RESEARCH PAPER

Growing Slowly 1 locus encodes a PLS-type PPR protein required for RNA editing and plant development in Arabidopsis

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Abstract

Most pentatricopeptide repeat (PPR) proteins are involved in organelle post-transcriptional processes, including RNA editing. The PPR proteins include the PLS subfamily, containing characteristic triplets of P, L, and S motifs; however, their editing mechanisms and roles in developmental processes are not fully understood. In this study, we isolated the \textit{Arabidopsis thaliana} Growing slowly 1 (AtGRS1) gene and showed that it functions in RNA editing and plant development. Arabidopsis null mutants of \textit{grs1} exhibit slow growth and sterility. Further analysis showed that cell division activity was reduced dramatically in the roots of \textit{grs1} plants. We determined that GRS1 is a nuclear-encoded mitochondria-localized PPR protein, and is a member of the PLS subfamily. GRS1 is responsible for the RNA editing at four specific sites of four mitochondrial mRNAs: \textit{nad1-265}, \textit{nad4L-55}, \textit{nad6-103}, and \textit{rps4-377}. The first three of these mRNAs encode for the subunits of complex I of the electron transport chain in mitochondria. Thus, the activity of complex I is strongly reduced in \textit{grs1}. Changes in \textit{RPS4} editing in \textit{grs1} plants affect mitochondrial ribosome biogenesis. Expression of the alternative respiratory pathway and the abscisic acid response gene \textit{ABI5} were up-regulated in \textit{grs1} mutant plants. Genetic analysis revealed that \textit{ABI5} is involved in the short root phenotype of \textit{grs1}. Taken together, our results indicate that \textit{AtGRS1} regulates plant development by controlling RNA editing in \textit{Arabidopsis}.

Key words: \textit{ABI5}, mitochondria, pentatricopeptide repeat proteins, RNA editing, root.

Introduction

Pentatricopeptide repeat (PPR) proteins are a class of RNA binding proteins characterized by the presence of a degenerate 35-amino-acid repeat, the PPR motif, which is arranged in tandem 2–50 times (Small and Peeters, 2000). The PPR motif (P motif) has another two variants, namely the S (short) motif with a length of 31 amino acids and the L (long) motif with a length of 35–36 amino acids. Based on their motifs, PPR proteins are divided into two subfamilies: the P subfamily has only P motifs, and the PLS subfamily contains characteristic triplets of P, L, and S motifs. Most members of the PLS subfamily contain extra conserved domains at their C-terminus, and these are designated the
E, E+, and DYW domains (Lurin et al., 2004; Cheng et al., 2016).

PPR proteins are involved in many aspects of RNA processing in mitochondria and chloroplasts, including RNA cleavage, splicing, editing, and translation, and play crucial roles in plant developmental processes and responses to environmental stresses (Andrèes et al., 2007; Zehrmann et al., 2009; Liu et al., 2010; Murayama et al., 2012; Zhu et al., 2012; Hailli et al., 2013; Mei et al., 2014; Yang et al., 2014; Hsieh et al., 2015). RNA editing is an important step in the post-transcriptional control of organelle gene expression. Most RNA editing in plants results in the conversion of cytidine (C) to uridine (U) (Covello and Gray, 1989; Gualberto et al., 1989; Hiesel et al., 1989; Shikanai, 2006; Chatteignier-Boutin and Small, 2010). In the mitochondria of Arabidopsis, approximately 500 C-to-U editing sites had been uncovered (Giege and Brennicke, 1999; Bentolila et al., 2005, 2008). The mechanism of the editing reaction puzzled researchers for many years, until the first PPR protein, CHLORORESPIRATORY REDUCTION 4, was found to be involved in chloroplast RNA editing (Kotera et al., 2005). Since then, PPR proteins have been found to be involved in RNA editing and all the discovered trans-factors involved in RNA editing in plants belong to the PLS subfamily (Takenaka et al., 2013; Shikanai, 2015). Although several PPR proteins target individual sites, some are found to recognize more than two and even as many as eight sites (Kim et al., 2009; Zehrmann et al., 2009, 2012; Zhu et al., 2012; Glass et al., 2015). Although recently bioinformatics, biochemical, and structural analyses have shown that PPR proteins recognize RNA in one-motif to one-nucleotide binding mode (Yagi et al., 2013; Yin et al., 2013; Barkan and Small, 2014), the mechanism of how a single PPR protein recognizes multiple target sequences still needs further investigation.

Mutations in many RNA-editing PPR proteins do not result in any evident developmental defect (Zehrmann et al., 2009; Verbiskiy et al., 2010; Härtel et al., 2013), although some PPRs are important in development (Yu et al., 2009; Koprivova et al., 2010; Liu et al., 2010; Murayama et al., 2012; Hailli et al., 2013; Yang et al., 2014). The relationship between mutant phenotype and RNA editing has not received much attention until recently. Mutations in PPR proteins involved in chloroplast RNA editing have been shown to impair chloroplast biogenesis (Yu et al., 2009). Several reports have shown that an increase in reactive oxygen species (ROS) is responsible for the developmental defects observed in the mitochondrial RNA editing by those mutant PPRs (Liu et al., 2010; Yang et al., 2014). The nature of other signaling pathways linking PPRs involved in mitochondrial RNA editing and plant development remains largely unknown.

