Evolutionary conservation and regulation of particular alternative splicing events in plant SR proteins

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ABSTRACT
Alternative splicing is an important mechanism for fine tuning of gene expression at the post-transcriptional level. SR proteins govern splice site selection and spliceosome assembly. The Arabidopsis genome encodes 19 SR proteins, several of which have no orthologues in metazoan. Three of the plant specific subfamilies are characterized by the presence of a relatively long alternatively spliced intron located in their first RNA recognition motif, which potentially results in an extremely truncated protein. In atRSZ33, a member of the RS2Z subfamily, this alternative splicing event was shown to be autoregulated. Here we show that atRSp31, a member of the RS subfamily, does not autoregulate alternative splicing of its similarly positioned intron. Interestingly, this alternative splicing event is regulated by atRSZ33. We demonstrate that the positions of these long introns and their capability for alternative splicing are conserved from green algae to flowering plants. Moreover, in particular alternative splicing events the splicing signals are embedded into highly conserved sequences. In different taxa, these conserved sequences occur in at least one gene within a subfamily. The evolutionary preservation of alternative splice forms together with highly conserved intron features argues for additional functions hidden in the genes of these plant-specific SR proteins.

INTRODUCTION
The accuracy of intron excision from pre-mRNAs relies on the precise recognition of rather degenerate splicing signals. Splice sites are defined by consensus sequences where only the two terminal nucleotides [GT at the acceptor (5') and AG at the donor (3') splice sites for the majority of introns] are highly conserved. The fidelity of the splicing process is attained through various exonic and intronic sequences acting as splicing enhancers or silencers thereby influencing splice site selection. The precise recognition of splice sites is achieved by the spliceosome, a large RNP particle consisting of 5 snRNPs and ~200 different proteins (1). Out of these, Ser/Arg-rich (SR) proteins play an important role in the regulation of splice site selection. SR proteins are a family of evolutionary conserved, structurally related, splicing factors which possess one or two RNA-recognition motifs (RRM) at the N-terminus and a C-terminal arginine/serine-rich (RS) domain (2,3). They are important for recognizing splice sites or exonic splicing enhancers and facilitate spliceosome assembly. It has been demonstrated that some SR proteins influence alternative splice site selection in a quantitative manner (4). Consequently, SR proteins are one of the determinants of splicing pattern present in different cells and at specific developmental stages or conditions.

Alternative splicing is an important mechanism for the generation of proteome complexity and fine tuning of gene expression at the post-transcriptional level both in the metazoan and plant species. Differential intron removal allows production of splice variants which may code for distinct protein isoforms affecting their post-translational modification, subcellular localization and the ability to interact with their binding partners. In addition, alternative splicing can also influence translational control and stability of mRNA transcripts.

Our analysis of the Arabidopsis genome has revealed 19 SR proteins which is almost twice as much as in humans (5,6). They fall into seven subfamilies (6) some of which have orthologues in metazoan (SF2/ASF, 9G8, SC35) but interestingly three of them seem to be plant-specific (RS, RS2Z and SCL). Many of the SR protein genes are alternatively spliced both in metazoan and in Arabidopsis (7–16). It has been noted that alternative splicing occurs mainly in
and around the long introns of the Arabidopsis SR genes ranging in size from ~400 to 1100 nt (6), while the typical size of plant introns is <150 nt (17). In atSRp30 and atSRp34/SR1, two Arabidopsis homologues of human SF2/ASF, such a long intron is situated near the 3’ end of the gene, and alternative splicing in this intron results in protein isoforms with shortened RS domains (12,15). Interestingly, all three plant-specific gene subfamilies possess a long intron at the beginning of the gene dividing the first RRM. Alternative splicing of this intron occurs in all three subfamilies and in a way that the potential protein is extremely truncated containing only a part of the RRM (6,13,14). Alternative splicing in the long intron of atRSZ33, a member of the RS2Z subfamily is autoregulated, as demonstrated by overexpression experiments (16). This autoregulation is crucial for correct gene expression levels, because overexpression of an intronless version of atRSZ33 is lethal.

In this study, we extend the analysis of alternative splicing and its regulation to the plant-specific RS subfamily, focusing on atRSp31. We also show that atRSZ33, a member of the RSZ subfamily, is involved in this regulation. In addition, we show that the position of the long intron and its capacity for alternative splicing is a characteristic feature of the plant-specific families of SR genes and is conserved from green algae to flowering plants. Moreover, sequences around the alternative splice sites in the long introns are much more conserved over large evolutionary distances than those in the region of the constitutive splice sites in the respective genes, indicating that they are under strong selective pressure.

MATERIALS AND METHODS

Accession numbers

Arabidopsis and rice sequences can be found at http://www.ncbi.nlm.nih.gov and http://www.tigr.org under following numbers: atRSp31 (At3g61860), atRSp31a (At2g46610), atRSp40 (At4g25500), atRSp41 (At5g52040), osRSp29 (Os04g02870), osRSp33 (Os02g03040), atRSZ32 (At3g35300), atRSZ33 (At2g37340), osRSZ36 (Os05g02880), osRSZ37a (Os01g06290), osRSZ37b (Os03g17710) and osRSZ39 (Os05g07000). Accession numbers of Arabidopsis, rice, maize, Pinus taeda, Physcomitrella patens and Chlamydomonas reinhardtii transcripts are in Supplementary Data.

