

REVIEW

Complexity of the Alternative Splicing Landscape in Plants

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Alternative splicing (AS) of precursor mRNAs (pre-mRNAs) from multiexon genes allows organisms to increase their coding potential and regulate gene expression through multiple mechanisms. Recent transcriptome-wide analysis of AS using RNA sequencing has revealed that AS is highly pervasive in plants. Pre-mRNAs from over 60% of intron-containing genes undergo AS to produce a vast repertoire of mRNA isoforms. The functions of most splice variants are unknown. However, emerging evidence indicates that splice variants increase the functional diversity of proteins. Furthermore, AS is coupled to transcript stability and translation through nonsense-mediated decay and microRNA-mediated gene regulation. Widespread changes in AS in response to developmental cues and stresses suggest a role for regulated splicing in plant development and stress responses. Here, we review recent progress in uncovering the extent and complexity of the AS landscape in plants, its regulation, and the roles of AS in gene regulation. The prevalence of AS in plants has raised many new questions that require additional studies. New tools based on recent technological advances are allowing genome-wide analysis of RNA elements in transcripts and of chromatin modifications that regulate AS. Application of these tools in plants will provide significant new insights into AS regulation and crosstalk between AS and other layers of gene regulation.

INTRODUCTION

Production of the right amount of protein in the right cells at the right time is crucial for growth and development of multicellular eukaryotes and their response to the environment. Hence, protein synthesis is tightly regulated with multiple layers of regulation. Transcriptional regulation of gene expression is a central component of this regulation. In recent years, it has become clear that regulation of cotranscriptional processes, such as splicing and polyadenylation, is also a major driving force of transcript complexity and abundance. Posttranscriptional gene regulation occurs at many levels, including transcript export, localization, mRNA stability, translation, posttranslational modifications of proteins, and protein stability and degradation, which ultimately dictate the amount and functionality of RNAs and proteins within the cell. The extent, complexity, and sophistication of posttranscriptional gene regulation are beginning to rival transcriptional regulation in eukaryotes. The first evidence for the significance of alternative splicing (AS) in plant development came from differential expression of Ser/Arg-rich (SR) protein splicing factors in different organs and during development (Lopato et al., 1996, 1999b; Kalyna et al., 2003; Palusa et al., 2007) indicating organ-specific regulation of AS in plants. Verification will come from genome-wide studies of AS in different organs and

during development (Loraine et al., 2013) as has been shown in animals (Wang et al., 2008; Kalsotra and Cooper, 2011; Barbosa-Morais et al., 2012; Ellis et al., 2012). In addition, screens for mutants in various pathways have frequently identified splicing factors as modulators of functional proteins, indicating that these pathways are regulated via differential splicing (Lee et al., 2006; Monaghan et al., 2009; Sugliani et al., 2010; Fouquet et al., 2011; Koncz et al., 2012). There is an ever-growing body of literature on how alternative splicing (AS) influences important developmental and signaling pathways, and many important examples have been discussed in the accompanying review by Staiger and Brown (2013). This review focuses on the current knowledge on splicing and genome-wide AS in plants, its regulation and potential functions, and the important outstanding questions and tools to address these.

The mechanism of splicing has been elucidated mainly by *in vitro* assays and genetic studies in mammals and yeast. The lack of an *in vitro* splicing assay in plants has been a major limitation in studying the mechanisms involved in intron recognition and spliceosome assembly in plants (Barta et al., 2012). However, the advent of the genomic era and the availability of whole-genome sequences of several plants allowed the identification of orthologous proteins and small nuclear RNAs (snRNAs) of the core components of the spliceosome (Wang and Brendel, 2004; Barta et al., 2012; Koncz et al., 2012) (see Supplemental Table 1 online), suggesting that the main principles of intron processing are also applicable to plants. Nevertheless, the fact that animal introns cannot be processed in plants made it clear that there is some specificity in the plant spliceosomal machinery and in the plant intronic sequences for their successful splicing (Barta et al., 1986; Brown et al., 1986; Hartmuth and Barta, 1986). Even though some