In this study, we analyzed the Arabidopsis T-DNA knock-out mutant grs1-1, which displays a phenotype of slow growth and sterility. Genetic and molecular analysis indicates that the GRS1 gene encodes a PPR protein. Further studies showed that GRS1 is required for the RNA editing of four mitochondrial transcripts. The upstream sequences of these editing sites share some conserved nucleotides. The lack of RNA editing at these sites leads to reduced levels of functional mitochondrial complex I and affects mitochondrial ribosome biogenesis. Abscisic acid (ABA) response gene ABI5 but not ROS is involved in the short root phenotype in grs1.

Materials and methods

Mutant library construction and selection of grs1-1

We generated an Arabidopsis mutant library with T-DNA encoding LAT52::EGFP, a cell-autonomous pollen-specific reporter (Twel et al., 1989; Sessions et al., 2002), and a hygromycin-resistance gene. T-DNA mutagenesis was carried out on qrt1 plants (Preuss et al., 1994), where mature pollen grains maintain male meiotic products in tetrads (Supplementary Fig. S1A, B) online. Hygromycin-resistant plants, heterozygous for a single locus T-DNA insertion, produced tetrads with two mutant pollen grains emitting green fluorescent protein (GFP) fluorescence, and two wild-type grains that did not display any GFP activity (Supplementary Fig. S1C, D). This simplified the process of determining whether a T2 plant was heterozygous (tetrads are two GFP+ to two GFP−, HYG resistant), homozygous (all four tetrads members are GFP+, HYG resistant) (Supplementary Fig. S1E, F) or wild-type (all four tetrads members are GFP−) for a T-DNA induced mutation.

For grs1-1 selection, T1 seeds were obtained by self-pollination of hygromycin-resistant grs1-1 plant and sown on 1/2 MS plates with hygromycin to select grs1-1 seedlings. Thirty-two hygromycin-resistant seedlings were grown on soil and the pollen grains of each plant were visualized under a fluorescence microscope to determining whether a T2 plant was heterozygotes, homozygotes, or wild-type. T1 seeds were sown on 1/2 MS plates for germination.

Plant materials and growth conditions

Arabidopsis thaliana qrt1 (Preuss et al., 1994) was used as a wild-type strain. The grs1-1 allele was isolated from our mutant library with hygromycin resistance (Wu et al., 2012, Supplementary data). The grs1-2 (CS428796) and gin1-3 lines were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio, USA). The mutant abf5-1 (Liu et al., 2012) was provided by Dr Lei Zhang (College of Life Sciences, Wuhan University). The transgenic line pCyclinB1:1-Dbox-GUS (Colon-Carmona et al., 1999) was provided by Dr Jian Xu (Temasek Life Sciences Laboratory, Singapore). Seeds were surface-sterilized with 20% bleach for 10min, and washed three times with sterile distilled water. Seeds were stratified for 3 d at 4 °C and then sown on 1/2 MS plates with 1.0% (w/v) sucrose. To decrease the ROS level in seedlings, diphenylenedio- nium (DPI, 100 μM, Sigma) or reduced glutathione (GSH, 300 μM, Sigma) was added to the culture media. Agar plates were placed in a growth room with a photoperiod of 16 h light/8 h dark. For kanamy- cin selection, 50 mg 1 L of kanamycin (Sigma) was supplemented to the media. Similarly, 50 mg 1 L of hygromycin (Roche) and 10 mg 1 L of sulfadiazin (Sigma) were added for hygromycin selection and sulfadiazin selection, respectively. Plants were grown on soil in a greenhouse under long-day conditions (16 h light/8 h dark) at 22 °C.

Cloning of the T-DNA flanking sequence and characterization of the grs1-1 and grs1-2 alleles

The T-DNA flanking sequence in the grs1-1 mutant was cloned by TAIL-PCR (Liu et al., 1995). The authenticity of the cloned sequence was confirmed by PCR using two pairs primes located around the T-DNA left border (GRSI-T1, TGGGACCAAGTTTCATCAGGGTCTTCT; LB-S, CCAAAATCCAGTACTAAATCAG) and right border (GRSI-T2, ATTCATGGTTGTGCATAAAAGAG; RB-S, CGCCCGTGGTCATCTAGT). For the grs1-2 allele, the T-DNA site was confirmed by PCR using the following primers: GRS1-RP, GTGAAATGGAGCAGAGCTG; and LB3, TAGCATCTGAAATTTCCATACCAATCTCAGATAC.
Vector construction and plant transformation

