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Two Novel Proteins, MRL7 and Its Paralog MRL7-L, Have Essential but Functionally Distinct Roles in Chloroplast Development and Are Involved in Plastid Gene Expression Regulation in Arabidopsis

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Chloroplast development requires the coordinated action of various proteins, many of which remain to be identified. Here, we report two novel genes, *Mesophyll-cell RNAi Library line 7* (*MRL7*) and *MRL7-Like* (*MRL7-L*), that are involved in this process. An Arabidopsis knock-down transgenic plant (*MRL7*-RNAi) with delayed-greening phenotype was isolated from an RNA interference (RNAi) transformant library. Cotyledons and young leaves of *MRL7*-RNAi were pale in seedlings and gradually greened as the plant matured, while a knock-out in the *MRL7* gene was seedling lethal. The MRL7 protein was shown to co-localize with a marker protein for nucleoids in chloroplasts, indicative of a role for the protein in chloroplast nucleic acid metabolism. Accordingly, chloroplast development was arrested upon loss of MRL7 function and the expression of plastid-encoded genes transcribed by plastid-encoded RNA polymerase (PEP) was significantly reduced in *MRL7* knock-down and knock-out plants. A paralog of *MRL7* (*MRL7-L*) was identified in the Arabidopsis genome. Both *MRL7* and *MRL7-L* are only found in land plants and encode previously uncharacterized proteins without any known conserved domain. Like *MRL7*, knock-down of *MRL7-L* also resulted in a virescent phenotype, and a similar effect on plastid gene expression. However, the MRL7-L protein was localized to the chloroplast stroma. Taken together, our data indicate that the two paralogous proteins MRL7 and MRL7-L have essential but distinct roles during early chloroplast development and are involved in regulation of plastid gene expression.

Keywords: Arabidopsis • Chloroplast • MRL7 • MRL7-L • Plastid gene expression.

Abbreviations: CaMV, cauliflower mosaic virus; ECFP, enhanced cyan fluorescent protein; GFP, green fluorescent protein; GUS, β-glucuronidase; MS, Murashige and Skoog; NEP, nucleus-encoded RNA polymerase; pTAC, plastid transcriptionally active chromosome; ORF, open reading frame; PEP, plastid-encoded RNA polymerase; PPR, pentatricopeptide repeat; QRT–PCR, quantitative real-time RT–PCR; RNAi, RNA interference; RT–PCR, reverse transcription–PCR.

Introduction

Chloroplasts are central organelles of plants and other eukaryotic photosynthetic organisms, in which photosynthesis and many other biosynthetic processes take place (Post-Beittenmiller et al. 1992, Lichtenthaler et al. 1997, Estévez et al. 2001). Chloroplasts develop from protoplastids and are the typical plastids in leaf mesophyll cells (Valkov et al. 2009, Yu et al. 2009). Owing to their endosymbiotic origin they are semi-autonomous and carry their own genome. The plastid genome (plastome) of higher plants encodes only about 100–150 genes whose products are mainly related to photosynthesis and plastid gene expression (Sato et al. 1999, Schmitz-Linneweber et al. 2001). However, most chloroplast-localized proteins (>2,000) are nucleus encoded and must be imported into the chloroplast post-translationally (Chi et al. 2009). The coordinated expression of proteins encoded by the chloroplast and those which are encoded by the nucleus but destined for the chloroplast is assumed to be regulated by retrograde and anterograde communication between the plastid and the nucleus (Nott et al. 2006).

In higher plants, the plastid genes are transcribed by three types of RNA polymerases, one plastid-encoded RNA polymerase (PEP) and two nucleus-encoded RNA polymerases (NEPs) (Swiatecka-Hagenbruch et al. 2008, Lerbs-Mache 2010). Although it appears that most plastid genes can principally be transcribed by both types of RNA polymerases (Krause et al. 2000, Legen et al. 2002) they traditionally have been divided into three groups (Class I, II and III) based on whether

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they are preferentially transcribed by PEP, NEP or both (Hajdukiewicz et al. 1997). NEPs are required during early stages of plastid development and mainly transcribe genes (called Class III genes) whose products are related to plastid gene transcription. PEP is composed of the core subunits encoded by the *rpoA*, *rpoB*, *rpoC1* and *rpoC2* genes in the plastome and other nucleus-encoded components. PEP mainly transcribes plastid genes involved in photosynthesis (called Class I genes) (Weihe and Börner 1999). Plastid genes whose products usually are non-photosynthetic and housekeeping are transcribed together by both PEP and NEP (called Class II genes) (Hanaoka et al. 2005, Swiatecka-Hagenbruch et al. 2007). Six nucleus-encoded sigma factors have been identified participating in the PEP-dependent transcription of plastid genes through their promoter recognition specificity in Arabidopsis (Privat et al. 2003, Waters and Langdale 2009, Lerbs-Mache 2010).

PEP now has been recognized as a large protein complex. Using proteomics approaches, a range of nucleus-encoded factors were identified as PEP-associated proteins through purification and determination of PEP complex components from Arabidopsis and mustard chloroplasts (Suzuki et al. 2004, Pfalz et al. 2006, Schröter et al. 2010). A total of 35 proteins were identified to be part of the plastid transcriptionally active chromosome (pTACs), although the function of many of these remains unknown (Pfalz et al. 2006). PTAC2, -6 and -12 might be involved in plastid gene expression as their knock-out phenotype suggests (Pfalz et al. 2006). Although for several pTACs a role in plastid gene expression has been suggested, the mechanism of regulation of progress is only poorly understood (Pfalz et al. 2006, Arsova et al. 2010, Lerbs-Mache 2010). It is likely that during chloroplast development at least some of the pTACs are recruited to the PEP core complex as auxiliary proteins to adjust PEP function according to the requirements of a functional chloroplast. Loss-of-function individuals of the PEP-associated proteins seem to be seedling lethal in many cases (Pfalz et al. 2006, Arsova et al. 2010).

Plastid gene expression is regulated at various levels including transcription, RNA metabolism and translation (Sugita and Sugiura 1996, Pfannschmidt and Liere 2005, Maier et al. 2008, Stern et al. 2010). It has been suggested that there may be up to several hundred regulatory proteins involved in plastid gene expression at different stages of plastid development (Wagner and Pfannschmidt 2006, Schwacke et al. 2007, Schröter et al. 2010).

Recently, a range of large-scale reverse genetics efforts have been launched to identify and characterize chloroplast functions in *Arabidopsis thaliana* (Ajjawi et al. 2010, Bryant et al. 2011, Myouga et al. 2010). Complementary to these approaches, we conducted a high-throughput RNAi (RNA interference) screening of a mesophyll cell-specific RNAi transformant collection of Arabidopsis. This identified a previously uncharacterized gene we termed *Mesophyll-cell RNAi Library line 7* (*MRL7*) and subsequently its paralog *MRL7-Like* (*MRL7-L*). In this study, we report that both the paralogous proteins MRL7 and MRL7-L play essential but non-redundant roles in chloroplast development.

Results

Isolation and phenotypic characterization of *MRL7* RNAi plants

In a genomics effort to identify novel factors essential for chloroplast function in *A. thaliana* we employed a high-throughput RNAi approach in transgenic Arabidopsis plants and screened for transformants with visual phenotypic changes in leaf development and growth. A cDNA library prepared from RNA that was isolated from laser micro-dissection-captured Arabidopsis mesophyll cells was converted into an RNAi library through a novel technology termed PARL (Phi29 Amplified RNAi Library; C. Ma et al. unpublished data). The resulting RNAi library comprised ~160,000 individual clones, with each clone carrying an inverted repeat of a cDNA fragment from the entry mesophyll cell cDNA library. A population of RNAi plants was generated by bulk transformation of Arabidopsis plants with the whole RNAi library. From a total of about 5,300 primary transformants, 285 were identified as albino or pale green lines (data not shown). An individual line grown on Murashige and Skoog (MS) medium displaying a delayed-greening seedling phenotype and slow growth (middle column in **Fig. 1**) was identified and subsequently named as *MRL7*-RNAi. The cotyledons of *MRL7*-RNAi displayed a severely pale phenotype after germination on soil (right column in **Fig. 1**), and retained the pale phenotype for about 2 weeks and then gradually greened at around the six-leaf stage. Their inner young leaves were pale and turned green as plants grew bigger, but plant growth and development were markedly retarded. When the *MRL7*-RNAi plants were allowed to self-pollinate, the phenotype was stably inherited over at least three generations.

