PRDA1, a Novel Chloroplast Nucleoid Protein, is Required for Early Chloroplast Development and is Involved in the Regulation of Plastid Gene Expression in Arabidopsis

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Chloroplast development requires accurate spatio-temporal expression of plastid genes. The regulation of plastid genes mediated by plastid-encoded RNA polymerase (PEP) is rather complex, and its related mechanism remains largely unclear. Here, we report the identification of a novel protein that is essential for plant development, PEP-Related Development Arrested 1 (PRDA1). Knock-out of *PRDA1* in Arabidopsis (*prda1* mutant) caused a seedling-lethal, albino phenotype and arrested the development of leaf chloroplasts. Localization analysis showed that PRDA1 was specifically targeted to chloroplasts and co-localized with chloroplast nucleoids, revealing that PRDA1 is a chloroplast nucleoid-associated protein. Gene expression analyses revealed that the PEP-dependent plastid transcript levels were greatly reduced in *prda1*. PRDA1 was co-expressed with most of the PEP-associated proteins. Protein interaction assays showed that PRDA1 clearly interacts with MRL7 and FSD2, both of which have been verified as essential for PEP-related chloroplast development. Reactive oxygen species scavenging through dimethylthiourea markedly alleviated the cotyledon-albino phenotypes of *PRDA1* and *MRL7* RNA interference seedlings. These results demonstrate that PRDA1 is required for early chloroplast development and involved in the regulation of plastid gene expression.

Keywords: Chloroplast • FSD2 • MRL7 • PEP • Plastid gene expression • PRDA1.

Abbreviations: BiFC, bimolecular fluorescence complementation; CFP, enhanced cyan fluorescent protein; cpChIP, chloroplast chromatin immunoprecipitation; DMTU, dimethylthiourea; LCI, luciferase complementation imaging; MS, Murashige and Skoog; NEP, nuclear-encoded polymerase; ORF, open reading frame; PAP, PEP-associated protein; PEP, plastid-encoded RNA polymerase; PRDA1, PEP-Related Development Arrested 1; QPCR, quantitative real-time-PCR; RNAi, RNA interference; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; YFP, yellow fluorescent protein.

Introduction

Although they evolved from cyanobacteria through endosymbiosis (Cattolico 1986, Cavalier-Smith 2000), the chloroplasts of higher plants only retained a small genome (plastome), encoding approximately 100–150 genes. A vast majority of the chloroplast proteins (>3,000 proteins) are encoded in the nucleus and are imported into the chloroplast after post-translational modification to exert various biological roles, such as photosynthetic pigment biosynthesis and photosynthesis, plastid gene expression and synthesis of essential metabolites (Robinson and Ellis 1984, Sato et al. 1999, Abdallah et al. 2000, Luo et al. 2012, Zhou et al. 2012, Luo et al. 2013). Chloroplast development and the normal execution of its function require the coordinated expression of the plastid-encoded and the related nuclear-encoded genes, and this coordination is regulated through the retrograde and anterograde signal transduction between chloroplasts and the nucleus (Susek et al. 1993, Nott et al. 2006, Sun et al. 2011, Chi et al. 2013). Chloroplasts have distinct machinery for DNA replication, gene transcription and protein expression (Mullet 1993, Sato et al. 1999), and numerous nuclear-encoded proteins are involved in these processes.

The transcription of plastid genes plays a critical role in chloroplast development from a proplastid in the meristem (Dreyfuss and Thornber 1994, Majeran et al. 2012). In higher plants, two types of chloroplast RNA polymerases, eubacterial type plastid-encoded polymerases (PEPs) and bacterial phage type nuclear-encoded polymerases (NEPs), have been identified and shown to be responsible for the transcription of genes of distinct groups classified according to their promoter specificity (Lerbs-Mache 1993, Hedtke et al. 1997, Chang et al. 1999, Azevedo et al. 2008, Swiatecka-Hagenbruch et al. 2008). NEPs (RPOTp and RPOTmp) are more active during chloroplast development in the youngest tissues, primarily transcribing housekeeping genes or genes associated with plastid transcription machinery, such as *accD* and *rpo* genes. On the other hand, PEPs are preferentially active in the developing and mature chloroplast and primarily transcribe the genes associated with

photosynthesis (Hanaoka et al. 2005, Swiatecka-Hagenbruch et al. 2007). In Arabidopsis, six sigma factors were identified and implicated in plastid promoter recognition during RNA polymerase-mediated transcription, of which SIG6 and SIG2 have been shown to play dominant roles in the PEP-dependent transcription of photosynthesis-related genes (Kanamaru et al. 2001, Ishizaki et al. 2005, Schweer et al. 2010, Lerbs-Mache 2011) and mediate plastid retrograde signals to control nuclear gene expression (Woodson et al. 2013). To date, the PEP-mediated transcriptional regulation of plastid gene expression has been reported to be much more complex than the NEP-dependent regulation of plastid gene expression (Schröter et al. 2010, Lerbs-Mache 2011, Zhelyazkova et al. 2012). It has been suggested that PEP plays an important role in mediating intracellular and extracellular signals to coordinate the chloroplast in response to environmental changes.

Several biochemical studies have revealed that the PEP of higher plants has a composition resembling that of bacterial multisubunit RNA polymerases. The PEP complex has a size of >1,000 kDa and comprises core subunits encoded by plastid *rpo* genes and many other peripheral nuclear-encoded proteins, which typically form DNA–protein binding complexes that are called transcriptionally active chromosome complexes (pTACs) (Hu and Bogorad 1990, Suzuki et al. 2004, Pfalz et al. 2006, Steiner et al. 2011). It has been suggested that a large proportion of certain nuclear-encoded proteins (e.g. the sigma factors) probably transiently or loosely interact with certain PEP subunits or bind to these proteins at substoichiometric levels. However, it is difficult to determine the detailed constituents of higher plant PEPs due to the technical limitation of protein complex identification and the dynamic conditional changes of this complex. Based on both the repeatability of biochemical identification and the deficient phenotypes of the respective mutants, Steiner et al. (2011) determined a number of proteins as the true and essential components of the PEP complex, including six *rpo* subunits and 10 additional nuclear-encoded PEP-associated proteins (PAPs). FSD2, FSD3, TRXz and FLN1 comprise one group, probably involved in the redox-mediated regulation of chloroplast development and the regulation of plastid gene expression (Myouga et al. 2008, Arsova et al. 2010).

Genetic and biochemical studies have shown that certain PAPs (and other PEP-related proteins), e.g. PTAC2, PTAC6 and PTAC12 (Pfalz et al. 2006), PTAC3 (Yagi et al. 2012), PTAC10 (Jeon et al. 2012), PTAC14 (Gao et al. 2011) and PTAC7 (Yu et al. 2013a), are essential for normal plastid gene expression, based on the analyses of their respective mutants. The distinct conserved domains in PTAC2, PTAC3, PTAC10 and PTAC14 indicated that these proteins might be associated with RNA/DNA binding or mRNA metabolism. Recent studies using chloroplast chromatin immunoprecipitation (cpChIP) assays have shown that PTAC3 binds not only the promoter regions of PEP-dependent genes but also the transcription elongation region of *psbA* during transcription, implying a wide-ranging regulatory function of PEP in the transcription of plastid genes during

initiation and other processes. Thus, it has been suggested that PTAC3 has an essential role in chloroplast development probably through the regulation of plastid mRNA transcription/metabolism. As many as four reactive oxygen secies (ROS)-related proteins (TRXz, FLN1, FSD2 and FSD3) have been identified as essential PEP subunits (Steiner et al. 2011), revealing that PEP activity is likely to be regulated through ROS signaling or the ROS state around chloroplast nucleoids. However, despite significant progress, the detailed functions and related molecular mechanisms of most PAPs remain obscure, and little is known about their upstream regulators/signals and downstream molecular/biochemical activities.