Plasmids P092, P093, and P094 were produced as described previously (Wu et al., 2012; Yan et al., 2016). To generate the pGRS1::GRS1 complementation construct, a 3876-bp wild-type genomic sequence containing the AT4G32430 gene, 1078-bp upstream of the ATG codon and 306-bp downstream of the TAG codon sequences, was PCR-amplified (primers: GRS1-F1, NNNNGTACCTGATTTTTGGAGCAGTCTC; and GRS1-R1, NNNNTCGAGAACCAACTCTACTTTAAGCCATC) from genomic DNA and was then cloned into the pP092 plasmid with T-DNA encoding pLATS2::DsRED and a kanamycin-resistance gene (Supplementary Fig. S2C). To examine the subcellular location of GRS1, we amplified and cloned the 35S promoters into pP094 to generate the 35S::EGFP construct. Then the GRS1 ORF was amplified (primers: GRS1::GRS1-EGFPGUS et al., 2010) and put under the control of 35S to generate 35S::GRS1-EGFP construct. Then we amplified and cloned the 129-bp DNA fragment containing the mitochondria-targeted pre-sequence of the located F1-ATPase (Shibata et al., 2010) and put it under the control of 35S to generate 35S::Rtcp. Then we amplified and cloned the 129-bp DNA fragment containing the mitochondria-targeted pre-sequence of the located F1-ATPase gene At5g12450 (Robison et al., 2009) (using primers Mito-1, NNNNGTGACCCGCACTGGCAATATCTCAGATCAGG; and Mito-2, NNNNTCTGAGATTTGAGCAGAGC ATGGCATATAAG) into 35S::RFP to generate the 35S::Mito-RFP construct. To investigate the expression pattern of GRS1, the GRS1 promoter was amplified (primers: GRS1-F1 as above, and GRS1-R2: NNNNTCTGAGGAAGACGACAACTACTCGGATTCTAATTG) and put upstream of GUS (β-glucuronidase) in P093 to generate pGRS1::GUS. All the gene constructs were transferred into Agrobacterium tumefaciens and put upstream of GUS (β-glucuronidase) in P093 to generate pGRS1::GUS. All the gene constructs were transferred into Arabidopsis plants by the floral dip method (Clough and Bent, 1998).

Genotype analysis of the genomic complemented lines

To identify the genotype of the genomic complemented lines, the DNA of these plants was extracted and PCR analysis was conducted using three pairs of primers (S1+Al, S2+Al, S1+Al) (Supplementary Fig. S2B, C). Primer S1, CATCTGTAAGAACTACTCACCACGAGGT located upstream of the T-DNA insertion site; Primer S2, CCAAAATCCGATCTCAAATCACGG; and Primer S3, NNNNTCTGAGATTTGAGCAGAGCATTTAAGCATCAG on the right border; Primer A1, CTTCTTCTCGTTTTTAACTGTC located downstream of the AT4G32430 gene and beyond the genomic fragment used for complementation; and Primer A2, TGACTTATTGATTGGAGGTTG located downstream of the genomic fragment used for complementation.

Histochemical analysis of GUS activity

For pCYCB1;1::Dbox-GUS staining, we crossed the pCYCB1;1::Dbox-GUS stable lines with grs1-1 mutant plants. F2 seeds were obtained by self-pollination of F1 and sown on 1/2 MS plates with hygromycin to select seedlings with the grs1-1 background. Individual F3 seeds were obtained by self-pollination of these seedlings and sown on 1/2 MS plates for germination. GUS activity analysis was performed with 8-d-old seedlings (with normal roots and short roots), and the lines with all normal roots with GUS activity were regarded as homozygous for both pCYCB1;1::Dbox-GUS and grs1-1. The histochemical analysis of GUS activity was performed according to Vieille-Calzada et al. (2000). Plant tissues were incubated at 37 °C in GUS-staining solution [2 mM 5-bromo-4-chloro-3-indolyl glucuronic acid (X-Gluc) in 50 mM sodium phosphate buffer, pH 7.0] containing 0.1% Triton X-100, 2 mM K3Fe(CN)6, and 2 mM K4Fe(CN)6. The stained tissues were then transferred to 70% (v/v) ethanol solution. Samples were mounted with traditional clearing solution and placed under a microscope (Olympus) fitted with differential interference contrast optics for imaging.

Analysis of subcellular localization of GRS1

The iPSORT Prediction program (Bannai et al., 2002) predicted that GRS1 is targeted to the mitochondria. To confirm its mitochondrial localization, transgenic plants containing the 35S::GRS1-EGFP construct were crossed with a transgenic mitochondrial marker line expressing 35S::mito-RFP. The petal cells of the F1 progeny were visualized using a FV1000 confocal laser-scanning microscope (CLSM; Olympus). GFP fluorescence was detected with excitation at 488 nm and emission at 510–530 nm; red fluorescent protein (RFP) fluorescence was detected with excitation at 568 nm and emission at 590–620 nm.

