plant Physiol.* 157, 1043–1055. doi: 10.1104/pp.111.184515

Stenbaek, A., Hansson, A., Wulff, R. P., Hansson, M., Dietz, K. J., and Jensen, P. E. (2008). NADPH-dependent thioredoxin reductase and 2-Cys peroxiredoxins are needed for the protection of Mg-protoporphyrin monomethyl ester cyclase. *FEBS Lett.* 582, 2773–2778. doi: 10.1016/j.febslet.2008.07.006

Strand, A., Asami, T., Alonso, J., Ecker, J. R., and Chory, J. (2003). Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. *Nature* 421, 79–83. doi: 10.1038/nature01204

Su, P. H., and Li, H. M. (2008). Arabidopsis stromal 70-kD heat shock proteins are essential for plant development and important for thermotolerance of germinating seeds. *Plant Physiol.* 146, 1231–1241. doi: 10.1104/pp.107.114496

Su, P. H., and Li, H. M. (2010). Stromal Hsp70 is important for protein translocation into pea and Arabidopsis chloroplasts. *Plant Cell* 22, 1516–1531. doi: 10.1105/tpc.109.071415

Sun, Y., and Zerges, W. (2015). Translational regulation in chloroplasts for development and homeostasis. *Biochim. Biophys. Acta* 1847, 809–820. doi: 10.1016/j.bbabio.2015.05.008

Susek, R. E., Ausubel, F. M., and Chory, J. (1993). Signal transduction mutants of Arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development. *Cell* 74, 787–799. doi: 10.1016/0092-8674(93)90459-4

Suzuki, K., Nakanishi, H., Bower, J., Yoder, D. W., Osteryoung, K. W., and Miyagishima, S. Y. (2009). Plastid chaperonin proteins Cpn60 alpha and Cpn60 beta are required for plastid division in *Arabidopsis thaliana*. *BMC Plant Biol.* 9:38. doi: 10.1186/1471-2229-9-38

Tadini, L., Pesaresi, P., Kleine, T., Rossi, F., Guljamow, A., Sommer, F., et al. (2016). GUN1 controls accumulation of the plastid ribosomal protein S1 at the protein level and interacts with proteins involved in plastid protein homeostasis. *Plant Physiol.* 170, 1817–1830. doi: 10.1104/pp.15.02033

Tanaka, R., Kobayashi, K., and Masuda, T. (2011). Tetrapyrrole metabolism in *Arabidopsis thaliana*. *Arabidopsis Book* 9:e0145. doi: 10.1199/tab.0145

Tiller, N., and Bock, R. (2014). The translational apparatus of plastids and its role in plant development. *Mol. Plant* 7, 1105–1120. doi: 10.1093/mp/ssu022

Tsugeki, R., and Nishimura, M. (1993). Interaction of homologues of Hsp70 and Cpn60 with ferredoxin-NADP+ reductase upon its import into chloroplasts. *FEBS Lett.* 320, 198–202. doi: 10.1016/0014-5793(93)80585-I

Van Wijk, K. J. (2015). Protein maturation and proteolysis in plant plastids, mitochondria, and peroxisomes. *Annu. Rev. Plant Biol.* 66, 75–111. doi: 10.1146/annurev-arplant-043014-115547

Woodson, J. D., and Chory, J. (2008). Coordination of gene expression between organellar and nuclear genomes. *Nat. Rev. Genet.* 9, 383–395. doi: 10.1038/nrg2348

Woodson, J. D., Perez-Ruiz, J. M., and Chory, J. (2011). Heme synthesis by plastid ferrochelatase I regulates nuclear gene expression in plants. *Curr. Biol.* 21, 897–903. doi: 10.1016/j.cub.2011.04.004

Zybailov, B., Friso, G., Kim, J., Rudella, A., Rodríguez, V. R., Asakura, Y., et al. (2009). Large scale comparative proteomics of a chloroplast Clp protease mutant reveals folding stress, altered protein homeostasis, and feedback regulation of metabolism. *Mol. Cell. Proteomics* 8, 1789–1810. doi: 10.1074/mcp.M900104-MCP200

