Two Alternatively Spliced Isoforms of the Arabidopsis SR45 Protein Have Distinct Roles during Normal Plant Development[1][W][OA]

Xiao-Ning Zhang and Stephen M. Mount*

Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20740

The serine-arginine-rich (SR) proteins constitute a conserved family of pre-mRNA splicing factors. In Arabidopsis (*Arabidopsis thaliana*), they are encoded by 19 genes, most of which are themselves alternatively spliced. In the case of *SR45*, the use of alternative 3′ splice sites 21 nucleotides apart generates two alternatively spliced isoforms. Isoform 1 (*SR45.1*) has an insertion relative to isoform 2 (*SR45.2*) that replaces a single arginine with eight amino acids (TSPQRKTG). The biological implications of *SR45* alternative splicing have been unclear. A previously described loss-of-function mutant affecting both isoforms, *sr45-1*, shows several developmental defects, including defects in petal development and root growth. We found that the *SR45* promoter is highly active in regions with actively growing and dividing cells. We also tested the ability of each *SR45* isoform to complement the *sr45-1* mutant by overexpression of isoform-specific green fluorescent protein (GFP) fusion proteins. As expected, transgenic plants overexpressing either isoform displayed both nuclear speckles and GFP fluorescence throughout the nucleoplasm. We found that *SR45.1-GFP* complements the flower petal phenotype, but not the root growth phenotype. Conversely, *SR45.2-GFP* complements root growth but not floral morphology. Mutation of a predicted phosphorylation site within the alternatively spliced segment, *SR45.1-S219A-GFP*, does not affect complementation. However, a double mutation affecting both serine-219 and the adjacent threonine-218 (*SR45.1-T218A + S219A-GFP*) behaves like isoform 2, complementing the root but not the floral phenotype. In conclusion, our study provides evidence that the two alternatively spliced isoforms of *SR45* have distinct biological functions.

Alternative splicing (AS) is common in multicellular eukaryotes, where it both contributes to protein diversity and quantitative regulation of gene expression. In Arabidopsis (*Arabidopsis thaliana*), at least 23.5% of genes show AS (Campbell et al., 2006). This makes AS less common in plants than in animals, but much more common than AS in unicellular eukaryotes. The mode of AS in plants differs from that in animals in that the majority of events are intron retention rather than alternative exon inclusion. Several splicing events have been connected to agricultural issues like seed quality, plant growth, and response to environment. For instance, a splice site mutation in the rice (*Oryza sativa*) *WAXY* gene is responsible for glutinous rice (Isshiki et al., 1998) and has been subject to strong selection in the recent past (Yamanaka et al., 2004; Olsen et al., 2006). Regulation of the plant disease resistance gene *RPS* by AS fine tunes its activity and limits damage inflicted by activated RPS4 protein (Zhang and Gassmann, 2007). Alternative processing of *FCA* (involving both polyadenylation and splicing) regulates flowering time (Macknight et al., 2002). A tobacco (*Nicotiana tabacum*) kinase (PK12) that phosphorylates Ser-Arg-rich (SR) proteins (Savaldi-Goldstein et al., 2000) has been attributed with conferring plasticity on organism-level traits (Marden, 2008). Thus, an understanding of AS regulation may contribute knowledge relevant to plant development and agricultural production.

The regulated splicing of precursors to mRNA generates functional protein diversity in evolutionarily diverse species, and many splicing regulators, including SR proteins, are conserved between plants and animals (Kalyna and Barta, 2004; Reddy, 2004). SR proteins function in both splice site recognition and spliceosome assembly. They are required for constitutive splicing and affect AS (Graveley, 2000; Long and Caceres, 2009). All SR proteins have one or two RNA-binding domains of the RNA recognition motif (RRM) type and a C-terminal RS domain rich in Arg-Ser dipeptides. There are 19 SR proteins identified in Arabidopsis and similar numbers in other plants (Kalyna and Barta, 2004; Reddy, 2004); this is about twice the number found in mammals. Several of these 19 SR protein genes arose during several genome duplication events in evolution (Kalyna and Barta, 2004). However, they are not simply functionally redundant. Arabidopsis SR proteins are distributed dif-

---

[1] This work was supported by the National Science Foundation (grant no. NSF 0544309 to S.M.M.).

* Corresponding author; e-mail smount@umd.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Stephen M. Mount (smount@umd.edu).

[W] The online version of this article contains Web-only data.

[OA] Open access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.109.138180

ferently in a spatial- and temporal-specific manner (Lopato et al., 1999, 2002; Lazar and Goodman, 2000; Reddy, 2004; Tanabe et al., 2007; Lorkovic et al., 2008). SR proteins are highly phosphorylated, primarily in their RS domains (Bourgeois et al., 2004; de la Fuente van Bentem et al., 2006). Phosphorylation may affect their RNA binding, splicing activity, subcellular localization, and protein-protein interactions (Lopato et al., 1996, 1999; Ali and Reddy, 2006; Shen and Green, 2006; Reddy, 2007). In addition, 15 of the 19 Arabidopsis SR proteins are alternatively spliced to produce about 95 different transcripts, which increases the complexity of the splicing regulation network by 6-fold (Palusa et al., 2007). It has been proposed that the protein products from these alternatively spliced SR protein transcripts may have distinct functions according to their domain structures predicted by the sequences of the transcripts (Palusa et al., 2007). However, experimental evidence elucidating functional differences between isoforms of SR proteins generated by AS has been lacking.

SR proteins also function in nuclear export and translation (Windgassen et al., 2004; Huang and Steitz, 2005). It is likely that shuttling SR proteins are displaced by the initial (pioneer) round of translation and then move back into the nucleus. In this regard, they resemble the exon junction complex (EJC), a defined set of proteins that is deposited on the mRNA about 25 nucleotides upstream of the site of each intron. The components of the EJC include a protein known as RNPS1 (Tange et al., 2004), which resembles SR proteins in many respects, including the presence of a related RRM and low complexity domains (both Ser rich and Arg-Ser rich). RNPS1 was initially identified as a general activator of pre-mRNA splicing (Mayeda et al., 1999) and was later shown to be a member of the EJC (Le Hir et al., 2001) that communicates with components of the mRNA surveillance (nonsense-mediated decay) machinery (Lykke-Andersen et al., 2001; Sakashita et al., 2004).