The cDNA that was inserted into the RNAi vector in the *MRL7*-RNAi was amplified and sequenced. A database search using BLAST (Altschul et al. 1990) indicated that it matched the 3′ end of the Arabidopsis At4g28590 cDNA. Reverse transcription–PCR (RT–PCR) results confirmed that the mRNA level of At4g28590 was significantly down-regulated in the *MRL7*-RNAi (**Fig. 2D**), suggesting that the virescent phenotype was caused by the down-regulation of At4g28590. The gene corresponding to the locus ID At4g28590 was referred to as *MRL7*.

A paralog of *MRL7* is present in Arabidopsis

MRL7 was annotated as encoding an unknown chloroplast protein in various databases. The corresponding polypeptide comprises 331 amino acids and has a predicted molecular mass of approximately 38 kDa. No conserved domain was found in its protein sequence. Searching the *MRL7* protein sequence against the Arabidopsis genome, a gene paralogous to *MRL7* was found (At2g31840) and named *MRL7-L* (*MRL7-Like*). *MRL7-L* was, just as *MRL7*, also annotated as encoding an unknown chloroplast

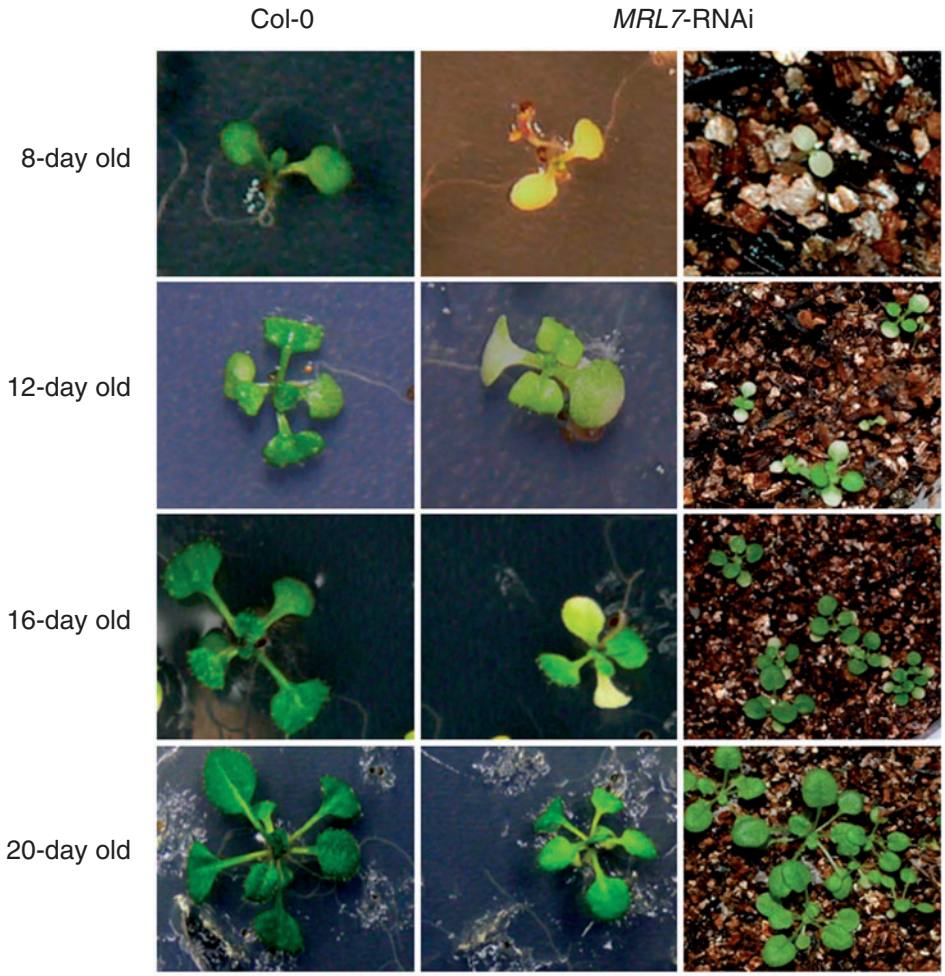

Fig. 1 Phenotype of *MRL7*-RNAi. Representative seedlings of *MRL7*-RNAi were grown on MS medium (supplemented with 2% sucrose) (middle column) and on soil (right column), respectively. Col-0 wild-type plants were grown on MS medium (left column) as control. Markers on the right indicate the ages of plants in each row.

protein. The MRL7-L polypeptide comprises 350 amino acids, slightly longer than MRL7, and has a predicted molecular weight of about 40 kDa. Protein sequence alignment through Blastp (NCBI) showed that they shared 42% identity and 56% similarity, respectively. No obvious functional domain or motif was predicted in MRL7-L either by any available bioinformatic tools, and no functional data about the two proteins could be found.

Protein sequence blast search analysis found *MRL7* and *MRL7-L* orthologs in other photosynthetic organisms including both dicot and monocot plants. These homologous proteins share one unknown conserved domain at their C-terminus and we named this domain the MRL7-related domain (MRL7R) (Supplementary Fig. S1). However, no obvious sequence similarities were found to the proteins of prokaryotic organisms and lower photosynthetic species, such as *Rhodobacter sphaeroides*, *Synechocystis* PCC6803 and *Clamydomonas reinhardtii*. In the lower land plants *Physcomitrella patens* and *Selaginella moellendorffii* only one protein sequence with similarities to both Arabidopsis *MRL7* and *MRL7-L* was found. A phylogenetic tree of MRL7 (MRL7-L) and its homologous sequences is shown in **Fig. 3**.

Knock-out of *MRL7* in Arabidopsis is seedling lethal

To establish further the relationship between the mutant phenotype and *MRL7* function, a T-DNA insertion line (Salk_075057) was obtained from the Arabidopsis Biological Resource Center (ABRC) and designated *mrl7*. Sequencing the T-DNA left border junction indicated that the T-DNA was inserted into the second intron of At4g28590 (**Fig. 2B**). When seeds of heterozygous *mrl7* were geminated on MS medium, the ratio of the green seedlings to pale seedlings was about 3 : 1, indicating that it is a single T-DNA insertion mutant. Homozygous plants were obtained by self-pollination (**Fig. 2C**), and RT–PCR analysis showed that the *MRL7* transcript was absent in these plants, indicating a complete knock-out (**Fig. 2D**).