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Ubiquitin–Proteasome-Dependent Regulation of Bidirectional Communication between Plastids and the Nucleus

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Plastids are DNA-containing organelles and can have unique differentiation states depending on age, tissue, and environment. Plastid biogenesis is optimized by bidirectional communication between plastids and the nucleus. Import of nuclear-encoded proteins into plastids serves as anterograde signals and vice versa, plastids themselves send retrograde signals to the nucleus, thereby controlling *de novo* synthesis of nuclear-encoded plastid proteins. Recently, it has become increasingly evident that the ubiquitin–proteasome system regulates both the import of anterograde plastid proteins and retrograde signaling from plastids to the nucleus. Targets of ubiquitin–proteasome regulation include unimported chloroplast precursor proteins in the cytosol, protein translocation machinery at the chloroplast surface, and transcription factors in the nucleus. This review will focus on the mechanism through which the ubiquitin–proteasome system optimizes plastid biogenesis and plant development through the regulation of nuclear–plastid interactions.

Keywords: proteasome, ubiquitin, plastid protein import, plastid biogenesis, retrograde signaling

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Plastids are DNA-containing organelles that have evolved from a cyanobacterial endosymbiont. Because most of the genes encoded by the bacterial ancestor have been transferred to the host nuclear genome, the expression of genes encoding plastid precursor proteins in the nucleus and the import of those proteins are indispensable for plastid biogenesis. The import of plastid precursor proteins is mediated by the translocon at the outer (or inner) envelope membrane of chloroplasts (TOC-TIC). Hence, plastid fate is largely controlled by the quality and quantity of plastid precursor proteins expressed in each cell and by their import into plastids (Inaba and Schnell, 2008; Jarvis and Lopez-Juez, 2013; Paila et al., 2015).

On the other hand, plastids also send feedback signals to regulate the expression of genes encoding plastid proteins in the nucleus. These signals are known as retrograde signals from plastids to the nucleus and are referred to as plastid signals. Plastid signals can be divided into two types: biogenic and operational (Pogson et al., 2008). Among them, biogenic signals are necessary to coordinate gene expression in two genomes, allowing cells to assemble the photosynthetic apparatus and to promote chloroplast development (Pogson et al., 2008; Inaba et al., 2011; Jarvis and Lopez-Juez, 2013). To date, several transcription factors have been shown to mediate biogenic signals from plastids to the nucleus (Koussevitzky et al., 2007; Ruckle et al., 2007; Kakizaki et al., 2009; Waters et al., 2009; Sun et al., 2011; Martin et al., 2016).

A number of studies have demonstrated the roles of *de novo* synthesis and the targeting of plastid precursor proteins in the regulation of nuclear–plastid interactions. However, it has become increasingly evident that the nuclear–plastid interaction is also regulated by the degradation of multiple components through the ubiquitin–proteasome system (Lee et al., 2013; Ling and Jarvis, 2015). Here, we focus on recent advances in our understanding

of how the ubiquitin–proteasome system regulates the nuclear–plastid interaction and plastid biogenesis. Other comprehensive reviews cover broad aspects of plastid protein import and plastid signaling (Li and Chiu, 2010; Inaba et al., 2011; Jarvis and Lopez-Juez, 2013; Pfannschmidt and Munné-Bosch, 2013; Paila et al., 2015; Chan et al., 2016), and space limitations prevent us from providing adequate coverage of all aspects of nuclear–plastid interaction.

DEGRADATION OF UNIMPORTED CHLOROPLAST PRECURSOR PROTEINS BY THE UBIQUITIN–PROTEASOME PATHWAY

It is well known that the expression of nuclear-encoded photosynthesis-associated genes are induced upon illumination and that mass transport of proteins encoded by these genes into plastids are indispensable for chloroplast development. Those plastid-targeted proteins are encoded as precursors in the nucleus, but only mature proteins are detectable under normal conditions *in vivo*. To avoid the accumulation of unimported proteins in the cytosol, plants have evolved at least two distinct mechanisms. One is feedback regulation of nuclear gene expression by plastid-derived signals, and the other is degradation of unimported precursor proteins by the ubiquitin–proteasome system (Lee et al., 2013; **Figure 1**).