Here, we report the reverse genetic identification of another novel factor that is essential for chloroplast development, PRDA1, based on the phenotypic characterization of a newly constructed RNA interference (RNAi) line. A series of analyses were performed, and the results demonstrated that PRDA1 is a novel chloroplast nucleoid protein required for the regulation of plastid gene expression, potentially in cooperation with MRL7, in an FSD2-mediated regulatory manner for PEP activity.

Results

Reverse genetic characterization of *PRDA1*

As a supplementation to the previous high-throughput RNAi plant library used in our study of MRL7 (Qiao et al. 2011), a number of target-off function-unknown genes, the products of which have been predicted to be targeted to chloroplasts and the expression profiles of which have been associated with the seedling development stage, were sorted, and their respective Arabidopsis RNAi lines were separately constructed (J. Qiao et al. unpublished results). Fortunately, RNAi silencing of one such target gene in Arabidopsis resulted in the typical delayed-greening seedling phenotypes, similar to the results obtained with *MRL7* (Qiao et al. 2011). Approximately four-fifths of the RNAi transformants exhibited consistent delayed-greening phenotypes, i.e. two cotyledons were nearly albino after seed germination and gradually greened at 3 weeks old (**Fig. 1A**). The Arabidopsis locus ID of the corresponding gene is AT5G48470, and we named this gene *PEP-Related Development Arrested 1* (*PRDA1*). Reverse transcription–PCR (RT–PCR) analysis confirmed the severe interference with *PRDA1* transcription in these RNAi plants (**Fig. 1B**).

PRDA1 encodes a novel protein conserved in photoautotrophic organisms

PRDA1 was annotated in public databases (e.g. TAIR) as encoding an unknown chloroplast protein. This gene contains nine exons and eight introns, and is located on chromosome 5. The open reading frame (ORF) of *PRDA1* encodes a polypeptide of 397 amino acids with a calculated molecular weight of approximately 44 kDa. No conserved functional domains were predicted in the PRDA1 sequence through any available
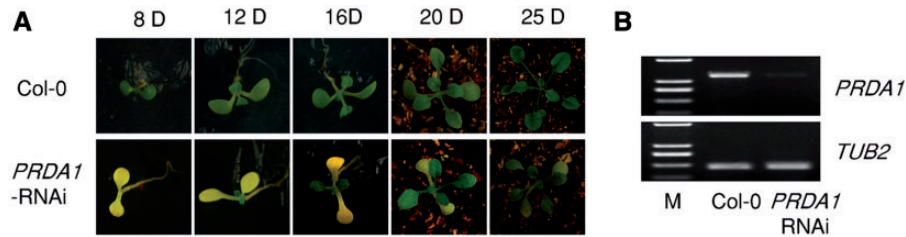
Fig. 1 Analyses of *PRDA1*-RNAi plants. (A) Phenotypes of *PRDA1*-RNAi plants. The representative *PRDA1*-RNAi seedlings and the wild-type (Col-0) controls were grown on MS medium (for plants 8, 12 and 16 d old) or in soil (for plants 20 and 25 d old). (B) RT–PCR analysis of *PRDA1* transcripts in *PRDA1*-RNAi plants. *TUB2* served as a control.
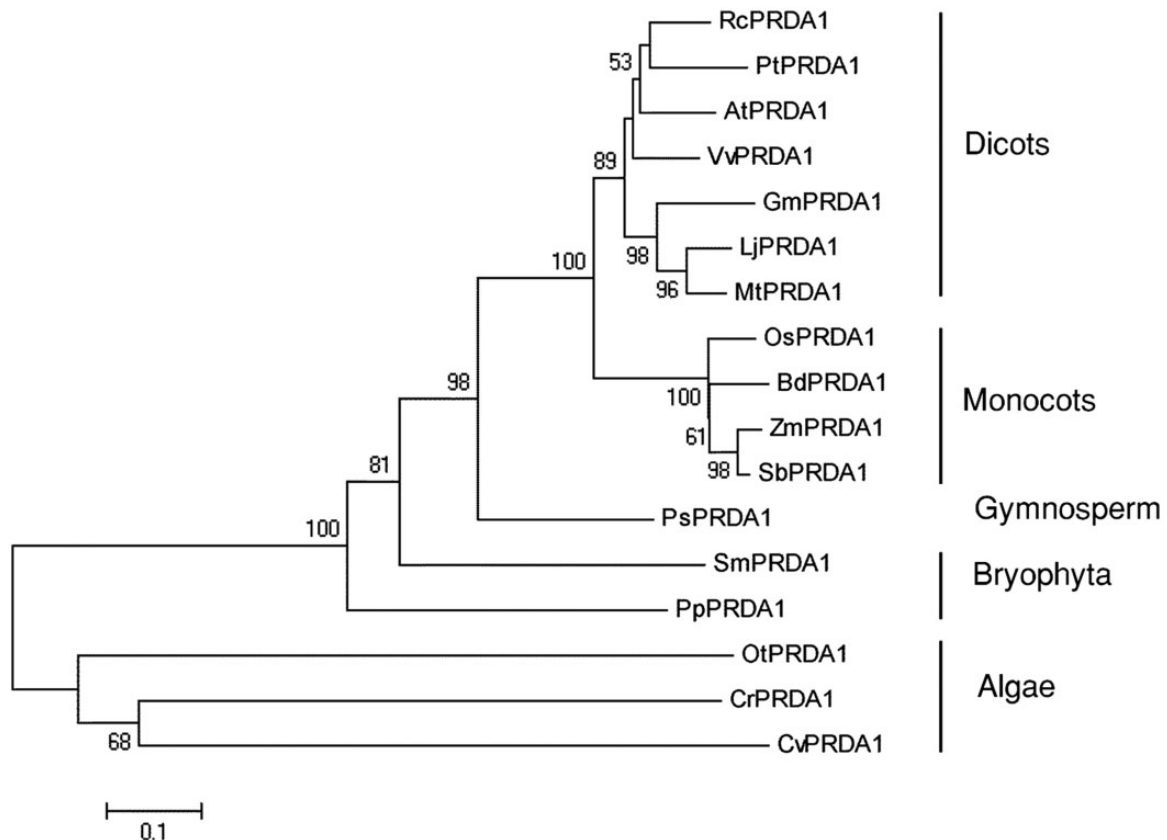

Fig. 2 Phylogenetic analysis of PRDA1 homologous proteins. The subclades representing evolutionary linkage are marked. The numbers at each node represent the bootstrap values (%) calculated from 1,000 trials. The length of branches indicates the extent of divergence according to the bar scale (relative units) at the bottom. The first two characters of each protein name represent the respective genus and species, respectively. At, *Arabidopsis thaliana* (NP_568697.1); Vv, *Vitis vinifera* (XP_002263438.2); Rc, *Ricinus communis* (XP_002527256.1); Pt, *Populus trichocarpa* (XP_002301824.1); Lj, *Lotus japonicas* (AFK43934.1); Gm, *Glycine max* (XP_003520998.1); Mt, *Medicago truncatula* (XP_003588461.1); Zm, *Zea mays* (AFW60715.1); Os, *Oryza sativa* (AAX94817.1); Sb, *Sorghum bicolor* (XP_002463643.1); Bd, *Brachypodium distachyon* (XP_003577592.1); Ps, *Picea sitchensis* (ABK22305.1); Sm, *Selaginella moellendorffii* (XP_002967187.1); Pp, *Physcomitrella patens* (XP_001779241.1); Ot, *Ostreococcus tauri* (XP_003080287.1); Cr, *Chlamydomonas reinhardtii* (XP_001694474.1); Cv, *Chlorella variabilis* (EFN50903.1).

bioinformatics tools. Blast searching the Arabidopsis genome revealed only one copy of the *PRDA1* gene in Arabidopsis, and no paralogs were identified. Sequence alignment analysis revealed that PRDA1 orthologs existed in nearly all known photosynthetic organisms, from lower green algae to various higher plants. PRDA1 and its homologs share high similarity in a region of approximately 300 amino acids in the C-terminus (**Supplementary Fig. S1**).

