The Pentratricopeptide Repeat Protein DELAYED GREENING1 Is Involved in the Regulation of Early Chloroplast Development and Chloroplast Gene Expression in Arabidopsis1[W][OA]

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An Arabidopsis (Arabidopsis thaliana) mutant that exhibited a delayed greening phenotype (dg1) was isolated from a population of activation-tagged Arabidopsis lines. Young, inner leaves of dg1 mutants were initially very pale, but gradually greened and mature outer leaves, more than 3 weeks old, appeared similar to those of wild-type plants. Sequence and transcription analyses showed that DG1 encodes a chloroplast protein consisting of eight pentratricopeptide repeat domains and that its expression depends on both light and developmental status. In addition, analysis of the transcript profiles of chloroplast genes revealed that plastid-encoded polymerase-dependent transcript levels were markedly reduced, while nucleus-encoded polymerase-dependent transcript levels were increased, in dg1 mutants. Thus, DG1 is probably involved in the regulation of plastid-encoded polymerase-dependent chloroplast gene expression during early stages of chloroplast development.

The formation of normal chloroplasts is crucial for higher plant growth and development. The chloroplast genomes of higher plants typically encode only about 100 genes, and the vast majority of the greater than 2,000 proteins that have functions in the chloroplast are encoded by nuclear genes, translated in the cytosol, and subsequently imported into the chloroplast (Abdallah et al., 2000). Thus, the development of functional chloroplasts (which is essential for the normal autotrophic growth and development of higher plants) is dependent on the coordinated expression of nuclear and chloroplast genes. The transcriptional levels of plastid-encoded genes change dramatically during the course of chloroplast development and are dependent on the stage of plastid development (Mullet, 1993; Emanuel et al., 2004; Zoschke et al., 2007), suggesting that adjustments to the transcriptional machinery play important roles in the regulation of chloroplast development. Two types of RNA polymerases have been identified in higher plant chloroplasts: plastid-encoded polymerases (PEPs) and nucleus-encoded polymerase (NEPs; Maliga, 1988; Hajdukiewicz et al., 1997; Hedtke et al., 1997). PEPs contain core subunits encoded by rpoA, rpoB, rpoC1, and rpoC2 genes, while NEPs are each composed of a single submit (Hedtke et al., 1997; Liere and Maliga, 1999). These two types of polymerases are responsible for the transcription of distinct sets of chloroplast genes (Allison et al., 1996; Hajdukiewicz et al., 1997). The chloroplast-encoded photosynthetic genes (such as psbA, psbD, and rbcL) are exclusively transcribed by PEPs, a few genes (mostly encoding components of the transcription/translation apparatus, such as rpoB) are exclusively transcribed by NEPs, and nonphotosynthetic housekeeping genes are mostly transcribed by both PEPs and NEPs. Since NEPs mainly transcribe plastid genes encoding proteins involved in transcription/translation, while PEP accounts for the transcription of photosynthetic genes (Kapoor et al., 1997; Liere and Maliga, 1999), correct timing of photosynthetic gene expression relies on an increase in PEP transcription activity during the course of chloroplast development (Mullet, 1993; Demarsy et al., 2006). Thus, PEPs may have important functions during early stages of chloroplast development.

Although PEPs are plastid encoded, the transcription of PEP-transcribed genes is also under the control of nuclear genes. A large number of proteins associated with the PEP catalytic core (Pfannschmidt et al., 2000; Loschelder et al., 2004; Suzuki et al., 2004; Pfalz...
et al., 2006) and others that transiently interact with PEPs, such as sigma factors, are encoded by nuclear genes and are likely to mediate the direct control of plastid gene expression. The replacement of sigma factors associated with PEPs has been proposed to account for the switching of transcription patterns during chloroplast development. In null mutants of Arabidopsis (Arabidopsis thaliana) sigma factor 6 (AtSig6), light-dependent chloroplast development has been found to be significantly delayed concomitantly with reductions in the accumulation of PEP-dependent transcripts, indicating that AtSig6 plays an essential role in the regulation of PEP-dependent gene expression (Ishizaki et al., 2005). In addition, knockout mutations of pTAC2, pTAC6, and pTAC12 (three components of the transcriptionally active plastid chromosome, pTAC, in Arabidopsis) have been found to be seedling lethal, and the affected genes all appear to be required for proper functioning of the PEP transcription machinery (Pfalz et al., 2006). These findings indicate that the regulation of PEP-dependent gene expression is much more complex than previously thought, and the nuclear genes involved in this process remain to be identified.

