The Plant Cell, Vol. 10, 1637–1647, October 1998, www.plantcell.org © 1998 American Society of Plant Physiologists

The Plant U1 Small Nuclear Ribonucleoprotein Particle 70K Protein Interacts with Two Novel Serine/Arginine–Rich Proteins

Maxim Golovkin and Anireddy S. N. Reddy[1]

Department of Biology and Program in Cell and Molecular Biology, Colorado State University, Fort Collins, Colorado 80523

The U1 small nuclear ribonucleoprotein particle (U1 snRNP) 70K protein (U1-70K), one of the three U1 snRNP–specific proteins, is implicated in basic and alternative splicing of nuclear pre-mRNAs. We have used the Arabidopsis U1-70K in the yeast two-hybrid system to isolate cDNAs encoding proteins that interact with it. This screening has resulted in the isolation of two novel plant serine/arginine–rich (SR) proteins, SRZ-22 and SRZ-21 (SRZ proteins). Neither the N-terminal region nor the arginine-rich C-terminal region of U1-70K alone interact with the SRZ proteins. The interaction of U1-70K with the SRZ proteins is confirmed further in vitro using a blot overlay assay. The plant SRZ proteins are highly similar to each other and contain conserved modular domains unique to different groups of splicing factors in the SR family of proteins. SRZ proteins are similar to human 9G8 splicing factor because they contain a zinc knuckle, precipitate with 65% ammonium sulfate, and cross-react with the 9G8 monoclonal antibody. However, unlike the 9G8 splicing factor, SRZ proteins contain a glycine hinge, a unique feature in other splicing factors (SC35 and ASF/SF2), located between the RNA binding domain and the zinc knuckle. SRZ-22 and SRZ-21 are encoded by two distinct genes and are expressed in all tissues tested with varied levels of expression. Our results suggest that the plant SRZ proteins represent a new group of SR proteins. The interaction of plant U1-70K with the SRZ proteins may account for some differences in pre-mRNA splicing between plants and animals.

INTRODUCTION

Most eukaryotic pre-mRNAs contain introns that are removed in the nucleus to produce functional mRNAs (Sharp, 1994; Staley and Guthrie, 1998). This process, known as pre-mRNA splicing, plays a key role in the regulation of gene expression (Smith et al., 1989; Guthrie, 1991; Manley and Tacke, 1996). Pre-mRNAs with multiple introns often undergo alternative splicing to produce structurally and functionally different protein isoforms from the same gene (Kramer, 1996; Manley and Tacke, 1996). Alternative splicing of pre-mRNAs is one of the important mechanisms in regulating gene expression and in controlling development and differentiation (Smith et al., 1989). For example, sex determination in Drosophila is controlled by alternative splicing of certain pre-mRNAs (Baker, 1989).

Although plant and animal introns share some common structural elements, there are several features in plant introns that are unique (Goodall et al., 1991; Luehrsen et al., 1994; Simpson and Filipowicz, 1996). The *cis*-acting signals that are necessary for removal of introns in plant pre-mRNAs differ from those in animal pre-mRNAs. High A and U nucleotide content in introns is necessary for efficient splicing and for 5′ and 3′ splice site selection in plants (Goodall and Filipowicz, 1989, 1991; Luehrsen and Walbot, 1994a, 1994b; Baynton et al., 1996; Egoavil et al., 1997; Merritt et al., 1997). In yeast, introns contain a highly conserved branch site sequence that is required for efficient splicing of pre-mRNAs (Simpson and Filipowicz, 1996). This branch site sequence is not conserved in plant introns. A polypyrimidine tract that is conserved in animals is also absent in plant introns (Luehrsen et al., 1994; Simpson and Filipowicz, 1996). Pre-mRNAs containing animal introns generally are either not spliced or incorrectly spliced in plant cells (Simpson and Filipowicz, 1996). Although certain plant introns are not processed in mammalian cells or in HeLa cell in vitro splicing extracts, some plant introns are processed efficiently and faithfully in animal systems (Brown et al., 1986; Hartmuth and Barta, 1986; van Santen and Spritz, 1987). These studies suggest some variations in splicing mechanisms between plants and animals.

The splicing of nuclear pre-mRNAs takes place in a multicomponent complex called the spliceosome, which consists of a pre-mRNA, five (U1, U2, U4/6, and U5) small nuclear ribonucleoprotein particles (snRNPs), and other non-snRNP splicing factors (Lührmann et al., 1990; Lamm and Lamond, 1993; Kramer, 1996). Assembly of a spliceosome is a highly

[1]To whom correspondence should be addressed. E-mail reddy@lamar.colostate.edu; fax 970-491-0649.

ordered process. The first step in the assembly is the binding of the U1 snRNP to the 5′ splice site, which is followed by binding of the other snRNPs (Mount et al., 1983; Black et al., 1985; Rosbash and Séraphin, 1991). There is also evidence that the U1 snRNP interacts with the U2 snRNP during splicing (Black et al., 1985). More recent studies suggest the involvement of U1 snRNP in alternative splicing of pre-mRNAs (Kuo et al., 1991). The mammalian U1 snRNP contains a molecule of U1 snRNA and 11 proteins, including three U1-specific proteins.

U1-70K, one of the three U1 snRNP–specific proteins, is implicated in both basic and alternative splicing. Recent studies have demonstrated direct interaction of animal U1-70K with two splicing factors, SC35 and ASF/SF2, that are involved in basic and alternative splicing of pre-mRNAs (Wu and Maniatis, 1993; Kohtz et al., 1994; Fu, 1995; Manley and Tacke, 1996). These factors are members of a family of serine/arginine–rich proteins (SR proteins) that function at multiple stages in splicesome assembly and splicing. The interaction of SC35 and ASF/SF2 with U1-70K takes place through a specific association of their serine/arginine–rich domains (Wu and Maniatis, 1993; Kohtz et al., 1994; Fu, 1995; Manley and Tacke, 1996). Furthermore, overexpression of the arginine-rich region of U1-70K has been shown to inhibit pre-mRNA splicing and nucleocytoplasmic transport of mRNA (Romac et al., 1994). These studies suggest a key role for U1-70K protein in both basic and alternative splicing through complex protein–protein interactions.

In plants, very little is known about the mechanisms involved in pre-mRNA splicing, and virtually nothing is known about the interaction of snRNP proteins with non-snRNP splicing factors. We recently isolated the Arabidopsis U1-70K gene, the only plant U1-70K gene that has been characterized thus far (Golovkin and Reddy, 1996). We have demonstrated that the Arabidopsis U1-70K gene produces two distinct (short and long) transcripts by alternative splicing of its pre-mRNA. The short transcript codes for a full-length functional U1-70K, whereas the long transcript encodes a truncated U1-70K because of an in-frame translational termination codon within the included intron. The plant U1-70K protein shares certain features with the animal U1-70K protein but differs in some structural features (Golovkin and Reddy, 1996). Previous reports on variations in pre-mRNA splicing between plants and animals as well as unique *cis*-acting elements in plant introns prompted us to identify the protein(s) that interacts with plant U1-70K. We used Arabidopsis U1-70K in a yeast two-hybrid screen to identify the proteins that interact with plant U1-70K. Here, we report that the plant U1-70K interacts specifically with two novel SR proteins, termed SRZ-21 and SRZ-22, that contain modular domains typical of different members of splicing factors in the SR family of proteins. We have confirmed the interaction of U1-70K with the SRZ proteins in vitro by using a blot overlay assay. It has not been shown previously that this group of SR proteins interacts with plant or animal U1-70K.