To investigate the evolutionary relationship among PRDA1 proteins, a phylogenetic tree was constructed. As shown in **Fig. 2**, the PRDA1 proteins are well differentiated and exhibit a clear evolutionary linkage from lower green algae to

angiosperms. PRDA1 proteins from algae and mosses form a subclade. The proteins from angiosperms form a large subclade, in which those from monocots and dicots are clearly divided. These data indicated that PRDA1 is likely to be a conserved protein in photoautotrophic organisms.

Knock-out of *PRDA1* in Arabidopsis is seedling lethal

To acquire further functional information on *PRDA1*, a T-DNA insertion line (*salk-069893c*) with an obvious albino phenotype was obtained from the Arabidopsis Biological Resource Center (ABRC) and designated as *prda1*. Genetic analysis showed that *PRDA1* controlled this mutant phenotype in a recessive manner and co-segregated with the kanamycin resistance marker. Sequencing of the T-DNA border junction showed that the T-DNA was inserted into the first intron of *PRDA1*. A PCR genotyping analysis of the plants developed from *prda1* heterozygous plant seeds showed that the segregation rate of green vs. albino plants was nearly 3 : 1, indicating that *prda1* was a single T-DNA insertion mutant (**Fig. 3A, C**). An RT–PCR analysis indicated that the transcription of *PRDA1* was completely lost in *prda1* (**Fig. 3D**). No *PRDA1* transcripts were found in *prda1* using quantitative real-time PCR (QPCR).

The homozygous *prda1* plants exhibited albino cotyledons without true primary leaves upon germination and survived no longer than 2 weeks when grown in soil or on Murashige and Skoog (MS) medium under standard conditions in a growth chamber, indicating that the *prda1* plants were unable to grow photoautotrophically. Nevertheless, on MS medium supplemented with sucrose as a carbon source, the *prda1* plants continuously grew and produced pale-yellow true leaves, but whole-plant development was greatly retarded

compared with wild-type plants (**Fig, 3B**), similar to the phenotypes of PAP mutant plants (Steiner et al. 2011). The results of this phenotypic characterization demonstrated that *PRDA1* plays an essential role in plant autotrophic growth. Thus, the mutant *prda1* was subjected to further detailed analysis to elucidate the molecular function of *PRDA1*.

The full-length *PRDA1* ORF, driven by the *Cauliflower mosaic virus* 35S (35S) promoter, was introduced into *prda1* heterozygous plants to perform complementation experiments. The successfully complemented *prda1* plants with a homozygous mutant background were identified through PCR genotyping, and the expression of the transgenic *PRDA1* ORF was verified through RT–PCR analysis (**Supplementary Fig. S2**). The complemented *prda1* plants grew normally and exhibited a wild-type-like phenotype (**Fig. 3B**), indicating that solely the disruption of *PRDA1* contributed to the seedling-lethal phenotype of *prda1*. In addition, *PRDA1* overexpression in a Col-0 background was also generated, but these plants were visually indistinguishable from wild-type plants, suggesting that the overexpression of *PRDA1* in Arabidopsis did not affect plant growth.

Expression profile analysis of *PRDA1*

To obtain more information associated with the molecular function of PRDA1, we investigated the tissue-specific expression of *PRDA1* in Arabidopsis. The transcript levels of *PRDA1* were evaluated in almost all tissues using both RT–PCR and QPCR analyses. The results showed that *PRDA1* transcripts were highly expressed in young leaves, shoots and flowers, but the expression was nearly absent in the roots and minimally detected in stems and siliques (**Fig. 4A, B**). The *PRDA1* expression profile is consistent with the data from Genevestigator
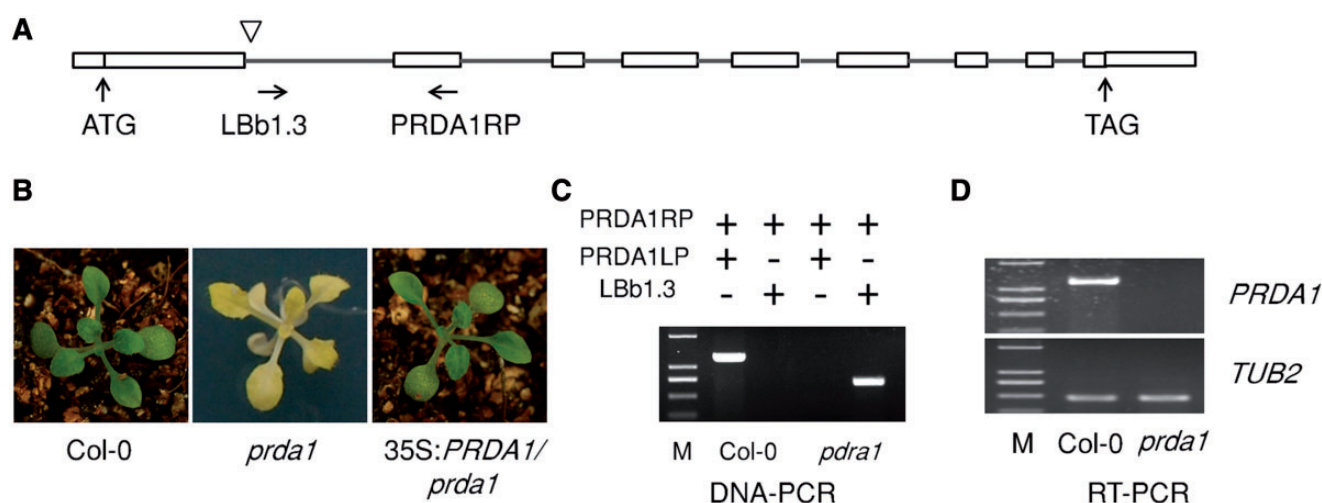

Fig. 3 Analyses of *PRDA1* mutants. (A) A schematic diagram for the gene structure of *PRDA1*. The triangle indicates the T-DNA insertion position in *prda1*. *PRDA1*RP and LBb1.3 show the positions of the genotyping primers. LBb1.3 is located in the T-DNA insertion. *PRDA1*LP is located at 408 bp upstream of the *PRDA1* start codon ATG (not shown). (B) Phenotypes of the 3-week-old representative *prda1* and its complemented plants. (C) PCR genotyping results of *prda1* and the control plants. Only the mutant allele (PCR products of the LBb1.3/*PRDA1*RP primer pair) was amplified in *prda1*. (D) RT–PCR analysis of *prda1* and the control plants. The results showed that *PRDA1* transcripts were totally absent in *prda1*.

(Zimmermann et al. 2004). These results indicated that *PRDA1* expression corresponds well to the plant developmental stage and potentially corresponds to the stage of plastid differentiation and leaf chloroplast development.
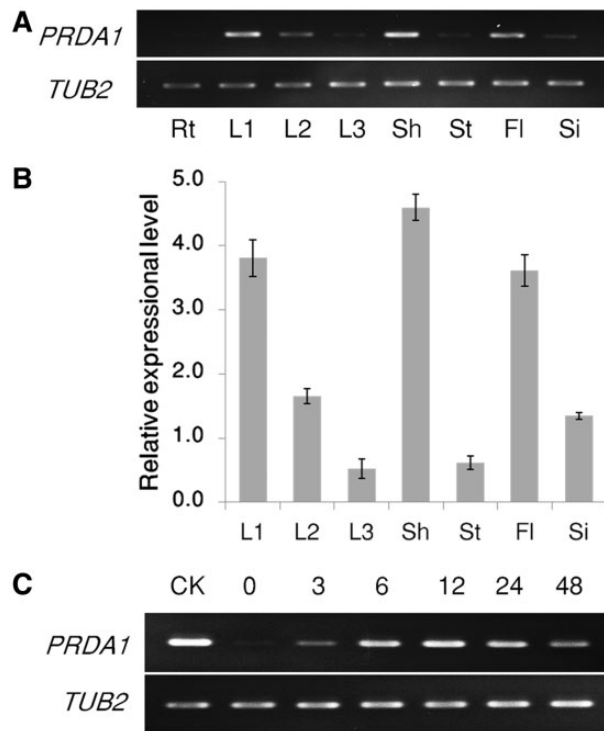

Fig. 4 Expression profiles of *PRDA1*. (A) RT–PCR analysis of *PRDA1* transcripts in Arabidopsis (Col-0) roots (Rt), juvenile leaves (L1), adult leaves (L2), senescent leaves (L3), shoots (Sh), stems (St), flowers (Fl) and siliques (Si). (B) QPCR analysis of the tissue-specific expression pattern of *PRDA1*. The relative expression was measured by QPCR and the changes were given in $\log_2$(sample/root) values, where 3.0 corresponds to 8-fold transcripts of *PRDA1* in the samples relative to the control tissue (roots). At least three independent biological replicates were used. Error bars represent the SD ($n = 3$). (C) Light-induced expression of *PRDA1*. The transcriptional levels of *PRDA1* in the etiolated plants exposed to light for 0, 3, 6, 12, 24 and 48 h were analyzed by RT–PCR.