Cytosolic heat shock cognate 70-4 (Hsc70-4) and carboxy terminus of Hsc70-interacting protein (CHIP) appear to be involved in the degradation of unimported precursor proteins in *Arabidopsis thaliana* (Lee et al., 2009). Hsc70-4 recognizes specific sequence motifs within the transit peptide of ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit protein and light-harvesting chlorophyll *a*/*b*-binding protein. Subsequently, CHIP interacts with Hsc70-4 and serves as an E3-ubiquitin ligase, thereby allowing unimported precursors to be degraded through the ubiquitin–proteasome system. This suggests that a transit peptide may function as both a chloroplast targeting signal and a degradation signal when unimported precursors accumulate in the cytosol. This idea is further substantiated by the findings of a recent proteomic study (Sako et al., 2014), in which certain plastid precursors were shown to interact with the proteasome both *in vivo* and *in vitro*.

The mechanism that discriminates between plastid-targeted precursors and proteasome-targeted precursors remains to be characterized in detail. Intriguingly, the *plastid protein import 2* (*ppi2*) mutant of *A. thaliana*, which lacks the atToc159 protein import receptor of plastids, accumulated *N*-acetylated plastid precursor proteins outside of plastids (Bischof et al., 2011). Although atToc159 plays key roles in the import of photosynthesis-associated proteins into plastids, it also participates in the import of constitutively expressed plastid proteins. As will be discussed later, the *ppi2* mutant has been known to exhibit down-regulation of genes encoding photosynthesis-associated proteins in the nucleus in response to plastid-derived signals, but not the expression of constitutive plastid proteins. In contrast, some constitutively expressed proteins were shown to be *N*-acetylated in the *ppi2* mutant. It has been shown that *N*-acetylation serves as a degradation signal for the ubiquitin–proteasome system in yeast (Hwang et al., 2010). Hence, one can speculate that excess precursors that cannot be controlled at the transcriptional level are subjected to *N*-acetylation and ubiquitin–proteasome-dependent degradation. As such, degradation of excess plastid precursors via the ubiquitin–proteasome system plays a key role in determining the amount of protein import and plastid biogenesis.

PLASTID PROTEIN IMPORT MACHINERY IS A DIRECT TARGET OF UBIQUITIN–PROTEASOME PATHWAY

The ubiquitin–proteasome system directly regulates the protein translocation machinery at the plastid surface (Ling and Jarvis, 2015; **Figure 1**). This unexpected link was demonstrated in an attempt to isolate a suppressor mutant of *plastid protein import 1* (*ppi1*). The *ppi1* mutant of *A. thaliana* exhibits a pale green phenotype due to the lack of atToc33 in the TOC complex, but can survive on soil. One suppressor mutant of *ppi1*, designated as *suppressor of ppi1 locus1* (*sp1*), possesses a lesion within the RING-type ubiquitin E3 ligase gene (Ling et al., 2012). TOC components are more abundant (1.5- to 2-fold) in the *sp1* mutant than in the wild-type. The wild-type SP1 protein interacts with components of TOC machinery. Furthermore, atToc159, atToc75, and atToc33 have been shown to be polyubiquitinated by SP1 activity. These findings indicate that the ubiquitin–proteasome system directly regulates the level of TOC components, thereby affecting the amount of protein import into plastids.

This mechanism also seems to play a key role in determining the fate of plastids within the cell (Ling et al., 2012). During the photomorphogenic response, the *sp1* single mutant displayed inefficient de-etiolation with reduced levels of photosynthesis-associated proteins and imbalanced TOC receptor levels. The *sp1* mutant also exhibited delayed senescence, and this was accompanied by the delayed transformation from chloroplasts to gerontoplasts within the cell. In contrast, overexpression of SP1 accelerated both de-etiolation and senescence. Hence, regulation of TOC components by the ubiquitin–proteasome system appears to be indispensable for determining both the quality and the quantity of plastid-targeted proteins, thereby affecting the fate of plastid and plant development.

