The Pentatricopeptide Repeat Protein SOT5/EMB2279 Is Required for Plastid rpl2 and trnK Intron Splicing

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Chloroplast biogenesis and development are highly complex processes requiring interaction between plastid and nuclear genomic products. Using a high-throughput screen for chloroplast biogenesis suppressors in Arabidopsis (Arabidopsis thaliana), we identified a suppressor of thf1 (sot5) that displays virescent and serrated leaves. Further characterization revealed that sot5 mutants are defective in leaf adaxial and abaxial polarity and act as enhancers of asymmetric leaves2. Map-based cloning identified SOT5 as a gene previously named EMB2279 that encodes a plastid-targeted pentatricopeptide repeat (PPR) protein with 11 PPR motifs. A G-to-A mutation in sot5 leads to a significant decrease in splicing efficiency, generating two additional mRNA variants. As reported previously, the sot5 null mutation is embryo lethal. SOT5 is predicted to bind to specific RNA sequences found in plastid rpl2 and trnK genes, and we found decreased splicing efficiency of the rpl2 and trnK genes in sot5 mutants. Together, our results reveal that the PPR protein SOT5/EMB2279 is required for intron splicing of plastid rpl2 and trnK, providing insights into the role of plastid translation in the coupled development between chloroplasts and leaves.

Chloroplast development, which is an essential event for the switching of plant growth from heterotrophy to autotrophy, relies on the coordinated expression of nuclear and plastid genes. Although significant progress has been made in the past few years toward understanding the molecular mechanisms underlying chloroplast development, it remains elusive how chloroplast gene expression is finely regulated in response to intracellular and environmental cues (Pogson et al., 2015; Chan et al., 2016). Since chloroplasts originated from endosymbiotic cyanobacteria and possess prokaryote-type circular DNA, the chloroplast remains the same machinery that carries out gene expression as prokaryotes (Jarvis and López-Juez, 2013). However, some novel events, such as RNA editing, intron splicing, and RNA cleavage and trimming (Stern et al., 2010), have evolved, making gene expression in chloroplasts distinct from that in prokaryotes.

In Arabidopsis (Arabidopsis thaliana) chloroplasts, 20 introns are distributed across 17 genes (de Longevialle et al., 2010); 19 of these introns belong to the group II family of introns, and only one, located in the trnL gene, belongs to group I. Original group I and II introns are large ribozymes that cleave phosphodiester bonds through two consecutive trans-esterification steps in a sequence-specific manner (Lehmann and Schmidt, 2003). The main differences between these two types of introns lie in their secondary structures and chemical reaction mechanisms for the first splicing step: group I introns are composed of 10 domains (P1–P10) that play a role in RNA folding for efficient splicing and ligation; group II introns have six domains (I–VI), each with distinct functions in intron self-splicing and/or retromobility (Lehmann and Schmidt, 2003; Dai et al., 2008; Pyle, 2016). The largest domain, domain I, functions as a structural scaffold and recognizes the exon positions for catalysis. Domains II and III enhance the catalysis of splicing. Domain IV, the most variable domain in different introns, contains the intron-encoded protein (IEP) sequence, which constitutes four different subdomains (reverse transcriptase, maturase, DNA binding, and endonuclease) that are required for RNA folding during splicing and intron retromobility after splicing. Chloroplast introns have no IEP sequence except for the trnK intron, which contains a limited coding sequence only for functional maturase (MatK; Zoschke et al., 2010) and the break point of the trans-splicing rps12-1 intron (Glanz and Kück, 2009). The highly conserved domain V contains the catalytic core that is essential for ribozyme activity. Domain VI...
contains the branch-point site adenosine required for the splicing reaction. Group II introns are further classified into several subclasses (IIA–IIF) that display distinct structural and functional differences (Pyle, 2016). Among the 19 group II introns in Arabidopsis chloroplasts, eight belong to group IIA and 11 belong to group IIB (de Longevialle et al., 2010). However, both group I and group II introns in plant organelles are degenerated and require additional trans-factors for efficient splicing in vivo (Hahn et al., 1998; de Longevialle et al., 2010). These trans-factors, including chloroplast IEP (MatK) and nucleus-encoded RNA-binding factors, are assembled with cis-elements (intron RNAs) into active ribonucleoprotein complexes for accurate and efficient self-splicing (Stern et al., 2010; Zoschke et al., 2010). The chloroplast matK gene was reported to be required for the splicing of seven group II A introns, trnA, trnI, trnV, atpF, rpl2, rps12-2, and trnK (Zoschke et al., 2010).

