RESEARCH PAPER

A 125 kDa RNase E/G-like protein is present in plastids and is essential for chloroplast development and autotrophic growth in *Arabidopsis* *

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Abstract

Endoribonuclease E (RNase E) is a regulator of global gene expression in *Escherichia coli* and is the best studied member of the RNase E/G ribonuclease family. Homologues are present in other bacteria but the roles of plant RNase E/G-like proteins are not known. *Arabidopsis thaliana* contains a single nuclear gene (At2g04270) encoding a product with the conserved catalytic domain of RNase E/G-like proteins. At2g04270 and the adjacent At2g04280 gene form converging transcription units with a 40 base overlap at their 3’ ends. Several translation products were predicted from the analyses of At2g04270 cDNAs. An antibody raised against a recombinant *A. thaliana* RNase E/G-like protein recognized a 125 kDa protein band in purified chloroplast preparations fractionated by SDS-PAGE. The 125 kDa RNase E/G-like protein was detected in cotyledons, rosette and cauline leaves. T-DNA insertions in exon 6 or intron 11 of At2g04270 result in loss of the 125 kDa band or truncation to a 110 kDa band. Loss of At2g04270 function resulted in the arrest of chloroplast development, loss of autotrophic growth, and reduced plastid ribosomal, *psbA* and *rbcL* RNA levels. Homozygous mutant plants were pale-green, contained smaller plastids with fewer thylakoids and shorter granal stacks than wild-type chloroplasts, and required sucrose at all growth stages following germination right up to flowering and setting seeds. Recombinant *A. thaliana* RNase E/G-like proteins rescued an *E. coli* RNase E mutant and cleaved an *rbcL* RNA substrate. Expression of At2g04270 was highly correlated with genes encoding plastid polyribonucleotide phosphorylase, S1 RNA-binding, and CRS1/YhbY domain proteins.

Key words: Arabidopsis, photosynthesis, polynucleotide phosphorylase, RNase E, ribonuclease.

Introduction

The conversion of chloroplasts from non-photosynthetic plastids is essential for autotrophic growth and plant development. Chloroplast development requires the coordinated expression of genes located in the nucleus and plastids (Lopez-Juez and Pyke, 2005) and is arrested by loss of plastid proteins encoded by the nuclear or plastid genomes. Albino plants arising from arrest of early chloroplast development can result from knockouts of expression-related plastid genes (Allison et al., 1996) or loss of plastid ribosomes (Zubko and Day, 1998, 2002). Deletions and knockouts in photosynthesis-related genes can block chloroplast development at a later stage resulting in pale-green plants in some cases (Klaus et al., 2003; Kode et al., 2006). The vast majority of proteins present in plastids are encoded by the nucleus (Lopez-Juez...
and Pyke, 2005). Genetic analyses can be used to distinguish between genes with essential roles from those with minor or redundant functions in chloroplasts. Mutations that give rise to albino or pale green cells have identified nuclear genes with important roles in chloroplast metabolism (Xu et al., 2002; Gutierrez-Nava et al., 2004) and gene expression (Chatterjee et al., 1996; Shirano et al., 2000; Wang et al., 2000; Bisanz et al., 2003; Albrecht et al., 2006; Motohashi et al., 2007).

Advances in chloroplast engineering have provided a new impetus for identifying nuclear genes affecting plastid gene expression. Promoters and ribosome-binding sites are known to be major determinants of transgene expression in transformed plastids (Maliga, 2004). Changes in mRNA stability also appear to modulate RNA levels (Shiina et al., 1998; Eibl et al., 1999; Zou et al., 2003) but the processes involved are poorly understood. Plastids are likely to be descendants of ancient cyanobacteria (Martin et al., 2002). Comparative genome analyses combined with reverse genetics provides a powerful approach for identifying the roles of plant proteins related to well-characterized bacterial proteins. The ribonuclease E/G (RNase E/G) family of proteins play important roles in RNA metabolism in bacteria (Grunberg-Manago, 1999; Callaghan et al., 2005a). Studies on RNase E, which was first identified as an enzyme required for ribosomal RNA processing (Apirion, 1978), underpinned rapid advances in our understanding of mRNA stability in bacteria. Escherichia coli RNase E makes the rate-limiting cleavage following 5′ pyrophosphate removal that initiates the decay of a large number of mRNA species (Mudd et al., 1990a, b; Babitzke and Kushner, 1991; Celenik et al., 2007; Deana et al., 2008). RNase E is now known to be an important regulator of global gene expression in E. coli (Callaghan et al., 2005a).

The role of RNase E/G-like proteins in eukaryotes is not known. Animals and fungi appear to lack genes encoding RNase E/G-like polypeptides. Genes for RNase E/G-like proteins are found in the nuclear genomes of flowering plants (Lin et al., 1999) and the chloroplast genomes of algae (Reith and Munholland, 1995; Douglas and Penny, 1999; Turmel et al., 1999). These observations are consistent with RNase E/G-like proteins playing a role in angiosperm chloroplasts. However, a number of proteomics studies have failed to detect RNase E/G-like proteins in angiosperm chloroplasts (Ferro et al., 2003; Friso et al., 2004; Kleffmann et al., 2004; Peltier et al., 2006; Pfalz et al., 2006; Baginsky et al., 2007). This work addresses whether RNase E/G-like proteins play a role in plastids using the model plant Arabidopsis thaliana. A. thaliana contains a single RNase E/G-like gene in its haploid genome (Lin et al., 1999) and is particularly amenable to reverse genetic analysis (Alonso et al., 2003; Rosso et al., 2003). The results in this paper show that the plant RNase E/G-like gene encodes a product present in chloroplasts that is essential for chloroplast development and autotrophic growth, displays ribonuclease activity, affects plastid RNA accumulation, and is co-expressed with nuclear genes involved in plastid RNA metabolism.