REGULATION OF PLASTID-TO-NUCLEUS RETROGRADE SIGNALING VIA THE UBIQUITIN–PROTEASOME PATHWAY

In addition to the anterograde signaling pathway, a recent study demonstrated that the retrograde signaling pathway from

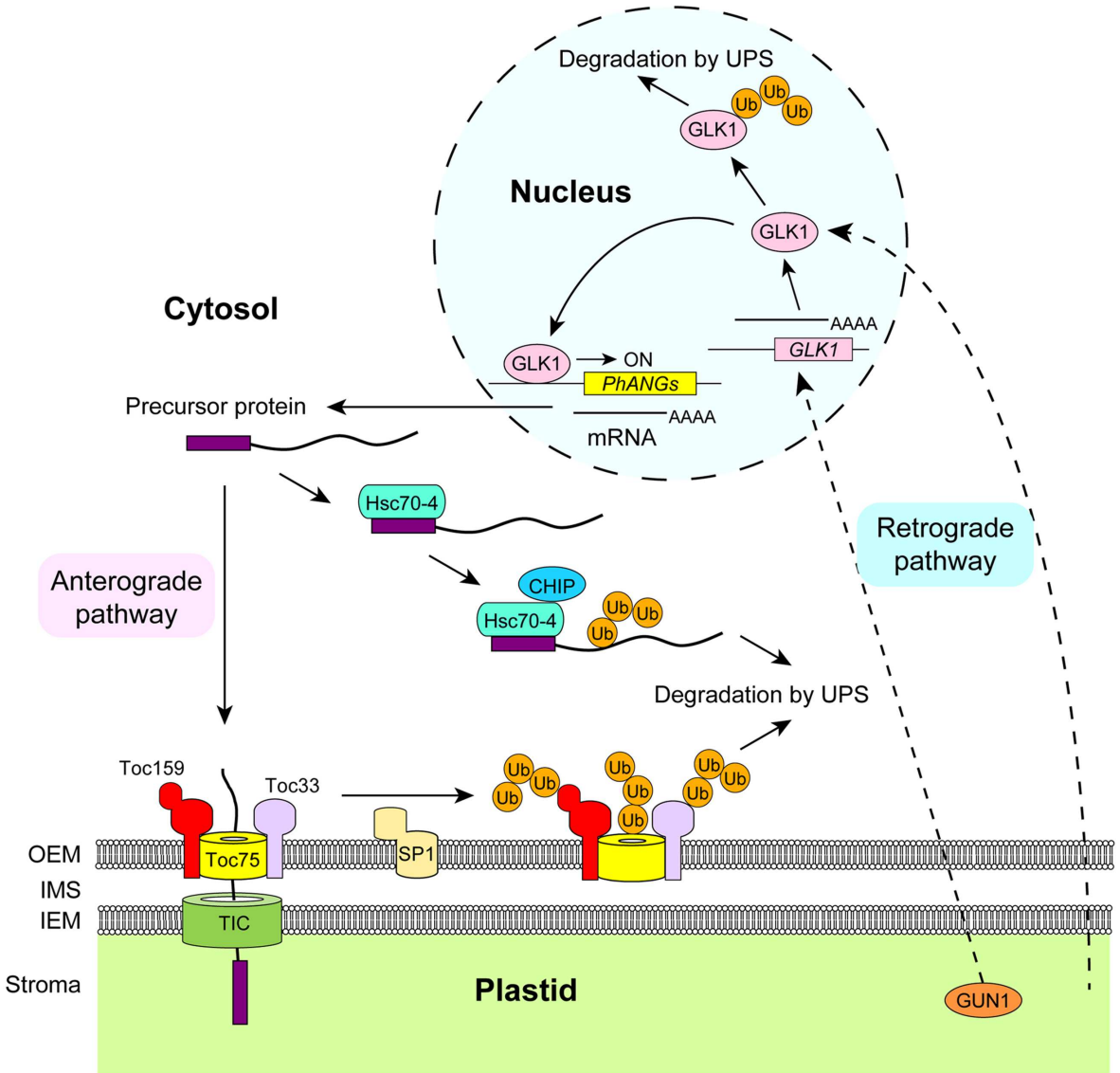

FIGURE 1 | Control of bidirectional signaling between plastids and the nucleus by the ubiquitin–proteasome system. Chloroplast development is promoted by the expression of nuclear-encoded *PhANGs* and the import of their products into chloroplasts. When excess precursors are produced, they are recognized by the heat shock cognate 70-4 (Hsc70-4) complex in the cytosol. Subsequently, they are polyubiquitinated by an E3 ubiquitin ligase, carboxy terminus of Hsc70-interacting protein (CHIP), resulting in their degradation by the proteasome. The translocon at the outer envelope membrane of chloroplasts (TOC) complex is also directly targeted by the ubiquitin proteasome system. At least three TOC components, Toc159, Toc75, and Toc33, are polyubiquitinated by a membrane-anchored E3 ubiquitin ligase, suppressor of *ppi1* locus1 (SP1). To further optimize the amount of protein import into chloroplasts, retrograde signals from chloroplasts regulate the level of the GOLDEN2-LIKE 1 (GLK1) transcription factor in the nucleus. Polyubiquitination of GLK1 is induced when chloroplast biogenesis is inhibited. The degradation of GLK1 results in the down-regulation of *PhANGs*, thereby preventing the accumulation of unnecessary precursor proteins in the cytosol. GLK1 is also regulated by retrograde signals at transcriptional level, and this regulation is mediated by GENOMES UNCOUPLED 1 (GUN1). Although this figure proposes a model for photosynthetic tissues, similar regulation by the ubiquitin–proteasome system appears to play key roles in plastid development in other tissues. Note that a number of other pathways between plastids and the nucleus have been identified, and those pathways are not shown in this figure due to space limitations but can be found in other adequate reviews. UPS, ubiquitin–proteasome system; OEM, outer envelope membrane; IEM, inner envelope membrane; IMS, intermembrane space; Ub, ubiquitin; *PhANGs*, photosynthesis-associated nuclear genes.

