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A role for SR proteins in plant stress responses

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Members of the SR (serine/arginine-rich) protein gene family are key players in the regulation of alternative splicing, an important means of generating proteome diversity and regulating gene expression. In plants, marked changes in alternative splicing are induced by a wide variety of abiotic stresses, suggesting a role for this highly versatile gene regulation mechanism in the response to environmental cues. In support of this notion, the expression of plant SR proteins is stress-regulated at multiple levels, with environmental signals controlling their own alternative splicing patterns, phosphorylation status and subcellular distribution. Most importantly, functional links between these RNA-binding proteins and plant stress tolerance are beginning to emerge, including a role in the regulation of abscisic acid (ABA) signaling. Future identification of the physiological mRNA targets of plant SR proteins holds much promise for the elucidation of the molecular mechanisms underlying their role in the response to abiotic stress.

SR (serine/arginine-rich) proteins constitute a highly conserved family of RNA-binding proteins, which plays key roles in the execution and regulation of precursor-mRNA (pre-mRNA) splicing. By affecting splice site selection in a concentration- and phosphorylation-dependent fashion, SR proteins significantly contribute to the alternative splicing process, which they appear to modulate in a tissue-specific, developmentally-regulated and stress-responsive manner.

The splicing of introns from the pre-mRNA is carried out by one of the largest molecular complexes of the cell, the spliceosome, which consists of five small nuclear ribonucleoproteins (snRNPs) and numerous additional proteins.[1,2] Members of the SR protein family are non-snRNP spliceosomal factors that have been shown in animal systems to play vital roles in the most crucial and early steps of spliceosome assembly.[3-6] These essential splicing factors share a multidomain structure typically characterized by the presence of one or two N-terminal RNA Recognition Motifs (RRMs) and a C-terminal reversibly phosphorylated arginine/serine-rich (RS) domain.[3,7] Binding of SR proteins to the pre-mRNA is mediated by the RRM, which recognizes short and degenerate sequences such as exonic splicing enhancers or silencers (ESEs or ESSs, respectively). The RRM confers RNA-binding specificity and each ESE/ESS is thought to be recognized by a unique set of one or more SR proteins.[8] On the other hand, the RS domain is involved in protein-protein interactions that promote recruitment of core splicing machinery components to nearby splice sites,[3,6,9] but has also been reported to contact directly with the pre-mRNA via the branchpoint and the 5' splice site to promote pre-spliceosome assembly.[5,10,11] Furthermore, the RS domain affects SR protein subcellular localization by acting as a nuclear localization signal via interaction with the nuclear import receptor, transportin-SR,[12-14] and can be highly phosphorylated at multiple serine residues by a number of specific cellular kinases.[7] Reversible phosphorylation of SR proteins is crucial for their ability to interact with RNA and other splicing factors, as well as for their nuclear localization and recruitment to sites of pre-mRNA synthesis.[7,15-17]

Studies in animal systems have also revealed that SR proteins that shuttle between the nucleus and the cytoplasm play additional roles in RNA metabolism. Some of these splicing factors function in mRNA export by interacting with the key nuclear export factor TAP/NFX1,[18,19] while overexpression of various SR proteins has been found to strongly enhance the nonsense-mediated mRNA decay (NMD) pathway.[20] Moreover, the nucleocytoplasmic SF2/ASF is able to associate with polyribosomes and stimulate protein synthesis[21] via recruitment of components of the mTOR (mammalian target of rapamycin) pathway,[22] and another two shuttling SR proteins have been shown to function in translation of specific mRNAs.[23,24] Recent findings have also linked SR proteins to active roles in promoting transcriptional elongation,[25] maintaining genome stability[26,27] and facilitating cell cycle progression.[28,29] Thus, at least in metazoan cells, specific SR splicing regulators have been emerging as critical factors in multiple additional steps of gene expression, from transcription to mRNA export, quality control and translation.[28,30,31]

Plant SR Proteins

Plant SR proteins, which in Arabidopsis range in size from 21–41 kDa, were first identified using a monoclonal antibody raised against a serine phosphoepitope in the RS domain.[32,33] Several of these proteins have been shown to restore splicing competency of HeLa cell cytoplasmic extracts deficient in SR proteins and to be active in heterologous alternative splicing assays,[32-37] suggesting conservation of the basic splicing mechanism in plants and metazoans. However, plant introns (which are considerably shorter and richer in U) are often inaccurately spliced in mammalian nuclear extracts and animal introns are not processed in plant nuclei.[38-41] Hence, intron recognition appears to differ in plants, which may partly explain the considerable expansion of the SR

