Title

The RNA Recognition Motif (RRM) of the *Arabidopsis* RS2Z32 and RS2Z33 splicing factors coordinates protein-protein and protein-RNA interactions, and contributes to their nucleocytoplasmic dynamics

Authors

Steven Fanara[1], Marie Schloesser[1], Méline Gérard[1], Simona De Franco[2], Marylène Vandevenne[2], Arnaud Vanden Broeck[3], Frédéric Kerff[4], Moreno Galleni[2], Frédéric Farnir[5], Marc Hanikenne[6,†] and Patrick Motte[1,†]

[1]InBioS-PhytoSystems, Functional Genomics and Plant Molecular Imaging, University of Liège, 4000, Liège, Belgium

[2]InBioS-Center for Protein Engineering, Laboratory of Biological Macromolecules, University of Liège, 4000, Liège, Belgium

[3]InBioS-Center for Protein Engineering, Laboratory of RNA Structural Biology and Biochemistry, University of Liège, 4000, Liège, Belgium

[4]InBioS-Center for Protein Engineering, Laboratory of Crystallography, University of Liège, 4000, Liège, Belgium

[5]FARAH, Biostatistics and Bioinformatics, University of Liège, 4000, Liège, Belgium

[6]InBioS-PhytoSystems, Translational Plant Biology, University of Liège, 4000, Liège, Belgium

[†]These two authors contributed equally.

Email address for each author

Steven Fanara: steven.fanara@univ-tlse3.fr

Marie Schloesser: mschloesser@uliege.be

Méline Gérard: melinegerard@gmail.com

Simona De Franco: sdefranco4@gmail.com

Marylène Vandevenne: mvandevenne@uliege.be

Arnaud Vanden Broeck: avbroeck@uliege.be

Frédéric Kerff: fkerff@uliege.be

Moreno Galleni: mgalleni@uliege.be
Frédéric Farnir: f.farnir@uliege.be
Marc Hanikenne: marc.hanikenne@uliege.be
Patrick Motte: patrick.motte@uliege.be

Corresponding authors
Steven Fanara (steven.fanara@univ-tlse3.fr)
Correspondence may also be addressed to Patrick Motte (patrick.motte@uliege.be)

Date of submission: 08/05/2025

Number of figures: 8

Number of supplementary figures: 10

Number of supplementary tables: 15

Running title

Functional characterization of RS2Z splicing factors

Highlight

Specific domains of the Arabidopsis RS2Z splicing factors contribute to their nuclear localization, nucleocytoplasmic dynamics, and ability to contact protein partners and specific pyrimidine-rich RNA motifs.

Abstract

The Arabidopsis splicing factors arginine/serine-rich zinc knuckle-containing proteins 32 and 33 (RS2Z32 and RS2Z33) are plant-specific members of the SR family. Here, we characterized both RS2Z32 and RS2Z33 by examining their expression profile at different stages of development and their spatial cellular distribution, as well as the contribution of their domains to the establishment of protein-protein interactions and to RNA binding specificity. We report that the *RS2Z32* and *RS2Z33* promoters are ubiquitously active during vegetative and reproductive growth, and that both RS2Z splicing factors localize in the nucleus (except the nucleolus). We show that the C-terminal arginine/serine-rich (RS) domain, but not the serine/proline-rich (SP) extension, is a determinant of nuclear localization. We demonstrate that their RNA recognition motif (RRM) domain specifically binds pyrimidine-rich RNA motifs via three residues (Y14, Y46, F48), and is also involved in protein-protein interactions with at least three SR proteins, namely SR45, SCL30, and SR34. Finally, we show that mutations in RNA-binding domains (i.e. RRM and zinc knuckles, ZnKs) affect the nucleocytoplasmic dynamics of both RS2Z proteins. Our findings provide molecular evidence for the involvement of plant-specific SR splicing factors into the regulation of the splicing process.

Keywords: Arabidopsis, RS2Z32, RS2Z33, serine/arginine-rich splicing factors, RNA recognition motif, zinc knuckles, pre-mRNA splicing, protein-RNA and protein-protein interaction, nucleocytoplasmic shuttling.

Introduction

The precursor messenger RNA (pre-mRNA) splicing process requires the precise recognition of splice sites at exon-intron boundaries. It is achieved by the spliceosome, consisting of small nuclear ribonucleoproteins (snRNPs) and many non-snRNP-associated proteins (Meyer *et al.,* 2015). Splicing may produce a single transcript from the pre-mRNA by excluding all introns or generates several mRNAs from a single precursor sequence through different alternative splicing events (Marquez *et al.,* 2015). When affecting coding sequences, alternative splicing may lead to proteomic diversity (Manuel *et al.,* 2023), or to nonsense-mediated mRNA decay (NMD) due to premature termination codons (PTCs) occurring in aberrant alternative transcripts (Chen and Manley 2009; Palusa and Reddy 2010; Kalyna *et al.,* 2012). Native and alternative protein isoforms usually diverge in their primary sequence or domain organization, which could alter the interaction network established with protein, DNA or RNA, as well as the protein function, stability, or intracellular localization (English *et al.,* 2010; Kalyna *et al.,* 2012; Drechsel *et al.,* 2013; Remy *et al.,* 2013; Kashkan *et al.,* 2022).

Serine/arginine-rich (SR) splicing factors are phylogenetically highly conserved in multicellular eukaryotes (Califice *et al.,* 2012). SR proteins typically share a modular structure defined by one or two N-terminal RNA recognition motifs (RRMs) and a C-terminal domain rich in arginine-serine dipeptide repeats (RS) (Barta *et al.,* 2010; Manley and Krainer 2010; Califice *et al.,* 2012). The RRM domain(s) support(s) RNA binding affinity and specificity (Maris *et al.,* 2005; Daubner *et al.,* 2013; Fanara *et al.,* 2024). The RS domain undergoes extensive reversible phosphorylation (Aubol *et al.,* 2016; Aubol *et al.,* 2017). The phosphorylation landscape of the RS domain modulates the ability of SR proteins to contact protein partners, as well as the nucleocytoplasmic dynamics, the subcellular localization, and the protein stability of SR proteins (Kataoka *et al.,* 1999; Lai *et al.,* 2000; Lai *et al.,* 2001; Xu *et al.,* 2011; Albuquerque-Martins *et al.,* 2023). A phylogenetic analysis of RRM-containing proteins highlighted the existence of nineteen SR proteins in *Arabidopsis thaliana* (Arabidopsis) (Califice *et al.,* 2012), including SR45 considered as a SR-like protein (Barta *et al.,* 2010). SR proteins are subdivided into several groups, defined by a distinct multidomain organization, among which the plant-specific RS2Z subfamily (Barta *et al.,* 2010; Califice *et al.,* 2012).

The zinc knuckle-containing SR splicing factors (RS2Z) bear two CCHC-type zinc knuckles (ZnKs) located between a unique RRM and the RS domain, as well as a C-terminal

serine/proline-rich (SP) domain of unknown function (Barta *et al.,* 2010; Califice *et al.,* 2012). The overexpression of *RS2Z33* was shown to lead to increased cell expansion, a modified polarization of cell elongation and division, and altered auxin signalling (Kalyna *et al.,* 2003). The involvement of RS2Z33 in cell patterning and root meristem function was recently further corroborated, depicting a role for RS2Z33 in primary and lateral root development (Thompson *et al.,* 2023). Both *RS2Z* genes undergo alternative splicing events leading to NMD features (e.g., PTC upstream of a splice junction) (Kalyna *et al.,* 2012; Thompson *et al.,* 2023). Post-translational modifications of native RS2Z isoforms were previously reported (Kim *et al.,* 2013; Liang *et al.,* 2019; Wang *et al.,* 2023). The detailed inventory of protein partners for RS2Z33 regroup many splicing factors (Lopato *et al.,* 2002; Gullerova *et al.,* 2006). Recently, SR45 was identified as a novel interactor of both RS2Z32 and RS2Z33 (Fanara *et al.,* 2024).

In Arabidopsis, RS2Z32 and RS2Z33 proteins remain poorly characterized at the molecular level. In this study, we aimed to characterize both proteins through the investigation of their spatio-temporal expression pattern, their subcellular distribution and nucleocytoplasmic dynamics, and their protein-protein interaction network. We further determined amino acid residues supporting these functions. We also identified the RNA motif bound by the RRM domain of each RS2Z proteins, observing slight modifications in the motifs specifically recognized by each protein. We showed how three amino acids (Y14, Y46, F48) define their RNA binding specificity. Finally, we showed that phosphorylatable serine residues are necessary and sufficient for establishing the RS2Z protein-protein interaction network. Our findings provide new insights into the molecular function of plant-specific splicing factors.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (Col-0 ecotype) plants were stably transformed by floral dipping, and at least three independent T3 homozygous lines were analyzed for each construct. Seeds were surface-sterilized, then sown on half-strength Murashige and Skoog (MS) medium (Duchefa Biochimie) containing 1% (w/v) sucrose (Duchefa Biochimie) and 0.8% (w/v) Select Agar (Sigma-Aldrich). After a 48-hour stratification period at 4°C in the dark, plates were transferred to a climate-controlled growth chamber (21°C) under an 8-h light (100 µmol m^{-2} sec^{-1})/16-h dark regime. Three-week-old seedlings were subsequently transferred in hydroponic trays (Araponics,

Liège, Belgium) and cultivated for 7 weeks in control Hoagland solution (Talke *et al.,* 2006; Hanikenne *et al.,* 2008; Fanara *et al.,* 2022) (until silique development) under a 16-h light (100 µmol m^{-2} sec^{-1})/8-h dark regime in a climate-controlled growth chamber (21°C). Expression profiling was conducted on 10-week-old plants hydroponically grown. The nutrient medium was renewed with fresh solution once a week.

Transient transformation of *Nicotiana tabacum* (cv. Petit Havana) leaves was carried out by *Agrobacterium*-mediated infiltration as described (Rausin et al., 2010; Stankovic et al., 2016).