RESULTS

Screening of the Arabidopsis Two-Hybrid Library for Proteins That Interact with U1-70K

For the yeast two-hybrid screening, the full-length Arabidopsis U1-70K (Figure 1A) was cloned into a yeast expression vector (pAS2) to produce a hybrid protein containing the DNA binding domain of the yeast transcription factor Gal4, and a cDNA library from 3-day-old seedlings was prepared in another yeast expression vector (pACT) by fusing the cDNAs to the transcription activation domain of the yeast Gal4 transcription factor (Durfee et al., 1993). The yeast Y190 strain, which contains two reporter genes (*lacZ* and *HIS3*), was transformed first with pAS2 containing U1-70K followed by the cDNA library.

Approximately 10^6 transformants from 16 independent transformations were plated separately on selection plates (synthetic complete media minus His, Leu, and Trp) containing 50 mM 3-aminotriazole. Transformants on these plates were then screened for β-galactosidase activity by using a filter assay (Schneider et al., 1996). Colonies that were His^+ and blue were selected for further analysis. Library-derived plasmids from these positives were isolated and reintroduced into Y190 either alone or with pAS2/U1-70K and assayed for β-galactosidase activity. Three cDNA clones (*SRZ-22*, *SRZ-21*, and *SRZ-21a*) from independent transformation events showed β-galactosidase activity only in the presence of the pAS2/U1-70K plasmid. The nucleotide sequences of two of the cDNAs (*SRZ-21* and *SRZ-21a*) are identical, except that the *SRZ-21* cDNA has a significantly longer 3′ untranslated region, suggesting that these two cDNAs are derived from the same gene. Yeast cells expressing U1-70K together with each of these clones grew on the selective medium (His^-, Leu^-, and Trp^-) and showed strong expression of the *lacZ* reporter gene (Figure 1B, left). These results clearly demonstrate in vivo interaction of SRZ-22 and SRZ-21 proteins with U1-70K. We have further corroborated the interaction of SRZ proteins with U1-70K in vitro using a blot overlay assay (see below). In control experiments, neither U1-70K nor SRZ proteins alone activated *lacZ* reporter gene expression (Figure 1B, right).

Interaction of SRZ Proteins with Different Regions of U1-70K

To determine whether the arginine-rich region in plant U1-70K interacts with these SRZ proteins, the N-terminal region (amino acids 1 to 172) and the C-terminal region (amino acids 179 to 427) containing the arginine-rich domain (see Figure 1A) were fused separately to the DNA binding domain of Gal4 and introduced into yeast cells together with either SRZ-22 or SRZ-21. Interestingly, neither the N-terminal or C-terminal region alone activated *lacZ* reporter gene expression (Figure

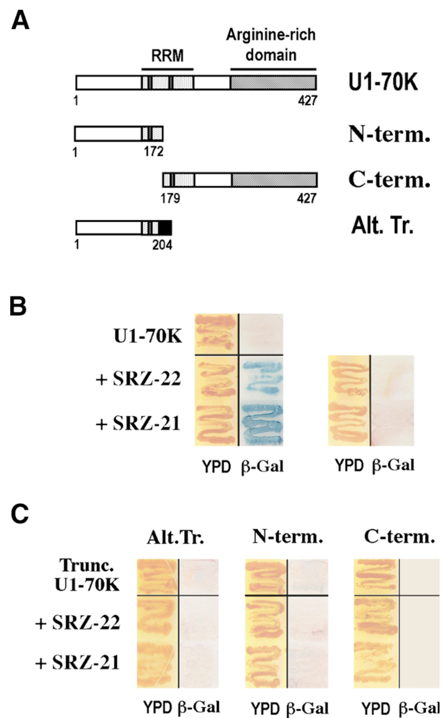

Figure 1. In Vivo Interaction of Arabidopsis U1-70K with SRZ-22 and SRZ-21 Proteins.

(A) Schematic diagram of the proteins encoded by a full-length (U1-70K), an N-terminal region (N-term.), a C-terminal region (C-term.), and an alternative transcript (Alt. Tr.) of the Arabidopsis U1-70K gene that were used in the yeast two-hybrid assays. The RNA recognition motif (RRM) and the arginine-rich domain are shown as light gray and dark gray areas, respectively. RNP2 and RNP1 consensus sequences within the RRM are indicated by double vertical lines. The filled area in the protein coded by an alternative transcript represents the region that is present only in this protein.

(B) At left, interaction of full-length U1-70K protein with SRZ proteins. Yeast cells containing U1-70K alone (U1-70K) or U1-70K together with *SRZ-22* (+SRZ-22) or *SRZ-21* (+SRZ-21) cDNAs were preselected and regrown on YPD medium (YPD) and then assayed for β-galactosidase activity (β-Gal). At right, yeast strains without U1-70K but containing SRZ clones were grown and assayed for β-galactosidase activity, as given for Figure 1B, left.

(C) Interaction of different regions of U1-70K with SRZ proteins. Yeast cells expressing a protein from U1-70K alternative transcript (Alt. Tr.), N-terminal region (N-term.), or C-terminal region (C-term.) either alone (Trunc. U1-70K) or with SRZ-22 (+SRZ-22) or SRZ-21 (+SRZ-21) were preselected, regrown on YPD medium (YPD), and then assayed for β-galactosidase activity (β-Gal).

1C). Furthermore, screening the yeast two-hybrid library with the C-terminal construct did not yield any *SRZ* clones. We have shown previously that Arabidopsis U1-70K pre-mRNA undergoes alternative splicing to produce two distinct mRNAs (Golovkin and Reddy, 1996). To test whether the truncated protein coded by the alternative transcript interacts with SRZ proteins, we transformed yeast cells with pAS2 containing the alternative transcript and either *SRZ-22* or *SRZ-21* cDNAs in pACT plasmid and assayed them for β-galactosidase activity. As shown in Figure 1C, the truncated protein encoded by the alternative transcript failed to interact with SRZ proteins.