Chloroplast differentiation and development are markedly induced and regulated by light. To determine the influence of light on *PRDA1* expression, we examined the accumulation of *PRDA1* transcripts during the light-induced greening of Arabidopsis etiolated seedlings. A semi-quantitative RT–PCR analysis of corresponding samples showed that *PRDA1* transcription maintained a low level of expression in etiolated seedlings, which gradually increased after light exposure, reaching a maximal stable level after approximately 12 h (**Fig. 4C**), suggesting that *PRDA1* expression is required for light-induced chloroplast development.

Chloroplast development was arrested in *prda1*

Both the phenotypic characterization of *PRDA1* mutants and the expression profile of *PRDA1* suggested that PRDA1 might function in chloroplast development. To test this supposition, the chloroplast morphological structure in *prda1* leaves was examined through transmission electron microscopy. As shown in **Fig. 5**, the leaf chloroplasts of *prda1* plants grown on sucrose-supplemented MS medium under normal conditions were severely shrunken, with abnormal shapes compared with the wild-type controls, and the thylakoid membrane structures were absent, indicative of deficient development and differentiation. Moreover, the shuttle-shaped starch granules in wild-type chloroplasts disappeared in *prda1* chloroplasts, suggesting that photosynthetic activity was impaired in *prda1* plants. No obvious changes in other organelles and the nucleus were observed in *prda1* leaves. These observations suggested that PRDA1 has essential function associated with early chloroplast development.

PRDA1 is localized to chloroplast nucleoids

PRDA1 is annotated in various public databases as encoding a chloroplast-localized protein and is predicted in ChloroP to possess an obvious chloroplast transit peptide (Emanuelsson et al. 1999). To verify these hypotheses, a chimeric gene expressing PRDA1 and the yellow fluorescent protein (YFP) fusion protein under the 35S promoter (35S:PRDA1–YFP) was constructed. The construct was introduced into *Nicotiana benthamiana* leaves, and the fluorescence was examined
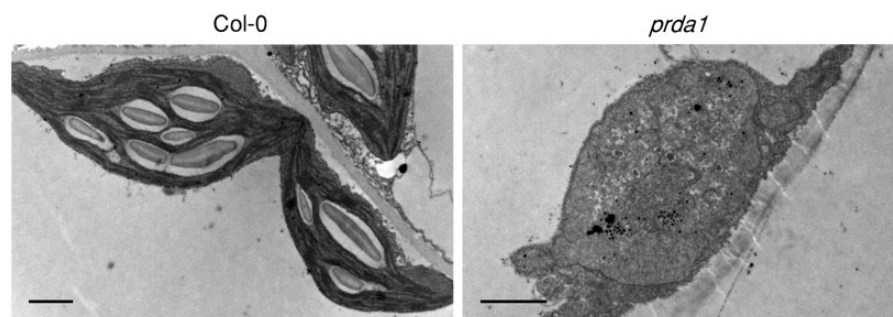

Fig. 5 Transmission electron micrographs of plastid ultrastructures in *prda1* and the wild-type plants. Plastids were from leaves of 3-week-old Col-0 plants and *prda1* plants grown on MS medium (supplemented with 2% sucrose). Bars = 1 μm.
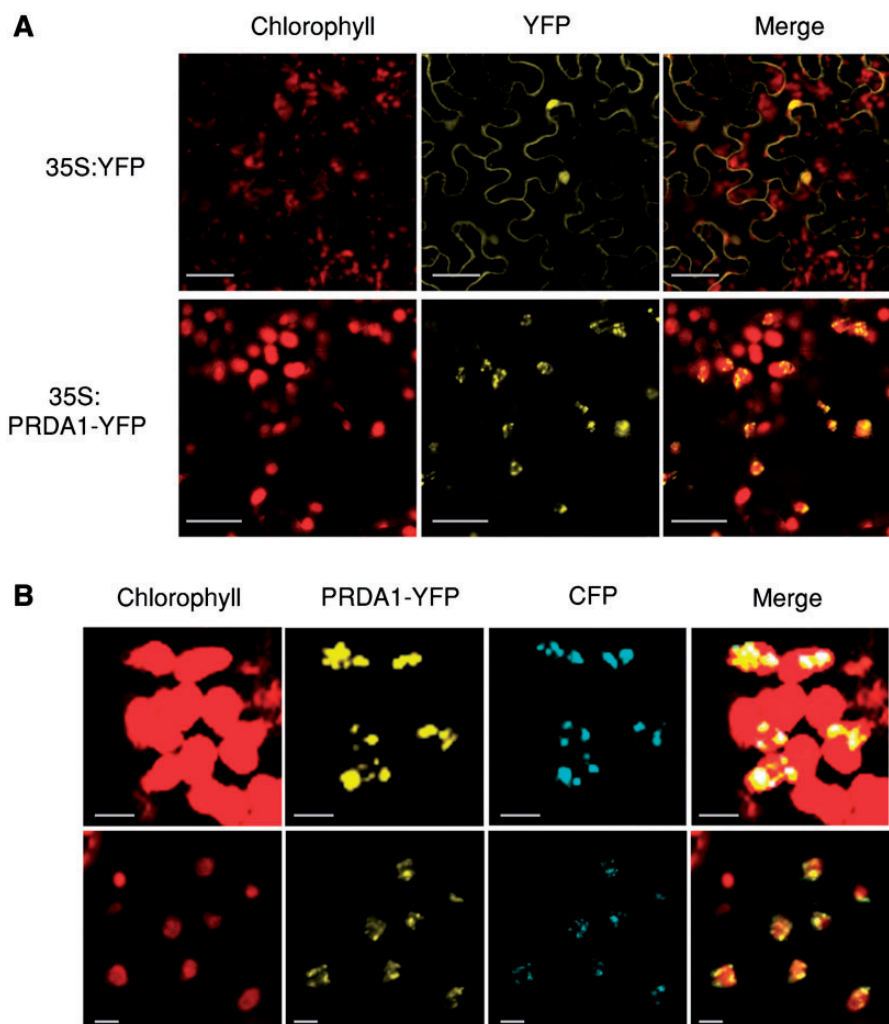
Fig. 6 Localization assays of PRDA1. The fluorescent proteins were transiently expressed in *N. benthamiana* leaves. The fluorescence signals were visualized by confocal laser-scanning microscopy. (A) Sublocalization of PRDA1 in chloroplasts. In contrast to the cell membrane- and nucleus-preferential distribution of the free YFP (upper row), the yellow fluorescence signals of PRDA1–YFP (lower row) merged well with the red Chl signals in chloroplasts. Bars = 10 μm. (B) PRDA1–YFP/PEND–CFP (upper row) co-localization and PRDA1–YFP/MRL7–CFP co-localization (lower row). Bars = 2 μm.

through confocal laser-scanning microscopy. Free YFP (35S:YFP) was used as a control. As shown in **Fig. 6A**, the yellow fluorescence of free YFP was distributed in regions close to the cell membrane and the nucleus. In contrast, the yellow fluorescence of the PRDA1–YFP fusion protein overlapped well with the red Chl autofluorescence, indicating that PRDA1 is specifically targeted to chloroplasts.