Pentraxicopeptide repeat (PPR) proteins, which are defined by the tandem array of a PPR motif consisting of 35 amino acids, are widely distributed in higher plants. There are 466 members in Arabidopsis and 480 in rice (Oryza sativa), but their functions are largely unknown (Small and Peeters, 2000; Lurin et al., 2004). Most of these PPR proteins, predicted to be located in the plastid or mitochondria, play essential roles in the posttranscriptional regulation of organelle gene expression. In the chloroplast, PPR proteins have been found to be involved in RNA splicing (Hashimoto et al., 2003; Meierhoff et al., 2003; Schmitz-Linneweber et al., 2006), RNA editing (Kotera et al., 2005; Okuda et al., 2007), RNA processing (Fisk et al., 1999), RNA stability (Yamazaki et al., 2004), and ribosome accumulation (Williams and Barkan, 2003). In the mitochondria, EMP4 is required for the correct expression of a small subset of mitochondrial transcripts in the maize (Zea mays) endosperm and OPT43 is required for the trans-splicing of the mitochondrial nad1 intron 1 in Arabidopsis (de Longevialle et al., 2007; Gutiérrez-Marcos et al., 2007).

Here, we describe a T-DNA insertion Arabidopsis mutant named dg1 (for delayed greening1) in which early chloroplast development is affected. The DG1 gene encodes a novel PPR protein that is probably involved in the regulation of PEP-dependent transcript accumulation.

RESULTS

Phenotype of dg1

The dg1 mutant was selected by its high chlorophyll fluorescence phenotype from a population of pSKI015 T-DNA-mutagenized Arabidopsis lines from the Arabidopsis Biological Resource Center (Peng et al., 2006). However, dg1 mutants exhibit high chlorophyll fluorescence, and associated phenotypic traits, in a developmentally regulated manner. As shown in Figure 1A, there were clear phenotypic differences between the young and old mature leaves in both 4- and 6-week-old plants. Growth of the dg1 mutants was retarded, and their young leaves exhibited a clearly chlorotic phenotype under normal growth conditions (Fig. 1A). In addition, $F_v/F_m$ ratios (indicating the maximum potential capacity of the photochemical reactions of PSII) of the young, chlorotic, central, 1- to 3-week-old leaves of the dg1 mutants were substantially lower than those of their wild-type counterparts. However, the $F_v/F_m$ ratios gradually increased and approached wild-type levels (0.85) in the more normal, mature, outer, older than 3-week-old green leaves (Fig. 1A). Thus, the mutant showed a delayed greening phenotype and (hence) was named dg1.

As shown in Figure 1B, the wild-type cotyledons were green when grown on Suc-supplemented Murashige and Skoog (MS) medium. However, the cotyledons of 3-d-old dg1 seedlings grown on this medium were white, then turned yellow at the 5th d. After 7 d, the cotyledons of dg1 became pale-green, and at 12 d, they had acquired an almost normal green coloration, albeit slightly paler than that of wild-type plants (Fig. 1B). The delayed greening phenotype of dg1 cotyledons suggested that DG1 may play a critical role in chloroplast development in early stages of seedling growth. To test this hypothesis, dg1 seeds were germinated on medium both with and without supplementary Suc, since exogenously supplied Suc is required to establish autotrophic growth when the early development of seedlings is arrested (Li et al., 1995). The development of dg1 seedlings on medium without supplementary Suc was severely inhibited compared with their development in the presence of exogenous Suc. After growth for 12 d without Suc, the cotyledons of dg1 mutants still remained yellow and no true leaves had appeared (Fig. 1C).

Impaired Chloroplast Development in dg1

To investigate the effects of the dg1 mutation on chloroplast development, we measured the chloroplasts and examined their ultrastructure in both wild-type and mutant plants by transmission electron microscopy. The observations showed that the chloroplasts were smaller in dg1 mutants than in wild-type plants in both the cotyledons of 5-d-old seedlings (wild type, 5.0 ± 0.3 μm; dg1, 3.0 ± 0.5 μm) and young, 3-week-old leaves of 4-week-old plants (Fig. 2). In addition, transmission electron microscopy observations of ultrathin sections of chloroplasts in 5-d-old cotyledons showed that those in wild-type plants had well-structured thylakoid membranes, composed of grana connected by stroma lamellae. However, the thylakoid membrane organization in the chloroplasts of 5-d-old dg1 cotyledons was disturbed, and their thylakoid membranes were much less abundant. In contrast, the thylakoid
membrane organization and abundance in mature 4-week-old leaves of $dg1$ mutants were similar to those in wild-type plants (Fig. 2).

Cloning of the $DG1$ Gene

The genetic analysis showed that $dg1$ was a single recessive mutant, and cosegregation of the phosphino-
tricin resistance marker of the T-DNA with the mutant phenotype indicated that the mutation was due to the T-DNA insertion. The insertion site in $dg1$ was identified by thermal asymmetric interlaced (TAIL)-PCR isolation of the genomic sequences flanking the T-DNA borders and subsequent sequence analysis, which showed that the T-DNA was inserted in the 5’ untranslated region of $At5g67570$, ~3 bp relative to the ATG start codon (Fig. 3A). No expression of the $At5g67570$ gene was detected in $dg1$ mutants by northern-blot analysis, although the levels of transcripts of its neighboring genes ($At5g67560$ and $At5g67580$) appeared to be similar to those of wild-type plants (Fig. 3B).