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Table 1. The Arabidopsis SR protein family

A. Mammalian orthologs

Subfamily	Gene/Protein symbol	Gene locus	Aliases
SR ASF/SF2 (SRSF1) orthologs	SR30	At1g09140	SRp30
	SR34	At1g02840	SRp34, SR1
	SR34a	At3g49430	SRp34a
	SR34b	At4g02430	SRp34b
RSZ 9G8 (SRSF7) orthologs	RSZ21	At1g23860	RSZp21, SRZ21
	RSZ22	At4g31580	RSZp22, SRZ22
	RSZ22a	At2g24590	RSZp22a
SC SC35 (SRSF2) ortholog	SC35	At5g64200	-

B. Plant specific

Subfamily	Gene/Protein symbol	Gene locus	Aliases
SCL	SCL28	At5g18810	-
	SCL30	At3g55460	-
	SCL30a	At3g13570	-
	SCL33	At1g55310	SR33
RS2Z	RS2Z32	At3g53500	RSZ32
	RS2Z33	At2g37340	RSZ33
RS	RS31	At3g61860	RSp31
	RS31a	At2g46610	RSp31a
	RS40	At4g25500	RSp40, RSp35
	RS41	At5g52040	RSp41

Nomenclature and classification recently proposed by Barta et al. [44]

protein gene family observed in the plant kingdom. In fact, flowering plants possess the highest number of SR proteins among eukaryotes, with a total of 24 in rice,[42] 17 in Brachypodium[43] and 18 in Arabidopsis,[44] whilst there are only seven SR protein genes in *C. elegans*[45] and 12 in humans.[46] Larger and more diverse families of these proteins in plants most likely resulted from genome amplification, particularly interchromosomal duplication events, and indeed at least 12 of the Arabidopsis SR genes are located on duplicated segments of the genome.[47] A key open question in the field is whether the proteins encoded by these six pairs of paralogs are redundant or have evolved different functions.

Owing to the presence of multiple paralogs, the Arabidopsis SR protein family can be divided into six subfamilies (**Table 1**),[44] three of which are constituted by true orthologs of human ASF/SF2, SC35 and 9G8 (recently renamed SRSF1, SRSF2 and SRSF7, respectively[46]). The RS, RS2Z and SCL subfamilies are plant-specific, presenting unique structural features not found in SR proteins from metazoan organisms. Proteins of the RS subfamily do not include the highly conserved SWQDLKD motif in their second RRM (characteristic of ASF/SF2 orthologs) and their RS domain is highly enriched in arginines rather than serine-arginine dipeptides.[35,37,44] The members of the RS2Z family contain two zinc knuckles (instead of one in 9G8 orthologs) separating the RRM from the RS domain as well as an acidic C-terminal extension rich in serine and proline residues.[44,48] Although similar to SC35, SCL subfamily members display a short N-terminal charged extension rich in arginines, prolines, serines, glycines and tyrosines.[44,48,49] The SR45 protein is a bona fide essential splicing factor, as it complements a splicing-deficient heterologous cell extract,[34] but displays a highly atypical SR protein structure—a single RRM flanked by two RS domains—and its exclusion from the Arabidopsis SR protein family was very recently proposed.[44] This protein seems to have appeared later in evolution in flowering plants[34] and is related to human RNPS1, a component of the exon-exon junction complex[50] involved in several aspects of RNA metabolism[51-53] and in alleviating genome instability.[54] In addition to SR45, 11 of the 18 Arabidopsis SR proteins have no direct counterparts in animal systems and present a unique domain organization, indicating that they may have evolved plant-specific functions. Nevertheless, the body of functional data available so far for plant SR proteins is scarce, stemming from Arabidopsis transgenic lines overexpressing SR30[55] or RS2Z33,[56] as well as a loss-of-function mutant for SR45.[34]

Numerous analyses in different plant tissues and organs have revealed both overlap and spatiotemporal diversification of SR gene expression patterns[32,37,48,55-60] and, until very recently, all Arabidopsis SR proteins analyzed had been found to be confined to the nucleus. The nucleoplasmic, nucleolar and nuclear speckle localization appears to depend on the developmental stage and the cell's type, cell cycle phase and physiological state, as well as the phosphorylation status of the SR protein,[57,61-66] but the mechanisms regulating the subcellular localization and nuclear dynamics of plant SR proteins remain virtually unknown. Interestingly, members of different Arabidopsis SR protein subfamilies were found to localize to distinct populations of speckles in the nuclei of tobacco protoplasts, suggesting specificity for splicing of particular pre-mRNAs.[67] Moreover, the first observation of nucleocytoplasmic shuttling of a plant SR protein has just been reported for the Arabidopsis RSZ22,[68] which thus represents a strong candidate for an SR protein also involved in post-splicing activities.

