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Altered expression of the *Arabidopsis* ortholog of *DCL* affects normal plant development

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Abstract The *DCL* (*defective chloroplasts and leaves*) gene of tomato (*Lycopersicon esculentum* Mill.) is required for chloroplast development, palisade cell morphogenesis, and embryogenesis. Previous work suggested that DCL protein is involved in 4.5S rRNA processing. The *Arabidopsis thaliana* (L.) Heynh. genome contains five sequences encoding for DCL-related proteins. In this paper, we investigate the function of AtDCL protein, which shows the highest amino acid sequence similarity with tomato DCL. *AtDCL* mRNA was expressed in all tissues examined and a fusion between AtDCL and green fluorescent protein (GFP) was sufficient to target GFP to plastids in vivo, consistent with the localization of AtDCL to chloroplasts. In an effort to clarify the function of *AtDCL*, transgenic plants with altered expression of this gene were constructed. Deregulation of *AtDCL* gene expression caused multiple phenotypes such as chlorosis, sterile flowers and abnormal cotyledon development, suggesting that this gene is required in different organs. The processing of the 4.5S rRNA was significantly altered in these transgenic plants, indicating that *AtDCL* is involved in plastid rRNA maturation. These results suggest that *AtDCL* is the *Arabidopsis* ortholog of tomato *DCL*, and indicate that plastid function is required for normal plant development.

Keywords *Arabidopsis* · *Defective chloroplasts and leaves* · Chloroplast development · Plastid ribosomal RNA

Abbreviations DCL: *Defective chloroplasts and leaves* · GFP: Green fluorescent protein

Introduction

As in the eubacteria, the plastid rRNA genes are arranged in an operon and are transcribed as a large precursor RNA, in the order 16S, 23S and 5S (Kössel et al. 1985). However, plastid rRNA operons have some features that are distinct from those of prokaryotic rRNA. For example, in *Chlamydomonas reinhardtii* the region corresponding to the 5' end of the eubacterial 23S gene is divided into 7S and 3S rRNAs (Rochaix and Darlix 1982). Sequence comparisons have revealed that the 3S and 7S rRNAs are not unique to plastids, but are equivalent to the 5' terminal regions of the eubacterial 23S rRNA (Rochaix and Darlix 1982).

In the chloroplasts of flowering plants the 7S and 3S rRNAs are absent, but the 3' terminus of the 23S gene is split off as a separate gene termed 4.5S rRNA (Edwards et al. 1981). This small rRNA is separated from the mature 23S gene by short spacer that is removed from the precursor rRNA posttranscriptionally (Kössel et al. 1982). The primary transcript containing the 16S, 23S, 4.5S and 5S rRNAs is processed into the 16S and the 5S rRNAs, and the 23S–4.5S rRNA precursor. The latter is subsequently cleaved into the 23S and 4.5S rRNAs (Kössel et al. 1982, 1985; Strittmatter and Kössel 1984).

While there is extensive information about the organization of plastid rRNA operons (reviewed by Harris et al. 1994), little is known about the enzymatic mechanisms of rRNA maturation. Mutants blocked at various stages of rRNA cleavage would be helpful to identify nuclear genes that play critical roles in plastid rRNA processing. A gene of tomato, *defective chloroplasts and*

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leaves (*DCL*), which is required for rRNA maturation has been identified in our laboratory. *DCL* is ubiquitously expressed and encodes a small protein that is required for chloroplast development, palisade cell morphogenesis, and embryogenesis (Keddie et al. 1996; Bellaoui et al. 2003). Examination of the expression of several nuclear and plastid genes has suggested that *DCL* protein is involved in the processing of 4.5S rRNA (Bellaoui et al. 2003).

The objective of the present work was to gain insight into *DCL* function in *Arabidopsis thaliana*, a particularly good model system for this study because of the many genetic resources available. We have characterized the *AtDCL* gene, one of the *Arabidopsis* homologs of *DCL*. We show that the *AtDCL* gene is expressed ubiquitously and that *AtDCL* protein is efficiently imported into chloroplasts in vivo, confirming that *AtDCL* is plastid-localized. We also demonstrate that several phenotypic changes are induced when the expression of *AtDCL* is deregulated in transgenic *Arabidopsis* plants. Furthermore, we show that 4.5S rRNA maturation is affected in these transgenic plants, implying that *AtDCL* is most likely the *Arabidopsis* ortholog of *DCL*.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. were sown in optima soil (<http://www.optima.magnet.ch>) and kept for 2 days at 4°C before being moved into the growth chamber. Plants were grown at 22°C under long-day growth conditions (16 h light/8 h dark). Transgenic *Arabidopsis* seeds were surface-sterilized, and plated on Murashige and Skoog medium containing hygromycin (35 µg ml⁻¹). Hygromycin-resistant seedlings were transferred to soil and grown in long-day conditions in the growth chamber.