Vector construction

All binary vector constructions and primers used in this study are listed and detailed in **Supplementary Tables S1-7**. Gene coding sequences and promoter regions were amplified from Arabidopsis cDNA libraries or genomic DNA, respectively, using the Q5 High-Fidelity DNA polymerase (NEB). Construct assemblies were verified by Sanger sequencing. For both transient transformation of tobacco leaves and stable transformations of Arabidopsis plants, all final plasmids were electroporated into *Agrobacterium tumefaciens* strain GV3101 (pMP90) and used for agroinfiltration or Arabidopsis floral dipping, respectively.

For directed yeast two-hybrid (dY2H) assays, coding sequences of potential interactors were cloned into pGBKT7/pGBKT7(+) and pGADT7/pGADT7(+) vectors (Clontech) using different restriction sites (**Supplementary Table S1** for primer lists) (Fanara *et al.,* 2024).

The promoter regions of *RS2Z32* and *RS2Z33* (701 bp and 1110 bp upstream of the ATG corresponding to the entire intergenic regions, respectively) were PCR-amplified from genomic DNA and inserted into the *Sbf*I/*Kpn*I sites of the pMDC32 vector (Curtis and Grossniklaus 2003) to generate pMDC32:pRS2Z vectors. Subsequently, the open reading frames of *RS2Z32* (852 bp) and *RS2Z33* (870 bp) (from the ATG to the last codon before the stop codon, respectively) were cloned into the *Asc*I/*Pac*I sites to obtain pMDC32:pRS2Z:RS2Z intermediate constructs. Finally, the *EGFP* coding sequence was inserted at the *Pac*I site to create the pMDC32:pRS2Z:RS2Z-EGFP vectors. For the pMDC32:pRS2Z:EGFP constructs, the 2X35S promoter of the pMDC32 vector (Curtis and Grossniklaus 2003) was replaced by the corresponding *RS2Z32* or *RS2Z33* promoter and *EGFP* was inserted at *Asc*I and *Eco*RI restriction sites **(Supplementary Table S2)**.

The *RS2Z32mutRRM* and *RS2Z33mutRRM* versions were obtained by PCR-based site-directed mutagenesis, introducing alanine substitutions in key RNA-contacting residues

(Y14A in RNP2 motif, and Y46A and F48A in RNP1 motif) (Daubner *et al.,* 2013), as previously described (Rausin *et al.,* 2010; Stankovic *et al.,* 2016) **(Supplementary Table S3)**. Additional mutant variants were assembled by PCR from partially overlapping amplicons covering specific domain of RS2Z32 or RS2Z33. These were amplified using either the native RS2Z genes, the *RS2ZmutRRM* template, or sequences harboring RS or SP domain mutations (named RA and AP, respectively) synthesized by GenScript (see **Supplementary Tables S4 and S5**). All assembled mutant versions **(Supplementary Figure S1)** were subsequently cloned into yeast vectors for Y2H (at *Eco*RI/*Bam*HI) or pMDC32 fused to EGFP for protein localization (at *Asc*I/*Pac*I sites), respectively.

Bimolecular fluorescence complementation (BiFC) constructs were generated as previously described (Stankovic *et al.,* 2016; Fanara *et al.,* 2024) **(Supplementary Table S6)**.

For SELEX experiments, coding sequences for the RRM or RRM+ZnKs of RS2Z32 and RS2Z33 were cloned into the pGEX6P-1 (at *Bam*HI/*Eco*RI sites) as described (De Franco *et al.,* 2019) **(Supplementary Table S7)**.

Yeast two-hybrid analyses

The vectors and yeast strains (*Saccharomyces cerevisiae*) provided in the Matchmaker Gold Yeast Two-Hybrid System (Clontech) were used to perform directed yeast two-hybrid (dY2H) analyses by co-transformation of the Y2HGold strain. Prior to (directed) Y2H experiments, toxicity and autoactivation of the wild-type RS2Z32 or RS2Z33 proteins and mutant variants were tested as previously described (Fanara *et al.,* 2024) **(Supplementary Figure S2)**.

The Y2HGold strain transformed with the bait vector (pGBKT7-RS2Z32 or pGBKT7-RS2Z33) was used for Y2H screening (including both commercial and custom Arabidopsis cDNA libraries) as previously described (Fanara *et al.,* 2024).

Analysis of mRNA levels

Total RNAs were extracted from 100 mg of plant tissues using NucleoSpin RNA Plant kit (Macherey Nagel). First-strand cDNA synthesis was carried out using 1 µg of total RNAs was used to synthesize cDNAs using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific) with oligo(dT) primers. Quantitative PCR reactions were performed as previously described (Fanara *et al.,* 2024). A total of three technical replicates were performed for

each combination of cDNA and primer pair **(Supplementary Table S8)**. Gene expression was normalized relative to *At1g58050* as described (Pfaffl 2001). *At1g58050* expression was the most stable gene among all tested references (*EF1α* and *UBQ10*) (Czechowski *et al.,* 2005; Spielmann *et al.,* 2020).

Confocal Microscopy

Live-cell imaging was performed on Leica TCS SP2 and SP5 inverted confocal laser-scanning microscopes (Leica Microsystems) equipped with a water-immersion objective. Images were acquired at a resolution of 512x512 pixels. EGFP or YFP fluorescence was imaged as previously described (Fanara *et al.,* 2024). The Fluorescence loss in photobleaching (FLIP)-shuttling experiments were carried out as previously described (Tillemans *et al.,* 2006; Rausin *et al.,* 2010; Stankovic *et al.,* 2016) and quantitative analyses were conducted as outlined in the statistical analysis section below **(Supplementary Table S9)**.

Production of recombinant proteins and SELEX experiments

Protein productions (native and mutated RRM of RS2Z32 or RS2Z33, or native RRM+ZnKs of RS2Z32 or RS2Z33) was performed using the expression vector pGEX6P-1 in *E. coli* BL21(DE3) cells as previously detailed (De Franco *et al.,* 2019; Fanara *et al.,* 2024). The Systematic Evolution of Ligands by EXponential enrichment (SELEX) experiment was performed using a randomized 25-nt DNA template library as previously reported (De Franco *et al.,* 2019; Fanara *et al.,* 2024), adding 100 µM $ZnCl_2$ in SELEX buffer when studying RRM+ZnKs recombinant proteins. At the end of the experience (round 4), final PCR products obtained from enriched RNA sequences were subcloned into the pJET vector (CloneJET PCR Cloning Kit, Thermo Fisher Scientific) and sequenced **(Supplementary Tables S10-15)**. The obtained sequences were analyzed using the MEME tool (version 5.5.1) (Bailey *et al.,* 2009). Logos were redesigned using WebLogo software (Crooks *et al.,* 2004).

Structural modeling of the RS2Z proteins in presence of Zn^{2+} ions

Three-dimensional model predictions of the RS2Z proteins were performed using the AlphaFold 3 server (https://alphafoldserver.com) (Abramson *et al.,* 2024). A total of 25 models were generated, all in the presence of two Zn^{2+} ions. All models were visually inspected, and the

ones with the best pTM and ipTM (predicted template modeling, and interface predicted template modeling) scores were further analyzed using ChimeraX (Pettersen *et al.,* 2021).

Statistical analysis

For quantitative RT-PCR, data evaluation and statistics were performed using GraphPad Prism 8 (GraphPad Software v8.0). The replication level of each experiment is described in the figure legends. For FLIP-shuttling assays, all statistical analyses have been performed using SAS 9.4. Percentages (relative to the initial level) of fluorescence have been analyzed using mixed linear models (MIXED procedure). Modeled factors included the protein (native or mutated versions), the time (sampled every 10 seconds from 0 to 120 seconds), the drug (presence or absence) and their 2- and 3-ways interactions. Since only 2-ways interactions between drug and protein, and drug and time were significant, other interactions were removed from the final model. Correlations between residuals from the same sample were modeled using a compound symmetry structure, and the degrees of freedom used in the tests were corrected using a Kenward-Roger correction. All relevant contrasts have been estimated and tested using the ESTIMATE function of the MIXED procedure. *p*-values lower than 0.05 were deemed significant. Statistics displayed on figures related to FLIP-shuttling assays, which compared –LMB and +LMB groups, were generated using means fluorescence intensities of each experimental group that were processed using a two-way repeated measures analysis of variance (ANOVA) approach followed by a Bonferroni multiple comparisons post-test.

Accession numbers and information

All gene sequences are available through The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/), with the following accession numbers: Arabidopsis *ACINUS* (AT4G39680), *AFC1* (AT3G53570), *AFC2* (AT4G24740), *AFC3* (AT4G32660), *ALY4* (AT5G37720), *Cyp59* (AT1G53720), *Cyp65* (AT5G67530), *Cyp71* (AT3G44600), *CypRS64* (AT3G63400), *CypRS92* (AT4G32420), *eIF4A3* (AT3G19760), *GRP7* (AT2G21660), *GRP8* (AT4G39260), *MAGO* (AT1G02140) *MOS12* (AT2G26430), *MOS14* (AT5G62600), *PININ* (AT1G15200), *PRP38* (AT2G40650), *RS2Z232* (AT3G53500), *RS2Z33* (AT2G37340), *RS31* (AT3G61860), *RS31a* (AT2G46610), *RS40* (AT4G25500), *RS41* (AT5G52040), *RSZ21* (AT1G23860), *RSZ22* (AT4G31580), *RSZ22a* (AT2G24590), *RZ-1B* (AT1G60650), *RZ-1C*

(AT5G04280), *SAP18* (AT2G45640), *SC35* (AT5G64200), *SCL28* (AT5G18810), *SCL30* (AT3G55460), *SCL30a* (AT3G13570), *SCL33* (AT1G55310), *SR30* (AT1G09140), *SR34* (AT1G02840), *SR34a* (AT3G49430), *SR34b* (AT4G02430), *SR45.1* (AT1G16610.1), *SR45.2* (AT1G16610.2), *SRPK1* (AT4G35500), *SRPK2* (AT2G17530), *SRPK3a* (AT5G22840), *SRPK3b/SRPK5* (AT3G44850), *SRPK4* (AT3G53030), and *Y14* (AT1G51510).