In addition to the trans-factor encoded by matK, a number of nucleus-encoded proteins involved in chloroplast splicing events have been identified, particularly from maize (Zea mays), through genetic and biochemical approaches. Some of the trans-factors are required for splicing multiple introns, while others are required for specific introns (Stern et al., 2010). Chloroplast RNA Splicing 1 (CRS1) and CRS2 (Jenkins et al., 1997) were the first reported trans-factors in maize. Whereas CRS1 contains three repeated chloroplast RNA splicing and ribosome maturation (CRM) domains and is essential solely for the splicing of the chloroplast atpF group II intron (Till et al., 2001; Keren et al., 2008), CRS2 is a homolog of bacterial peptidyl tRNA hydrolase and required for the splicing of nine group II introns (Jenkins et al., 1997; Ostheimer et al., 2003). CRS2 forms a complex with CRS2-Associated Factor 1 (CAF1) and/or CAF2, both of which are CRM proteins with two CRM domains, to facilitate the splicing of the CRS2-dependent introns (Ostheimer et al., 2003). The splicing specificity of CRS2/CAF-dependent introns is determined by CRM Family Member 2 (CFM2) and CFM3 (Asakura et al., 2008). Two general splicing factors, an RNase III domain protein (RNCl) and WTF1 (WHAT IS THIS FACTOR1), were identified from CAF1- and/or CAF2-immunoprecipitated ribonucleoprotein particles by mass spectrometry (Watkins et al., 2007). RNCl, which contains two RNase III domains without RNA cleavage activity, and WTF1, which has the plant organelle RNA recognition domain (PORR), form heterodimers that splice most of chloroplast group II introns in a CAF1/CAF2-dependent or -independent manner (Kroeger et al., 2009). It remains unclear whether a core spliceosome, as in the nucleus, is present for the in vivo splicing of all group II introns in chloroplasts.

In addition to these general splicing factors, another group of proteins involved in chloroplast intron splicing events are pentatricopeptide repeat (PPR) proteins, which bind to their single-stranded RNA targets in a modular and base-specific fashion (Yin et al., 2013; Barkan and Small, 2014). For example, PPR4 and PPR5 are responsible for the splicing of the rpl12 and trnG-UCC group II introns in maize, respectively (Schmitz-Linneweber et al., 2006; Beick et al., 2008); in Arabidopsis, ORGANELLE TRANSCRIPT PROCESSING 70 (OTP70) is required for the splicing of the rpc1 intron (Chateignier-Boutin et al., 2011), OTP51 for ycf3 intron 2 and several other group II A introns as well (de Longevialle et al., 2008), THYLAKOID ASSEMBLY8 for ycf3 intron 2 and trnA (Krouchitchova et al., 2012), EMBRYO DEFFECTIVE2654 (EMB2654) for trans-splicing of rps12 intron 1 (Aryamanesh et al., 2017); in rice (Oryza sativa), WHITE STRIPE LEAF (WSL) is required for rpl2 intron splicing (Tan et al., 2014) and WSL4 for four group II introns, atpF, rpl2, rps12-2, and ndhA (Wang et al., 2017). Generally, PPR proteins are characterized by a one-repeat-one-nucleotide recognition mechanism (Prikryl et al., 2011; Yin et al., 2013). Thus, the binding specificity and affinity of a PPR protein to RNA targets are associated with the number of PPR repeats. It has been known that over 450 PPR proteins are present in Arabidopsis and divided into two subfamilies, designated P and PLS, according to the characteristics of the PPR motifs (Lurin et al., 2004). Most PPRs are involved in organellar gene expression, including RNA transcription and stability, RNA editing, RNA maturation, RNA translation, and RNA splicing (Schmitz-Linneweber and Small, 2008). However, the roles of most PPR proteins in RNA processing still remain unknown. It is thought that, during the group II intron splicing process, the intron-specific PPRs act in an initial sequence-specific binding step that alters RNA conformation ready for the recruitment of the relevant general splicing complex, such as the WTA/RNC1 complex (de Longevialle et al., 2010). This hypothesis still remains to be validated by experimental data.

Variegation mutants are valuable for the study of chloroplast biogenesis mechanisms. The study of suppressors of variegation mutants, such as var2 (variegation2) and thf1 (thylakoid formation1), has provided lots of insights into the mechanisms of chloroplast development (Yu et al., 2008; Wu et al., 2013, 2016; Hu et al., 2015; Ma et al., 2015). Here, we report another suppressor of thf1, namely sot5 (suppressor of thf1 5), whose gene encodes one of the Arabidopsis PPR proteins, EMB2279. We found that EMB2279/SOT5 is required specifically for plastid rpl2 and trnK intron splicing. The sot5 defects in plastid translation and leaf morphogenesis may indicate a role of plastid gene expression in regulating leaf development.

RESULTS

Identification and Characterization of the sot5 Mutant

To investigate the molecular mechanisms by which THF1 mutations lead to defects in chloroplast development, we mutagenized thf1 seeds with ethyl
methanesulfonate and screened for suppressors of thf1 leaf variegation, as reported previously (Wu et al., 2013, 2016; Hu et al., 2015; Ma et al., 2015). The sot5 single mutant was isolated from the F2 generation of the cross between a suppressor line, 130-1, and Columbia-0 (Col-0). Both sot5 and 130-1 displayed the same phenotype, such as virescent and serrated leaves (Fig. 1A). sot5 cotyledons were yellowish and contained about 74% of the chlorophyll (Chl) content of wild-type cotyledons (Supplemental Fig. S1, A and B). By contrast, young true leaves of sot5 were initially pale yellow but gradually became light green (Fig. 1A; Supplemental Fig. S1C). Total Chl analysis showed that sot5 had about 15% and 64% of the Chl content of the wild type in young and mature leaves, respectively (Fig. 1B). Transmission electron microscopy analysis of chloroplast ultrastructure demonstrated that chloroplasts in sot5 leaves were initially smaller and had fewer thylakoid membranes than those in wild-type leaves (Fig. 1C), but this difference was not apparent in mature leaves (Fig. 1C). Taken together, these results indicate that SOT5 is required for chloroplast development.