A striking feature of both plant and animal genes encoding SR proteins and other splicing components is that they often undergo alternative splicing themselves. In Arabidopsis, it appears that only two SR protein genes, *RSZ22a* and *SCL28*, produce a single pre-mRNA (http://www.arabidopsis.org; Palusa et al.[60]), the remainder reportedly producing together up to over 90 transcripts, thus increasing dramatically the complexity of the SR gene family transcriptome.[60,69] The majority of these splice variants contains a premature stop codon (PTC) and may encode either nonfunctional or truncated proteins with altered functions, but a recent study has shown that roughly half of these PTC-containing transcripts are targeted to degradation by NMD.[69] In mammalian cells, the coupling of alternative splicing and NMD provides an effective means of downregulating physiological transcripts and is frequently employed for autoregulation

of SR and other splicing-related proteins.[70] Indeed, gain of in vivo function of the Arabidopsis SR proteins SR30 and RS2Z33 not only affected splicing of other SR gene transcripts, but also of their own pre-mRNAs,[55,56] which have been shown to generate NMD-targeted transcripts.[69]

Alternative Splicing and Abiotic Stress

By selectively joining different exons and generating different transcripts from a single gene, alternative splicing pathways provide a key mechanism for generating proteome diversity and functional complexity, as well as regulating gene expression. In contrast to transcriptional control, alternative splicing changes the structure of transcripts and can influence almost all aspects of protein function, such as binding properties, enzymatic activity, intracellular localization, post-translational modification or protein stability. As noted above, alternative splicing may also be coupled to NMD to regulate functional transcript levels.[70] The past decade has witnessed the emergence of alternative splicing as a major feature of several transcriptomes, including those of higher plants. The most recent estimates, based on next generation sequencing analyses, indicate that over 90% of human genes[71,72] and at least 42% of Arabidopsis intron-containing genes[73] produce more than one transcript. The prevalence of alternative splicing in many genomes suggests that this mechanism plays crucial roles in biological processes, as is emphasized by the fact that its misregulation can lead to many human diseases.[74]

The sessile growth habit of plants has empowered them with unique adaptive developmental and physiological strategies to cope with environmental stress, which range from morphological modifications to physiological adaptation at the cellular level. However, the basis of the capacity for adaptation lies ultimately at the level of the genome, and the exceptional versatility associated with gene regulation by alternative splicing is likely to play a prominent role in these adaptive processes. Interestingly, comparative analyses of mammalian genomes indicated that evolutionary change occurs at a faster rate in genes that are subject to alternative splicing,[75] which in plants could have been useful in the acquisition of specific adaptive benefits essential for survival under adverse environmental conditions.[76] Although the vast majority of plant alternative splicing events has not been functionally characterized, two major lines of evidence support the notion that they participate in important plant functions such as the response to abiotic stress. Firstly, plant genes with regulatory functions and associated with various stresses are particularly prone to alternative splicing, appearing overrepresented in plant alternative splicing databases.[76-79] Consistent with this, the functional distribution of Arabidopsis genes coding for transcripts with retained introns was found to be biased towards stress-related functions.[80] Secondly, alternative splicing in plants is often associated with abiotic stress. Indeed, genomewide analyses in Arabidopsis have found altered alternative splicing profiles under different stress conditions.[73,81] Moreover, there are numerous reports of individual genes from various species and implicated in diversified functions whose alternative pre-mRNA splicing is affected by stress.