3' and 5' rapid amplification of cDNA ends (3' and 5' RACE)

Total RNA was extracted from *Arabidopsis* leaves. First-strand cDNA from total RNA was prepared using the Advantage RT-for-PCR Kit (Clontech). 3' and 5' RACE experiments were performed using the Marathon cDNA Amplification kit (Clontech) and the following *AtDCL*-specific primers:

- For 5' RACE:
 - 5'-CTC TTA AAA TCC GGG TGA TGC CCT ACC-3'
 - 5'-GCG AAA ATG TCT GAG GAT GAA ACT GTC-3'
 - 5'-TGG CTG AGG TCT CAC CCC AAA ACC-3'.

– For 3' RACE:

- 5'-GAT GCT ACT TCC TTA TCA TCC TGA ATG-3'
- 5'-GAC AGT TTC ATC CTC AGA CAT TTT CGC-3'
- 5'-CTC GGT TCC ACT ACA AGG TTT TGG GGT-3'.

Construction of the *AtDCL::GFP* fusion and the transformation vectors

The open reading frame of the *AtDCL* gene was PCR-amplified using primers that contained an extra restriction site for *NcoI*. The amplified fragment was digested with *NcoI* and cloned in-frame into the *NcoI* site of the green fluorescent protein (GFP) expression vector pGFP-MRC (Rodriguez-Concepcion et al. 1999) to express the fusion protein *AtDCL::GFP*. The nucleotide sequence of the resulting construct (named p*AtDCL::GFP*) was checked by sequencing.

For overexpression of *AtDCL::GFP* in *A. thaliana*, the p*AtDCL::GFP* construct was digested with *HindIII* to isolate the 35S::TL::*AtDCL::GFP*::Ter fragment. This fragment was then ligated into the pGPTV-HPT plant vector. The generated construct was introduced into *Agrobacterium tumefaciens* cells for plant transformation. Plants were inoculated with *A. tumefaciens* by dipping aerial parts of the plants for 20 s in dipping solution as described by Clough and Bent (1998).

To generate the antisense *AtDCL* construct, the open reading frame of the *AtDCL* gene was PCR-amplified by using primers that contained extra restriction sites. The amplified fragment was digested and cloned in antisense orientation into *HindIII*–*XbaI* sites of the plant vector pGPTV-HPT. The generated construct was further sequenced for confirmation and introduced into *A. tumefaciens* cells for plant transformation.

Subcellular localization

Protoplasts were isolated as described by Spangenberg and Potrykus (1995). Confocal imaging was performed using a Leica confocal laser-scanning microscope.

RNA gel blot analysis

RNA was extracted as described by Bonhomme et al. (1991). Northern blot experiments were conducted as described by Ausubel et al. (1990). 10 µg of RNA was hybridized to random-primed *AtDCL* probe. The probe was generated by PCR amplification of the coding region of *AtDCL*.

Fig. 1 Alignment of the predicted amino acid sequences of tomato (*Lycopersicon esculentum*) *DCL* and *Arabidopsis thaliana*-related genes. The alignment was created using MultAlin and Boxshade (ExPASy Molecular Biology Server and Boxshade server). Gaps that were introduced to improve the alignment are represented by dashes. Dark-shaded boxes indicate amino acids that are identical in at least three sequences and lighter shaded boxes indicate conserved amino acids. Stars indicate the DCL-domain