We also analyzed the photosynthetic capacity of sot5. The $F_v/F_m$ value, which represents the maximum quantum yield of PSII photochemistry, was significantly lower in young leaves of sot5 than those of the wild type but was comparable in the mature leaves between sot5 and the wild type (Fig. 2, A and B). NPQ, which is an important photoprotective mechanism to dissipate excessive light energy absorbed by antennae as heat, increased more rapidly in sot5 than in the wild type in both mature and young leaves, whereas there was no obvious difference in NPQ relaxation in the dark between sot5 and the wild type (Fig. 2B), and young sot5 leaves maintained a higher level of NPQ than wild-type leaves in light (Fig. 2B). Immunoblot analysis showed that all photosynthetic proteins examined, such as D1, Lhca1, Lhcb6, PsaD, and RbcL, accumulated to apparently significantly lower levels in sot5 young leaves (Fig. 2C) compared with the wild type, but the difference of these proteins between the wild type and sot5 disappeared in mature leaves. Further analysis of photosynthetic complexes in sot5 leaves using blue-native (BN) PAGE assays showed that sot5 contained the same pattern of photosynthetic complexes as the wild type (Fig. 2D). Together, these results indicate that loss-of-function SOT5 reduces photosynthetic efficiency and capacity.

Leaf Morphology and Cell Proliferation Were Altered in sot5

In addition to photosynthesis-related defects, sot5 mutants displayed leaf morphology defects. The first
true leaf of sot5 had serrations and simplified vascular bundles (Fig. 3A), and cross sections of the first leaf showed that the epidermis of sot5 was not as smooth as that of the wild type and that the air spaces between mesophyll cells were larger in sot5 than in the wild type (Fig. 3A). In addition, the anatomical identity of palisade and spongy cells disappeared in sot5 leaves (Fig. 3A), indicating that the leaf adaxial-abaxial polarity was altered in sot5 mutants. Using scanning electron microscopy to examine the structure of abaxial and adaxial cells in sot5 leaves (Fig. 3A), we found that both adaxial and abaxial sides of sot5 leaves were severely wrinkled and that adaxial epidermal cells resembled abaxial ones (Fig. 3B), suggesting reduced adaxial cell identity in the sot5 mutant. Since sot5 leaves are small and their mesophyll cells are loosely arranged, we hypothesized that the number of mesophyll cells was reduced in sot5. As expected, the number of mesophyll cells in sot5 was about 60% of the wild-type value (Fig. 3C), indicating that cell proliferation is inhibited in sot5 leaves. Together, these results indicate that SOT5 plays an important role in establishing abaxial-adaxial leaf polarity.

In Arabidopsis leaf development, ASYMMETRIC LEAVES1 (AS1) and AS2 form an epigenetic repressor complex that plays a critical role in repressing abaxial-determining genes and promotes adaxial polarity formation (Husbands et al., 2015). AS1 or AS2 loss-of-function mutants exhibit mild abaxialized leaves, such as lotus-like leaves (Xu et al., 2003). Previous work has demonstrated that the Arabidopsis mutants with altered leaf cell proliferation and leaf adaxial-abaxial patterning (asymmetric leaves1/2 enhancer7, elongator, and scabra1 [sca1]) can enhance the as2 phenotype, resulting in narrow and even needle-like (abaxialized) leaves (Yuan et al., 2010; Xu et al., 2012; Mateo-Bonmati

Figure 2. Photosynthetic characterization of sot5 mutants. A, Analysis of $F_v/F_m$ of 30-d-old wild-type (WT) and sot5 plants. The graph at right shows quantified $F_v/F_m$ of the mature and young leaves shown at left (Chl fluorescence image). The data represent means ± se (n = 6). Asterisks indicate a significant difference between the wild type and sot5 (Student’s t test, **, P < 0.05). B, Nonphotochemical quenching (NPQ) measurement of mature and young leaves from 30-d-old wild-type and sot5 plants. The data represent means ± se (n = 6). C, Immunoblot analysis of photosynthetic proteins accumulated in mature and young leaves from 30-d-old wild-type and sot5 plants. Coomassie Brilliant Blue (CBB) staining is shown to check the difference in sample loading. D, BN-PAGE analysis of photosynthetic complexes in thylakoid membranes isolated from 30-d-old wild-type and sot5 plants. Each lane was loaded with 6 μg of Chl.
et al., 2015). To genetically test whether leaf polarity is altered in sot5 mutants, we generated double sot5 as2 mutants and found that they displayed many narrow or needle-like leaves (Fig. 3D), supporting our hypothesis that sot5 is an enhancer of as2. Together, these results indicate that SOT5 has a role in promoting leaf adaxial identity formation.