plastids to the nucleus is also subjected to ubiquitin–proteasome-dependent regulation in *A. thaliana* (Tokumaru et al., 2017). The key mechanism involves the regulation of the GOLDEN2-LIKE 1 (GLK1) transcription factor by the ubiquitin–proteasome system (**Figure 1**).

The GLK family of transcription factors was originally isolated in maize (Hall et al., 1998; Rossini et al., 2001). The *GLK* genes positively regulate the expression of photosynthesis-associated genes in numerous plants, thereby strongly promoting chloroplast development (Fitter et al., 2002; Yasumura et al., 2005;

Waters et al., 2009). Overexpression of *GLK* has been shown to be sufficient to induce chloroplast development in rice calli (Nakamura et al., 2009) and *Arabidopsis* root cells (Kobayashi et al., 2012; Tokumaru et al., 2017). Two separate studies reported that the expression of *GLK* genes responds to inhibitor treatment thus compromising chloroplast development (Kakizaki et al., 2009; Waters et al., 2009). The findings of those studies concluded that *GLK* gene expression responds to plastid signals, resulting in the regulation of photosynthesis-associated genes in response to plastid signals. Intriguingly, impaired chloroplast development caused by the *ppi2* mutation also suppress *GLK1* expression in the nucleus (Kakizaki et al., 2009). This regulation is mediated by the retrograde signaling pathway, because the GENOMES UNCOUPLED 1 (GUN1) protein, which is localized in plastids, appears to act upstream of *GLK1*. From those studies, it becomes clear that plastids transmit signals to determine the amount of anterograde protein import, thereby avoiding the accumulation of excess levels of precursors within the cytosol.