Notably, heat stress changes the alternative splicing patterns of both the *waxy* gene encoding a rice starch synthase[82] and the Arabidopsis heat shock factor *HSFA2*,[83] while cold-dependent changes in alternative transcripts have been reported for a potato invertase gene,[84] the black spruce β-hydroxyacyl ACP dehydratase gene involved in fatty acid biosynthesis,[85] the trifoliate orange *CTL* gene expressed exclusively at low temperatures,[86] a durum wheat gene encoding a putative ribokinase,[87] and a tomato alternative oxidase (*AOX*) gene involved in the removal of stress-induced reactive oxygen species.[88] In rice, alternative splicing of an *AOX* gene is also changed, but in response to salt stress.[89] Other plant genes have their alternative splicing patterns affected by more than one type of abiotic stress, such as an ubiquitin ligase durum wheat gene whose retention of a 3'UTR-located intron is promoted by cold and drought stress,[87] and the maize NADPH oxidase B gene where splicing of intron 11 is enhanced by salt, temperature and radiation stress.[90]

Transcriptional control of the expression of stress-responsive genes is a pivotal component of abiotic stress response in plants. Importantly, several plant genes encoding transcription factors also undergo alternative splicing in a stress-dependent fashion, thereby potentially ensuring appropriate downstream stress-related gene expression. An illustrative example is that of *DREB2*-type transcription factors involved in controlling cold- and drought-responsive gene expression in Arabidopsis. Grass family orthologs of these genes often undergo alternative splicing, which in the case of the wheat *Wdreb2*,[91] the maize *ZmDREB2A*[92] and the rice *OsDREB2B*[93] is affected by salt, drought and temperature stress.

The results described above strongly suggest that splicing mechanisms play an important role in regulating gene activity under stress conditions, and indeed a couple of recent studies have provided compelling evidence that stress-induced changes in alternative splicing may be functionally relevant. For both the maize DREB2A[92] and the rice DREB2B[93] transcription factors, Shinozaki and coworkers have shown that abiotic stresses specifically induce the splice variant encoding the full-length protein, which when functionally expressed in Arabidopsis confers enhanced target gene expression and improved drought and heat-shock stress tolerance. On the other hand, the accumulation of a non-functional alternative transcript encoded by the Arabidopsis heat shock factor gene *HSFA2* that is targeted to NMD is a feature of the cytosolic protein response (CPR), a subcomponent of the heat shock response, pointing to a mechanism for posttranscriptional regulation of the production of active protein.[83]

SR Proteins and Plant Stress Responses

As key factors in the early recognition of splice sites and being highly conserved in all genomes undergoing alternative splicing, SR proteins are widely recognized as the major regulators of this versatile gene regulation mechanism. Hence, detailed characterization of the SR protein family in plants should be able to substantiate the role of alternative splicing in plant stress responses.

A comprehensive RT-PCR analysis of Arabidopsis SR gene expression showed no dramatic stress-dependent changes in

overall transcript levels, except perhaps for *SCL33*, which was repressed by salt and temperature stress as well as exogenous application of the stress phytohormone abscisic acid (ABA).[60] However, the alternative splicing pattern of several members of the Arabidopsis SR protein family has been shown to change strikingly under various abiotic stress conditions, including temperature stress,[59,60,73] high salinity[60,94] and high light irradiation.[73,94] In the absence of functional data on the different splice isoforms, the biological significance of the observed changes is difficult to assess. Nevertheless, stress-induced changes in SR protein gene products could in turn alter the splicing of downstream targets resulting in adaptive transcriptome changes in response to environmental cues. In support of this, the relative levels of the splice variant encoding the full-length SR30 protein, which has also been shown to affect splicing of its own pre-mRNA,[55] were recently reported to increase markedly under heat, light and salt stress.[73] The multidomain structure of SR proteins may also allow alternative splicing to generate isoforms that differ in their domain organization and hence in function.[95] On the other hand, shifts in SR gene splicing patterns may be coupled to NMD as a negative feedback mechanism to regulate the amounts of functional SR protein in response to stress. Indeed, intron retention—the most prevalent form of alternative splicing in plants[73,80] and an important generator of PTC-containing isoforms—is frequently associated with different abiotic stresses, which induced dramatic changes in the abundance of unproductive transcripts of both *SR30* and *SR34* in Arabidopsis.[73]