Structural Organization of SRZ Proteins

The *SRZ-22* and *SRZ-21* cDNAs have open reading frames that encode proteins of 200 and 187 amino acids with a calculated molecular mass of 22 and 21 kD, respectively (Figure 2A). The predicted amino acid sequences of SRZ-22 and SRZ-21 proteins show ~70% amino acid identity and >80% similarity to each other. A BLAST search (Altschul et al., 1990; Gish and States, 1993) of the protein databases with the deduced amino acid sequence of SRZ-22 and SRZ-21 showed sequence similarity to domains in different members of the SR protein family. The SRZ proteins contain several well-defined modular domains characteristic of the SR family (Figures 2B and 3). Figure 2B shows the alignment of SRZ-22 and SRZ-21 with two SR proteins (9G8 and ASF/SF2), which exhibit the highest sequence identity (44 and 37%, respectively). SRZ-22 and SRZ-21 contain a single RNA binding domain with RNP2 and RNP1 consensus sequences, a glycine hinge, a zinc knuckle (CCHC motif), and a serine/arginine–rich region (Figures 2B and 3).

The SRZ proteins are more similar to the human 9G8 splicing factor because it also has a CCHC motif (zinc knuckle). This motif is a feature found only in 9G8 splicing factor (Cavaloc et al., 1994). The RNA binding domain in SRZ proteins shows the highest sequence similarity to human 9G8, Drosophila RBP1, and human splicing factor SRp20 (Zahler et al., 1992; Cavaloc et al., 1994; Heinrichs and Baker, 1995). However, the serine/arginine–rich region in the SRZ proteins differs from 9G8 splicing factor in lacking the consensus sequences (RRSRSXSX) (Cavaloc et al., 1994). Both SRZ proteins, however, contain a glycine-rich region (glycine hinge) that is present in ASF/SF2, RSp55, SC35, and SR1 splicing factors but not in 9G8 (Manley and Tacke, 1996). These unique structural features of the SRZ proteins and the fact that the interaction occurs with the full-length U1-70K indicate that the SRZ proteins represent a new group of serine/arginine–rich proteins. Search of a Prosite database with the SRZ proteins revealed the presence of potential nuclear localization signals, including four bipartite motifs in each SRZ protein, suggesting that these are nuclear proteins.

Expression of SRZ Proteins

The expression of the genes encoding SRZ-22 and SRZ-21 was examined in different plant tissues by RNA gel blot

Figure 2. Sequence Analysis and Structural Characterization of SRZ-22 and SRZ-21.

(A) Nucleotide and predicted amino acid sequences of *SRZ-22* and *SRZ-21* cDNAs. The amino acid sequence is shown under the nucleotide sequence. Numbers at right correspond to nucleotides and deduced amino acids. Arrows indicate the position of introns. *SRZ-21a* is identical to *SRZ-21* except that it is shorter and does not contain 163 nucleotides, shown in boldface. The sequence data for SRZ-22 and SRZ-21 were submitted to the EMBL, GenBank, and DDBJ databases as accession numbers AF033586 and AF033587, respectively.

(B) Comparison of predicted amino acid sequence of SRZ-22 and SRZ-21 with the SR proteins ASF/SF2 (ASF2) and 9G8 (Cavaloc et al., 1994; accession number AF001035). RNP2, RNP1, and the glycine hinge sequences are indicated by a solid line above the alignment. Conserved CCHC residues in the zinc knuckle are indicated with asterisks. Identical amino acids are shown by reverse lettering. Dashes indicate gaps in the alignment.

analysis. As shown in Figure 4, the *SRZ-22* probe hybridized with a transcript of ∼0.85 kb, whereas the *SRZ-21* probe hybridized with a major transcript of ∼1.0 kb and a minor transcript of ∼0.8 kb, which was visible in autoradiograms exposed for 1 week. The lengths of the hybridizing bands are close to the lengths of the cDNAs isolated, suggesting that the cDNAs are full length. Although both genes were found to be expressed in all tissues tested, the level of expression in different tissues varied (Figure 4). *SRZ-22* expression was very high in cell suspension but not in roots, whereas *SRZ-21* was highly expressed in roots. Both *SRZ-22* and *SRZ-21* transcripts were relatively abundant in flowers. Among the tissues tested, leaves showed the lowest accumulation of *SRZ* transcripts (Figure 4).

DNA gel blot analysis indicated that SRZ-22 and SRZ-21 are encoded by two distinct genes (Figure 5). Additional hybridizing bands were detected with each of the digests under low-stringency conditions (data not shown), implying that there are other *SRZ*-like genes in Arabidopsis. *SRZ*-like sequences found in the Arabidopsis expressed sequence tags database also indicate the presence of other *SRZ*-like genes. The sequences of two recently submitted Arabidopsis bacterial artificial chromosome clones (GenBank accession numbers AL031004 and ACOO2423) from chromosomes 4 and 1 contain *SRZ-22* and *SRZ-21* sequences, respectively. Comparison of the nucleotide sequence of *SRZ-22* and *SRZ-21* cDNAs with the corresponding genomic sequences indicates the presence of five introns in *SRZ-22* and *SRZ-21*. The location of introns in the *SRZ* genes is shown in Figure 2A. One of the introns is in the 5′ untranslated region, and the other four introns are in the coding region. The location of all introns in the *SRZ-22* and *SRZ-21* genes is conserved.

Expression and Purification of SRZ Proteins

To characterize plant SRZ proteins and further substantiate the in vivo interaction of U1-70K with SRZ proteins, SRZ-22 and SRZ-21 were expressed as His-T7.tag fusions by using the pET28 system. Analysis of the soluble and pellet fractions of total protein extracts showed recovery of fusion protein in both fractions. The gene fusion from the cDNA clones was expected to produce polypeptides of ∼27 kD (21 to 22 kD from the cDNAs plus 5 kD from the His-T7.tag). With the T7.tag monoclonal antibody, we detected a polypeptide of ∼32 kD in soluble proteins from cells containing SRZ-22 (Figure 6A, lanes 1). The size of the protein is larger than the size expected from the deduced amino acid sequence. This is most likely due to the presence of the serine/arginine–rich region because proteins containing this region migrate abnormally (Query and Keene, 1987; Manley and Tacke, 1996). Similar results were obtained with the SRZ-21 protein (data not shown).

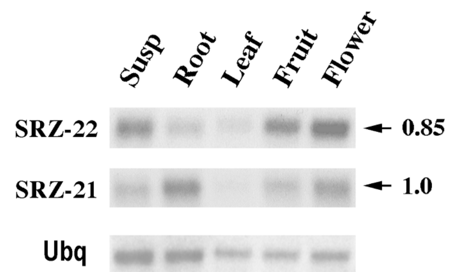

Figure 4. Expression of the *SRZ-22* and *SRZ-21* Genes in Different Tissues.

Five micrograms of poly(A)$^+$ RNA from the indicated tissues and organs was loaded in each lane. Duplicate blots were probed with *SRZ-22* or *SRZ-21* cDNAs. The amount of mRNA in each lane was verified by probing one of the blots with a constitutively expressed ubiquitin cDNA (Ubq). Numbers at right indicate the length of size markers in kilobases. Susp, suspension cultured plants.