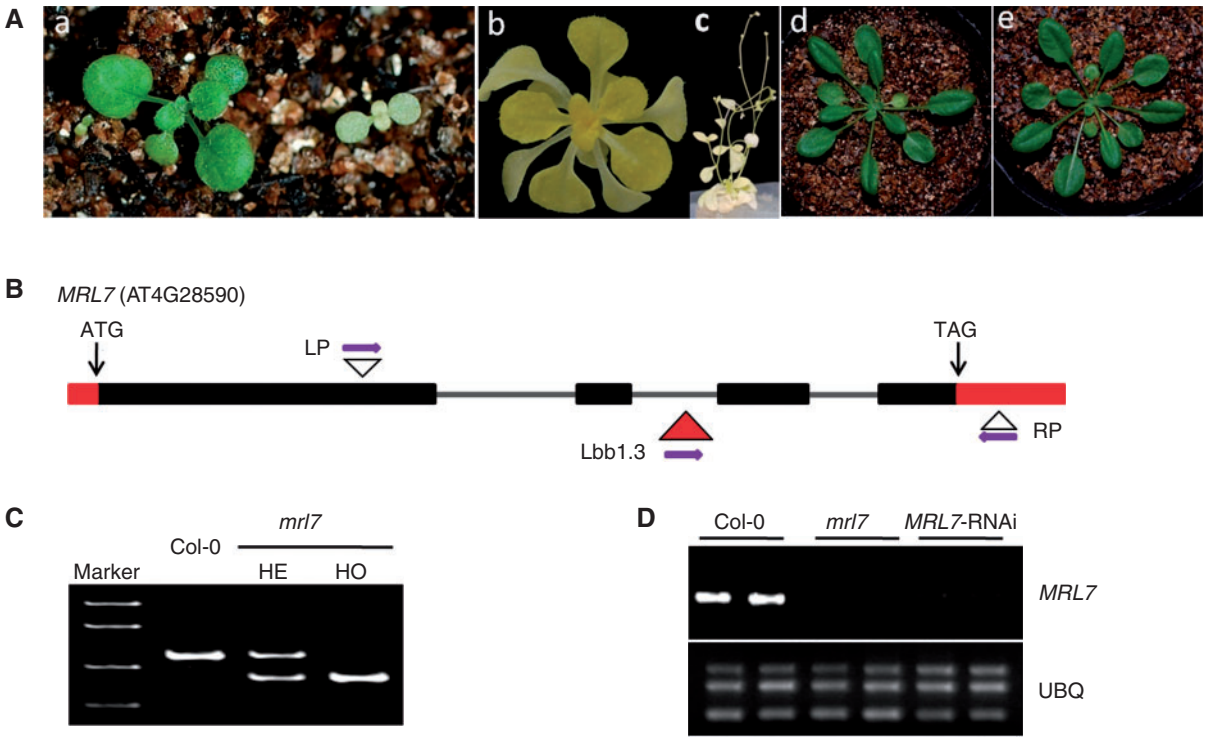

Fig. 2 Characterization of *mrl7*. (A) Phenotypes of *mrl7* and its complementation. Two-week-old Col-0 (left) and *mrl7* (right) seedlings grown on soil (a), 1- (b) and 2- (c) month-old *mrl7* grown on MS medium (supplemented with 2% sucrose), 4-week old wild-type control plant (d) and a representative *mrl7*-complemented plant (e). (B) Gene structure of *MRL7* and the T-DNA insertion position in *mrl7*. A red triangle indicates the insertion position of the T-DNA; LP, RP and LBb1.3 are the primers used for genotyping PCR. (C) A representative genotyping PCR result of *mrl7*. Offspring of the heterozygous *mrl7* were segregated into three genotypes, wild types (Col-0), heterozygous types (HE) and homozygous types (HO). (D) RT–PCR analysis of *MRL7* transcripts in *mrl7*, *MRL7*-RNAi plants and wild-type control. Ubiquitin (UBQ) served as a control.

When grown on soil under normal conditions, the homozygous *mrl7* failed to develop true leaves and could not survive longer than 2 weeks (**Fig. 2A-a**). However, on MS medium supplemented with 2% sucrose, the homozygous *mrl7* could continuously grow, develop true leaves (**Fig. 2A-b**) and even flower (**Fig. 2A-c**), but the whole plants always kept the pale phenotype and their development was greatly retarded. These results indicate that the MRL7 is essential for leaf greening and to establish autotrophic growth.

Ectopic expression of *MRL7* complemented the pale phenotype in the *mrl7* mutant

To prove further that the lack of *MRL7* transcript was responsible for the *mrl7* mutant phenotype, we performed a complementation experiment. As the homozygous *mrl7* are unable to grow autotrophically on soil, the full-length *MRL7* open reading frame (ORF) driven by the cauliflower mosaic virus 35S (CaMV35S) promoter was introduced into *mrl7* heterozygous plants. From 58 transformants, seven were found with an *mrl7* homozygous background by PCR genotyping. All those seven transformants could grow normally on soil and displayed a wild-type-like phenotype (**Fig. 2A-e**), suggesting that the loss-of-function phenotype in the homozygous *mrl7* was complemented by the ectopic expression of the *MRL7* cDNA. RT–PCR using primers specific for the *MRL7* coding region confirmed that the transcript of *MRL7* was present in those plants (Supplementary Fig. S2), further demonstrating that the *MRL7* ORF under the CaMV35S promoter is sufficient to restore the *mrl7* mutant phenotype and the disruption of *MRL7* is solely responsible for the *mrl7* mutant phenotype. However, when the same overexpression construct was introduced into wild-type Arabidopsis, all the transgenic plants were indistinguishable from the wild type (Supplementary Fig. S3).

Chloroplast development was impaired in *mrl7*

The apparent phenotype of *MRL7* knock-down (*MRL7*-RNAi) and knock-out (T-DNA insertion mutant *mrl7*) plants suggests that MRL7 might be essential for chloroplast development. Thus, we examined the ultrastructure of chloroplasts from 20-day-old *mrl7* plants grown on MS medium (supplemented with 2% sucrose) by transmission electron microscopy. In wild-type plants chloroplasts are crescent shaped and with well-developed thylakoids (**Fig. 4A, C**). However, in *mrl7*, the chloroplasts were abnormally shaped with much smaller sizes and lacked internal membrane structures, such as stromal thylakoids or stacked grana thylakoids (**Fig. 4B, D**). These results suggest that MRL7 is indeed required for an early step during chloroplast development.

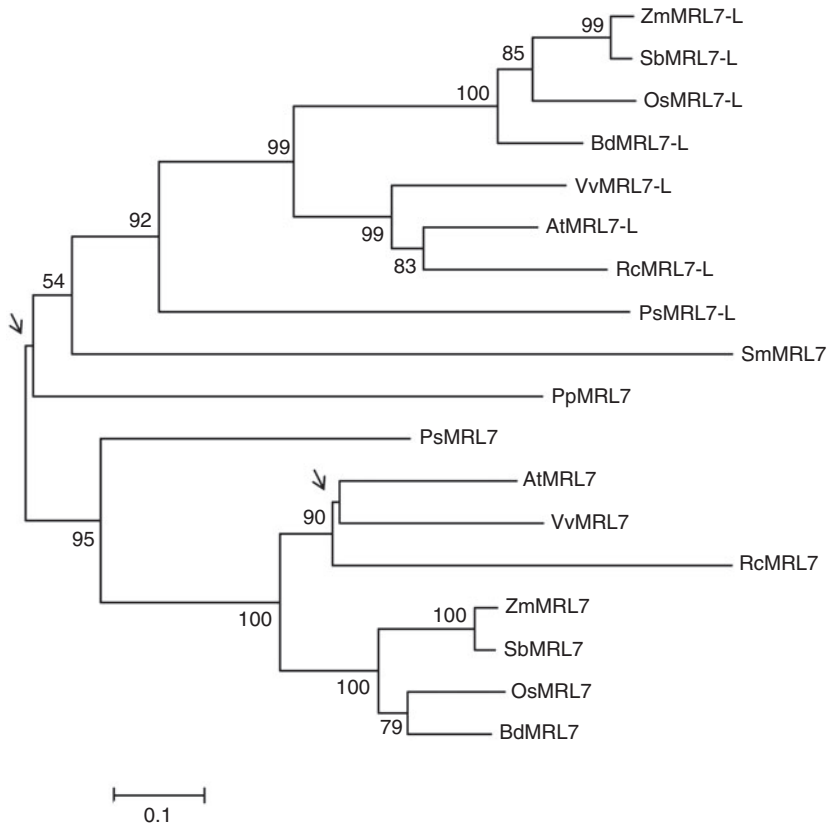

Fig. 3 The phylogenetic tree of MRL7 (MRL7-L) and its homologous proteins. The first two characters before the protein names MRL7 and MRL7-L are the abbreviations of the respective genus and species. At, *Arabidopsis thaliana*; Vv, *Vitis vinifera*; Zm, *Zea mays*; Os, *Oryza sativa*; Sb, *Sorghum bicolor*; Rc, *Ricinus communis*; Ps, *Picea sitchensis*; Pp, *Physcomitrella patens*; Sm, *Selaginella moellendorffii*. The unrooted phylogenetic tree was constructed by the Neighbor–Joining method with genetic distance calculated by MEGA 5. The numbers at each node indicate the bootstrap values (%) from 1,000 replications; the arrows indicate that the values <50% were hidden. The length of branches represents the extent of divergence according to the scale at the bottom.