Stress signals are also known to control both the phosphorylation status and the subcellular localization of plant SR proteins, pinpointing potential mechanistic links between abiotic stress and the regulation of alternative splicing. In Arabidopsis, the noncanonical SR protein SR45 is preferentially localized in enlarged nuclear speckles upon heat shock, while cold induces its relocalization to a diffuse nucleoplasmic pattern.[62] This intranuclear redistribution in response to temperature stress was shown to be dependent on protein phosphorylation.[62] The phosphorylation status of the cell also affected the subcellular distribution of RSZ22, which concentrates in the nucleolus upon experimental stress (prolonged observation periods), probably as a result of ATP depletion.[66,68] However, the effect of different stresses on the nucleocytoplasmic dynamics of this Arabidopsis shuttling SR protein is unknown. Interestingly, an earlier study showed that the ethylene-inducible tobacco PK12 kinase phosphorylates and specifically interacts with the Arabidopsis SR34,[96] suggesting a role for PK12 in the transduction to the splicing machinery of environmental signals that trigger the biosynthesis of the ethylene phytohormone, such as drought, chilling or anoxia. Finally, heterologous expression of an Arabidopsis RS domain in yeast conferred tolerance to salt stress, which required phosphorylation by the Sky1p SR protein kinase.[97] Importantly, overexpression of this RS domain also conferred increased salt tolerance to transgenic Arabidopsis plants.[97]

The first functional studies involving plant SR proteins revealed pleiotropic developmental and morphological changes in transgenic plants overexpressing SR30,[55] and RS2Z33,[56] as well as in a loss-of-function mutant for the non-canonical SR protein SR45,[34] but did not address the response to environmental cues. A functional link between SR proteins and stress responses has been provided by the recent report that, in Arabidopsis, SR45 negatively regulates glucose signaling during early seedling development by downregulating the ABA pathway.[98] Indeed, the *sr45-1* knockout mutant, which is hypersensitive to both glucose and ABA, displays enhanced glucose-induced accumulation of endogenous ABA as well as glucose overinduction of ABA biosynthesis and signaling gene expression.[98] Interestingly, the molecular mechanism underlying the action of SR45 appears to be independent of the hexokinase 1 (HXK1) sugar sensor and to involve modulation of the levels of KIN10/SnRK1.1 (Carvalho RF, et al. unpublished results), a protein kinase implicated in sensing/signaling of stress-associated energy deprivation.[99]

Identification of the physiological transcripts targeted by SR proteins will be crucial for unraveling their precise roles in plant stress responses. Previous work has shown that overexpression of SR30[55] and RS2Z33[56] in Arabidopsis, as well as of *Os*RS2Z36 and *Os*SR33 in transgenic rice,[100] alters the splicing patterns of their own pre-mRNAs and those of several other SR protein genes. SR45 displays splicing activity in vitro and has also been shown to affect alternative splicing of five other SR genes.[34] In addition, microarray and RT-PCR experiments have revealed upregulation of a key flowering repressor[34] and ABA-related genes[98] in the *sr45-1* mutant. Finally, a recently developed tool to monitor multiple Arabidopsis alternative splicing events simultaneously by RT-PCR has identified significant alternative splicing changes induced by ectopic expression of SR30 and RS2Z33 in 13 additional genes.[101] Despite the importance of these findings in providing valuable functional clues, they do not represent a comprehensive analysis of the gene expression and splicing changes induced by SR proteins and may include direct targets, further downstream targets or a combination of both. Ultimately, formal demonstration of the endogenous mRNAs directly targeted by individual plant SR proteins will come from biochemical approaches for which these studies may provide candidate genes.

Conclusions and Perspectives

Their unique developmental and physiological plasticity suggests that plants offer exceptional opportunities to reveal alternative splicing mechanisms, which appear to be especially important in adapting to environmental stress. Recent studies have clearly shown that plant transcriptome complexity is significantly increased by alternative splicing, which undergoes remarkable changes with potential functional relevance in response to abiotic stress. Genomewide comparison of alternative splicing profiles in an ecotype adapted to moderate environments versus a stress-tolerant one could strongly corroborate the biological significance of these changes. Furthermore, functional analyses of the splice isoforms generated by SR and closely related genes are beginning to substantiate a role for alternative splicing in plant stress tolerance. However, the possibility that, like their animal counterparts, plant SR proteins are involved in other steps of gene expression should not be excluded, as is underscored by the

nucleocytoplasmic shuttling of at least one Arabidopsis SR protein.[68] Bioinformatics analyses and in vitro selection from pools of random RNA sequences may provide important information on the high affinity binding sites recognized by individual SR proteins, but elucidation of the precise molecular mechanisms underlying the functions of plant SR proteins in stress responses will require the identification of their physiological targets. This may be accomplished through reversible crosslinking combined with immunoprecipitation approaches to analyze RNA-protein interactions in stressed plants and subsequent validation of the candidate molecular targets in plants where individual SR proteins have been mutated or overexpressed.

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