Both SRZ proteins were precipitated by 20 mM magnesium chloride (Figure 6A, lanes 2; data not shown for SRZ-21), which is a characteristic of SR proteins (Zahler et al., 1992; Fu, 1995). However, unlike most animal SR proteins that are soluble in 65% ammonium sulfate (Zahler et al., 1992), both SRZ proteins were precipitated completely by 65% ammonium sulfate (Figure 6A; data not shown for SRZ-21). Furthermore, a monoclonal antibody raised against the 9G8 splicing factor cross-reacted strongly with *Escherichia coli*–expressed SRZ-22 and SRZ-21 (Figure 6B). In these respects, the SRZ proteins are similar to human 9G8 protein because this is the only known SR protein that precipitates with 65% ammonium sulfate.

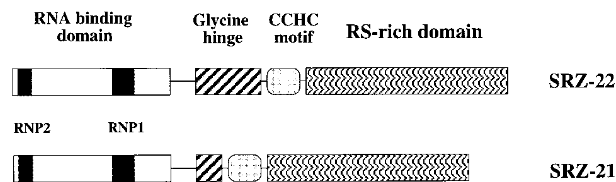

Figure 3. Schematic Diagram Showing the Structural Organization of SRZ-22 and SRZ-21 Proteins.

RS, arginine/serine.

In Vitro Interaction of SRZ Proteins with U1-70K

The Arabidopsis U1-70K protein expressed as a fusion to S.tag and SRZ proteins expressed as His-T7.tag fusions were used in an in vitro blot overlay assay (Figure 7). The amount of BSA and U1-70K present on the blots is shown in Figure 7A. The detection of U1-70K with S.tag and SRZ proteins with T7.tag is shown in Figures 7B and 7D, respectively. The U1-70K protein is not detected by T7.tag antibodies (Figure 7D). To confirm the interaction of U1-70K

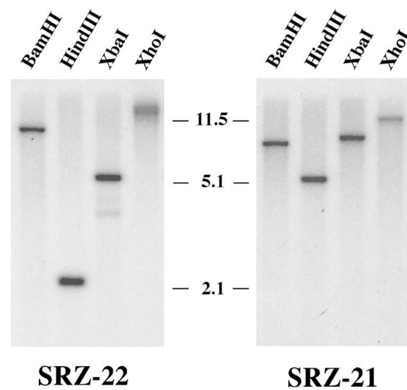

Figure 5. Genomic DNA Blot Analysis.

Duplicate blots containing Arabidopsis genomic DNA digested with BamHI, HindIII, XbaI, and XhoI were prepared and probed with ^{32}P-labeled *SRZ-22* or *SRZ-21* cDNA probes. Numbers in the center indicate the length of size markers in kilobases.

with SRZ proteins, we performed a blot overlay assay. Protein blots containing bacterially expressed plant U1-70K protein were incubated with purified SRZ-22 or SRZ-21 proteins (Kohtz et al., 1994) (Figure 7C). The binding of SRZ proteins to U1-70K was determined by T7.tag antibodies, which recognize SRZ proteins but not U1-70K protein (Figures 7C and 7D). Both proteins interacted with U1-70K in blot overlay assays (Figure 7C), supporting our results obtained with the yeast two-hybrid system. In blot overlay assays, the SRZ proteins did not interact with either BSA or *E. coli* proteins (Figure 7C), indicating that the observed interaction between U1-70K and SRZ proteins is specific.

DISCUSSION

SR proteins are a family of essential splicing factors that function in early steps of spliceosome assembly and modulate splice site selection (Zahler et al., 1993; Fu, 1995; Zahler and Roth, 1995; Manley and Tacke, 1996). Several studies in recent years have indicated a critical role for SR proteins in both constitutive and regulated splicing (Sun et al., 1993; Zahler et al., 1993; Tacke and Manley, 1995; Zahler and Roth, 1995; Caceres et al., 1997; Jumaa and Nielsen, 1997; Tacke et al., 1997). During the past 5 years, several SR proteins with a molecular mass ranging from 20 to 75 kD have been characterized in animals. The sequences of individual SR proteins are highly conserved between species. Recently, it was demonstrated that two SR proteins (SC35 and ASF/SF2) interact directly with the U1-70K and that this interaction occurs through their serine/arginine–rich domains (Wu and Maniatis, 1993; Kohtz et al., 1994). Furthermore, SC35 and ASF/SF2 can interact simultaneously with U1-70K and U2AF35, which associates with U2AF65 (Wu and Maniatis, 1993), suggesting the involvement of SR proteins in bridging 5′ and 3′ splice sites.

In plants, very little is known about SR proteins and their role in pre-mRNA splicing (Lopato et al., 1996a). Recently, four SR proteins were isolated from Arabidopsis (Lazar et al., 1995; Lopato et al., 1996b). These are the only four SR

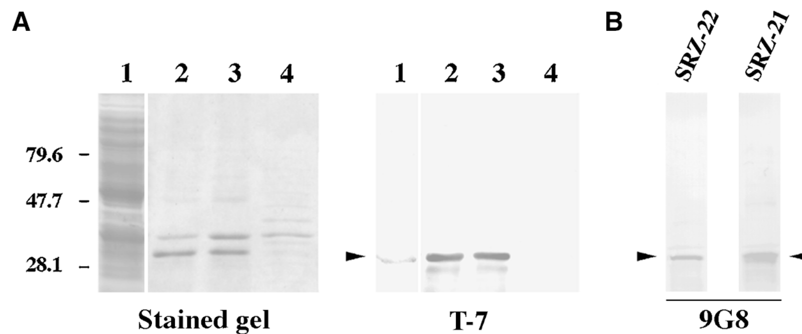

Figure 6. Expression, Salt Precipitation, and Detection of SRZ Proteins Expressed in *E. coli.*

(A) Soluble proteins from induced cultures containing SRZ-22 were separated on duplicate gels. One gel was stained with Coomassie Brilliant Blue R 250 (Stained gel), and a duplicate gel was blotted and probed with T7.tag monoclonal antibodies (T-7). Lanes 1 contain soluble proteins; lanes 2, proteins precipitated with 20 mM $MgCl_2$; lanes 3, proteins precipitated first with 65% ammonium sulfate followed by precipitation with 20 mM $MgCl_2$; and lanes 4, proteins precipitated first with 65 to 90% ammonium sulfate followed by precipitation with 20 mM $MgCl_2$.
(B) SRZ proteins were separated on a gel, blotted, and probed with human anti-9G8 monoclonal antibody (9G8).
Molecular mass markers are shown at left in kilodaltons. Arrowheads indicate the SRZ proteins.

