An SMU Splicing Factor Complex Within Nuclear Speckles Contributes to Magnesium Homeostasis in Arabidopsis

Zhihang Feng, Hiroshi Nagao, Baohai Li, Naoyuki Sotta, Yusuke Shikanai, Katsushi Yamaguchi, Shuji Shigenobu, Takehiro Kamiya, and Toru Fujiwara

Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113–8657, Japan
National Institute for Basic Biology, Okazaki 444–8585, Japan
Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Saitama 332–0012, Japan

ORCID IDs: 0000-0003-1802-0635 (Z.F.); 0000-0003-5558-5155 (N.S.); 0000-0002-5363-6040 (T.F.); 0000-0001-5558-5155 (N.S.); 0000-0001-8429-3389 (Y.S.); 0000-0001-6871-7882 (K.Y.); 0000-0003-6460-2323 (S.S.); 0000-0001-3790-7119 (T.K.); 0000-0002-5363-6040 (T.F.)

Mg$^{2+}$ is among the most abundant divalent cations in living cells. In plants, investigations on magnesium (Mg) homeostasis are restricted to the functional characterization of Mg$^{2+}$ transporters. Here, we demonstrate that the splicing factors SUPPRESSORS OF MEC-8 AND UNC-52 1 (SMU1) and SMU2 mediate Mg homeostasis in Arabidopsis (Arabidopsis thaliana). A low-Mg sensitive Arabidopsis mutant was isolated, and the causal gene was identified as SMU1. Disruption of SMU2, a protein that can form a complex with SMU1, resulted in a similar low-Mg sensitive phenotype. In both mutants, an Mg$^{2+}$ transporter gene, Mitochondrial RNA Splicing 2 (MRS2-7), showed altered splicing patterns. Genetic evidence indicated that MRS2-7 functions in the same pathway as SMU1 and SMU2 for low-Mg adaptation. In contrast with previous results showing that the SMU1-SMU2 complex is the active form in RNA splicing, MRS2-7 splicing was promoted in the smu2 mutant overexpressing SMU1, indicating that complex formation is not a prerequisite for the splicing. We found here that formation of the SMU1-SMU2 complex is an essential step for their compartmentation in the nuclear speckles, a type of nuclear body enriched with proteins that participate in various aspects of RNA metabolism. Taken together, our study reveals the involvement of the SMU splicing factors in plant Mg homeostasis and provides evidence that complex formation is required for their intranuclear compartmentation.

Mg$^{2+}$ is among the most abundant divalent cations in living cells, and it is involved in a number of physiological and biochemical processes, such as energy metabolism, DNA replication, transcription, and ribosome aggregation (Wolf and Cittadini, 2003; Klein et al., 2004; Shenvi et al., 2005; Karley and White, 2009). In addition to the conserved functions among all organisms, the most well-known function of magnesium (Mg) in plants is to serve as the central ion of chlorophyll, which acts in pigment-protein complexes to harvest photons in PSI as well as PSII (Jansson, 1994; Cowan, 2002; Shaul, 2002).

To maintain appropriate Mg concentrations in cells and tissues, plants use multiple transport systems for Mg$^{2+}$ uptake, translocation, and compartmentation. The Mitochondrial RNA Splicing 2 (MRS2) transporter family has been extensively studied in plants. In Arabidopsis (Arabidopsis thaliana), a total of nine MRS2 genes have been identified (Schock et al., 2000; Li et al., 2001). Complementation tests in yeast and bacteria mutants confirmed the Mg$^{2+}$ transport activity of these MRS2 proteins (Drummond et al., 2006; Mao et al., 2008; Gerbit et al., 2009). Currently, increasing lines of evidence demonstrated that the plant MRS2 family members show distinct spatial and temporal expression patterns and display functional diversity throughout the plant lifecycle (Hermans et al., 2013; Kobayashi and Tano, 2015; Chen et al., 2017). Among the nine Arabidopsis MRS2 members, MRS2-1, MRS2-5, and MRS2-10 are involved in leaf mesophyll...
Mg maintenance (Conn et al., 2011a, 2011b). The endoplasmic reticulum—localized MRS2-4 and MRS2-7 are required for shoot Mg accumulation (Mao et al., 2014; Oda et al., 2016; Yan et al., 2018). MRS2-11, the most diversified member compared with other MRS2 paralogs, is localized to the chloroplast envelope membrane and plays an important role in maintaining Mg concentrations in the chloroplast stroma (Drummond et al., 2006; Liang et al., 2017; Sun et al., 2017). At the reproductive stage, MRS2-2, MRS2-3, and MRS2-6 are highly expressed in anthers. They are essential for pollen development probably through the regulation of Mg homeostasis (Li et al., 2008; Chen et al., 2009; Li et al., 2015; Xu et al., 2015).

Currently, relative to the extensive studies focusing on transporter function characterization, little is known about the regulatory mechanisms of these Mg$^{2+}$ transporters. It has been previously identified that one of the Arabidopsis MRS2 genes, MRS2-7, encodes mRNA with distinct splicing variants (Mao et al., 2008). One of the variants encodes a low-affinity Mg$^{2+}$ transporter when expressed in bacteria; the other one, with a 15 amino acid deletion at a coiled coil region, however, does not show Mg$^{2+}$ transport activity when expressed (Mao et al., 2008). Until now, despite the characterization of the functional diversification of MRS2-7 splicing variants, the underlying upstream regulator(s) remains unknown.

In eukaryotic cells, the nucleus is a membrane-surrounded organelle containing several types of nuclear bodies or compartments in addition to chromatin (Lamond and Sleeman, 2003). These self-organized structures are accepted as machinery involved in various activities of the nucleus (Misteli, 2001; Carmo-Fonseca, 2002; Staněk and Fox, 2017). Splicing factors are proteins that contribute to a complex molecular machine called the spliceosome, which is engaged in catalyzing precursor mRNA (pre-mRNA) splicing (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski et al., 1985). In the nucleus, splicing factors are often enriched in one of the nuclear bodies called nuclear speckles (Lamond and Spector, 2003; Lorković and Barta, 2004). Several lines of evidence demonstrate that nuclear speckles can serve as the storage and/or recycling sites for splicing factors (Lamond and Spector, 2003). When gene transcription is inhibited, either by the use of inhibitors (Melčák et al., 2000; Fang et al., 2004), or as a result of a heat shock (Spector et al., 1991), splicing factors in the nucleoplasm tend to accumulate in nuclear speckles. In comparison, when transcription is induced by viral infection (Jiménez-Garcia and Spector, 1993; Bridge et al., 1995), or expression of intron-containing genes is increased (Huang and Spector, 1996; Misteli et al., 1997), splicing factors can be released from nuclear speckles to the nearby transcription sites, where gene transcription and pre-mRNA splicing are carried out. Shuttling of splicing factors between nuclear speckles and nucleoplasm is a limiting step in the RNA splicing process (Duncan et al., 1997; Hartmann et al., 2001). However, in contrast with the well-documented spliceosome assembly process during splicing (Fica and Nagai, 2017; Shi, 2017), much less is known about the regulatory mechanism and the gene(s) that participates in the storage and recycling of splicing factors between the spliceosome and nuclear speckles.