Species	AGI number	Sequence
DCL	6014934	1 -----MASICTSNFHCDCKNISSEFHHLLS--PSSLSESRCCGLLRCRCAAVKIGS
AtDCL	15220071	1 -MELASIPSSPVASPYFRCTYIFSFSSSPGLYFPRGDSTSRPRVVALRTES--DGA
DCL-related	15232596	1 -MISLLLRRLPLLNRNGELNRRHVGIVAGGL--LHRRRLLOSADRPOFYENNDVSPV
DCL-related	15225617	483 AWCGRNKTSSEIESGAGAWGSWGSPPTAEDKDTNEDDRNPSVSLKETSRREKDKERSQ
DCL-related	25404390	1222 -FSSPQCFLKAAKEGVRDDLQGSIDALANGKVPFGFGDCEETIISPKVHGFTTVDVY
DCL-related	20385491	1 -----MAEEQIVDSLSAE
DCL	6014934	53 GGGIISDNAELRREVSSTL---EITSESEELVKKESDDEVGKSGDGGVVDWEDTI
AtDCL	15220071	58 KIGNSESYGSELRRPRASE---EISEEPEEEENSE-----GEPVVDWEDTI
DCL-related	15232596	59 GSGAAAMN---VTSFVVDNSW---RYEPEPYRK-----LKNIEDETI
DCL-related	15225617	544 WCNPAKFPSSGGWENGGGAVWKGNNRNTFRPRSEENLAPMFTATRQRLSSTSEDETI
DCL-related	25404390	1279 GLLSSTKTRMRTNSAPKSKA----VQVDFGLLHSAFLKDKIKVLDGKGIPIMSLRTIFT
DCL-related	20385491	15 VNPDQIVDMEVETAPKASGDEKRERESEEEENGGEKQKQVGEEEKSGPVKLGPKRF
DCL	6014934	110 LEITVPLRQFVVMILHSEKYAIGDRLSEPHORTLEMLRLLPYHPEKPKIGEGVDVIVVY
AtDCL	15220071	105 LEVTVPLRQFVVMILHSEKYANRDRLSPEHEFTLEMLLPYHPEKPKIGEGDVIWVGH
DCL-related	15232596	95 LRDEPESLRLAKELHSEKYLDGRLDFDEKIVIKELLPYHPEKPKIGEGDVIWVGH
DCL-related	15225617	603 LSDIEPVRTLRKIHESAYPDGDESDDDKTFVLEKILNHPHPEKPKIGEGVDVIVVY
DCL-related	25404390	1336 WKNTEPLSQSLRILHSE--YEINELLNERDEGLV-KMVLQHPNSVEKIGEGVKGIRVAK
DCL-related	20385491	75 VTSIAMFDYFVK-FLHF--KPTDLLDNKYHMHLLLKKKCHSEKPKIGEGKRTFOVRT
DCL	6014934	170 HPRFENSRCFFVVRKDGETVDFSYKCKGGLTRKKNYPLMAYSFTHRFRKRRRND----
AtDCL	15220071	165 HPRFENSRCFFVVRKDGGEVDFSYKCKGGLTRKKNYPLMAYSFTHRFRKRRRND----
DCL-related	15232596	155 HPRFENSRCFFVVRKDGWVDFSYKCKGGLTRKKNYPLMAYSFTHRFRKRRRND----
DCL-related	15225617	663 HPRFENSRCFFVVSVDGAKQDFSYKCKGGLTRKKNYPLMAYSFTHRFRKRRRND----
DCL-related	25404390	1393 -SKHGSCCFEIVVRKDGTFDFSYKCKGGLTRKKNYPLMAYSFTHRFRKRRRND----
DCL-related	20385491	132 HPMK-SRCFFVVRKDDTADDFSRKCKDGLPLENMTPTGANGNGHGGEGGGGGRRG

Protein gel blot analysis

Total Protein extracts were prepared as described by Barkan (1998) and resuspended in Laemmli buffer (Laemmli 1970). Protein electrophoresis and western blotting were conducted as described by Grelon et al. (1994). Immunoblots were developed using the Super Signal kit (Pierce Chemical Co.).

Results

Arabidopsis homologous of DCL

Sequence comparison of tomato DCL with proteins in current databases revealed significant similarity to five sequences from the *Arabidopsis* genome-sequencing

project (Fig. 1). These sequences showed no significant homology to any protein sequence in the database other than DCL, except for a *DCL*-related gene, which is much larger than tomato *DCL* (AGI 2504390) and encodes a protein related to RNA polymerase subunit. One of these *DCL*-related sequences exhibited the highest amino acid sequence similarity with tomato DCL and was designated AtDCL. It shows 68% similarity and 60% identity over the entire open reading frame (Fig. 1). The highest sequence identity was seen at the C-terminus (92% similarity and 83% identity), which contains the DCL-domain found in all DCL-related proteins (Bellaoui et al. 2003). Analysis of these *Arabidopsis* DCL-related proteins using common targeting prediction programs (<http://www.inra.fr/Internet/Produits/Predotar/>) suggested that only AtDCL is localized in the chloroplast. We suggest, therefore, that AtDCL is the *Arabidopsis* ortholog of DCL.