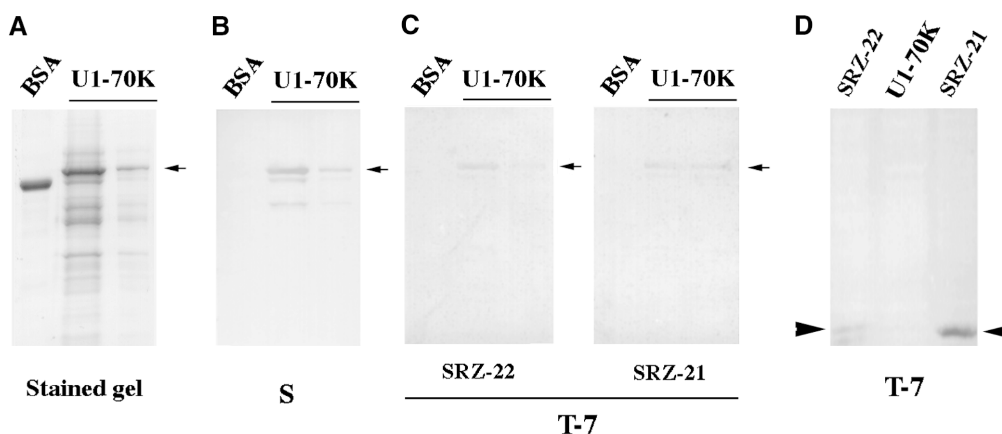

Figure 7. Blot Overlay Assay Showing the in Vitro Interaction of SRZ-22 and SRZ-21 Proteins with the Plant U1-70K Protein.

The U1-70K protein expressed in pET32 and SRZ-22 and SRZ-21 proteins expressed in pET28 were separated on SDS-containing gels and transferred onto a nitrocellulose filter. The blots were used for detection of the fusion proteins or were incubated with either SRZ-22 or SRZ-21 protein followed by detection with T7.tag antibody.
(A) Coomassie Brilliant Blue R 250–stained gel showing BSA and two concentrations of *E. coli*–expressed U1-70K (U1-70K) protein.
(B) A blot containing the same proteins as given in **(A)** was probed with S.tag protein (S) to detect U1-70K protein.
(C) Blots containing the same proteins as given in **(A)** were used in a blot overlay assay with either SRZ-22 or SRZ-21. The binding of SRZ proteins to U1-70K was detected by monoclonal antibodies to the T7.tag (T-7).
(D) A blot containing SRZ-22, U1-70K, and SRZ-21 fusion proteins was incubated with the T7.tag monoclonal antibody (T-7).
The arrows indicate the positions of the U1-70K protein, and the arrowheads indicate the positions of the SRZ proteins.

proteins that have been characterized so far in plants. Although the plant SR proteins share some sequence similarity with animal SR protein, they have some unique structural features. The two plant SR proteins (SRZ-22 and SRZ-21) that we have isolated using the yeast two-hybrid screen with Arabidopsis U1-70K are different from all previously reported plant and animal SR proteins. The plant SRZ proteins, like all known SR proteins, contain an RNA recognition motif at the N terminus and a serine/arginine–rich domain at the C terminus. However, the domain organization of plant SRZ proteins is clearly different from known SR proteins because they contain conserved modules present in different members of splicing factors in the SR family. The plant SRZ proteins share structural features present in the 9G8 (CCHC motif) and ASF/SF2 (glycine hinge) splicing factors. Furthermore, SRZ proteins are similar to 9G8 splicing factors because they precipitate with 65% ammonium sulfate and cross-react with 9G8 monoclonal antibodies. However, unlike human 9G8 splicing factor, SRZ proteins contain a glycine hinge, a unique feature present in other splicing factors such as ASF/SF2 and SC35. Moreover, SRZ proteins lack an amino acid consensus sequence that is repeated six times in the serine/arginine–rich region of 9G8 protein. The serine/arginine–rich domain in SRZ proteins, unlike the human 9G8 protein, has a high proportion of proline and tyrosine residues.

Currently, 9G8, a splicing factor characterized only in humans, is not known to interact with U1-70K. Although there are no published reports on zinc knuckle–containing SR proteins in plants, several cDNA and genomic sequences encoding proteins that are very similar to the SRZ proteins have been deposited recently in GenBank.

Interaction studies with the full-length U1-70K, as well as with N-terminal, C-terminal, and alternative transcripts of U1-70K, have shown that the full-length U1-70K protein, but not the truncated versions, can interact with SRZ proteins in vivo. These results suggest one of the following possibilities. First, the SRZ proteins interact with only the full-length protein. Second, separation of U1-70K into N-terminal and C-terminal regions may have disrupted the site of interaction. Finally, truncation of U1-70K protein may have affected the folding of the resulting proteins. However, animal splicing factors ASF/SF2 and SC35 have been shown to interact directly with human U1-70K through their serine/arginine–rich domains (Wu and Maniatis, 1993; Kohtz et al., 1994). Although the C terminus of Arabidopsis U1-70K is rich in arginine, it does not have distinct serine/arginine–rich regions (Golovkin and Reddy, 1996). This may reflect some variation in the function of plant U1-70K. More recently it has been reported that the serine/arginine–rich domain alone in animal ASF/SF2 is necessary, but not sufficient, for binding to U1-70K (Xiao and Manley, 1997). Our in vivo and in vitro data show that U1-70K interacts with two new members of the plant SR protein family that contain a CCHC zinc knuckle motif.

The steady state levels of *SRZ* expression are quite variable among tissues. Leaves had the lowest level of transcripts

for both *SRZ-22* and *SRZ-21*. Furthermore, in some tissues, *SRZ-21* transcripts were more abundant than were the *SRZ-22* transcripts. For example, in roots, *SRZ-21* transcripts were far more abundant than were *SRZ-22* transcripts.

Pre-mRNAs of several SR proteins, such as SC35, ASF/SF2, and 9G8, undergo alternative splicing to generate multiple mature mRNAs that encode distinct isoforms (Ge and Manley, 1990; Fu and Maniatis, 1992; Popielarz et al., 1995). However, the function of each of these isoforms in pre-mRNA splicing is not yet clear. *SRZ-22* probes hybridized with a single transcript, suggesting that the *SRZ-22* pre-mRNA does not undergo alternative splicing. In the case of *SRZ-21*, two transcripts (1.0 and 0.8 kb), which are of the same size as the two cDNAs we isolated, were detected. However, it is not clear whether the two transcripts are derived by alternative splicing or differential polyadenylation of transcripts.

Several studies indicate that phosphorylation plays an important role in pre-mRNA splicing (Tacke et al., 1997; Xiao and Manley, 1997; Wang et al., 1998). Human U1-70K and animal SR proteins are known to be phosphoproteins, and phosphorylation of these proteins affects the activity and localization of these proteins (Colwill et al., 1996b; Misteli et al., 1997; Xiao and Manley, 1997). Two families of kinases, SRPK and Clk/Sty, have been shown to phosphorylate serine/arginine–rich domain–containing splicing factors (Gui et al., 1994; Colwill et al., 1996a, 1996b). It is not known if the Arabidopsis U1-70K or SRZ proteins are phosphoproteins. However, there are several potential phosphorylation sites in U1-70K as well as in the SRZ proteins. Recently, genes encoding Clk/Sty–type (also known as LAMMER-type) kinases have been isolated from Arabidopsis (Bender and Fink, 1994). It would be interesting to test whether the Arabidopsis Clk/Sty–type protein kinases phosphorylate the SRZ proteins.