**SOT5 Encodes a Chloroplast-Localized PPR Protein**

Genetic analysis showed that the sot5 phenotype was controlled by a single nuclear recessive mutation. To clone sot5, we produced an F2 population from the cross between 130-1 and Landsberg erecta (Ler) for map-based cloning. Using 900 recombinant plants, we mapped the sot5 gene on the upper arm of chromosome 1 within an ~70-kb region between F26G16-a and T5I8-d markers, in which there are 20 annotated genes (Fig. 4A). Bioinformatic analysis revealed that, among these 20 candidates, six genes encode chloroplast-localized proteins, one of which is a PPR protein (AT1G30610), named EMB2279. DNA sequencing showed a G-to-A substitution at the first nucleotide of the seventh intron in AT1G30610 (Fig. 4B). Reverse

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**Figure 3.** Leaf morphology and adaxial-abaxial polarity are altered in sot5. A, Morphology and cross section of the first true leaves from 25-d-old wild-type (WT) and sot5 plants. Bars = 5 mm at left and 100 µm at right. B, Scanning electron microscopy analysis of adaxial and abaxial surfaces of the first true leaves from 25-d-old wild-type and sot5 plants. Enlargements of the larger and smaller squares in the top row are shown in the middle and bottom rows, respectively. Bars = 1 mm in the top row, 200 µm in the middle row, and 50 µm in the bottom row. C, Comparison of palisade cell size (left) and number (right) from the first true leaves of 25-d-old wild-type and sot5 plants. The data represent means ± so of 10 leaves. Asterisks indicate a significant difference between the wild type and sot5 (Student’s t test, ** P < 0.05). Bars = 50 µm. D, sot5 enhances the leaf adaxial-abaxial polarity of as2-1. White arrows indicate filamental leaves. Bars = 1 cm.
transcription (RT)-PCR analysis using the primer pair shown in Figure 4B detected three variants of the transcripts in sot5 but only one in the wild type (Fig. 4C). Sequencing of these three transcripts revealed that the longest transcript (a in Fig. 4C) contained the seventh intron, the middle transcript (b in Fig. 4C) was the same as the wild type, and the shortest transcript (c in Fig. 4C) lacked 22-bp nucleotides at the 3′ end of the seventh exon. The 22-bp nucleotide deletion resulted in a truncated form of SOT5. Thus, the sot5 point mutation leads to a low efficiency of the intron splicing and missplicing of the exon. By contrast, we did not detect any alternative splicing events in the seventh intron of the SOT5 gene in the wild type, which is contrary to recorded data in TAIR, where two transcriptional variants with or without the seventh intron of the SOT5 gene are described. Based on our data, SOT5 encodes only one protein with 978 amino acid residues.

To confirm the map-based cloning result, we transformed the SOT5 cDNA under the control of the cauliflower mosaic virus 35S promoter into the sot5 mutant. All the sot5 phenotypes were complemented by over-expressing SOT5 (Fig. 4D), confirming that SOT5 encoding the 978-amino-acid polypeptide is functional. A previous study reported that the null mutation emb2279-2 is embryo lethal (Lurin et al., 2004; Cushing et al., 2005), causing embryo development to stop at the globular stage (Tzafrir et al., 2003). To determine whether emb2279 and sot5 are alleles of the same gene, we first identified emb2279-2 (SALK_088420) from the Arabidopsis Biological Resource Center (ABRC) stock. Indeed, about 25% of the seeds were albino and aborted in the emb2279-2 heterozygote siliques (Fig. 4E), confirming the embryo-lethal phenotype of emb2279. We then crossed sot5 with the emb2279-2 heterozygote. We found that half of the F1 plants were almost albino and half of them were wild type like when they were cultured on one-half-strength Murashige and Skoog medium. The albino plants could turn a bit green when cultured on one-half-strength Murashige and Skoog medium but could not survive after transfer to the soil, since their leaves were severely pale (Fig. 4F). Furthermore, we generated SOT5 knockdown lines by an artificial microRNA technique. Transgenic plants with low expression levels of SOT5 showed a virescent phenotype (Fig. 4G). Taken together, our data suggest that sot5 is a weak allele of emb2279.

Although SOT5/EMB2279 was predicted to target chloroplasts, no evidence supports this prediction. To test this, we made a construct in which the first N-terminal 120 amino acids containing the transit peptide of SOT5 were fused to yellow fluorescent protein (YFP) driven by the cauliflower mosaic virus 35S promoter and transformed it into protoplasts. Confocal microscopy analysis showed that YFP signal was totally overlapped with Chl autofluorescence (Fig. 5A), indicating that SOT5 is localized in chloroplasts. RT-PCR analysis showed that SOT5 was expressed ubiquitously in all examined tissues (Fig. 5B): in 5-d-old green or etiolated seedlings and in etiolated seedlings that had been exposed to light for 4 h (Fig. 5B). These results indicate that SOT5 functions in both green and non-green tissues.