Database analysis of the Arabidopsis genome revealed that two partially overlapping cDNA clones...
(AK22212 and NM-126157) with different lengths occur at this locus. The 3' region of the NM-126157 clone overlaps with the 5' region of the AK22212 clone. Reverse transcription (RT)-PCR and sequence analysis of PCR products revealed that the full-length cDNA of At5g67570 does indeed consist of these two combined clones (Supplemental Fig. S1) and that two types of transcripts are present due to alternative splicing (Fig. 3C). One (designated transcript 1) appears to be functional, with an open reading frame that putatively encodes a polypeptide of 798 amino acids, while the other (transcript 2) encodes a truncated polypeptide with 205 amino acids. Transcript 2 has three additional exons, which act as introns (introns 1, 5, and 8) in transcript 1, and a nine-nucleotide insertion (5'-TCACTTTAG-3') in the 5' region of exon 4 (Fig. 3C). Sequence alignment revealed that the nine-nucleotide short sequence corresponds to the 3' region of intron 3.

Figure 3. Molecular cloning of DG1. A, Schematic diagram (not to scale) showing the T-DNA inserted into the 5' untranslated region of At5g67570, genes (in boxes), and the T-DNA insertion locus (bar topped by a triangle). LB, Left border; RB, right border. B, Northern-blot analysis of At5g67570 and its neighboring genes in dg1 mutant and wild-type (WT) plants. 25S rRNA stained with ethidium bromide is shown as a loading control. C, Schematic diagrams of DG1 genomic organization with exons (black boxes) and introns (lines between exons) and its alternatively spliced transcripts. The three additional exons of transcript 2 are shown as gray boxes. The black triangle represents the nine-nucleotide insertion in transcript 2. Arrows indicate the locations of primers used for PCR analysis. D, RT-PCR analysis of DG1 transcripts. The expression of two forms of transcripts with different lengths was examined using primers spanning intron 1 (PU and PD, shown in C). 1 and 2 indicate the positions of transcripts 1 and 2, respectively. E, Relative quantities of two transcripts of DG1 by real-time RT-PCR analysis. Transcript 1 is given a value as 100, and values are means ± se of three replicates.
which indicated that two different splicing sites exist at the 3’ end of intron 3 and that the nine-nucleotide sequence may be due to its alternative splicing. Such a phenomenon of alternative splicing was also observed in rice SDHB and peach (Prunus persica) ETR1, which are associated with differences in tissue localization and responses to environmental stresses, respectively (Kubo et al., 1999; Bassett et al., 2002). Besides the alternative splicing above, DG1 had unusual GC/AG borders at the 5’ and 3’ splicing sites in intron 7 (see www.arabidopsis.org/servlets/tairobject), rather than the GT/AG borders in the vast majority of eukaryotic introns. In Arabidopsis, unconventional splicing sites amount to 0.7% of all splice sites (Alexandrov et al., 2006). Unusual borders of CA/CC and AT/AC in splicing sites have also been detected in rice AGPP and Arabidopsis AtLIM15, respectively (Anderson et al., 1991; Sato et al., 1995). The sequences of transcripts 1 and 2 are provided in Supplemental Figure S1.

Only one signal, corresponding to transcript 1, was detected in northern-blot analyses of wild-type plants, and no signal was detected at the expected position of transcript 2 (Fig. 3B), which may be due to the very low-level expression of transcript 2. To further examine the expression of these two transcripts, RT-PCR using primers spanning intron 1 was performed (Fig. 3C). RT-PCR analysis revealed that transcript 2 accumulated to very low levels compared with transcript 1 in wild-type plants and that neither transcript was expressed in dg1 mutants (Fig. 3D), in accordance with the results of northern-blot analysis (Fig. 3B). To quantify the relation between transcript 1 and transcript 2, real-time RT-PCR with specific primers was performed. Our results showed that the abundance of transcript 2 was about 7% of that of transcript 1.

To confirm that the inactivity of At5g67570 was responsible for the mutant phenotype of dg1, we genetically complemented the line with the full-length At5g67570 cDNA under the control of the cauliflower mosaic virus 35S promoter and obtained seven independent transgenic plants. Subsequent phenotypic observations and chlorophyll fluorescence analyses confirmed that wild-type traits had been restored in the complemented mutant (Fig. 1, A and C). Thus, it can be concluded that the disruption of At5g67570 is indeed responsible for the dg1 mutant phenotype.

DG1 Encodes a PPR Protein Targeted to the Chloroplast

BLAST searches of the complete Arabidopsis sequence revealed that only one copy of the DG1 gene is present in the nuclear genome, which encodes a putative polypeptide of 796 amino acids with a calculated molecular mass of 92 kD, containing eight PPR motifs (Fig. 4). Protein alignments showed that it shares significant identity with the Medicago truncatula protein MtrDRAFT_AC147000g14V1 (73% similarity) and the rice protein Os05g0315100 (64% similarity). Another (hypothetical) Arabidopsis protein, At1g30610, was also found to have high similarity (59%) to DG1.

