Plant Mol Biol (2009) 70:241–252
DOI 10.1007/s11103-009-9469-y

Plant-specific SR-related protein atSR45a interacts with spliceosomal proteins in plant nucleus

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Received: 29 September 2008 / Accepted: 8 February 2009 / Published online: 24 February 2009

Abstract Serine/arginine-rich (SR) protein and its homologues (SR-related proteins) are important regulators of constitutive and/or alternative splicing and other aspects of mRNA metabolism. To clarify the contribution of a plant-specific and stress-responsive SR-related protein, *atSR45a,* to splicing events, here we analyzed the interaction of atSR45a with the other splicing factors by conducting a yeast two-hybrid assay and a bimolecular fluorescence complementation analysis. The atSR45a-1a and -2 proteins, the presumed mature forms produced by alternative splicing of *atSR45a*, interacted with U1-70K and U2AF^{35}b, splicing factors for the initial definition of 5′ and 3′ splice sites, respectively, in the early stage of spliceosome assembly. Both proteins also interacted with themselves, other SR proteins (atSR45 and atSCL28), and PRP38-like protein, a homologue of the splicing factor essential for cleavage of the 5′ splice site. The mapping of deletion mutants of atSR45a proteins revealed that the C-terminal arginine/serine-rich (RS) domain of atSR45a proteins are required for the interaction with U1-70K, U2AF^{35}b, atSR45, atSCL28, PRP38-like protein, and themselves, and the N-terminal RS domain enhances the interaction efficiency. Interestingly, the distinctive N-terminal extension in atSR45a-1a protein, but not atSR45a-2 protein, inhibited the interaction with these splicing factors. These findings suggest that the atSR45a proteins help to form the bridge between 5′ and 3′ splice sites in the spliceosome assembly and the efficiency of spliceosome formation is affected by the expression ratio of atSR45a-1a and atSR45a-2.

Keywords SR-related protein · atSR45a · Spliceosome assembly · *Arabidopsis*

Abbreviations

BiFC	Bimolecular fluorescence complementation
His	Histidine
Leu	Leucine
PCR	Polymerase chain reaction
Py	Polypyrimidine
RRM	RNA-recognition motif
snRNP	Small nuclear ribonucleoprotein particle
RFP	Red fluorescence protein
RS	Arginine/serine-rich
SR	Serine/arginine-rich
Trp	Tryptophan
Ura	Uracil
YFP	Yellow fluorescence protein

Introduction

Pre-mRNA splicing is an essential step in the expression of the vast majority of protein-coding genes in higher eukaryotes. Production of functional mRNAs from pre-mRNAs requires the accurate removal of introns in the nucleus (Sharp 1994). Both the constitutive and alternative splicing of nuclear pre-mRNAs takes place in a large macromolecular complex called the spliceosome, which consists of *cis*-acting elements of pre-mRNA, five small nuclear ribonucleoproteins (snRNPs), U1, U2, U4/6, and U5, and other

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non-snRNP splicing factors as *trans*-acting regulators, including serine/arginine-rich (SR) proteins (Zhou et al. 2002). A fully assembled, catalytically active spliceosome contains at least 300 different proteins and is the most complex cellular machine characterized so far (Rappsilber et al. 2002; Zhou et al. 2002). snRNPs with the assistance of non-snRNPs assemble onto the intron of pre-mRNA in a coordinated manner. In the early spliceosomal complex, U1 and U2 snRNPs are required for the initial definition of 5′ and 3′ splice sites, respectively. The mature spliceosome is formed by the U4/U6-U5 tri-snRNP, which finally leads to the displacement of U1 and U4 snRNPs (Burge et al. 1999; Kramer 1996; Reed 2000). Among non-snRNP splicing factors, the family of SR proteins is required for an accurate spliceosome assembly (Graveley 2000).

In animals, 11 SR proteins have been characterized and classified into several subfamilies, such as *SF2/ASF*, *9G8/SRp20*, *SRp55*, and *SC35*, based on their domain construction (Sanford et al. 2003). SR proteins share a distinctive domain structure, which consists of one or two copies of a RNA-recognition motif (RRM), followed by a characteristic C-terminal arginine/serine-rich (RS) domain (Birney et al. 1993). These domains are primarily responsible for promoting the protein-RNA and protein–protein interactions during spliceosome assembly (Caceres and Krainer 1993; Graveley 2001). In particular, the RS domains were essential for protein–protein interactions of SR proteins with each other and with other components of the splicing machinery (Wu and Maniatis 1993; Kohtz et al. 1994; Zhu and Krainer 2000), and also for nuclear and subnuclear localization and nucleocytoplasmic shuttling (Bourgeois et al. 2004). In humans, the binding of SR proteins to exonic (intronic) splicing enhancers (silencers) in pre-mRNAs resulted in recruitment of U1 snRNP to the 5′ splice site and U2 snRNP to the branch point sequence. Subsequently, the first reaction for spliceosome assembly was mediated by the interaction of SR proteins with U1-70K, the U1 snRNP-specific protein. In animals, three SR proteins (SC35, ASF/SF2, and SRp38) interact with U1-70K (Manley and Tacke 1996; Wu and Maniatis 1993; Shin et al. 2004). It has been shown that the interaction of SR proteins with $U2AF^{35}$, the small subunit of heterodimeric U2AF, stabilizes U2AF itself at the polypyrimidine (Py) tract in pre-mRNAs (Burge et al. 1999; Kramer 1996; Reed 2000; Hastings and Krainer 2001). In addition, SR proteins play an important role in the splicing of minor, AT-AC class introns (Hastings and Krainer 2001).

In higher plants, there are a large number of genes encoding SR protein homologues, some of which are homologues of the metazoan SR protein family, whereas others are unique to plants (Golovkin and Reddy 1999; Lorkovic and Barta 2002; Reddy 2004). Several SR proteins have been shown to interact with other members of the family and with other spliceosomal proteins (Golovkin and Reddy 1998, 1999; Lopato et al. 2002). U1-70K in *Arabidopsis thaliana* interacted with, at least, five SR proteins, namely atSRZ21/RSZ21, atRSZ22, atSR34/SR1, atSR45, and atSR33/SCL33 (Lopato et al. 1999, 2002; Golovkin and Reddy 1998, 1999; Lorkovic et al. 2004; Ali et al. 2008).

We have previously characterized a plant-specific SR-related protein, *atSR45a* (At1g07350), from *Arabidopsis* (Tanabe et al. 2007). Six types of mRNA variants (atSR45a-1a–e and atSR45a-2) were produced by the alternative splicing of *atSR45a*. Among proteins encoded by these mRNA variants, the presumed mature forms, atSR45a-1a and -2, had both RRM and RS domains like the other SR proteins, but are unique in having two RS domains, one at the C-terminus and the other at the N-terminus, separated by a RRM. The other mRNAs (atSR45a-1b–e) encoded truncated proteins lacking the C-terminal RS domain. By the yeast two-hybrid assay, it was shown that the atSR45a-1a and -2 proteins interacted with U1-70K protein, suggesting that the proteins function as a factor involved in splicing events in *Arabidopsis*. Notably, the expression of *atSR45a* was responsive to stressful conditions and thus the atSR45a-1a and atSR45a-2 mRNAs were markedly increased by the induction of transcription and the alteration of the alternative splicing efficiency (Tanabe et al. 2007).