Materials and methods

Plant propagation and media

T-DNA mutants were generated in the context of the Salk (Alonso et al., 2003) and GABI-KAT programs (Rosso et al., 2003) and provided by the Nottingham Arabidopsis Stock Centre (UK), the Arabidopsis Biological Resource Centre (USA), and Bernd Weisshaar (MPI for Plant Breeding Research, Cologne, Germany). Arabidopsis thaliana seeds were sterilized in 70% ethanol for 2 min followed by sodium hypochlorite (3% active chlorine) for 5 min, then rinsed with four washes of sterile water before placing on MS medium solidified with 0.7–0.8% agar or 0.25% Phytagel (Sigma-Aldrich, Poole, UK) without or with the following supplements: 2% sucrose; 2% sucrose and 25–50 mg l⁻¹ kanamycin (Melford, Ipswich, UK); 2% sucrose and 20 mg l⁻¹ sulphathiazole (Sigma-Aldrich, Poole, UK). Seedlings and plants were propagated on solidified MS medium with 2% sucrose.

Analyses of T-DNA insertion lines

Supplementary Table 1 lists all primers and can be found at JXB online. WT and mutant alleles were identified using the following combination of PCR primers: 15 and gene-specific primers 16 and 17 for SALK_093546; 15 and gene-specific primers 21 and 22 for SALK_025999; 15 and gene-specific primers 23 and 24 for SALK_070705; 20 and gene-specific primers 18 and 19 for Gkn187E08. The SALK_093546 and Gkn187E08 mutations segregate as single recessive loci in progeny seedlings of heterozygous plants grown on media lacking sucrose. The SALK_093546 AtRne E/G mutant was kanamycin sensitive, probably due to silencing of the nptII gene used to select T-DNA insertions. Amongst 199 seedlings germinated on sucrose-free MS media from seeds obtained from selfed heterozygous SALK_093546 parents, 153 were green and 46 were white. This fits a 3:1 segregation of a single recessive locus. Plating Gkn187E08 seeds from selfed heterozygote parents on: (i) MS media with 20 mg l⁻¹ sulphathiazole gave rise to 107 resistant and 35 sensitive seedlings consistent with a single dominant resistance locus; (ii) MS medium lacking sucrose gave rise to 63 green and 23 white seedlings consistent with a single recessive locus. White SALK_093546 and Gkn187E08 seedlings were arrested in growth at the cotyledon stage while green seedlings formed true green leaves. White SALK_093546 and Gkn187E08 seedlings transferred to MS medium with sucrose gave rise to plants with pale-green leaves on sucrose-medium. Seeds from heterozygous SALK_093546 and Gkn187E08 parents germinated directly on MS medium with sucrose (2%) gave rise to approximately a quarter of pale-green plants, which were unable to grow on MS media lacking sucrose. However, the pale-green phenotype was more difficult to score for segregation purposes compared with the white phenotype on sucrose-free medium. All pale-green Gkn187E08 plants were sulphathiazole resistant, reflecting co-segregation of the mutant phenotype with the T-DNA insert containing Km. Seeds from a homozygous SALK_093546 mutant pale-green plant grown in vitro only gave rise to pale-green plants on sucrose medium. PCR analyses showed that all pale-green SALK_093546 and Gkn187E08 plants tested were homozygous mutants at the At2g04270 locus whereas dark-green plants were heterozygote or WT. DNA blot analysis of SALK_093546 lines confirmed the homozygote status of pale-green knockout plants (see Supplementary Fig. 1 at JXB).
online). Homozygous At2g04280-disrupted Salk_025999 lines were similar in appearance to WT plants and grew in soil. Of 110 seeds from selfed homozygous SALK_025999 plants germinated on MS medium lacking sucrose, all were green.

**Microscopy**

Sections were prepared as described for transmission electron microscopy (Kode et al., 2005) using a FEI Tecnai 12 Biotwin transmission electron microscope (FEI company, Eindhoven, The Netherlands).

**Cloning in E. coli**

Plasmids were constructed and propagated in *E. coli* strains DH5α, TOP10, or BL21(DE3)pLysS (Invitrogen, Paisley, UK). Strains with plasmids were propagated in Luria-Bertani (LB) medium containing ampicillin (50 μg ml⁻¹), kanamycin (40 μg ml⁻¹), or chloramphenicol (34 μg ml⁻¹). A cloned 1.35 kb cDNA probe, amplified with primers 6 and 7 from cDNA prepared using PolyA RNA from *A. thaliana* WT seedlings and oligo dt, was used to isolate a 2.4 kb cDNA (Fig. 2) from a Lambda Zap II library (Kieber et al., 1993). A 1.7 kb cDNA was amplified using outer primers 2 and 3, and inner primers 4 and 5 on oligo dT primed (primer 1) cDNA made using poly A-enriched leaf RNA. cDNAs were cloned into pGEM-T Easy (Promega, Southampton, UK). The 1.7 kb cDNA sequence was cloned between the *Nco*I and *Sal*I sites of the pTc99a expression vector (Pharmacia Biotech) to express the 64 kDa recombinant protein (Fig. 2). Tests for rescue of an *rne* mutation were carried out in *E. coli* KW086 [MC1061 recA rne<sup>trc</sup> zec-726: Tn10] (Wang and Cohen, 1994) grown at 43 °C. A 460 bp *Nco*I–*Sal*I fragment from the 1.7 kb cDNA was joined to a 1621 bp *Spl*-*Nco*I fragment from the 2.4 kb cDNA and cloned into pET30a (Novagen, Madison, USA). The resulting sequence contains 669 bp *AtRne* E/G codons and 45 upstream codons from pET30a and encodes a 79 kDa recombinant protein (Fig. 2). *AtRne* E/G cDNAs isolated in this work have been deposited in the EBI database with accession: AJ252122, AJ508604.