To examine the cellular localization of the DG1 protein, we constructed a chimeric gene expressing a fusion protein consisting of the 300 N-terminal amino acids of DG1 and GFP under the control of the 35S promoter. The plasmid containing the chimeric gene was transformed into wild-type Arabidopsis plants, and leaves of stably transformed plants were examined by confocal laser-scanning microscopy. The fusion protein was colocalized with the chloroplastic chlorophyll in the mesophyll cells, in accordance with results obtained when the GFP was fused to the transit peptide of the small subunit of Arabidopsis ribulose bisphtate carboxylase (Fig. 5E; Lee et al., 2002). In contrast, GFP signals accumulated specifically in the nucleus when GFP was fused to the nuclear localization signal of the fibrillarin protein from Arabidopsis (Pih et al., 2000). In addition, GFP signals were found to accumulate in both the cytoplasm and the nucleus when Arabidopsis was transformed with the control vector without a specific targeting sequence, and no GFP signal was detected in wild-type, untransformed plants. Thus, these findings suggest that DG1 is targeted to the chloroplast.

Embryogenesis Was Affected in dg1

BLAST searches of the Arabidopsis database revealed that At5g67570 was cataloged as the candidate gene EMB1408 (for EMBRYO-DEFECTIVE1408) involved in embryo development. To confirm its involvement in the development of embryos, siliques from homozygous dg1 plants were dissected approximately 7 d after pollination, and cleared ovules were examined by differential interference contrast microscopy using a Nomarski optics microscope. The results showed that about 40% of the ovules were arrested at various stages, from the early globular stage to the heart stage, in dg1 plants, while the remaining 60% apparently developed normally, similar to wild-type ovules (Table I). The arrested embryos subsequently shriveled and finally degenerated. Impaired embryo development has also been observed in other mutants in which plastid development is affected (Uwer et al., 1998; Apuya et al., 2001; Hörmann et al., 2004; Baldwin et al., 2005; Kovacheva et al., 2005; Kobayashi et al., 2007). Thus, genes that affect the function and/or development of plastids may also be required for embryo development (Uwer et al., 1998; Apuya et al., 2001; Hörmann et al., 2004; Baldwin et al., 2005; Kovacheva et al., 2005; Kobayashi et al., 2007).

Expression Profiles of DG1

Light, which triggers the differentiation of nonphotosynthetic proplastids into fully functional photosynthetic chloroplasts, is one of the most important signals influencing chloroplast development (López-Juez and Pyke, 2005). To examine the effects of light on the expression of DG1 in wild-type plants, the accumulation of DG1 transcripts during the light-induced greening of...
etiolated seedlings was investigated. DG1 transcripts accumulated at very low levels in the etiolated seedlings, increased after illumination for 4 h, and reached maximal levels after 16 h of illumination (Fig. 6A).

To study the expression of DG1 at different developmental stages, levels of DG1 transcripts in leaves ranging from 1 to 4 weeks old were evaluated by northern-blot analysis. The expression level of DG1 decreased as the age and developmental state of both the plants and leaves increased. The DG1 transcript content of 4-week-old plants was only about 10% of that detected in 1-week-old plants (Fig. 6B), and levels of DG1 transcripts in the mature leaves were only approximately 20% of those in the young leaves of 6-week-old plants (Fig. 6C).

Expression of Chloroplast Genes in dg1

To assess the possibility that the delayed chloroplast development in dg1 mutants may be reflected at the level of photosynthetic gene expression, we also examined the expression of photosynthetic genes in dg1 by northern-blot analysis. Since there was a clear difference in $F_v/F_m$ ratios between young leaves (1–3 weeks old) and mature green leaves (>3 weeks old) in dg1 plants, differences in plastid transcript profiles were compared between young and old mature leaves as well as between mutant and wild-type plants. As shown in Figure 7A, there were no significant differences in the contents of PEP-dependent transcripts in mature green leaves of dg1 mutants, apart from slight increases in $psbA$ and $psaA$ transcripts. However, the levels of $psbA$, $psbB$, $psbC$, $psbEFJ$, $psaA$, and $petA$ transcripts were significantly lower in young leaves of dg1 mutants than in those of wild-type plants (Fig. 7A). Furthermore, $psbA$ transcripts were detected in the cotyledons of wild-type plants after growth for 3 d, and their levels increased with further development, while in dg1 cotyledons $psbA$ was expressed much more weakly until the plants were 7 d old (Fig. 7B).