Expression pattern of *AtDCL*

To begin the study of the function of *AtDCL*, expression analysis of the *AtDCL* gene was undertaken. A screen of the EST database identified two expressed sequence tags

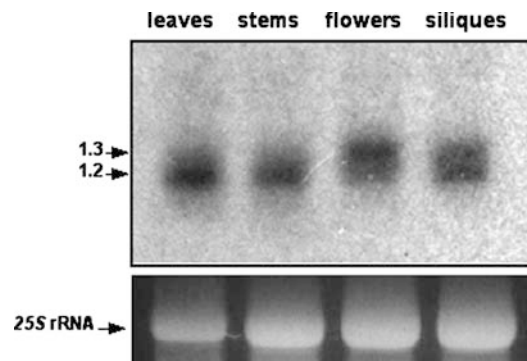


Fig. 2 RNA-blot analysis of the *AtDCL* gene. A 10- μ g sample of total RNA from each organ was loaded in each lane. The blot was hybridized with *AtDCL* cDNA probe. The numbers on the left indicate the approximate length of each transcript in kilobases. After ethidium bromide coloration of the gel, the cytosolic 25S rRNA is shown in the lower panel as a loading control

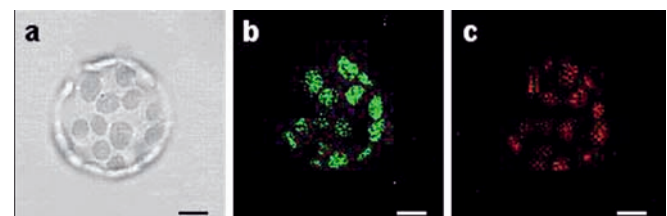


Fig. 3a-c The AtDCL protein imports the GFP into chloroplasts in vivo. Bright-field (a), fluorescence (b) and chlorophyll autofluorescence (c) images of a protoplast from a stably transformed *Arabidopsis* plant with AtDCL::GFP fusion. Bars = 10 μ m

Fig. 4a–c Phenotype of *A. thaliana* plants harboring the *35S::AtDCL::GFP* transgene. **a** Four-week-old wild-type (*top*) and *AtDCL::GFP-2* transgenic (*bottom*) plants. **b** Two-month-old wild-type (*left*) and *AtDCL::GFP-2* mutant (*right*) plants. **c** Variation in cotyledon number and shape in the progeny of the *AtDCL::GFP-2* line



(accession numbers: AI994762 and N38199). Neither of these cDNA sequences is complete (391 and 417 bp), and so 5' and 3' RACE experiments were performed using total RNA from leaves. The sequence of the full-length cDNA of *AtDCL* was then determined (1,151 bp).

Using *AtDCL* cDNA as probe, the expression pattern of *AtDCL* was examined by northern blot experiments. The *AtDCL* gene was expressed in all organs examined, including leaves, stems, flowers, and siliques (Fig. 2). This is consistent with the expression pattern of tomato *DCL*, which is also expressed in all types of tissues and stages of development examined (Keddie et al. 1996). A 1.2-kb transcript was detected in the four organs examined, consistent with the size determined by sequencing the full-length cDNA of *AtDCL*. However, a second transcript of 1.3 kb was detected in flowers, and siliques. Since *AtDCL* genes are quite different from each other at the level of DNA sequence, we assume that this 1.3-kb transcript in flowers and siliques corresponds

to differentially spliced transcript or resulted from an alternative transcript termination.

AtDCL targets GFP to the chloroplast in vivo

To confirm that *AtDCL* is targeted to plastids in vivo, an in-frame fusion between the coding regions of *AtDCL* and the gene for GFP was made and used for stable transformation of *Arabidopsis*. The expression of *AtDCL::GFP* fusion in protoplasts isolated from a stably transformed *Arabidopsis* plant was examined by confocal laser-scanning microscopy. The results of these experiments clearly show that the fusion protein colocalized with the chloroplast chlorophyll (Fig. 3). Without the putative transit peptide, GFP signals were dispersed in the cytoplasm and nucleus (data not shown). Thus, *AtDCL* is able to target proteins to plastids in vivo, confirming that *AtDCL* is a plastid-localized protein.

Analysis of AtDCL::GFP fusion overexpression

To explore the function of the *AtDCL* gene, phenotypic and molecular analyses of transgenic *Arabidopsis* lines overexpressing the *AtDCL::GFP* fusion were performed. For this, the *AtDCL::GFP* fusion was cloned downstream of the CaMV 35S promoter. This promoter was chosen because it is known to be constitutively expressed in *Arabidopsis*, an expression pattern that matches that of the *AtDCL* gene. This construct was transformed into *Arabidopsis* wild-type plants (ecotype Columbia).