Here, we report the identification and characterization of genes involved in Mg$^{2+}$ transporter pre-mRNA splicing, namely SUPPRESSORS OF MEC-8 AND UNC-52 1 (SMU1) and SMU2. We demonstrate here, in Arabidopsis, that SMU1 and SMU2 are required for low-Mg tolerance through regulating the pre-mRNA splicing of the Mg$^{2+}$ transporter gene MRS2-7. In contrast with previous knowledge that formation of the SMU1SMU2 complex is required for the involvement of both proteins in RNA splicing (Spartz et al., 2004; Chung et al., 2009; Fournier et al., 2014), we showed that SMU1 is able to act as a functional splicing factor in the absence of SMU2. In addition, we revealed that complex formation between SMU1 and SMU2 is essential for their accumulation in the nuclear speckles. The results imply that protein interaction drives the intranuclear compartmentation of SMU splicing factors.

RESULTS

The kudo4 Mutant Exhibited Reduced Shoot Mg Accumulation under low-Mg Conditions

To identify genes required for low-Mg stress adaptation, we conducted a mutant screening for Arabidopsis mutants defective in shoot growth under low-Mg/high-Ca conditions as previously described (Oda et al., 2016). One of the mutants, named kudo4, was isolated from an ethyl methanesulfonate (EMS)–mutagenized M2 population of Arabidopsis Col-0 (Fig. 1A). To confirm and characterize the mutant phenotype in detail, kudo4 together with Col-0 were grown on normal (1500 μM) and low-Mg (15 μM) agar media for 14 d. Shoot fresh weights as well as Mg concentrations in shoots and roots were measured. Under the normal condition, shoot biomass and Mg accumulation of the kudo4 mutant were comparable with Col-0 (Fig. 1, B and C). In contrast, when exposed to the low-Mg condition, both shoot growth and Mg accumulation of kudo4 were significantly reduced compared with Col-0 (Fig. 1, B and C). In roots, Mg accumulation in the kudo4 mutant was less than in Col-0 under the normal Mg condition, but no significant difference was detected under the low-Mg condition (Fig. 1C). These data demonstrate that the kudo4 mutant is a low-Mg sensitive mutant, which exhibits differential Mg accumulation in shoot and root tissues depending on the Mg conditions.

SMU1 Is the Causal Gene for the Low-Mg Sensitive Phenotype of the kudo4 Mutant

To identify the causal gene for the shoot growth defects of the kudo4 mutant, map-based cloning was
Figure 1. The splicing factors SMU1 and SMU2 are identified to be required for plant growth under low-Mg condition. A, Representative images of the wild-type (WT; Col-0) and kudo4 mutant. Seedlings were grown on MGRL agar medium with normal Mg (1500 μM) and low-Mg (15 μM) for 14 d. Scale bar = 1 cm. B, Shoot fresh weight of Col-0 and kudo4 in A. In the box-plots, boxes, middle lines and error bars represent the first quartile to third quartile, median, and maximum and minimum, respectively (n = 5). Asterisks indicate a significant difference from Col-0 by Welch’s t-test (**P < 0.001). ns, No significance. C, Shoot and root Mg concentrations of Col-0 and kudo4 in (A). Dot plots and mean ± SD are shown (n = 5). Asterisks indicate a significant difference from Col-0 by Welch’s t-test (**P < 0.001). DW, Dry weight. D, Map-based cloning of kudo4. Molecular
conducted. The kudo4 mutant was crossed with the Arabidopsis Landsberg (Ler) ecotype, and the F2 population was used for genetic mapping. The segregation of the phenotype in the F2 individuals was 144:46 (wild-type: mutant), which was not significantly different from the Mendelian segregation ratio of 3:1(χ² test, P > 0.05), suggesting that kudo4 carries a single recessive mutation responsible for the phenotype. Genetic mapping with simple sequence length polymorphism markers narrowed down the location of the casual gene to be between the markers F25P22 and T1111 (27.0–29.4 Mbp) on chromosome 1 (Fig. 1D). Among the genes in this region, the kudo4 mutant had a nonsynonymous mutation in only one gene named SMU1, an auxiliary mRNA splicing factor. A point mutation was found at a predicted splicing donor site in the 5’ end of the 12th intron of this gene.

To determine the effect of this point mutation on SMU1 mRNA processing in the kudo4 mutant, a pair of reverse transcription PCR (RT-PCR) primers (Fig. 1D, primers 1 and 3) spanning the 12th intron was designed to detect SMU1 mature mRNA, but not pre-mRNA nor contaminating genomic DNA. In the kudo4 mutant, two aberrant SMU1 splicing variant (SV) transcripts (SV1 and SV2) were detected, but the wild-type SMU1 transcript was absent (Fig. 1E). Sequencing analysis revealed that the SV2 and SV1 transcripts contain the full length of the 12th intron or a part of the 5’ end of the 12th intron, leading to a premature stop codon or an insertion of eight additional amino acids without a frameshift, respectively (Fig. 1F).

To confirm that the mutation in SMU1 is responsible for the kudo4 phenotype, we obtained a SMU1 transfer DNA (T-DNA) insertion line named smu1-2. The smu1-2 line was chosen despite a partially functional allele, since a loss-of-function mutation (smu1-1) is lethal (Chung et al., 2009). The growth of smu1-2 without Mg supplementation was poor compared with wild type, similar to the case of the kudo4 mutant (Fig. 1G). F1 crosses between kudo4 and smu1-2 plants did not recover the growth, suggesting that the causal gene is SMU1 (Fig. 1G). To further support this conclusion, we also established SMU1 overexpression (OX) lines in the kudo4 background. Two independent T3 lines with about 3-fold higher SMU1 mRNA expression levels compared with wild type were obtained (Supplemental Fig. S1). When grown under the low-Mg condition, shoot growth of the OX lines was similar to the wild-type (Fig. 1, H and I), further supporting that SMU1 is the casual gene for the poor growth of kudo4 under low-Mg conditions. Furthermore, the overexpression of SMU1 resulted in the recovery of shoot Mg accumulation under low-Mg conditions, but the difference in root Mg accumulation under normal conditions did not recover (Fig. 1J). Until now, four independent smu1 mutants have been reported (Chung et al., 2009; Kanno et al., 2017); hereafter, we designated the kudo4 mutant as smu1-5.

