Two alternatively spliced isoforms of the *Arabidopsis thaliana* SR45 protein have distinct roles during normal plant development

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Abstract

The serine-arginine-rich (SR) proteins constitute a conserved family of pre-mRNA splicing factors. In *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 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.
Introduction

Alternative splicing (AS) is common in multicellular eukaryotes, where it both contributes to protein diversity and quantitative regulation of gene expression. In Arabidopsis, at least 23.5% of genes show alternative splicing (Campbell et al., 2006). This makes alternative splicing less common in plants than in animals, but much more common than alternative splicing in unicellular eukaryotes. The mode of alternative splicing 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 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 alternative splicing 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 kinase (PK12) that phosphorylates SR proteins (Savaldi-Goldstein et al., 2000) has been attributed with conferring plasticity on organism-level traits (Marden, 2008). Thus, an understanding of alternative splicing 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 alternative splicing (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 arginine-serine 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 differently in a spatial and temporal-specific manner (Lopato et al., 1999; Lazar and Goodman, 2000; Lopato et al., 2002; 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; Lopato et al., 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 six-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 alternative splicing 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 serine-rich and arginine-serine-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 \textit{SR45} (\textit{sr45-1}) has pleiotropic phenotypes, including narrow petals and leaves, altered number of petals and stamens, delayed root growth and flowering (Ali \textit{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 \textit{et al.}, 2003; Reddy, 2004; Pendle \textit{et al.}, 2005; Ali and Reddy, 2006; Ali \textit{et al.},
2008). 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 serine and arginine-serine repeats and a C-terminal low complexity domain rich in arginine-serine repeats and SPXR motifs (Figure 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 (Figure 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 thaliana 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. by referring to it as an SR protein.

Two SR45 isoforms are similarly expressed in a tissue-specific manner

Extensive analysis by reverse transcription and PCR (Palusa et al. 2007; data not shown) reveals two SR45 isoforms which 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 (Figure 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 (Figure 2A) we found that the relative level of the two isoforms was significantly altered in response to sucrose (there is less SR45.2 in the absence of sucrose) 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 in order 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 inflorescence, and only slightly less (0.87) in leaf tissue (Figure 2B). Both SR45 isoforms are present at much higher levels (10-fold or more) in root and inflorescence tissues than in leaves (Figure 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 (Figure 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. In order to examine the transcriptional regulation of SR45, we fused the SR45 promoter sequence to a beta-glucuronidase (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 (Figure 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 serine 219 (S219) that is predicted by both the NetPhos 2.0 Server (http://www.cbs.dtu.dk/services/NetPhos) (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). In order to assess the potential functional difference between SR45.1 and SR45.2 we introduced a point mutation at this position (S219A). In addition, threonine 218 (T218) was also predicted as a possible phosphorylation site (score = 0.913) by NetPhos 2.0 Server but not PhosphAt. We mutated the threonine to alanine together with serine 219 (T218A+S219A) to see if T218 has any impact on SR45 function in addition to serine 219. 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-terminal of each proteins transiently in *sr45-1* mutant mesophyll protoplasts and detected GFP signal in the nucleus of transfected protoplasts for all the constructs (Figure 3A). Then we generated stable transgenic lines with these constructs in the *sr45-1* mutant background for *in vivo* functional study (Figure 3B and C). In transgenic plants overexpressing *SR45.1-GFP* (*OX1*), the narrow flower petal phenotype in *sr45-1* mutants was rescued (Figure 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 (Figure 3D). Nor was this phenotype rescued by the T218A, S219A double mutant (*OX1TSAA*; Figure 3D). However, a single mutation of S219A (*OX1SA*) behaved like wild-type *OX1* with respect to the flower petal phenotype (Figure 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 (Figure 3E). Neither *OX1* nor *OX1SA* were able to rescue the mutant root growth on MS agar medium (Figure 3E). However, *OX2* and *OX1TSAA* seedlings exhibited normal root growth compared to the mutant and other transgenic seedlings.
(Figure 3E). These results suggest that although both isoforms were expressed in all tissues in the overexpression lines (Figure 3B 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 alternative splicing of other SR Protein Genes**