In total, 15 transformants were obtained after selection on Murashige and Skoog medium containing hygromycin. When transferred to soil, 12 T1 transformants showed similar developmental defects and were further analyzed. The transformants were pale green (Fig. 4a), had chlorotic and poorly fertile flowers and their growth was extremely retarded compared to the wild type (Fig. 4b). The results presented here are all derived from studies of one of these transgenic lines, named *AtDCL::GFP-2*. Segregation analysis of the T2 generation indicated that a single T-DNA was inserted into the *AtDCL::GFP-2* line. Northern blot analysis revealed that this line accumulated a very high level of *AtDCL::GFP* transcript and GFP fluorescence indicated that the *AtDCL::GFP* fusion protein accumulated to high levels and was correctly localized in the plastids (Fig. 3 and data not shown). Therefore, these results suggest that the developmental defects observed in transgenic lines resulted from an overexpression of *AtDCL::GFP* fusion protein.

When germinated in vitro, the T2 progeny of the *AtDCL::GFP-2* line contained seedlings with abnormal cotyledon development (seedlings with one, three, fused or unequal cotyledons) (Fig. 4c). This phenotype is consistent with the role of tomato *DCL* during embryogenesis (Bellaoui et al. 2003).

To test whether *AtDCL* protein is involved in *4.5S* rRNA processing, we examined the expression of the small rRNA in the transgenic plants. As expected, the expression pattern of *4.5S* rRNA was severely affected in the *AtDCL::GFP-2* mutant (Fig. 5). In contrast, *5S* rRNA accumulated normally (Fig. 5). Together, these data suggest that overexpression of *AtDCL::GFP* affects *4.5S* rRNA formation, which in turn affects normal plastid function and plant development. Therefore, we suggest that overexpression of *AtDCL::GFP* behaves as a dominant negative mutation and indicates that *AtDCL* might play the same function as tomato *DCL*.

Analysis of antisense *AtDCL* expression

To further analyze *AtDCL* function, we generated transgenic *Arabidopsis* plants that constitutively expressed antisense *AtDCL* transcript. When selected on Murashige and Skoog medium containing hygromycin, true transformants with healthy roots segregated into normal green and albino seedlings. The latter grew