The PRDA1–YFP yellow fluorescence exhibited a clear punctate distribution pattern, similar to *MRL7*, *FLN1*, *FLN2* and *FSD3* (Myouga et al. 2008, Arsova et al. 2010, Qiao et al. 2011), all of which have been previously identified as chloroplast nucleoid-localized proteins, suggesting that PRDA1 is also a chloroplast nucleoid-localized protein. To confirm these results, two pairs of co-localization experiments (PRDA1/PEND and PRDA1/MRL7) were performed in which PEND (Sato et al. 1993) and MRL7 were used as two

nucleoid-localized control proteins. The results showed that the fluorescent signals from both PRDA1–YFP/PEND–enhanced cyan fluorescent protein (CFP) and PRDA1–YFP/MRL7–CFP merged well with nucleoids in leaf chloroplasts (**Fig. 6B**), indicating that PRDA1 is finely localized to chloroplast nucleoids.

PEP activity was seriously impaired in *prda1*

Initially, we used web-based analyses to predict the molecular function of PRDA1. The co-expression data from ATTED-II (Obayashi et al. 2007) showed that *PRDA1* is co-expressed with genes related to chloroplast development, of which *PRIN2*, *TRXz*, *FLN1*, *FLN2* and *FSD2* have been identified as essential for the regulation of PEP-dependent plastid gene expression (Steiner et al. 2011) (**Supplementary Fig. S3, Table S1**). These findings implied that PRDA1 is potentially

involved in the regulation of plastid gene expression. To examine this hypothesis further, the transcript levels of plastid genes were investigated in *PRDA1* knock-out mutants. Eight genes (*psaA, psbA, psbD, psbH, rbcL, petB, ndhA* and *rps14*) were chosen as PEP-dependent genes (Class I), six genes (*accD, rpoB, rpoA, rpoC1, rpoC2* and *ycf2*) were chosen as NEP-dependent genes (Class III) and six genes (*rrn23, atpI, rps16, ndhB, clpP* and *ycf1*) were selected to represent both PEP- and NEP-dependent genes (Class II). In addition, the transcript levels of certain nuclear-encoded genes encoding chloroplast proteins were also examined. The seedling-lethal albino and chloroplast development-arrested mutant, *salk_026379* (Zhang et al. 2006), was also used as a control. The *salk_026379* mutant is a single T-DNA insertion mutant of magnesium chelatase ChlD (Luo et al. 1999), the transcripts of which were absent in *salk_026379*. This mutant could be completely complemented through the ectopic expression of the ChlD ORF (J. Qiao et al. unpublished results).

As shown in **Fig. 7**, the analysis of plastid gene expression revealed that the transcript levels of all eight PEP-dependent genes were significantly decreased in *prda1*, while the transcript levels of all six NEP-dependent genes were markedly increased. For the representative Class II genes, only the transcript level of *rrn23* was decreased, while the transcript levels of the other five genes were obviously increased. However, the transcript accumulation of all investigated plastid genes in *salk-026379* maintained nearly the same levels as observed in wild-type plants. The transcript accumulation of the genes encoding photosynthesis-related proteins (*psaG, psaL, psbO, psbR* and

LHCA1) was not affected in both *PRDA1* and *ChlD* mutants (**Fig. 7**). The transcript accumulation of plastid genes in *prda1* showed high similarity to that in previously characterized PEP-deficient mutants, such as *dg1, sig6, ptac2, patc6, ptac12, ptac14, trxz* and *mrl7* plants (Pfalz et al. 2006, Chi et al. 2008, Schweer et al. 2010, Gao et al. 2011, Qiao et al. 2011). These results indicated that plastid gene expression was severely disturbed in Arabidopsis through *PRDA1* knock-out, and PRDA1 is essentially required for the regulation of PEP-dependent plastid gene expression.

PRDA1 interacts with FSD2, an essential subunit of the PEP complex

Interestingly, as shown above, PRDA1 was co-expressed with many nuclear-encoded PEP essential subunits, e.g. TRXz, FLN1, FLN2, FSD2, pTAC6, pTAC10 and pTAC12, based on the results of the public co-expression analysis (Manfield et al. 2006, Obayashi et al. 2007). In addition, both *PRIN2* (Kindgren et al. 2012) and *MRL7* (Qiao et al. 2011) were also found to be co-expressed with *PRDA1*. These results suggested that PRDA1 might functionally associate with the PEP polymeric complex through interactions with certain PEP components.

To assess the putative association between PRDA1 and PEP, protein–protein interaction assays were performed between PRDA1 and the co-expressed PAPs using bimolecular fluorescence complementation (BiFC) (Waadt et al. 2008). Here, the PAPs include all the nuclear-encoded PEP essential subunits (Steiner et al. 2011) and three other proteins, FLN2, PRIN2
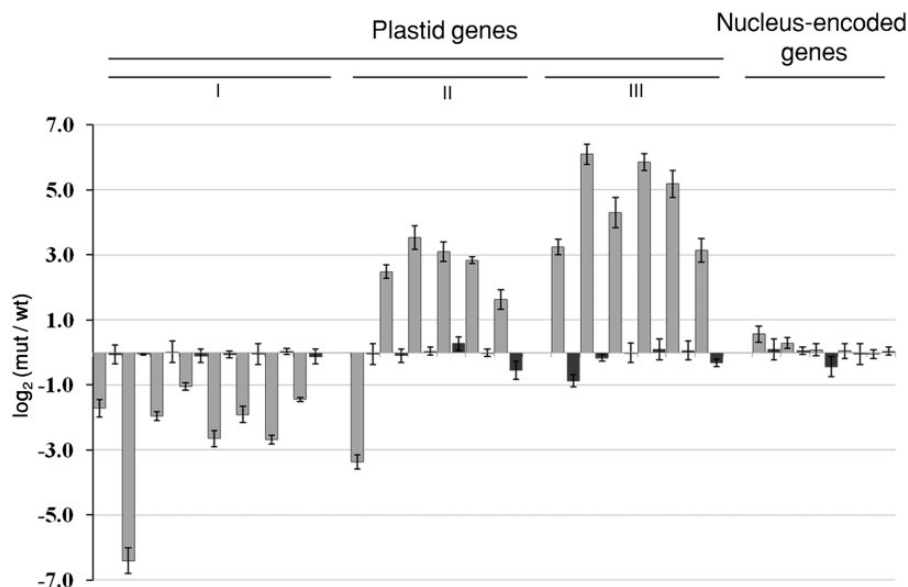
Fig. 7 Analysis of transcriptional levels of plastid genes and nuclear-encoded genes in *prda1* (gray bars) and *salk_026379* (dark bars). QPCR was performed and the results are displayed by $\log_2$(mut/wt) values as in **Fig. 4B**. Mut, *prda1* or *salk_026379* mutants; wt, Col-0 plants. The investigated plastid genes include Class I (I) genes (from left to right: *psaA, psbA, psbD, psbH, rbcL, petB, ndhA* and *rps14*), Class II (II) genes (from left to right: *rrn23, atpI, rps16, ndhB, clpP* and *ycf1*) and Class III (III) genes (from left to right: *accD, rpoB, rpoA, rpoC1, rpoC2* and *ycf2*). The investigated nuclear-encoded genes include photosynthesis-related genes (from left to right: *psaG, psaL, psbO, psbR* and *LHCA1*).

and MRL7 (**Supplementary Table S2**). The ORFs of PRDA1 and the PAPs were fused to the C- and N-terminal moieties of YFP, respectively, to generate PRDA1–YFP^C and PAP–YFP^N constructs, respectively. Each control combination (YFP^C/PAP–YFP^N and YFP^N/PRDA1–YFP^C) produced no fluorescence. In contrast, strong punctate yellow fluorescent signals, merging well with the red Chl autofluorescence, were observed only from the combination of both FSD2–YFP^N/PRDA1–YFP^C and MRL7–YFP^N/PRDA1–YFP^C (**Fig. 8A**; **Supplementary Fig. S4**), indicative of the formation of PRDA1–FSD2 and PRDA1–MRL7 complexes in chloroplast nucleoids. As MRL7 has been identified as an interaction partner of PRDA1, the interaction between MRL7 and the above PAPs was also analyzed.