Different localization of MRL7 and MRL7-L in chloroplasts

To examine the subcellular localization of MRL7 and MRL7-L, the respective full-length protein was fused with the green fluorescent protein (GFP) at the C-terminus. Driven by the CaMV35S promoter, the two MRL:GFP fusion constructs were individually introduced into tobacco leaf cells by particle bombardment, and the fluorescence was examined by confocal laser scanning microscopy. Both fusion proteins were exclusively localized within chloroplasts (**Fig. 5A**), indicating that MRL7 and MRL7-L proteins indeed are chloroplast-localized proteins. However, the fluorescent MRL7:GFP fusion protein displayed an apparently different distribution pattern as compared with the MRL7-L:GFP fusion protein (lower panel in **Fig. 5A**). In contrast to the evenly dispersed MRL7-L:GFP fluorescence in chloroplasts, the MRL7:GFP fluorescence exhibited a punctate distribution pattern (upper panel in **Fig. 5A**), which resembles the appearance of plastid nucleoids that are located within the stroma as small particles mostly associated with thylakoids (Sato et al. 1993, Arsova et al. 2010).

To test further whether MRL7 co-localizes with plastid DNA in nucleoids, a well-characterized chloroplast DNA-binding protein PEND (Sato et al. 1993) was fused with ECFP (enhanced cyan fluorescent protein) and served as a control. MRL7:GFP and PEND:ECFP plasmids were co-transformed into tobacco leaf cells by particle bombardment. As shown in **Fig. 5B**, the fluorescence signals of PEND:ECFP were observed in the chloroplast nucleoids and overlapped exactly with the MRL7:GFP fluorescence. The co-localization of the MRL7:GFP signals with the PEND:ECFP signals in the chloroplast indicates that MRL7 co-localizes with nucleoids in leaf chloroplasts. It was also examined whether MRL7-L co-localized with PEND like MRL7; however, the fluorescence of MRL7-L:GFP was distributed throughout the chloroplast stroma (data not shown), indicating that MRL7-L is probably a stromal protein.

Expression profiles of *MRL7* and *MRL7-L*

To elucidate further the physiological roles of *MRL7* and *MRL7-L*, we examined their organ-specific expression pattern in Arabidopsis. Expression data from Genevestigator

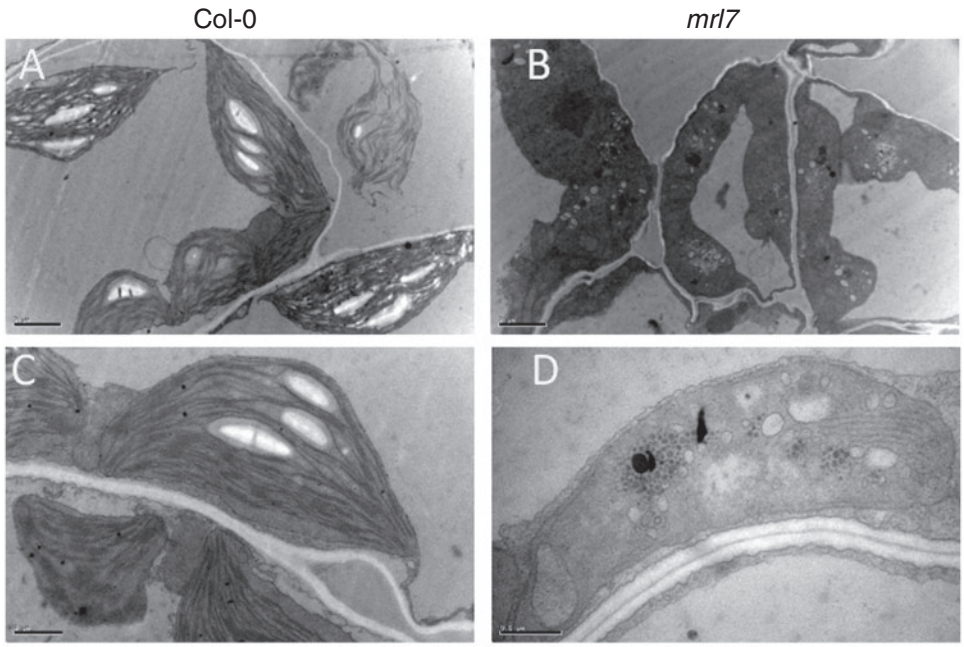

Fig. 4 Ultrastructure of chloroplasts in wild-type Arabidopsis (A and C) and *mrl7* (B and D). Transmission electron microscopy was carried out on 20-day-old leaves of *mrl7* and a wild-type plant. Bars = 0.5 μm.

(Zimmermann et al. 2004) showed that high expression of *MRL7* and *MRL7-L* was observed in seedlings, rosette, flowers and seeds (data not shown). The expression profile of *MRL7* was analyzed in detail. RT–PCR analysis using gene-specific primers showed that *MRL7* was highly transcribed in young leaves, shoots, flowers and young stem, but weakly expressed in adult leaves and roots (**Fig. 6A**). The results were confirmed by β-glucuronidase (GUS) staining of the transgenic plants expressing GUS driven by the *MRL7* promoter. High GUS activity was observed in seedlings, young rosette leaves, flowers and the developing seeds (**Fig. 6B**). These results indicate that MRL7 and MRL7-L expression is developmentally controlled and their expression pattern aligns well with stages in which plastid differentiation takes place.

Plastid gene expression was disturbed in both *MRL7* knock-down and knock-out plants

The fact that MRL7 has no known conserved domain poses a big challenge in deciphering its biological functions. However, the co-localization of MRL7 together with nucleoids might suggest an involvement of MRL7 in plastid DNA or RNA metabolism. Hence, quantitative real-time RT–PCR (QPCR) was performed in *MRL7* knock-out and knock-down plants to investigate the level of the transcripts of the plastid-encoded genes which are transcribed by PEP and (or) NEP. As shown in **Fig. 7A**, the analysis revealed a consistent decrease in mRNA levels of Class I genes (*psaA*, *psbA*, *psbK* and *rbcL*) and an increase in those of Class III genes (*accD*, *rpoA* and *rpoB*). The expression of nucleus-encoded genes (*psaE*, *psaH* and *psbO*) whose products are destined for the chloroplast was not affected in *MRL7*-RNAi and *mrl7* plants. The expression changes in *MRL7*-RNAi (white bars in **Fig. 7A**) were a little weaker than that in *mrl7* (gray bars in **Fig. 7A**), reflecting the different severity of the phenotype observed in the two genotypes. These results suggested that MRL7 is required for proper plastid gene expression.

Knock-down of *MRL7-L* phenocopies a down-regulation of *MRL7*

To evaluate the biological roles of *MRL7-L* in plant development, an *MRL7-L* knock-down transgenic line (named *MRL7-L*-RNAi) was created by using the artificial microRNA (Schwab et al. 2006) directed against the *MRL7-L* transcript. Thirty-nine of a total of 42 *MRL7-L* RNAi transformants also exhibited a consistent delayed-green phenotype like *MRL7*-RNAi. Cotyledons and inner young leaves were pale and then gradually greened (**Fig. 8**). These phenotypic characterization results showed that lack of MRL7 and MRL7-L resulted in the same phenotype, suggesting that MRL7-L is also essential for chloroplast development.

Since *MRL7* and *MRL7-L* are paralogous genes, we analyzed the expression of the two genes in the respective single mutants through QPCR (**Fig. 7**) to investigate whether their expression is mutually dependent. The results showed that the expression of each gene was not clearly affected by that of the other.