Results

RS2Z genes encode nuclear proteins and are expressed at all developmental stages

Quantitative RT-PCR profiling showed that the *RS2Z32* and *RS2Z33* genes were expressed in vegetative (roots, leaves, stems and seedlings) and reproductive (inflorescences and siliques) organs examined during plant development. *RS2Z32* was significantly more expressed compared to *RS2Z33* in all organs but roots, with a significant greater expression for both genes in siliques and young seedlings **(Figure 1)**. This corroborates and expands the previous quantification available for these genes (Palusa *et al.,* 2007; Cruz *et al.,* 2014). The expression profile of both *RS2Z* genes and the subcellular and tissular distribution of the two splicing factors were also further determined using promoter:reporter lines [*pRS2Z32:EGFP* **(Supplementary Figure S3A-M)** or *pRS2Z33:EGFP* **(Supplementary Figure S3a-m)**] and GFP translational fusions [*pRS2Z32:RS2Z32-EGFP* **(Figure 2A-Q)** or *pRS2Z33:RS2Z33-EGFP* **(Figure 2a-q)**], respectively. Translational fusion lines displayed a nuclear localization of both RS2Z members in the seed coat **(Figure 2A/a)** and in embryos freshly dissected from seeds **(Figure 2B/b)**. During vegetative growth, the hypocotyl and cotyledons displayed fluorescence **(Figure 2C/c-D/d)**, which was also detected in roots and root hairs **(Figure 2E/e-F/f)**, as well as leaves, trichomes and stomata **(Figure 2G/g-I/i)**. Immature floral buds showed intense fluorescence signals in the receptacle, the abscission zone, sepals, petals, stamens and the pistil **(Figure 2J/j)**. During flowering, mature styles and stigmas **(Figure 2K/k)**, as well as stamens (anthers, filaments and pollen grains) **(Figure 2L/l-N/n)** displayed fluorescence signals. Fluorescence was also observed in ovules, funiculi **(Figure 2O/o)**, and fully developed petals and sepals (including veins) **(Figure 2P/p-Q/q)**. The observations in reporter lines were in agreement with those of the translational fusion lines, and also largely complete previous results depicting the activity of the promoter of *RS2Z33* in the embryo, leaves, roots, ovules and pollen grains (Kalyna *et al.,* 2003) **(Supplementary Figure S3A/a-M/m)**.

Structural modeling of the RS2Z proteins

Prior to directed mutagenesis of their different constitutive domains, structural modeling was performed for both RS2Z32 and RS2Z33 proteins. During the analysis of their full-length models, the predicted local distance difference test (pLDDT) —a per-atom confidence estimate— was used to evaluate model reliability. As anticipated, each protein model showed the presence of

one RRM domain (residues 10–79) predicted with very high confidence (pLDDT >90), followed by two consecutive Zn^{2+}-bound zinc knuckles (residues 100–138; hereafter referred as ZnK1 and ZnK2, and ZnKs if discussed simultaneously) predicted with high confidence (pLDDT >70). Additionally, both proteins featured an extended unstructured C-terminal region (residues 143–284 in RS2Z32 and 143–290 in RS2Z33) corresponding to their respective RS and SP domains, predicted with low confidence (pLDDT <50) **(Figure 3A-B)**. As observed in the models, the RS2Z canonical RRM was defined by a β1–α1–β2–β3–α2–β4 topology containing the residues thought to mediate RNA recognition and specificity (Y14 within the β1-strand, and Y46 and F48 within the β3-strand) (Daubner *et al.,* 2013). The ZnKs were structured through Zn^{2+} metallic coordination that was mediated by C101, C104, H109 and C114 (ZnK1) and C123, C126, H131 and C136 (ZnK2) within 3 Å **(Figure 3A-B)**. These results suggest that substitutions of all serine to alanine residues within either the RS domain or the SP domain would not affect the global structure of the RS2Z proteins as these domains appeared to be intrinsically disordered. On the other hand, Cys-to-Ala substitutions would likely inhibit the ability of ZnKs to bind Zn^{2+}, leading to the abolition of their presumed RNA-binding activity. Finally, the canonical RRM (residues 10 to 79) would likely maintain its three-dimensional structure upon Y14A, Y46A and F48A mutations.

RS2Z localization depends on native serine residues within the RS domain

The subcellular localization of the two RS2Z proteins, as well as mutant variants for each of their domains, was next examined. Twelve mutant variants were engineered **(Supplementary Figure S1)**. First, the RS2ZmutRNP2 and RS2ZmutRNP1 variants were obtained by substituting to alanines conserved residues mediating the RNA specificity [Y14 (RNP2), Y46 (RNP1) and F48 (RNP1)] of the canonical RRM (Cléry *et al.,* 2008), and combining these substitutions generated the RS2ZmutRRM variants. Second, the 3 cysteine residues from each CCHC-type zinc knuckle were substituted into alanine residues to generate the RS2ZmutZnK1 and RS2ZmutZnK2 variants, whose mutations were combined to obtain the RS2ZmutZnKs variants. Mutations within the RRM and both ZnKs were merged to produce the RS2ZmutRRM+ZnKs variants. Third, all serine were substituted to alanine residues within the RS (17 S-to-A substitutions) and/or SP domains (15 and 16 S-to-A substitutions for RS2Z32 and RS2Z33, respectively), generating three variants (mutRS, mutSP, mutRS+SP), respectively. Finally, RRM

and RS or SP mutations were combined, producing two more mutant variants (mutRRM+RS and mutRRM+SP) **(Supplementary Figure S1)**.

The intracellular localization of the native and variant RS2Z proteins was determined after transient expression in tobacco leaf cells, confirming the localization of RS2Z32 and RS2Z33 within the nucleoplasm and speckles-like structures but not in the nucleolus (Lorković and Barta 2004; Tillemans *et al.,* 2005) **(Figure 4A/a)**, similarly to what was observed in leaves of Arabidopsis stable plants expressing the GFP translational fusions **(Figure 4J/j)**. This localization was maintained in the mutant variants RS2ZmutRRM **(Figure 4B/b)** (or lower order mutant variants; mutRNP1 and mutRNP2) **(Supplementary Figure S4A-B)**, RS2ZmutZnKs **(Figure 4C/c)** (or lower order mutant variants; mutZnK1 and mutZnK2) **(Supplementary Figure S4C-D)**, RS2ZmutRRM+ZnKs **(Figure 4D/d)**, RS2ZmutSP **(Figure 4F/f)** and RS2ZmutRRM+SP **(Figure 4I/i)** mutant variants. In contrast, RS2ZmutRS **(Figure 4E/e)**, RS2ZmutRS+SP **(Figure 4G/g)** and RS2ZmutRRM+RS **(Figure 4H/h)** mutant variants partially accumulated in the cytoplasm in addition to a nuclear and nucleolar localization. Of note, the RS2ZmutRRM+RS displayed a fainter cytoplasmic signal compared with the two other mutant variants bearing the mutated RS **(Figure 4H/h)**. Altogether, the presence of native serine residues within the RS domain appears necessary to ensure a strict nucleoplasmic localization of both RS2Z32 and RS2Z33.

RS2Z dynamics depends mostly on the RRM integrity to be partly controlled by XPO1

We previously determined that RSZ22, SR34 and SR45 are shuttling splicing factors whose nuclear export might be partly controlled by the XPO1-dependent export pathway (Tillemans *et al.,* 2006; Rausin *et al.,* 2010; Stankovic *et al.,* 2016). We further showed that the canonical SR34 RRM (RRM1) contributes to such nucleocytoplasmic dynamics (Stankovic *et al.,* 2016). On the contrary, the canonical RRM or ZnK of RSZ22 are dispensable for nucleocytoplasmic shuttling (Rausin *et al.,* 2010). In this context, we aimed to determine whether (i) the RS2Z proteins displayed a shuttling behavior, (ii) whether it may be dependent on the XPO1 export pathway, and (iii) whether the RRM and/or the ZnKs contributed to shuttling. We specifically characterized the dynamics of RS2Z32 and RS2Z33 using FLIP-shuttling assays (Tillemans *et al.,* 2006), focusing on the mutants harboring mutations within the RNP motifs of the RRM and/or within the CCHC-type zinc knuckle(s) **(Supplementary Figure S1)**. The native

RS2Z proteins were highly dynamic factors, as indicated by the rapid loss of fluorescence upon photobleaching **(Figure 5A/a)**. Treatment with leptomycin B (LMB), a XPO1-specific inhibitor, led to a statistically significant lower shuttling kinetics, suggesting a XPO1-dependent nuclear export **(Figure 5A/a)**. Mutations in the RNP1, RNP2 or ZnK2 significantly altered the shuttling activity of the RS2Z proteins in absence of LMB treatment compared to the native RS2Z protein **(Figure 5B/b-E/e, insets; Supplementary Table S9)**. In contrast, the RS2ZmutZnK1 displayed similar dynamics than the native RS2Z proteins **(Figure 5D/d, insets; Supplementary Table S9)**. Combining either RNP1 and RNP2 or ZnK1 and ZnK2 mutations (i.e. mutRRM and mutZnKs, respectively) did not impair more the shuttling dynamics of these mutant variants in absence of LMB **(Supplementary Figure S5A/a-B/b, insets)**; however, the RS2ZmutRRM+ZnKs variant remained as dynamic as the native RS2Z proteins **(Supplementary Figure S5C/c, Supplementary Table S9)**. The RS2ZmutZnK2 variant showed no significant impairment compared to the native RS2Z proteins upon LMB treatment, in contrast to the RS2ZmutRNP1 variant that appeared more sensitive to LMB. All other mutant variants displayed an increased shuttling kinetics compared to the native RS2Z proteins, resulting in a faster rate of fluorescence loss when subjected to LMB **(Figure 5 and Supplementary Figure S5, and Supplementary Table S9)**. Interestingly, the RS2ZmutRNP2 variant was not sensitive to LMB treatment, displaying shuttling kinetics similar to what was observed without LMB treatment **(Figure 5C/c, Supplementary Table S9)**.