We observed that MRL7, similarly to PRDA1, clearly interacts with FSD2 but not with other PAPs. These interactions were further validated through firefly luciferase complementation imaging (LCI) assays (Chen et al. 2008, Shang et al. 2010) (**Fig. 8B**). The PEP essential subunit FSD2 is a chloroplast stroma-localized iron superoxide dismutase, which interacts with the chloroplast nucleoid-localized FSD3 and, together, these proteins act as ROS scavengers to protect chloroplast nucleoids (Myouga et al. 2008). The possible interaction of both PRDA1 and MRL7 with FSD2 suggested that PRDA1 and MRL7 probably act as components of the PEP complex and might be involved in the redox control of plastid gene expression.
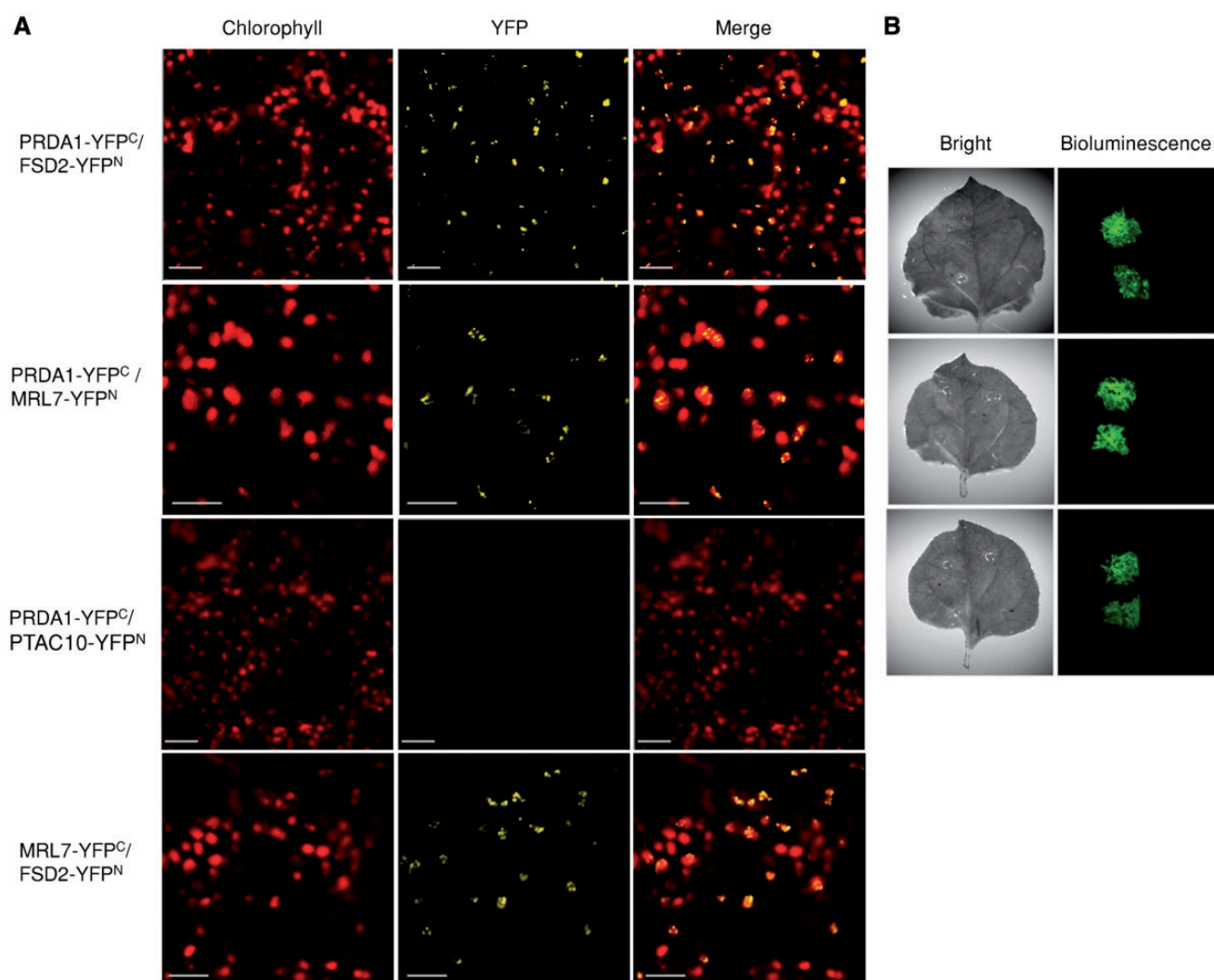

Fig. 8 Interactions among PRDA1, MRL7 and FSD2. The fusion proteins were transiently expressed in *N. benthamiana* leaves. (A) Visualization of each interaction in chloroplasts by BiFC analysis. The controls are shown in **Supplementary Fig. S4**. Bars = 10 µm. (B) Validation of interactions by LCI assays. The left column indicates the bright light field. The right column indicates the bioluminescence (green) signals produced by the catalysis of luciferin by the combinational luciferase. The left half of each leaf shows the combinations of PRDA1–LUC^N/MRL7–LUC^C (upper row), PRDA1–LUC^N/FSD2–LUC^C (middle row) and MRL7–YFP^N/FSD2–YFP^C (lower row). The right half of each leaf shows the control combinations of PRDA1–LUC^N/pTAC10–LUC^C (upper row), PTAC10–LUC^N/MRL7–LUC^C (middle row) and FeCh–YFP^N/FSD2–YFP^C (lower row).

PRDA1-deficient plants are sensitive to oxidative stress

The interactions identified above suggested that, similarly to FSD2 and FSD3 (Myouga et al. 2008), PRDA1 and MRL7 might function in scavenging ROS around chloroplast nucleoids. Thus, we examined whether the PRDA1-deficient plants are sensitive to oxidative stress. To this end, we investigated the greening progress of PRDA1-RNAi plants, prda1 mutants and the Col-0 control plants growing under high light (200 μmol m^{-2} s^{-1}) and low light (15 μmol m^{-2} s^{-1}) conditions. As shown in **Fig. 9A**, Col-0 plants exhibited nearly the same growth under low and high light conditions. In contrast, the cotyledons of PRDA1-RNAi plants growing under low light conditions typically greened earlier and grew more strongly than those under high light conditions. The prda1 mutants growing under low light conditions developed rapidly, with more yellowish leaves, while those under high light conditions grew slowly, with leaves exhibiting ivory phenotypes. These analyses indicated that both the PRDA1-RNAi and mutant plants showed light intensity-dependent responses consistent with the case of FSD2 mutants. Nitroblue tetrazolium (NBT) staining and subsequent quantification analyses were performed on 2 d dark-acclimated PRDA1-deficient plants, and the results showed that the accumulation of O$_2^-$ in dark-acclimated PRDA1-deficient plants was significantly higher than that in wild-type control plants (**Supplementary Fig. S5**), but was similar to that in FSD2 mutants. These results imply that the PRDA1-deficient plants are sensitive to oxidative stress, similarly to FSD2 mutants (Myouga et al. 2008).