**Mutation of SMU2 also Causes Low-Mg Sensitivity**

In Arabidopsis, SMU1 and SMU2 interact with each other, and they are involved in splicing of a set of common pre-mRNA targets (Chung et al., 2009). To test whether SMU2 is also involved in low-Mg tolerance, we obtained a SMU2 knock-out mutant named smu2-1 (endogenous SMU2 protein cannot be detected; Chung et al., 2009) and characterized the phenotype under both the control and low-Mg conditions. Similar to the case of the smu1-5 mutant, smu2-1 also exhibited a reduced shoot growth and a reduction of Mg accumulation compared with wild-type under the low-Mg condition (Fig. 1, K–M). These findings show that SMU2 is also required for low-Mg tolerance. Taken together with the physical interaction relationship between SMU1 and SMU2 (Chung et al., 2009), it is likely that SMU1 and SMU2 function in the same pathway.

**The Mg²⁺ Transporter Gene MRS2-7 Is Mis-spliced in smu1 and smu2 Mutants**

Considering the involvement of SMU1 and SMU2 in pre-mRNA splicing (Spike et al., 2001; Spartz et al.,...
The abundance of exon 7 skipped transcript (SV4) probably be-
detected in all three
was reduced (Fig. 2C, bottom; Supplemental Fig. S3A). Therefore, we tested the splicing
MRS2-7
We first examined whether the mrs2-7 mutant shows a similar low-Mg sensitive phenotype in our experimental conditions. A previously reported MRS2-7 knock-out mutant [mrs2-7(1); Gebert et al., 2009] was grown under normal Mg supply medium and low-Mg medium. Consistently, when compared with the wild type, the mrs2-7(1) mutant displayed a suppressed shoot growth as well as a decreased shoot Mg concentration under the low-Mg condition (Supplemental Fig. S2).

We then investigated the effects of SMU proteins on MRS2-7 mRNA splicing. According to the TAIR10 annotation (Fig. 2A) as well as a previous report (Mao et al., 2008), splicing variants generated by the combination of exons 6 to 11 refer to SV1. To semiquantify the splicing variants, a pair of primers (Fig. 2A, F1 and R1) was designed and PCRs were performed using complementary DNA templates. Several bands with different intensities appeared, and the band patterns were affected by genotypes (Fig. 2B). Based on sequencing, the band with the smallest size (Fig. 2B, mature mRNA) represents the transcript in which all of the introns between the 6th and 11th exons of SV1 are spliced out. When compared with wild-type, splicing patterns of MRS2-7 were altered in all smu mutants examined, and the mutants accumulated unspliced MRS2-7 transcripts at a higher abundance (Fig. 2B, pre-mRNA). In both the wild-type and smu mutants, splicing patterns of MRS2-7 were not evidently different between the normal and low-Mg supply (Fig. 2B). These results indicate that splicing of MRS2-7 is regulated by both SMU1 and SMU2 irrespective of Mg conditions.

Among the different patterns of splicing, we paid specific attention to exon 7 in MRS2-7, due to its critical role for the Mg2+ transportation capability (Mao et al., 2008). A pair of primers located on the neighboring exons (exon 6 and exon 8) was designed to detect the splicing pattern within that region (Fig. 2C, top). In agreement with the above result, intron-retained transcripts were more abundant in the smu mutants, whereas accumulation of precisely spliced transcripts was reduced (Fig. 2C, bottom; Supplemental Fig. S3A). Besides, a portion of transcripts without exon 7 was detected in all three smu mutants, whereas the same band was not detected in the wild type (Fig. 2C, bottom, triangle; Supplemental Fig. S3B). This is probably because the abundance of exon 7 skipped transcript (SV4) is below the detection limit of PCR. These results demonstrate that both SMU1 and SMU2 are important for correct splicing to retain exon 7 in the mature mRNA of MRS2-7.

In addition to the previously annotated variable region (Fig. 2A, sequence between exon 6 and exon 11), we further checked the splicing of MRS2-7 from the first to last intron. Ten pairs of primers located at the upstream and downstream exons of every intron were designed to measure the relative abundance of spliced and unspliced transcripts (Fig. 2D, bottom). Results showed that all of the ten introns were retained at higher levels to various extents in the smu mutants compared with wild-type plants under both the control and low-Mg treatments (Fig. 2D, bottom; Supplemental Fig. S4B). This indicates that SMU1 and SMU2 contribute to the splicing of all examined introns in the MRS2-7 pre-mRNA.

To obtain quantitative data of MRS2-7 pre-mRNA splicing, the abundance of mature mRNA (Fig. 2A, primers F2/R2, F3/R3) and the total of mature and pre-mRNA (Fig. 2A, primers F4/R4) was measured in Col-0 and the smu mutants by reverse transcription quantitative PCR (RT-qPCR). The results demonstrated that the abundance of total MRS2-7 mRNA was comparable between mutants and wild-type (Fig. 2E). Conversely, accumulation of precisely spliced transcripts (i.e. Intron 10 removed and “xnon 7 retained transcripts) were significantly decreased in the smu mutants compared with the wild type (Fig. 2E). Furthermore, we examined the splicing pattern of MRS2-7 in the established SMU1 OX lines, and the results demonstrated that SMU1 over-accumulation significantly promotes the pre-mRNA splicing of MRS2-7 (Supplemental Fig. S3). Taken together, our results indicate that SMU1 and SMU2 are involved in MRS2-7 pre-mRNA splicing.

In addition, we found that MRS2-7 splicing defects were different among the three smu mutants (Fig. 2E). The partial loss-of-function smu2-1 mutation is a strong allele compared with smu1-5 (Fig. 2, D and E). In the smu1-2 mutant, the splicing defect of MRS2-7 is more severe than in the smu2-1 knock-out mutant (Fig. 2, D and E). These results suggest that the effects of SMU1 and SMU2 on pre-mRNA splicing are different.

MRS2-7 Functions in the Same Pathway as SMU1 and SMU2 in Low-Mg Stress Adaptation

Given the facts that the mrs2-7 mutant showed low-Mg sensitivity similar to the smu1-5 and smu2-1 mutants (Fig. 1; Supplemental Fig. S2), and that splicing patterns of MRS2-7 were altered in smu1-5 and smu2-1 mutants (Fig. 2; Supplemental Fig. S4), we hypothesized that the defects in MRS2-7 pre-mRNA splicing may contribute to the suppressed shoot growth and decreased shoot Mg accumulation in the smu mutants. To test this genetically, smu1-5 and smu2-1 mutants
were crossed with the mrs2-7(1) mutant to establish smu1-5 mrs2-7(1) and smu2-1 mrs2-7(1) double mutants. The growth of these double mutants was compared with that of single mutants and wild-type Col-0 under different Mg concentrations (1500, 20, 15, and 10 μM). Results showed that under different low-Mg conditions, shoot fresh weights of the double mutants were similar to the mrs2-7(1) single mutant (Fig. 3, A and B). Shoot and root Mg concentrations in the double mutants were also comparable with those in the mrs2-7(1) single mutant (Fig. 3C). These results suggest that MRS2-7 and SMU1 genes function in the same genetic pathway in adaptation to low-Mg conditions.