In this paper, to clarify the roles of the atSR45a proteins in spliceosome assembly, we identified factors interacting with atSR45a and the domains required for the interactions with the yeast two-hybrid assay. In vivo interaction of the atSR45a proteins with identified proteins was confirmed by bimolecular fluorescence complementation (BiFC) assay. The present findings suggest that the atSR45a proteins affect the efficiency of pre-mRNA splicing as an essential component for the formation of the spliceosome assembly.

Materials and methods

Materials and plant growth conditions

Arabidopsis thaliana ecotype Columbia was grown under long-day conditions (16 h light, 25°C/8 h dark, 22°C) on Murashige and Skoog's (MS) medium under a light intensity of 100 $\mu E/m^2/s$. Two-week-old seedlings were collected and frozen in liquid nitrogen and stored at −80°C for further preparation.

Restriction enzymes and modifying enzymes were purchased from TaKaRa (Kyoto, Japan). All other chemicals were of analytical grade and used without further purification. Protein concentrations were determined by the method of Bradford (1976).

Yeast two-hybrid assay

A yeast two-hybrid assay was carried out to analyze protein–protein interactions according to the manufacturer's instructions (The Hybrid Hunter™; Invitrogen). The full-length cDNAs encoding atSR45a-1a and atSR45a-2 were cloned into the vector pHybLex (carrying the Zeocin resistance gene), containing the LexA DNA binding domain, for the production of a bait protein. They were amplified using the following primer sets; atSR45a-1a *Eco*RI F (5′-GAA TTCATGGGGAAACGTGA-3′), atSR45a-2 *Eco*RI F (5′-GA ATTCATGTCTTACTCAAG-3′), atSR45a *Sal*I R (5′-GTC GACCTGTTATGCTGA-3′). The cDNAs encoding atSR 45a-1a, atSR45a-2, atSR30, atSR34a, atRS31, atRS31a, atRS40, atRS41, atSC35, atSCL28, atSCL30, atSR33/SCL33, atSRZ21/RSZ21, atRSZ22, atRSZ22a, atRSZ32, atRSZ33, U2AF^{35}a, and U1-70K were also cloned into the vector pYES Trp2 (carrying the *TRP1* gene), containing the B42 activation domain, for the production of a prey protein. They were amplified using the following primer sets; atSR30 *Kpn*I F (5′-GGTACCATGAGTAGCCGATG-3′), atSR30 *Sac*I R (5′-GAGCTCATTCGGGTACAGCC-3′), atSR34a *Kpn*I F (5′-GGTACCATGAGTGGGCGATT-3′), atSR34a *Sac*I R (5′-CTCTCACACACTGCCTT-3′), atRS31 *Xho*I F (5′-CT CGAGATGAGGCCAGTGTT-3′), atRS31 *Sph*I F (5′-GC ATGCTCAAGGTCTTCCTC-3′), atRS31a *Kpn*I F (5′-GGT ACCGTCGTCTCTTCAGA-3′), atRS31a *Sac*I R (5′-GAGC TCTCAACCTCTTGCTC-3′), atRS40 *Sac*I F (5′-GAGCTC AGCATGAAGCCAGT-3′), atRS40 *Xho*I R (5′-CTCGAGT CACTCGTCAGCTG-3′), atRS41 *Xho*I F (5′-CTCGAGGGA ATC ATGAAGCC-3′), atRS41 *Sph*I R (5′-GCATGCCTG CATCTCCAATC-3′), atSR 45a-1a *Kpn*I F (5′-GGTACCAT GGGGAAACGTGAAA-3′), atSR45a-2 *Kpn*I F (5′-GGTA CCATGTCTTACTCAAGAA-3′), atSR45a *Sac*I R (5′-GA GCTCTTATGGGCTGACGGAT-3′), atSC35 *Kpn*I F (5′-G GTACCATGTCGCACTTCGG-3′), atSC35 *Sac*I R (5′-GA GCTCATTCCGCAGCATAA-3′), atSR33/SCL33 *Hin*dIII F (5′-AAGCTTGACTCAATGAGGGG-3′), atSR33/SCL33 *Xho*I R (5′-CTCGAGTGACGAATCAATCA-3′), atSCL30 *Eco*RI F (5′-GAATTCTATGGTAGGTGATG-3′), atSCL30 *Xho*I R (5′-CTCGAGGGCCACTTCTTCAC-3′), atSCL28 *Kpn*I F (5′-GGTACCATGGCTAGAGCGAG-3′), atSCL28 *Sac*I R (5′-GAGCTCCTAAGGAAGTTGCC-3′), atSRZ21/RSZ21 *Kpn*I F (5′-GGTACCAACATGACGAGGGT-3′), atSRZ21/RSZ21 *Sac*I R (5′-GAGCTCCTTCAGGGTCCAG T-3′), atRSZ22 *Kpn*I F (5′-GGTACCAGCTCTTCCCACA A-3′), atRSZ22 *Xho*I R (5′-CTCGAGATCACTCACTCA GC-3′), atRSZ22a *Eco*RI F (5′-GAATTCCAGGTAACTTG AGG-3′), atRSZ22a *Sph*I R (5′-GCATGCTAACACTCAGC TCC-3′), atRSZ32 *Kpn*I F (5′-GGTACCGTACGAGATGT GGA-3′), atRSZ32 *Xho*I R (5′-CTCGAGTCAAGGTGACT CAC-3′), atRSZ33 *Eco*RI F (5′-GAATTCTGGATATGA AGCGA-3′), atRSZ33 *Xho*I R (5′-CTCGAGGCAAGCCGT GGCCA-3′), U2AF^{35}a *Sac*I F (5′-GAGCTCATGGCGGAG CATTT-3′), U2AF^{35}a *Xho*I R (5′-CTCGAGTTATGCTCCT CCCT-3′), U1-70K *Kpn*I F (5′-GGTACCATGGGAGACTC CGG-3′), U1-70K *Sac*I R (5′-GAGCTCTCAACGAACAT ACT-3′).

Interaction between bait and prey proteins was analyzed by introducing appropriate plasmids into the yeast strain *L40* (*MATa his Δ200trp1-901 leu2-3112 ade2 LYS2::(4lexAop-HIS3) URA3::(8lex Aop-lacZ) LexA*; Hollenberg et al. 1996). The transformed yeast cells bearing both plasmids for the production of bait and prey proteins were selected using the YC medium (in 150 mm plates) containing 300 μg/ml of Zeocin and lacking Tryptophan (Trp), Uracil (Ura), and Histidine (His), at 30°C for 3 days. Simultaneously, the transformed yeast cells were grown on YC medium containing 300 μg/ml of Zeocin and lacking Trp and Ura, at 30°C for 3 days, and then used for a colony-lift filter assay to check the activity of β-galactosidase produced from the reporter gene. Briefly, fresh colonies grown to about 1–3 mm in diameter were transferred completely to a sterile filter and submerged in a pool of liquid nitrogen for 10 s and thawed at room temperature, then placed on a pre-soaked filter in 1.5 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCL, 1 mM $MgSO_4 \cdot 7H_2O$, pH7.0) containing 30 μl of 50 mg/ml 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The filters were incubated at 30°C, and the colors of colonies were checked periodically. A liquid assay to quantify β-galactosidase activity was performed by growing the transformants to a mid-exponential phase in the appropriate selection medium, lacking Trp and Ura, using *o*-nitrophenylgalactoside (ONPG). ONPG was used as the substrate of β-galactosidase for the liquid culture assay. In brief, at least three independent clones were selected, grown, harvested, centrifuged, resuspended in Z buffer, frozen in liquid nitrogen, and thawed at 37°C in a water bath. Then the reaction systems (ONPG + Z buffer + β-mercaptoethanol + yeast cells resuspension) were placed in a 30°C incubator. After the yellow color developed Na_2CO_3 1 mol/l was added to the reaction and blank tubes. Relapsed time was recorded in minutes. Reaction tubes were centrifuged at 17,800*g* for 10 min and supernatants were carefully transferred to clean cuvettes and OD_{420} of the samples relative to the blank was recorded. At last, the β-galactosidase units were calculated as: β-galactosidase units $= 1000 \times OD_{420}/(t \times V \times OD_{600})$ where t is elapsed time (in min) of incubation, $V = 0.1$ ml $\times$ concentration factor (the concentration factor is 5), OD_{600} is Optical density at 600 nm of 1 ml of culture.