The identification of viable mutants makes it possible to study SR protein function using reverse genetics. A T-DNA insertion mutant of *SR45* (*sr45-1*) has pleiotropic phenotypes, including narrow petals and leaves, altered number of petals and stamens, delayed root growth, and flowering (Ali et al., 2007), indicating that SR45 may regulate genes that are involved in different growth and developmental processes. Recently, several studies have described SR45's interaction with other proteins and its dynamic localization within the nucleus (Ali et al., 2003, 2008; Reddy, 2004; Pendle et al., 2005; Ali and Reddy, 2006). However, the mechanisms by which SR45 participates in different processes are still largely unknown. SR45 itself is alternatively spliced, producing two isoforms by use of alternative 3′ splice sites (Palusa et al., 2007), as indicated in Figure 1A. Here we have investigated this difference by genetic analysis. We find that the two alternatively spliced isoforms of *SR45* have distinct biological functions in plant development, with SR45.1 playing a major role in flower petal development and SR45.2 playing a major role in root growth.

RESULTS

SR45 Is Likely to Be an Ortholog of RNPS1

SR45 has, in addition to an RRM domain with similarity to other SR proteins, an N-terminal low complexity domain rich in Ser and Arg-Ser repeats and a C-terminal low complexity domain rich in Arg-Ser repeats and SPXR motifs (Fig. 1B). This domain structure differs from that found in other SR proteins, which uniformly lack an amino-terminal RS domain (Kalyna and Barta, 2004), but resembles the domain structure in RNPS1 (Fig. 1B). RNPS1 and SR45 are listed as orthologs on the inparanoid Web site (O'Brien et al., 2005), and have been treated as such in previous publications (Pendle et al., 2005), but Ali et al. (2007) describe SR45 as a novel, plant-specific splicing factor. When the Arabidopsis SR45 RRM (At1g16610.1; amino acids 99–173) is used as a query in a blast search, RNPS1 is identified as the most similar human protein. Conversely, when the human RNPS1 RRM is used as a query, SR45 is found to be the most similar Arabidopsis protein. This reciprocal best hit relationship indicates, but does not establish, orthology. On the other hand, the functions of RNPS1 and SR proteins are related, and Arabidopsis SR45 has been shown to function as a splicing factor in human extracts (Ali et al., 2007). In the absence of data that address the critical question of whether the Arabidopsis SR45 protein functions, like RNPS1, as part of the Arabidopsis EJC, we follow Ali et al. (2007) by referring to it as an SR protein.

Two SR45 Isoforms Are Similarly Expressed in a Tissue-Specific Manner

Extensive analysis by reverse transcription (RT) and PCR (Palusa et al., 2007; data not shown) reveals two *SR45* isoforms that differ by a 21-nucleotide sequence that is present in isoform 1 (SR45.1) but missing in isoform 2 (SR45.2) due to an alternative acceptor splice site at the beginning of the seventh exon (Fig. 1A). Because the alternatively spliced segment is in frame, it is likely that both isoforms are functional. We first examined the response of isoform abundance to different plant hormones and environmental cues. Using a common set of flanking primers (Fig. 2A) we found that the relative level of the two isoforms was significantly altered in response to Suc (there is less SR45.2 in the absence of Suc) and temperature changes (the level of SR45.2 is reduced in cold, and increased in warm, temperatures). We next examined the level of these two isoforms in different plant tissues using isoform-specific primers to avoid competition between overlapping products during amplification. Quantitative RT-PCR results showed that the ratio of the two isoforms (SR45.1/SR45.2) is close to 1.0 in root and

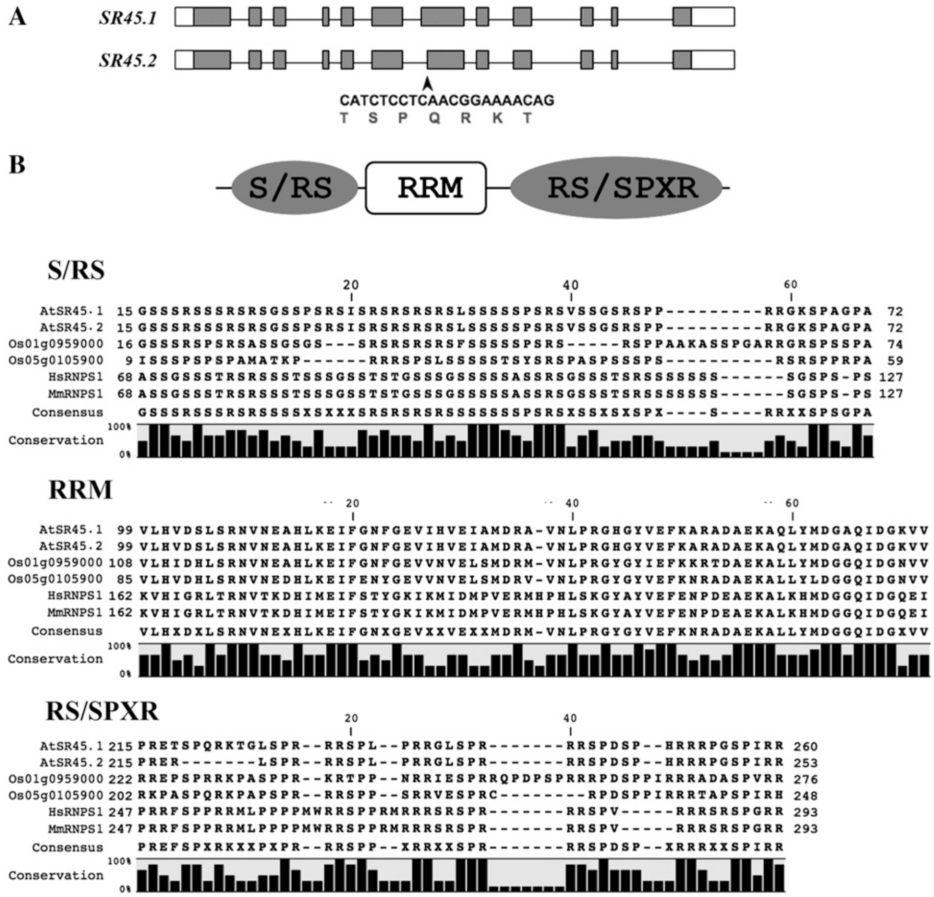

Figure 1. *SR45* gene structure and protein sequence. A, *SR45* gene structure demonstrates two splicing isoforms: *SR45.1* and *SR45.2*. Exons are shown as gray boxes; introns are shown as straight lines; untranslated regions are shown as white boxes. The alternative acceptor site is indicated by an arrowhead, and the 21-nucleotide sequence that is present in *SR45.1* but missing in *SR45.2* is shown with its deduced amino acid sequence. B, The domain structure of SR45 protein and the alignment of each domain of the six given protein sequences: SR45.1 (At1g16610.1), SR45.2 (At1g16610.2), rice SR45 homologs (Os01g0959000 and Os05g0105900), and RNPS1 from human (HsRNPS1) and mouse (MmRNPS1). The alignment was done with CLC Sequence Viewer 5. Five SPXR sequences were found in the C-terminal RS/SPXR domain with the first one absent in AtSR45.2.

inflorescence, and only slightly less (0.87) in leaf tissue (Fig. 2B). Both *SR45* isoforms are present at much higher levels (10-fold or more) in root and inflorescence tissues than in leaves (Fig. 2B). Thus, despite large differences in the overall level of expression, there is not a significant difference between tissues with respect to the ratio of the two spliced isoforms.