MRL7-L affects plastid gene expression in a similar way to MRL7

Considering the paralogous relationship between *MRL7-L* and *MRL7*, we also examined the expression of the plastid-encoded

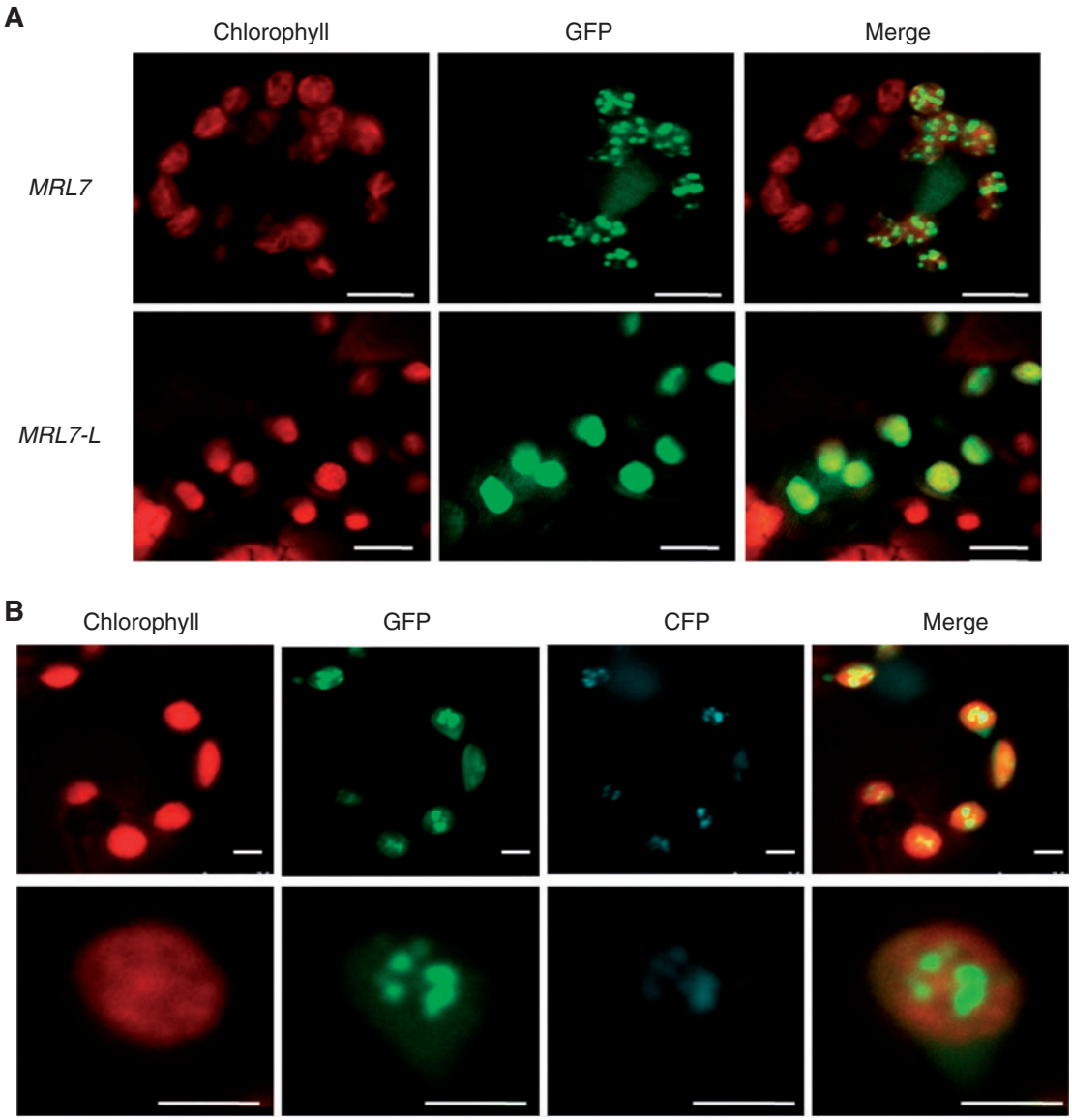

Fig. 5 MRL7 and MRL7-L localization in chloroplasts of tobacco epidermal leaf cells. The fluorescence signals were visualized using a confocal laser scanning microscope. (A) Subcellular localization of MRL7 (upper row) and MRL7-L (lower row); the merge shows the overlay of green fluorescence of the GFP fusion protein and Chl red autofluorescence. Bars = 10 μm. (B) Co-localization of MRL7:GFP fusion proteins (green) and PEND:ECFP (blue). Bars = 2.5 μm.

genes in *MRL7-L*-RNAi with QPCR. As in *MRL7*-RNAi and *mrl7*, the PEP-dependent transcription of all the genes examined was significantly reduced, while NEP-dependent transcription of the genes examined was increased in *MRL7-L*-RNAi (**Fig. 7B**), indicating that MRL7-L also plays an important role related to plastid gene expression like MRL7.

Chl fluorescence analysis of the *MRL7* and *MRL7-L* knock-down plants

In order to investigate the impact of MRL7 and MRL7-L on photosynthetic capacity during development, Chl fluorescence analysis was carried out on their RNAi knock-down plants with the typical virescent phenotype. Four-week-old *MRL7* and *MRL7-L* RNAi plants were used in the experiment. As shown in **Fig. 9**, all leaves of the wild-type Arabidopsis plants showed normal F_v/F_m ratios (maximal PSII quantum yield) of about 0.7–0.8. However, the inner young leaves of both *MRL7*-RNAi and *MRL7-L*-RNAi had abnormal F_v/F_m ratios of about 0.3–0.5, while the outer mature leaves had normal values of 0.7–0.8 like the wild type. The F_v/F_m ratios of the inner young leaves were much lower than those of their wild-type counterparts. As the young leaves grew bigger the F_v/F_m ratios could gradually increase to the normal values (0.7–0.8). These results revealed that photosynthetic activity was impaired in the inner young

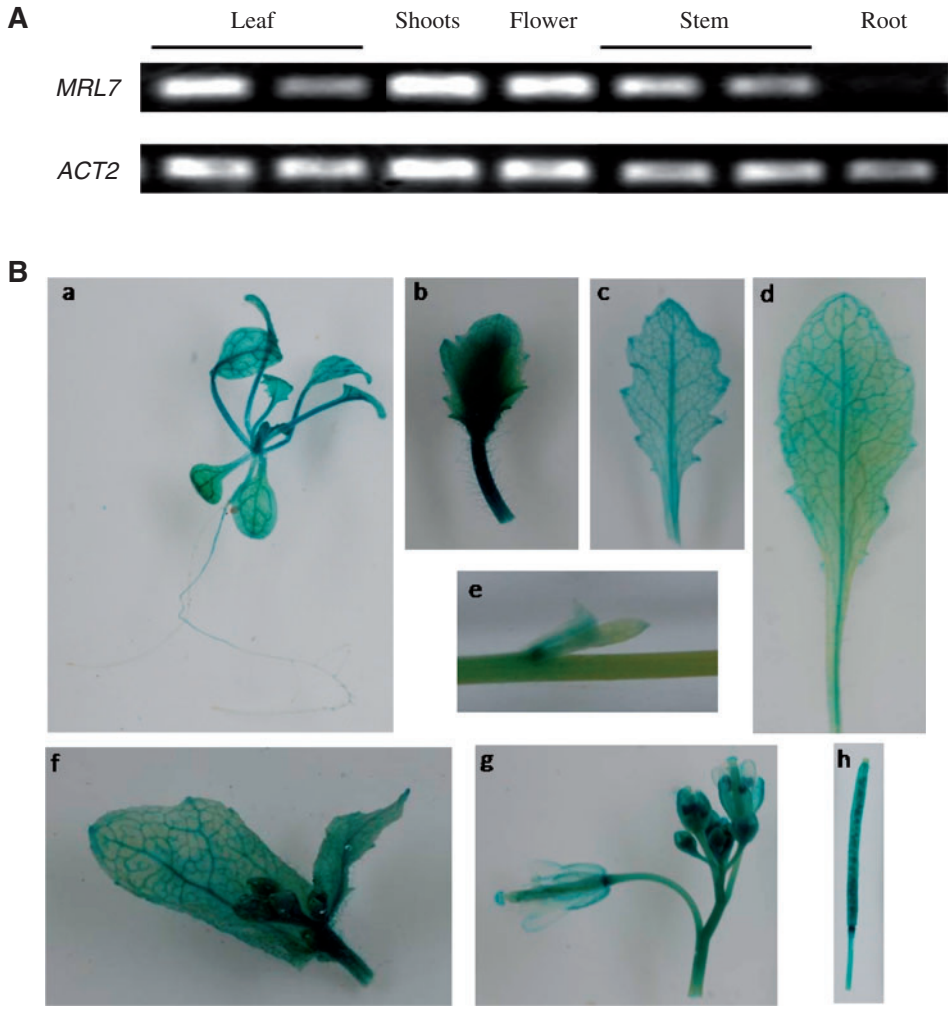

Fig. 6 The spatial expression pattern of the *MRL7* gene. (A) RT–PCR analysis of *MRL7* transcripts in various organs of wild-type Arabidopsis plants. For leaf and stem, both adult (left) and young (right) plants were used. (B) GUS staining of transgenic plants expressing GUS driven by the *MRL7* promoter. GUS staining was observed in whole seedling (a), shoot (b), young rossette leaves (c), adult rossette leaves (d), young cauline leaf (e), florescent buds (f), flowers (g) and siliques (h).