Besides transcriptional regulation, a recent study showed that plastid signals also directly regulate the level of GLK1 protein (Tokumaru et al., 2017). The *GLK1* gene is fully expressed in *gun1* mutants treated with norflurazon. In contrast, the level of GLK1 protein is much lower than that expected from the *GLK1* mRNA levels in the norflurazon-treated *gun1* mutant. The discrepancy between GLK1 protein and mRNA levels is in part attributable to the degradation of the GLK1 protein by the ubiquitin–proteasome system (Tokumaru et al., 2017). When norflurazon-treated plants were further treated with MG-132, a proteasome inhibitor, the accumulation of GLK1 was partially restored. Because the *gun1* mutant also exhibited the same response, it appears that GUN1 is not required for the proteasome-mediated regulation of GLK1. Likewise, MG-132 treatment partially restored the level of GLK1 protein in the *ppi2* mutant. Hence, this mechanism is also used to optimize the expression of nuclear genes encoding photosynthesis-associated proteins when plastid protein import is compromised.

The level of GLK is also regulated by the ubiquitin–proteasome system in fruit tissues of tomato. The *Solanum lycopersicum* GLK2 protein, SlGLK2, regulates chloroplast development in tomato fruit tissues, and fruits of the *slglk2* mutant exhibit uniformly light green coloration (Powell et al., 2012; Nguyen et al., 2014). SlGLK2 was found to be degraded by the ubiquitin E3 ligase complex containing CULLIN4 (CUL4) and UV-DAMAGED DNA BINDING PROTEIN 1 (DDB1; Tang et al., 2016). Consistent with this observation, a mutation in DDB1 significantly increased the pigment contents and chloroplast/chromoplast size in tomato fruits (Cookson et al., 2003), presumably due to the excess accumulation of SlGLK2. Although the roles of SlGLK2 in retrograde signaling remains to be established, these studies further support the idea that the ubiquitin–proteasome pathway is indispensable for the regulation of GLKs.

CONCLUSION AND PERSPECTIVE

Although the *de novo* synthesis and targeting of plastid precursor proteins are indispensable for plastid biogenesis, it becomes clear that ubiquitin–proteasome-dependent protein degradation also plays a key role in the regulation of plastid biogenesis. Meanwhile, a number of questions remains to be solved: Are there any other ubiquitin–proteasome regulated transcription factors involved in the retrograde signaling from plastids to the nucleus? Is ubiquitin–proteasome system indispensable for the retrograde signaling from plastids other than chloroplasts? Does operational control of retrograde signaling also requires ubiquitin–proteasome system? In fact, other studies start addressing these questions. Proteasome-regulated transcription factors, such as ELONGATED HYPOCOTYL 5 (HY5) and PHYTOCHROME INTERACTING FACTORS (PIFs), have been shown to participate in retrograde signaling, as well as in light signaling (Ruckle et al., 2007; Martin et al., 2016). Reactive oxygen species-producing chloroplasts appear to be ubiquitinated and subsequently degraded (Woodson et al., 2015). Further investigation will provide novel insight into the roles of the ubiquitin–proteasome system in regulating plastid biogenesis and plant development.

AUTHOR CONTRIBUTIONS

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REFERENCES

Bischof, S., Baerenfaller, K., Wildhaber, T., Troesch, R., Vidi, P. A., Roschitzki, B., et al. (2011). Plastid proteome assembly without Toc159: photosynthetic protein import and accumulation of N-acetylated plastid precursor proteins. *Plant Cell* 23, 3911–3928. doi: 10.1105/tpc.111.092882

Chan, K. X., Phua, S. Y., Crisp, P., Mcquinn, R., and Pogson, B. J. (2016). Learning the languages of the chloroplast: retrograde signaling and beyond. *Annu. Rev. Plant Biol.* 67, 25–53. doi: 10.1146/annurev-arplant-043015-111854

Cookson, P. J., Kiano, J. W., Shipton, C. A., Fraser, P. D., Romer, S., Schuch, W., et al. (2003). Increases in cell elongation, plastid compartment size and phytoene synthase activity underlie the phenotype of the high pigment-1 mutant of tomato. *Planta* 217, 896–903. doi: 10.1007/s00425-003-1065-9

Fitter, D. W., Martin, D. J., Copley, M. J., Scotland, R. W., and Langdale, J. A. (2002). GLK gene pairs regulate chloroplast development in diverse plant species. *Plant J.* 31, 713–727. doi: 10.1046/j.1365-313X.2002.01390.x