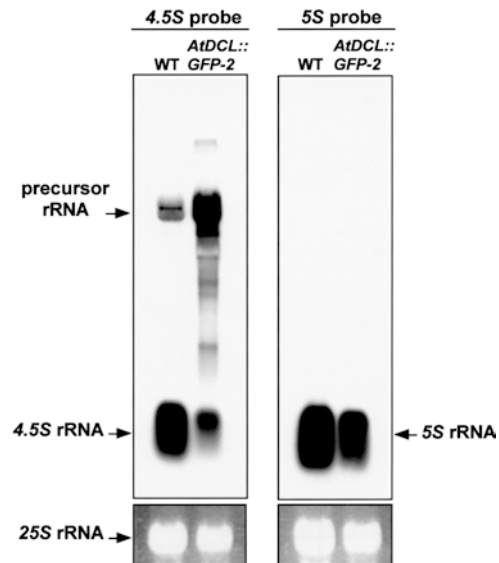


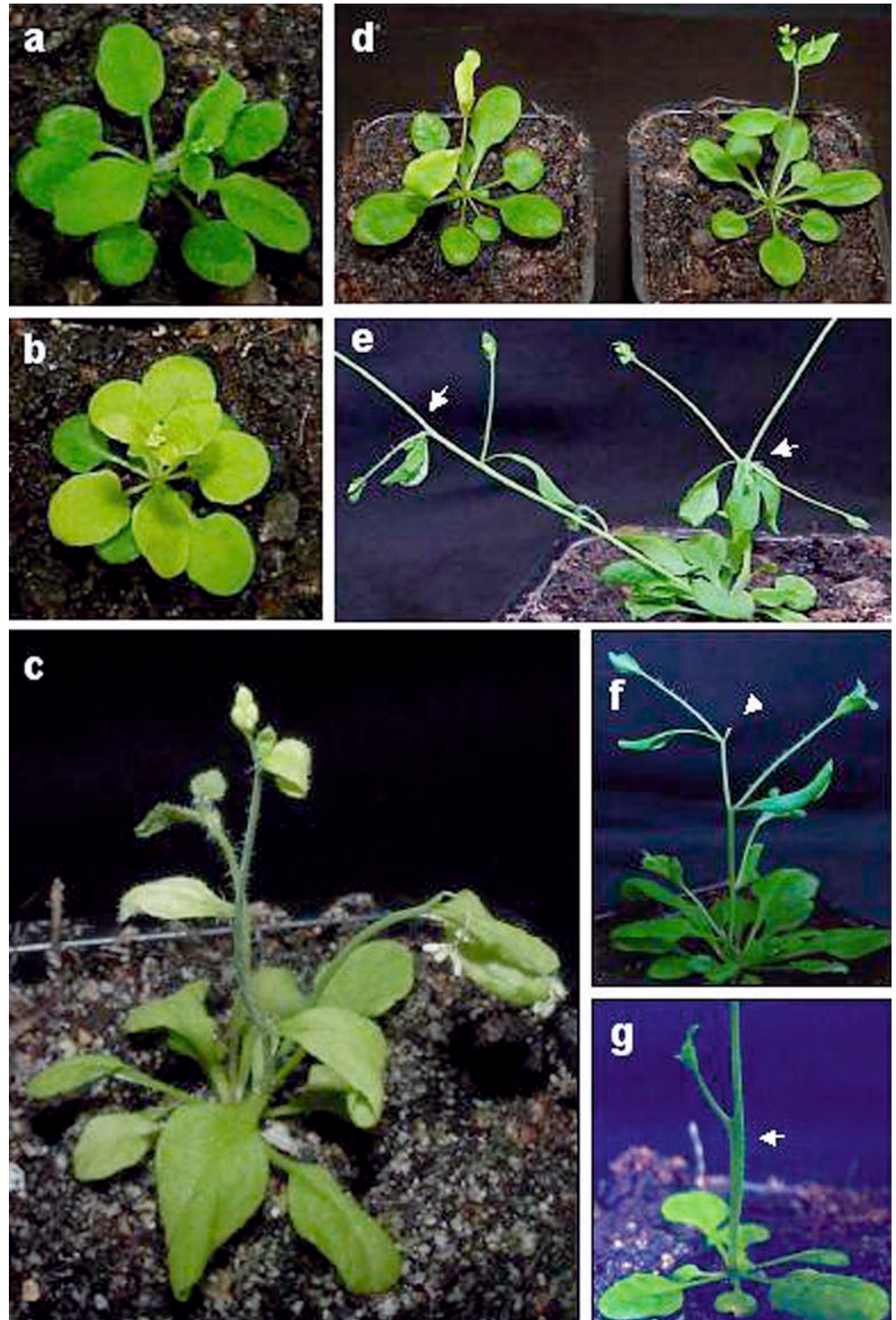
Fig. 5 Effect of overexpression of *AtDCL::GFP* on chloroplast gene expression in *A. thaliana*. Northern analysis of *4.5S* and *5S* rRNA expression. Total RNA samples from wild-type (*WT*) and *AtDCL::GFP-2* mutant (*AtDCL::GFP-2*) leaves were fractionated on agarose gels, transferred to nylon membrane and hybridized with labeled probes for *4.5S* and *5S* rRNA. After ethidium bromide coloration of the gel, the cytosolic *25S* rRNA is shown in the lower panels as a loading control

slowly and died before true leaves were formed which suggests that expression of antisense *AtDCL* is lethal.

The survivor T1 transformants were transferred to soil. From 26 T1 independent transgenic lines, 12 became chlorotic after 2 weeks and were further analyzed. They gave rise to few seeds and their T2 progeny segregated into normal and chlorotic seedlings characterized by variation in cotyledon number. Five-week-old siblings of the T2 progeny of one of the 12 transformants are shown in Fig. 6a (normal plant) and 6b (mutant plant). Mutant plants grew extremely slowly, had chlorotic flower buds and died after 7–8 weeks (Fig. 6c). In contrast, the normal progeny showed developmental defects at a more advanced stage: yellow inflorescence (Fig. 6d), initiation of many axillary meristems at the same place (Fig. 6e), shoot apical meristem arrested (Fig. 6f), stem fasciation (Fig. 6g) and fasciated siliques (data not shown). We assume that these plants still express the *AtDCL* gene albeit at reduced levels, while it is completely off in the mutant progeny exhibiting chlorosis at an early stage. Together, these data show that overexpression of antisense *AtDCL* causes chlorosis throughout the vegetative cycle of the plant and affects normal plant development.