Figure 4. Amino acid sequence alignment of DG1 PPR domains. Comparison of the amino acid sequence of the DG1 PPR domains with homologous sequences from O. sativa, M. truncatula, and Arabidopsis. Black boxes indicate strictly conserved amino acids among these proteins. Bars above the sequences labeled A to H indicate the eight PPR domains of DG1.
The delayed accumulation of PEP-dependent transcripts was consistent with the delayed greening phenotype of dg1. In addition, our results showed that the contents of rpoB transcripts were almost identical in mature green leaves of dg1 and wild-type plants, but they were considerably higher in the young leaves of dg1 plants. In contrast, levels of clpP and accD transcripts were higher in both young and mature leaves of dg1 plants (Fig. 7C). These findings are interesting, since clpP is transcribed by both PEP and NEP, while rpoB and accD are transcribed exclusively by NEP. Unlike the plastid genes mentioned above, transcript levels of the nuclear genes were largely unaltered in both young and mature leaves of dg1 plants (Fig. 7D).

DISCUSSION

Nucleus-encoded factors play essential roles in the regulation of chloroplast development, which requires the coordinated expression of both nucleus-encoded and plastid-encoded genes. In the study reported here, we identified a novel Arabidopsis mutant with delayed greening, dg1, and isolated the affected gene, which encodes a PPR protein targeted to the chloroplast. Subsequent genetic and molecular analyses showed that DG1 is probably involved in the regulation of early stages of chloroplast development and plastid gene expression.

Normal Chloroplast Development Was Delayed in dg1

The most distinct characteristic of dg1 was retarded greening of both its cotyledons and rosette leaves. The young leaves were initially chlorotic, then gradually greened during development in dg1 plants, and the mature, green leaves appeared similar to those of wild-type plants. Analyses of chlorophyll fluorescence in dg1 mutants revealed that photosynthetic activity was reduced in their young leaves (Fig. 1). In addition, ultrastructural observations showed that thylakoid membranes were less abundant in the young leaves of the mutants than in wild-type counterparts, but...
their abundance gradually increased as the leaves developed and eventually reached levels similar to wild-type levels (Fig. 2). These results suggest that DG1 plays important roles in early stages of chloroplast development and that functional chloroplast development was dependent on leaves reaching a mature or close to mature developmental state in the dg1 mutants.

Several mutants with impaired chloroplast development have been described. Sig6-1 mutants, which have no functional copies of the plastid sigma factor gene AtSIG6, have chlorotic young cotyledons, but a wild-type phenotype is restored in 8-d-old seedlings (Ishizaki et al., 2005). Repression of the ATP-dependent Clp proteases, Clp4 and Clp6, of Arabidopsis reportedly results in a chlorotic phenotype in young leaves, which moderates upon maturity (Sjögren et al., 2006; Zheng et al., 2006), but no such chlorosis has been observed in the cotyledons of Clp6 antisense plants (Sjögren et al., 2006). The severity of the chlorotic phenotype in young leaves in the cited mutants suggests that the affected chloroplast proteins may play important roles in early stages of chloroplast development. A possible explanation for the delayed chloroplast development is that other proteins may partly compensate for their absence during late stages of development. For instance, another sigma factor is likely to have overlapping functions with AtSIG6 when they are 8 or more days old (Ishizaki et al., 2005). Given the complexity of Clp protease complements, it is also

Table 1. The frequency of aborted seeds in dg1 siliques

Embryos about 7 d after pollination were examined by whole-mount seed clearing. Siliques from five different plants were scored for the presence of normal and aborted seeds at different stages.

<table>
<thead>
<tr>
<th>Phenotypic Class</th>
<th>Normal Seeds</th>
<th>Aborted Seeds</th>
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<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>dg1</td>
</tr>
<tr>
<td>Preglobular</td>
<td>51 (19%)</td>
<td>81 (11%)</td>
</tr>
<tr>
<td>Globular</td>
<td>64 (24%)</td>
<td>103 (14%)</td>
</tr>
<tr>
<td>Heart</td>
<td>75 (28%)</td>
<td>125 (17%)</td>
</tr>
<tr>
<td>Torpedo</td>
<td>40 (15%)</td>
<td>74 (10%)</td>
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<tr>
<td>Cotyledon</td>
<td>33 (13%)</td>
<td>66 (9%)</td>
</tr>
<tr>
<td>Total</td>
<td>263 (99%)</td>
<td>449 (61%)</td>
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Figure 7. Expression of chloroplast genes in dg1 mutant and wild-type (WT) plants. Total RNA samples from wild-type and dg1 mutant plants were size fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and probed with 32P-labeled cDNA probes. A, PEP-dependent chloroplast genes. B, psbA transcription accumulation during early seedling development. C, NEP-dependent chloroplast genes. D, Nuclear genes.