**Nucleic acid manipulations**

Procedures for blot analyses, hybridization probe preparation, phosphorimager analyses of band intensities using Aida software and PCR analyses have been described (Zubko and Day, 2002; Kode et al., 2005). The plastid rRNA probe (Zubko and Day, 2002) contains 1.5 kb of 16S rRNA and 1.6 kb of 23S rRNA sequences. Nested PCR primer sets 33 + 34 and 35 + 36 on plastid DNA. RNA for RT-PCR experiments was treated with RNase-free DNase I (Roche, Lewes, UK). A cloned 1.35 kb cDNA was amplified using outer primers 2 and 3, and inner primers 4 and 5 on oligo dt primed (primer 1) cDNA made using poly A-enriched leaf RNA. cDNAs were cloned into pGEM-T Easy (Promega, Southampton, UK). The 1.7 kb cDNA sequence was cloned between the *Nco*I and *Sal*I sites of the pTc99a expression vector (Pharmacia Biotech) to express the 64 kDa recombinant protein (Fig. 2). *AtRne* E/G cDNAs isolated in this work have been deposited in the EBI database with accession: AJ252122, AJ508604.

**Protein manipulations**

The Vector NTI AlignX program was used to compare RNase E/G-like proteins in pairwise alignments. Protein blot analysis was as described (Zubko and Day, 2002). Leaves were ground in liquid nitrogen and 300 μl of 1× sample buffer (62.5 mM TRIS-HCl, pH 6.8, 10% (v/v) glycerol, 3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue) was added per 100 mg of leaf powder. The homogenate was measured by optical density (at 630 nm) before loading on pre-cast 10% (w/v) polyacrylamide ready gels with 4% stacking gels (Bio-Rad Laboratories, Hemel Hempsted, UK) for SDS-PAGE in a Bio-Rad MiniProtein II system. The over-expressed 714 aa AtRnase E/G-like recombinant peptidase was purified by fractionation on preparative SDS–polyacrylamide gels, electroeluted from Coomasie-stained gel slices, or eluted from sonicated potassium-chloride-stained gel slices by diffusion at 4 °C. After concentration using Microcon centrifuge filter devices (Millipore UK Ltd, Watford, UK), approximately 400 μg of the purified polypeptide was used to make antibodies in sheep (Diagnosics Scotland, Edinburgh, UK). Prior to use against western blots, the antibodies were affinity-purified using overexpressed AtRnase E/G-like protein bound to nitrocellulose strips. The nitrocellulose strips were washed and affinity purified antibodies eluted as described (Mudd and Higgins, 1993). Proteins from SDS-PAGE gels were transferred to Hybond-ECL nitrocellulose (Amersham, Little Chalfont, UK) by electroblotting in western transfer buffer (25 mM TRIS, 192 mM glycine, 20% (v/v) methanol, pH 8.3) in a Bio-Rad mini transblot cell (50 min at 100 V). After staining with Ponceau red to ensure even transfer, blotted nitrocellulose sheets were incubated with primary antibodies against *AtRnase* E/G, LS Rubisco CO or α-tubulin (Zubko and Day, 2002). Binding of the primary antibody was detected using secondary antibodies linked to alkaline phosphatase (*Sigma*-Aldrich, Poole, UK) and staining with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) liquid substrate (Sigma-Aldrich, Poole, UK). Highly purified intact chloroplasts were prepared on Percoll (Amersham, Little Chalfont, UK) gradients (lower layer 85% Percoll mix; upper layer 40% Percoll mix) as described by Gruissem et al. (1986).

**In vitro cleavage assays**

The template for RNA synthesis was made by PCR with oligonucleotides 31 and 32 on a cloned *Brassica napus* plastid DNA sequence (A Day and K van Zuilen, unpublished data) containing the *rbcL* gene. These primers would amplify the region between co-ordinates 54 778 and 56 778 in the *A. thaliana* plastid genome (Sato et al., 1999). The inclusion of the T7 promoter region in primer 31 allows the purified PCR product to be used as a template to synthesize RNA using T7 RNA polymerase (Milligan and Uhlenbeck, 1989). Preparation of radiolabelled RNA with T7 RNA polymerase (*Roche*, Lewes, UK) and 400 Ci mmol⁻¹[^32P]-UTP (GE-Healthcare, Little Chalfont, UK) and conditions for induction of expression, preparation of cell extracts, *in vitro* assays, and fractionation of products on 6% urea-polyacrylamide gels have been described (Mudd and Higgins, 1993).