Root and inflorescence tissue both contain meristematic regions with active cell division, while leaf cells are rather differentiated. This suggested that *SR45* may be preferentially expressed in actively dividing cells. Organ-specific microarray expression profiles collected by Genevestigator (Zimmermann et al., 2004) showed that the expression of *SR45* was detected in all plant organs, with the highest levels in imbibed seeds, root tip, shoot apex, and inflorescence tissues (Supplemental Fig. S1). Previous microarray data on pollen showed that *SR45* expression is high in both unicellular and bicellular pollen (Honys and Twell, 2004). All of this indicates that the expression of *SR45* is associated with both root and flower development, perhaps pollen development in particular. To examine the transcriptional regulation of *SR45*, we fused the *SR45* promoter sequence to a *GUS* reporter gene. Transgenic lines carrying this reporter showed the strongest GUS activity in pollen, primary and secondary root meristem, shoot apical meristem, and leaf primordia (Fig. 2C). The GUS activity was also detected in vascular tissue, hydathode, and cells close to the root meristem. These results are consistent with the idea that *SR45* transcription is enriched in, and perhaps limited to, actively dividing and rapidly growing cells.

Two SR45 Isoforms Function Differently in a Tissue-Specific Manner

The eight amino acid segment found in SR45.1 but not SR45.2, TSPQRKTG, contains a potential phosphorylation site, at Ser 219 (S219) that is predicted by both the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos; cite_bib>Blom et al., 1999) with significantly high score (0.994) and by the Arabidopsis Protein Phosphorylation Site Database (PhosPhAt) with the prediction score of +0.932 (Heazlewood et al., 2008). To assess the potential functional difference between SR45.1 and SR45.2 we introduced a point mutation at this position (S219A). In addition, Thr 218 (T218) was also predicted as a possible phosphorylation site (score = 0.913) by NetPhos 2.0 Server but not PhosphAt. We mutated the Thr to Ala together with S219 (T218A + S219A) to see if T218 has any impact on SR45 function in addition to S219. We individually expressed SR45.1-GFP, SR45.1-S219A-GFP, SR45.1-T218A + S219A-GFP, and SR45.2-GFP with GFP fused at the C terminus of

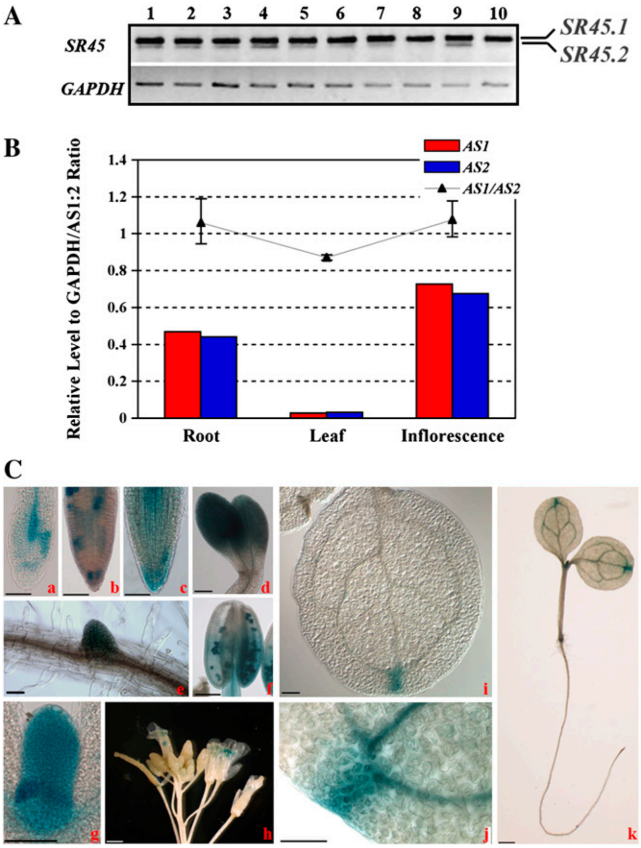

Figure 2. The expression and splicing pattern of *SR45*. A, RT-PCR results show the AS pattern of *SR45* from 8-d-old wild-type Columbia-0 seedlings in response to different conditions. All seedlings (1–9) were grown on Murashige and Skoog plates with 3% Suc except for the last treatment number 10. From 1 to 10: untreated control, 10 μM GA$_3$, 2 μM jasmonic acid, 1 μM indole-3-acetic acid, 100 μM 1-aminocyclopropane-1-carboxylic acid, 50 mM NaCl, 100 μM abscisic acid for 4 h, 7°C for 24 h, 37°C for 24 h, and without Suc. *GAPDH* was used as control. B, Two replicates were used in quantitative RT-PCR. The expression level of *SR45.1* (*AS1*) and *SR45.2* (*AS2*) in root, leaf, and inflorescence tissue is shown as bars (red bar for *AS1*; blue bar for *AS2*). The ratio of two isoforms (*AS1*/*AS2*) was plotted as a line in contrast to the expression levels. Error bars indicate SDS calculated from the PCR results. C, *SR45* promoter activity was examined in *SR45*::*GUS* transgenic wild-type plants by GUS staining at 4-d-old dark-grown seedling stage (primary root tip [a] and cotyledon [d]); primary root tip at 2-d-old seedling stage (b); 4-d-old seeding stage (primary root tip [c], secondary root tip [e], cotyledon [i and j], shoot apex [g], and the whole seedling [k], inflorescence [h], and anthor [f]). Three independent lines were used in the staining analysis. Bar = 100 μm for a to g and i and j. Bar = 1 mm for h and k.

each proteins transiently in *sr45-1* mutant mesophyll protoplasts and detected GFP signal in the nucleus of transfected protoplasts for all the constructs (Fig. 3A). Then we generated stable transgenic lines with these constructs in the *sr45-1* mutant background for in vivo functional study (Fig. 3, B and C). In transgenic plants overexpressing *SR45.1-GFP* (*OX1*), the narrow flower petal phenotype in *sr45-1* mutants was rescued (Fig. 3D). Similar rescue was observed by using an N-terminal GFP fusion (GFPSR45.1) in an earlier independent study (Ali et al., 2007). Putting these results together, it appears that SR45 can tolerate a GFP fusion at either terminus. All of our studies were conducted with the C-terminal GFP fusion.