Analysis of RNA editing

The status of Arabidopsis mitochondrial RNA editing in grs1 plants was examined as described by Zehrmann et al. (2008). Total RNA was extracted from 20-d-old grs1 and wild-type seedlings. Complementary DNA fragments of all mitochondrial transcripts containing RNA editing sites were amplified by RT-PCR. The primers used in this experiment are given in Supplementary Table S3. The amplified PCR products were directly sequenced and the results were compared to the corresponding DNA sequence for each transcript.

Phenotypic characterization

For the determination of the root meristem size, root tips were excised from seedlings 8 d after germination, and examined with a differential interference contrast (DIC) microscope (Olympus).

Measurement of ROS in roots

For nitroblue tetrazolium (NBT) staining to detect superoxide, seedlings were incubated in a reaction buffer containing 1 mM NBT (Sigma-Aldrich) and 20 mM K-phosphate at pH 6.0 for 20 min. The seedlings stained by NBT were washed three times with water and then transferred to acetic acid:ethanol (1:3, v/v) solution. To enable the detection of superoxide, the seedlings were stained by NBT were washed three times with water and were then examined in 10% glycerol with a Olympus microscope.

Quantitative RT-PCR

Total RNAs of seeds before germination and 7-d-old seedlings were extracted using the RNAqueous® Phenol-free total RNA Isolation kit (Ambion) according to the manufacturer’s protocol. After digestion with RNase-free DNase I (Promega), the first strand of cDNA was synthesized using oligo-dT and M-MLV reverse transcriptase (Invitrogen). Quantitative PCR analysis was performed using FastStart Essential DNA Green Master (Roche) on a CFX ConnectTM Real-Time System (BioRad). Each experiment was repeated three times and samples were normalized using UBQ10 expression. Data acquisition and analyses used Bio-Rad CFX Manager software; the relative expression levels were measured using the 2–ΔΔCt analysis method and the error bars in the figures represent the variance of three replicates. The genes and the primers used for detection of the mRNA expression are listed in Supplementary Table S4.

Detection of enzyme activity of complex I

Analysis of the NADP dehydrogenase activity of mitochondrion complex I was performed according to Wu et al. (2015). Proteins of crude organelle extract from young seedlings were solubilized with
1% (v/v) digitonin and resolved by Blue Native-PAGE. After PAGE, the NADH dehydrogenase activity of complex I was visualized by incubation of the gel in the presence of 1 mM nitroblue tetrazolium (NBT) and 0.2 mM NADH in 0.05 M MOPS (pH 7.6).

Results

GRS1 plays an essential role in vegetative and reproductive development

We generated an Arabidopsis mutant library to simplify the process of screening mutants whose homozygotes were lethal or exhibited growth retardation (Supplementary Fig. S1). One mutant displaying an extremely slow growth phenotype was isolated and named growing slowly1 (grs1-1). When we analyzed the effect of grs1-1 on plant development, we found that grs1-1/+ heterozygous plants had no visible morphological abnormalities in vegetative and reproductive organs compared with wild-type plants. grs1-1 homozygous plants, however, exhibited multiple phenotypes as shown in Fig. 1. Thirty-two hygromycin-resistant T2 plant were heterozygous grs1-1, suggesting that the grs1-1 homozygotes are either lethal or exhibited growth retardation. T1 seeds of grs1-1 were sown on 1/2 MS plates for germination and about 25% of 11-d-old seedlings showed an extremely slow growth phenotype (Supplementary Fig. S2A). The DNA of these slow-growth seedlings was extracted and PCR analysis confirmed that they were homozygous for grs1-1 (Supplementary Fig. S2D). grs1-1 homozygous seedlings only survived on MS medium plates, and their vegetative growth was strongly affected (Fig. 1A, B). Opening the siliques of grs1-1 two days after flowering revealed the absence of developed seeds. To determine which parent was responsible for the aborted phenotype, we performed reciprocal crosses of grs1-1 and wild-type plants. Both females and males were found to be sterile in grs1-1 mutant plants. Further analysis showed that the number of pollen grains in grs1-1 was much lower than in the wild-type; female gametophyte development in grs1-1

Fig. 1. Several developmental processes are impaired in grs1. (A) Root growth of 8-d-old seedlings in wild-type (WT), grs1-1, and grs1-2 plants. (B) Appearance of 35-d-old plants in wild-type, grs1-1, and grs1-2. (C, D) Anther of wild-type (C) and grs1-1 (D) plants. The amount of pollen grains in grs1-1 is much lower compared to the wild-type. (E, F) Aniline blue staining of pollen tube guidance in ovules. Ovules attract pollen tubes (indicated by arrows) in the wild-type (E), but no pollen tubes are observed in the ovules of grs1-1 homozygous plants (F). The stars indicate the micropylar end of the ovules.
was also found to be retarded and did not appear to be able to attract wild-type pollen tubes into the ovules (Fig. 1C–F).