To screen for proteins interacting with the atSR45a proteins from the *Arabidopsis* cDNA library, the full-length atSR45a-2 protein was expressed as a construct fused to the Gal4 DNA binding domain in a pGBKT7

vector for the production of bait protein (Clontech). The construct, designated pGBKT7/atSR45a-2, was generated using primers 5′-GAATTCGAATTCATGTCTTACT-3′ and 5′-GTCGACTTATGGGCTGACGGAT-3′, containing an *Eco*RI and a *Sal*I restriction site, respectively. Total RNA was isolated from the seedlings of 2-week-old *Arabidopsis* plants as previously described (Yoshimura et al. 1999). Poly(A)$^+$ RNA was then obtained with poly A tract mRNA isolation system IV (Promega), and used for the preparation of cDNA libraries with MATCHMAKER Two-Hybrid System 3 (Clontech). An *Arabidopsis* cDNA library was prepared as a construct fused to the transcription activation domain of the yeast transcription factor Gal4 in the vector pGADT7-Rec. The yeast strain *AH109* (James et al. 1996) was transformed with pGBKT7/atSR45a-2 and with the cDNA library through the LiAc transformation method and selected with SD medium lacking Trp, Leusine (Leu), and His. Colonies that grew on the selection medium were further assayed for β-galactosidase activity (Schneider et al. 1996). DNA from positives colonies was amplified by PCR with the pGADT7-Rec 5′ and 3′ primers. The PCR product was sequenced (ABI PRISM^{TM} 3100, Applied Biosystems). To verify the interaction, pGADT7-Rec plasmids bearing full-length cDNA of respective splicing factors isolated in the screening were introduced back into strain *AH109* with pGBKT7/atSR45a-2 and tested for β-galactosidase activity. Recombinant hybrid proteins were tested for self-activation and nonspecific protein-binding properties.

Domain mapping experiments with the yeast two-hybrid assay

To identify the domain(s) of atSR45a proteins involved in the interaction with U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, and atSR45a itself, pHybLex vectors containing each of the atSR45a domain(s), the N-terminal RS domain (RS1), RS1 and RRM (RS1 + RRM), RRM, RRM and the C-terminal RS domain (RRM + RS2), or the C-terminal RS domain (RS2) were prepared. Each of the atSR45a segments was amplified using the following primer sets; atSR45a-1a *Eco*RI F (5′-GAATTCATGGGGAAACGTGA-3′), atSR45a *Sal*I R (5′-GTCGACCTGTTATGGGCTGA-3′), atSR45a RRM *Kpn*I F (5′-GGTACCAGTTTATATGTAACTG-3′), atSR45a RRM *Sac*I R (5′-GAGCTCCTCAACAGTGATGACG-3′), and atSR45a SR2 *Kpn*I F (5′-GGTACCAAGGTAAAAA GACTAA-3′). pHybLex and pYESTrp2 bearing each of the proteins were introduced into the yeast strain *L40*. The transformants were then grown on the selection plates and assayed for β-galactosidase activity, as described above.

Data analysis

The significance of differences between data sets was evaluated by *t*-test. Calculations were carried out with Microsoft Excel software.

Bimolecular fluorescence complementation analysis

The cDNA fragments of atSR45a-2 were subcloned into the *Sal*I/*Bam*HI sites of the vector pSY736 containing the N-terminal fragment of YFP (YN) and pSY735 containing the C-terminal fragment of YFP (YC), and also into the *Sal*I/*Not*I sites of pSY728 containing YN and pSY738 containing YC (Bracha-Drori et al. 2004). The cDNAs of U1-70K, U2AF^{35}b, and PRP38-like protein were subcloned into the *Sal*I/*Spe*I sites of pSY736 and pSY735, and also subcloned into the *Sal*I/*Not*I sites of pSY728 and pSY738. The cDNA of atSR45 was subcloned into the *Nde*I/*Bam*HI sites of pSY736 and pSY735, or the *Not*I/*Sal*I sites of pSY728 and pSY738. The cDNA of atSCL28 was subcloned into the *Nde*I/*Bam*HI sites of pSY736 and pSY735, and into the *Not*I/*Nco*I sites of pSY728 and pSY738. These constructs were amplified by PCR using the following primer sets: atSR45a-2 NterY *Not*I R (5′-GCGGCCGCGGGCTGAC GGAT-3′), atSR45a-2 CterY *Sal*I F (5′-GTCGACGATGT CTTACTCAAGA-3′), atSR45a NterY *Bam*HI R (5′-GG ATCCTTATGGGCTGACGG-3′), atSR45a NterY *Not*I R (5′-CGCCGGCGCCCGTGACGGAT-3′), atSCL28 NterY *Nde*I F (5′-CATATGCGATGGCTAGAGCG-3′), atSCL28 CterY *Nco*I F (5′-CCATGGCGATGGCTAGAGCG-3′), atSCL28 NterY *Bam*HI R (5′-GGATCCGCCATTCCTTC TTC-3′), atSCL28 CterY *Not*I R (5′-GCGGCCGCCGACT TAAGGATCG-3′), U1-70K NterY *Sal*I F (5′-GTCGAC CATGGGAGACTCCG-3′), U1-70K CterY *Not*I R (5′-GC GGCCGCCGAACATACTCTCG-3′), atSR45 CterYN *Sal*I F (5′-CGGTCGACCATGGCGAAACCAAGTCG-3′), at SR45 CterN *Bam*HI R (5′-CCGGGATCCTTAAGTTTTA CGAGGTGG-3′), atSR45 NterY *Not*I R (5′-GCGGCC GCCGAGGTGGAGGTGGT-3′), U2AF^{35}b NCterY *Sal*I F(5′-GTCGACAATGGCAGAGCATT-3′), U2AF^{35}b NterY *Spe*I R (5′-ACTAGTTTAAACTCCCTCAT-3′), U2AF^{35}b CterY *Not*I R (5′-GCGGCCGCTAAACTCCCTCA-3′), PRP38-like NCterY *Sal*I F (5′-GTCGACAATGGCAAAC AGAA-3′), PRP38-like NterY *Spe*I R (5′-ACTAGTTCAG TCCCTGAGGG-3′), PRP38-like CterY *Not*I R (5′-GCGG CCGCCAGTCCCTGAGG-3′). To clone the full-length U1-70K into the plant expression vector pUGW2, the coding regions were PCR amplified U1-70K NterY *Sal*I F (5′-GT CGACCATGGGAGACTCCG-3′), U1-70K-pUGW2 *Kpn*I R (5′-GGTACCCAACGAACATACTC-3′), and expressed as a fusion protein with a red fluorescence protein (RFP).