**Results**

**Structure and transcription products of the AtRnase E/G gene**

An RNase E/G-like protein is encoded by gene At2g04270 on *A. thaliana* chromosome 2 (Lin et al., 1999) adjacent to a gene (At2g04280) of unknown function (Fig. 1). Transcripts from both genes overlap by ~40 bases in their 3′ untranslated regions, which might
influence the polyadenylation site (Jen et al., 2005; Wang et al., 2005). The At2g04270 RNase E/G-like gene (AtRNase E/G-like) was poorly expressed, hindering the analysis of transcription products. Analyses of cDNAs predict four polypeptides of 111, 97, 79, and 69 kDa (Fig. 2). A 2.55 kbp cDNA (AY093213) from a full-length cDNA collection (Seki et al., 2002) would encode a 79 kDa protein starting with the amino acids MQTD. Overlap of the 5’ ends of RACE products with EST AV529253 gives a contig (RNA 2 in Fig. 2) encoding a 97 kDa protein with amino acids MIMN at the amino terminus. A splice variant extending exon 4 was detected by RT-PCR using a primer located at the start of Est AV529253. The frame shift resulting from the 23 base insertion predicts an alternative start codon located in exon 1 (RNA 1 in Fig. 2) which would give rise to a 111 kDa polypeptide starting with amino acids MDVT. This 111 kDa polypeptide was co-linear with sequence accession AF450479 (M Walter and J Kudla, unpublished results). RT-PCR using primers located in intron 12 and exon 5 amplified a 1.7 kb cDNA product. This truncates the C-terminus and would shorten each protein; the 79 kDa protein would be reduced to 69 kDa. The presence of intron 12 in a cDNA might reflect alternative splicing or amplification of a partially processed pre-mRNA species. Targeting programs predict a plastid location for the 111 kDa protein and a cytosolic location for the 79 kDa protein (Emanuelsson et al., 2000; Small et al., 2004; Hoglund et al., 2006). Protein analyses (see below) are required to confirm the presence of one or more of these predicted translation products.

Structure of the AtRNase E/G-like protein

The domains of E. coli RNase E have been located in the 2.9 Å crystal structure (Callaghan et al., 2005a). Only the N-terminal catalytic domain of E. coli RNase E aligns with residues 327–839 of the 996 aa AtRNase E/G-like protein (Fig. 3). The highest identity (42%) was found in the DNase I-like domain. Large N-terminal extensions preceding the catalytic domain were found in the plant proteins. The C-terminal regions of the A. thaliana and rice proteins exhibit greater sequence identity (53%) than the N-terminal extensions (11%). Unlike the E. coli and Synechocystis proteins, the Chlorobium tepidum and plant RNase E/G-like proteins contain an insertion in the region corresponding to the S1 domain (Fig. 3), which might affect interactions with RNA substrates (Lee and Cohen, 2003). A variant of the Zn-link motif present in bacterial RNase E/G proteins (Callaghan et al., 2005b) with consensus CXCCH(A/G) (I/T)G was present in plant
RNase E/G-like proteins (Fig. 3). The motif is required for co-ordinating a zinc ion in the quaternary structure of the catalytic domain (Callaghan et al., 2005a).

The AtRneE/G-like gene is required for chloroplast development and autotrophic growth

Heterozygous plants from the Salk (Alonso et al., 2003) and GABI-KAT (Rosso et al., 2003) collections with T-DNA insertions in exon 6 (SALK_093546) or intron 11 (GK187E08) (Fig. 1) were identified by PCR and DNA blot analysis. When seedlings from these heterozygous plants were grown on media lacking sucrose they segregated to give a 3:1 ratio of green to white seedlings. White SALK_093546 and GK187E08 seedlings were arrested at the cotyledon stage (Fig. 4A, B, labelled W) and easily distinguished from green WT and heterozygous seedlings on sucrose-free medium (Fig. 4A, B, labelled G). The white phenotype could be rescued by transferring mutant seedlings to media containing sucrose whereupon new pale-green leaves emerged between the white cotyledons (Fig. 4C, D, left plant; F, left plant). All the resulting pale-green plants tested by PCR and DNA blot analyses were homozygote for the mutant At2g04270 allele whereas normal green plants were either homozygote or heterozygote for the WT At2g04270 allele. RT-PCR with primers located in exons 12 and 14 indicated AtRne E/G transcript levels downstream of the T-DNA insertions were reduced in SALK_09346 and elevated in GK187E08 mutants (not shown). The 3:1 segregation of green to white seedlings on sucrose-free media for both At2g04270 disrupted lines (Salk_093546 and GK187E08) was consistent with this phenotype resulting from a mutation at a single locus. The observation that the white (on sucrose-free medium) and pale-green (on sucrose medium) phenotypes co-segregate with the disrupted At2g04270 alleles in homozygote mutants in two independent lines (Salk_093546 and GK187E08) strongly suggests these phenotypes result from loss-of-function of the At2g04270 gene rather than a second T-DNA insertion in an unrelated gene. Homozygous plants with an insertion in the adjacent At2g04280 gene (SALK_025999) grew on media lacking sucrose and were similar in appearance to WT plants (not shown).

Transfer of pale-green mutant Salk_093546 or GK187E08 (not shown) plants to media lacking sucrose (Fig. 4G) or to soil (Fig. 4H) arrested growth and resulted in death whereas WT and a T-DNA insertion in an intron of gene At3g03710 (Salk_070705) encoding a polynucleotide phosphorylase-like (AtPNP) protein were able to grow without sucrose (Fig. 4G). These results (Fig. 4A–H), based on two independent T-DNA insertions, suggest that homozygous At2g04270 mutant plants are unable to grow autotrophically and require sucrose at all stages of growth. Homozygous SALK_093546 mutant plants flowered in vitro on sucrose medium allowing the collection of seeds (Fig. 4I, J). Seeds from self-fertilized SALK_093546 homozygous mutant plants germinated on sucrose medium gave rise to seedlings with pale-green cotyledons (Fig. 4E, labelled CO). Whilst some pale-green mutants are viable in soil (Jarvis et al., 1998) sucrose-dependent growth combined with pale green leaves is a phenotype associated with photosynthetic mutants unable to fix carbon dioxide (Kode et al., 2006). To examine plastid ultrastructure in Salk_093546 mutants, sections were prepared from cauline leaves of plants grown on sucrose medium for electron microscopy. Chloroplasts packed with thylakoids and large granal stacks were predominant in WT leaf cells (Fig. 5A, labelled cp). Small less-developed plastids were also found in WT leaves (Fig. 5B) but were less common. The most common plastids in mutant leaves were smaller
than WT chloroplasts with fewer thylakoids and short granal stacks (Fig. 5C, D) indicating an arrest in chloroplast development. Fully developed chloroplasts were not observed in mutant leaf sections. The morphology of mitochondria appeared similar in WT and mutant plants (Fig. 5B, C, D; labelled mt). These results suggest that the AtRne E/G-like gene is required for converting small non-photosynthetic plastids to chloroplasts allowing photosynthesis and sucrose-independent growth.