In summary, using yeast two-hybrid screening, we have isolated two novel plant SR proteins that interact with plant U1-70K protein. We demonstrate a direct interaction between a specific snRNP protein (U1-70K) and SR proteins in plants. These SRZ proteins possess unique structural features with conserved modular domains typical of different splicing factors. Our results suggest that the plant SRZ proteins represent a new group of SR proteins. Interaction of the U1-70K with this novel group of SR proteins may explain the differences in splicing of plant and animal pre-mRNAs.

METHODS

Plant Material

Arabidopsis thaliana (ecotype Columbia) plants were grown on Metromix 200 (American Clay Works, Denver, CO) under continuous light. Leaves, fruits, and flowers were harvested from 6-week-old plants. Roots were grown in liquid culture and harvested as described previously (Reddy et al., 1994). Arabidopsis suspension cultures were grown in Murashige and Skoog media (Life Technologies, Gaithersburg, MD), and cells were harvested 5 to 7 days after transfer to new media (Day et al., 1996).

Preparation of Plasmid Constructs

A full-length U1-70K and different regions of the U1-70K (N-terminal region, C-terminal region, and an alternative transcript) were expressed as a fusion to Gal4 DNA binding domain in the pAS2 vector (Clonetech, Palo Alto, CA). A 1.5-kb NcoI-PstI fragment containing the entire coding region of Arabidopsis U1-70K (amino acids 1 to 427) was cloned in-frame in the pAS2 vector. To prepare the N-terminal construct, we cloned a fragment (~0.5 kb; NcoI-PvuII) containing the coding region for amino acids 1 to 172 in frame in the pAS2 vector. The C-terminal construct containing the coding region for amino acids 179 to 427 was prepared by releasing an NcoI-PvuII fragment from the full-length construct. An 0.8-kb NcoI fragment corresponding to the 5′ region of a cDNA corresponding to an alternatively spliced transcript of U1-70K (Golovkin and Reddy, 1996) was cloned into pAS2. The constructs were sequenced to verify the junction areas.

Screening of the Yeast Two-Hybrid Library

An Arabidopsis cDNA library was prepared as a fusion to the transcription activation domain of the yeast transcription factor Gal4 in λACT vector, as described previously (Durfee et al., 1993). mRNA from 3-day-old etiolated seedlings was used to prepare the cDNA. The conversion of a λACT cDNA library into a plasmid library was performed according to Durfee et al. (1993). Plasmid DNA was prepared and purified using a CsCl density gradient (Sambrook et al., 1989). The yeast Y190 strain was first transformed with the pAS2 containing the full-length U1-70K and then transformed with the cDNA library via the LiAcetate transformation method. Approximately 10^6 transformants were plated on selection (Leu^-, Trp^-, and His^-) plates. Colonies able to grow on selection plates were assayed for β-galactosidase activity (Schneider et al., 1996). Total yeast DNA from the positives was isolated and electroporated into *Escherichia coli* HB101. Colonies containing the pACT plasmid were identified by polymerase chain reaction amplification with pACT 5′ and 3′ primers. To verify the interaction, we transformed the pACT plasmid back into the yeast Y190 strain or a Y190 strain containing the pAS2/U1-70K plasmid, and the transformants were tested for β-galactosidase activity. To test the interaction of different regions of U1-70K with SRZ proteins, we transformed Y190 strains containing each of the U1-70K constructs (N terminal, C terminal, or alternative transcript) with pACT plasmid, grew them on selection plates, and assayed them for β-galactosidase activity, as described above.

Sequence Analysis

Both strands of cDNA clones were sequenced by the dideoxy nucleotide chain termination method, using double-stranded DNA as a template (Sanger et al., 1977). Primer walking was used to obtain complete sequences of the clones. Sequence analysis was performed using Sequencher (Gene Codes Corporation, Ann Arbor, MI), Lasergene (DNA Star Inc., Madison, WI), and MacVector (International Biotechnologies Inc., New Haven, CT) sequence analysis

software. Database searches were performed at the National Center for Biotechnology Information by using the BLAST network service.

Protein Expression in *E. coli* and Immunodetection

U1-70K cDNA was digested with DraI and NcoI to release a 1.3-kb fragment. The cDNA fragment (NcoI blunt ended) was then inserted in frame into the pET32 plasmid (Novagen, Madison, WI) that contained a NcoI and a HindIII blunt-ended site. A 0.7-kb XhoI fragment of *SRZ-22* cDNA and a 1.0-kb XhoI fragment of *SRZ-21* cDNA were cloned in frame in pET28 (Novagen, Madison, WI). The orientation of the cDNAs was confirmed by restriction analysis. The plasmid constructs were transformed into BL21(DE3)pLysS host cells for expression of fusion protein. Bacterial cultures were allowed to grow at 37°C to an OD_{600} of 0.6. Expression of fusion protein was induced by adding isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM, and the culture was allowed to grow further for ~8 hr at 20°C. Soluble and insoluble proteins from induced and uninduced cultures were prepared, separated on 12% SDS–polyacrylamide gels, and transblotted onto a nitrocellulose membrane, as described previously (Reddy et al., 1996). The filters were blocked for 1 hr at 30°C in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) containing 3% gelatin. After rinsing with TBST, the filters were incubated with either T7.tag monoclonal antibody conjugated to alkaline phosphatase (1:10,000 dilution) or human 9G8 monoclonal antibody for 30 min. The filters were washed, and the blot that was treated with the 9G8 antibody was incubated with secondary antibody (goat anti–mouse) conjugated to alkaline phosphatase (1:10,000 dilution). Immunoreactive protein bands were detected colorimetrically by immersing the filters in a substrate solution (Bowser and Reddy, 1997).

Salt Precipitation of SRZ Proteins

Proteins in the soluble fraction were first precipitated by adding ammonium sulfate to 65%. After centrifugation, the supernatant was collected, and ammonium sulfate was added to 90% saturation (Zahler et al., 1992). Resulting protein pellets were dissolved in TE (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) and reprecipitated with 20 mM $MgCl_2$, according to methods published previously (Zahler et al., 1992). The protein pellet was dissolved in TE and used in blot overlay assays. Alternatively, fusion proteins in the soluble fraction were precipitated directly with 20 mM $MgCl_2$ and processed as described above.

Blot Overlay Assay

The interaction of U1-70K with the SRZ proteins was analyzed by a blot overlay assay (Kohtz et al., 1994; Narasimhulu et al., 1997). BSA (0.5 μg) and an *E. coli* lysate containing U1-70K protein was electrophoresed in an SDS-containing polyacrylamide gel. The gel was incubated in transblotting buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) for 1 hr and transferred onto a nitrocellulose membrane. The membrane was blocked overnight in TBS buffer (10 mM Tris, pH 8.0, and 150 mM NaCl) containing 3% gelatin at 30°C. The membranes were then incubated in 5 mL of TBS containing salt-purified SRZ proteins (1 μg/mL) for 2 hr and washed five times (5 min each) with TBS buffer. The binding of SRZ proteins to U1-70K was detected with T7.tag antibodies conjugated to alkaline phosphatase, as described above. The U1-70K protein was detected with S protein conjugated to alkaline phosphatase (McCormick and Mierendorf, 1994).