Sequence retrieval and analysis

To identify splice variants of Arabidopsis SR proteins, corresponding genomic sequences were used in BLAST search against EST database limited to Arabidopsis thaliana at http://www.ncbi.nlm.nih.gov/BLAST/. ESTs with matches to corresponding gene were selected and then aligned using Genesequer (18) at http://www.plantdb.org.

To identify splicing events in rice, orthologues of atRSp31 and atRSZ33 were searched using Arabidopsis protein sequences at The TIGR Rice Database (http://tigrblast.tigr.org). Genomic sequences for retrieved proteins were used to get corresponding transcripts. Selected transcripts were aligned to genomic sequences using Genesequer.

Splice variants of maize orthologues of atRSp31 were retrieved at Genesequer using genomic sequences of zmRSp31A and zmRSp31B (19).

To identify orthologues of either atRSp31 or atRSZ33 in the distant species Arabidopsis protein sequences were used to pre-screen translated EST databases at http://www.ncbi.nlm.nih.gov/BLAST/ limited to Coniferophyta, Bryophyta or algae. A second screen was done using either atRSp31 or atRSZ33 protein sequences in a BLAST search limited to species detected in the pre-screen. EST sequences were assembled into contigs using the CAP2 program (20) at http://www.infobiogen.fr. Obtained contigs were analysed by blastx against Arabidopsis proteins. In addition, translated contigs were compared to Arabidopsis and rice SR proteins using ClustalW program (21) at http://clustalw.genome.jp. Shading of multiple alignment files was done with BOXSHADE at http://www.ch.embnet.org/.

Contigs were used to retrieve genomic sequences at NCBI and species specific databases (PHYSOCBase, http://moss.nibb.ac.jp; ChlamyDB http://www.chlamy.org/chlamydb.html). Genomic sequences were used in screen for additional ESTs representing splice variants. Gene structures and alternative splicing events were finally analysed using Genesequer.

Plant growth and protoplast transformation

Wild-type plants of A.thaliana (Col-0) and plants over-expressing atRSZ33 (16) were germinated and grown either on GM medium (22) or in soil under 16 h light/8 h dark photoperiod at 23°C. Arabidopsis cell suspension growth conditions and protoplasts preparation and transformation were as described previously (23). Protoplasts were collected by centrifugation and frozen in liquid nitrogen for RNA isolation or resuspended in SDS–PAGE loading buffer for analysis by western blotting, 24 h after transformation.

RNA isolation and analysis of alternative splicing forms

Total RNA from Arabidopsis plants and transformed cell culture protoplasts was isolated using RNAeasy Plant Mini Kit (Qiagen) and treated with DNase I (Promega). cDNAs of mRNA isoforms of atRSp31 were obtained by RT–PCR (24).

Primer 1, 5'-AAATGAGCTCCATTATGAAGTTTCTA-CTG-3', containing a SacI restriction site (underline), was used to prime reverse transcriptase.

Primer 2, 5'-AAACTGGATCCAGTCTCGTCAACTGCTG-CATAAGGG-3', and primer 3, 5'-ATATAGGGATCCCATGAGTCTCCTCTTGGGACCTGAGCAG-3', both of which contain an additional BamHI restriction site, were used for PCR. Splicing events in the second intron were investigated by PCR using primers from the adjacent exons: primer 4, 5'-ATATGAGCTCAGATTGCGAGTTAATA-GAGCACAG-3', and primer 5, 5'-ATATGAGCTCTTGGCCATTTCAACTGATAAAG-3', containing a SacI and BamHI restriction site, respectively.

To analyse regulation of the splicing profile in Arabidopsis cell suspension protoplasts transfected with either pHA-catRSp31 or pHA-gatRSp31 (see below), reverse transcription was carried out using primer 5'-CTTGAGTAGCTCTCAAGG-3'. PCR was performed using the same reverse primer and a direct primer either to the HA tag 5'-GATCTTACAAGATGGACTGTTGAAC-3' (to detect
transgenic atRSp31), or 5'-CGAATTACGGATCAAAGATG-
AGGCCA-3' (to detect endogenic atRSp31; the position of
the HA tag insertion in the pHA-catRSp31 or pHA-gatRSp31
is indicated by an arrow).

To control loading, RT–PCR of ubiquitin with following
primers 5'-CTCCCTTTTTCGGATACGGT-3' and 5'-CTC-
CTTCTTCTGTGGATAACGT-3' (25) was used. All PCR
products, except ubiquitin, were sequenced.

In vitro transcription and splicing in HeLa
splicing extracts

A DNA template for in vitro transcription of the complete
second intron was obtained by PCR amplification on the
genomic clone using primers 4 and 5. The PCR product was
subcloned into the SacI and BamHI sites of the vector pSP65
(Promega), linearized with BamHI and used for in vitro
transcription as described previously (26). RNA was purified and
stored in aliquots at −70°C. Splicing reactions were carried
out using the HeLa cell RNA Splicing System (Promega).
Products of splicing were analysed by RT–PCR using primers
4 and 5 and sequenced.