A 125 kDa AtRNase E/G-like protein is present in chloroplasts

To express the A. thaliana RNase E-like protein in E. coli, the N-terminus was removed and isoleucine at position 327 changed to the N-terminal methionine of the recombinant protein. The N-terminus of the truncated protein aligns with the start of E. coli RNase E (Fig. 2). The modified AtRne E/G-like cDNA cloned in pET30a encodes a 79 kDa protein containing the catalytic domain and C-terminus (Fig. 2). Induction of pET30a expression in E. coli BL21(DE3)pLysS cells gave rise to a 90 kDa band on SDS-PAGE gels (Fig. 6A, lane 2) that was not detected in extracts from uninduced cells (Fig. 6A, lane 1). This slower-than-expected electrophoretic mobility of the recombinant AtRNase E/G-like protein is also a property of E. coli RNase E (Mudd and Higgins, 1993). The specificity of a polyclonal antibody raised against the recombinant 79 kDa AtRNase E/G-like protein was tested by western blot analysis using uninduced and induced E. coli extracts. The antibody bound strongly to the induced 90 kDa protein and smaller polypeptides, which were likely to be degraded or prematurely terminated translation products of the AtRne E/G-like cDNA expressed in pET30a (Fig. 6B, lane 2). Weakeness of the pET30a vector gave rise to weak bands in the uninduced lane (Fig. 6B, lane 1).

The antibody raised against the recombinant AtRNase E/G-like protein recognized a 125 kDa band in total soluble protein from wild-type plants fractionated by SDS-PAGE (Fig. 7A, lane 7). If this 125 kDa protein is the product of the AtRne E/G gene it should be altered in T-DNA insertion mutants. Two homozygous mutant plants were used for the SALK_093546 and GK187E08 T-DNA insertion lines (T-DNA insertion sites shown in Fig. 1). The 125 kDa band was not detected in SALK_093546 lines (Fig. 7A, lanes 2, 3) and was replaced by a smaller band of 110 kDa in GK187E08 lines (Fig. 7A, lanes 4, 5). A green heterozygote plant with the WT and mutant GK187E08 alleles accumulated both 110 and 125 kDa proteins (Fig. 7A, lane 6). The 110 kDa band can be explained by termination of translation at a stop codon located at the start of intron 11, which would remove the C-terminal 155 aa of the AtRNase E/G-like protein in T-DNA insertion line GK187E08 and result in a reduction of 18 kDa. In summary, the results indicate that the major protein product of the AtRne E/G-like gene migrated as a 125 kDa protein band on SDS/PAGE gels, and was not detectable or was altered in size in T-DNA insertion lines. Only one of the proteins predicted from the

Fig. 5. Ultrastructure of WT and Salk_093546 mutant cells. (A) WT chloroplasts (cp), (B) mitochondrion (mt) and immature plastid (pl) in WT leaves, (C) plastids, mitochondria, and nucleus (nu) in SALK_093546 mutant, and (D) magnified SALK_093546 mutant plastid. Scale bar 1 μm.

Fig. 6. pET30a-based overexpression of a 714 aa AtRNase E/G-like recombinant protein in E. coli. (A) SDS-PAGE fractionated total soluble proteins from uninduced and induced E. coli cells. The induced 90 kDa RNase E/G-like protein is arrowed. (B) Western blot analysis using antibodies raised against the purified recombinant 90 kDa RNase E/G-like protein.
analysis of transcripts (Fig. 2) accumulates to detectable levels in WT plants. The larger than expected size of 125 kDa might reflect low mobility on SDS-PAGE gels. However, the possibility that the protein is larger than predicted from cDNA analyses cannot be ruled out. In agreement with the findings that the AtRNase E/G-like gene product was required for sucrose-independent growth right up to flowering and setting seeds, the 125 kDa RNase E/G-like protein was detected in cotyledons (Fig. 7B, lower panel, lane 1), rosette leaves of different ages (Fig. 7B, lanes 2–5), and cauline leaves (Fig. 7B, lane 6). The levels of the large subunit of ribulose bisphosphate carboxylase/oxygenase (LS RuBisCO) are shown in Fig. 7B (upper panel) for comparison.

The AtRNase E/G-specific antibody detected a 125 kDa band in solubilized proteins from purified WT chloroplasts (Fig. 7C, top panel, lane 7). Importantly, the band co-migrates with the bands detected in total soluble protein samples from wild-type and heterozygous SALK_093546 plants (Fig. 7C, top panel, lanes 2, 3). Moreover, no co-migrating band was present in total soluble protein from two SALK_093546 mutant plants, which lack the AtRNase E/G-like protein (Fig. 7C, lanes 4, 5). These results strongly suggest that the 125 kDa RNase E/G band detected in total protein extracts was also present in purified chloroplasts. An antibody raised against the large subunit of ribulose bisphosphate carboxylase/oxygenase (LS RuBisCO) showed this plastid-localized photosynthetic protein was present in WT, heterozygote and SALK_093546 mutant plants (Fig. 7C, lanes 2–5) and purified chloroplasts (lane 7). The levels of LS RuBisCO appeared to be reduced in SALK_093546 mutant plants. High levels of α-tubulin were found in total soluble protein samples from WT, heterozygote, and SALK_093546 mutant plants (Fig. 7C, lanes 2–5) but not in purified WT chloroplasts (Fig. 7C, lane 7) indicating successful purification of chloroplasts from cytosolic proteins.