DNA and RNA Gel Blot Analyses

Arabidopsis genomic DNA was isolated using urea–phenol—containing buffer, digested with different restriction endonucleases, electrophoretically separated in an 0.8% agarose gel, and transferred to a nylon membrane. The DNA was cross-linked to the filter by exposing it to UV light in a Stratalinker (Stratagene, La Jolla, CA). The blots were hybridized with ^{32}P-labeled cDNAs. Prehybridization and hybridization were performed using Rapid-Hyb buffer (Amersham), according to the instructions provided by the supplier. The filters were washed under high-stringency conditions and exposed to x-ray film.

Total RNA from different organs and tissues was isolated and purified on a CsCl cushion (Sambrook et al., 1989). Poly(A)$^+$ mRNA was isolated by an oligo(dT) cellulose column. Five micrograms of poly(A)$^+$ RNA from each tissue was separated in a 1.5% formaldehyde-containing agarose gel, transferred to a Hybond N+ membrane, and cross-linked using a Stratalinker. Prehybridization and hybridization were performed as described above.

ACKNOWLEDGMENTS

We thank Dr. James Stevenin for providing 9G8 antibodies; the Arabidopsis Biological Resource Center (Columbus, OH) for the cDNA library in the yeast vector; and Dr. Donald Mykles, Dr. June Medford, Dr. Irene Day, and Jonathan Bowser for critical comments on the manuscript. This work was supported by grants from the U.S. Department of Agriculture National Research Initiative Competitive Grant Program (Nos. 9400849 and 9701310) to A.S.N.R.

Received March 30, 1998; accepted August 4, 1998.

REFERENCES

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. **215,** 403–410.

Baker, B.S. (1989). Sex in flies, the splice of life. Nature **340,** 521–524.

Baynton, C.E., Potthoff, S.J., McCullough, A.J., and Schuler, M.A. (1996). U-rich tracts enhance 3′ splice site recognition in plant nuclei. Plant J. **10,** 703–711.

Bender, J., and Fink, G. (1994). AFC1, a LAMMER kinase from *Arabidopsis thaliana*, activates STE12-dependent processes in yeast. Proc. Natl. Acad. Sci. USA **91,** 12105–12109.

Black, D.L., Chabot, B., and Steitz, J.A. (1985). U1 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. Cell **42,** 737–750.

Bowser, J., and Reddy, A.S.N. (1997). Localization of a kinesin-like calmodulin-binding protein in dividing cells of *Arabidopsis* and tobacco. Plant J. **12,** 1429–1438.

Brown, J.W.S., Feix, G., and Frendewey, D. (1986). Accurate in vitro splicing of two pre-mRNA plant introns in a HeLa cell nuclear extract. EMBO J. **5,** 2749–2758.

Caceres, J.F., Misteli, T., Screaton, G.R., Spector, D.L., and Krainer, A.R. (1997). Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J. Cell Biol. **138,** 225–238.

Cavaloc, Y., Popielarz, M., Fuchs, J.-P., Gattoni, R., and Stevenin, J. (1994). Characterization and cloning of the human splicing factor 9G8: A novel 35 kDa factor of the serine/arginine protein family. EMBO J. **13,** 2639–2649.

Colwill, K., Feng, L.L., Yeakley, J.M., Dish, G.D., Caceres, J.F., Pawson, T., and Fu, X.-D. (1996a). SRPK1 and Clk/Sty protein kinases show distinct substrate specificities for serine/arginine–rich splicing factors. J. Biol. Chem. **271,** 24569–24575.

Colwill, K., Pawson, T., Andrews, B., Prasad, J., Manley, J., Bell, J.C., and Duncan, P.I. (1996b). The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. EMBO J. **15,** 265–275.

Day, I., Reddy, A.S.N., and Golovkin, M. (1996). Isolation of a new mitotic cyclin from *Arabidopsis*: Complementation of a yeast cyclin mutant with a plant cyclin. Plant Mol. Biol. **30,** 565–575.

Durfee, T., Becherer, K., Chen, P.-L., Yeh, S.-H., Yang, Y., Kilburn, A.E., Lee, W.-H., and Elledge, S.J. (1993). The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7,** 555–569.

Egoavil, C., Marton, H.A., Baynton, C.E., McCullough, A.J., and Schuler, M.A. (1997). Structural analysis of elements contributing to 5′ splice site selection in plant pre-mRNA transcripts. Plant J. **12,** 971–980.

Fu, X.-D. (1995). The superfamily of arginine/serine–rich splicing factors. RNA **1,** 663–680.

Fu, X.-D., and Maniatis, T. (1992). Isolation of a complementary DNA that encodes the mammalian splicing factor SC35. Science **256,** 535–538.

Ge, H., and Manley, J.L. (1990). A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. Cell **62,** 25–34.

Gish, W., and States, D.J. (1993). Identification of protein coding regions by database similarity search. Nature Genet. **3,** 266–272.

Golovkin, M., and Reddy, A.S.N. (1996). Structure and expression of a plant U1 snRNP 70K gene: Alternative splicing of U1 snRNP 70K pre-mRNAs produces two different transcripts. Plant Cell **8,** 1421–1435.

Goodall, G.J., and Filipowicz, W. (1989). The AU-rich sequences present in the introns of plant nuclear pre-mRNAs are required for splicing. Cell **58,** 473–483.

Goodall, G.J., and Filipowicz, W. (1991). Different effects of intron nucleotide composition and secondary structure on pre-mRNA splicing in monocot and dicot plants. EMBO J. **10,** 2635–2644.

Goodall, G.J., Kiss, T., and Filipowicz, W. (1991). Nuclear RNA splicing and small nuclear RNAs and their genes in higher plants. Ox. Surv. Plant Mol. Cell Biol. **7,** 255–296.

Gui, J.F., Lane, W.S., and Fu, X.-D. (1994). A serine kinase regulates intracellular localization of splicing factors in the cell cycle. Nature **369,** 678–682.

Guthrie, C. (1991). Messenger RNA splicing in yeast: Clues to why the spliceosome is a ribonucleoprotein. Science **253,** 157–163.

Hartmuth, K., and Barta, A. (1986). In vitro processing of a plant pre-mRNA in HeLa cell nuclear extract. Nucleic Acids Res. **14,** 7513–7528.

Heinrichs, V., and Baker, B.S. (1995). The Drosophila SR protein RBP1 contributes to the regulation of doublesex alternative splicing by recognizing RBP1 RNA target sequences. EMBO J. **14,** 3987–4000.

Jumaa, H., and Nielsen, P.J. (1997). The splicing factor SRp20 modifies splicing of its own mRNA and ASF/SF2 antagonizes this regulation. EMBO J. **16,** 5077–5085.

Kohtz, J.D., Jamison, S.F., Will, C.L., Zuo, P., Lührmann, R., Garcia-Blanco, M.A., and Manley, J.L. (1994). Protein–protein interactions and 5′-splice-site recognition in mammalian mRNA precursors. Nature **368,** 119–124.