Protein purifications

The coding region of atRSp31 cDNA was amplified by PCR
using primers 6, 5'-ATATCCATGGGGGATGTTGTCG-
CGG-3', and primer 3, containing NcoI and BamHI restriction
sites, respectively. Following cleavage of the PCR prod-
uct with these enzymes, it was subcloned into the pET-3d expression vector (Novagen). To obtain the NcoI restriction
site, the fourth nucleotide of the coding region was changed
to G (see above primer 6, boldface G). Thus, in the expressed protein the second amino acid is changed from an arginine to
a glycine. Induction of recombinant protein expression in the
Escherichia coli strain BL21(DE3) pLysS was done according
to the Novagen protocol. atRSp31 was purified from inclu-
sion bodies as described previously (12). The SR proteins
from Arabidopsis, carrot and rabbit were purified using a two-step salt precipitation method as described in (26).

Preparation of constructs of HA-tagged atRSp31 for
protoplast transformation

The coding and genomic sequences of atRSp31 were ampli-
fied using primers 5'-CATGCCATGCGTTACCCATAT-
GAGTTCAGATTACGGTATGGGATGTTGTCGCGG-3'
(NcoI site, underlined; HA epitope tag, in boldface) and
5'-AAAACGTGCATGGTACAAAAGGTCTTCTC-3' (PstI
site is underlined). The resulting PCR fragments were cloned
into NcoI and PstI sites of pDEDH-Nco (27). Next, transla-
tion initiation sequence of pDEDH-Nco was replaced by the
second intron to allow monitoring the expression of alternative splice forms is regulated in a tissue/
organ-specific manner, it was interesting to check if a protein
is produced from these alternatively spliced mRNAs. Arabidopsis cell culture protoplasts were transiently trans-
fected with a genomic construct of atRSp31 under the control of a 35S CaMV promoter. A hemagglutinin (HA)-tag was
introduced just after the start codon to allow monitoring the
protein production. As shown in Figure 2A, lane 2, upper
panel, both alternatively spliced mRNAs could be detected,

RESULTS

Gene structure and alternative splicing forms of
atRSp31

The five introns of atRSp31 are arranged such that they pre-
dominantly separate protein domains (Figure 1). The first
intron is situated in the 5'-UTR, whereas the much longer
second intron divides the first RRM, separating the highly
conserved RNP2 and RNP1 domains. The third and the fourth
introns border the second RRM. Furthermore, the RS region
is delimited by the fourth and fifth introns so that the last
exon contains the remaining nine amino acids and the
3'-UTR. Previously, northern blot analysis of poly(A)+
RNA from various tissues of A. thaliana plants revealed at least three transcripts of atRSp31 which were differentially
expressed in roots, leaves, stems and flowers of wild-type
plants (11). RT–PCR amplification using total RNA was
performed, and the three main products of 1320, 1199
and 791 bp were subcloned and sequenced (Figure 1). The
791 bp form corresponds to the correctly spliced cDNA
(mRNA1) which was published earlier (11) and which
gives rise to a full-length 31 kDa protein. The longest form
(1320 bp) arises from the usage of an alternative 3' splice
site in the long intron (mRNA3) and encodes a short protein
of 71 amino acids (8.5 kDa) due to a new in frame stop codon
in the included intron (Figure 1). The hypothetical protein
contains the first 35 amino acids from the RRM1 domain
comprising the conserved RNP2 and 36 amino acids from
the included sequence of the second intron. In the 1199 bp
product, the same alternative 3' splice site is used as in
mRNA3 but an additional alternative 5' splice site is recog-
nized in this intron resulting in a new alternative exon
(mRNA2, Figure 1). However, this splicing event gives rise
to the same potential protein product as the one encoded
by mRNA3. Additionally, both mRNA2 and mRNA3
possess partial sequences of the third intron due to the use
of an alternative 3' splice site.

According to the RT–PCR analysis presented in the
Figure 1, all three splice forms of atRSp31 are expressed in
Arabidopsis flowers. In contrast, in wild-type suspension protoplasts (Figure 2A, middle panel) and wild-type seedlings
(Figure 2C, lane 1), only mRNA1 encoding the full-length
protein is produced. Interestingly, alternative mRNA2 and
mRNA3 are the only transcripts of atRSp31 detected by
northern blotting in the leaves and stems (11). Because expression of alternative splice forms is regulated in a tissue/
organ-specific manner, it was interesting to check if a protein
is produced from these alternatively spliced mRNAs. Arabidopsis cell culture protoplasts were transiently trans-
fected with a genomic construct of atRSp31 under the control of a 35S CaMV promoter. A hemagglutinin (HA)-tag was
introduced just after the start codon to allow monitoring the
protein production. As shown in Figure 2A, lane 2, upper
panel, both alternatively spliced mRNAs could be detected,
but no 8.5 kDa protein was visible in a western blot analysis with HA-antibodies (Figure 2B, lane 2). This experiment shows that under the conditions employed no protein from mRNA2 and mRNA3 is detectable.