leaves of *MRL7* and *MRL7-L* knock-down plants and that each MRL7 (MRL7-L) protein function is required for early chloroplast development but becomes less relevant as leaves mature.

Discussion

Here we used a high-throughput RNAi approach to screen for genes that might have an essential function in leaves of Arabidopsis. The advantage of using RNAi or related approaches like antisense to identify novel factors with essential functions over the otherwise widely used T-DNA/transposon insertional mutagenesis is that the latter approach is untargeted and requires the generation of large mutant populations. Moreover, a homozygous knock-out in an essential gene is often lethal to the plant and thus precludes further functional analysis. In the case of RNAi, the efficiency of silencing varies considerably between individual transformants. This allows the isolation of 'leaky' transformants which retain sufficient expression of the target gene to enable the survival of the plant on soil but create a phenotype that can point towards a possible gene function.

Using this approach, we isolated a virescent RNAi transgenic plant (*MRL7*-RNAi) and subsequently identified two novel genes, *MRL7* and *MRL7-L*, which encode chloroplast-targeted proteins without any known conserved domain. Silencing of either *MRL7* or *MRL7-L* in Arabidopsis led to a delayed greening phenotype, and a knock-out of *MRL7* is seedling lethal. Plastid gene expression was disturbed in the *mrl7* mutant as well as in RNAi plants of both *MRL7* and *MRL7-L*. MRL7 and MRL7-L are paralogous proteins only present in land plants. They have different subplastidial localizations, with MRL7 co-localizing with nucleoids and MRL7-L being evenly dispersed throughout the stroma. Collectively, our results indicate that both proteins

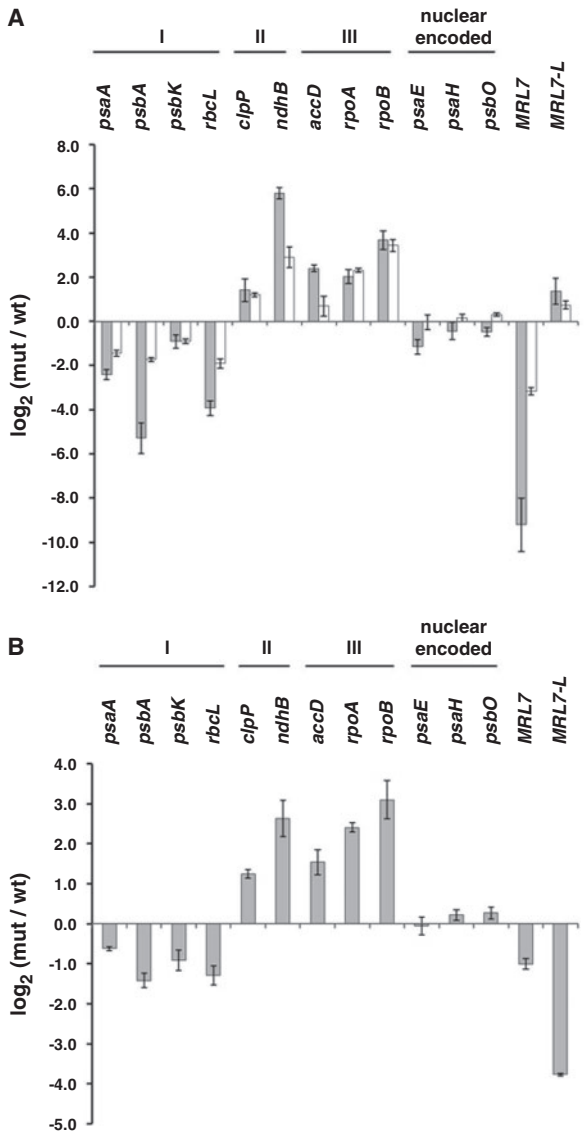

Fig. 7 Plastid gene expression changes in *mrl7*, *MRL7*-RNAi and *MRL7-L*-RNAi plants. (A) Changes of transcript abundance of the chosen genes in *mrl7* (gray bars) and *MRL7*-RNAi (white bars). (B) Changes of transcript abundance of the chosen genes in *MRL7-L*-RNAi. The log2 (mutant [mut]/wild type [wt]) value is shown as in Arsova et al. (2010), where 3.32 represents a 10-fold up-regulation and −3.32 a 10-fold down-regulation in the mutants compared with the control. I, Class I genes; II, Class II genes; III, Class III genes. 18S rRNA was used as a reference.

probably have distinct functional roles during early chloroplast development.

MRL7 possesses no conserved domain that could suggest a biological activity. *MRL7* was recently isolated as a suppressor of the *var2* mutation in Arabidopsis and named *SVR4* (for *SUPPRESSOR OF VARIEGATION4*) in that study (Yu et al. 2011). The *var2* mutant is characterized by green and white sectors (variegated) on photosynthetic organs, with white sectors having disturbed chloroplast development (Chen et al. 1999), and the *svr4-1* allele restores normal greening of *var2* (Yu et al. 2011). The *VAR2* gene encodes a plastid-localized homolog of the FtsH class of ATP-dependent metalloproteases (Chen et al. 2000). The exact function of VAR2 and the reason why its loss leads to a variegated phenotype is currently unclear but the protein has been implicated in a range of processes essential for early chloroplast development (Yu et al. 2004, Yu et al. 2007). Nor is it known why *SVR4*/*MRL7* is able to suppress the *var2* phenotype. A range of other *var2* suppressors thus far have been isolated and the corresponding gene functions are involved in a range of different processes (Park and Rodermel 2004, Miura et al. 2007, Yu et al. 2008, Liu et al. 2010), and a number of suppressors have been shown to negatively affect chloroplast rRNA processing or chloroplast protein synthesis. One hypothesis which has been put forward to explain the suppressor effect of these mutations is that defects in the corresponding gene functions slow down chloroplast biogenesis allowing VAR2 levels to rise above a certain threshold level sufficient for normal chloroplast development (Yu et al. 2004, Yu et al. 2007). The mutant allele *svr4-1* that has been isolated from an ethyl methane sulfonate mutagenesis carries a missense mutation giving rise to a non-conservative amino acid substitution (R291W) in the SVR4/MRL7 protein (Yu et al. 2011). The substitution obviously leads to a reduced function as the *svr4-1* allele displays a virescent phenotype in the wild-type background. This is similar to the phenotype of the *MRL7*-RNAi plants which also display a virescent phenotype and is in contrast to the lethal phenotype of the respective null allele (Yu et al. 2011; this study). Yu et al. (2011) showed that *svr4-1* mutants have enhanced non-photochemical quenching capacity which led to the proposal that SVR4/MRL7 acts to specify the parameters of non-photochemical quenching capacity early in chloroplast development perhaps indirectly by altering the rigidity and flexibility of PSII macro-organization. The localization of MRL7 to nucleoids and the changes in chloroplast gene expression we observed in *MRL7*-RNAi plants as well as in *mrl7* mutants leads us to hypothesize that MRL7 is necessary for proper plastid gene expression.