Rescue of a temperature-sensitive RNase E mutation
A cDNA encoding a 576 aa recombinant protein (Fig. 2) containing the entire catalytic domain was cloned into the Trc99a expression vector. Thirty min of IPTG induction of pTrc99A-AtRne E/G gave rise to a polypeptide of 75 kDa (Fig. 8A, lane 2) that was not detected in extracts from uninduced cells (Fig. 8A, lane 1) or induced cells containing the pTrc99a vector without an insert (Fig. 8A, lane 4). The 75 kDa band exhibits a lower-than-expected mobility for this 64 kDa recombinant protein, which was consistent with the lower-than-expected electrophoretic mobilities of other AtRNase E/G-like polypeptides (see above).

Four plasmids were used in rescue experiments with the temperature-sensitive RNase E mutant strain E. coli KW086 (Wang and Cohen, 1994): (i) pCH220, (ii) pGEM-T-AtRne E/G, (iii) pTrc99a, (iv) pTrc99a-AtRne E/G. Plasmid pCH220 contains a truncated 830 aa long E. coli RNase E protein, which was known to rescue the rne58 mutation and provided a positive control (Mudd and
allow growth at 43 °C (Fig. 8B). Six randomly-picked transformants from two independent transformations of the KW086 strain with plasmid pTrc99a-AtRne E/G restored growth at the non-permissive temperature. Plasmid preparations from these transformants and restriction enzyme analysis of the plasmid DNA confirmed that they all contained the expected plasmid pTrc99a-AtRne E/G (data not shown). The specific ability of Trc99a-AtRne E/G but not pGEM-T-AtRne E/G or pTrc99a to rescue growth, rules out reconstitution of functional E. coli RNase E, by recombination or reversion of the rne<sup>ts</sup> mutation, in Trc99a-AtRne E/G containing KW086 strains. This rescue of the rne<sup>ts</sup> mutation by the recombinant plant AtRNase E/G-like protein supports conservation of function.

**Ribonuclease activity of a recombinant 79 kDa AtRNase-E/G protein**

*In vitro* studies have shown bacterial RNase E/G homologues containing the conserved ribonucleolytic catalytic domain to be ribonucleases (Jager et al., 2001; Lee and Cohen, 2003). Protein extracts were prepared from *E. coli* strains either containing (pET30a with AtRne E/G coding sequence) or lacking (pET30a vector without insert) the overexpressed recombinant 79 kDa AtRne E/G-like protein (Fig. 2). If the AtRnase E/G is a ribonuclease it should give rise to RNA cleavage products that are not detected in the pET30a vector-only *E. coli* extracts. Moreover, overexpression of a ribonuclease should result in more rapid degradation of the RNA substrate. This overexpression approach was used successfully to identify cleavage products of *E. coli* RNase E (Mudd and Higgins, 1993). The use of *E. coli* strains with and without recombinant plant enzymes overcomes the technical problems of excluding contaminating RNase activities present in purified plant proteins. Equal amounts of a radiolabelled RNA substrate transcribed from a 2 kbp plastid region (map coordinates 54 778–56 788) containing the *rbcL* gene (Sato et al., 1999) were incubated with buffer alone, or protein preparations either containing or lacking the overexpressed recombinant AtRNase E/G-like protein. RNA samples incubated with buffer or cell extracts were fractionated by denaturing gel electrophoresis and visualized by autoradiography. The high radioactivity of the substrate RNA incubated in buffer alone resulted in saturation and blackening of the film by large >1.63 kb RNA molecules and smaller less abundant molecules; the latter probably representing incomplete transcription products (Fig. 9A, lane 1). Ribonuclease activity in the *E. coli* extracts degraded most of the substrate RNA (Fig. 9A, lanes 2, 3). At longer autorgraphic exposures, bands were visible in the RNA samples treated with *E. coli* extracts. A reduction in the sizes and intensities of the high MW RNA molecules at

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**Fig. 8.** Rescue of an *E. coli* rne<sup>ts</sup> mutant with the catalytic domain of the AtRNase E-like protein. (A) Cell lysates from *E. coli* strains containing plasmid Trc99a-AtRne E/G coding for a 576 aa polypeptide or Trc99a vector (control), at time 0 and 30 min after addition of 1 mM IPTG, were fractionated on a 7% (w/v) SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Molecular weight markers are indicated on the right. The induced 75 kDa RNase E/G-like polypeptide is arrowed. (B) Four *E. coli* KW086 strains containing the temperature-sensitive RNase E mutation and plasmids (1) pCH220 (positive control), (2) pGEM-T AtRne E/G (negative control), (3) pTrc99a AtRne E/G, (4) pTrc99a (negative control). Cells were streaked out on LB plates containing ampicillin plates containing IPTG to induce expression of the promoter in the pTrc99a expression vector. Plates were incubated at the permissive 30 °C or the non-permissive 43 °C temperature. All strains grew at 30 °C/C or the non-permissive temperature.
the top of the gel suggest the extract containing the overexpressed AtRNase E/G-like protein (Fig. 9B, lane 2) degrades the RNA substrate more effectively than the extract lacking this overexpressed protein (Fig. 9B, lane 3). Unique RNA bands of 0.26 and 0.12 nucleotides accumulate in the lane corresponding to RNA substrate treated with the AtRNase E/G-like protein (Fig. 9, lane 1) that were not found in the pET30a vector only lane. The overexpressed AtRNase E/G-like protein was likely to be responsible for these ribonucleolytic cleavage events, which were not detected in the RNA substrate treated with E. coli extracts lacking the recombinant enzyme. The increased ribonucleolytic activity and unique cleavage events associated with the overexpressed AtRNase E/G-like protein suggest it is a ribonuclease.