A

WT       dg1-young   dg1-mature
psbA
psbB
psaA
25S rRNA

B

WT       dg1
3       5       7       12 d
25S rRNA

C

WT       dg1-mature   dg1-young
accD
rpoB
clpP
25S rRNA

D

WT       dg1-mature   dg1-young
Rbccs
psbO
Cub
25S rRNA
Involvement of DG1 in Chloroplast Development

Swiatecka-Hagenbruch et al., 2008). In

dg1

expression (Hricová et al., 2006; Courtois et al., 2007; Baba et al., 2004; Pfalz et al., 2006). Inactivation of pTAC2, a PPR repeat protein, Nac2, is strictly required for the stabilization of psbD transcripts via a large protein complex that is associated with RNA (Boudreau et al., 2000), and a peptide chain release factor 2 has been shown to affect the stability of UGA-containing transcripts in Arabidopsis chloroplasts (Meurer et al., 2002). Thus, DG1 may be involved in regulation of the stability of PEP-dependent transcripts via its PPR motif associating with its mRNA targets. Finally, it is also possible that the reduced levels of PEP-dependent transcripts are due to inefficient assembly of the plastid ribosome, since (for instance) translation of PEP subunits is limited, and may account for reduced PEP activities, in ribosome-deficient mutants (Hess et al., 1993; Zubko and Day, 2002).

The developmental status of the chloroplast has been shown to control a set of nuclear genes that encode chloroplast-localized proteins via a process known as retrograde signaling (Surpin et al., 2002). However, as shown in Figure 7D, the transcript levels of the nuclear genes were not affected, although the chloroplast development was delayed, in dg1 mutants, suggesting that the retrograde signaling mechanisms controlling nuclear gene expression are intact in dg1. A similar absence of effects on the expression of nuclear genes encoding chloroplast proteins has also been observed in pac and dcl mutants, which have defective chloroplasts (Reiter et al., 1994; Meurer et al., 1998; Bellaloui et al., 2003), and the retrograde signaling mechanisms controlling palisade cell development are affected, but not those regulating nuclear gene expression.

PEP-Dependent Transcripts Were Dramatically Reduced in dg1

The expression of both nucleus- and chloroplast-encoded photosynthetic genes was tightly linked with the developmental status of the chloroplasts. In dg1 mutants, the levels of PEP-dependent transcripts were reduced, levels of NEP-dependent transcripts were increased, and levels of nuclear gene transcripts were not clearly altered (Fig. 7). It is generally accepted that NEP activities are largely responsible for the transcription of housekeeping genes during early stages of chloroplast development and that PEP activities increase while NEP activities decrease during subsequent chloroplast development (Hanaoka et al., 2005). Thus, NEP activities play important roles in the early stages of chloroplast development. Accordingly, the mutations of RpoT2, a NEP polymerase that is dually targeted to chloroplasts and mitochondria, result in only moderate defects in chloroplast gene expression and delayed greening of the leaves (Baba et al., 2004; Courtois et al., 2007), while the knockout mutants and transgenic lines with low levels of solely chloroplast-targeted NEP polymerase reportedly exhibit more severe defects in chloroplast development and gene expression (Hricová et al., 2006; Courtois et al., 2007; Swiatecka-Hagenbruch et al., 2008). In dg1 mutants, however, levels of PEP-dependent transcriptions were reduced (Fig. 7A), but not levels of NEP-dependent transcripts, suggesting that NEP functions efficiently in dg1. Thus, PEP is likely to have an important role in early stages of chloroplast development as well as NEP. In accordance with this hypothesis, the PEP transcription system is present in very early stages of plant development, even in dry seeds and during germination (Demarsy et al., 2006).

Three interpretations can be made to the observation of decreased accumulation of PEP-dependent transcripts. PEP is a multisubunit (bacterial-type) polymerase. Recently, in addition to the chloroplast-encoded catalytic core subunits, a number of polypeptides have been shown to be associated with transcriptionally active chloroplast chromosomes (Plantschmidt et al., 2000; Pfalz et al., 2006). Inactivation of pTAC2, a PPR protein, resulted in a seedling-lethal phenotype, resembling those of rpo mutants (Pfalz et al., 2006). This suggests that pTAC2 is an intrinsic component of PEP. DG1 is not detected in the transcriptionally active chromosome (Pfalz et al., 2006). Thus, DG1 may be involved in the regulation of PEP transcription machinery during early stages of chloroplast development, and the decrease of PEP-dependent transcripts might be due to impairment of the PEP transcription machinery in dg1. Another possible explanation for the reduced levels of PEP-dependent transcripts in the dg1 mutants is that the stability of the transcripts may be lower, or the rate of mRNA turnover enhanced, in them. In support of this hypothesis, a number of nucleus-encoded factors that affect the stability of chloroplast gene transcripts have been identified. For example, in Chlamydomonas, a 140-kD tetratricopeptide repeat protein, Nac2, is strictly required for the stabilization of psbD transcripts via a large protein complex that is associated with RNA (Boudreau et al., 2000), and a peptide chain release factor 2 has been shown to affect the stability of UGA-containing transcripts in Arabidopsis chloroplasts (Meurer et al., 2002). Thus, DG1 may be involved in regulation of the stability of PEP-dependent transcripts via its PPR motif associating with its mRNA targets. Finally, it is also possible that the reduced levels of PEP-dependent transcripts are due to inefficient assembly of the plastid ribosome, since (for instance) translation of PEP subunits is limited, and may account for reduced PEP activities, in ribosome-deficient mutants (Hess et al., 1993; Zubko and Day, 2002).