**Figure 2.** Regulation of alternative splicing in *atRSp31*. (A) Analysis of *atRSp31* splicing in Arabidopsis cell culture protoplasts. Protoplasts were transiently transformed with either the cDNA or the genomic construct of *atRSp31* under 35S CaMV promoter (lanes 1 and 2). Lanes 3 and 4: control transformations with empty vector and water, respectively. Total RNA was analysed by RT–PCR. Upper panel: detection of the transgenic *atRSp31* using the primer to HA tag and the primer to the fourth exon of *atRSp31*. Middle panel: detection of the endogenous *atRSp31* using the primer to the sequence which is disrupted in transgene by insertion of HA tag and the primer to the fourth exon of *atRSp31*. Lower panel: RT–PCR of ubiquitin used as a loading control. (B) Immunodetection of HA-tagged *atRSp31*. Arabidopsis cell culture protoplasts were transformed with HA-tagged the cDNA or the genomic construct of *atRSp31* under 35S CaMV promoter (lanes 1 and 2, respectively). Total protein extracts were analysed by western blot using a monoclonal antibody against HA-tag. Lane M, molecular weight markers. (C) Regulation of alternative splicing in *atRSp31* by *atRSZ33*. Upper panel: total RNA from wild-type (lane 1) and seedlings overexpressing *atRSZ33* (lane 2) was analysed by RT–PCR using the primers to the beginning of the second and the end of the fourth exons of *atRSp31*. Lower panel: RT–PCR of ubiquitin used as a loading control.

**Regulation of alternative splicing in *atRSp31***

Experiments with another *Arabidopsis* SR protein, *atRSZ33*, had uncovered that this protein regulates splicing of its own pre-mRNA by changing alternative splicing in the similarly
positioned long intron in the middle of the RRM (16). We therefore asked whether atRSp31 might also regulate its own splicing. Previously, we had shown that recombinant atRSp31 can stimulate splicing activity of a HeLa cell S100 extract (11). As no plant splicing extract is available, we used the HeLa cell system to test the ability of recombinant atRSp31 to influence splice site choice in its own pre-mRNA. A construct containing the long intron of atRSp31 with adjacent exon sequences was used for \textit{in vitro} transcription (Supplementary Figure S1). Obtained pre-mRNA was spliced in a HeLa cell nuclear extract, and the RNA products were characterized by RT–PCR using primers from the adjacent exon regions. A time course of the splicing reaction (Supplementary Figure S1) revealed the production of correctly spliced mRNA but not of alternatively spliced mRNAs. Neither the addition of recombinant atRSp31 nor of SR protein preparations from \textit{Arabidopsis} (arabSR), carrot (carSR) or rabbit (rabSR) influenced this splicing pattern. These data show that the two splice sites of the long intron leading to the correct mRNA are strong splice sites in an animal splicing system. Therefore, if atRSp31 would indeed be able to influence splicing of its own pre-mRNA \textit{in vivo}, then more plant-specific splicing factors might be needed for autoregulation. Moreover, these alternative splicing events might be controlled by \textit{cis}-acting sequences not present on this short pre-mRNA construct.

To test these possibilities we transformed \textit{Arabidopsis} cell culture protoplasts transiently with either a genomic or cDNA construct of atRSp31 containing an N-terminal HA-tag, and analysed splicing \textit{by RT–PCR}. The RT reaction primer was from the end of the fourth exon, and the same reverse primer was used for the PCRs. To distinguish expression of transgenic or endogenous atRSp31 transcripts, the direct primer was either to the HA-tag or to the sequence which is disrupted in the transgene by the insertion of the HA-tag, respectively. The upper panel on Figure 2A shows expression of the transgenic cDNA construct (lane 1) and the genomic construct (lane 2) of atRSp31. Lanes 3 and 4 are control transformations with empty vector or water, respectively. The middle panel shows the expression of endogenous atRSp31 in the same samples, and in this case only correctly spliced mRNA was detected. Western blot analysis with an HA-tagged antibody confirmed that a protein is produced from both constructs (Figure 2B, lane 1, cDNA construct; lane 2, genomic construct). The amount of protein is much lower in the case of the genomic construct which conforms to the lower level of mRNA1. As the mRNA patterns of the endogenous transcripts were unchanged by overexpression of atRSp31, these experiments show that atRSp31 does not influence splicing of its own pre-mRNA.

Because atRSZ33 regulates splicing of a similarly positioned intron of its own pre-mRNA (16), we decided to check whether atRSZ33 is capable to affect also splicing in the long intron of atRSp31. Total RNA from 10 days old wild-type seedlings and seedlings overexpressing atRSZ33 was isolated and analysed by RT–PCR using primers to the second and fourth exons of atRSp31. In wild-type seedlings, mRNA1 coding for the full-length protein is the only splice form detected (Figure 2C, lane 1). Overexpression of atRSZ33 drastically affected splicing in the long intron of atRSp31 as mRNA1 is decreased and the production of the alternative splice variants mRNA2 and mRNA3 is greatly enhanced (Figure 2C, lane 2), strongly resembling the splicing pattern of atRSp31 in wild-type flowers (compare to Figure 1). The effect of atRSZ33 on alternative splicing of the long intron in atRSp31 is similar to the one in its own pre-mRNA, suggesting that atRSZ33 also regulates alternative splicing of atRSp31.