Oddly, plants overexpressing *SR45.2-GFP* (*OX2*) did not rescue the narrow petal phenotype (Fig. 3D). Nor was this phenotype rescued by the T218A, S219A double mutant (*OX1TSAA*; Fig. 3D). However, a single mutation of S219A (*OX1SA*) behaved like wild-type *OX1* with respect to the flower petal phenotype (Fig. 3D). These results suggested that S219 is not required for the normal flower petal development, but T218 is required, either as an alternative phosphorylation site or by itself. We did not test a mutation of only T218.

The root growth of *sr45-1* mutant seedlings was significantly delayed compared to wild-type seedlings (Fig. 3E). Neither *OX1* nor *OX1SA* were able to rescue the mutant root growth on Murashige and Skoog agar medium (Fig. 3E). However, *OX2* and *OX1TSAA* seedlings exhibited normal root growth compared to the mutant and other transgenic seedlings (Fig. 3E). These results suggest that although both isoforms were expressed in all tissues in the overexpression lines (Fig. 3, B and C), SR45.1 function is more important for flower development while SR45.2 function is more important for root growth. Interestingly, introducing S219A in SR45.1 did not change root growth, but introducing mutations on both T218 and S219 sites in SR45.1 mimicked the SR45.2 function. This indicates that T218 may be most important in distinguishing SR45.1 and SR45.2. Two independent lines were used for each transgene, and expression in both root and floral tissue was verified by examination of GFP, so it is unlikely that the observed differences can be attributed to positional effects of T-DNA insertion.

SR45 Affects the AS of Other SR Protein Genes

In several animal species, splicing factors have been identified as AS regulation targets during development (Barberan-Soler and Zahler, 2008). Similar observations have been obtained in plants as well (Lopato et al., 1999; Isshiki et al., 2006; Palusa et al., 2007; Reddy, 2007). Fifteen of the Arabidopsis SR protein genes are alternatively spliced and their AS patterns change in a developmental and tissue-specific manner (Palusa et al., 2007). In both inflorescence and root tissues, the AS pattern of *SRP30*, *RSP31*, *RSP31a*, *SR34*, and *SRP34b* was altered in *sr45-1* mutants compared to wild type (Ali et al., 2007). This suggests that SR45 may directly or indirectly regulate the splicing pattern of these SR protein genes in both root and inflorescence tissues. We examined the AS pattern of these five SR protein genes in wild type, *sr45-1*, and all transgenic plants. In inflorescence tissues, the ratio of two *SRP30* isoforms (isoform5/isoform1) was increased in *sr45-1* relative to wild type and this ratio was restored toward wild type in transgenic plants that also show phenotypic rescue (those overexpressing SR45.1GFP or SR45.1-S219A-GFP but not SR45.1-T218A + S219A-