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animal introns are as small as or smaller than plant introns, plants do not have the long introns characteristic of many animal species. There is a clear difference in average size of introns between plants and animals; while animal introns can be quite long with an average size of 5 kb (Sakharkar et al., 2004), plant introns are relatively small with an average size of 160 bp (Iwata and Gotoh, 2011; Marquez et al., 2012), suggesting that recognition of exons and introns might differ in plants. The consensus sequences for the 5' splice site (5'SS), 3' splice site (3'SS), and branch point (BP) are comparable in animals and plants (Simpson et al., 2002; Reddy, 2007; Iwata and Gotoh, 2011), indicating that core splicing factors recognizing such sequences are acting in similar ways. However, in plants, the presence of uridine-rich sequences toward the 3'SS was found to be an important determinant of splicing efficiency as was a clear difference in the adenine-uridine (AU) and guanine-cytosine (GC) content of introns and exons especially in dicotyledons where introns are more than 60% AU rich (Goodall and Filipowicz, 1989). Differences can also be seen between different plant species; for example, there is a higher AU content in the introns of dicotyledons species in comparison with those in monocotyledons (Baek et al., 2008). Differences in the GC/AU content of exons and introns were lately reported to occur as well in mammals supporting a shared principle for exon and intron definition (Amit et al., 2012). The difference in GC/AU content might explain differences in nucleosome positioning of exons and introns that may influence RNA polymerase II elongation rates and have an effect on splicing (reviewed in Gómez Acuña et al., 2013).

SPLICEOSOME COMPOSITION AND ASSEMBLY

One of the major advances over the past years was the insight that most RNA splicing events occur cotranscriptionally and hence are dependent on DNA and chromatin structure and modifications (reviewed in Braunschweig et al., 2013). Up to 90% of protein-coding and many non-protein-coding genes contain introns in photosynthetic eukaryotes (Hirsch et al., 2006; Szarynska et al., 2009; Labadorf et al., 2010). Generation of mature mRNAs from these genes requires precise removal of introns from precursor mRNAs (pre-mRNAs) and joining of exons. Four loosely conserved core sequence elements in introns, the 5'SS with a conserved GT, the 3'SS with a conserved AG, a BP with a conserved A residue 18 to 40 nucleotides upstream of the 3'SS, and a polypyrimidine tract following the BP, are necessary for spliceosome assembly. Splicing requires the precise recognition of splice sites and spliceosome assembly with numerous RNA-RNA, RNA-protein, and protein-protein interactions and conformational rearrangements of these interactions in a precise sequential manner. An in-depth discussion on spliceosome composition and dynamics during different stages of splicing in yeast and metazoans is presented in recent reviews (Will and Lührmann, 2011; Hoskins and Moore, 2012).

Plant spliceosomes have not been isolated so far; hence, their assembly and composition are not known. Extensive studies with yeast and human spliceosomes that are assembled *in vitro* on model pre-mRNA substrates have revealed that the spliceosome is a highly dynamic and complex macromolecular machine consisting of five snRNAs and over 300 proteins (Jurica and Moore,

2003; Will and Lührmann, 2011). The yeast *Saccharomyces cerevisiae* spliceosome has ~160 proteins (Fabrizio et al., 2009), and the homologs of all of these are present in the human spliceosome, indicating the conservation of core spliceosomal proteins across eukaryotes. However, human spliceosomes have over 100 additional proteins, which are thought to play a role in splice site selection and regulation of AS (Will and Lührmann, 2011).

The analyses of splicing homologs in *Arabidopsis* have led to the identification of almost twice the number of splicing regulatory factors in comparison to human (see Supplemental Table 1 online) (Wang and Brendel, 2004; Barta et al., 2012; Koncz et al., 2012). The presence of multiple paralogs in plants seems to be the result of whole-genome duplications followed by massive genomic rearrangements in the course of evolution (Kalyna and Barta, 2004; Adams and Wendel, 2005; Cui et al., 2006). The existence of multigene families of splicing factors including those involved in AS, like the multimember family of the SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) that share distinct levels of identity, raises the question of how many of the family members have redundant roles or have acquired new functions. The higher complexity of plant core spliceosomal proteins might be the reason that mutations in several proteins associated with plant small nuclear ribonucleoprotein particles (snRNPs) are not lethal but cause various defects in the development and response to environmental conditions (Staiger and Brown, 2013).