Alternative splicing in the \textit{Arabidopsis} plant-specific RS subfamily

atRSp31 is a member of the plant-specific RS subfamily, which includes also atRSp31a (6), atRSp40 and atRSp41 (11). atRSp31/atRSp31a and atRSp40/atRSp41 are two pairs of paralogues located at duplicated regions of the \textit{Arabidopsis} genome (6). All four genes contain long introns at the same position between RNP2 and RNP1 of the first RR. We were interested to analyse whether similar alternative splicing occurs in the other genes of this family as well. To identify potential alternative splicing events in atRSp31a, RT–PCR was performed with RNA from 6 days old seedlings, leaves, flowers and roots. Two main products were obtained (data not shown), one of them was the normally spliced mRNA and the other was a splice variant utilizing an alternative 5' splice site in the long intron of atRSp31a with the sequence matching the alternative 5' splice site in atRSp31 (Figure 3A and C). We did not detect a splice form with the alternative 3' splice site in the long intron of atRSp31a although this sequence is almost identical to atRSp31 (Figure 3B). In \textit{silico} search in the available cDNA and EST databases revealed also only the transcript using the alternative 5' splice site (Supplementary Table S1). Because of the high sequence conservation of the 3' alternative splice sites in both genes, we suppose that this splice site might be utilized in tissues and/or conditions different to those used in our analysis. However, we cannot exclude that certain \textit{cis}-elements necessary for the utilization of the alternative 3' splice site are absent in atRSp31a.

According to our previous data (11), only atRSp40 but not atRSp41 undergoes alternative splicing. In agreement with these data, database searches revealed alternative splice forms involving the long intron of atRSp40 only (Supplementary Table S1). Similar to atRSp31, alternative 3' and 5' splicing occurs in the long intron of atRSp40 (Figure 3A). However, comparison of the atRSp40 and atRSp31/atRSp31a sequences did not reveal any sequence similarity at the regions of their alternative splice sites. There is no experimental indication for alternative splicing in the long intron of atRSp41, and analysis of its intronic sequence did not reveal any potential splice sites similar to those in atRSp40 or in atRSp31/atRSp31a.

Evolutionary conservation of alternative splicing in the RS subfamily

Alternative splicing in most of the genes of the \textit{Arabidopsis} RS subfamily prompted us to examine the splicing profile of their orthologues in other species. As rice genome sequencing is finished, we decided to analyse alternative splicing events in this species. A BLAST search showed that the rice genome encodes only two proteins which belong
to the RS subfamily, Os02g03040 and Os04g02870. In a recent paper also these two proteins from this subfamily were identified, osRSp29 (Os04g02870) and osRSp33 (Os02g03040) (28). Both rice genes contain long introns at the identical positions in the first RRM (Figure 4). Moreover, analysis of available transcript data revealed alternative splicing events similar to those found in Arabidopsis (Figure 3A and Supplementary Table S1). For osRSp29, we were able to retrieve a single type of differential transcript utilizing alternative 3' and 5' splice sites at the same time. In osRSp33, there are two types of splice variants, one which also utilizes alternative 3' and 5' splice sites simultaneously and one which uses only the alternative 3' splice site (Figure 3A and Supplementary Table S1). However, only osRSp29 revealed a surprisingly high conservation of the sequences adjacent to the alternative 3' and 5' splice sites in the long introns. In comparison, sequence conservation around any constitutive splice site is poor (Figure 3B and C).

Recently, orthologues of atRSp31 in maize were identified and termed zmRSp31A and zmRSp31B. It has been shown that they produce multiple alternatively spliced transcripts (19). We compared the genomic sequences of the maize genes with their orthologues in Arabidopsis and rice, and found again the conserved sequences around the alternative 3' and 5' splice sites in the long introns (Figure 3B and C). Analysis performed using Geneseqer revealed the splice variants described by (19) as well as some new splice forms (Supplementary Table S1). Interestingly, different combinations of both conserved and non-conserved splice sites are utilized in the long introns of maize genes from RS subfamily (Figure 3A).

As both monocot species possessed the conserved sequences utilized for alternative splicing in the long intron we wanted to investigate how far this feature was conserved in more distant taxa. To find proteins from the RS subfamily we used the atRSp31 protein sequence in BLAST searches vs. translated EST databases of gymnosperms (P. taeda), bryophytes (P. patens) and algae (C. reinhardtii) (Materials and Methods).

We have identified five proteins that belong to the RS subfamily in P. taeda (Figure 4). In two of these genes, ptRSp34 and ptRSpNN, the intron position is conserved and the intron is alternatively spliced (Supplementary Figure S2 and Table S2). Because we lack genomic sequences for P. taeda we do not know if the sequences surrounding these splice sites are still conserved.