**Cell division is impaired in grs1-1**

After germination, the growth rate of the primary root was dramatically reduced in grs1-1 plants compared to the wild-type. To determine the cellular basis for the observed defects in the root development of grs1-1 plants, we examined the size of the root meristem in seedlings 8 d after germination. It was observed that the size of root meristem in grs1-1 was much shorter than that of the wild-type (Fig. 2A). To further substantiate the role of GRS1 in controlling root cell division, we crossed pCyclin B1;1:Dbox-GUS stable lines (Colon-Carmona et al., 1999) with grs1-1 mutant plants. The pCyclin B1;1:Dbox-GUS reporter allows the visualization of cells at the G2-M phase of the cell cycle, and thus to monitor mitotic activity in the root meristem (Colon-Carmona et al., 1999). In contrast to the wild-type, we found that there was almost no GUS signal in grs1-1 roots (Fig. 2B). The results indicate that the number of dividing cells was reduced dramatically in grs1-1 compared to wild-type roots.

**Molecular characterization of grs1-1**

Arabidopsis grs1-1 plants were generated by T-DNA insertion with resistance to hygromycin. All the grs1-1/+ heterozygous plants were resistant to hygromycin, suggesting that the mutant phenotype was caused by a T-DNA insertion. We cloned the T-DNA flanking sequence by using the thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) technique (Liu et al., 1995). The grs1-1 mutant was shown to carry a T-DNA insertion in the gene AT4G32430 located 1325 bp downstream of the ATG start codon (Fig. 3A, Supplementary Fig. S2B). Another allele containing a T-DNA insertion in the GRS1 gene, CS428796, was obtained from the Arabidopsis Biological Resource Center. We verified that the CS428796 mutant carries a T-DNA insertion in the AT4G32430 gene at 850 bp downstream of the ATG start codon (Fig. 3A). We then renamed the CS428796 allele grs1-2. Homozygous grs1-2 plants were found to phenocopy grs1-1 homozygous plants (Fig. 1A, B).

To confirm that the grs1-1 mutant phenotypes were indeed caused by knockout of the AT4G32430 gene, we performed a complementation test with the genomic sequence of AT4G32430. Fifty-nine T1 transgenic plants were screened on double-resistance plates with hygromycin and kanamycin (for the transformed genomic sequence). Among them, eleven plants were homozygous for grs1-1. All these grs1-1 homozygous plants carrying the fragments of the exogenous genomic sequence (resistance to kanamycin) showed no obvious differences compared to the wild-type, and were named the genomic complemented lines (homozygous for grs1-1, heterozygous for exogenous genomic fragment) (Supplementary Fig. S2A). Genotype analysis confirmed the genomic complemented lines contained both the mutated grs1-1 version and expression of the wild-type version (Supplementary Fig. S2D). These results indicate that the AT4G32430 gene can successfully complement the grs1-1 phenotype. The AT4G32430 gene was therefore renamed as GRS1.

GRS1 encodes a mitochondria-targeted pentatricopeptide repeat protein

To investigate the expression pattern of GRS1, we fused the GRS1 promoter sequence to a GUS reporter gene, and transformed this construct into the wild-type. In seedlings, GRS1::GUS was preferentially expressed in the meristematic region of both roots and stems. In flowers, GUS activity was detected in the sepal, stigma, stamen, and pollen grains (Fig. 3B).
BLAST analysis identified GRS1 as a member of the PPR family, more specifically belonging to the PLS subfamily. Thus, GRS1 encodes a PLS-type pentatricopeptide repeat protein, as proposed by Lurin et al. (2004). It consists of six PPR-like S, six PPR-like L, and five P motifs with E1, E2, and DYW C-terminal extensions (Lurin et al., 2004; Barkan and Small, 2014; Cheng et al., 2016) (Fig. 3A, Supplementary Fig. S3, and Table S1). The iPSORT Prediction program (Bannai et al., 2002) predicted that GRS1 is targeted to mitochondria and, indeed, GRS1-GFP was found to co-localize with the mitochondria-localized Mito-RFP (Fig. 3C), indicating that GRS1 is a nuclear-encoded mitochondrial protein.

GRS1 is required for mitochondrial RNA editing

Since GRS1 encodes a DYW-type PPR protein, we tested its involvement in mitochondrial RNA editing. We identified several unedited sites in the mitochondrial RNA in the grs1-1 mutants. Our results revealed that C-to-U editing at the positions of nad1-265, nad4L-55, nad6-103, and rps4-377 was specifically blocked in the grs1-1 plants. Editing of these four sites is also inhibited in grs1-2 mutants (Fig. 4). The C-to-U editing in the nad1 mRNA results in an arginine-to-tryptophane amino acid change (R89W) in the NAD1 protein. The C-to-U editing in the nad4L mRNA results in an arginine-to-tryptophane amino acid change (R19W) in the NAD4L protein. The C-to-U editing in the nad6 mRNA results in an arginine-to-cystine amino acid change (R35C) in the NAD6 protein. The C-to-U editing in the rps4 mRNA results in a proline-to-leucine amino acid change (P126L) in the RPS4 protein. Editing of the four mRNAs at these four editing sites was highly efficient in the wild-type, as shown by the detection of a single peak equivalent to the T nucleotide at these positions, whereas editing of these sites was totally abolished in grs1-1 and grs1-2 mutants (Fig. 4). Editing deficiencies of the mutant alleles were restored in the grs1-1 complemented lines (Fig. 4). These results confirmed that mutation in the GRS1 gene was responsible for the defect of mitochondrial RNA editing in the grs1-1 mutants.