In several animal species, splicing factors have been identified as alternative splicing 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). 15 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 towards wild-type in transgenic plants that also show phenotypic rescue (those overexpressing SR45.1-GFP or SR45.1-S219A-GFP) but not SR45.1-T218A+S219A-GFP or SR45.2-GFP. The AS pattern of RSP31, RSP31a, SR34 and SRP34b was rescued in all transgenic plants (Figure 4A). In root tissues, the altered isoform5+3/isoform1 ratio of SRp30 seen in sr45-1 was restored towards 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 (Figure 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 alternative splicing have distinct, tissue-specific, biological functions. SR45.1 is required for normal flower 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 post-translational 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; de la Fuente van Bentem et al., 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 OXI-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 threonine 218 is the site of phosphorylation or that serine 219 is normally the site of phosphorylation but can be replaced by threonine 218. Whatever the situation within this region, it is very likely that phosphorylation plays an important role in the function of the serine-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 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 exon junction complex (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 (NMD) (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 alternative splicing regulator that interacts with other splicing factors, U1 snRNP component hLUcA, pinin and UPF complex to trigger NMD (Lykke-Andersen et al., 2001; Sakashita et al., 2004). Phosphorylation at serine 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 (U1-70K) and kinase AFC2 in vitro (Golovkin and Reddy, 1998; Reddy, 2004). In vivo interaction was observed between U1-70K 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 (Figure 4A 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.
Material and Methods

Plant Growth Condition

All Arabidopsis plants used in this study are in Colombia (Col) background. Mutant plants sr45-1 (SALK_004132) were originally from Arabidopsis Biological Resource Center (ABRC) and was previously described by Ali et al. (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: GATTGGAGATCTTCTTGAGG, and LBb1.3: ATTTTGCCGATTTCGGAAC. All plants were either grown in soil with 16/8 hr photoperiod or sowed on MS 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 ºC.

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; NCBI accession NM_101523.3) and isoform 2 (SR45.2; NCBI 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 NcoI_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: GGCGCCCAAGAGAGCATCTCCTCAACGG, 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: SR45pro5NotI: GCGGCCGCATTTCTGCTCCAAAATTCTATAA and SR45pro3BglII: 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

RNasey Mini Kit (Qiagen) was used to isolate total RNA. Five microgram of total RNA from each sample was digested by DNase I (Invitrogen) and applied for reverse transcription 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 (Table S1)

Plant Screening and Morphology
All T1 transgenic plants were screened by 1:1000 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 days. Pictures were taken from all the petals. Width and length of each petal were measured in Image J software. All width-to-length ratios were calculated and plotted using Microsoft Excel.

Pictures were taken of 4-day-old seedlings grown on MS agar medium. Root length was measured by image J and plotted in Microsoft Excel.

*Confocal Imaging*

The SR45-GFP signal in nucleus was examined with Zeiss LSM 510 Confocal Microscope (Leica Microsystems, Germany). The excitation wavelength for GFP was 488 nm, and the emission filter wavelength was 505-530 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 40x oil-immersion objective at 1024x1024 pixel resolution. Each image was scanned 4 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, pH7.0) then submerged in GUS staining solution with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-GlcU A (X-gluc). For inflorescences, trapped air was removed by pulling a vacuum for 5 min followed by releasing vacuum. The process was repeated till 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 till 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.
Supplementary Materials

Table S1. Sequences of the gene specific primers that were used in Real-Time PCRs and RT-PCRs.