Figure 3. Conservation of alternative splicing events in the RS plant-specific subfamily. (A) Gene structures and alternative splicing. Domain structure typical for this subfamily of proteins is shown on the top. Gene structures are shown in green. ESTs/cDNAs representing each type of splicing are shown in red. Long introns are boxed. Blue and green arrows indicate conserved alternative 3' and 5' splice sites, respectively. Black arrow in atRSp31a indicates the presence of the conserved sequence of the alternative 3' splice site; however, in this case alternative splicing is not supported by EST/cDNA data. (B) Alignments of the nucleotide sequences around 3' alternative splice site in the long introns and around the 3' splice site of a constitutively spliced exon. Sequence of atRSp31a at the alternative 3' splice site is shown in italics to indicate the lack of EST data supporting this splicing event. (C) Alignments of nucleotide sequences around the 5' alternative splice site in the long introns and around the 5' splice site of a constitutively spliced exon. Intronic and exonic sequences are shown in small and capital letters, respectively. Nucleotides identical at all positions are shaded.
In *Physcomitrella*, a single protein from the RS subfamily was detected, which we named ppRSp27 (Figure 4). Comparison of the genomic sequence to ESTs revealed the presence of the long intron at the conserved position. However, no ESTs were found supporting alternative splicing in this gene. Alignment of the *Physcomitrella* and *Arabidopsis/rice/maize* genomic sequences also did not show any conserved alternative splice sites in the long intron.

We have also detected a single protein for the RS subfamily in *C. reinhardtii*, crRSp35 (Figure 4 and Supplementary Table S2). Interestingly, in crRSp35, the number and positions of the majority of introns are not conserved. The only introns which have conserved positions and phases in *Chlamydomonas* are the one corresponding to the long intron and the next one separating the RRM1 and RRM2. In addition, most introns in the *Chlamydomonas* gene have symmetrical phases (Figure 4). Nevertheless, existing EST data support alternative 3' splicing in the long intron of crRSp35 situated in the RRM similar to the one in atRSp31 (13,16). Analysing the second RS2Z gene in *Arabidopsis*, atRSZ32, we found that it is also alternatively spliced and produces similar splice variants as atRSZ33. One of them originates from the usage of the 3' alternative splice site in the long intron [Figure 5A and Supplementary Table S1 (accession nos BX823668 and BX825053)].

These analyses demonstrate that, in the RS subfamily, the presence of an alternatively spliced long intron at the position between RNP2 and RNP1 of the first RRM is highly conserved from green algae to angiosperms. Furthermore, the sequences of particular alternative splice sites in this intron are highly conserved between monocots and dicots (and possibly in gymnosperms) arguing for the evolution of a conserved alternative splicing mechanism for these genes in higher plant species.

Evolutionary analysis of alternative splicing in the plant specific RS2Z subfamily

The fact that atRSZ33 could equally modulate the conserved alternative splicing in the long intron of *atRSp31* as well as in its own pre-mRNA prompted us to investigate the evolutionary conservation of the long introns in the RRM of the plant specific RS2Z subfamily. Proteins of this subfamily are characterized by a single N-terminal RRM, two zinc knuckles, followed by an RS- and SP-rich domain (13). *Arabidopsis* has a paralogous pair of genes, *atRSZ33/atRSZ32*, which are located on duplicated regions of the *Arabidopsis* genome (6). We have published previously that alternative 3' splicing occurs in the long intron of *atRSZ33* situated in the RRM similar to the one in *atRSp31* (13,16). Analysing the second RS2Z gene in *Arabidopsis*, *atRSZ32*, we found that it is also alternatively spliced and produces similar splice variants as *atRSZ33*. One of them originates from the usage of the 3' alternative splice site in the long intron [Figure 5A and Supplementary Table S1 (accession nos BX823668 and BX825053)]. Another splice variant found in both *Arabidopsis* genes is created by the same event in the long intron and the retention of the following intron [Figure 5A and Supplementary Table S1 (accession nos BX823668 and BX825053)].

The BLAST search for proteins of this subfamily in rice revealed four genes: *osRSZ36* (Os05g02880), *osRSZ37a*
In Figure 5, Conservation of alternative splicing events in the RS2Z subfamily (A) Gene structures and alternative splicing. Domain structure typical for this subfamily of proteins is shown on the top. Gene structures are shown in green. ESTs/cDNAs representing each type of splicing are shown in red. Long introns are boxed. Blue arrows indicate the conserved alternative 3' splice sites. The black arrow in ppRSZ38 indicates the presence of the conserved sequences around the alternative 3' splice site; however, alternative splicing is not supported by EST data. (B) Alignments of nucleotide sequences around the alternative 3' splice site in the long introns and around a 3' constitutive splice site. Sequence of ppRSZ38 at the alternative 3' splice site is shown in italics to indicate the lack of EST data supporting this splicing event. Intronic and exonic sequences are shown in small and capital letters, respectively. Nucleotides identical at all positions are shaded.