The RRM of RS2Z proteins contributes, together with the RS domain, to contacting splicing factors

A previous study described a yeast two-hybrid (Y2H) screen to identify RS2Z33 protein interactors (Lopato *et al.,* 2002). It used a truncated version of the protein (RS2Z33ΔSP) due to the toxicity of the full-length protein in yeast. In this screen, RS2Z33ΔSP was found to interact with SR34, RSZ21, RSZ22, SC35, SCL28, SCL30, and SCL33 (Lopato *et al.,* 2002). As the native untruncated RS2Z32 protein was recovered during a recent Y2H screen using SR45 as a bait (Fanara *et al.,* 2024), we used here full-length versions of the two RS2Z proteins in new Y2H analyses using two Arabidopsis cDNA libraries (a commercially available and a custom-made) in yeast two-hybrid (Y2H) assays. This confirmed known interactions of RS2Z33 with SCL30 (Lopato *et al.,* 2002), SR45.1 and SR45.2 (Zhang and Mount 2009; Fanara *et al.,* 2024), and led

to the identification of previously unidentified partners of RS2Z32 (SCL30 and CypRS64). Directed yeast two-hybrid assays (dY2H) allowed the confirmation of these interactions **(Figure 6A, Supplementary Figure S6 and Supplementary Figure S7)**.

The dY2H set-up was further used to examine associations between all Arabidopsis SR proteins and the native RS2Z proteins. Contrary to RS2Z interactions with RSZ21, SR34a, SCL28 or SCL30a, which appeared to be relatively weak, RS2Z strongly associated to SR45 isoforms, SR34, SCL30 and SCL33 **(Supplementary Figure S7A-B)**. Upon testing all mutant variants engineered in this study, the RS2Z contacts with the two SR45 isoforms were disrupted (e.g., no growth of yeast cells) upon RS mutations, and the RS2ZmutRRM variants showed a weakened (e.g., fewer colonies able to grow) association to both SR45 isoforms **(Figure 6A)**. In contrast, we observed a number of distinct interaction patterns of RS2Z mutant variants and the two SR45 isoforms: (i) the association of RS2ZmutRRM variants with SR45.2 was weaker than with SR45.1, as shown by the absence of growth of yeast cells upon serial dilutions (e.g., OD 0.025) **(Supplementary Figure S6)**; and (ii) the RRM+ZnKs mutations abolished contacts with SR45.2 but not with SR45.1 **(Figure 6A)**. We further showed that the sole RS2Z RRM (RS2ZΔZnKs+RS+SP) sustained a weakened interaction with SR45 isoforms **(Supplementary Figure S7C)**, and that the RS2Z proteins were predominantly contacted by the RS1 domain of SR45.1, in which mutations impacted more strongly the association to RS2Z32 compared to RS2Z33 **(Supplementary Figure S8)**. A complete abolition of these contacts was observed upon mutations applied to both RS domains of SR45.1 **(Supplementary Figures S8)**. Similarly, the RS2Z-SCL30 interactions were also weakened or abolished upon mutations applied to the RRM or ZnKs and to the RS domain, respectively. Mutations of the RS domain of RS2Z32 and RS2Z33, respectively, abolished and weakened the association to SR34. The RS2Z33-SR34 association was fully disrupted when combining RS and RRM mutations **(Figure 6A)**.

Putative interactions with kinases potentially involved in the phosphorylation of serine residues of the RS2Z RS and SP domains were also analyzed using dY2H. While none of the 3 CLK kinases (AFC1-3) (Bender and Fink 1994) interacted with the RS2Z proteins **(Figure 6B)**, 3 out of 5 SRPK kinases (SRPK3a, SRPK3b [also named SRPK5] and SRPK4) were found to strongly interact with RS2Z32 and RS2Z33, which confirmed a recent report (Wang *et al.,* 2023) **(Figure 6A and Supplementary Figure S6)**. Mutations applied to the RS domain abolished the association to SRPK3a, SRPK3b and SRPK4 **(Figure 6A)**. Those results indicated that the RS

domain, but not the SP, is involved in interactions with kinases of the SRPK family, suggesting that phosphorylation of serine residues in the RS domain may take place.

As nuclear cyclophilins are involved in pre-mRNA maturation and are known to interact with SR proteins (Lorković and Barta 2004; Gullerova *et al.,* 2006; Barbosa Dos Santos and Park 2019; Jo *et al.,* 2022), we tested the associations of RS2Z proteins to Cyp59, Cyp65, Cyp71, CypRS64 and CypRS92 **(Figure 6A-B)**. Cyp59, CypRS64 and CypRS92 were found to interact with the two RS2Z proteins, however, the mutRRM variants were unable to sustain these contacts. Mutations affecting the RS domain were also responsible for the complete disruption of the RS2Z-Cyp59 association, hence only the native proteins and the mutant variants harboring altered ZnKs or SP domains were able to contact Cyp59 **(Figure 6A)**. The interaction between Cyp59 and RS2Z32 appeared to be weaker than the one involving RS2Z33, as depicted by the lack of resilience of yeast cells to grow upon serial dilutions (also noticeable at the starting OD of 0.25) **(Supplementary Figure S6)**.

It was shown that RS2Z33 interacts with MOS14, a transportin-SR (TRN-SR) thought to drive the nuclear import of SR proteins (Xu *et al.,* 2011). Here, we found that both RS2Z32 and RS2Z33 were able to interact with MOS14, which required an intact RS domain, as well as an intact SP domain **(Figure 6A)**.

Finally, no interaction was observed between hnRNP-like proteins (GRP7, GRP8, RZ-1B, and RZ-1C), PRP38, MOS12, ALY/REF ortholog ALY4, EJC core components (MAGO, Y14, eIF4AIII), or the peripheral EJC components (ACINUS, SAP18, and PININ) and the full-length versions of RS2Z proteins **(Figure 6B)**.

Our observations depict the involvement of serine residues in contacting kinases and splicing factors, which also requires in some extent the presence of a native canonical RRM (containing unaltered RNP1 and RNP2 motifs).

RS2Z proteins strongly interact with mRNA processing factors in planta

Interactions observed in yeast cells showed that the RS2Z proteins interact with few SR proteins that are known to interact between themselves (e.g., SCL30-SR34 (Lorković *et al.,* 2008), SR34-SR45 (Stankovic *et al.,* 2016), and SR45-SCL30 (Fanara *et al.,* 2024)) and that are all contacted by CypRS64 (Lorković *et al.,* 2004; Fanara *et al.,* 2024). We used Yellow Fluorescent Protein (YFP)-based bimolecular fluorescence complementation (BiFC) to confirm

in planta these specific interactions. RS2Z proteins fused to the C-terminal fragment of YFP (^{C}YFP) and interactors fused to the N-terminal fragment of YFP (^{N}YFP) were transiently co-expressed in tobacco leaf cells. This allowed to determine that RS2Z proteins interacted with SR45.1, SCL30, SR34 and CypRS64 within the nucleoplasm and inside speckles-like granules in tobacco leaf cells **(Figure 7)**. The interaction between the RS2Z proteins and SR45 was also dependent on their respective RS domain(s) **(Supplementary Figure S9)**, confirming yeast results.

RNA binding specificity of RNA-contacting domains of the RS2Z proteins

We then determined the RNA sequences bound by the RS2Z factors. Recombinant RRM domains of RS2Z32 and RS2Z33 fused to a glutathione-S-transferase were used in a Systematic Evolution of Ligands by EXponential enrichment (SELEX) experiment. Among the 10 independent clones sequenced after SELEX and used in a MEME analysis, a common central motif, 5'-G[G/C]UA-3', flanked by variable regions was found to be recognized by the two RS2Z proteins **(Figure 8A-B)**. While both RRM recognized a U/C-rich stretch at the 3'-end of their respective RNA motif, the 5'-end was highly variable. We studied the occurrence of 'CC' dinucleotides in the sequences used by MEME to identify each motif, which showed that 60% and 71% of the sequences contained the motif 5'-CCGGUA-3' for RS2Z32 and RS2Z33, respectively **(Supplementary Tables S10-11)**.

Substitutions to alanines of Y14, Y46 and F48 residues, that are thought to control RNA binding specificity, disrupted the recovery of a significant motif by SELEX **(Figure 8C-D)**. Indeed, two different sets of ten sequences obtained with the mutated RS2Z32 or RS2Z33 RRM were analyzed **(Supplementary Tables S12-13)** using the MEME tool, and no enriched motif was discovered **(Figure 8C-D).** Interestingly, among the sequences selected through binding to the native RRM of RS2Z32 and RS2Z33, purine and pyrimidine contents were approximately even (with a 2.5-3% enrichment in purine bases) **(Supplementary Tables S10-11)**. In contrast, upon Y14A-Y46A-F48A substitutions, purine bases were enriched by ~14% to ~22% on average in sequences contacted by RS2Z32 and RS2Z33 **(Supplementary Tables S12-13)**. This also confirmed the importance of these residues in the selectivity of sequences connected by the native RRM.

Although Zn knuckles were shown to contribute to the RNA binding specificity of many splicing factors (De Franco *et al.,* 2019), including SRSF7 (Cavaloc *et al.,* 1999; Königs *et al.,* 2020), no significant RNA motif were uncovered when SELEX experiments were performed using recombinant proteins including both RRM and ZnKs **(Supplementary Figure S10** and **Supplementary Tables S14-15)**. Altogether, our results demonstrate that the amino acids Y14, Y46 and F48 of the RRM govern the RNA binding specificity of each RS2Z protein for an RNA motif characterized by a U/C-rich stretch at its 3' end.