Figure 2. The Mg2+ transporter gene MRS2-7 is mis-spliced in smu1 and smu2 mutants. A, Schematic diagram of MRS2-7 splicing variants 1 to 4 (SV1–SV4) annotated by TAIR10 (https://www.arabidopsis.org). Black and gray boxes indicate exons and untranslated regions, respectively; positions of primers used for RT-PCR and RT-qPCR are labeled with arrows. B, MRS2-7 splicing patterns in Col-0, smu1-5, smu1-2, and smu2-1 plants. Total RNA was prepared from seedlings 14 d after germination, and RT-PCR was performed using the primers F1/R1 indicated in A. EF1α was used as a control. Arrow and asterisks indicate mature and pre-mRNA, respectively. C, Exon skipping detection. RT-PCR was performed using a pair of primers (red arrows, ES_F/R) located on exon 6 and 8. The black arrow and asterisk indicate intron-removed and intron-containing transcripts, respectively. The triangle indicates transcripts without exon 7. D, Overall detection of MRS2-7 intron removal. Ten pairs of primers located on the neighboring exons (red arrows, IR_F/R) were designed for RT-PCR analysis. The numbers in the primer name indicate the respective intron. Arrows and asterisks indicate spliced and unspliced transcripts, respectively. E, Quantitative measurement of spliced and unspliced MRS2-7 transcripts. RT-qPCR was performed to measure the relative abundance (compared with Col-0) of intron-removed (F2/R2), exon-retained (F3/R3), and total (F4/R4) MRS2-7 mRNA. Positions of primers are indicated in A. Values represent means ± SD (n = 3). Different lowercase letters indicate significance differences by two-way ANOVA, Tukey’s HSD test (P < 0.05).
Overexpression of SMU1 or SMU2 Promotes MRS2-7 pre-mRNA Splicing in the *smu* Mutants

Due to the lack of conserved RNA binding motifs in the SMU1 and SMU2 protein sequences, it is generally believed that they serve as auxiliary splicing regulators, linking spliceosome and RNA targets through the interaction with certain spliceosomal proteins (Agafonov et al., 2011; Hegele et al., 2012). Specifically, these two molecules were found to physically interact with each other in various eukaryotes (Spartz et al., 2004; Sugaya et al., 2006; Chung et al., 2009; Agafonov et al., 2011; Sugaya et al., 2011), suggesting that SMU1-SMU2 complex formation is essential for their respective functions. It is intriguing to note that in our study, as mentioned above, the effects on MRS2-7 mis-splicing caused by the disruption of *SMU1* and *SMU2* are not identical (Fig. 2, B, D, and E; Supplemental Fig. S4B). This suggests that, although these two factors share a similar function, they have differences in their detailed functions in pre-mRNA splicing.

To further evaluate the individual contribution of SMU1 and SMU2 to MRS2-7 pre-mRNA splicing, we used a transient expression system based on protoplasts isolated from different genetic backgrounds of Arabidopsis rosette leaves. Plasmids for the expression of GFP fusion proteins SMU1-GFP, SMU2-GFP, or GFP alone were introduced into protoplasts, isolated from wild-type Col-0 and the three *smu* mutants: *smu1-2* and *smu1-5* are partial loss-of-function alleles and *smu2-1* is a knock-out allele. After 16 h incubation, successful transformation was confirmed by GFP fluorescence observation (Supplemental Fig. S5). Total RNA was extracted and used for the determination of the MRS2-7 splicing pattern. In all three *smu* mutants, the splicing pattern of MRS2-7 was altered by the expression of
either SMU1- or SMU2-GFP (Fig. 4A). RT-qPCR results demonstrated that, in the smu mutants, expression of either SMU1- or SMU2-GFP increased the accumulation of spliced MRS2-7 transcripts, whereas the abundance of total MRS2-7 was not significantly changed (Fig. 4B). This result suggests that formation of the SMU1-SMU2 complex is not indispensable for their mRNA splicing activity, and SMU1 alone is sufficient for splicing, at least for MRS2-7.

Complex Formation between SMU1 and SMU2 Is Essential for their Compartmentation in Nuclear Speckles

In Caenorhabditis elegans, it has been shown that SMU1 dimerizes and the N-terminal region (NTR) of the dimer interacts with RED [homolog of SMU2 in C. elegans, named after a region rich in Arg (R) or Arg/Asp (D) repeats], resulting in the heterotetrameric SMU1-RED complex (Fig. 5A, left, Ulrich et al., 2016). Unlike the case in C. elegans, in the purified human spliceosomal B complex, a SMU1-RED (homolog of SMU2 in Homo sapiens) hetero-dimer is detected (Fig. 5A, right, Bertram et al., 2017). In plants, the SMU1-SMU2 interaction has been demonstrated but the protein self-interactions remain unclear. We tested the interaction with bimolecular fluorescence complementation (BiFC) in Arabidopsis protoplasts. N-terminal yellow fluorescent protein (nYFP) or C-terminal YFP (cYFP) was fused with SMU1 or SMU2, and reconstituted YFP signals were observed by confocal microscopy. In addition to the SMU1-SMU2 interaction, we found SMU1-SMU1 self-interaction (Fig. 5B and C). In contrast, self-interaction of SMU2 was not detected (Fig. 5B; Supplemental Fig. S6).

In the BiFC assay, reconstituted YFP signals, which represent the SMU1-SMU2 complex, are observed as spherical forms in the nucleus (Fig. 5B), whereas similar patterns were not observed in protoplasts expressing either SMU1-GFP or SMU2-GFP alone, or coexpressing both SMU1-GFP and SMU2-RFP (Fig. 5D), which reflect the monomer, dimer and oligomer forms of these two proteins. The uniform distribution patterns of SMU1 and SMU2 may represent their presence in both nucleoplasm and the spherical-form compartments in comparable levels.