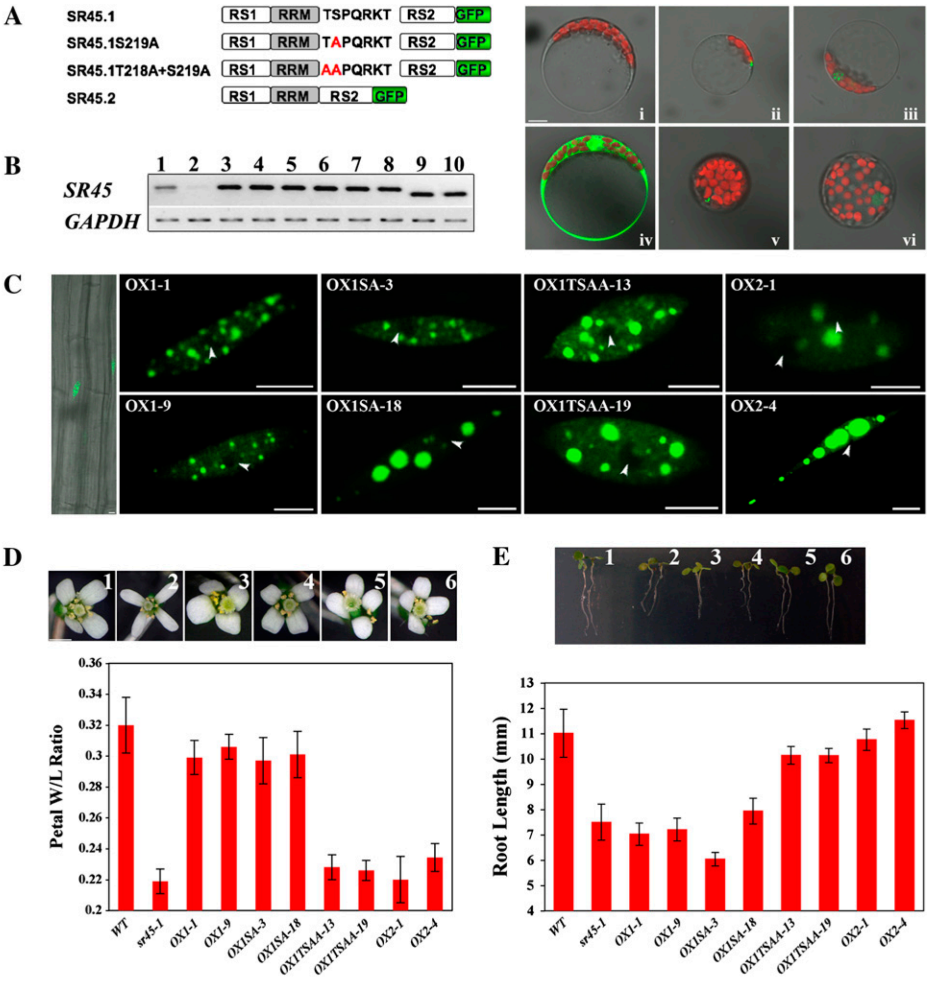

Figure 3. Transgenic plants overexpressing different SR45 constructs. A, Transient expression of SR45 constructs. Cartoon on the left shows the domain arrangement of SR45.1, SR45.2, and protein substitution in the mutant constructs. On the right, GFP transient expression is observed in *sr45-1* mesophyll protoplasts that were transfected with i: empty vector; ii: SR45.1-GFP (OX1); iii: SR45.1-T218A-S219A-GFP (OX1TSAA); iv: GFP control; v: SR45.2-GFP (OX2); and vi: SR45.1-S219A-GFP (OX1SA). Bar = 10 μm. B, RT-PCR results show expression level of *SR45* in each plant in the order (1–10) of wild type, *sr45-1*, OX1-1, OX1-9, OX1SA-3, OX1SA-18, OX1TSAA-13, OX1TSAA-19, OX2-1, and OX2-4. *GAPDH* was used as control. C, GFP signal was detected in nucleus of root cells from the elongation zone of each 8-d-old transgenic seedling as indicated. Arrowheads show nucleoli. Bar = 5 μm. D, Floral phenotype. Top section: Label numbers 1 to 6 show flowers from wild type (WT), *sr45-1*, transgenic plants overexpressing OX1, OX1SA, OX1TSAA, and OX2, respectively. Bottom section: Ratio of petal width (W) to length (L) from wild-type, *sr45-1*, and two independent lines for each transgene in the order as indicated in the figure. Error bars show the SD from at least 20 flower petals. E, Root phenotype. Top section: Label numbers 1 to 6 show 4-d-old seedlings in the same order as in D (top section). Bottom section: Root length measured from 4-d-old seedlings from wild type, *sr45-1*, and two independent lines from each transgene in the order as indicated in the figure. Error bars show the SD from 20 seedlings from one plate. Similar pattern was observed in three independent experiments.

GFP or SR45.2-GFP). The AS pattern of *RSP31*, *RSP31a*, *SR34*, and *SRP34b* was rescued in all transgenic plants (Fig. 4A). In root tissues, the altered isoform5+3/isoform1 ratio of *SRP30* seen in *sr45-1* was restored toward wild-type levels in transgenic plants that show phenotypic rescue in the root (those expressing SR45.1-T218A + S219A-GFP or SR45.2-GFP, but not those expressing SR45.1-GFP or SR45.1-S219A-GFP). As for *RSP31*, the wild-type ratio of isoform 2 to isoform 1 was restored in all transgenic plants except for those overexpressing SR45.1-GFP. The AS pattern of *RSP31a* and *SR34b* was rescued in all transgenic plants, but to differing extents (Fig. 4B). These AS profiles suggested that regulation of the AS pattern of *SRP30* may be directly associated with the flower petal morphology change and the root growth difference in *sr45-1* mutant plants.

DISCUSSION

Here we have found that two isoforms of SR45 generated by AS have distinct, tissue-specific, biological functions. SR45.1 is required for normal flower

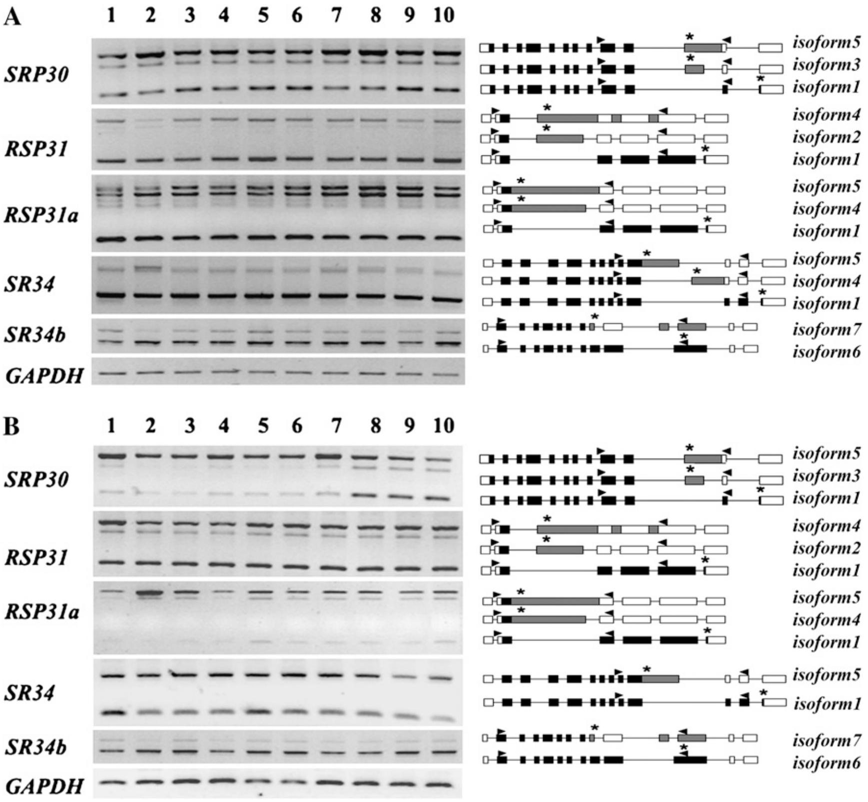

Figure 4. AS pattern of SR protein genes in wild-type, *sr45-1*, and different transgenic plants. AS pattern was examined on *SRP30*, *RSP31*, *SRP31a*, *SRP34*, and *SR34b*. Lanes 1 to 10 present RT-PCR results from plants in the same order as in Figure 3B. *GAPDH* was used as control. A, Inflorescence tissues. The splicing pattern of each isoform is shown as cartoon next to each band position. B, Root tissues. Each band was corresponding to each splicing isoform. The untranslated regions are represented by white boxes, the exons by black boxes, the alternatively spliced exons by gray boxes, and the introns by lines. The position of gene-specific primers is shown by black arrowheads and the stop codons are shown as *.

petal development while SR45.2 is required for normal root growth. To our knowledge, this is the first demonstration that natural splicing variants of SR proteins are required for distinct biological functions. We also find that a mutant form of the longer isoform resembles the shorter isoform with respect to genetic complementation. Our data consistently show that the mutant *SR45.1-T218A + S219A* (*OX1-TSAA*) functions similarly to *SR45.2*, while the single mutant *SR45.1-S219A* functions as does *SR45.1*. Both *OX1* and *OX1-S219A* restore wild-type petal development to the *sr45-1* mutant flowers, while *OX2* and *OX1-T218A-S219A* restore normal root growth when overexpressed in *sr45-1* mutant seedlings. It is notable that although SR45.1 and SR45.2 are expressed in all tissues in our overexpression lines and similarly distributed throughout the nucleoplasm, they complement *sr45-1* defects only in specific tissues. It is possible that some type of posttranslational modification selectively represses SR45.1 activity in root and SR45.2 activity in inflorescence, or activates SR45.2 in root and SR45.1 in inflorescence. Alternatively, SR45.1 or SR45.2 may alter the processing of RNA from a different subset of genes transcribed in root versus in inflorescence.