The SR protein family from *Arabidopsis* includes three plant-specific subfamilies (RS, RS2Z and SCL) characterized by the presence of an alternatively spliced relatively long intron in their N-terminal RRM (6). Analysis of the alternative splice forms in all three subfamilies shows that they encode truncated putative proteins which would contain only a part of the RRM due to premature termination codons (PTC) generated by the inclusion of intronic sequences (13,14). If produced, these proteins should have no influence on splicing activity as they lack both RNA and protein interaction domains. To investigate the significance of these alternative splicing events we have analysed the regulation of alternative splicing of *atRSp31* from the RS subfamily. We found that its alternative splicing is regulated by atRSZ33, a member of the RS2Z subfamily. As this regulation is reminiscent of the autoregulation of the similar long intron in *atRSZ33* (16) we were interested to follow the evolutionary conservation of these splicing events in both plant-specific subfamilies in different lineages.

**DISCUSSION**

Further, we retrieved sequences of RS2Z proteins in gymnosperms, bryophytes and algae in a similar way as for the RS subfamily. The alignment of proteins which belong to RS2Z subfamily is shown in Figure 6. Intron positions and phases are conserved in all detected genes.

In *P. taeda*, we have found a protein, ppRSZ35, which belongs to this subfamily (Figure 6). Comparison of the ESTs revealed alternative splicing in the conserved position between RNP2 and RNP1 (Supplementary Figure S3 and Table S2). We conclude that the position and alternative splicing of this intron in RS2Z subfamily is conserved in angiosperms and gymnosperms; however, as genomic sequences are not available in *P. taeda* we cannot draw any conclusions about the conservation of the sequences preceding the alternative 3' splice site.

In *Physcomitrella*, at least one protein with all the domain features as well as exon–intron structure of the RS2Z subfamily is present, which we named ppRSZ38 (Figure 6). This protein contains an unusual glycine-rich stretch in front of the RNP2. ppRSZ38 contains a long intron at the conserved position. Alignment of genomic sequences revealed highly conserved alternative 3' splice site in the long intron of *ppRSZ38* almost identical to the corresponding *Arabidopsis*/*rice* sequences (Figure 5A and B). However, no EST was found in the available databases which would support alternative splicing in this intron. Whether this splice site is utilized in *Physcomitrella* needs to be proven.

Interestingly, in *C. reinhardtii* we found a single protein with a N-terminal RRM, an SP and RS regions, but no zinc knuckles (data not shown). This protein might therefore not be considered a true member of the RS2Z subfamily.

In summary, the RS2Z subfamily possesses a long intron in a conserved position in the RRM which contains intron sequences highly conserved from mosses to angiosperms. These conserved sequences are used for alternative splicing in monocots and dicots and most likely also in gymnosperms and mosses.
RS and RS2Z subfamilies. In addition, introns are arranged such that they predominantly separate protein domains. This is in agreement with the hypothesis of a modular assembly of functional or structural domains in the evolution of complex genes (29). The only exception is a Chlamydomonas member of RS subfamily, crRSp35. Beside two introns at conserved positions in the first RRM, this gene has additional introns, most of them are in symmetrical phases. At present it is not clear whether Chlamydomonas has gained the additional introns or if the exon–intron structure of the Chlamydomonas gene represents an ancestral arrangement and the other orthologues of the RS subfamily went through loss of introns.

One of the most conserved intron positions in both subfamilies is occupied by the relatively long intron separating RNP2 and RNP1. In addition to its particular length and conserved position, we have observed alternative splicing of these introns in some members of the plant-specific SR subfamilies. The functional significance of alternative transcripts can be best assessed by comparison of splicing profiles of orthologues in different species. Interestingly, in both subfamilies alternative splicing was found to be highly conserved in evolution in all species tested from angiosperms to green algae for the RS subfamily, and most likely down to the mosses for the RS2Z subfamily. In addition, a subset of alternative splicing events involves conserved 3' and/or 5' alternative splice sites, which are specific for each subfamily. These alternative splice sites conform to the splice site consensus; however, the surrounding sequences are much more conserved than in any of the constitutive splice sites in the corresponding genes. In the RS subfamily, the conserved intronic sequences are found both in monocots and dicots. In gymnosperms, the lack of genomic sequences does not allow to prove whether the observed alternative splicing events utilize the same conserved sequences. In contrast, the conserved intron sequences are present in the RS2Z family from angiosperms to mosses, although supporting experimental data are still missing for the latter class. Taken together the evolutionary preservation of these particular alternative splicing events suggests a functional, yet unknown significance. During preparation of this manuscript, an in silico analysis of alternative splicing events in plant SR proteins revealed that also the SCL subfamily possess conserved alternative splicing events although no hint for conserved intron sequences was presented (30).