Fig. 3. Structural features, expression patterns, and subcellular localization of GRS1. (A) Diagram showing the relative position of the T-DNA insertion in the GRS1 gene and the structural features of the GRS1 protein. Various protein domains are indicated below the diagram. (B) GUS expression patterns in different plant parts of transgenic proGRS1::GUS lines. Top row: 7-d-old seedling. GUS signal is observed in root and shoot meristems. Scale bar = 5 mm. Bottom row, left: an inflorescence, scale bar = 3 mm. right: an anther with pollen grains, scale bar = 50 µm. (C) Localization of GRS1-GFP protein in the mitochondria. Petal cells of plant co-expressing GRS1-GFP and the mitochondrial marker Mito-RFP were examined with confocal laser scanning microscopy. From left to right: green fluorescent signal from GRS1-GFP; red fluorescent signal from the mitochondrial marker Mito-RFP; merged picture with green and red signals showing co-localization. Scale bar = 20 µm.

Fig. 4. GRS1 is responsible for RNA editing of four sites in Arabidopsis mitochondria. Wild-type plants show that RNA editing of the mitochondrial editing sites nad1-265, nad4L-55, nad6-103, and rps4-377 is efficient, while in grs1-1 and grs1-2 these sites are not edited. Editing deficiencies were restored in the grs1-1 complemented lines (Fig. 4). These results confirmed that mutation in the GRS1 gene was responsible for the defect of mitochondrial RNA editing in the grs1-1 mutants.
Complex I function and mitoribosomal biogenesis are impaired in grs1-1 mutants

The proteins NAD1, NAD4L, and NAD6 are components of the mitochondrial electron transport chain complex I (NADH dehydrogenase). Having observed that RNA editing of these genes was altered in grs1-1 mutants and resulted in amino acid changes, we hypothesized that RNA editing defects of these transcripts may lead to complex I malfunction in grs1-1 mutants. To test this hypothesis, we isolated crude mitochondria from seedlings of wild-type, grs1-1 mutants, and grs1l-1 complemented lines. Separation of mitochondrial complexes by blue-native PAGE and NADH dehydrogenase activity staining showed that both protein levels and activity of complex I could barely be detected in grs1-1 mutants (Fig. 5A, B).

Since the RPS4 protein is a component of the small subunit (SSU) of the mitoribosome, we tested whether the change in RPS4 editing in the grs1 mutants affects mitochondrial ribosomal biogenesis. As rRNAs are unstable when unassembled, rRNA levels can serve as a marker for the accumulation of ribosomal subunits (Walter et al., 2010; Kwasniak et al., 2013). We determined the abundance of mitochondrial (mt 18S and mt 26S), chloroplast (chl 16S and chl 23S) and cytosolic (cyt 18S and cyt 25S) rRNAs. The mt 18S showed no evident difference between grs1-1 and the wild-type, while a significant increase was observed for mt 26S rRNA in grs1-1 plants compared to the wild-type (Fig. 5C), with the increased ratio of mt 26S to mt 18S indicating an imbalance between mitoribosomal subunits. The chl 16S, chl 23S, cyt 18S, and cyt 25S showed no obvious differences between grs1-1 and the wild-type (Fig. 5C), suggesting the grs1 mutation only affects mitochondrial ribosome biogenesis.

An alternative respiratory pathway is activated in grs1-1 mutants

Lack of complex I activities is known to result in elevated levels of an alternative respiratory pathway in Arabidopsis (Yuan and Liu, 2012). The components of this alternative respiratory pathway include several alternative NAD(P)H dehydrogenases (NDs) and alternative oxidases (AOXs). To determine whether grs1-1 mutants had the same phenotype, we performed quantitative RT-PCR assays for the transcripts levels of six ND genes and three AOX genes in wild-type and grs1-1 plants. As shown in Fig. 6, the expression levels of the nine examined genes in grs1-1 increased significantly relative to the wild-type. These results indicate that the alternative respiratory pathway is activated in grs1-1. grs1-2 mutants had a similar phenotype with up-regulation of transcripts for alternative respiration compared with the wild-type. (Supplementary Fig. S4).