Hall, L. N., Rossini, L., Cribb, L., and Langdale, J. A. (1998). GOLDEN 2: a novel transcriptional regulator of cellular differentiation in the maize leaf. *Plant Cell* 10, 925–936. doi: 10.1105/tpc.10.6.925

Hwang, C. S., Shemorry, A., and Varshavsky, A. (2010). N-terminal acetylation of cellular proteins creates specific degradation signals. *Science* 327, 973–977. doi: 10.1126/science.1183147

Inaba, T., and Schnell, D. J. (2008). Protein trafficking to plastids: one theme, many variations. *Biochem. J.* 413, 15–28. doi: 10.1042/BJ20080490

Inaba, T., Yazu, F., Ito-Inaba, Y., Kakizaki, T., and Nakayama, K. (2011). Retrograde signaling pathway from plastid to nucleus. *Int. Rev. Cell Mol. Biol.* 290, 167–204. doi: 10.1016/B978-0-12-386037-8.00002-8

Jarvis, P., and Lopez-Juez, E. (2013). Biogenesis and homeostasis of chloroplasts and other plastids. *Nat. Rev. Mol. Cell Biol.* 14, 787–802. doi: 10.1038/nrm3702

Kakizaki, T., Matsumura, H., Nakayama, K., Che, F. S., Terauchi, R., and Inaba, T. (2009). Coordination of plastid protein import and nuclear gene expression by plastid-to-nucleus retrograde signaling. *Plant Physiol.* 151, 1339–1353. doi: 10.1104/pp.109.145987

Kobayashi, K., Baba, S., Obayashi, T., Sato, M., Toyooka, K., Keranen, M., et al. (2012). Regulation of root greening by light and auxin/cytokinin signaling in *Arabidopsis*. *Plant Cell* 24, 1081–1095. doi: 10.1105/tpc.111.092254

Koussevitzky, S., Nott, A., Mockler, T. C., Hong, F., Sachetto-Martins, G., Surpin, M., et al. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. *Science* 316, 715–719. doi: 10.1126/science.1140516

Lee, D. W., Jung, C., and Hwang, I. (2013). Cytosolic events involved in chloroplast protein targeting. *Biochim. Biophys. Acta* 1833, 245–252. doi: 10.1016/j.bbamcr.2012.03.006

Lee, S., Lee, D. W., Lee, Y., Mayer, U., Stierhof, Y. D., Lee, S., et al. (2009). Heat shock protein cognate 70-4 and an E3 ubiquitin ligase, CHIP, mediate plastid-destined precursor degradation through the ubiquitin-26S proteasome system in *Arabidopsis*. *Plant Cell* 21, 3984–4001. doi: 10.1105/tpc.109.071548

Li, H. M., and Chiu, C. C. (2010). Protein transport into chloroplasts. *Annu. Rev. Plant Biol.* 61, 157–180. doi: 10.1146/annurev-arplant-042809-112222

Ling, Q., Huang, W., Baldwin, A., and Jarvis, P. (2012). Chloroplast biogenesis is regulated by direct action of the ubiquitin-proteasome system. *Science* 338, 655–659. doi: 10.1126/science.1225053

Ling, Q., and Jarvis, P. (2015). Functions of plastid protein import and the ubiquitin-proteasome system in plastid development. *Biochim. Biophys. Acta* 1847, 939–948. doi: 10.1016/j.bbabio.2015.02.017

Martin, G., Leivar, P., Ludevid, D., Tepperman, J. M., Quail, P. H., and Monte, E. (2016). Phytochrome and retrograde signalling pathways converge to antagonistically regulate a light-induced transcriptional network. *Nat. Commun.* 7:11431. doi: 10.1038/ncomms11431

Nakamura, H., Muramatsu, M., Hakata, M., Ueno, O., Nagamura, Y., Hirochika, H., et al. (2009). Ectopic overexpression of the transcription factor OsGLK1 induces chloroplast development in non-green rice cells. *Plant Cell Physiol.* 50, 1933–1949. doi: 10.1093/pcp/pcp138