In metazoans, there are two types of spliceosomes: a major U2 type with U1, U2, U4, U5, and U6 snRNPs involved in splicing of most pre-mRNAs with GT-AG splice sites and a minor U12 type with U11, U12, U4atac, U5, and U6atac snRNPs that splice a small fraction of pre-mRNAs with so-called U12 type introns. These introns are characterized by a more conserved 5'SS and BP sequence and have both canonical (GT-AG) and noncanonical splice sites (Friend et al., 2008; Steitz et al., 2008). They are not very abundant with ~800 in human and mice and 300 in plants (Alioto, 2007); however, a recent deep-sequencing analysis resulted in identification of ~2000 introns with U12-type signatures in *Arabidopsis thaliana* (Marquez et al., 2012). These introns, which occur usually only once in a transcript, are spliced mainly cotranscriptionally with a slower kinetics. Furthermore, these are often evolutionarily conserved and important for developmental processes (recently reviewed in Turunen et al., 2013). Interestingly, 20 U12-type introns are conserved in orthologous positions between human and *Arabidopsis* (Zhu and Brendel, 2003; Basu et al., 2008). Analysis of the large pool of newly discovered U12-type introns might change this number (Zhu and Brendel, 2003; Basu et al., 2008). Based on the presence of most of the RNA and protein homologs of mammalian spliceosomes (see Supplemental Table 1 online), it appears that plants, like animals, have both major and minor spliceosomes (Shukla and Padgett, 1999; Reddy, 2001; Wang and Brendel, 2004; Lorkovic et al., 2005; Ru et al., 2008; Simpson and Brown, 2008; Barta et al., 2012; Koncz et al., 2012).

CONSTITUTIVE SPLICING AND AS

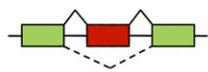
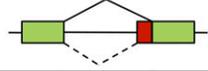
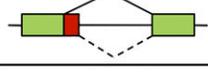
A multiexon gene can produce a single mature mRNA by constitutive splicing (CS), where only one set of splice sites is used.

Failure to select or differential usage of some of these splice sites can lead to intron retention (IR) or exon skipping events. In addition, alternative splice sites (5'SS, 3'SS, or both) can be employed, and all these events generate multiple mRNA isoforms from the same gene (Figure 1) (reviewed in Reddy, 2007; Syed et al., 2012). A given splice variant may show more than one of these events. The long-standing question of how higher organisms achieve their complexity despite having similar gene numbers is at least partly explainable by the complexity of RNA transcripts created by AS. Deep sequencing of human transcriptomes from different organs, tissues, and cell lines indicates that almost every intron-containing gene generates multiple splice variants under certain conditions, and many AS events are regulated in a cell-, tissue-, or condition-specific manner (Pan et al., 2008; Wang et al., 2008; Merkin et al., 2012). Furthermore, recent studies indicate that the extent of AS increases as the complexity of tissues and species increases (Barbosa-Morais et al., 2012; Merkin et al., 2012).

The discovery of alternative transcripts in many organisms, including plant species, has been accomplished mainly using ESTs. The estimations of AS based on ESTs suggested that 11 to 30% of the multiexonic genes were alternatively spliced in *Arabidopsis* (Iida et al., 2004; Campbell et al., 2006; Barbazuk et al., 2008). Nevertheless, the discovery of new isoforms depended highly on the depth of the EST and cDNA libraries. Innovations in sequencing RNA (RNA-seq) led to the generation of millions of short sequence reads of cDNAs derived from poly(A)-enriched mRNA from different tissues or conditions in a cost effective manner, which were then mapped onto unique locations on the genome to predict AS events (Wang et al., 2009a; Filichkin et al., 2010; Hallegger et al., 2010; Marquez et al., 2012). This has been used to analyze the extent of AS and to identify novel introns and splice variants in *Arabidopsis* (Filichkin et al., 2010; Marquez et al., 2012; Syed et al., 2012), rice (*Oryza sativa*; Zhang et al., 2010b),

cucumber (*Cucumis sativus*; Guo et al., 2010), grape (*Vitis vinifera*; Zenoni et al., 2010), and *Brachypodium distachyon* (Walters et al., 2013). These studies have revealed that up to 60% of intron-containing genes are alternatively spliced.