The resulting 28 plasmids were co-bombarded under various combinations with gold particles (Bio-Rad) into onion epidermal cell layers as described (Yap et al. 2005). After incubation at 25°C for 48 h in darkness, epidermal cell layers were viewed under a microscope (Olympus FV1000) equipped with a fluorescence module.

Results

Interaction of atSR45a with splicing factors

Among six types of alternatively spliced mRNA variants (atSR45a-1a–e and atSR45a-2) of *atSR45a*, the atSR45a-1a and atSR45a-2 mRNAs encoded the domains of a plant-specific SR-related protein and were most abundant in various tissues under normal and stressful conditions (Tanabe et al. 2007). Therefore, atSR45a-1a and atSR45a-2 seem to be mature and functional products of *atSR45a*. To determine whether the atSR45a-1a and atSR45a-2 proteins serve as a component of the spliceosome assembly, the interaction of proteins with splicing factors including the other SR proteins in *Arabidopsis* was analyzed by yeast two-hybrid assay (The Hybrid Hunter^{TM}; Invitrogen) (Fig. 1a, b). Yeast cells expressing atSR45a-1a or atSR45a-2 as a bait protein together with U1-70K, atSCL28, atSR45a-1a, or atSR45a-2, as a prey protein, were capable of growth on the selection plate lacking Trp, His, and Ura (data not shown). β-galactosidase activity was detected in the extracts prepared from the cells expressing atSR45a-1a or atSR45a-2 together with U1-70K, atSCL28, or the atSR45a proteins (Fig. 1a, b). The activity in cells expressing the atSR45a-2 protein together with the U1-70K, atSCL28, atSR45a-1a, or atSR45a-2 protein was greater than that in cells expressing the atSR45a-1a protein (Fig. 1b). Cells expressing atSR45a-1a or atSR45a-2 together with the other splicing factors (atSR30, atSR34a, atSR31, atSR31a, atSR40, atSR41, atSC35, atSCL30, SCL33, atRSZ21, atRSZ22, atRSZ22a, atRSZ32, atRSZ33 and U2AF^{35}a) showed very low activity (Fig. 1b). As a control experiment, neither the bait protein nor the prey protein alone activated the expression of the reporter gene (data not shown).

Screening for proteins interacting with atSR45a from Arabidopsis cDNA library

To identify novel proteins interacting with the atSR45a proteins, yeast two-hybrid screening (Clontech) was carried out using atSR45a-2 as bait and the proteins encoded by the *Arabidopsis* cDNA library as prey. The cDNA library was prepared from 2-week-old *Arabidopsis* seedlings and subcloned into the vector pGADT7-Rec for the expression of proteins fused with the Gal4 activation domain. Approximately 10^6 yeast transformants from 46 independent experiments were plated on selection medium lacking Trp, Leu, and His. Transformants grown on the plates were then screened for β-galactosidase activity in a colony-lift filter assay. Sequencing identified clones encoding four different proteins with sequence homology to pre-mRNA splicing factors known in animals, yeast, or plants. They were atSR45a-2 itself, atSR45, U2AF^{35}b as a 3′-splice site recognition factor, and PRP38-like protein as a homologue of the splicing factor in *S. cerevisiae* (Table 1). Transformants expressing full-length cDNA encoding each positive clone as a prey protein grew on the selection medium, lacking Trp, Leu, and His, and showed β-galactosidase activity only in the presence of the bait protein, atSR45a-2 (Fig. 2a, b).

Mapping of domains in atSR45a involved in the interaction with splicing factors

We carried out a yeast two-hybrid assay to determine the domain required for the interaction of the atSR45a proteins with U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, and themselves (Fig. 3). Judging from the β-galactosidase activity in the yeast two-hybrid assay (Fig. 3b), the segments containing the C-terminal RS domain (RRM + RS2 and RS2) common to both atSR45a-1a and atSR45a-2 effectively interacted with U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, or themselves. The segments lacking the C-terminal RS domain (RS1, RS1 + RRM, and RRM) showed weak interaction. As compared to the respective segments of the proteins, the full-length version of atSR45a proteins interacted strongly with U1-70K and atSR45a-1a, while the C-terminal RS domain (RS2) of atSR45a proteins interacted with U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, and atSR45a-2, which was efficiency similar to that of the full-length atSR45a-1a (Fig. 3c). The interaction of the full-length atSR45a-2 protein with U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, atSR45a-1a, and atSR45a-2 was much stronger than those of the segments (RS1, RS1 + RRM, RRM, and RRM + RS2) or the full-length atSR45a-1a protein (Fig. 3c).

In vivo interaction of atSR45a with splicing factors

In vivo interactions between the atSR45a proteins and the splicing factors identified by the yeast two-hybrid assay were directly examined in a BiFC analysis, in which the active YFP was reconstituted only when the non-fluorescent N-terminal (YN) and C-terminal (YC) YFP fragments were brought together by the protein–protein interactions (Bracha-Drori et al. 2004). We determined the optimal