To identify in which nuclear compartment(s) the SMU1-SMU2 complex is enriched, we checked the nuclear distribution patterns of the SMU1-SMU2 complex with several markers representing the typical nuclear bodies including a cajal body marker (U2B''), a nucleolus marker (PRH75), and three nuclear speckle markers (CypRS64, SRp34, and SCL30; Lorkovic et al., 2004a; Reddy et al., 2012). Red fluorescent protein (RFP) fusions of these marker proteins were introduced into protoplasts with SMU1/2-nYFP/cYFP. The SMU1-SMU2

![Figure 4. Formation of the SMU1-SMU2 complex is not a prerequisite for their function, as splicing regulators. A, Splicing patterns of MRS2-7 in protoplasts expressing GFP, SMU1-GFP, and SMU2-GFP. Arabidopsis mesophyll protoplasts were isolated from Col-0, smu1-5, smu1-2, and smu2-1 rosette leaves. After plasmid transformations followed by overnight cultivation, samples were collected, and total RNA was extracted. RT-PCR analysis was performed to check the MRS2-7 pre-mRNA splicing patterns (F1/R1 indicated in Fig. 2A). The arrow and asterisks indicate mature and pre-mRNA, respectively. EF1a was used as a control. B, Quantification of the abundance of spliced and unspliced MRS2-7 transcripts in the total RNA extracted from protoplasts expressing GFP, SMU1-GFP, and SMU2-GFP. RT-qPCR was performed to measure the relative abundance (compared with Col-0 expressing GFP) of intron-removed (F2/R2), exon-retained (F3/R3), and total (F4/R4) MRS2-7 mRNA. Positions of primers are indicated in Figure 2A. Values represent means ± SD (n = 3). Different lowercase letters indicate significance differences by one-way ANOVA, Tukey’s HSD test (P < 0.05).](image-url)
complex did not colocalize with the Cajal body and nucleolus markers (Supplemental Fig. S7, B and C). Among the three nuclear speckle marker proteins, the SMU1-SMU2 complex perfectly colocalized with CypRS64 (Fig. 5E, top; Supplemental Fig. S7A), which is a cyclophilin family protein that interacts with a subset of Arabidopsis Ser/Arg (SR)-rich proteins and specific U1 and U11 small nuclear ribonucleoproteins (snRNPs; Lorković et al., 2004b). In comparison, SRp34 was compartmented in the nuclear speckles other than those where the SMU1-SMU2 complex was localized (Fig. 5E, middle), and the accumulation of SCL30 was...
less abundant in the regions where the SMU1-SMU2 complex was enriched (Fig. 5E, bottom). It has been reported that different splicing factors can be compartmented in different nuclear speckles (Lorković et al., 2008; Reddy et al., 2012), although the biological significance of this remains unclear.

Unlike the SMU1-SMU2 complex, the SMU1 homo-oligomer was localized to structures with an irregular and flocculent appearance (Fig. 5B). This result suggests that, without SMU2 proteins, SMU1 homo-oligomers would not be properly targeted to the nuclear speckles. To test this, we checked the distribution patterns of the SMU1-SMU2 complex and SMU1-SMU1 homo-oligomer with or without SMU2 introduction. YFP signals representing the SMU1-SMU2 complex were observed in the nuclear speckles irrespective of RFP or SMU2-RFP accumulation (Fig. 5F, top). In contrast, the SMU1 homo-oligomer was mainly enriched in the nuclear speckles when SMU2-RFP was expressed (Fig. 5F, bottom). When only RFP was expressed, YFP signals were observed as a flocculent appearance with fuzzy boundaries (Fig. 5F, bottom). Taken together, these results indicate that complex formation between SMU1 and SMU2 is essential for the compartmentation of both proteins in the nuclear speckles.

DISCUSSION

SMU1 and SMU2 Regulate MRS2-7 pre-mRNA Splicing in a Mg-Independent Manner

Through a forward genetic screening, we showed that the splicing regulator SMU1 and its interacting partner SMU2 play essential roles in shoot growth and Mg accumulation under low-Mg conditions (Fig. 1). These two genes were originally identified in C. elegans and function together to regulate the pre-mRNA splicing of a set of genes at the initial 5' splice site recognition step (Spike et al., 2001; Spartz et al., 2004; Dassah et al., 2009). Homologs in mammalian cells were also found to act as auxiliary factors in the regulation of the RNA splicing process (Sugaya et al., 2006; Sugaya et al., 2011). In Arabidopsis, SMU1 and SMU2 are expressed ubiquitously and especially in actively dividing tissues (Chung et al., 2009). Lack of either gene causes altered splicing of a set of common target genes, and the mutants exhibit similar developmental defects (Chung et al., 2009).

In our study, MRS2-7 was identified as a target gene whose splicing is regulated by SMU1 and SMU2 (Fig. 2B). A detailed analysis revealed that SMU proteins are required for efficient intron removal and precise splice site selection of MRS2-7 pre-mRNA (Fig. 2, C–E; Supplemental Fig. S4). Similar functions have also been found for the nematode and mammal SMU homologs (Spike et al., 2001; Spartz et al., 2004; Sugaya et al., 2006). In a recent genome-wide analysis of RNA splicing in an Arabidopsis smu1 mutant, intron retention accounted for the most common occurrence (72.2%) in the smu1 mutant among various types of splicing events, suggesting a central role of SMU1 in intron removal processing (Kanno et al., 2017).

In our study, MRS2-7 transcripts showed an altered splicing pattern in smu mutants compared with wild type, but the splicing pattern in either wild-type or the smu mutants is independent of the Mg condition (Fig. 2, B–E; Supplemental Fig. S3). The results suggest that, rather than functioning as alternative splicing regulators in response to low-Mg, SMU proteins could be in charge of mediating the constitutive splicing of MRS2-7 pre-mRNA.

Due to the lack of RNA binding motifs in SMU proteins, they are generally considered to function in pre-mRNA splicing by association with other spliceosomal proteins (Chung et al., 2009; Ulrich et al., 2016; Kanno et al., 2017). Proteomic analysis of the human spliceosome shows that SMU1 and RED are enriched in the spliceosome B complex (Agafonov et al., 2011). In addition, cryogenic electron microscopy structure and cross-linking experiments of the human spliceosomal B complex revealed the structural organization of SMU1 and SMU2. In the B complex, SMU1 bridges U2- and U5-snRNP components (Bertram et al., 2017). In purified B complexes, crosslinks are detected between the SMU1 NTR and a short helix region (i.e. amino acids 209–222) of RED (Bertram et al., 2017). Moreover, numerous crosslinks are detected between the N-terminal part of RED and several U2 snRNP component proteins, whereas the SMU2 C-terminal region is crosslinked to Prp8, one of the core component proteins of the U5 snRNP (Bertram et al., 2017). These results imply that, similar to SMU1, RED also plays a role in bridging U2 with U5 proteins in the B complex. Considering the evolutionary conservation of these two splicing factors among multicellular eukaryotes, plant homologs of SMU1 and SMU2 could be responsible for similar functions as in the human B complex and contribute to pre-mRNA splicing.