Figure S1. Microarray data from Genevestigator showing SR45 gene expression level in different tissues. Data were obtained from the Genevestigator web site (Zimmermann et al., 2004) and summarized. Arabidopsis thaliana was chosen as the target organism. All the 4076 experiments producing high quality arrays on ATH1 were selected for further analysis. The Affymetrix probe corresponding to SR45 is 246324_at. Data from anatomy were viewed and exported as scatterplot.
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Figure Legends

Figure 1. SR45 gene structure and protein sequence.
(A) SR45 gene structure demonstrates two splicing isoforms – SR45.1 and SR45.2. Exons were shown as red boxes; introns were shown as straight lines; UTRs were shown as white boxes. The alternative acceptor site was indicated by arrow and the 21-nucleotide sequence that is present in SR45.1 but missing in SR45.2 was 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 Os05g 0105900), RNPS1 from human (HsRNPS1) and mouse (MmRNPS1). The alignment was done with CLC Sequence Viewer 5. Five SPXR sequences were found in the C-terminus RS/SPXR domain with the first one absent in AtSR45.2.

Figure 2. The expression and splicing pattern of SR45.
(A) RT-PCR results showed the alternative splicing pattern of SR45 from 8-day-old wild-type (Col-0) seedlings in response to different conditions. All seedlings (1-9) were grown on MS plates with 3% sucrose except for the last treatment #10. From 1 to 10: untreated control, 10 μM GA3, 2 μM JA, 1 μM IAA, 100 μM ACC, 50 mM NaCl, 100 μM ABA for 4 hours, 7 °C for 24 hrs, 37 °C for 24 hrs, and without sucrose. 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 was shown as bars (red bar for AS1; blue bar for AS2). The ration of two isoforms (AS1/AS2) was plotted as
a line in contrast to the expression levels. Standard deviation was calculated from the PCR results. (C) SR45 promoter activity was examined in SR45::GUS transgenic wild-type plants by GUS staining at 4-day-old dark-grown seedling stage (primary root tip (a) and cotyledon (d)); primary root tip at 2-day-old seedling stage (b); 4-day-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 anther (f). Three independent lines were used in the staining analysis. Bar = 100 µm for a-g and i-j. Bar = 1 mm for h and k.

Figure 3. Transgenic plants overexpressing different SR45 constructs.

(A) Transient expression of SR45 constructs. Cartoon on the left showed the domain arrangement of SR45.1, SR45.2 and protein substitution in the mutant constructs. On the right, GFP transient expression was observed in sr45-1 mesophyll protoplasts that were transfected with: i: empty vector; ii: SR45.1-GFP; iii: SR45.1-T218A-S219A-GFP; iv: GFP control; v: SR45.2-GFP; vi: SR45.1-S219A-GFP. Bar= 10 µm. (B) RT-PCR results showed 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-day-old transgenic seedling as indicated. Arrowhead showed nucleoli. Bar= 5 µm. (D) Floral phenotype. Upper panel: label number 1 to 6 showed flowers from wild-type (WT), sr45-1, transgenic plants overexpressing SR45.1-GFP (OX1), SR45.1-S219A-GFP (OX1SA), SR45.1-T218A+S219A-GFP (OX1TSAA), and SR45.2-GFP (OX2), respectively. Lower panel: ratio of petal width to length from wild-type, sr45-1 and two independent lines for each transgene in the order as indicated in the
figure. Error bar showed the standard deviation from at least 20 flower petals. (E) Root phenotype. Upper panel: Label number 1-6 showed 4-day-old seedlings in the same order as in (D) upper panel. Lower panel: Root length measured from 4-day-old seedlings from wild-type, *sr45-1* and two independent lines from each transgene in the order as indicated in the figure. Error bar showed the standard deviation from 20 seedlings from one plate. The similar pattern was observed in three independent experiments.

**Figure 4. Alternative splicing pattern of SR protein genes in wild-type, *sr45-1* and different transgenic plants.**

Alternative splicing pattern was examined on *SRP30, RSp31, SRP31a, SRP34* and *SR34b*. Lane 1-10 presented RT-PCR results from plants in the same order as in Figure 3(B). *GAPDH* was used as control. (A) Inflorescence tissues. The splicing pattern of each isoform was shown as cartoon next to each band position. (B) Root tissues. Each band was corresponding to the each splicing isoform. The UTRs were presented by open boxes; the exons were presented by black boxes; the alternatively spliced exons were presented gray boxes, the introns were presented by lines. The position of gene-specific primers was shown by black arrowhead and the stop codons were shown as *.