The grs1-1 mutant does not accumulate higher amounts of ROS than the wild-type

Reports have shown that impaired activity of the mitochondrial electron transport chain of complex I can cause a redox imbalance and increases in ROS accumulation, leading to the accumulation of more ROS in mutants than in the wild-type (Liu et al., 2010; Yang et al., 2014). We analyzed the ROS levels in grs1-1 mutants and wild-type plants and showed that grs1-1 mutants do not accumulate higher amounts of ROS than the wild-type (Fig. 7A, B). Consistent with these results, addition of the reducing agent glutathione (GSH) or diphenyleneiodonium chloride (DPI) was not able to complement the root growth defects of grs1-1 mutant plants (Fig. 7C).

Fig. 5. Complex I activity and mitoribosomal biogenesis are affected in grs1-1 mutants. (A) Proteins of crude organelle extractions from young seedlings of wild-type (WT), grs1-1, and grs1-1 complemented lines were stained by Coomassie blue. (B) In-gel assay of NADH dehydrogenase activity in WT, grs1-1, and grs1-1 complemented lines. Activity of complex I could hardly be detected in grs1-1. The activity staining bands on the lower part of the gel correspond to the activity of the dehydrodiploamide dehydrogenase, which can serve as a loading control. I, II, and III, mitochondrial complex I and complex II super-complex; I, mitochondrial complex I; sub I, mitochondrial sub-complex I. (C) Accumulation of rRNAs as a proxy for corresponding ribosomal subunits in grs1-1 compared with wild-type plants. Levels of rRNA transcripts of large subunits and small subunits in mitochondrial, chloroplast, and cytosolic ribosomes are shown. The values obtained were averaged for three biological replicates, with error bars representing SD. Statistically significant differences between grs1-1 and the wild-type are indicated: **P<0.01 (Student’s t-test).
abi5 partially rescues the post-germination growth arrest of grs1-1

Since grs1 mutant display defects in seed germination and post-germination growth, it is possible that the ABA signaling pathway is activated in these mutants. Given that the transcription factors AB13 and AB15 are key proteins in the ABA signaling pathway (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001), expression of AB13 and AB15 was analyzed in grs1-1 mutant and wild-type seedling plants 8 d after germination. Expression of AB15 was found to be significantly up-regulated in grs1-1 mutants, whereas expression levels of AB13 were not significantly altered (Fig. 8A), implying that AB15, but not AB13, is activated in grs1-1 mutants and is involved in the short-root phenotype. To test this hypothesis, the grs1-1 abi5-1 double-mutant was generated, and it showed longer roots than those of the grs1-1 mutants (Fig. 8B, Supplementary Table S2). While only about 10 cells could be observed in the meristems of in grs1-1 mutants, approximately 20 cells were established in the meristem of grs1-1 abi5-1 double-mutant plants (Fig. 8C). These results indicate that abi5-1 partially rescues the post-germination growth arrest of the grs1-1 mutants.

We then tested whether a decrease in the ABA content in grs1-1 mutants can rescue the post-germination growth arrest of these plants. The gin1-3 mutant line is a knockout allele of the ABA2 gene, one of the key genes involved in ABA synthesis. However, the grs1-1 gin1-3 double-mutant did not show any evident differences compared with the grs1-1 single-mutant plants in post-germination growth (Fig. 8B, C).

Discussion

Putative cis-acting elements recognized by GRS1

Recently bioinformatics, biochemical, and structural analyses have shown that PPR proteins recognize RNA in one-motif to one-nucleotide binding mode (Kim et al., 2009; Yagi et al.,
The major determinant is the amino acid at position 5 of the motif (Yagi et al., 2013; Yin et al., 2013; Barkan and Small, 2014). The second major determinant is at position 2 of the motif and position 35 of the following motif (Yagi et al., 2013; Yin et al., 2013; Barkan and Small, 2014; Cheng et al., 2016). The site-specific RNA editing factors PPR and the RNA target sequences show optimal correlations when the PPR domains are aligned with the nucleotide sequences upstream of RNA editing sites up to the fourth nucleotide (nucleotide −4). The last S motif of GRS1 is accordingly positioned at the −4 nucleotides site of all the editing sites (Supplementary Fig. S3 and Table S1). In this way, the conserved A nucleotide at position −12 and G nucleotide at position −6 are consistent with the predictions of bioinformatics (Kim et al., 2009; Yagi et al., 2013; Yin et al., 2013; Barkan and Small, 2014).

Cis-elements located between 20 to 25 nucleotides upstream and one to three nucleotides downstream of the edited C are known to be important in the context of RNA editing in mitochondria and plastids (Zehrmann et al., 2009; Barkan and Small, 2014). When comparing the context of the four RNA sites edited by GRS1, five nucleotides are identical in addition to the edited C (Supplementary Fig. S3), suggesting that these positions are important for guiding editing through GRS1 in the mitochondria. These five nucleotides, however, are not sufficient to specify a unique site in the plant mitochondrial transcriptome. An in silico screen identified NAD4-403, another editing site with the same RNA context in the mitochondrial genome (Supplementary Fig. S3). NAD4-403 is edited normally in the wild-type and in the grs1 mutant, confirming that the five shared nucleotide positions are not sufficient to guide editing through GRS1. More information may be provided by other nucleotides inside the context of RNA editing of the four sites to ensure GRS1 specifically binds to them. It was reported that PPR proteins distinguish purines from pyrimidines much better than they distinguish between C/U or A/G (Yagi et al., 2014; Kindgren et al., 2015). The conservation between these four sequences is better than shown when this is taken into account, with several other nucleotide positions, such as −4, −7, −9, −14, and −15, showing expected matches to the protein sequence in addition to the ones that have been indicated. The correlations of the amino acid codes in GRS1 and the diversity of its targeted RNA bases can offer more information for predicting whether a PPR protein can bind a particular RNA.