Nguyen, C. V., Vrebalov, J. T., Gapper, N. E., Zheng, Y., Zhong, S., Fei, Z., et al. (2014). Tomato GOLDEN2-LIKE transcription factors reveal molecular gradients that function during fruit development and ripening. *Plant Cell* 26, 585–601. doi: 10.1105/tpc.113.118794

Paila, Y. D., Richardson, L. G., and Schnell, D. J. (2015). New insights into the mechanism of chloroplast protein import and its integration with protein quality control, organelle biogenesis and development. *J. Mol. Biol.* 427, 1038–1060. doi: 10.1016/j.jmb.2014.08.016

Pfannschmidt, T., and Munné-Bosch, S. (2013). Plastid signaling during the plant life cycle. *Adv. Photosynth. Respir.* 36, 503–528. doi: 10.1007/978-94-007-5724-0_22

Pogson, B. J., Woo, N. S., Forster, B., and Small, I. D. (2008). Plastid signalling to the nucleus and beyond. *Trends Plant Sci.* 13, 602–609. doi: 10.1016/j.tplants.2008.08.008

Powell, A. L., Nguyen, C. V., Hill, T., Cheng, K. L., Figueroa-Balderas, R., Aktas, H., et al. (2012). Uniform ripening encodes a Golden 2-like transcription factor regulating tomato fruit chloroplast development. *Science* 336, 1711–1715. doi: 10.1126/science.1222218

Rossini, L., Cribb, L., Martin, D. J., and Langdale, J. A. (2001). The maize golden2 gene defines a novel class of transcriptional regulators in plants. *Plant Cell* 13, 1231–1244. doi: 10.1105/tpc.13.5.1231

Ruckle, M. E., Demarco, S. M., and Larkin, R. M. (2007). Plastid signals remodel light signaling networks and are essential for efficient chloroplast biogenesis in *Arabidopsis*. *Plant Cell* 19, 3944–3960. doi: 10.1105/tpc.107.054312

Sako, K., Yanagawa, Y., Kanai, T., Sato, T., Seki, M., Fujiwara, M., et al. (2014). Proteomic analysis of the 26S proteasome reveals its direct interaction with transit peptides of plastid protein precursors for their degradation. *J. Proteome Res.* 13, 3223–3230. doi: 10.1021/pr401245g

Sun, X., Feng, P., Xu, X., Guo, H., Ma, J., Chi, W., et al. (2011). A chloroplast envelope-bound PHD transcription factor mediates chloroplast signals to the nucleus. *Nat. Commun* 2:477. doi: 10.1038/ncomms1486

Tang, X., Miao, M., Niu, X., Zhang, D., Cao, X., Jin, X., et al. (2016). Ubiquitin-conjugated degradation of golden 2-like transcription factor is mediated by CUL4-DDB1-based E3 ligase complex in tomato. *New Phytol.* 209, 1028–1039. doi: 10.1111/nph.13635

Tokumaru, M., Adachi, F., Toda, M., Ito-Inaba, Y., Yazu, F., Hirosawa, Y., et al. (2017). Ubiquitin-proteasome dependent regulation of the GOLDEN2-LIKE 1 transcription factor in response to plastid signals. *Plant Physiol.* 173, 524–535. doi: 10.1104/pp.16.01546

Waters, M. T., Wang, P., Korkaric, M., Capper, R. G., Saunders, N. J., and Langdale, J. A. (2009). GLK transcription factors coordinate expression of the photosynthetic apparatus in *Arabidopsis*. *Plant Cell* 21, 1109–1128. doi: 10.1105/tpc.108.065250

Woodson, J. D., Joens, M. S., Sinson, A. B., Gilkerson, J., Salome, P. A., Weigel, D., et al. (2015). Ubiquitin facilitates a quality-control pathway that removes damaged chloroplasts. *Science* 350, 450–454. doi: 10.1126/science.aac7444

Yasumura, Y., Moylan, E. C., and Langdale, J. A. (2005). A conserved transcription factor mediates nuclear control of organelle biogenesis in anciently diverged land plants. *Plant Cell* 17, 1894–1907. doi: 10.1105/tpc.105.033191

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