These observations support the hypothesis that the predicted phosphorylation sites found in the alternatively spliced segment contribute to functional differences between the two isoforms. However, global proteomic studies of Arabidopsis cells in suspension did not detect phosphorylation in this region, even though phosphorylation was found at other sites within SR45 in the same studies (de la Fuente van Bentem et al., 2006, 2008; Heazlewood et al., 2008). The lack of support from proteomics does not mean that this site is not phosphorylated. Any single phosphopeptide could be missed in such a study, functionally important phosphorylation could be transient, or the phosphorylation status of proteins in dark-grown root cell culture may differ from that of light-grown plant tissues. Of course, it is also possible that the effect of the *OX1-TSAA* mutation is not through an effect on phosphorylation. Even if phosphorylation is responsible for the effect of the alternatively spliced segment, our observation that the S219A mutation alone has no effect could mean either that T218 is the site of phosphorylation or that S219 is normally the site of phosphorylation but can be replaced by T218. Whatever the situation within this region, it is very likely that phosphorylation plays an important role in the function of the Ser-rich SR45 protein. We observed the similar nuclear localization pattern for all transgenes, so it is unlikely that either of T218 and S219 is required for protein mobility in nucleus even though they are required for the activity of SR45.1.

What is that activity? SR45 has splicing factor activity in vitro (Ali et al., 2007) and our observation that the effect of *SR45* transgenes on the relative amounts of two *SRP30* isoforms correlates with their ability to rescue distinct *sr45-1* is consistent with a role in splicing. However, our analysis indicates that SR45 may be orthologous to animal RNPS1, and RNPS1 has been assigned additional functions, particularly as a component of the EJC. Prior work on animals has shown that the EJC is a conserved multiprotein complex that mediates mRNA localization, export, and nonsense-mediated mRNA decay (Tange et al., 2004). Interestingly SR45, as well as other EJC components, was found in the plant nucleolus (Pendle et al., 2005), suggesting the possibility that SR45 may be a component of the EJC. In addition, RNPS1 may be a versatile AS regulator that interacts with other splicing factors, U1 snRNP component hLUcA, pinin, and UPF complex to trigger nonsense-mediated mRNA decay (Lykke-Andersen et al., 2001; Sakashita et al., 2004). Phosphorylation at Ser 53 by caseine kinase 2 may activate its splicing regulation function (Trembley et al., 2005). There is also evidence showing that Arabidopsis SR45 interacted with another SR protein, SCL33, a subunit of U1 snRNP (U1-70 K) and kinase AFC2 in vitro (Golovkin and Reddy, 1998; Reddy, 2004). In vivo interaction was observed between U1-70 K and both RS domains of SR45 in nuclear speckles supports the idea that SR45 may recruit U1 snRNP directly (Ali et al., 2008). It was also found that adding phosphorylation inhibitors influenced SR45 mobility (Ali and Reddy, 2006).

In conclusion, we found that two isoforms of SR45 influence biological functions in a tissue-specific manner. SR45.1 may mostly function in flower petal development by directly regulating the AS pattern of splicing factor genes, while SR45.2 may play a major role in root growth by directly regulating the AS pattern of splicing factor genes (Fig. 4, A and B). To our knowledge, this is the first experimental evidence showing that splicing variants of SR proteins may function distinctively. In fact, interestingly, both transcript variants were detected in both inflorescences and roots. This indicates a possible posttranslational regulation mechanism in controlling SR45 activity. It will be very encouraging if such supporting evidence becomes available.

MATERIALS AND METHODS

Plant Growth Condition

All Arabidopsis (*Arabidopsis thaliana*) plants used in this study are in Colombia background. Mutant plants *sr45-1* (SALK_004132) were originally from the Arabidopsis Biological Resource Center and were previously described by Ali et al. (2007). Primers that were used for examining T-DNA insertion in *sr45-1* mutants were designed by the T-DNA primer design tool powered by Genome Express Browser Server: 004132LP: ATGGATCGAGCTGTAAGTTGC, 004132RP: GATTGGAGATCTTCTGGGAGG, and LBb1.3: ATTTTGCCGATTTCGGAAC. All plants were either grown in soil with 16/8 h photoperiod or sowed on Murashige and Skoog plates with 1% agar in continuous light treatment at 100 μmol m^{-2} s^{-1}. For the plants growing in soil, Peter's fertilizer (Griffin Greenhouse & Nursery Supplies, 67–2030) was applied at the concentration of 3 g L^{-1}. All plants were grown at 22°C.

Cloning of SR45 Isoform cDNAs and Mutant cDNAs

SR45 cDNA was amplified from wild-type cDNA and then cloned into pCR2.1 vector from Invitrogen. After sequencing, cDNA clones with isoform 1 (*SR45.1*; National Center for Biotechnology Information accession NM_101523.3) and isoform 2 (*SR45.2*; National Center for Biotechnology Information accession NM_202115.2) were identified separately. Digestion sites were added at 5′ and 3′ of the cDNAs by using primers: SR45PstATG: CTGCAGATGGCGAAACCAAGTCGT and SR45 *Nco*I_3: CCATGGGAGTTTTACGAGGTGGAGGT. About 1.3 kb *SR45.1*/*SR45.2* fragments were then isolated and inserted into *Kpn*I/*Nco*I sites in an GFP overexpression vector (generated from pND1 and pDN393) maintained in the lab to generate *SR45.1*/*2* cDNAs with C-terminal *GFP* fusion. *35S*::*SR45.1*/*2-GFP-NOS3′* was isolated by *Not*I and cloned into a binary vector pMLBart. Point mutations were introduced into *SR45.1* by QuickChange XL site-directed mutagenesis kit (STRATAGENE). The primers used for T to A mutation are: SR45T218AF: GGCGCCCAAGAGAGGCATCTCCTCAACGG, and SR45T218AR: CCGTTGAGGAGATGCCTCTCTTGGGCGCC; and the primers used for S to A mutation are: SR45S219AF: CGCCCAAGAGAGACAGCTCCTCAACGGAA, and SR45S219AR: TTCCGTTGAGGAGCTGTCTCTCTTGGGCG. All sequences were confirmed by sequencing. All sequencing was done by Genewiz Inc.

SR45 Promoter::GUS

SR45 upstream region of 1.6 kb was cloned by PCR with additional restriction enzyme sites added to primers: SR45pro5*Not*I: GCGGCCGCATTTCTGCTCCAAAATTCTATATAA and SR45pro3*Bgl*I: CCGGCCATGGTGGCGAGAGATATCGAGAAAAT. The PCR product was cloned into pCR2.1 vector for sequence confirmation and inserted into vector pRITA I by using *Not*I and *Kpn*I sites. The region containing *SR45* promoter and *GUS* gene was subcloned into the binary vector pMLBart by using *Not*I sites. The resulting construct was named *SR45*::*GUS* for examination of *SR45* promoter activity by GUS staining.