Comparison of grs1-1 plants with other Arabidopsis complex I mutant lines

Loss of GRS1 directly affects the editing of three components of complex I: nadl-265, nad4L-55, and nad6-103, which in turn impair the function of complex I. Most complex I mutants show a retarded growth phenotype, such as ahgl1 (Murayama et al., 2012), abo5 (Liu et al., 2010), abo8 (Yang et al., 2014), birh (Kopriovova et al., 2010), cssl (Nakagawa and Sakurai, 2006), indh (Wydro et al., 2013), mtsfl (Haili et al., 2013), nMat1 (Keren et al., 2012), nMat2 (Keren et al., 2009), nMat4 (Cohen et al., 2014), otp43 (de Longevialle et al., 2007), otp439 and tang2 (Colas des Francs-Smith et al., 2014), slg1 (Sung et al., 2010), slo2 (Zhu et al., 2012), slo3 (Hsieh et al., 2015), and also asamk1 (small kernel 1), which has been shown to be responsible for loss of editing of NAD7-448 transcripts in maize and rice (Li et al., 2014).

The phenotype of grs1-1 plants, however, cannot be fully explained by the loss of function of complex I. The defects observed in grs1-1 plants are much stronger than those of mutants defective in complex I activity such as the slo2 (Zhu et al., 2012), otp43 (de Longevialle et al., 2007), nMat1 (Keren et al., 2012) and ndufs4 mutants (Meyer et al., 2009). Impaired activity of the mitochondrial electron transport chain of complex I can cause a redox imbalance and increases in ROS accumulation, leading to the accumulation of more ROS in mutants than in the wild-type (Liu et al., 2010; Yang et al., 2014); however, the grs1-1 mutants do not accumulate more ROS than the wild-type. Consistent with these results, addition of GSH or DPI could not restore the root growth defects of grs1-1 mutant plants. The results indicate that other signals must be responsible for the retarded growth phenotype observed in grs1-1 plants.

ABA is a well-established key player in seed germination and post-germination growth. Furthermore, some reports have shown that mutations of PPR proteins result in mutant plants that are more sensitive to ABA than wild-type plants (Liu et al., 2010; Murayama et al., 2012; Yang et al., 2014). Expression of ABI5 was found to be significantly up-regulated...
in the grs1-1 mutants compared to the wild-type plants, while expression of \( AB13 \) was not up-regulated in grs1-1 mutant plants compared to the wild-type. The results indicate that the up-regulated expression of \( AB15 \) is independent of the ABA signal. The grs1-1 ab15-1 double-mutant displayed higher root meristem cell numbers than the grs1-1 single-mutant plants. The results indicate that ab15-1 partially rescued the post-germination growth arrest of grs1-1 mutant plants. The grs1-1 gin1-3 double-mutant, however, could not partially rescue the post-germination growth arrest of grs1-1 mutant plants. These findings suggest that \( AB15 \), but not ABA, is involved in the post-germination growth arrest of grs1-1 mutant plants. The mechanism through which grs1-1 mutant plants activate \( AB15 \) remains an interesting question for future investigation.

Other factors must be involved in the root growth defects of the grs1-1 mutant plants, since ab15-1 only partially rescued their post-germination growth arrest. One possibility is that the mutation of GRS1 also impairs the function of mitoribosomes, leading to a dysfunction of mitochondria in addition to the loss of function of complex I. This scenario is found in mcsfi mutants, where the activity of complexes I and IV are both reduced, leading to severe defects in embryo development, which is arrested at the early globular stage (Zmudjak et al., 2013).

### Supplementary data

Supplementary data are available at JXB online.

**Figure S1.** Rapid identification of heterozygous and homozygous mutants through pollen fluorescence.

**Figure S2.** Genomic complement fragment of \( At4g32430 \) rescues the phenotype of grs1-1.

**Figure S3.** Putative coordination of PPR motifs of GRS1 and RNA nucleotides around the editing sites targeted by GRS1.

**Figure S4.** Relative expression of alternative pathway genes in wild-type and grs1-2.

**Table S1.** PLS repeat structure of \( At4g32430 \).

**Table S2.** The root length of 8-d-old seedlings.

**Table S3.** Primers used for RNA editing analysis.

**Table S4.** Primers used for quantitative RT-PCR.

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