The expression pattern of plastid genes in *MRL7*-RNAi plants as well as in *mrl7* mutants resembles that of Δ*rpo* (for subunits of the RNA polymerase) mutants which lack a functional PEP (Allison et al. 1996, De Santis-MacIossek et al. 1999). In all of them, the expression of Class I genes transcribed by PEP is down-regulated, while the expression of Class III genes transcribed by NEP is up-regulated. The same situation is also found in *MRL7-L* RNAi plants, suggesting that both MRL7 and MRL7-L proteins somehow affect PEP function. So far, neither of the two proteins has been identified as a component of the PEP complex and thus it is currently unclear whether MRL7 or MRL7-L directly affects PEP function or whether this particular phenotype is a pleiotropic effect owing to a more general impact on chloroplast development in these plants. The localization data we obtained for MRL7 suggest that it is associated with plastid nucleoids, indicating that MRL7 could be involved in an early

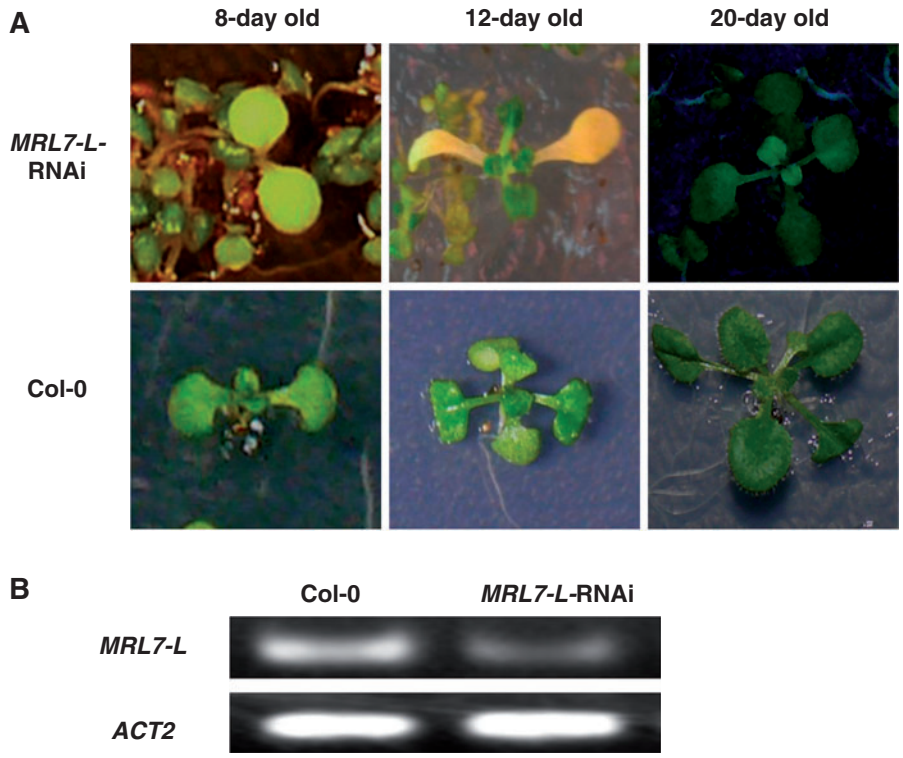

Fig. 8 Phenotypes of *MRL7-L*-RNAi. (A) Phenotypes of representative *MRL7-L*-RNAi plants at various ages. (B) RT–PCR analysis of *MRL7-L* transcripts in *MRL7-L*-RNAi plants and wild-type plants (as control). Actin2 gene was used as a control.

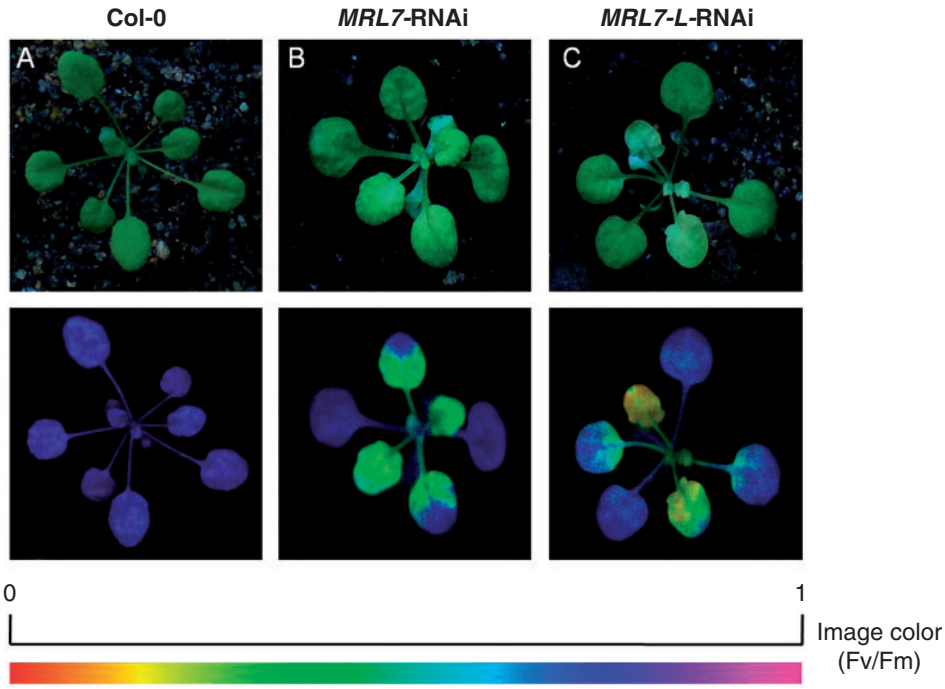

Fig. 9 Photographs and Chl fluorescence images of representative 1-month-old *MRL7*-RNAi and *MRL7-L*-RNAi plants. Fluorescence was measured by a MINI-Imaging-PAM Chlorophyll Fluorometer, and visualized using a pseudocolor index as indicated at the bottom.

step of plastid gene expression such as transcription or RNA maturation. Given the apparent stromal localization of the MRL7-L:GFP fusion protein it seems that MRL7-L affects plastid gene expression at a step different from MRL7. Given the likely functional specificity of the two proteins it would be interesting to see whether a reduced function of MRL7-L would also lead to suppression of *var2* as the leaky *svr4-1* allele does (Yu et al. 2011).

MRL7 and *MRL7-L* RNAi knock-down plants exhibited a virescent phenotype (**Figs. 1**, **8A**). This is a feature of, for instance, mutants impaired in RNA processing in chloroplasts mediated by pentatricopeptide repeat (PPR) proteins. A strong delay in greening can be observed in the *clb19* mutant, in which RNA editing of *rpoA* and *clpP* transcripts is defective, leading to a loss of PEP activity (Chateigner-Boutin et al. 2008). CLB19 is a PPR protein required for plastid RNA metabolism. Loss of other PPR proteins, such as YS1 (Zhou et al. 2009) or DG1 (Chi et al. 2009), has also been shown to result in a virescent phenotype and impaired PEP function. Neither MRL7 nor MRL7-L possesses obvious PPR or other RNA binding motifs and thus it is currently not known whether they could be directly involved in RNA metabolism. However, one explanation for the observed virescent phenotype could be that these two proteins might somehow affect the function of PPR proteins and thereby indirectly affect RNA metabolism. The virescent phenotype further suggests that the MRL7 and MR7-L protein function is particularly important during early chloroplast development. Chlorophyll fluorescence analysis of *MRL7*-RNAi plants showed that fully differentiated leaves had almost wild-type like fluorescence levels while young leaves were severely affected. This suggests that once the chloroplast is fully differentiated MRL7 function is dispensable under laboratory growth conditions.