Formation of the SMU1-SMU2 Complex Is Not a Prerequisite for pre-mRNA Splicing

Protein interactions between SMU1 and SMU2 have been detected conservatively among different eukaryotic organisms, and it is generally accepted that the SMU1-SMU2 complex represents the functional form of the two proteins in pre-mRNA splicing (Spartz et al., 2004; Chung et al., 2009; Fournier et al., 2014). However, we found that either SMU1 or SMU2 is able to facilitate pre-mRNA splicing of MRS2-7 without forming a complex with the other one (Fig. 4). The smu2-1 mutation is a complete loss-of-function allele (Chung et al., 2009). However, expression of SMU1 proteins can rescue the splicing defect of MRS2-7 to some extent in a SMU2-independent manner (Fig. 4). This indicates that, even without forming a complex with SMU2, SMU1 alone is able to act as a functional splicing regulator and contribute to pre-mRNA splicing, at least in the case of MRS2-7. On the other hand, it
is difficult to judge whether this applies to SMU2 since both of the smu1 mutants (smu1-2 and smu1-5) used in this study are not complete loss-of-function mutants. In the smu1-2 allele, a T-DNA is inserted in the last intron and transcripts before the insertion site were detected (Chung et al., 2009). In smu1-5, a point mutation is present in the splicing junction of the 12th intron and two splicing variants are detected (Fig. 1D). Therefore, in both mutants, the NTR of SMU1 could be correctly translated. In a previous report, endogenous SMU2 could not be detected in the smu1-2 mutant (Chung et al., 2009). In our experiments, overexpression of SMU2 made it possible for SMU2 protein to accumulate in both smu1 mutants (Supplemental Fig. S5), enabling the interaction between them. In combination with the results that SMU2 interacts with the NTR of SMU1 (Ulrich et al., 2016; Bertram et al., 2017), the improved splicing of MRS2-7 in smu1 mutants overexpressing SMU2-GFP could result from the interaction between SMU2 and the truncated SMU1 proteins, thus forming a partial loss-of-function SMU1-SMU2 complex. Of course, we cannot rule out the possibility that SMU2 is able to function as a splicing regulator without forming a complex with SMU1.

**SMU Proteins Could Stabilize Each Other by Forming a Complex**

In this study, the presence of the SMU1-SMU2 complex as well as a SMU1 homo-oligomer in Arabidopsis was confirmed through a BiFC assay (Fig. 5, B and C). Crystal structure determination of the *C. elegans* SMU1-RED complex reveals its presence as a hetero-tetramer consisting of two molecules of SMU1 as well as RED (Ulrich et al., 2016). Two SMU1 molecules first dimerize via a hydrophobic interface involving its LisH motif, and the NTR of each protomer is able to recruit a RED protein by binding one of their short, central helices, thus resulting into a SMU1-RED hetero-tetramer (Ulrich et al., 2016). Unlike the case in *C. elegans*, in the purified human spliceosomal B complex, a SMU1-RED hetero-dimer is detected (Bertram et al., 2017). Our results suggest that the hetero-tetrameric complex found in *C. elegans* could also be present in plants (Fig. 5, B and C; Supplemental Fig. S6).

What could be the benefit of forming a SMU1-SMU2 complex? In different organisms, the disruption or knock-down of one SMU gene resulted in the reduction of the other one at the protein level. In a *C. elegans* smu2 knock-out mutant, SMU1 protein accumulation is lower compared with wild type (Spartz et al., 2004). In human cells, knock-down of SMU1 results in a 90% reduction of RED protein, and vice versa (Fournier et al., 2014). In the Arabidopsis smu1-2 mutant, SMU2 protein accumulation is not detectable (Chung et al., 2009). In all cases, transcription of the indicated genes is not affected (Spartz et al., 2004; Chung et al., 2009; Fournier et al., 2014). These findings suggest that SMU proteins could stabilize each other to prevent degradation by forming a complex.

**Figure 6.** Proposed working models of SMU1 and SMU2 in RNA splicing. The proposed models are integrations of the findings described in this study with the present knowledge on the SMU1-SMU2 complex of *C. elegans* (Ulrich et al., 2016) and *H. sapiens* (Bertram et al., 2017). Two schematic diagrams were prepared depending on the potential function of nuclear speckles as either the storage sites of splicing factors (left; Lamond and Spector, 2003; Lorković and Barta, 2004; Lorković et al., 2004a; Spector and Lamond, 2011; Reddy et al., 2012) or the places where RNA splicing events occur (right; Dias et al., 2010; Girard et al., 2012). During the formation of the spliceosomal B complex, SMU1 and SMU2 stored in nuclear speckles are recruited to the spliceosome by associating with each other (as a hetero-dimer) as well as with numerous specific spliceosomal components, which function as splicing regulators in bridging U2 and U5 snRNPs. After releasing to the nucleoplasm, SMU1 dimerizes first and associates with SMU2. The ensuing hetero-tetrameric SMU1-SMU2 complex can be efficiently targeted to the nuclear-speckles. When necessary, SMU proteins within the nuclear speckles can be efficiently recruited to the spliceosome for the next round of pre-mRNA splicing.
Intraneural Distribution of SMU Protein would be a Determinant of RNA Splicing Efficiency

In this study, SMU proteins were observed to be diversified within the nucleus (Fig. 5D), whereas their complexes were enriched in the nuclear speckles (Fig. 5E; Supplemental Fig. S7A). Nuclear speckles were initially discovered as subnuclear compartments enriched with splicing factor proteins (Perraud et al., 1979; Spector et al., 1983). Further studies have identified additional nuclear speckle-localized proteins involved in various aspects of RNA metabolism, such as transcription, mRNA maturation, and export (Spector and Lamond, 2011; Galganski et al., 2017). One of the functions of nuclear speckles is to serve as a reservoir that supplies splicing factors to the active transcription sites (Lamond and Spector, 2003; Lorković and Barta, 2004; Lorković et al., 2004a; Spector and Lamond, 2011; Reddy et al., 2012). In addition, there are several reports suggesting that posttranscriptional splicing, which accounts for 10% to 20% of total pre-mRNA splicing events, might occur within specific nuclear speckles (Dias et al., 2010; Girard et al., 2012). Taken together, SMU1-SMU2 complex formation would be important for their efficient targeting to the subnuclear compartments, which may act as reservoirs of splicing factors or places where RNA splicing events occur.

In RNA processing, release and recruitment of splicing factors between nuclear speckles and nucleoplasm determine the splicing efficiency of target genes (Duncan et al., 1997; Hartmann et al., 2001). We revealed that the intranuclear distribution of SMU proteins depends on their quaternary structures (Fig. 5, B, D, and F; Supplemental Fig. S7). In addition, pre-mRNA splicing of a same set of genes (Chung et al., 2009) including MR52-7 (Fig. 2) are affected in either the smu1 or smu2 mutant. These results further indicate that the identified complex formation-dependent nuclear speckle localization of SMU proteins could determine the splicing efficiency through maintaining the intranuclear distribution of these two splicing factors.