SOT5 Is Required for Splicing of the Plastid rpl2 and trnK Introns

SOT5 is a P-subfamily PPR protein composed of 978 amino acid residues. ScanProsite (https://prosite.expasy.org/) analysis showed that SOT5 has 11 PPR motifs at the C terminus and no other function-known domains at the N terminus (Fig. 6A). BLAST searches demonstrated that SOT5 is a single-copy gene in Arabidopsis and is conserved in monocots and dicots, but not in Bryophyta, Lycopodiophyta, Chlorophyta, and Rhodophyta (http://plantsensembl.org/Arabidopsis_thaliana/Gene/Compara_Otholog?db=core;g=AT1G30610;r=x:10846513-10850724). The 11-PPR motifs of SOT5 also are present in the mosses Physcomitrella patens and Marchantia polymorpha, in the fern Selaginella moellendorffii, but not in green algae (Supplemental Fig. S2).

Since PPR proteins participate in RNA processing, such as RNA splicing, editing, maturation, and stabilization, through directly recognizing specific RNA sequences, we propose that the target of SOT5 also might be conserved in higher plants. To identify the targets of SOT5, we first predicted its binding target, the single-stranded RNAs, according to the PPR code and previously identified single-stranded RNAs targeted by different PPRs (Barkan et al., 2012; Yin et al., 2013; Yagi et al., 2014). Our analysis revealed that the 11 ribonucleotides targeted by the 11 PPR motifs of SOT5 are X(G/U)C(A/C)XXAUXC, with X representing any nucleotide that cannot be precisely predicted and nucleotides in parentheses being optional (Fig. 6B). We then searched the plastome using this potential SOT5-binding sequence and found that 14 plastid genes, ATTCG00860 (ycf2.1), ATTCG01280 (ycf2.2), ATTCG00170 (rpoC2), ATTCG00950 (23S.1 rRNA), ATTCG01180 (23S.2 rRNA), ATTCG00030 (trnK), ATTCG01130 (ycf1.2), ATTCG00100 (trnG), ATTCG00360 (ycf3), ATTCG00570 (psbF), ATTCG00680 (psbB), ATTCG00830.1 (rpl2.1), ATTCG01310 (rpl2.2), and ATTCG01100 (ndhA), contain the sequence (Supplemental Table S1). Based on these data, we designed primers flanking the binding sites and analyzed the expression levels of these candidate genes by reverse transcription-quantitative PCR (RT-qPCR). None of these genes were down-regulated significantly in sot5 (Fig. 6C), but some NER genes, such as rpoC2, ycf1, and ycf2, were up-regulated in sot5 (Fig. 6C). These results indicate that SOT5 is not required for the RNA stability of these candidate genes.

Among these candidate genes, rpl2, ndhA, trnK, and trnG contain the group II intron. Therefore, we investigated whether SOT5 was involved in the intron splicing of these chloroplast genes. RT-qPCR analysis showed that the intron splicing efficiency for only rpl2 was reduced significantly in sot5 (Fig. 6D). Further RT-qPCR analysis using the primers flanking the group II intron showed that the precursor levels of...
rpl2 transcripts were accumulated in sot5 and thf1 sot5 but not in the wild type and thf1, but no difference was detected in transcript levels of other intron-containing genes between the wild type and sot5 (Fig. 6E). Consistently, the levels of unspliced rpl2 transcripts accumulated in the SOT5 knockdown line (amiR-SOT5) but were restored to wild-type levels in complementary lines (35S:SOT5/sot5; Fig. 6, F and G). Since mature tRNAs are small and have strong secondary structure and base modifications, they are difficult to detect precisely by RT-PCR (de Longevialle et al., 2008). To assess mature tRNA and precursor
levels, we employed northern-blot analysis. Our data demonstrated that only trnK unspliced precursors accumulated significantly in sot5, sot5 thf1, and amiR-SOT5, and trnK spliced mature products were reduced dramatically in these genotypes compared with the wild type and 35S:SOT5/sot5 (Fig. 6H), suggesting that SOT5 mutations result in the reduced splicing efficiency of trnK. We were unable to verify the binding site of SOT5 in vitro, because the recombinant SOT5 protein was not expressed in prokaryotic and eukaryotic systems.

Since the matK gene, which is required for the splicing of seven group IIA introns in trnA, trnI, trnV, atpF, rpl2, rps12-2, and trnK (Zoschke et al., 2010), is located in the trnK intron, it is possible that lowered trnK intron splicing efficiency in sot5 would affect the level of matK transcripts and subsequently affect the intron splicing of all group IIA genes. Moreover, decreased rpl2 and trnK might affect plastid protein translation, leading to a decrease in MatK activity in sot5. However, our results demonstrated that the relative level of matK transcripts was enhanced in sot5 (Fig. 6C), probably due to the higher accumulation of trnK precursors in sot5, and the splicing efficiency of atpF, rps12-2, trnA, trnI, and trnV was not affected significantly by SOT5 mutations (Fig. 6, C and H). These results suggest that, in sot5 mutants, MatK activity is high enough to maintain the splicing of these introns, and the reduced splicing efficiency of rpl2 and trnK should be caused directly by SOT5 mutations.