Kramer, A. (1996). The structure and function of proteins involved in mammalian pre-mRNA splicing. Annu. Rev. Biochem. **65,** 367–409.

Kuo, H., Nasim, F.H., and Grabowski, P.J. (1991). Control of alternative splicing by differential binding of U1 small nuclear ribonucleoprotein particle. Science **251,** 1045–1050.

Lamm, G.M., and Lamond, A.I. (1993). Non-snRNP protein splicing factors. Biochim. Biophys. Acta **1173,** 247–265.

Lazar, G., Schall, T., Maniatis, T., and Goodman, H. (1995). Identification of a plant serine/arginine–rich protein similar to the mammalian splicing factor SF2/ASF. Proc. Natl. Acad. Sci. USA **92,** 7672–7676.

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

Lopato, S., Waigmann, E., and Barta, A. (1996b). Characterization of a novel arginine/serine–rich splicing factor in Arabidopsis. Plant Cell **8,** 2255–2264.

Luehrsen, K.R., and Walbot, V. (1994a). Addition of A- and U-rich sequences increases the splicing efficiency of a deleted form of a maize intron. Plant Mol. Biol. **24,** 449–463.

Luehrsen, K.R., and Walbot, V. (1994b). Intron creation and polyadenylation in maize are directed by AU-rich RNA. Genes Dev. **8,** 1117–1130.

Luehrsen, K.R., Taha, S., and Walbot, V. (1994). Nuclear pre-mRNA processing in higher plants. Prog. Nucleic Acid Res. **47,** 149–193.

Lührmann, R., Kastner, B., and Bach, M. (1990). Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. Biochim. Biophys. Acta **1087,** 265–292.

Manley, J.L., and Tacke, R. (1996). SR proteins and splicing control. Genes Dev. **10,** 1569–1579.

McCormick, M., and Mierendorf, R. (1994). S.Tag: A multipurpose fusion peptide for recombinant proteins. inNovations **1,** 4–7.

Merritt, H., McCullough, A.J., and Schuler, M.A. (1997). Internal AU-rich elements modulate activity of two competing 3′ splice sites in plant nuclei. Plant J. **112,** 937–943.

Misteli, T., Caceres, J.F., and Spector, D.L. (1997). The dynamics of a pre-mRNA splicing factor in living cells. Nature **387,** 523–527.

Mount, S.M., Pettersson, I., Hinterberger, M., Karmas, A., and Steitz, J.A. (1983). The U1 small nuclear RNA–protein complex selectively binds a 5′ splice site in vitro. Cell **33,** 509–518.

Narasimhulu, S.B., Kao, Y.-L., and Reddy, A.S.N. (1997). Interaction of *Arabidopsis* kinesin-like calmodulin-binding protein with tubulin subunits: Modulation by Ca^{2+}-calmodulin. Plant J. **12,** 1139–1149.

Popielarz, M., Cavaloc, Y., Mattei, M.-G., Gattoni, R., and Stevenin, J. (1995). The gene encoding human splicing factor 9G8: Structure, chromosomal localization, and expression of alternatively processed transcripts. J. Biol. Chem. **270,** 17830–17835.

Query, C.C., and Keene, J.D. (1987). A human autoimmune protein associated with U1 RNA contains a region of homology that is cross-reactive with retroviral $p30^{gag}$ antigen. Cell **51,** 211–220.

Reddy, A.S.N., Safadi, F., Beyette, J., and Mykles, D.L. (1994). Calcium-dependent proteinase activity in root cultures of Arabidopsis. Biochim. Biophys. Res. Commun. **199,** 1089–1095.

Reddy, A.S.N., Safadi, F., Narasimhulu, S.B., Golovkin, M., and Hu, X. (1996). A novel plant calmodulin-binding protein with a kinesin heavy chain motor domain. J. Biol. Chem. **271,** 7052–7060.

Romac, J.M.-J., Graff, D.H., and Keene, J.D. (1994). The U1 small nuclear ribonucleoprotein (snRNP) 70K protein is transported independently of U1 snRNP particles via a nuclear localization signal in the RNA-binding domain. Mol. Cell. Biol. **14,** 4662–4670.

Rosbash, M., and Séraphin, B. (1991). Who's on first? The U1 snRNP–5′ splice site interaction and splicing. Trends Biochem. Sci. **16,** 187–190.

Sambrook, J., Fritsch, F.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain termination inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schneider, S., Buchert, M., and Hovens, C.M. (1996). An in vitro assay of β-galactosidase from yeast. BioTechniques **20,** 960–962.

Sharp, P.A. (1994). Split genes and RNA splicing. Cell **77,** 805–815.

Simpson, G.G., and Filipowicz, W. (1996). Splicing of precursors to mRNA in higher plants: Mechanism, regulation, and sub-nuclear organization of the spliceosomal machinery. Plant Mol. Biol. **32,** 1–41.

Smith, C.W.J., Patton, J.G., and Nadal-Ginard, B. (1989). Alternative splicing in the control of gene expression. Annu. Rev. Genet. **23,** 527–577.

Staley, J.P., and Guthrie, C. (1998). Mechanical devices of the spliceosome: Motors, clocks, springs and things. Cell **92,** 315–326.

Sun, Q., Mayeda, A., Hampson, R.K., Krainer, A.R., and Rottman, F.M. (1993). General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. Genes Dev. **7,** 2598–2608.

Tacke, R., and Manley, J.L. (1995). The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. EMBO J. **14,** 3540–3551.

Tacke, R., Chen, Y., and Manley, J.L. (1997). Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: Creation of an SRp40-specific splicing enhancer. Bioessays **19,** 189–192.

van Santen, V.L., and Spritz, R.A. (1987). Splicing of plant pre-mRNAs in animal systems and vice versa. Gene **56,** 253–265.

Wang, H.-Y., Lin, W., Dyck, J.A., Yeakley, J.M., Songyang, Z., Cantley, L.C., and Fu, X.-D. (1998). SRPK2: A differentially expressed SR protein–specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. J. Cell Biol. **140,** 737–750.

Wu, J.Y., and Maniatis, T. (1993). Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell **75,** 1061–1070.

Xiao, S.-H., and Manley, J.L. (1997). Phosphorylation of the ASF/SF2 RS domain affects both protein–protein and protein–RNA interactions and is necessary for splicing. Genes Dev. **11,** 334–344.

Zahler, A.M., and Roth, M.B. (1995). Distinct functions of SR proteins in recruitment of U1 small nuclear ribonucleoprotein to alternative 5′ splice sites. Proc. Natl. Acad. Sci. USA **92,** 2642–2646.

Zahler, A.M., Lane, W.S., Stolk, J.A., and Roth, M.B. (1992). SR proteins: A conserved family of pre-mRNA splicing factors. Genes Dev. **6,** 837–847.

Zahler, A.M., Neugebauer, K.M., Lane, W.S., and Roth, M.B. (1993). Distinct functions of SR proteins in alternative pre-mRNA splicing. Science **260,** 219–222.