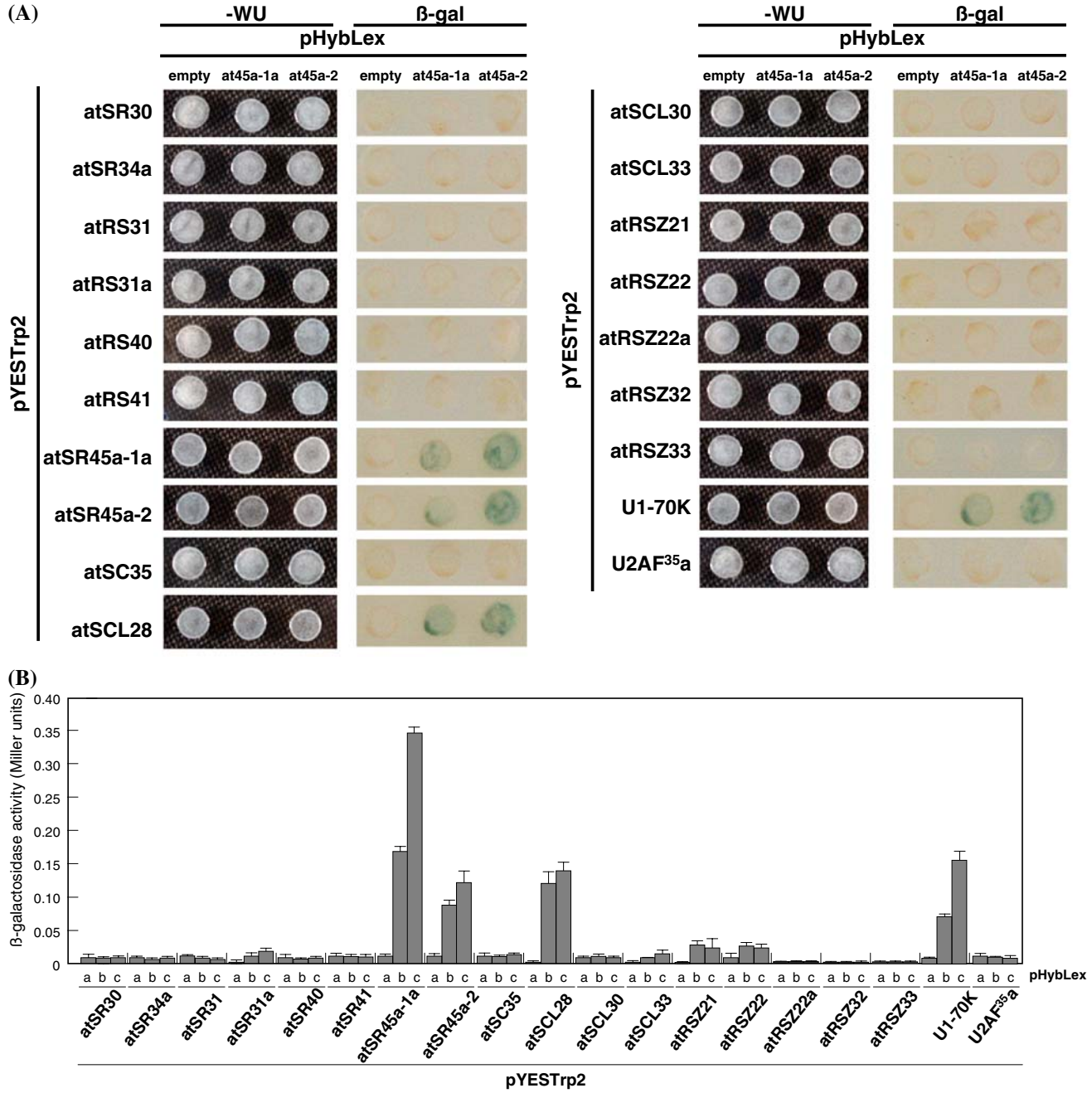


Fig. 1 Interaction of the atSR45 proteins with splicing factors. **a** Analysis of interactions of the atSR45 proteins with various splicing factors by the yeast two-hybrid assay. Yeast cells were co-transformed with the prey (pYESTrp2/atSR30, pYESTrp2/atSR34a, pYESTrp2/atRS31, pYESTrp2/atRS31a, pYESTrp2/atRS40, pYESTrp2/atRS41, pYESTrp2/atSR45a-1a, pYESTrp2/atSR45a-2, pYESTrp2/atSC35, pYESTrp2/atSCL28, pYESTrp2/atSCL30, pYESTrp2/SCL33, pYESTrp2/RSZ21, pYESTrp2/atRSZ22, pYESTrp2/atRSZ22a, pYESTrp2/atRSZ32, pYESTrp2/atRSZ33, pYESTrp2/U1-70K, or pYESTrp2/U2AF^{35}a) and bait (pHybLex, pHybLex/atSR45a-1a, or pHybLex/atSR45a-2) plasmids. The cells obtained were grown on the selective medium without Trp and Ura (-WU) at 30°C for 3 days. The β-galactosidase activity in the cells was determined by the colony-lift filter assay (β-gal). **b** Strength of interactions of atSR45a proteins with the splicing factors. The β-galactosidase activity in each transformant was monitored by the liquid ONPG assay and shown in Miller units. (**a**) empty; (**b**) atSR45a-1a; (**c**) atSR45a-2. Detailed procedures are described in the Materials and Methods section. Data are mean values ± SD for three individual experiments ($n = 3$)

conditions for various combinations of vectors and inserts by transient co-expression using onion epidermal cell layers through particle bombardment. Eight combinations such as atSR45a-2 fused with the C-terminus of YN fragment (YN-atSR45a-2) or atU2AF^{35}b fused with the C-terminus of YC (YC-atU2AF^{35}b) and vice versa were

Table 1 Functional information for clones identified by yeast two-hybrid screening

Gene	Name	Function	Localization	References
At1g07350	atSR45a-2	Spliceosome formation	Nucleus	Tanabe et al. (2007)
At1g16610	atSR45	Spliceosome formation	Nuclear speckles	Ali et al. (2003, 2008)
At5g42820	U2AF^{35}b	Recognition of the 3′-splice site	Nuclear speckles	Domon et al. (1998) Wang and Brendel (2006)
At2g40650	PRP38-like protein	Unwinding of the U4/U6 snRNA	Spliceosome	Blanton et al. (1992)

used. Cells co-expressing YN-U1-70K and atSR45a-2-YC clearly showed YFP fluorescence in the nucleus (Fig. 4A–C). The same results were obtained with other combinations, U2AF^{35}b-YN and atSR45a-2-YC (Fig. 4D–F), atSR45a-2-YN and YC-PRP38-like protein (Fig. 4G–I), YN-atSR45 and atSR45a-2-YC (Fig. 4J–L), atSCL28-YN and atSR45a-2-YC (Fig. 4M–O), and YN-atSR45a-2 and YC-atSR45a-2 (Fig. 4P–R). The YFP fluorescence was observed throughout the cells expressing YN-CaMXMT1 and YC-CaMWMT1 as a positive control (Kodama et al. 2007) (Fig. 4S). Nuclear localization of the interactions was confirmed by the co-expression of U1-70K fused with RFP as described previously (Golovkin and Reddy 1999) (Fig. 4B, E, H, K, N, Q). These findings clearly indicate that atSR45a-2 interacts with these splicing factors in the nucleus in vivo.

Discussion

Constitution of spliceosomal assembly involving atSR45a

Previously, we have reported that, among the proteins produced by alternative splicing of *atSR45a*, atSR45a-1a and -2 are the presumed mature forms, are distributed in the nucleus, and interact with U1-70K which is an important component of U1snRNP required for the initial definition of 5′ splice sites of introns in pre-mRNAs, suggesting that the atSR45a-1a and -2 proteins are involved in the pre-mRNA splicing events (Tanabe et al. 2007).

It has been thought that the complex networks of plant SR proteins are closely associated with the regulation of splicing efficiency (Reddy 2004). Here, we showed that atSR45a proteins, plant-specific SR-related proteins, interact with various types of splicing factors, including themselves. To gain further insight into how the atSR45a proteins function in the pre-mRNA splicing events, here we identified the other splicing factors interacting with the atSR45a proteins by the yeast two-hybrid assay and BiFC analysis. A schematic representation of the interaction between the atSR45a proteins and the other splicing factors in the spliceosome assembly is shown in Fig. 5. The atSR45a proteins interacted with U2AF^{35}b as well as U1-70K in the nucleus (Figs. 1, 2, 4). In metazoans, the recruitment of U1snRNP to the 5′ splice site is facilitated by members of the SR protein family, such as SC35 and ASF/SF2 (Kohtz et al. 1994; Wu and Maniatis 1993). In plants, several types of SR protein, such as atRSZ21/RSZ21, atRSZ22, atSR34/SR1, atSR45, and atSR33/SCL33, have interacted with U1-70K (Lopato et al. 1999, 2002; Golovkin and Reddy 1998, 1999; Lorkovic et al. 2004; Ali et al. 2008). These findings suggest that the selection of 5′ splice site in plants is different from that in animals. U2AF^{35} is a subunit of U2AF comprising U2snRNP required for the initial definition of 3′ splice sites of introns in pre-mRNAs, (Zamore and Green 1989; Zamore et al. 1992; Merendino et al. 1999; Wu et al. 1999; Zorio and Blumenthal 1999). In mammals, U2AF^{35} functions in the promotion of binding of U2AF^{65}, the other subunit for U2snRNP, to the Py tract in pre-mRNAs by interacting simultaneously with the U2AF^{65} and SR proteins (Zuo and Maniatis 1996). In *Arabidopsis* plants, atU2AF^{35}a and atU2AF^{35}b are thought to be the metazoan counterparts (Wang and Brendel 2006). So far, no SR protein interacting with the atU2AF^{35} proteins has been identified. Our results suggest that the atSR45a proteins function in the assembling of spliceosomal components at the 5′ and 3′ splicing sites through binding with U1-70K and U2AF^{35}b, respectively, at the early stage of spliceosome assembly and in the bridging of these components (early spliceosomal complex in Fig. 5).