**Loss-of-function of the AtRNase E/G-like protein influences plastid RNA levels**

The SALK_093546 mutant, which contains no detectable AtRNase E/G-like protein, was used to examine the effects of the AtRne E/G-like gene on plastid RNA accumulation (Fig. 10A). Total RNA from above-ground parts of WT and mutant plants grown in vitro was used for blot analyses (Fig. 10A). Band intensities were standardized using hybridization signals of cytosolic 25S and 18S ribosomal RNAs. Quantitation of the 25S and 18S band intensities (Fig. 10A, nuc rRNA) show the SALK_093546 lane was loaded with less RNA (0.66) than the WT lane (1.00). Limited variation of the 18S mitochondrial rRNA (mt18S rRNA) band intensities was
found between WT and mutant plants. In contrast, taking into account the reduced 0.66× loadings of the SALK_093546 lanes, the levels of plastid-encoded RNAs tested were reduced overall by greater than 2-fold in mutant relative to WT plants. These significantly reduced hybridization signals were observed for the plastid encoded ribosomal RNAs (pt rRNA), rbcL and psbA mRNAs (Fig. 10A). A prominent smear was visible below the rbcL and psbA main bands in mutant lanes indicating elevated levels of incomplete or degraded transcripts. Processing of an unstable bacteriophage T4 gene 32 mRNA into a stable species requires E. coli ribonuclease E (Mudd et al., 1988). Similarly, the reduction in levels of mature plastid RNA transcripts can be explained if the AtRNase E/G-like protein is required to process primary transcripts to stable forms. Reduced rbcL mRNA levels correlate with a reduction in LS RuBisCO protein levels (Fig. 7C). Differences in plastid RNA levels were not due to template abundance. DNA blot analyses with a plastid rbcL probe indicated similar levels of plastid DNA in WT and mutant plants (Fig. 10B). Lower plastid RNA levels correlate with the reduced plastid volume per cell (Fig. 5) resulting from the arrest of chloroplast development in the SALK_093546 mutant.

**Analysis of genes co-expressed with the AtRne E/G-like gene**

Co-expression of genes provides a powerful tool to identify genes involved in common cell and developmental pathways (Brown et al., 2005). The Arabidopsis co-expression tool allows the identification of genes with correlated expression patterns amongst a genome set of 22,000 genes (Jen et al., 2006). Correlation coefficients for co-expression (r) values above 0.7 are of interest. Table 1 shows that three of the top four genes co-expressed with the AtRne E/G-like gene are plastid proteins and are likely to play a role in RNA metabolism. Genes co-expressed with the AtRNase E/G-like protein include a CRS1/YhbY domain protein implicated in group II intron splicing in plastids (Till et al., 2001), an unknown protein with an S1 RNA binding domain, and polynucleotide phosphorylase. RNase E and polynucleotide phosphorylase are found in an RNA degrading multi-protein complex in E. coli and act in concert to degrade RNA (Py et al., 1994; Grunberg-Manago, 1999). Co-expression of the plant AtRne E/G-like and plant polynucleotide phosphorylase (Hayes et al., 1996; Li et al., 1998) genes provides a possible mechanism for coordinating the activities of their products in plastids.

**Discussion**

The AtRne E/G gene (At2g04270) in A. thaliana encodes an RNase E/G-like protein present in chloroplasts that is required for plastid RNA accumulation, plastid development, and autotrophic growth. Recombinant plant RNase E/G-like proteins containing the evolutionary-conserved catalytic domain rescue an E. coli RNase E mutation and possess ribonucleolytic cleavage activity. Loss-of-function of the At2g04270 gene results in plants with pale-green cotyledons and pale-green true leaves, in the presence of sucrose, and white cotyledons and white true leaves in the absence of sucrose. The similar phenotypes of cotyledons and leaves in At2g04270 mutants contrasts with mutations in genes that have a more marked effect on the pigmentation of cotyledons relative to true leaves (Yamamoto et al., 2000; Albrecht et al., 2006). Pale-green leaves of mutant plants contained small plastids with a reduced number of thylakoids and short granal stacks compared to chloroplasts. These results support an important role for the AtRNase E/G-like protein in plastid function and development. The observation that loss-of-function of the AtRne E/G gene was not lethal in homozygous mutant plants provided they were grown on sucrose might indicate that absence of the AtRNase E/G-like protein has a limited impact on extra-plastidic processes.