The developmental status of the chloroplast has been shown to control a set of nuclear genes that encode chloroplast-localized proteins via a process known as retrograde signaling (Surpin et al., 2002). However, as shown in Figure 7D, the transcript levels of the nuclear genes were not affected, although the chloroplast development was delayed, in dg1 mutants, suggesting that the retrograde signaling mechanisms controlling nuclear gene expression are intact in dg1. A similar absence of effects on the expression of nuclear genes encoding chloroplast proteins has also been observed in pac and dcl mutants, which have defective chloroplasts (Reiter et al., 1994; Meurer et al., 1998; Bellaloui et al., 2003), and the retrograde signaling mechanisms controlling palisade cell development are affected, but not those regulating nuclear gene expression.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Mutant and wild-type Arabidopsis (Arabidopsis thaliana ecotype Columbia) plants were grown in soil under short-day conditions (10-h-light/14-h-dark cycles) with a photon flux density of 120 μmol m⁻² s⁻¹ at a constant temperature of 22°C. To ensure synchronized germination, the seeds were incubated in darkness for 48 h at 4°C after sowing. For growth on agar plates, seeds were surface sterilized and sown on MS medium containing 2% Suc (except in the Suc-free treatment) and 0.8% agar.
Chlorophyll Fluorescence Analysis

Chlorophyll a fluorescence images were obtained at room temperature using a modulated imaging fluorometer (FluorCam; Photon System Instruments) according to the instructions provided by the manufacturer. Values of $F_0$ (for minimum fluorescence yield) and $F_m$ (for maximum fluorescence yield) were averaged to improve the signal-to-noise ratio. Image data acquired in each experiment were normalized to a false color scale, with arbitrarily assigned extreme values of 0.3 (lowest) and 0.85 (highest). This resulted in the highest and lowest $F_0/F_m$ values being represented by the red and blue extremes of the color scale, respectively.

Chlorophyll fluorescence was measured using a PAM 2000 portable chlorophyll fluorometer (Walz) connected by a leaf-clip holder (2000-B; Walz) with a trifurcated fiber optic cable (2010-F; Walz; Peng et al., 2006). Before each measurement, leaves were dark adapted for 10 min. The $F_m$ was measured using light (650 nm) with very low intensity (0.8 $\mu$mol m$^{-2}$ s$^{-1}$). To estimate the $F_0$, a saturating pulse of white light (3,000 $\text{mol m}^{-2}$ s$^{-1}$ for 1 s) was applied. The maximal photochemical efficiency of PSII was calculated from the ratio of $F_m$ to $F_0$ ($F_v/F_m = (F_m - F_0)/F_m$).

TAIL-PCR and RT-PCR

DNA fragments flanking the T-DNA were obtained by a series of three nested TAIL-PCRs (Liu et al., 1995). The products of the final PCR were subcloned into the pGEM-T Easy vector (Promega), sequenced, and subjected to BLAST analysis.

Total RNA isolation and RT-PCR were performed according to the manufacturer’s instructions. The specific primers used to amplify the cDNA of DG1 were as follows: sense primer, 5'-CTTTGAACGCTCTTCAGGTGAC-3'; antisense primer, 5'-TCATTCTTTCTTACTACTCTC-3'. The primers spanning intron 1 were as follows: PU, 5'-AGAGTGGCAATCCATTAGCAC-3'; and PD, 5'-TCTCCGTGGCCCTCTTGCCAAAC-3'.

Real-Time RT-PCR

Two different primer pairs were used to quantify the expression of transcript 1 and transcript 2. One primer pair was designed to specifically amplify transcript 2 (forward, 5'-CCACCATACCGACACCGAC-3'; reverse, 5'-ACATGACCACCGTTCA-3'). The other primer pair amplifies both transcripts (forward, 5'-CACCTGCCTGGACTACGCACT-3'; reverse, 5'-CATCA-AACATGACCACCGTCA-3'). The amplification of ELONGATION FACTOR-1α was used as an internal control for normalization. Experimental process and data analysis were performed according to Livak and Schmittgen (2001). The primers were designed using Primer Express software. PCRs were performed with an ABI 7900 system (Applied Biosystems) according to the manufacturer’s protocol.