Discussion

Splicing is a crucial event in the regulation of gene expression and many *trans*-acting protein factors, including SR proteins, are involved in splice-site selection through the binding to specific *cis*-elements (Chen and Manley 2009). The functional relationship between RRM and ZnK of the plant RSZ (one RRM and one ZnK) was previously explored in the context of the nucleocytoplasmic shuttling activity and subcellular localization of RSZ22 (Rausin *et al.,* 2010). In contrast, little was known about RS2Z32 and RS2Z33, which belong to a plant-specific subfamily characterized by the presence of a canonical RRM domain, directly followed by two CCHC-type zinc knuckles (ZnKs), and RS and SP domains (Barta *et al.,* 2010).

Using mutant variants, we evaluated how each of the RRM, ZnKs, RS and SP domains modulate the molecular functions played by the RS2Z proteins. The impact of mutations introduced in each domain of RS2Z proteins was evaluated by assessing several parameters defining a typical SR splicing factor, i.e. (i) their intracellular localization; (ii) their nucleocytoplasmic dynamics, showing that shuttling kinetics were differently affected depending on the mutations; (iii) the protein–protein interactions, highlighting that different mutations abolished the association to different partners; and (iv) the protein–RNA interactions, proving the existence of contacts between the mutated RRM and RNA molecules despite an alteration of specificity (Yang *et al.,* 2011; Pabis *et al.,* 2019; Ni *et al.,* 2023; Fanara *et al.,* 2024). While we showed changes in the molecular functions of the engineered mutant variants compared to the native RS2Z proteins, these changes were likely not linked to structural impairments of the mutated domains as the RS2Z proteins are largely predicted to be poorly structured outside of the RNA recognition motif (RRM), therefore these intrinsically disordered region are expected not to be affected upon alanine substitutions **(Figure 3A-B)**.

Alteration of the RS domain of RS2Z proteins leads to their nucleolar sequestration and disrupts their ability to contact splicing factors

We observed that the RS domain of both RS2Z proteins often reinforces the contact between their RRM and other splicing factors. Indeed, the substitution of all serine residues within the RS domain by alanine residues can either severely diminish or even abolish the contact with splicing partners (e.g., SR45 isoforms, SCL30 and SR34). Interestingly, S-to-A substitutions within the RS domain also affect the subcellular localization of the RS2Z members, which leads to nucleolar sequestration and to a partial cytoplasmic accumulation of the RS2ZmutRS variants suggesting a role for the RS domain in the control of the nucleoplasmic localization of SR proteins. This is further supported by the observation of the cytoplasmic retention of both a SR34 mutant variant displaying R/S-to-G/T substitutions (Stankovic *et al.,* 2016) and a SR45 mutant variant displaying S-to-A substitutions within the RS2 domain (Fanara *et al.,* 2024). This suggests that RS-mutated variants are less effectively imported in the nucleus, which is in agreement with the absence of interactions of RS2ZmutRS variants with the plant transportin-SR (MOS14) thought to ensure the nuclear import of plant SR proteins (Xu *et al.,* 2011). Furthermore, the absence of interactions between the RS2ZmutRS variants and the SRPK kinases (SRPK3a, SRPK3b and SRPK4) also supports the observation of the cytoplasmic accumulation of these mutant variants, as the nuclear import of SR proteins by MOS14 requires phosphorylated residues (Lai *et al.,* 2000; Lai *et al.,* 2001). These SRPK kinases (SRPK3a, SRPK3b and SRPK4) might therefore contribute to the nuclear import of the RS2Z members conjointly with MOS14. This hypothesis is also supported by a recent study that links an exclusive cytoplasmic localization of RS2Z32 to the down-regulation of phosphorylation levels of specific serine residues within the RS domain (but not within the SP domain) in an *srpk3 srpk4 srpk3b/srpk5* triple mutant (Wang *et al.,* 2023). All of these observations thus suggest that nuclear import of RS2Z32 and RS2Z33 by the transportin-SR MOS14 requires prior phosphorylation of RS domain serine residues, likely by SRPK3a, SRPK3b and SRPK4. Interestingly, the RS2ZmutRRM+RS variant displays a fainter cytoplasmic fluorescent signal than the RS2ZmutRS, suggesting a diminution of the nuclear export of the RS2Z proteins, which is corroborated by the observed diminution of shuttling kinetics of this mutant variant compared to the native protein, mostly due to the alteration of the RNP2 motif.

RS2Z proteins contact splicing partners through their RRM

Our results indicate that many residues of the RRM of RS2Z proteins (including the mutated residues Y14, Y46 and F48) are involved in the association with other splicing factors, such as SR45 (SR45.1 and SR45.2, **Supplementary Figure S7C**), SCL30 and SR34 **(Figure 6A and Supplementary Figure S6)**. This corroborates a previous report showing that the RNA-binding domains (e.g., RRM and ZnKs) of RS2Z33 (truncated protein harboring RRM+ZnKs only) were involved in the interactions with a second SCL subfamily member, namely SCL33 (Lopato *et al.,* 2002). Interestingly, our dY2H assays showed that the RS2Z32-SR45 dimer is more affected by mutations applied to the RRM (mutRRM variant) than the RS2Z33-SR45 dimer, which implies that mutated residues (Y14A, Y46 and F48) might be involved in the formation of the RS2Z32-SR45 complex. This challenges the general idea that the canonical RRM domain is solely involved in the recognition of RNA molecules. The contribution of the RRM to the formation of spliceosomal complexes has already been described for Y14-MAGO that heterodimerize notably through the β-sheet surface of the RRM of Y14, ultimately unabling its RNA binding ability (Shi and Xu 2003). Our dY2H and BiFC results additionally indicate that the association of the RS2Z proteins to SR45 involves both RS domains of SR45. Indeed, neither RS2Z32 nor RS2Z33 can interact with the SR45mutRS1+RS2 in yeast and plant cells **(Supplementary Figures S8 and S9)**. Reciprocally, the RS domain of RS2Z proteins also proved important to maintain this interaction in yeast and plant cells **(Figure 6A, Supplementary Figure S6 and Supplementary Figure S9)**.

RNA binding specificity of the RRM of RS2Z proteins

Cis-elements (or RNA motifs) bound by plant SR proteins have been studied through several strategies: (i) *in silico* studies performed on RNA-Seq data of *sr* loss-of-function mutants searching for motifs common to differentially expressed genes, (ii) SELEX experiments, (iii) individual-nucleotide crosslinking and immunoprecipitation (iCLIP) assay, and (iv) RNAcompete approach (Yan *et al.,* 2017; Laloum *et al.,* 2023; Fanara *et al.,* 2024; Köster *et al.,* 2025). Motifs associated to SCL30, SR45, SR34, SR34a and RS31 binding to RNAs were determined, all of which display purine-rich features (Yan *et al.,* 2017; Fanara *et al.,* 2024; Köster *et al.,* 2025), with the exception of the 5'-GCU-3' motif of SR34a (Laloum *et al.,* 2023). In contrast, RS2Z proteins

recognize a common *cis*-element displaying a pyrimidine-rich feature (U/C stretch) at the 3' end of a central motif 5'-G[G/C]UA-3'. Interestingly, the sequences bound by RS2Z32 and RS2Z33 upstream and downstream of this central motif were more variable. These distinct specificities may stem from sequence divergence between the RRM of the two RS2Z proteins, e.g. the RS2Z32 Y62 residue is replaced by a histidine in RS2Z33, or the RS2Z32 A78 and N89 residues are replaced by phenylalanine residues capable of connecting RNA (Zeke *et al.,* 2022) in RS2Z33. However, these additional residues are not the main drivers of the RNA binding specificity of RS2Z proteins as mutations of three conserved residues in RNP2 and RNP1 motifs (Y14, Y46 and F48) were sufficient to disrupt the recovery of a significant motif with each RS2Z RRM. The enrichment in pyrimidine bases in an RNA motif, as observed at the 3' end of the RS2Z RNA motifs, is usually associated to the control of AS events (Ule *et al.,* 2006; Goers *et al.,* 2010; Uemura *et al.,* 2017). The binding of RS2Z33 to the *CALLOSE SYNTHASE5* (*CalS5*) pre-mRNA has been proposed to positively regulate the splicing removal of its intron 6 (Huang *et al.,* 2013). While the motif associated with both RS2Z32 and RS2Z33 (5'-CCGCUA-3') is absent from the *CalS5* pre-mRNA sequence, the 5'-CUGCUA-3' motif specifically bound by the RRM of RS2Z33 is present in both the exon 5 and the exon 27 of *CalS5*. The observation of a RS2Z33 binding motif in an exon upstream of the regulated intron 6 suggests that this SR protein might indeed regulate the splicing pattern of its putative target, *CalS5*, which supports the working hypothesis of Huang and colleagues (Huang *et al.,* 2013). The interactors of RS2Z33 (SR45, SCL30, and SR34) might not regulate this specific intron removal as their RNA binding sites (5'-AGAAGA-3' for SR45 and SCL30, and 5'-AGGGAG-3' for SR34) are present in relatively distant positions from the intron 6 (Yan *et al.,* 2017; Fanara *et al.,* 2024).

Do RS2Z proteins play redundant molecular functions?