Moreover, PRDA1- and MRL7-RNAi seedlings were subjected to ROS-related drug treatments. The delayed-greening phenotype of these plants is convenient to observe ROS-induced phenotypic changes. The well-known ROS scavenger, dimethylthiourea (DMTU) (Garretón et al. 2002, Kabeya and Miyagishima. 2013), was used here. A series of final concentrations (from 0.1 to 1 mM) of DMTU were added to the MS culture to examine plant tolerance against this antioxidant. Higher DMTU concentrations of up to 1 mM inhibited seed germination and delayed seedling growth, and 0.5 mM DMTU was chosen to treat both RNAi plants. As shown in **Fig. 9B**, the phenotypic investigation indicated that the cotyledons of either PRDA1- or MRL7-RNAi plants treated with 0.5 mM DMTU generally exhibited nearly green (pale) phenotypes, distinctly different from the yellowish phenotypes of the untreated RNAi plants. Chl determination assays showed that
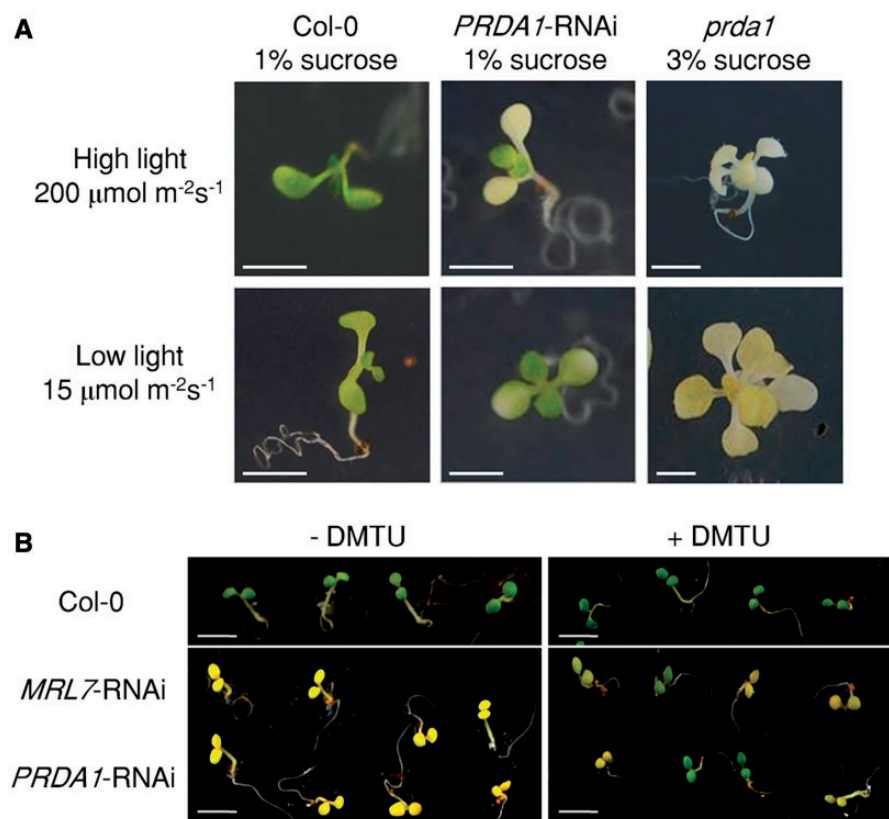

Fig. 9 PRDA1-deficient plants are sensitive to oxidative stress. (A) Visible phenotypes of PRDA1-RNAi plants (2 weeks old), prda1 plants (4 weeks old) and their wild-type control plants (1 week old) under high light and low light conditions on MS plates supplemented with 1% or 3% sucrose. Bars = 0.5 cm. (B) Phenotypes of representative DMTU-treated PRDA1- and MRL7-RNAi seedlings (8 d old). The seedlings were grown on MS medium with or without 0.5 mM DMTU. Bars = 0.5 cm.

DMTU-treated *PRDA1-* and *MRL7-*RNAi seedlings contained higher Chl levels than untreated seedlings (**Supplementary Fig. S6**). These results suggested that ROS accumulation in *PRDA1-* and *MRL7-*RNAi plants impeded plant greening, indicating that PRDA1 is likely to be involved in the maintenance of the redox state around chloroplast nucleoids.

Discussion

PRDA1 is essential for early chloroplast development and PEP activity

Currently, a number of PEP-related proteins have been identified as essential to plant development (Schröter et al. 2010, Lerbs-Mache 2011, Steiner et al. 2011), and these proteins might function in mediating signal transduction from the extracellular environment to chloroplast nucleoids to control PEP-dependent plastid gene expression. However, the systemic and complete elucidation of the extremely complex mechanism underlying the regulation of plastid gene expression has not been achieved.

Here, PRDA1 was identified through reverse genetics as a novel chloroplast nucleoid protein essential for PEP-related chloroplast development, similar to MRL7 (Qiao et al. 2011). Disabling PRDA1 in Arabidopsis arrested normal chloroplast development and severely impaired PEP activity, resulting in a seedling-lethal, albino phenotype. These results are highly similar to those observed for many other PEP-associated proteins (Steiner et al. 2011). The examination of the plastid mRNA editing showed that all 34 editing sites were correctly edited in *prda1*, suggesting that PRDA1 is not responsible for the editing of plastid mRNA. In addition, embryonic lethality was not observed for the seeds of *prda1* heterozygous plants as observed in other mutants, such as *dg1* (Chi et al. 2008). These results revealed that PRDA1 is essentially required for early chloroplast development and PEP activity.

PRDA1 is a chloroplast nucleoid-associated protein

Chloroplast nucleoids are multiple copies of plastid chromosome folded together with proteins and RNA molecules. Chloroplast nucleoids are the major location of plastid DNA/RNA metabolism, ribosome assembly and many other processes associated with chloroplast development (Majeran et al. 2012). The PEP-mediated transcriptional machinery forms a typical subdomain in chloroplast nucleoids and represents an early developmental bottleneck, leading to the abortion of proper chloroplast biogenesis when essential components are disturbed (Pfalz and Pfannschmidt 2013). Sublocalization experiments demonstrated that PRDA1 together with MRL7 are localized with chloroplast nucleoids (**Fig. 6**), identical to the localization pattern of pTAC3, FSD3, FLN1 and FLN2, all of which have been identified as essential PEP subunits (Myouga et al. 2008, Arsova et al. 2010, Yagi et al. 2012, Pfalz and Pfannschmidt 2013).

The nucleoid-localized proteins FLN1 and FLN2 are targets of the plastidial thioredoxin, TRXz, involved in the thiol-dependent redox control of plastid gene expression regulation (Arsova et al. 2010). FSD2 and FSD3, two iron superoxide dismutases with important roles against oxidative stress, may form a heterodimer involved in the regulation of PEP-dependent plastid gene expression in chloroplast nucleoids (Myouga et al. 2008). Moreover, the screening of high-light-insensitive mutants showed that PRIN2 is a ROS-mediated retrograde signaling factor. In addition, PRIN2 localized with chloroplast nucleoids and was required for normal PEP-dependent plastid gene expression (Kindgren et al. 2012). These studies potentially suggest that ROS/redox-related signaling probably plays an essential role in regulating plastid gene expression. As chloroplast nucleoid-localized and light-induced proteins PRDA1 and MRL7 are co-expressed with PRIN2, FSD2, FLN1 and FLN2 (**Supplementary Table S1**) and interact with FSD2, it is highly possible that PRDA1 might be involved in the ROS-related signaling pathway responsible for plastid gene expression.

Interactions between PRDA1 and FSD2

Identifying interacting proteins is typically used to unravel the functional mechanisms of target proteins. PEP has recently been demonstrated to be one large protein complex (Steiner et al. 2011). However, the mechanistic information underlying the combination of its subunits remains poorly understood. Among these subunits, TRXz interacts with FLN1 and FLN2 (Arsova et al. 2010), pTAC14 interacts with PTAC12 (Gao et al. 2011), pTAC3 associates with the α core subunit in cpChIP assays (Yagi et al. 2012), and PTAC7 interacts with up to four TAC components, including FLN1, PTAC10, PTAC12 and PTAC14, in yeast two-hybrid assays (Yu et al. 2013a). These results provided valuable information for the mechanical assembly of the PEP complex, although further studies are needed to demonstrate the detailed biological significance of these interactions.