SOT5 Mutations Also Suppress the Leaf Variegation of var2 and Inhibit Plastid rRNA Processing

Since thf1 leaf variegation is attributed to a low level of var2 (Zhang et al., 2009), we tested whether sot5 can suppress the variegation phenotype of var2. Genetic analysis showed that var2 sot5 double mutants displayed a similar phenotype to sot5 mutants (Fig. 7A), suggesting that sot5 is a suppressor of var2. This is consistent with the previously reported results that a decrease of plastid translation efficiency can suppress leaf variegation (Yu et al., 2008; Ma et al., 2015; Wu et al., 2016).

It was reported previously that mutants deficient in plastid translation display a reduced efficiency of plastid rRNA processing (Yu et al., 2008; Ma et al., 2015; Wu et al., 2016). Based on this, we investigated the effect of sot5 mutations on rRNA processing. Northern-blot analysis showed that levels of mature 16S, 23S, and 4.5S rRNA were reduced in sot5 compared with the wild type, whereas their precursors overaccumulated (Fig. 7B), similar to our previously reported mutants, prpl11-2 and prps9 (Supplemental Fig. S3), which were defective in plastid ribosomal protein large subunit 11 and ribosomal protein small subunit 9, respectively (Wu et al., 2013; Ma et al., 2015). These results indicate that plastid rRNA processing is linked to ribosome biogenesis and that the sot5 mutant is indeed defective in ribosome biogenesis and protein translation in plastids due to the reduced splicing of rpl2 and trnK genes.
Figure 6. SOT5 is required for the splicing of the plastid rpl2 and trnK introns. A, Schematic SOT5 protein containing 11 PPR motifs. B, Predicted 11 ribonucleotides targeted by the 11 PPR motifs shown in A. In each repeat, the two key amino acid residues and their nucleotide targets are shown. X indicates unpredictable; two nucleotides with a slash mean optional; the red nucleotides are precisely predicted. C, RT-qPCR analysis of relative expression levels of the plastid genes containing the predicted target sequences or group IIA introns in sot5. Three biological replicates were analyzed. D, Splicing efficiency analysis of 14 plastid introns in sot5 by RT-qPCR. Three biological replicates were analyzed. E, RT-PCR analysis of intron retention in eight plastid genes. Genomic DNA (gDNA) was used as a template control. S, Spliced; U, unspliced. F, RT-PCR analysis of rpl2
reduced efficiency of plastid rRNA processing may be the indirect effect of a translation defect in sot5. However, since 23S rRNA also was predicted to be the target of SOT5, we cannot rule out the possibility that SOT5 also can bind directly to 23S rRNA and facilitate its processing.

DISCUSSION

A Role of SOT5 in rpl2 and trnK Intron Splicing

In this study, we identified a PPR protein, SOT5/EMB2279, that is involved in the splicing of the plastid rpl2 and trnK introns. Since both Rpl2 and trnK are essential for plastid protein translation, complete disruption of SOT5 would lead to embryo lethality in Arabidopsis, similar to previously reported results for EMB2279 (Lurin et al., 2004; Cushing et al., 2005). The sot5 mutant contains a point mutation that leads to a low efficiency of the seventh intron splicing and missplicing of the seventh exon, resulting in a hypomorphic allele of emb2279. In TAIR, the SOT5 locus (AT1G30610) is recorded to produce two types of mRNA depending on whether the seventh intron is removed or not: the longer transcript encodes a 1,006-amino acid-long peptide with 10 PPR motifs,
whereas the shorter one encodes a 978-amino acid-long peptide with 11 PPR motifs. However, we only detected the shorter transcript, which lacks 84 bp compared with the longer one, indicating that SOT5 has 11 PPR motifs. Consistent with the previous reports (Lurin et al., 2004; Cushing et al., 2005), total disruption of SOT5 leads to embryo lethality in Arabidopsis, whereas knocking down SOT5 expression through the artificial microRNA technique or partially knocking out the function of SOT5 by a point mutation resulted in a virescent phenotype. These results are consistent with the fact that most of the suppressors for thf1 and var2 leaf variegation are mutants defective in the plastid translation system (Miura et al., 2007; Yu et al., 2008; Ma et al., 2015; Wu et al., 2016).