While most of our results largely suggest a functional redundancy between RS2Z32 and RS2Z33 (e.g., identical expression profile and interactors, or similar dynamics and RNA motifs bound), our dY2H assays showed some noticeable differences between the RS2Z32 and RS2Z33 complexes. Although the association of RS2Z32 to partners also contacted by RS2Z33 appeared to be weaker (e.g., Cyp59), the putative functional redundancy of the RS2Z proteins remains to be confirmed by complementation experiment of a double mutant. However, as a quintuple *rsz rs2z* mutant (Yan *et al.*, 2017) displays no phenotypic impairments, it is challenging to propose a

specific biological function for the RS2Z members. One could speculate on the physiological role(s) of the RS2Z proteins by examining how the expression of their respective genes is regulated by multiple stresses. Using available Arabidopsis RNA-seq resources (Zhang *et al.,* 2020; Yu *et al.,* 2022) (https://plantrnadb.com/athrdb/) suggests that *RS2Z* genes are responsive to specific stresses for which they are distinctively transcriptionally regulated (e.g., dehydration, low red/far-red ratio or salt), sometimes in opposite directions (cold or methyl jasmonate) (Feng *et al.,* 2020), even though they can be co-regulated in some instances (e.g., dark treatments) (Pietzenuk *et al.,* 2016).

Conclusion

In this study, we characterized the contribution of the RS, SP, RRM and two ZnKs domains, and their constitutive residues, to the function of the Arabidopsis RS2Z32 and RS2Z33 proteins investigating the many aspects defining a splicing factor. Serines of the RS domain control the nucleoplasmic diffusion of RS2Z32 and RS2Z33. On the other hand, the RNA-binding domains of both RS2Z proteins are involved in the shuttling activity of the RS2Z proteins, and the RRM itself is also involved in both protein-protein and protein-RNA interactions. The RRM specifically binds RNA molecules via the residues Y14, Y46 and F48, and the RNA motifs identified appear to be different from the generally observed purine-rich sequences recognized by SR45, SCL30 and SR34. Many charged residues in the RRM likely serve to contact splicing factors that also displays a shuttling dynamic (e.g., SR45 and SR34). A BiFC approach allowed the confirmation of several interactions observed in yeast cells. Among them, the connection between SR45, SCL30, SR34 or CypRS64 and the RS2Z proteins was visualized *in planta*. Collectively, our observations suggest that RS2Z proteins play highly redundant functions.

Supplementary data

Supplementary Figure S1. Scaled schemes depicting the native RS2Z proteins and their corresponding mutant variants.

Supplementary Figure S2. Toxicity and autoactivation assays for yeast vectors harboring the native *RS2Z32* and *RS2Z33* or corresponding mutant variants coding sequences.

Supplementary Figure S3. Reporter lines depicting the activity of the native *RS2Z32* or *RS2Z33*

promoters (*pRS2Z:EGFP*) at different developmental stages.

Supplementary Figure S4. Neither RNP motifs nor ZnK domains control the nuclear distribution of RS2Z proteins.

Supplementary Figure S5. Nucleocytoplasmic shuttling of mutant variants altered in the RRM, ZnKs or RRM+ZnKs.

Supplementary Figure S6. Study of the strength of interactions established between RS2Z32 or RS2Z33 and kinases, splicing-associated factors or the nuclear import factor.

Supplementary Figure S7. Full-length RS2Z proteins interact with only few Arabidopsis SR proteins.

Supplementary Figure S8. RS2Z proteins associate to SR45, partly though its RS1 domain.

Supplementary Figure S9. The *in planta* interaction between RS2Z proteins and SR45 is dependent on their respective phosphorylatable RS domain(s).

Supplementary Figure S10. The RRM and zinc-knuckles of the RS2Z proteins do not conjointly provide a specific RNA motif binding.

Supplementary Tables S1-S8. List of primers used in this study, subdivided into experimental categories.

Supplementary Table S9. Statistical analysis of FLIP-shuttling assays.

Supplementary Tables S10-S15. List of sequences submitted to MEME to identify motif consensus(es) for RNA-binding sites of the native RS2Z RRMs and the corresponding mutated RRMs or RRM+ZnKs recombinant proteins.

Acknowledgments

We thank Dr Julien Spielmann, and Dr Marine Joris for helpful discussions.

Author contributions

PM: conceptualization and directing the research; PM and MH: supervision; SF: conducting most experiments, with contributions of MS, SDF, MV, and MGa for SELEX experiments, and of MGe for FLIP-shuttling assays; SF and AVB: performing AlphaFold computational modeling with initial comments of FK; PM and SF: data analysis, with the help of FF for FLIP-shuttling statistical analyses; SF: making the figures, with the help of AVB for figures related to AlphaFold. SF, MH, and PM wrote the manuscript, with comments of all authors.

Conflict of interest

No conflict of interest is declared.

Funding

Funding was provided by the 'Fonds de la Recherche Scientifique–FNRS' (FRFC-1.E049.15, PDR-T.0206.13, CDR-J.0009.17, PDR-T0120.18, CDR-J.0082.21, PDR-T.0104.22, PDR-T.0121.22). FK is Senior Research Associate of the FRS-FNRS. AVB is supported by a FRS–FNRS MISU grant (MISU F.6005.25) and ERC Starting grant (TranSplice 101162011). SF was a doctoral fellow of the FRIA ('Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture').

Data availability

All data supporting the findings of the study are available within the paper and within its supplementary data published online.

References

Abramson J, Adler J, Dunger J, Evans R, Green T, Pritzel A, Ronneberger O, Willmore L, Ballard AJ, Bambrick J, Bodenstein SW, Evans DA, Hung CC, O'Neill M, Reiman D, Tunyasuvunakool K, Wu Z, Žemgulyte A, Arvaniti E, Beattie C, Bertolli O, Bridgland A, Cherepanov A, Congreve M, Cowen-Rivers AI, Cowie A, Figurnov M, Fuchs FB, Gladman H, Jain R, Khan YA, Low CMR, Perlin K, Potapenko A, Savy P, Singh S, Stecula A, Thillaisundaram A, Tong C, Yakneen S, Zhong ED, Zielinski M, Žídek A, Bapst V, Kohli P, Jaderberg M, Hassabis D, Jumper JM. 2024. Accurate structure prediction of biomolecular interactions with AlphaFold 3. Nature 630, 493-500.

Albuquerque-Martins R, Szakonyi D, Rowe J, Jones AM, Duque P. 2023. ABA signaling prevents phosphodegradation of the SR45 splicing factor to alleviate inhibition of early seedling development in *Arabidopsis*. Plant Communications 4, 100495.

Aubol BE, Hailey KL, Fattet L, Jennings PA, Adams JA. 2017. Redirecting SR Protein Nuclear Trafficking through an Allosteric Platform. Journal of Molecular Biology 429, 2178-2191.

Aubol BE, Wu G, Keshwani MM, Movassat M, Fattet L, Hertel KJ, Fu XD, Adams JA. 2016. Release of SR Proteins from CLK1 by SRPK1: A Symbiotic Kinase System for Phosphorylation Control of Pre-mRNA Splicing. Molecular Cell 63, 218-228.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. Nucleic Acids Research 37, W202-208.

Barbosa Dos Santos I, Park SW. 2019. Versatility of Cyclophilins in Plant Growth and Survival: A Case Study in *Arabidopsis*. Biomolecules 9.

Barta A, Kalyna M, Reddy AS. 2010. Implementing a rational and consistent nomenclature for serine/arginine-rich protein splicing factors (SR proteins) in plants. The Plant Cell 22, 2926-2929.

Bender J, Fink GR. 1994. AFC1, a LAMMER kinase from *Arabidopsis thaliana*, activates STE12-dependent processes in yeast. Proceedings of the National Academy of Sciences, USA 91, 12105-12109.

Califice S, Baurain D, Hanikenne M, Motte P. 2012. A single ancient origin for prototypical serine/arginine-rich splicing factors. Plant Physiology 158, 546-560.

Cavaloc Y, Bourgeois CF, Kister L, Stévenin J. 1999. The splicing factors 9G8 and SRp20

719 transactivate splicing through different and specific enhancers. RNA 5, 468-483.
720 **Chen M, Manley JL.** 2009. Mechanisms of alternative splicing regulation: insights from
721 molecular and genomics approaches. Nature Reviews Molecular Cell Biology 10, 741-754.
722 **Cléry A, Blatter M, Allain FH.** 2008. RNA recognition motifs: boring? Not quite. Current
723 Opinion in Structural Biology 18, 290-298.
724 **Crooks GE, Hon G, Chandonia JM, Brenner SE.** 2004. WebLogo: a sequence logo generator.
725 Genome Research 14, 1188-1190.
726 **Cruz TM, Carvalho RF, Richardson DN, Duque P.** 2014. Abscisic acid (ABA) regulation of
727 *Arabidopsis* SR protein gene expression. International Journal of Molecular Sciences 15, 17541-
728 17564.
729 **Curtis MD, Grossniklaus U.** 2003. A gateway cloning vector set for high-throughput functional
730 analysis of genes in planta. Plant Physiology 133, 462-469.
731 **Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR.** 2005. Genome-wide
732 identification and testing of superior reference genes for transcript normalization in Arabidopsis.
733 Plant Physiology 139, 5-17.
734 **Daubner GM, Cléry A, Allain FH.** 2013. RRM-RNA recognition: NMR or crystallography...and
735 new findings. Current Opinion in Structural Biology 23, 100-108.
736 **De Franco S, Vandenameele J, Brans A, Verlaine O, Bendak K, Damblon C, Matagne A,**
737 **Segal DJ, Galleni M, Mackay JP, Vandevenne M.** 2019. Exploring the suitability of RanBP2-
738 type Zinc Fingers for RNA-binding protein design. Scientific Reports 9, 2484.
739 **Drechsel G, Kahles A, Kesarwani AK, Stauffer E, Behr J, Drewe P, Rätsch G, Wachter A.**
740 2013. Nonsense-mediated decay of alternative precursor mRNA splicing variants is a major
741 determinant of the *Arabidopsis* steady state transcriptome. The Plant Cell 25, 3726-3742.
742 **English AC, Patel KS, Loraine AE.** 2010. Prevalence of alternative splicing choices in
743 *Arabidopsis thaliana*. BMC Plant Biology 10, 102.
744 **Fanara S, Schloesser M, Hanikenne M, Motte P.** 2022. Altered metal distribution in the *sr45-1*
745 Arabidopsis mutant causes developmental defects. The Plant Journal 110, 1332-1352.
746 **Fanara S, Schloesser M, Joris M, De Franco S, Vandevenne M, Kerff F, Hanikenne M,**
747 **Motte P.** 2024. The Arabidopsis SR45 splicing factor bridges the splicing machinery and the
748 exon-exon junction complex. Journal of Experimental Botany 75, 2280-2298.
749 **Feng G, Yoo MJ, Davenport R, Boatwright JL, Koh J, Chen S, Barbazuk WB.** 2020.