Transmission Electron Microscopy

Samples of wild-type and mutant leaves were prepared for transmission electron microscopy by cutting them into small pieces, fixing in 2.5% glutaraldehyde in phosphate buffer for 4 h at 4°C, rinsing and incubating them in 1% OsO$_4$ overnight at 4°C, rinsing again in phosphate buffer, dehydrating in an ethanol series, infiltrating with a graded series of epoxy resin in epoxy propylene and then embedding them in Epon 812 resin. Thin sections were obtained using a diamond knife and a Reichert OM2 ultramicrotome, stained in propane, and then embedding them in Epon 812 resin. Thin sections were viewed with a transmission electron microscope (JEM-1230; JEOL).

Analysis of Embryo Development

Siliques from wild-type and mutant plants were dissected with hypodermic needles and cleared in Hoyer’s solution (7.5 g of gum arabic, 100 g of chloral hydrate, and 5 mL of glycerol in 30 mL of water) as described by Meinke (1994). Embryo development was studied microscopically using an Olympus BH-2 microscope equipped with Nomarski optics.

Complementation of the dg1 Mutant

The full-length DG1 cDNA amplified using PCR was cloned into the pBI121 vector under the control of the cauliflower mosaic virus 35S promoter. The resultant construct was transferred into Agrobacterium tumefaciens strain CS8, then introduced into dg1 homozygous plants using the floral dip method (Clough and Bent, 1998). Transformed plants were selected on MS medium containing 50 $\mu$g/mL kanamycin monosulfate. Seven resistant plants were transferred to soil and grown in a greenhouse to produce seeds. The success of the complementation was confirmed by measurements of chlorophyll fluorescence.

Subcellular Localization of GFP Proteins

DNA encoding the N-terminal region of the DG1 protein (amino acids 1–300) was amplified by PCR and ligated into the GFP fusion vector p221-GFP, which encodes a GFP under the constitutive control of the cauliflower mosaic virus 35S promoter. Two variants were then constructed: one in which the nuclear localization signal of the fibrillarin protein (Pih et al., 2000) and the other in which the transit peptide of the small subunit of ribulose biphosphate carboxylase of Arabidopsis (Lee et al., 2002) were also fused to the vector. The resulting fusion constructions and the control vector (GFP with no additional coding sequence) were introduced into wild-type Arabidopsis. The GFP in the examined samples was excited with the 488-nm laser line, and GFP emission was detected using a 505- to 530-nm bandpass filter.

Northern-Blot Analysis

Total RNAs were extracted, fractionated, and transferred onto nylon membranes, which were probed with $^3P$-labeled cDNA probes. Following high-stringency hybridization and washing, all of the blots were exposed to x-ray film for 1 to 3 d. The hybridization probes were prepared using a random priming labeling kit (Promega) with the PCR fragments of encoding regions of target genes. The sequences of the PCR primers used to amplify the genes are presented in Supplemental Table S1.

Supplemental Data

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

Supplemental Figure S1. Sequences of two transcripts of DG1.

Supplemental Table S1. Primers used to prepare the hybridization probes.

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LITERATURE CITED

Chloroplast development in Arabidopsis is involved in various processes, including seed development and plant growth. The NAC2 gene, homologue of the ethylene receptor, PpETR1, family. Plant Physiol 147: 715–733.


Chi W., Ma J., Zhang D., Guo J., Chen F., Lu C., and Zhang L. The Pentatricopeptide Repeat Protein DELAYED GREENING1 Is Involved in the Regulation of Early Chloroplast Development and Chloroplast Gene Expression in Arabidopsis.

The revised Figure 5D shown below is the result of an independent replicate experiment that corrects a copy-and-paste error that occurred during the preparation of Figure 5D for the published article. The legend was modified accordingly. The original conclusions of this article are not affected by this correction.

For ease of comparison, the original and corrected versions of Figure 5 affected by this mistake are presented below with the positions of the errors and corrections marked with a red box.

Figure 5. Original: Cellular localization of DG1 by GFP assays. Fluorescence signals were visualized using confocal laser-scanning microscopy. Green fluorescence indicates GFP, red fluorescence shows chloroplast autofluorescence, and orange/yellow fluorescence shows images with the two types of fluorescence merged. A, GFP signals from the DG1-GFP fusion protein. B, Control with the transit peptide of ribulose bisphosphate carboxylase small subunit. C, Control with the nuclear localization signal of fibrillarin. D, Control lacking the transit peptide. E, Wild type with no transformation. Bars = 5 μm.

Figure 5. Corrected: Cellular localization of DG1 by GFP assays. Fluorescence signals were visualized using confocal laser-scanning microscopy. Green fluorescence indicates GFP, red fluorescence shows chloroplast autofluorescence, and orange/yellow fluorescence shows images with the two types of fluorescence merged. A, GFP signals from the DG1-GFP fusion protein. B, Control with the transit peptide of ribulose bisphosphate carboxylase small subunit. C, Control with the nuclear localization signal of fibrillarin. D, Control lacking the transit peptide. Images were derived from an independent replicate experiment that was not performed in parallel with the other samples. E, Wild type with no transformation. Bars = 5 μm.