Materials and Methods

Plant materials and growth conditions

Wild-type Arabidopsis ecotype Col-0 and its transgenic plants were grown on soil at 22°C under 16 h light/8 h dark photoperiod (photon flux density of 120 μmol m^{-2} s^{-1}) light conditions with 50–70% humidity. Seeds of Arabidopsis *MRL7*-RNAi, *MRL7-L*-RNAi and *mrl7* were surface sterilized and sown on MS medium (Sigma) supplemented with 2% sucrose (w/v) and 0.8% agar. Gene primers MRL7LP (5′-TCGGAGTATTTCGATACACATCC-3′) and MRL7RP (5′-GCACAGATTGACGAGTAAAATCC-3′), and the T-DNA primer Lbb1.3 were used for identifying the *mrl7* mutant. The tobacco seeds were germinated on soil and cultivated in a greenhouse at 25°C under a 14 h light/10 h dark photoperiod with 50–60% relative humidity.

Plasmid construction and plant transformation

The entire ORF of Arabidopsis *MRL7* was amplified from Arabidopsis cDNA using the primers MRL7F (5′-GGTACCATGAGTTTCTTCGCTGTTGCT-3′) and MRL7R (5′-CTGCAGACTAGTACAGTACGGGGTTACATTAGC-3′). The amplified PCR fragment was cloned into the *Kpn*I and *Pst*I sites of pCAMBIA1300S (Xiong and Yang 2003). The resultant construct was transformed into heterozygous *mrl7* for *mrl7* complementation or into wild-type plants for overexpression using the floral dip method (Clough and Bent 1998). Artificial microRNA for *MRL7-L* was designed according to the web-based tool (http://wmd.weigelworld.org) and integrated into miR319a by overlapping PCR using primers MRL7-LR1 (5′-GATCTTATGCTTAATTCACGCTTTCTCTCTTTTGTATTCC-3′), MRL7-LR2 (5′-GAAAGCGTGAATTAAGCATAAGATCAAAGAGAATCAATGA-3′), RL7-LR3 (5′-GAAAACGTGAATTAACCATAAGTTCACAGGTCGTGATATG-3′) and MRL7-LR4 (5′-GAACTTATGGTTAATTCACGTTTTCTACATATATATTCCT-3′) according to Chen et al. (2009). After sequencing the PCR fragment was cloned into pCAMBIA1300S at the *Kpn*I and *Pst*I sites; the resultant silencing construct was transformed into wild-type Arabidopsis.

Microscopy analysis

To create the GFP subcellular localization vector, a *Hin*dIII/*Eco*RI fragment carrying the double-enhanced CaMV35S promoter and the nopaline synthase A terminator from pCAMBIA1300S was inserted into pUC19 to create pUC19S. The eGFP coding region fragments were amplified from pCAMBIA1381xb-GFP (Hu et al. 2006) using primers GFPF (5′-ACTAGTATGGTGAGCAAGGGCGAGGAGCT-3′) and GFPR (5′-CTGCAGTTACTTGTACAGCTCGTCCATGC-3′). *MRL7* and *MRL7-L* ORF fragments (with *Kpn*I and *Spe*I sites), amplified using primers MRL7F and MRL7R as above, MRL7-LF (5′-GGTACCATGATTCTTCCATTTTCGACACAGTT-3′) and MRL7-LR (5′-ACTAGTATTCACACTTACATCGACTAAAGGAA-3′) and the eGFP coding region fragment (with *Spe*I and *Pst*I sites) were subsequently cloned into pUC19S to generate the pUC19S-MRL7:GFP and pUC19S-MRL7-L:GFP constructs, respectively.

The resultant constructs were transiently transformed into tobacco (*Nicotiana tabacum* var Samsun NN) leaf cells and the GFP fluorescence was observed as described in Arsova et al. (2010). The co-localization experiments were performed as described in Arsova et al. (2010). pUC19S-MRL7:GFP and pUC19S-MRL7-L:GFP plasmids were each co-transformed with PEND:ECFP (Arsova et al. 2010) into tobacco leaves. Double-labeled cells were scanned sequentially to prevent any cross-talk between fluorescence channels.

For the transmission electron microscopy analysis, leaves from the wild type and *mrl7* were cut into 0.5–1.0 mm^3 pieces, and then prepared and viewed as described in Yi et al. (2010).

RNA extraction and expression analysis

Total RNA were isolated from 3-week-old Arabidopsis plants using TRIzol reagents (Invitrogen) and then treated with RNase-free DNase (Fermentas) following the manufacturer's

instructions. For each sample, about 2 μg of total RNA was used to synthesize the first stand cDNA. The primers used for the first strand cDNA synthesis were oligo$(dT)_{20}$ for RT–PCR and random primers for QPCR using the Revert-Aid reverse transcriptase (Fermentas). RT–PCRs were performed to examine the *MRL7* transcript level in *MRL7*-RNAi and *mrl7*. For QPCR, the cDNAs were amplified using Brilliant II SYBR Green QPCR Mastermix (Stratagene) in an MX3000P real-time PCR instrument (Stratagene). PCR was optimized, and reactions were performed in triplicate. The transcript level was standardized based on cDNA amplification of 18S rRNA as a reference. Relative gene expression data were generated using the wild type as a calibrator. Fold induction values of target genes were calculated with the ΔΔCP equation according to Pfaffl (2001). Primer sequences for plastid and nuclear genes were as previously described (Arsova et al. 2010). The primer sequences for *MRL7s* used here are: RTMRL7F (5′-CGTGGTTGGAGATGATGG GAGT-3′), RTMRL7R (5′-TTCATCCGCCGTGCGAAAGTCT-3′), RTMRL7-LF (5′-TGTTCACAACCGCTACAAGAGG-3′), RTMRL7-LR (5′-TCTTGCGAATTCACGACACCAT-3′), ACT2F (5′-CCACAACAGCAGAGCGGGAAAT-3′) and ACT2R (5′-TGCT GGAATGTGCTGAGGGAAG-3′).

Phylogenetic analysis

Protein sequences of Arabidopsis MRL7 and MRL7-L were used to blast search their homologous protein sequences in the GenBank database and in the MIPS plant genomics group database (http://mips.helmholtz-muenchen.de/plant/). Multiple sequence alignment of these full-length protein sequences was performed using the ClustalW2 tool and displayed using Boxshade 3.21. An unrooted phylogenetic tree was constructed using MEGA 5 (Kumar et al. 2008) based on the Neighbor–Joining method.

Sequence data used in this article can be found in GenBank and in the MIPS plant genomics group database under the following accession numbers: the resultant sequences were AtMRL7, At4g28590; VvMRL7, XP_002272044; ZmMRL7, NP_001149252; OsMRL7, NP_001044686; SbMRL7, XP_002456577; RcMRL7, XP_002529828; PsMRL7, ABK26269; PpMRL7, XP_001783068; SmMRL7, XP_002962566; BdMRL7, Bradi2g53890; AtMRL7-L, At2g31840; VvMRL7-L, CAN75809; ZmMRL-7, NP_001140302; OsMRL7-L, NP_001055561; SbMRL7-L, XP_002441104; RcMRL7-L, XP_002533350; PsMRL7-L, ADE75897; BdMRL7-L, Bradi2g25250.

GUS activity assay

The 2,531 bp fragment upstream of the translation start point of *MRL7* was amplified using the primers pMRL7F (5′-GTCGAC TTTTTATTGCCTCTTTGTTTGGTTGATGTT-3′) and pMRL7R (5′-CCATGGTTCTCTCGTTTCCTAAATTCAAAACGAAAT-3′), and then cloned into *Sal*I and *Nco*I sites of pCAMBIA1391Z (http://www.cambia.org) to generate the pMRL7:GUS construct. The resultant construct was transformed into Arabidopsis and the pMRL7:GUS transformants were selected on MS medium with 30 μg ml^{-1} hygromycin (Roche), and GUS staining was performed as described in Caissard et al. (1994).

Chl fluorescence analysis

Chl fluorescence imaging and analysis was performed with a Chl imaging system (MINI-Imaging-PAM Chlorophyll Fluorometer, Walz); the photosynthetic parameters were determined as described in Horst et al. (2008). Before each measurement, plants were dark-adapted for 20 min.

Supplementary data

Supplementary data are available at PCP online.

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