Furthermore, the atSR45a proteins interacted with the PRP38-like protein in the nucleus (Figs. 2, 4). In *S. cerevisiae*, Prp38 was shown to be necessary for dissociation of U4/U6 intermolecular helices, an essential maturation step that occurred prior to the cleavage of the 5′ splice site of the first exon of pre-mRNA (Blanton et al. 1992). Recently, it has been reported that a wheat (*Triticum aestivum*) SR protein, TaRSZ38, interacted with TaPrp38 (Lopato et al. 2006). These findings suggest that the atSR45a proteins remain in the spliceosome assembly at least until the end of cleavage of the first exon (mature spliceosomal complex in Fig. 5).

It has been reported that several SR proteins in plants interact with other SR proteins. atSR33/SCL33 interacted with atSR45 and atSR33/SCL33 itself (Golovkin and Reddy

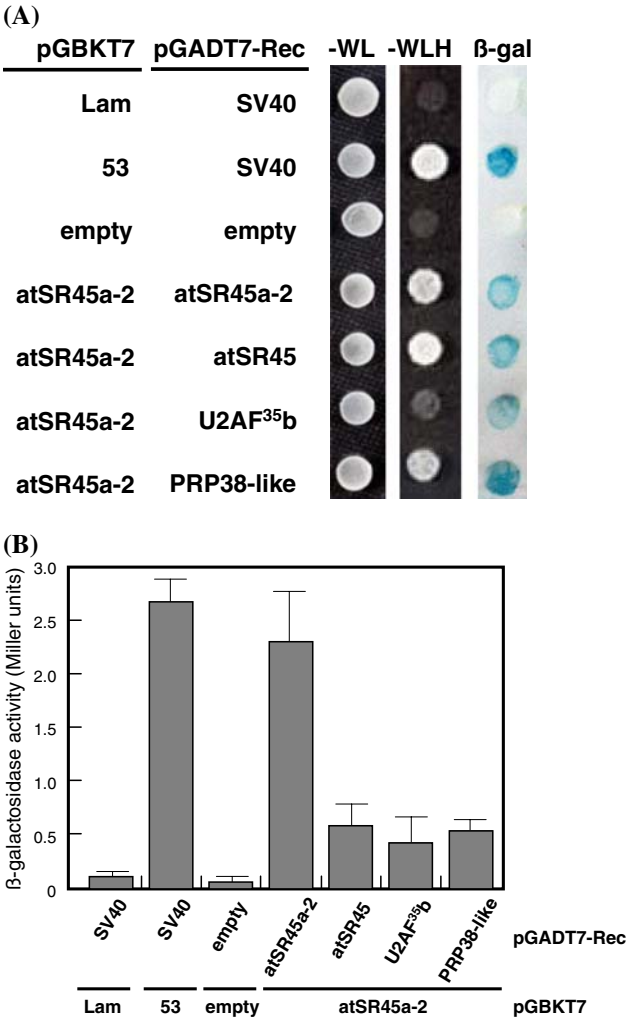


Fig. 2 Interaction of the atSR45a proteins with splicing factors identified by yeast two-hybrid screening. **a** Reconstitution of two-hybrid interactions found in yeast two-hybrid screening. Yeast cells were co-transformed with prey plasmids (pGADT7-Rec/atSR45a-2, pGADT7-Rec/atSR45, pGADT7-Rec/U2AF^{35}b, or pGADT7-Rec/PRP38-like protein) bearing full-length cDNA of the splicing factors isolated in the yeast two-hybrid screening and bait plasmid (pGBKT7/atSR45a-2). The cells were grown on the selective medium without Trp, and Leu (-WL) or without Trp, Leu, and His (-WLH) at 30°C for 3 days. The β-galactosidase activity in the cells was determined as described in Fig. 1. Constructs pGBKT7/53 and pGBADT7-Rec/SV40 were used as a pair of positive controls, while pGBKT7/Lam and pGBADT7-Rec/SV40 were used as a pair of negative controls. **b** Strength of the interactions of atSR45a-2 protein with the splicing factors. The β-galactosidase activity in the extracts prepared form yeast cells was determined as described in Fig. 1. Detailed procedures are described in the Materials and Methods section. Data are mean values ± SD for three individual experiments ($n = 3$)

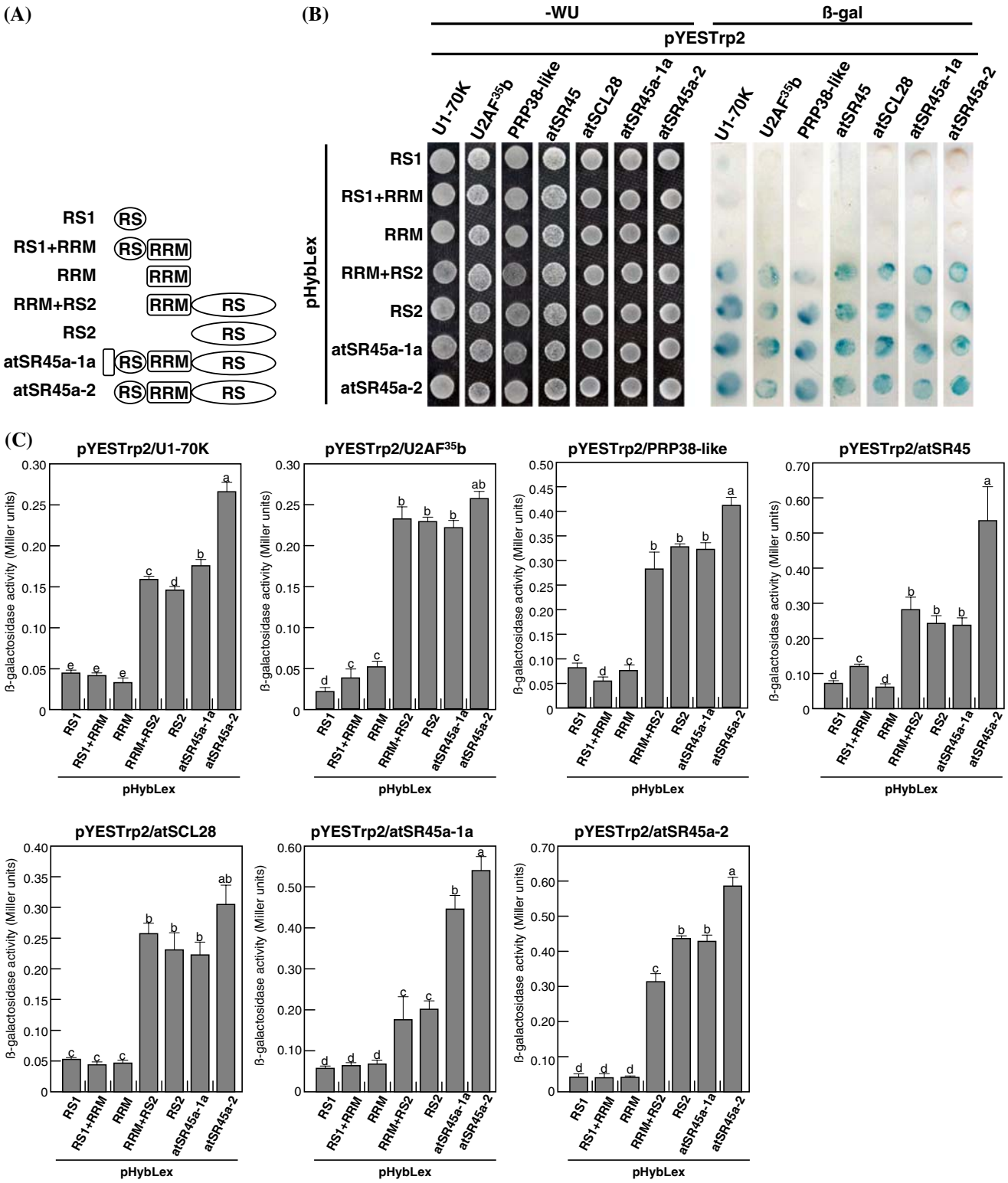
1999), while atRSZ33 interacted with atSR34/SR1, atSRZ21/RSZ21, atRSZ22, atSCL28, atSCL30, and atSR33/SCL33 (Lopato et al. 2002). In addition, five SCL-type SR proteins, atSC35, atSCL28, atSCL30, atSCL30a, and atSR33/SCL33, can form homo/heterodimers with various SR proteins (Lopato et al. 2002). We demonstrated that the