In summary, this study identifies a genetic pathway that links pre-mRNA splicing and Mg homeostasis in Arabidopsis. The finding of SMU1 and SMU2 as the splicing regulators of the Mg2+ transporter gene MR52-7 offers the potential for crop breeding with improved Mg use efficiency and enhanced resistance to Mg limitations. In addition, we reveal that the formation of the SMU1-SMU2 complex is essential for their enrichment in the nuclear speckles, pointing out protein complex formation as a strategy to control the intranuclear distribution of splicing factors (Fig. 6). The underlying mechanism(s) that drives the complex formation-dependent protein distribution within the cell nucleus will be addressed in future works.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The kudo4 mutant was isolated from ethyl methanesulfonate–mutagenized Columbia (Col-0) ecotype plants of Arabidopsis (Arabidopsis thaliana). T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). The accession numbers are as follows: SALK_055163 (smu1-2), SALK_039202 (smu2-1), SALK_064741 [msu2-7]. Primers used for establishing homozygous T-DNA lines are listed in Supplemental Table S1.

MGRL medium (Fujikawa et al., 1992) with a modified Ca concentration [3.0 mM Ca(NO3)2] was used to grow the plants. Sigma Type A agar (0.9% [v/v], catalog no. A4550, Sigma-Aldrich) was used to solidify the plates. For the allelism test result shown in Figure 2D, MGRL medium (Fujikawa et al., 1992) with the default Ca concentration [2.0 mM Ca(NO3)2] was used to grow plants. Sigma Type E agar (0.8% [v/v], catalog no. A4657, Sigma-Aldrich) was used to solidify the plates. Seeds were surface-sterilized with 10% (v/v) commercial bleach and washed 4 to 5 times using autoclaved ultra-pure water. After being sown on media and incubated at 4°C for 2 to 4 d, seeds were placed in a growth chamber at 22°C under a 16-h/8-h light/dark cycle.

Genetic Mapping

For genetic mapping, the kudo4 mutant was crossed with the Landsberg erecta ecotype and a self-fertilized F2 population was used for mapping. Simple sequence length polymorphism markers were used to detect the genotype. After rough mapping, a whole genome resequencing was performed with the SOLID system (Life Technologies) to identify mutations in mapped regions as previously described (Tabata et al., 2013). The identified mutation was further confirmed by Sanger sequencing.

Plasmid Construction and Transformation

To generate a plasmid for SMU1 overexpression (35S:SMU1), the SMU1 coding sequence (CDS) with stop codon was amplified with the primers SMU1_CDS_F1 and SMU1_CDS_R1. The amplified fragment was cloned into the pENTR/D-TOPO vector (Life Technologies) and then subcloned to the Gateway destination vector pGW511 (Nakagawa et al., 2007) using LR clonase (Life Technologies).

For the transient expression analysis and BiFC assay, protoplasts were isolated from Arabidopsis rosette leaves as described previously (Wu et al., 2009). Protoplasts were transformed with plasmids by a polyethylene glycol–mediated transfection procedure (You et al., 2007).

For the generation of stable transgenic plants, the respective binary vector was introduced into Agrobacterium tumefaciens strain GV3101::pMP90 (Bechtold and Pelletier, 1998) and then introduced into Arabidopsis using the floral dip method (Zhang et al., 2006). Transformants were selected on half-strength Murashige and Skoog medium containing 20 μg mL−1 hygromycin B (Wako) and 250 μg mL−1 Claron (Sanofi).
RNA Extraction and Expression Analysis

Total RNA was prepared from 14-d-old seedlings (after germination) with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. Approximately 500 ng total RNA was reverse-transcribed to complementary DNA using a PrimeScript RT reagent kit (Takara). Products were diluted 10-fold and used as PCR templates. To determine gene expressions, semiquantitative (semiqu) and qPCR were performed with MightyAmp DNA Polymerase (Ver.3; Takara) and SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara), respectively. EF1a and Actin2 were used as internal controls for semiqPCR and qPCR, respectively. Detailed information of all of the primers used in this study is listed in Supplemental Table S1.

Confocal Laser Scanning Microscopy Observation

For the fluorescence observation in protoplasts, cells were first incubated in W5 solution (Yoo et al., 2007) at 22°C for 16 to 20 h. GFP, RFP, YFP, and chlorophyll autofluorescence were detected using confocal microscopy (FV1000, Olympus). Excitation and emission wavelengths were set as follows: 473 and 500 to 525 nm for GFP; 559 and 600 to 660 nm for RFP and mCherry; 473 and 515 to 540 nm for YFP; 559 and 650 to 710 nm for chlorophyll autofluorescence.

Element Measurement

Shoots and roots of 14-d-old seedlings (after germination) were separated and washed with ultrapure water. After they were dried at 60°C for 2 to 3 d, the dry weights were measured. Samples were then digested with 61% (v/v) HNO3 (Wako) and dissolved in 0.08M HNO3 containing 1 ppb indium as an internal standard. Element concentrations were determined by inductively coupled plasma mass spectrometry (Agilent 7800).

BiFC Quantification

YFP and mCherry signals of protoplasts were observed by confocal microscopy (FV1000, Olympus), and six to nine images were taken for each BiFC plasmid combination. The images with 8 bit/channel were analyzed by Fiji (https://fiji.sc) with a custom macro. Mean fluorescence intensity per area was calculated for each protoplast. To determine the area of each protoplast, the “Analyze Particles” function was applied after binarization with auto thresholding by Yen’s method. Obtained regions of interest were redirected to the fluorescent image, and mean intensity per area was calculated for each particle. The ratios of YFP/mCherry fluorescence intensity were presented as the BiFC fluorescence index.

Statistical Analysis

All statistical analyses performed in this study were carried out in R software version 3.5.2 (https://www.r-project.org/). Welch’s two sample t-test (t.test function from package stats) was used to compare the means between two groups of values. ANOVA with a post hoc Tukey Honest Significance Difference (HSD) was used to compare the means between more than two groups (aov and TukeyHSD functions from package stats).

Accession Numbers

Sequence data from this article can be found in the The Arabidopsis Information Resource (https://www.arabidopsis.org/) under accession numbers: AT1G73720 (SMU1), AT2G26460 (SMU2), AT3G96960 (MRS2-7), AT3G63400 (CypR564), AT1G02840 (SRp34), AT3G55460 (SCL30), AT2G30260 (U2B*), AT3G62190 (PRH75), AT3G60390 (EF1a), and AT3G18780 (Actin2).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Quantitative measurement of wild-type SMU1 transcripts in SMU1-ox lines.

Supplemental Figure S2. Growth phenotype and Mg accumulation pattern of the mrs2-7(I) mutant.

Supplemental Figure S3. Splicing patterns of MRS2-7 pre-mRNA in SMU1-ox lines.