Jasmonate induced alternative splicing responses in *Arabidopsis*. Plant Direct 4, e00245.

Goers ES, Purcell J, Voelker RB, Gates DP, Berglund JA. 2010. MBNL1 binds GC motifs embedded in pyrimidines to regulate alternative splicing. Nucleic Acids Research 38, 2467-2484.

Gullerova M, Barta A, Lorković ZJ. 2006. AtCyp59 is a multidomain cyclophilin from *Arabidopsis thaliana* that interacts with SR proteins and the C-terminal domain of the RNA polymerase II. RNA 12, 631-643.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Krämer U. 2008. Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of *HMA4*. Nature 453, 391-395.

Huang XY, Niu J, Sun MX, Zhu J, Gao JF, Yang J, Zhou Q, Yang ZN. 2013. CYCLIN-DEPENDENT KINASE G1 is associated with the spliceosome to regulate *CALLOSE SYNTHASE5* splicing and pollen wall formation in *Arabidopsis*. The Plant Cell 25, 637-648.

Jo SH, Park HJ, Lee A, Jung H, Park JM, Kwon SY, Kim HS, Lee HJ, Kim YS, Jung C, Cho HS. 2022. The Arabidopsis cyclophilin CYP18-1 facilitates PRP18 dephosphorylation and the splicing of introns retained under heat stress. The Plant Cell 34, 2383-2403.

Kalyna M, Lopato S, Barta A. 2003. Ectopic expression of atRSZ33 reveals its function in splicing and causes pleiotropic changes in development. Molecular Biology of the Cell 14, 3565-3577.

Kalyna M, Simpson CG, Syed NH, Lewandowska D, Marquez Y, Kusenda B, Marshall J, Fuller J, Cardle L, McNicol J, Dinh HQ, Barta A, Brown JW. 2012. Alternative splicing and nonsense-mediated decay modulate expression of important regulatory genes in *Arabidopsis*. Nucleic Acids Research 40, 2454-2469.

Kashkan I, Timofeyenko K, Růžička K. 2022. How alternative splicing changes the properties of plant proteins. Quantitative Plant Biology 3, 1–11.

Kataoka N, Bachorik JL, Dreyfuss G. 1999. Transportin-SR, a nuclear import receptor for SR proteins. Journal of Cell Biology 145, 1145-1152.

Kim DY, Scalf M, Smith LM, Vierstra RD. 2013. Advanced proteomic analyses yield a deep catalog of ubiquitylation targets in *Arabidopsis*. The Plant Cell 25, 1523-1540.

Königs V, de Oliveira Freitas Machado C, Arnold B, Blümel N, Solovyeva A, Löbbert S, Schafranek M, Ruiz De Los Mozos I, Wittig I, McNicoll F, Schulz MH, Müller-McNicoll M. 2020. SRSF7 maintains its homeostasis through the expression of Split-ORFs and nuclear body

781 assembly. Nature Structural & Molecular Biology 27, 260-273.
782 **Köster T, Venhuizen P, Lewinski M, Petrillo E, Marquez Y, Fuchs A, Ray D, Nimeth BA,**
783 **Riegler S, Franzmeier S, Rodriguez FS, Aballay FE, Tognacca RS, Zheng H, Hughes T,**
784 **Morris Q, Barta A, Staiger D, Kalyna M.** 2025. At-RS31 orchestrates hierarchical cross-
785 regulation of splicing factors and integrates alternative splicing with TOR-ABA pathways. New
786 Phytologist 247, 738-759.
787 **Lai MC, Lin RI, Huang SY, Tsai CW, Tarn WY.** 2000. A human importin-beta family protein,
788 transportin-SR2, interacts with the phosphorylated RS domain of SR proteins. Journal of
789 Biological Chemistry 275, 7950-7957.
790 **Lai MC, Lin RI, Tarn WY.** 2001. Transportin-SR2 mediates nuclear import of phosphorylated
791 SR proteins. Proceedings of the National Academy of Sciences 98, 10154-10159.
792 **Laloum T, Martín G, Lewinski M, Yanez RJR, Köster T, Staiger D, Duque P.** 2023. An
793 *Arabidopsis* SR protein relieving ABA inhibition of seedling establishment represses ABA-
794 responsive alternative splicing. BioRxiv doi: 10.1101/2023.12.19.572415 [Preprint].
795 **Liang Q, Geng Q, Jiang L, Liang M, Li L, Zhang C, Wang W.** 2019. Protein methylome
796 analysis in *Arabidopsis* reveals regulation in RNA-related processes. Journal of Proteomics 213,
797 103601.
798 **Lopato S, Forstner C, Kalyna M, Hilscher J, Langhammer U, Indrapichate K, Lorković ZJ,**
799 **Barta A.** 2002. Network of interactions of a novel plant-specific Arg/Ser-rich protein, atRSZ33,
800 with atSC35-like splicing factors. Journal of Biological Chemistry 277, 39989-39998.
801 **Lorković ZJ, Barta A.** 2004. Compartmentalization of the splicing machinery in plant cell
802 nuclei. Trends in Plant Science 9, 565-568.
803 **Lorković ZJ, Hilscher J, Barta A.** 2008. Co-localisation studies of Arabidopsis SR splicing
804 factors reveal different types of speckles in plant cell nuclei. Experimental Cell Research 314,
805 3175-3186.
806 **Lorković ZJ, Lopato S, Pexa M, Lehner R, Barta A.** 2004. Interactions of *Arabidopsis* RS
807 domain containing cyclophilins with SR proteins and U1 and U11 small nuclear
808 ribonucleoprotein-specific proteins suggest their involvement in pre-mRNA Splicing. Journal of
809 Biological Chemistry 279, 33890-33898.
810 **Manley JL, Krainer AR.** 2010. A rational nomenclature for serine/arginine-rich protein splicing
811 factors (SR proteins). Genes & Development 24, 1073-1074.

Manuel JM, Guilloy N, Khatir I, Roucou X, Laurent B. 2023. Re-evaluating the impact of alternative RNA splicing on proteomic diversity. Frontiers in Genetics 14, 1089053.

Maris C, Dominguez C, Allain FH. 2005. The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. The FEBS Journal 272, 2118-2131.

Marquez Y, Höpfler M, Ayatollahi Z, Barta A, Kalyna M. 2015. Unmasking alternative splicing inside protein-coding exons defines exitrons and their role in proteome plasticity. Genome Research 25, 995-1007.

Meyer K, Koester T, Staiger D. 2015. Pre-mRNA Splicing in Plants: *In Vivo* Functions of RNA-Binding Proteins Implicated in the Splicing Process. Biomolecules 5, 1717-1740.

Ni X, Joerger AC, Chaikuad A, Knapp S. 2023. Structural insights into a regulatory mechanism of FIR RRM1-FUSE interaction. Open Biology 13, 230031.

Pabis M, Popowicz GM, Stehle R, Fernández-Ramos D, Asami S, Warner L, García-Mauriño SM, Schlundt A, Martínez-Chantar ML, Díaz-Moreno I, Sattler M. 2019. HuR biological function involves RRM3-mediated dimerization and RNA binding by all three RRMs. Nucleic Acids Research 47, 1011-1029.

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

Palusa SG, Reddy AS. 2010. Extensive coupling of alternative splicing of pre-mRNAs of serine/arginine (SR) genes with nonsense-mediated decay. New Phytologist 185, 83-89.

Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morris JH, Ferrin TE. 2021. UCSF ChimeraX: Structure visualization for researchers, educators, and developers. Protein Science 30, 70-82.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29, e45.

Pietzenuk B, Markus C, Gaubert H, Bagwan N, Merotto A, Bucher E, Pecinka A. 2016. Recurrent evolution of heat-responsiveness in *Brassicaceae* COPIA elements. Genome Biology 17, 209.

Rausin G, Tillemans V, Stankovic N, Hanikenne M, Motte P. 2010. Dynamic nucleocytoplasmic shuttling of an Arabidopsis SR splicing factor: role of the RNA-binding domains. Plant Physiology 153, 273-284.

Remy E, Cabrito TR, Baster P, Batista RA, Teixeira MC, Friml J, Sá-Correia I, Duque P. 2013. A major facilitator superfamily transporter plays a dual role in polar auxin transport and drought stress tolerance in *Arabidopsis*. The Plant Cell 25, 901-926.

Shi H, Xu RM. 2003. Crystal structure of the *Drosophila* Mago nashi-Y14 complex. Genes & Development 17, 971-976.

Spielmann J, Ahmadi H, Scheepers M, Weber M, Nitsche S, Carnol M, Bosman B, Kroymann J, Motte P, Clemens S, Hanikenne M. 2020. The two copies of the zinc and cadmium ZIP6 transporter of *Arabidopsis halleri* have distinct effects on cadmium tolerance. Plant, Cell & Environment 43, 2143-2157.

Stankovic N, Schloesser M, Joris M, Sauvage E, Hanikenne M, Motte P. 2016. Dynamic Distribution and Interaction of the Arabidopsis SRSF1 Subfamily Splicing Factors. Plant Physiology 170, 1000-1013.

Talke IN, Hanikenne M, Krämer U. 2006. Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. Plant Physiology 142, 148-167.