It is generally recognized that binding sites of PPR proteins that are involved in intron splicing are located in the intron (Schmitz-Linneweber et al., 2006; Beick et al., 2008; de Longevialle et al., 2008, 2010), which is helpful for maintaining RNA conformation and the subsequent recruitment of general splicing factors to facilitate intron splicing. Consistently, the predicted SOT5-binding site is located in the intron of trnK. In contrast, the predicted SOT5-binding site is located in the exon of rpl2, although there is a shorter motif containing eight nucleotides that overlapped with the predicted SOT5-binding site in the rpl2 intron (523–530; Supplemental Table S1). Based on this, it is possible that SOT5 can facilitate both rpl2 intron splicing and transcript stabilization. Considering that the plastid rpl2 intron was independently lost in several species, such as in the Caryophyllales and Lythraceae (Downie et al., 1991; Gu et al., 2016), it will be interesting to examine whether the role of SOT5 homologs in these species is associated with rpl2 transcript stabilization. In addition, we cannot exclude the possibility that the binding of SOT5 to the rpl2 exon also is required for intron splicing. This hypothesis is consistent with the notion that the specific pattern of OTP51 action cannot be explained by an intron feature, because no conserved RNA motif was found in the ycf3.2, atpF, trnV, and trnK group IIA introns (de Longevialle et al., 2008). Whether PPR protein-binding sites can be located in an exon needs to be investigated further in the future.

Retrograde Signaling from Plastids to the Nucleus Regulates Leaf Development

Besides the leaf virescent phenotype, sot5 displays multiple defects in leaf development, such as serrated leaves and less vascular bundles (Fig. 3). It is well documented that abnormal leaf morphology occurs frequently in mutants with dysfunctional chloroplasts: for example, in DEFECTIVE CHLOROPLAST AND LEAVES, functioning in plastid rRNA maturation (Keddie et al., 1996; Bellaoui et al., 2003); SCA3, encoding a plastidic RpoTp RNA polymerase (Hricová et al., 2006); SCA1, encoding a plastid-type ribosomal protein S5 (Mateo-Bonmati et al., 2015); ENF2, encoding a chloroplast-localized and functionally unknown protein, with low similarity to the bacterial polyamine transporters PotD and PotF (Tameshige et al., 2013); and ANGULATAT7, encoding a DnaJ-like zinc finger domain protein localized in chloroplasts (Muñoz-Nortes et al., 2017). It has been suggested that the effect of chloroplast development on leaf morphology is associated with improper cell proliferation and expansion during leaf development (Mateo-Bonmati et al., 2015). Recent studies showed that the serrated leaves and abnormal vascular development in initiation factor3, encoding a plastid translation initiation factor, were linked to altered auxin homeostasis and auxin-regulated pathways (Zheng et al., 2016); however, it remains unknown how chloroplast gene expression affects auxin biosynthesis and/or distribution. Our study also demonstrated that the layer of vertically elongated palisade cells beneath the adaxial epidermis was missing in sot5 (Fig. 3), suggesting that adaxial domain formation is inhibited in sot5 leaf primordia. Furthermore, the as2 sot5 double mutant had more severe abaxialized filamentous leaves, which were similar to those of sca1 as2 mutants. Both sca1 and sot5 are defective in a plastid ribosomal subunit (Mateo-Bonmati et al., 2015), indicating that plastid translation may trigger a retrograde signal that regulates leaf polarity development. The sot5 mutant provides ideal material in which to investigate the molecular mechanisms by which leaf development is feedback regulated by plastid gene expression for improved photosynthesis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Arabidopsis (Arabidopsis thaliana) ecotype Col-0 was used as the wild type in this study. Single mutants used in this study were described previously: thf1-1 (Huang et al., 2006), var2 (Yu et al., 2004) and as2-1 (Xu et al., 2003). The eml2279-2 (SALK_088420) mutant that was reported previously (Cushing et al., 2005) was obtained from the ABRC stock center (https://abrc.osu.edu/) and was genotyped according to the guidelines described there. Double mutants were identified in F2 generations derived from crosses between single mutants by the PCR-based genotyping procedure. Seeds were surface sterilized by 0.1% (w/v) ethyl methanesulfonate in the dark at 37 °C and then sown onto one-half-strength Murashige and Skoog medium with 1% Suc, or seeds were sown directly into soil and grown in a phytotron with long-day conditions (16 h of light/8 h of dark) and light intensity (100 μmol photons m−2 s−1) at 22 °C.

Map-Based Cloning, Plasmid Construction, and Transformation

Suppressors were identified by screening M2 seeds that were mutagenized by 0.1% (w/v) ethyl methanesulfonate in the thf1 genetic background. The suppressor line was crossed to Ler, and the F2 seedlings were used as mapping populations. The mutation was mapped using simple sequence length polymorphism markers, and information is available at FAIR (http://www.arabidopsis.org). The SOT5/EMB2279 coding DNA sequence (CDS) and the CDS for the first 120 amino acids including transit peptide sequence (fragment CDS) were cloned into the pENTR SD/D-TOPO entry vector (Invitrogen). The SOT5/EMB2279 CDS was then recombined into the pGW2 destination vector (Nakagawa et al., 2007). Artificial microRNA of SOT5 was built according to Schwab et al. (2006). The destination vectors were transformed into Col-0 or sot5 mutants according to the method described by Clough and Bent (1998). For transient subcellular localization analysis, the coding sequence of the first 120 amino acids including transit peptide sequence was recombined into the
SOT5 Regulates Plastid Intron Splicing

The Patmatch tool (https://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) was used to predict the binding sequences according to the SOT5 PPR code, XGKCMXATCX (where X means any base, K means G or T, and M means A or C) by searching TAILR genes (+introns, +UTRs; DNA database, with other parameters at default settings (Supplemental Table S1)).