Specific antibodies against a recombinant AtRNase E/G-like protein recognized a 125 kDa protein in total soluble protein from above-ground parts at all developmental stages tested from cotyledons to cauline leaves. The 125 kDa protein was not detected in soluble protein from SALK_093546 mutants with a T-DNA insertion in exon 6. A truncated 110 kDa AtRNase E/G protein was found in GK187E08 mutants with a T-DNA insertion in intron 11. This suggests that the activities of the 125 kDa AtRNase E/G protein required for chloroplast development and autotrophic growth are not functional in the 110 kDa mutant protein. These results confirm the presence of only one AtRNase E/G-polypeptide in plants and its size (125 kDa) does not correspond to any of the four 111 kDa,

**Table 1. Top four genes co-expressed with the AtRne-E/G like gene**

Analysis using ACT (Jen et al., 2006) on public microarray datasets.

| Gene         | Function                              | Predicted location | r     | References               |
|--------------|---------------------------------------|--------------------|-------|--------------------------|
| At3g18390    | CRS1/YhbY domain possible RNA binding | Plastid            | 0.89  | Till et al., 2001        |
| At1g02150    | Pentatricopeptide repeat protein      | Mitochondrion      | 0.88  | Cheuk et al., unpublished data |
| At1g12800    | S1 RNA-binding domain-containing protein | Plastid            | 0.87  | Rhee et al., 2003        |
| At3g03710    | Polynucleotide phosphorylase (PNPase) | Plastid            | 0.87  | Hayes et al., 1996      |
97 kDa, 79 kDa and 69 kDa polypeptides predicted from the analysis of AtRne E/G transcription products (Fig. 2). The larger than expected size of the 125 kDa RNase E/G protein might reflect aberrant electrophoretic mobility on SDS/PAGE gels. Recombinant 64 kDa and 79 kDa AtRNase E/G-like polypeptides migrated as larger-than-expected bands on SDS/PAGE gels and this lower-than-expected electrophoretic mobility might be a property of the protein. The 118 kDa *E. coli* RNase E protein migrates as a 180 kDa band on SDS/PAGE gels (Mudd and Higgins, 1993). Mapping the 5’ end of the full-length message was made difficult by the relatively low abundance of AtRne E/G RNA products. Accumulation of unspliced pre-mRNA, differential splicing (Alexandrov et al., 2006), non-AUG start codons (Hedtke et al., 2002; Christensen et al., 2005) or multiple AUG start sites (Chabregas et al., 2003; Hanfrey et al., 2005; Sunderland et al., 2006) make accurate predictions of the N-termini of proteins from genomic and cDNA sequences more difficult and the possibility cannot be excluded that the N-terminus of the 125 kDa protein lies upstream of the predicted start site in exon 1.

The predicted 111 kDa protein contains a plastid targeting sequence which was predicted by the ChloroP program (Emanuelsson et al., 2000) to be cleaved to a 104 kDa mature protein following import into plastids. Western blot analysis with an AtRNase E/G-specific antibody demonstrated the presence of the 125 kDa protein in purified chloroplasts. These results do not exclude the presence of AtRne E/G products at other locations within the cell. Low abundance and intraplastidic location might explain the failure of a number of proteomics studies to identify the 125 kDa RNase E/G-like protein in chloroplasts (Ferro et al., 2003; Friso et al., 2004; Kleffmann et al., 2004; Peltier et al., 2006; Baginsky et al., 2007). Targeting studies involving fusions to GFP provide an alternative to immunolocalization to identify the subcellular location of the AtRNase E/G-like protein. However, this approach can give rise to contradictory results (Christensen et al., 2005) and at present was not suitable for the AtRNase E/G-like protein. This was because of the discrepancy in sizes of the proteins predicted from cDNA sequences and the 125 kDa protein found in chloroplasts, which raises the possibility that the true N-terminus has not been identified. A plastid location for RNase E/G-like proteins was supported by partial sequencing of a 126 kDa protein present in a Triton-insoluble fraction from pea chloroplasts (Phinney and Thelen, 2005). The sequenced peptides from the 126 kDa pea protein share high identity with the *A. thaliana* RNase E/G-like protein (Phinney and Thelen, 2005) translated from deposited cDNA sequence AJ252122 (this work).

Loss-of-function of the AtRNase E/G-like protein was associated with reduced accumulation of plastid rRNA, psbA and *rbcL* mRNA. This could result from a direct effect of the AtRNase E/G-like protein on plastid RNA metabolism, or indirectly, for example as a consequence of arrested plastid development. Reduced plastid RNA levels were also found in yellow DAL (Bisanz et al., 2003) and pale-green, pale cress (Meurer et al., 1998) mutants of *A. thaliana* in which chloroplast development was blocked. In pale-green *A. thaliana* mir1 mutants, *rbcL* and *psbA* levels accumulate to WT levels but ribosomal RNA processing was impaired (Kishine et al., 2004). Support for a direct role of the AtRNase E/G-like in RNA metabolism is indicated by the presence of the conserved RNase E/G ribonucleolytic domain and the ribonucleolytic activity of the recombinant protein. Further work is required to determine whether the phenotypic consequences of loss-of-function of the AtRNase E/G-like protein results from a global effect on RNA metabolism (many targets) or lack of processing of a limited number of targets, which remain to be identified. In this regard, it is interesting to note that the lesion(s) underlying the non-viability of *E. coli* rne mutants remain(s) unclear (Takada et al., 2005) despite 20 years of productive research on the role of *E. coli* RNase E in mRNA decay (Mudd et al., 1988, 1990b; Callaghan et al., 2005a). It remains possible that the essential nature of the *E. coli* RNase E and plant RNase E/G-like proteins might be due to functions (Taghbalout and Rothfield, 2007) unrelated to RNA metabolism. The finding that the AtRne E/G-like gene was co-expressed with genes encoding a plastid-targeted polynucleotide phosphorylase and plastid proteins with S1 RNA binding and CRS1/YhbY domains raises the possibility that these genes are involved in common pathways in plastids.

**Supplementary data**

Supplementary data can be found at *JXB* online.

All oligonucleotides are listed in Supplementary Table 1. The figure shows the location of the primers on the At2g04270 sequence. DNA blot analyses of the Salk_093546 mutant is shown in Supplementary Figure 1.

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