In this study, both PRDA1 and MRL7 were shown to interact with FSD2 in planta through a one-to-one test using BiFC, and this interaction was further confirmed using LCI. The chloroplast-localized iron superoxide dismutase FSD2 was also identified as an essential PEP subunit (Steiner et al. 2011) that protects chloroplast nucleoids against ROS damage (Myouga et al. 2008). The phenotypic alleviation of *PRDA1-*RNAi seedlings could be induced in response to ROS scavenging through DMTU treatment. Thus, the molecular function of PRDA1 for PEP-mediated chloroplast development is likely to be associated with ROS metabolism around chloroplast nucleoids.

The regulation of PEP-dependent plastid gene expression is rather complex, and currently neither the detailed biochemical constituents of the PEP holoenzyme nor the exact roles of each known component have been clearly elucidated. The transcriptional regulation of plastid genes through PEP might be mediated via signal transduction from the nucleus or

environmental cues. Together with the results from Myouga et al. (2008), FSD2 have been shown to interact with nucleoid-localized proteins, FSD3, PRDA1 and MRL7. However, FSD2 is uniformly distributed throughout the entire chloroplast (Myouga et al. 2008). This localization discrepancy was also observed for the interaction of the nucleoid-localized FLN1 and FLN2 with the chloroplast-localized TRXz (Arsova et al., 2010). The chloroplast nucleoids are typically distributed in puncta throughout the stroma. These results suggested that FSD2 and TRXz might function by mediating/transmitting certain signals (e.g. ROS-related signals) from the stroma to chloroplast nucleoids. Recently, MRL7 was shown also to interact with TRXz and FSD3 in a yeast two-hybrid assay (Yu et al. 2013b), further implying that MRL7 is involved in redox-mediated regulation of chloroplast development. Further genetic and biochemical analyses are required to elucidate the underlying molecular mechanisms of these interactions.

Materials and Methods

Plant materials and growth conditions

Arabidopsis plants (wild-type Columbia-0 ecotype, the mutants and the transgenic plants) were grown in soil in a growth chamber under a 16 h light/8 h dark long-day condition, with a photon flux density of 120 μmol m^{-2} s^{-1} and 50–70% humidity at a constant temperature of 22°C. For plants grown on agar plates, the seeds were surface sterilized and sown on MS medium (Sigma) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar. Subsequently, the seeds were vernalized at 4°C for 3 d and transferred to a growth chamber. For drug treatment, DMTU (Sigma) was directly added to the MS medium at different final concentrations, and the surface-sterilized seeds were planted and transferred to the growth chamber. For N. benthamiana plants, the seeds were germinated in soil and cultivated in a growth chamber at 25°C under a 14 h light/10 h dark photoperiod.

Generation and analyses of RNAi lines

A PRDA1 cDNA fragment was amplified using primers (PRDA1-RNAiF and PRDA1-RNAiR) and cloned into pRNAi-LIC (Xu et al. 2010) to create the RNAi constructs expressing the artificial PRDA1-specific hairpin RNA under the control of the 35S promoter. The resulting PRDA1-silencing construct was transformed into wild-type Arabidopsis plants using the floral dip method (Clough and Bent 1998). The transformants were screened on MS medium containing 50 mg l^{-1} kanamycin, and pictures were taken at the corresponding developmental stages. RT–PCR analysis was performed to evaluate the level of PRDA1 transcription in these plants.

Identification and complementation of T-DNA insertion mutants

The PRDA1 T-DNA insertion line (salk_069893c) was identified through PCR genotyping analysis using primers PRDA1LP and PRDA1RP, and the T-DNA primer LBb1.3. RT–PCR was performed to examine the transcriptional level of PRDA1 in prda1. The full-length ORF of PRDA1 was cloned into the KpnI and PstI sites of pCAMBIA1300S (Xiong and Yang 2003), and the resulting pCAMBIA1300S-PRDA1 constructs were introduced into prda1 heterozygous plants for complementation. The successful complementation was identified through PCR genotyping and RT–PCR as described above. The gene-specific primers are listed in **Supplementary Table S2**.

Protein alignment and phylogenetic analysis

The sequences of PRDA1 homologous proteins were obtained through a BLASTP alignment in the GenBank database (http://blast.ncbi.nlm.nih.gov/). The multiple sequence alignments and phylogenetic tree construction were performed according to the methods described in a previous study (Qiao et al. 2011).

Microscopic analyses

For the transmission electron microscopy analysis of the chloroplast ultrastructures, the leaves from 3-week-old wild-type and prda1 plants were used as described in Qiao et al. (2011). The examination of the sublocalization of PRDA1 and control proteins was performed according to the methods described in a previous study (Qiao et al. 2011), with minor modifications. The binary vector pCAMBIA1300S was adopted instead of pUC19 as the skeleton sequence to create the pCAMBIA1300S-YFP vector. The DNA fragments encoding PRDA1–YFP and MRL7/PEND–CFP fusion proteins were cloned into pCAMBIA1300S (with KpnI and PstI sites) to generate the pCAMBIA1300S-PRDA1-YFP, pCAMBIA1300S-MRL7-CFP and pCAMBIA1300S-PEND-CFP constructs, respectively. These plasmids were introduced into Agrobacterium GV3101 and used to transform N. benthamiana leaves transiently through infiltration. YFP and CFP fluorescence was visualized through confocal laser-scanning microscopy (LSM 510 META-ConfoCor 2 system). For the co-localization experiments, equal amounts of Agrobacterium cultures containing the corresponding plastids were mixed before infiltration. A distinct excitation wavelength was used for the analysis, and the fluorescence was sequentially scanned to avoid any cross-talk between fluorescence channels.

RNA isolation, cDNA synthesis and gene expression analyses

Total RNA isolation and the first-strand cDNA synthesis were performed according to the methods described in a previous study (Qiao et al. 2011). Random Hexamer primers (Thermo Scientific) were used in the first-strand cDNA synthesis for the QPCR analysis of plastid gene expression, and oligo(dT)$_{20}$ primers (Thermo Scientific) were used for other analyses. RT–PCR was performed to examine the PRDA1 transcript level in PRDA1-RNAi and prda1 plants. For QPCR, the cDNA was amplified using the SYBR Green Real-time PCR Master Mix (Toyobo) in an IQ5 real-time PCR detection instrument (Bio-Rad).

The PCR optimization, design of the replicates and calculation of the relative gene expression ratios were performed as previously described in Arsova et al. (2010). The 18S rRNA gene (AT3G41768) was used as a reference. The primer sequences used here are listed in Supplementary Table S2.

Protein interaction assays

The full-length ORF of *MRL7* was cloned into *Bam*HI and *Kpn*I sites of SPYNE173, and the full-length ORF of *PRDA1* was cloned into *Bam*HI and *Kpn*I sites of SPYCE(M) (Waadt et al. 2008). The related PAPs were cloned into SPYNE173 at distinct sites in the corresponding primer sequences. The resulting PCAMBIA1300S-based BiFC constructs were introduced into *N. benthamiana* leaves through infiltration, as described in Waadt et al. (2008). The fluorescent signals were monitored and viewed as previously described for the localization experiments. For the LCI assay, fragments encoding PRDA1-CLuc, MRL7-NLuc and relative PAP-CLuc fusion proteins were cloned into pCAMBIA1300S and introduced into *N. benthamiana* leaves for transient expression. The imaging was performed as previously described in Chen et al. (2008).

Measurements of ROS and Chl

Visualization and quantification assays were performed according to Myouga et al. (2008), with minor modifications. For the quantification of superoxide, formazan-precipitated blue plants were ground and solubilized in the corresponding volume ($1\,ml\,g^{-1}$ FW) of 2 M KOH–dimethylsulfoxide (DMSO; 1 : 1.16, v/v). After centrifuging to remove debris, the blue supernatant was measured spectrophotometrically at A_{680}. Chl determination assays were performed according to Pruzinská et al. (2005).

mRNA editing analysis

The investigation of plastid mRNA editing was performed according to Yu et al. (2009). All known plastid mRNA editing sites were amplified from the associated cDNA and directly sequenced three times using specific primers.

Supplementary data

Supplementary data are available at PCP online.

Disclosures

The authors have no conflicts of interest to declare.

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