Fig. 3 Mapping of domains in the atSR45a proteins involved in the interaction with splicing factors. **a** Schematic diagram of the domains encoded by segments of atSR45a cDNAs (RS1, RRM, RS2, RS1 + RRM, RRM + RS2), and full-length atSR45a-1a and atSR45a-2 cDNAs. **b** Analysis of interactions of the segments or full-length versions of atSR45a proteins with U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, atSR45a-1a, and atSR45a-2 by the yeast two-hybrid assay. Yeast cells were co-transformed with pHybLex bearing cDNAs for the segments or full-length version of atSR45a and pYESTrp2 bearing cDNAs encoding U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, atSR45a-1a, and atSR45a-2. The cells were grown on the selective medium without Trp and Ura (-WU) at 30°C for 3 days. β-galactosidase activity in the cells was determined as described in Fig. 1. **c** Strength of the interactions of the segments or full-length versions of atSR45a proteins with the U1-70K, U2AF^{35}b, PRP38 like protein, atSR45,a tSCL28, atSR45a-1a, and atSR45a-2. β-galactosidase activity of the extract prepared from yeast cells was determined as described in Fig. 1. Detailed procedures are described in the Materials and Methods section. Data are mean values ± SD for three individual experiments ($n = 3$). Values without a common letter are significantly different according to t-test ($P < 0.05$)

atSR45a proteins interacted with not only themselves but also the other *Arabidopsis* SR proteins, atSCL28 and atSR45, in the nucleus (Figs. 1, 2, 4, 5). Therefore, the atSR45a proteins might form homo/heterodimers as the other SR proteins do. It has been reported that atSCL28 exists in the vicinity of 5′ splice sites by interacting with atSCL33/SR33, atSCL30 and CypRS92 (Lopato et al. 2002; Lorkovic et al. 2004) (Fig. 5). This supports the function of atSR45a proteins at the 5′ splice site as described above. In addition, the homo/heterodimerization of atSR45a proteins are closely related to the bridging of the spliceosomal components of the 5′ and 3′ splice sites through their binding with U1-70K and U2AF^{35}b.

Domains in atSR45a involved in protein–protein interaction

Both atSR45a-1a and atSR45a-2 have one RRM and two distinct RS domains, one each in the N- and C-terminus (Tanabe et al. 2007). The RS domains of various proteins have been primarily implicated in protein-protein interaction (Blencowe and Lamond 1999; Valcarcel and Green 1996). The RS domain of SR proteins participated in both protein-RNA and protein-protein interactions (Zhu and Krainer 2000). In metazoans, the RS domain of SR proteins interacted with U1snRNP and pre-mRNA simultaneously and, hence, promoted the identification of the 5′ splice site (Graveley 2000; Reddy 2004). It has been assumed that, due to RS-repeated structures within the domain, protein binding occurs in several regions within this domain (Lopato et al. 2002). On the other hand, the RS domain of atRSZ33 was not sufficient for the interaction with atSR33/SCL33 and the interaction required the presence of the zinc knuckle domain and a part of the RRM of atRSZ33. The domain



mapping experiments showed that all of the segments lacking the C-terminal RS domain of atSR45a proteins failed to interact with U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, atSR45a-1a, and atSR45a-2 (Fig. 3). In addition, the binding efficiency of the C-terminal RS domain of atSR45a proteins to U2AF^{35}b, PRP38-like protein, atSCL28, atSR45, and atSR45a-2 was similar to that of the full-length atSR45a-1a (Fig. 3c). Among six types of atSR45a variants, the atSR45a-1b–e proteins lack the C-terminal RS domain (Tanabe et al. 2007). SR proteins are