Plant Transformation

The final binary vectors with *SR45.1*, *SR45.1S219A*, *SR45.1T218A + S219A*, *SR45.2*, and *SR45*::*GUS* were transformed into *Agrobacterium tumefaciens* GB3101 individually. Then the resulting Agrobacteria were used to transform Arabidopsis plants by flower-dipping methods described before (Bent, 2000). Basta resistance was used for stable transgenic plants selection. At least two independent transgenic lines were selected from each transformation for further analysis. All characterization was done at T3 generation. All constructs except for *SR45*::*GUS* were transiently expressed in Arabidopsis mesophyll protoplasts (Kovtun et al., 2000) and examined after 24 h.

RNA Isolation and RT-PCR

RNeasy mini kit (Qiagen) was used to isolate total RNA. Five micrograms of total RNA from each sample was digested by DNase I (Invitrogen) and applied for RT with Superscript II system from Invitrogen. The transcription level of candidate genes was verified by quantitative RT-PCR on Roche LightCycler 480. Roche SYBR green master mix was used to prepare all reactions. *GAPDH* was used for normalization purpose. Splicing pattern of SR protein genes was examined using gene-specific primers (Supplemental Table S1).

Plant Screening and Morphology

All T1 transgenic plants were screened by 1:1,000 diluted Finale. Plants expressing *Basta* resistance gene from the plasmids were selected and allowed to self in T2 generations. Plants from T3 generation for each transgene were used for all the examinations.

Flower petals were picked from plants that already flowered for around 10 d. Pictures were taken from all the petals. Width and length of each petal were measured in ImageJ software. All width-to-length ratios were calculated and plotted using Microsoft Excel.

Pictures were taken of 4-d-old seedlings grown on Murashige and Skoog agar medium. Root length was measured by ImageJ and plotted in Microsoft Excel.

Confocal Imaging

The SR45-GFP signal in nucleus was examined with Zeiss LSM 510 confocal microscope (Leica Microsystems). The excitation wavelength for

GFP was 488 nm, and the emission filter wavelength was 505 to 530 nm. Protoplasts incubated in buffer (0.5 mannitol, 4 mM MES, pH 5.7, 20 mM KCl) were directly mounted on a glass slide. Whole seedlings were mounted in water for visualization of the GFP-fusion proteins in root tissues. Digitized images were acquired using a 40× oil-immersion objective at 1,024 × 1,024 pixel resolution. Each image was scanned four times using LCS510 software. Captured images were exported as TIFF format files and further processed in Adobe Photoshop.

GUS Staining

Three to five independent transgenic lines were examined for GUS activity. Plant tissues were fixed in 80% acetone on ice for 30 min, washed in GUS staining solution (0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 100 mM potassium phosphate buffer, pH 7.0), then submerged in GUS staining solution with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcU A. For inflorescences, trapped air was removed by pulling a vacuum for 5 min followed by releasing vacuum. The process was repeated until the flowers were completely submerged. All tissues were incubated at 37°C for 24 h. Chlorophyll was removed by replacing the stain solution with 70% ethanol several times until the green color disappeared and blue GUS stain was clearly visible. Photographs of the stained tissues were taken by a Nikon stereoscopic zoom microscope SMZ1000 or with differential interference contrast using a Nikon E600 microscope.

Sequence data used in this article can be found in the GenBank/EMBL data libraries under accession numbers NP_173107 (SR45.1), NP_973844 (SR45.2), NP_001045458 (Os01g0959000), NP_001054414 (Os05g0105900), NP_542161 (HsRNPS1), and NP_033096 (MmRNPS1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Microarray data from Genevestigator showing *SR45* gene expression level in different tissues.

Supplemental Table S1. Sequences of the gene specific primers that were used in real-time PCRs and RT-PCRs.

ACKNOWLEDGMENTS

We thank Dr. Caren Chang and Heven Sze for their intensive reading and constructive comments on the manuscript, the Arabidopsis Biological Resource Center for T-DNA mutant seeds, core facilities in the Department of Cell Biology and Molecular Genetics at University of Maryland for their assistance with confocal microscopy and quantitative PCR, and Dr. Jason Edmonds for generating the GFP overexpression vector.

Received March 6, 2009; accepted April 16, 2009; published April 29, 2009.

LITERATURE CITED

Ali GS, Golovkin M, Reddy AS (2003) Nuclear localization and in vivo dynamics of a plant-specific serine/arginine-rich protein. Plant J **36:** 883–893

Ali GS, Palusa SG, Golovkin M, Prasad J, Manley JL, Reddy AS (2007) Regulation of plant developmental processes by a novel splicing factor. PLoS One **2:** e471

Ali GS, Prasad KV, Hanumappa M, Reddy AS (2008) Analyses of in vivo interaction and mobility of two spliceosomal proteins using FRAP and BiFC. PLoS One **3:** e1953

Ali GS, Reddy AS (2006) ATP, phosphorylation and transcription regulate the mobility of plant splicing factors. J Cell Sci **119:** 3527–3538

Barberan-Soler S, Zahler AM (2008) Alternative splicing regulation during C. elegans development: splicing factors as regulated targets. PLoS Genet **4:** e1000001

Bent AF (2000) Arabidopsis in planta transformation: uses, mechanisms, and prospects for transformation of other species. Plant Physiol **124:** 1540–1547

Blom N, Gammeltoft S, Brunak S (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol **294:** 1351–1362

Bourgeois CF, Lejeune F, Stevenin J (2004) Broad specificity of SR (serine/arginine) proteins in the regulation of alternative splicing of pre-messenger RNA. Prog Nucleic Acid Res Mol Biol **78:** 37–88

Campbell MA, Haas BJ, Hamilton JP, Mount SM, Buell CR (2006) Comprehensive analysis of alternative splicing in rice and comparative analyses with Arabidopsis. BMC Genomics **7:** 327

de la Fuente van Bentem S, Anrather D, Dohnal I, Roitinger E, Csaszar E, Joore J, Buijnink J, Carreri A, Forzani C, Lorkovic ZJ, et al (2008) Site-specific phosphorylation profiling of Arabidopsis proteins by mass spectrometry and peptide chip analysis. J Proteome Res **7:** 2458–2470

de la Fuente van Bentem S, Anrather D, Roitinger E, Djamei A, Hufnagl T, Barta A, Csaszar E, Dohnal I, Lecourieux D, Hirt H (2006) Phosphoproteomics reveals extensive in vivo phosphorylation of Arabidopsis proteins involved in RNA metabolism. Nucleic Acids Res **34:** 3267–3278

Golovkin M, Reddy AS (1998) The plant U1 small nuclear ribonucleoprotein particle 70K protein interacts with two novel serine/arginine-rich proteins. Plant Cell **10:** 1637–1648

Graveley BR (2000) Sorting out the complexity of SR protein functions. RNA **6:** 1197–1211