Supplemental Figure S4. Splicing pattern of MRS2-7 pre-mRNA in smu1 and smu2 mutants under the low-Mg condition.

Supplemental Figure S5. Expression confirmation of GFP, SMU1-GFP and MRS2-GFP in protoplasts prepared from wild-type and smu mutants.

Supplemental Figure S6. Effect of SMU1 overexpression on SMU2 self-interactions.

Supplemental Figure S7. Colocalization confirmation of SMU1-SMU2 hetero-oligomers with nuclear body marker proteins.

Supplemental Table S1. Primers used in this study.

ACKNOWLEDGMENTS

We thank Tsuyoshi Nakagawa for kindly providing the BiFC vectors and pGWB-series vectors. Yoko Kawara and Emiko Yokota for technical assistance, and Dr. Marcel Beier for critical reading of the manuscript. T-DNA insertion lines were provided by the Arabidopsis Biological Resource Center.

Received January 30, 2020; accepted June 11, 2020; published June 29, 2020.

LITERATURE CITED

Agafonov DE, Deckert J, Wolf E, Odenwälder P, Bessonov S, Will CL, Urlaub H, Lührmann R (2011) Semiquantitative proteomic analysis of the human spliceosome via a novel two-dimensional gel electrophoresis method. Mol Cell Biol 31: 2667–2682

Bechtold N, Pelletier G (1998) In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol Biol 82: 259–266

Bertram K, Agafonov DE, Dybkov O, Haselbach D, Leelaram MN, Will CL, Urlaub H, Kastner B, Lührmann R, Stark H (2017) Cryo-EM structure of a pre-catalytic human spliceosome primed for activation. Cell 170: 701–713

Bridge E, Xia DX, Carmo-Fonseca M, Cardinali B, Lamond AI, Pettersson U (1995) Dynamic organization of splicing factors in adenovirus-infected cells. J Virol 69: 281–290

Brody E, Abelson J (1985) The “splicesome”: Yeast pre-messenger RNA associates with a 40S complex in a splicing-dependent reaction. Science 228: 963–967

Carmo-Fonseca M (2002) The contribution of nuclear compartmentalization to gene regulation. Cell 108: 513–521

Chen J, Li LG, Liu ZH, Yuan YJ, Guo HL, Mao DD, Tian LF, Chen LB, Luan S, Li DP (2009) Magnesium transporter AtMGT9 is essential for pollen development in Arabidopsis. Cell Res 19: 887–898

Chen ZC, Peng WT, Li J, Liao H (2017) Functional dissection and transport mechanism of magnesium in plants. Semin Cell Dev Biol 74: 142–152

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants by vacuum infiltration. Methods Mol Biol 82: 259–266

Chung T, Wang D, Kim CS, Yadegari R, Larkins BA (2009) Plant SMU1 and SMU2 homologues regulate pre-mRNA splicing and multiple aspects of development. Plant Physiol 151: 1498–1512

Conn SJ, Conn V, Tyerman SD, Kaiser BN, Leigh RA, Gilliam M (2011a) Magnesium transporters, MGT2/MRS2-1 and MGT3/MRS2-5, are important for magnesium partitioning within Arabidopsis thaliana mesophyll vacuoles. New Phytol 190: 583–594

Conn SJ, Gilliam M, Ahlman A, Schreiber AW, Baumann U, Moller I, Cheng NH, Stancombe MA, et al (2011b) Cell-specific vacuolar calcium storage mediated by CAX1 regulates apoplastic calcium concentration, gas exchange, and plant productivity in Arabidopsis. Plant Cell 23: 240–257

Cowan JA (2002) Structural and catalytic chemistry of magnesium-dependent enzymes. Biometals 15: 225–235

Dassah M, Patzek S, Hunt VM, Medina PE, Zahler AM (2009) A genetic screen for suppressors of a mutated 5’ splice site identifies factors associated with later steps of spliceosome assembly. Genetics 182: 725–734
Spector DL, Lamond AI (2011) Nuclear speckles. Cold Spring Harb Perspect Biol 3: a000646
Spector DL, Schrier WH, Busch H (1983) Immunoelectron microscopic localization of snRNPs. Biol Cell 49: 1–10
Spike CA, Shaw JE, Herman RK (2001) Analysis of smu-1, a gene that regulates the alternative splicing of unc-52 pre-mRNA in Caenorhabditis elegans. Mol Cell Biol 21: 4985–4995
Stanek D, Fox AH (2017) Nuclear bodies: News insights into structure and function. Curr Opin Cell Biol 46: 94–101
Sugaya K, Hongo E, Ishihara Y, Tsuji H (2006) The conserved role of Smu1 in splicing is characterized in its mammalian temperature-sensitive mutant. J Cell Sci 119: 4944–4951
Sugaya K, Ishihara Y, Sugaya K (2011) Enlargement of speckles of SF2/ASF due to loss of function of Smu1 is characterized in the mammalian temperature-sensitive mutant. RNA Biol 8: 488–495
Sun Y, Yang R, Li L, Huang J (2017) The magnesium transporter MGT10 is essential for chloroplast development and photosynthesis in Arabidopsis thaliana. Mol Plant 10: 1584–1587
Tabata R, Kamiya T, Shigenobu S, Yamaguchi K, Yamada M, Hasebe M, Fujiwara T, Sawa S (2013) Identification of an EMS-induced causal mutation in a gene required for boron-mediated root development by low-coverage genome re-sequencing in Arabidopsis. Plant Signal Behav 8: e22534

Ulrich AKC, Schulz JF, Kamprad A, Schütze T, Wahl MC (2016) Structural basis for the functional coupling of the alternative splicing factors Smu1 and RED. Structure 24: 762–773
Wolf FI, Cittadini A (2003) Chemistry and biochemistry of magnesium. Mol Aspects Med 24: 3–9
Wu FH, Shen SC, Lee LY, Lee SH, Chan MT, Lin CS (2009) Tape-Arabidopsis sandwich—a simpler Arabidopsis protoplast isolation method. Plant Methods 5: 16
Xu XF, Wang B, Lou Y, Han WJ, Lu JY, Li DD, Li LG, Zhu J, Yang ZN (2015) Magnesium Transporter 5 plays an important role in Mg transport for male gametophyte development in Arabidopsis. Plant J 84: 925–936
Yan YW, Mao DD, Yang L, Qi JL, Zhang XX, Tang QL, Li YP, Tang RJ, Luan S (2018) Magnesium Transporter MGT6 plays an essential role in maintaining magnesium homeostasis and regulating high magnesium tolerance in Arabidopsis. Front Plant Sci 9: 274
Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: A versatile cell system for transient gene expression analysis. Nat Protoc 2: 1565–1572
Zhang X, Henries R, Lin SS, Niu QW, Chua NH (2006) Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nat Protoc 1: 641–646