Accession Numbers

Sequence data from this article can be found in the TAIR data libraries under the following accession numbers: EMBER279 (ATIG06102.2), rpl2.2 (ATCG00830.1), rpl2.2 (ATCG01310.1), trnK (ATCG00030.1), ycf2.1 (ATCG0860.1), ycf2.2 (ATCG01280.1), rpc2 (ATCG00170.1), 25.S1 ATCG00950.1), 25.S2 (ATCG01130.1), ycf3 (ATCG00360.1), psbF (ATCG00570.1), psbF (ATCG00680.1), ndhA (ATCG01100.1), trnG (ATCG00940.1 and ATCG0190), trnl (ATCG00930.1 and ATCG01200.1), trnV (ATCG00450.1), and trnl (ATCG04040.1).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Phenotypic comparison between wild-type and sot5 seedlings.

Supplemental Figure S2. The PPR motifs of SOT5 are conserved in higher plants.

Supplemental Figure S3. Northern-blot analysis of plastid rRNA processing in sot5, prpl11, and prps9 mutants.

Supplemental Table S1. Plastid genes that are predicted to be targets of SOT5.

Supplemental Table S2. Primer used in this study.

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p2GW7 vector (http://gateway.psb.ugent.be/vector/show/p2GWY7/search/index/) and transformed into the Arabidopsis protoplasts according to the method of Wu et al. (2009).

Analyses of Chl Content, Chl Fluorescence, and Chloroplast Ultrastructure

Total Chl was extracted with 80% acetone at 4°C for 24 h in darkness. The levels of Chl a and Chl b were determined as described by Porra et al., 1989. Chl fluorescence was analyzed using Imaging PAM 101 (Walz), and F_0/F_m values were determined according to the manufacturer’s instructions. For chloroplast ultrastructure analysis, the first true leaves of 15- and 30-d-old plants were sampled and fixed in a solution of 4% glutaraldehyde, then processed, embedded, and viewed via transmission electron microscopy as described (Zhou et al., 2009).

Protein Extraction, Immunoblot, and BN-PAGE Analyses

Protein extraction, immunoblot, and BN-PAGE analyses were performed as described previously (Huang et al., 2013).

Microscopy

Thin-section specimens and leaf samples for scanning electron microscopy were prepared according to our previous report (Huang et al., 2016). Samples for measuring and observing leaf mesophyll cells were processed as described by Xue et al. (2015).

RNA Isolation and RT-qPCR

Total RNA was extracted from leaves of 25-d-old plants using an RNasea plant mini kit (Qiagen), according to the manufacturer’s instructions. Downstream DNase I treatment and RT-PCR steps were conducted as described previously by Huang et al. (2013). Quantitative and semi-quantitative RT-PCR of chloroplast genes was carried out using the gene-specific primers designed previously by de Longevialle et al. (2008) and designed by ourselves (Supplemental Table S2). Three biological repeats were analyzed, and each sample was run in triplicate. The data set was normalized using ACTIN2 as a reference. The method to quantify the transcript level and splicing efficiency of plastid genes was described previously (de Longevialle et al., 2008).

Northern-Blot Analysis

For northern-blot analysis of plastid rRNA, the following biotinylated oligomer probes, which were described previously by de Longevialle et al. (2008), were synthesized by Thermo Fisher Scientific: trnK, 5′-AAAGC-CGAGTACTCTACCGTTG-3′; trnl, 5′-TGGGATAGGGACTTGAAC-3′; trnG, 5′-CTATCGTTAGCTTGGAAGGCTAAGGG-3′; trnl, 5′-CTACCCT-GAAGTATAAGCCGCC-3′; trnV′, 5′-GTGTTAAACGGGTTGCTTACCTAA-3′; and trnG, 5′-GGACTCAGCACCGCTGACATCCGCC-3′. Each probe sequence is in its exon. Total RNAs used for northern blot were extracted from 15-d-old seedlings according to the manufacturer’s instructions (Promega Denaturating Solution; Z5651). A total of 1 to 2 μg of RNA was run on 2% agarose/formaldehyde gels, transferred to positively charged nylon membranes (Roche; 1141724001), and hybridized with biotinylated oligomer probes at 42°C. The North2South Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific; 17097) was used for the downstream steps according to the kit instructions. Northern-blot analysis of plastid rRNAs was performed as described previously (Wu et al., 2016).

Prediction of SOT5-Binding Sequences

SOT5-binding sequences were predicted as described previously (Barkan et al., 2012; Yin et al., 2013; Yagi et al., 2014; Shen et al., 2015, 2016). In brief, each PPR motif coordinates with one RNA base, and the combinations of the fifth and 35th amino acids (known as the PPR code) within each motif confer RNA-specific binding. The PPR motif sequences from SOT5 were used to search against the previously defined RNA recognition database for PPRs, leading to the following predicted recognition sequence: XG(G/U)C(A/C) XXAUXC, where X indicates a nucleotide that cannot be predicted precisely.
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