RNA gel blot analysis was used to investigate the effect of overexpression of antisense *AtDCL* on the steady-state level of *4.5S* rRNA. Leaf tissue was collected for total RNA extraction from wild-type and antisense *AtDCL* mutant plants. Figure 7a shows that *4.5S* rRNA accumulation was dramatically reduced in antisense *AtDCL* mutant plants in comparison with the

Fig. 6a–g T2 progeny of the antisense *AtDCL-11* line of *A. thaliana* harboring the antisense *AtDCL* transgene. **a** Five-week-old normal plant. **b** Five-week-old mutant plant. **c** Eight-week-old mutant plant. **d** Six-week-old normal plant with yellow inflorescence (*left*) compared to wild-type plant (*right*). **e** Eight-week-old normal plant with many axillary meristems initiated at the same place (*arrows*). **f** Eight-week-old normal plant with arrested apical meristem (*arrowhead*). **g** Eight-week-old normal plant with stem fasciation (*arrow*)



wild type, which is consistent with previous data from overexpression of the *AtDCL::GFP* fusion.

We then tested the effect of the overexpression of antisense *AtDCL* on the accumulation of plastid proteins. Total leaf protein was extracted from mutant plants of two independent lines (antisense *AtDCL-11* and *AtDCL-21*), and protein gel blot analysis was performed using antibodies raised against plastid proteins.

While POR protein accumulated to near normal level, a strong reduction was observed in the abundance of CF1 α , PSI-D and LHCII proteins (Fig. 7b). This is consistent with the effect of *dcl-m* mutation on the accumulation of plastid proteins in tomato (Bellaoui et al. 2003).

Together, these results demonstrate that overexpression of antisense *AtDCL* affects 4.5S rRNA processing

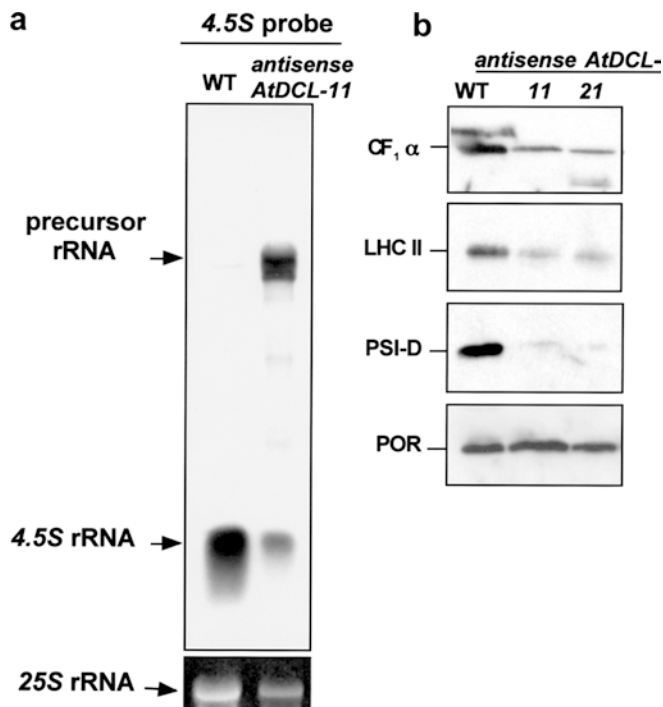


Fig. 7a, b Effect of overexpression of antisense *AtDCL* on chloroplast gene expression. **a** Total RNA was prepared from wild-type (*WT*) and antisense *AtDCL* mutant (line 11) leaves and analyzed by northern blot hybridization for the expression of 4.5S rRNA. After ethidium bromide coloration of the gel, the cytosolic 25S rRNA is shown in the lower panel as a loading control. **b** Immunoblot analysis of plastid proteins. Equal amounts of total protein from wild-type (*WT*) and antisense *AtDCL* mutant (lines 11 and 21) plants were separated on 12 polyacrylamide-SDS gels and immunoblotted with antisera raised against the α -subunit of ATP synthase (*CF1 α*), the light-harvesting chlorophyll *a/b*-binding protein of photosystem II (*LHCII*), the D subunit of photosystem I (*PSI-D*), and protochlorophyllide oxidoreductase (*POR*)

and strongly suggest that *AtDCL* protein is involved in plastid rRNA maturation.