Heazlewood JL, Durek P, Hummel J, Selbig J, Weckwerth W, Walther D, Schulze WX (2008) PhosPhAt: a database of phosphorylation sites in Arabidopsis thaliana and a plant-specific phosphorylation site predictor. Nucleic Acids Res **36:** D1015–1021

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol **5:** R85

Huang Y, Steitz JA (2005) SRprises along a messenger's journey. Mol Cell **17:** 613–615

Isshiki M, Morino K, Nakajima M, Okagaki RJ, Wessler SR, Izawa T, Shimamoto K (1998) A naturally occurring functional allele of the rice waxy locus has a GT to TT mutation at the 5′ splice site of the first intron. Plant J **15:** 133–138

Isshiki M, Tsumoto A, Shimamoto K (2006) The serine/arginine-rich protein family in rice plays important roles in constitutive and alternative splicing of pre-mRNA. Plant Cell **18:** 146–158

Kalyna M, Barta A (2004) A plethora of plant serine/arginine-rich proteins: redundancy or evolution of novel gene functions? Biochem Soc Trans **32:** 561–564

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci USA **97:** 2940–2945

Lazar G, Goodman HM (2000) The Arabidopsis splicing factor SR1 is regulated by alternative splicing. Plant Mol Biol **42:** 571–581

Le Hir H, Gatfield D, Izaurralde E, Moore MJ (2001) The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay. EMBO J **20:** 4987–4997

Long JC, Caceres JF (2009) The SR protein family of splicing factors: master regulators of gene expression. Biochem J **417:** 15–27

Lopato S, Forstner C, Kalyna M, Hilscher J, Langhammer U, Indrapichate K, Lorkovic ZJ, Barta A (2002) Network of interactions of a novel plant-specific Arg/Ser-rich protein, atRSZ33, with atSC35-like splicing factors. J Biol Chem **277:** 39989–39998

Lopato S, Kalyna M, Dorner S, Kobayashi R, Krainer AR, Barta A (1999) atSRp30, one of two SF2/ASF-like proteins from Arabidopsis thaliana, regulates splicing of specific plant genes. Genes Dev **13:** 987–1001

Lopato S, Mayeda A, Krainer AR, Barta A (1996) Pre-mRNA splicing in plants: characterization of Ser/Arg splicing factors. Proc Natl Acad Sci USA **93:** 3074–3079

Lorkovic ZJ, Hilscher J, Barta A (2008) Co-localisation studies of Arabidopsis SR splicing factors reveal different types of speckles in plant cell nuclei. Exp Cell Res **314:** 3175–3186

Lykke-Andersen J, Shu MD, Steitz JA (2001) Communication of the position of exon-exon junctions to the mRNA surveillance machinery by the protein RNPS1. Science **293:** 1836–1839

Macknight R, Duroux M, Laurie R, Dijkwel P, Simpson G, Dean C (2002) Functional significance of the alternative transcript processing of the *Arabidopsis* floral promoter FCA. Plant Cell **14:** 877–888

Marden JH (2008) Quantitative and evolutionary biology of alternative splicing: how changing the mix of alternative transcripts affects phenotypic plasticity and reaction norms. Heredity **100:** 111–120

Mayeda A, Badolato J, Kobayashi R, Zhang MQ, Gardiner EM, Krainer

AR (1999) Purification and characterization of human RNPS1: a general activator of pre-mRNA splicing. EMBO J **18:** 4560–4570

O'Brien KP, Remm M, Sonnhammer EL (2005) Inparanoid: a comprehensive database of eukaryotic orthologs. Nucleic Acids Res **33:** D476–480

Olsen KM, Caicedo AL, Polato N, McClung A, McCouch S, Purugganan MD (2006) Selection under domestication: evidence for a sweep in the rice waxy genomic region. Genetics **173:** 975–983

Palusa SG, Ali GS, Reddy AS (2007) Alternative splicing of pre-mRNAs of Arabidopsis serine/arginine-rich proteins: regulation by hormones and stresses. Plant J **49:** 1091–1107

Pendle AF, Clark GP, Boon R, Lewandowska D, Lam YW, Andersen J, Mann M, Lamond AI, Brown JW, Shaw PJ (2005) Proteomic analysis of the Arabidopsis nucleolus suggests novel nucleolar functions. Mol Biol Cell **16:** 260–269

Reddy AS (2004) Plant serine/arginine-rich proteins and their role in pre-mRNA splicing. Trends Plant Sci **9:** 541–547

Reddy AS (2007) Alternative splicing of pre-messenger RNAs in plants in the genomic era. Annu Rev Plant Biol **58:** 267–294

Sakashita E, Tatsumi S, Werner D, Endo H, Mayeda A (2004) Human RNPS1 and its associated factors: a versatile alternative pre-mRNA splicing regulator in vivo. Mol Cell Biol **24:** 1174–1187

Savaldi-Goldstein S, Sessa G, Fluhr R (2000) The ethylene-inducible PK12 kinase mediates the phosphorylation of SR splicing factors. Plant J **21:** 91–96

Shen H, Green MR (2006) RS domains contact splicing signals and promote splicing by a common mechanism in yeast through humans. Genes Dev **20:** 1755–1765

Tanabe N, Yoshimura K, Kimura A, Yabuta Y, Shigeoka S (2007) Differential expression of alternatively spliced mRNAs of Arabidopsis SR protein homologs, atSR30 and atSR45a, in response to environmental stress. Plant Cell Physiol **48:** 1036–1049

Tange TO, Nott A, Moore MJ (2004) The ever-increasing complexities of the exon junction complex. Curr Opin Cell Biol **16:** 279–284

Trembley JH, Tatsumi S, Sakashita E, Loyer P, Slaughter CA, Suzuki H, Endo H, Kidd VJ, Mayeda A (2005) Activation of pre-mRNA splicing by human RNPS1 is regulated by CK2 phosphorylation. Mol Cell Biol **25:** 1446–1457

Windgassen M, Sturm D, Cajigas IJ, Gonzalez CI, Seedorf M, Bastians H, Krebber H (2004) Yeast shuttling SR proteins Npl3p, Gbp2p, and Hrb1p are part of the translating mRNPs, and Npl3p can function as a translational repressor. Mol Cell Biol **24:** 10479–10491

Yamanaka S, Nakamura I, Watanabe KN, Sato Y (2004) Identification of SNPs in the waxy gene among glutinous rice cultivars and their evolutionary significance during the domestication process of rice. Theor Appl Genet **108:** 1200–1204

Zhang XC, Gassmann W (2007) Alternative splicing and mRNA levels of the disease resistance gene RPS4 are induced during defense responses. Plant Physiol **145:** 1577–1587

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR: Arabidopsis microarray database and analysis toolbox. Plant Physiol **136:** 2621–2632