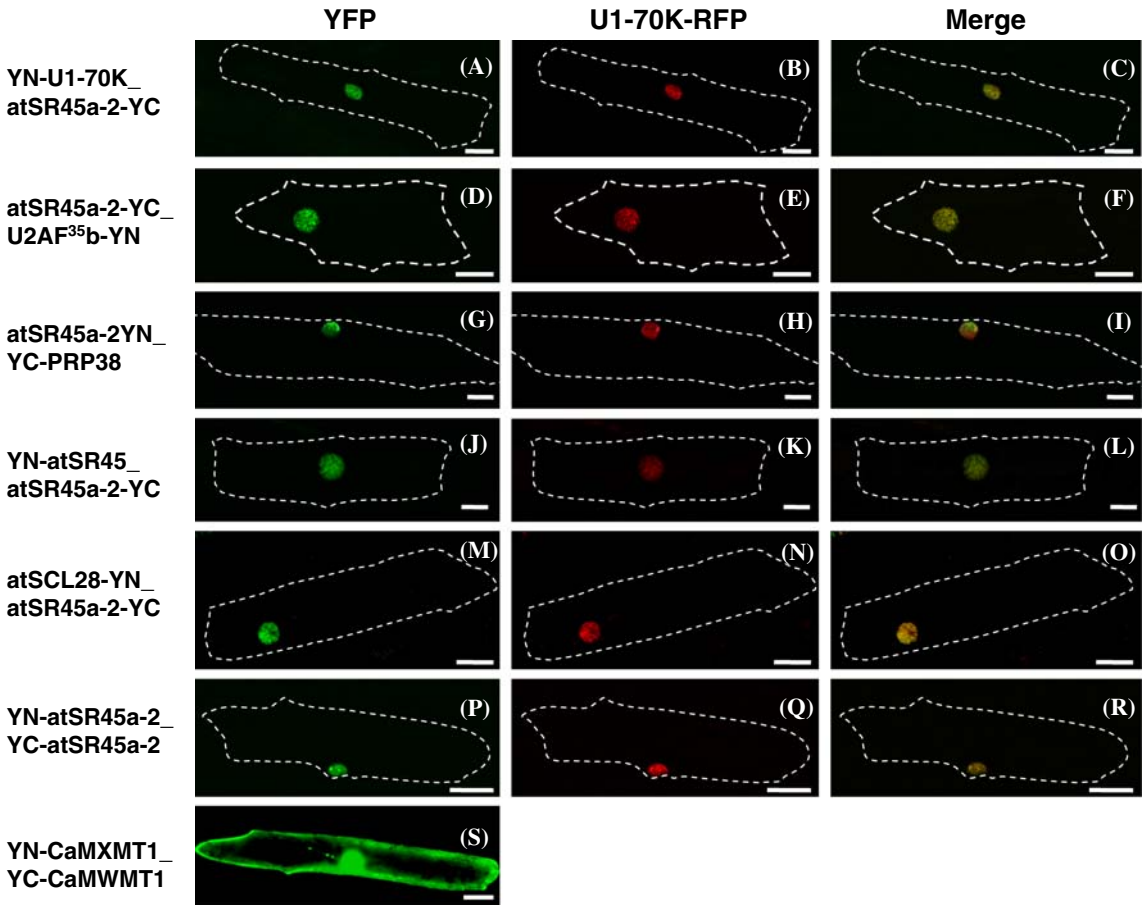


Fig. 4 In vivo BiFC analysis of interaction of the atSR45a-2 protein with splicing factors. The plasmids bearing atSR45a-2, U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, or atSCL28 fused with the N-terminal (YN) or C-terminal (YC) YFP fragments were co-expressed transiently in various combinations into onion epidermal cell layers. The broken line delineates the cell. The nucleus was detected by the co-expression of U1-70K fused with RFP (U1-70K-RFP). YFP and U1-70K images are merged (Merge). Confocal images of the cells co-expressing YN-U1-70K, atSR45a-2-YC, and U1-70K-RFP (**A**, **B**, and **C**), U2AF^{35}b-YN, atSR45a-2-YC, and U1-70K-RFP (**D**, **E**, and **F**), YC-PRP38-like protein, atSR45a-2-YC, and U1-70K-RFP (**G**, **H**, and **I**), YN-atSR45, atSR45a-2-YC, and U1-70K-RFP (**J**, **K**, and **L**), atSCL28-YN, atSR45a-2-YC, and U1-70K-RFP (**M**, **N**, and **O**), YN-atSR45a-2, YC-atSR45a-2, and U1-70K-RFP (**P**, **Q**, and **R**), and YN-CaMXMT1 and YC-CaMWMT1 as a positive control (**S**) are shown. Yellow fluorescent indicates interaction with corresponding fusion proteins. Scale bars are 50 μm. Detailed procedures are described in the Materials and Methods section

phosphoproteins characterized by the presence of a RS dipeptide that serves as a substrate for phosphorylation (Golovkin and Reddy 1999; Misteli et al. 1998). It has been demonstrated that atSR45a is a phospohorylation substrate of activated mitogen-activated protein kinase 3 (MPK3) in vitro and the phosphorylation sites in the C-terminal RS domain (Feilner et al. 2005; Bentem et al. 2006). Although more detailed studies are required for an understanding of the importance of the phosphorylation of the C-terminal RS domain in atSR45a, it seems unlikely that the truncated atSR45a proteins (atSR45a-1b–e) are functional for the spliceosome assembly.

The C-terminal RS domain of atSR45a proteins was not sufficient for the binding of U1-70K and atSR45a-1a. The full-length version of atSR45a proteins was necessary for strong interaction with U1-70K and atSR45a-1a (Fig. 3b, c), suggesting that the N-terminal RS domain is required for the efficient interaction. Interestingly, the full-length version of atSR45a-2 was more effectively bound to U1-70K, U2AF^{35}b, PRP38-like protein, atSR45, atSCL28, atSR45a-1a, and atSR45a-2 than the full-length atSR45a-1a and the segments of atSR45a proteins. As to the structural difference between the atSR45a-1a and atSR45a-2 proteins, there is an N-terminal extension sequence in atSR45a-1a, but not in atSR45a-2 (Tanabe et al. 2007). Therefore, it is possible that the N-terminal RS domain common to atSR45a-1a and atSR45a-2 proteins functions as an enhancer for the binding and the N-terminal extension in the atSR45a-1a protein inhibits the action of N-terminal RS domain. Consequently, the alternative splicing of *atSR45a* may contribute to modulate the efficiency of spliceosome assembly through regulation of the expression ratio of atSR45a-1a and atSR45a-2.

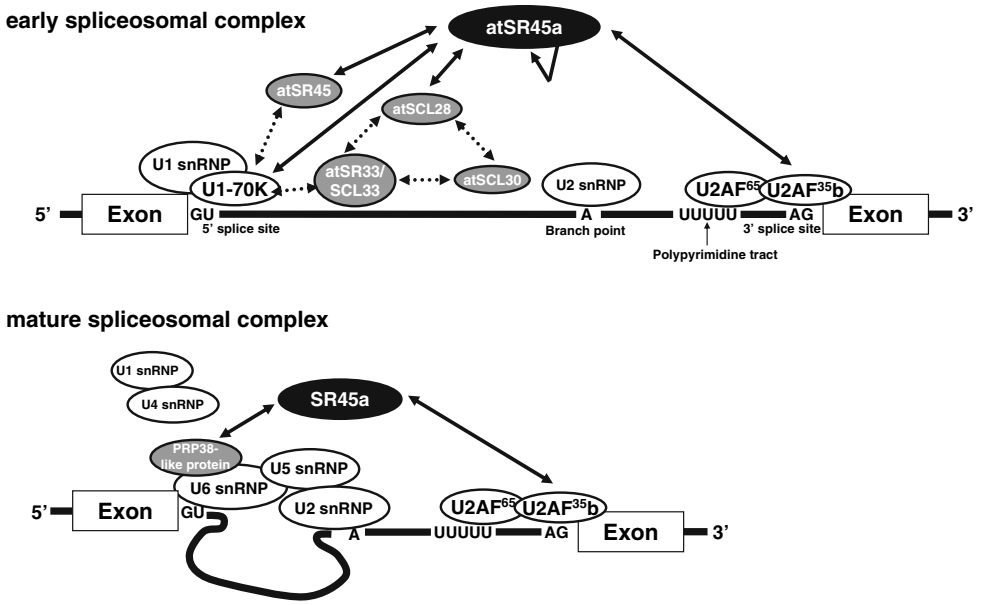


Fig. 5 Schematic representation of interaction of the atSR45a proteins (*black circle*) with snRNPs (*white circle*) and the other splicing factors (*gray circle*) in the spliceosome assembly at the process of pre-mRNA splicing. The exons are shown as boxes and the introns as lines. Double-headed arrows indicate the protein-protein interactions obtained from the present study. An arrow turning back on itself indicates the interaction of atSR45a with itself. The binding of snRNPs, PRP38-like protein, atSCL28, atSCL30, and atSR33/SCL33 to the other proteins and respective regions of pre-mRNA molecule based on the data reported previously (Lopato et al. 2002, 2006; Wang and Brendel 2006; Ali et al. 2008) is indicated by dotted line arrows

Acknowledgment This work was supported by Scientific Research for Plant Graduate Students from the Nara Institute of Science and Technology (N. T), by a Grant-in-Aid for Scientific Research (K. Y: 19770037 and S. S: 19208031) and Priority Areas (19039032) from the MEXT, JAPAN, in part by CREST, JST (S. S: 2005-2010), and the "Academic Frontier" Project for Private Universities: matching fund subsidy from MEXT (S. S: 2004-2008).

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