Thompson HL, Shen W, Matus R, Kakkar M, Jones C, Dolan D, Grellscheid S, Yang X, Zhang N, Mozaffari-Jovin S, Chen C, Zhang X, Topping JF, Lindsey K. 2023. MERISTEM-DEFECTIVE regulates the balance between stemness and differentiation in the root meristem through RNA splicing control. Development 150.

Tillemans V, Dispa L, Remacle C, Collinge M, Motte P. 2005. Functional distribution and dynamics of Arabidopsis SR splicing factors in living plant cells. The Plant Journal 41, 567-582.

Tillemans V, Leponce I, Rausin G, Dispa L, Motte P. 2006. Insights into nuclear organization in plants as revealed by the dynamic distribution of Arabidopsis SR splicing factors. The Plant Cell 18, 3218-3234.

Uemura Y, Oshima T, Yamamoto M, Reyes CJ, Costa Cruz PH, Shibuya T, Kawahara Y. 2017. Matrin3 binds directly to intronic pyrimidine-rich sequences and controls alternative splicing. Genes to Cells 22, 785-798.

Ule J, Stefani G, Mele A, Ruggiu M, Wang X, Taneri B, Gaasterland T, Blencowe BJ, Darnell RB. 2006. An RNA map predicting Nova-dependent splicing regulation. Nature 444, 580-586.

Wang T, Wang X, Wang H, Yu C, Xiao C, Zhao Y, Han H, Zhao S, Shao Q, Zhu J, Zhao Y,

Wang P, Ma C. 2023. *Arabidopsis* SRPKII family proteins regulate flowering via phosphorylation of SR proteins and effects on gene expression and alternative splicing. New Phytologist 238, 1889-1907.

Xu S, Zhang Z, Jing B, Gannon P, Ding J, Xu F, Li X, Zhang Y. 2011. Transportin-SR is required for proper splicing of *Resistance* genes and plant immunity. PLoS Genetics 7, e1002159.

Yan Q, Xia X, Sun Z, Fang Y. 2017. Depletion of *Arabidopsis* SC35 and SC35-like serine/arginine-rich proteins affects the transcription and splicing of a subset of genes. PLoS Genetics 13, e1006663.

Yang Q, Coseno M, Gilmartin GM, Doublié S. 2011. Crystal structure of a human cleavage factor CFI(m)25/CFI(m)68/RNA complex provides an insight into poly(A) site recognition and RNA looping. Structure 19, 368-377.

Yu Y, Zhang H, Long Y, Shu Y, Zhai J. 2022. Plant Public RNA-seq Database: a comprehensive online database for expression analysis of ~45 000 plant public RNA-Seq libraries. Plant Biotechnology Journal 20, 806-808.

Zeke A, Schád E, Horváth T, Abukhairan R, Szabó B, Tantos A. 2022. Deep structural insights into RNA-binding disordered protein regions. Wiley Interdisciplinary Reviews: RNA 13, e1714.

Zhang H, Zhang F, Yu Y, Feng L, Jia J, Liu B, Li B, Guo H, Zhai J. 2020. A Comprehensive Online Database for Exploring approximately 20,000 Public *Arabidopsis* RNA-Seq Libraries. Molecular Plant 13, 1231-1233.

Zhang XN, Mount, SM. 2009. Two alternatively spliced isoforms of the Arabidopsis SR45 protein have distinct roles during normal plant development. Plant Physiology 150, 1450-1458.

Figure legends

Figure 1. Expression profiling of *RS2Z* genes in Arabidopsis organs. Quantitative RT-PCR analyses of *RS2Z* genes expression in Arabidopsis vegetative and reproductive organs. Values represent means ± SEM (from three biological replicates, each consisting of pools of organs from three plants) and are relative to *AT1G58050*. Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post-test. Statistically significant differences between means of all genes within one tissue are indicated by asterisks (**P<0.01, ***P<0.001). Statistically significant differences between means for one gene between tissues are indicated by letters (P<0.05). n.s., not significant.

Figure 2. Localization of RS2Z proteins in all vegetative and reproductive organs. Translational fusions are expressed in seed coat **(A/a)**, embryo **(B/b)**, hypocotyl **(C/c)** and cotyledons **(D/d),** two-week-old root epidermis **(E/e)** including root hairs **(F/f)**, leaf epidermis **(G/g-I/i)** including trichomes **(G/g-H/h)** and stomata **(I/i)**, immature floral bud (including sepals, petals, stamens and pistil) **(J/j)**, mature gynoecium (valves, stigma and style) **(K/k)**, mature androecium (anther and filament) **(L/l)**, pollen grains [**(M/m-N,n)**, arrow], ovules **(O/o)** and funiculi [**(O/o)**, arrow], petals (including veins) **(P/p)** and sepals (including veins) **(Q/q)**. Red signals represent chlorophyll autofluorescence. At least three independent T3 homozygous lines were generated and analyzed, depicting similar fluorescence profiles.

Figure 3. Structure of the native RS2Z32 and RS2Z33 proteins. AlphaFold models of RS2Z monomers. Cartoon representations of the RS2Z32 **(A)** and RS2Z33 **(B)** monomers colored according to the pLDDT value from red (<50), white (>70) and blue (>90) **(left)**. RS and SP domains, which present pLDDT values below 50, are considered unstructured and are not displayed. Predicted structured domains are shown: RRM (with Y14, Y46 and F48 shown as sticks) in red and ZnK1/2 in yellow and orange, respectively **(right)**. Purple spheres represent Zn^{2+} ions that are coordinated by amino-acid residues shown as sticks: Cys101, Cys104, His109 and Cys114 in ZnK1 and Cys123, Cys126, His131 and Cys136 in ZnK2 (within 3 Å).

Figure 4. Structural determinants modulating the localization of RS2Z members. Subcellular fluorescence distribution in transient expression assays in tobacco leaf cells upon N-terminal

EGFP-tagging of the native RS2Z32 or RS2Z33 and their respective mutant variants: wild-type RS2Z proteins **(A/a)**, RS2ZmutRRM **(B/b)**, RS2ZmutZnKs **(C/c)**, RS2ZmutRRM+ZnKs **(D/d),** RS2ZmutRS **(E/e)**, RS2ZmutSP **(F/f)**, RS2ZmutRS+SP **(G/g)**, RS2ZmutRRM+RS **(H/h)**, RS2ZmutRRM+SP **(I/i)**, and *Arabidopsis thaliana* T3 homozygous plants expressing the GFP translational fusions (*pRS2Z32:RS2Z32-EGFP* and *pRS2Z33:RS2Z33-EGFP*) **(J/j)**. Scale bars = 10 µm. Red signals represent chlorophyll autofluorescence. Whenever cytoplasmic and nuclear signals were detected, insets depict the detailed intranuclear localization. At least three independent transient events were generated and analyzed, depicting similar fluorescence profiles.

Figure 5. Nucleocytoplasmic shuttling of native RS2Z proteins and mutant variants. FLIP-shuttling was assessed without (−LMB) or with LMB (+LMB) treatment in tobacco epidermal leaf cells. One hundred percent fluorescence indicates prebleach fluorescence intensity. As a control of the dynamic nature of native RS2Z proteins, cells were repeatedly scanned under no photobleaching conditions and fluorescence was monitored. Insets show the overlay of wild-type and mutant curves. Values are means ± SEM for at least 12 nuclei. A significant inhibitory effect of LMB is indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). n.s., not significant.

Figure 6. RS2Z32 and RS2Z33 associate with kinases, splicing-associated factors, and the nuclear import factor. **(A)** The baits tested correspond to native and mutant variants. **(B)** Absence of interaction between RS2Z32 or RS2Z33 with kinases (SRPK1, SRPK2, and AFC1-3), nuclear cylophilins (Cyp65 and Cyp71), hnRNP-like proteins (GRP7, GRP8, RZ-1B, and RZ-1C), PRP38, MOS12, ALY/REF ortholog ALY4, EJC core components (MAGO, Y14, eIF4AIII), or the peripheral EJC components (ACINUS, PININ, and SAP18). From the initial culture, dilutions to an OD600 of 0.25 were spotted on -Trp/-Leu/-His/X-α-Gal/AurA agar plates. Positive interactions were confirmed by growth and blue staining as seen with the positive control (pGADT7-T x pGBKT7-53). An abolished interaction is characterized at most by a shadow of dead cells (similar to the negative control pGADT7-T x pGBKT7-Lam). Serial dilutions (OD 0.25, 0.025 and 0.0025) are presented in **Supplementary Figure S6**.

959 **Figure 7.** RS2Z proteins interact with mRNA processing factors *in planta*. Bimolecular
960 fluorescence complementation (BiFC) in transient expression assay in tobacco leaf cells through
961 co-expression of RS2Z32 or RS2Z33 fused to the C-terminal half of YFP (RS2Z32:^{C}YFP or
962 RS2Z33:^{C}YFP) and either SR45.1., SCL30, SR34 and CypRS64 fused to the N-terminal half of
963 YFP (SR45.1, SCL30, SR34, or CypRS64:^{N}YFP). For all observations, representative images of
964 fluorescence reveal the interactions of RS2Z32 or RS2Z33 with the respective proteins in the
965 nucleus, and in speckle-like structures (arrowheads). At least three independent transient events
966 were generated and analyzed, depicting similar fluorescence profiles. Scale bars = 10 µm. Red
967 signals represent chlorophyll autofluorescence.
968
969 **Figure 8.** The RRM of RS2Z proteins binds to a pyrimidine-rich RNA motif. RNA motifs were
970 identified through 4 rounds of SELEX selection using either the native RRM of RS2Z32 **(A)** and
971 RS2Z33 **(B)** or the mutated versions of the RRM of RS2Z32 **(C)** and RS2Z33 **(D)**. Two non-
972 significant RNA motifs (#1-2) were identified using the mutated versions. The statistical
973 significance (*E*-value) is indicated at the bottom of each consensus. Logos were redesigned using
974 WebLogo (Crooks *et al.*, 2004). All RNA motifs were discovered using the MEME tool (version
975 5.5.1) with 10 sequences.