It is worth noting that not all members of the subfamily in a species possess this particular alternative splicing event. For example, in the Arabidopsis RS subfamily, two genes, atRSp31 and atRSp31a, utilize these conserved alternative splicing signals, but not atRSp40 and atRSp41. Similarly, both rice orthologues, osRSp29 and osRSp33, are alternatively spliced, but only in osRSp29 alternative splicing involves the conserved sequences. In maize, both orthologues, zmRSp31A and zmRSp31B, produce multiple splice variants ([19] and this study), and some of them are generated by the simultaneous use of both conserved and non-conserved alternative splice signals. Similarly, among the four rice genes in the RS2Z subfamily, two have the conserved alternative splicing event at least in one member of the subfamily. These findings corroborate the significance of these highly conserved alternative splicing events. It therefore seems that in a given species selective pressure has preserved conserved alternative splicing events at least in one member of the subfamily. These findings corroborate the significance of these highly conserved alternative splicing events. They might be also a good example for the notion that gene function is more often correlated with splicing profile similarity between orthologues in different species than with sequence similarity of paralogues in the same species (31).

**Functional significance and regulation of the alternative splicing events**

Alternative splicing is an important mechanism for generation of proteome diversity and regulation of gene expression at the post-transcriptional level. In both RS and RS2Z subfamilies, all conserved alternative splicing transcripts potentially encode extremely truncated proteins containing only a
part of the RRM and some sequences from the included introns due to the generation of PTC. Our current data from the analysis of atRSp31 suggest that no truncated protein is made from these variant transcripts. Similarly, no truncated protein could be detected for atGRP7 which also contains an alternatively spliced long intron in the RRM domain (32). For maize orthologues of the RS subfamily, it has been shown that PTC-containing alternatively spliced transcripts associate with polysomes, but there is no evidence of their translation (19). Even if the alternative splice forms are translated, these short proteins will certainly have no splicing activity as they lack a complete RRM and RS domains. These alternative splicing events are different from those leading to the production of diverse protein isoforms. For example, alternative splicing events in the Arabidopsis homologues of human ASF/SF2 occur in long introns located in the 3' ends of genes. Here, splice variants encode protein isoforms with a shortened RS domains (12,15), which might affect the phosphorylation status of the protein and/or its ability to interact with other proteins.

Generally, alternative splicing events preserving the reading frame are more conserved than those leading to frameshifts. It has been shown that only 5% of cassette alternative exons are both conserved and have the potential to introduce PTC (33). It is therefore very unlikely that the evolutionary conserved alternative splicing events seen in the plant SR protein subfamilies have occurred just by chance as aberrant splicing events, as they are preserved in different subfamilies of genes and across distant taxa.

Given that no or only inactive proteins are made from the alternative transcripts, what functional significance could these conserved alternative splicing events in the long introns of the plant specific SR proteins have? Either the alternative splicing event is crucial for modulating the level of the splicing factor or the alternative transcript itself encodes an yet unknown activity. Although there is no evidence for the latter case, it has been shown previously that the presence of PTC in splice isoforms can promote nonsense-mediated decay (NMD) of such transcripts, and some splicing factors are subjected to such regulation (34,35). However, it has been shown recently by quantitative microarray profiling that NMD does not significantly influence the overall steady-state levels of transcripts with PTC generated by alternative splicing events (36). The alternative transcripts of atRSp31 are readily detectable by northern blotting and in some tissues they represent the only type of transcripts (26). It is therefore unlikely that the variant transcripts of atRSp31 are controlled by NMD, however, experimental proof is required.

Tight control of the atRSZ33 protein levels by autoregulation of alternative splicing has been demonstrated previously (16). Autoregulation seems to be conserved in the RSZ family as the rice homologue, osRSZ36, has also been shown to cause changes in its splicing pattern (28). Autoregulatory circuits have been shown for several non-plant splicing factors, such as SXL (37), TRA-2 (38), SWAP (39) and SRp20 (40). On the other hand, we have demonstrated here that overexpression of atRSp31, either in vitro or in vivo, does not stimulate usage of the alternative splice sites. Interestingly, also in rice none of the two homologues of atRSp31, osRSp29 and osRSp33, can change the splicing pattern of their pre-mRNA (28). Together with this, we show that atRSZ33 is involved in the regulation of alternative splicing in the long intron of atRSp31. However, atRSZ33 does not interact with atRSp31 in pull-down assays (13) which would imply that regulation of splicing in the long intron of atRSp31 by atRSZ33 occurs without direct interaction of these two proteins.

How and why are these alternative splicing events regulated? It has been noted that similar to animals, many of the alternatively spliced genes in Arabidopsis encode proteins with regulatory functions and many stress related genes are alternatively spliced (41). A genome wide analysis in A.thaliana uncovered many alternative splicing events in splicing factors and several of them were strongly influenced by cold stress (42). This is especially interesting as these alternatively spliced splicing regulators might coordinate alternative splicing of a particular set of stress response genes. Our unpublished data indicate that expression of atRSp31 is tightly regulated both transcriptionally and post-transcriptionally during plant development and in response to hormones, sugars and different light conditions. It would be extremely interesting to find out whether the highly conserved alternative transcripts of plant-specific SR genes have a specific role in development and/or environmental responses. Preservation of alternative splicing events that have often been attributed to accidents or erroneous action of splicing machinery argues for basic plant cell specific regulatory circuits established early in the plant evolution.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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