Discussion

Five sequences with homology to tomato *DCL* have been identified in the *Arabidopsis* genome. A very high degree of conservation was seen at the *DCL*-domain-containing C-terminus of these sequences. However, no significant amino acid sequence similarity between these proteins was found at the N terminus, which suggests that the *Arabidopsis* homologs of *DCL* do not have identical activities. We assume that the different *DCL*-like proteins are functionally distinct members of a gene family sharing a common domain. Among those sequences, *AtDCL* exhibited the highest amino acid sequence similarity throughout its length with tomato *DCL*. We have demonstrated that, like tomato *DCL*, *AtDCL* is localized in the plastid. Expression analysis showed that *DCL* and *AtDCL* are normally expressed not only in photosynthetic but also in non-photosyn-

thetic tissue, suggesting that these genes are required for the development of different plastid types in different organs. Our findings that *AtDCL* and tomato *DCL* have the same pattern of expression and that are both localized in the same organelle suggest that they perform the same function.

To clarify the *in vivo* function of the *AtDCL* gene we investigated the physiological consequence of overexpressing *AtDCL* antisense RNA and *AtDCL::GFP* fusion in transgenic *Arabidopsis* plants. In both strategies, the deregulation of *AtDCL* gene expression caused multiple phenotypes. Chlorosis is the main developmental defect phenotype observed in transgenic plants. The chlorosis is the result of reduced chlorophylls and carotenoids, which reflect a block of chloroplast development. Deregulation of *AtDCL* gene expression caused other phenotypes such as sterile flowers, abnormal cotyledon development, fasciated siliques, arrested shoot apical meristem, and stem fasciation. This defect in chloroplast biogenesis that affects plant organ development is similar to that observed in many chloroplast developmental mutants. For instance, null mutations of the nuclear-encoded chloroplast genes *EDD1*, *SLP*, *Emb506* and *DCL* cause embryo abortion (Uwer et al. 1998; Albert et al. 1999; Apuya et al. 2001; Despres et al. 2001; Bellaoui et al. 2003). Similarly, mutations that block chloroplast biogenesis and alter palisade cell morphogenesis have been identified, and suggest that chloroplast development also regulates leaf differentiation (Reiter et al. 1994; Chatterjee et al. 1996; Babiychuk et al. 1997; Keddie et al. 1996; Wang et al. 2000). Because the primary defect in all of these mutants appears to be in chloroplast development, we suggest that these genes are involved in the initial events of plastid biogenesis, and that essential compounds for organ development are produced by the early-developed plastid.

We have previously shown that a defect in plastid rRNA processing is associated with *dcl-m* mutation, and therefore we suggested that the alteration of rRNA maturation is the primary lesion responsible for the blockage of chloroplast biogenesis in the *dcl-m* mutant (Bellaoui et al. 2003). In this paper we have shown that overexpressing the antisense RNA of the *AtDCL* gene affected rRNA processing. The same defect was observed when *AtDCL::GFP* fusion protein was overexpressed in transgenic *Arabidopsis* plants. Our data suggest that *AtDCL* activity might be required for rRNA maturation and provide further support for the proposed role of tomato *DCL* gene in rRNA processing (Bellaoui et al. 2003). In *Chlamydomonas*, a mutant defective in chloroplast 23S rRNA maturation has been reported (Boynton et al. 1970; Herrin et al. 1990). Holloway et al. (1998) have shown that the primary defect in this mutant, termed *ac20*, is associated with the maturation of the 23S rRNA itself.

A defect in chloroplast rRNA processing was also observed in other mutants such as *hcf7* in maize and *dall-2* in *Arabidopsis* (Barkan 1993; Bisanz et al. 2003). In the *hcf7* mutant, maturation of the 16S rRNA is blocked and

the *16S* RNA precursor accumulated in the monosome fraction of a polysome gradient, thereby reducing chloroplast translation efficiency (Barkan 1993). In *dall-2*, a defect in the maturation of *16S* and *23S* rRNAs was observed, suggesting that Dall protein is involved in plastid rRNA processing (Bisanz et al. 2003). While chloroplast mRNA processing has been extensively investigated, relatively little is known about plastid rRNA maturation. To our knowledge, *hcf7*, *ac20*, *dall-2* and *dcl-m* are the only examples of mutants that are defective in chloroplast rRNA processing, and are ideal tools for determining the enzymes that are involved in this process. Like tomato DCL, AtDCL does not contain obvious RNA-binding domains and does not show similarity to any known nuclease. Preliminary two-hybrid screening experiments show that AtDCL protein interacts with proteins known to be involved in RNA metabolism and ribosome biogenesis. When these interactions are confirmed, genetic and molecular analyses of the candidate enzymes should be helpful for dissecting the molecular mechanism of rRNA processing.

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