Analysis of different AS events in *Arabidopsis* and several other plants indicated that IR is the predominant mode of AS in land plants (Ner-Gaon et al., 2004; Wang and Brendel, 2006; Baek et al., 2008; Filichkin et al., 2010; Marquez et al., 2012; Syed et al., 2012). In metazoans, the predominant AS event is exon skipping and IR is least prevalent (Figure 1), suggesting differences in regulation of splice site recognition in metazoans and plants. Although IR is the most prevalent AS event in *Arabidopsis*, more than 51% of genes producing AS transcripts do not show IR events (Marquez et al., 2012). This indicates that the significance of IR for AS in plants has been overestimated. The analysis also showed that many IR events have very low sequence coverage and some annotated IR may not exist. A recent study has indicated that most of the IR events in *Arabidopsis* that are predicted targets of nonsense-mediated decay (NMD) escape this mechanism, provoking the question as to the fate of these transcripts and the function of gene expression regulation by IR events (see below) (Kalyna et al., 2012). One answer comes from investigations into gametophyte development in the fern *Marsilea vestita*, where it was shown that IR transcripts are stored in the spore until their splicing and translation are initiated upon spermatogenesis in a regulated temporal pattern (Boothby et al., 2013). In mammals, regulated IR in neuron-specific genes was shown to inhibit the export of partially spliced mRNAs to the cytoplasm and trigger their degradation in the nucleus independently of the NMD machinery and to play an important role during neurogenesis (Yap et al., 2012). It is possible that regulated retention of introns in the nucleus is widespread and serves to store precursor RNAs. These results are exciting as they indicate that IR might be used to regulate other developmental processes or to enhance the response to environmental signals.

Alternative splicing event	Human	<i>Arabidopsis thaliana</i>
 Exon skipping/ inclusion	>40%	~ 8%
 Alternative 3' splice site	~18.4%	~15.5%
 Alternative 5' splice site	~7.9%	~7.5%
 Intron retention	<5%	~40%

 Constitutive region  Alternative region

Figure 1. Frequency of Common Types of AS Events in Humans and *Arabidopsis*.

Proportion of common types of AS events in human (Keren et al., 2010; Reddy et al., 2012b) and *Arabidopsis* (Barta et al., 2012; Marquez et al., 2012). [See online article for color version of this figure.]

Cell-, tissue-, and condition-specific AS is highly prevalent in animals, and the physiological significance of many such events has been confirmed (Pan et al., 2008; Kalsotra and Cooper, 2011). So far, only limited global studies on AS with different cell types and tissues and under different conditions have been performed in plants that show regulated AS in different tissues or in response to stresses (Jiao and Meyerowitz, 2010; Loraine et al., 2013; reviewed in Staiger and Brown, 2013). As more RNA-seq studies are now being performed with different tissues and individual cell types and under different biotic and abiotic stresses, our knowledge of AS in plants is likely to further increase in the near future.

AS at Tandem Acceptor Sites

Numerous intron-containing genes in animals and land plants have tandem acceptor sites (3' SS) that are separated by three nucleotides (NAGNAG; N is any nucleotide) (Hiller et al., 2004; Iida et al., 2008; Schindler et al., 2008; Sinha et al., 2010). AS at these acceptor sites that preserve the reading frame generates mRNA isoforms that include or exclude a triplet thereby contributing to transcriptome and proteome diversity. A majority of NAGNAG splicing events lead to an insertion or a deletion of a single amino acid, mostly polar amino acids (Hiller et al., 2004; Iida et al., 2008; Bradley et al., 2012). Global RNA-seq studies in humans and mouse in 24 tissues have shown that ~20% of reading frame-preserving AS events are due to AS of tandem acceptors. Furthermore, about one-quarter of alternatively spliced NAGNAG sites show tissue-specific regulation in mammals, suggesting that NAGNAG AS contributes to differences in proteomes of different cell types. Furthermore, tissue-specific NAGNAG AS is highly conserved (Bradley et al., 2012). In *Arabidopsis*, close to 7000 introns in over 5000 genes contain tandem acceptors and occur predominantly in DNA binding proteins and splicing regulators such as SR and SR-like proteins (Iida et al., 2008; Schindler et al., 2008). There is evidence for both mRNA isoforms for several hundred genes that contained tandem acceptor sites in *Arabidopsis*, rice, and moss (Iida et al., 2008; Schindler et al., 2008; Sinha et al., 2010). Interestingly, NAGNAG AS in pre-mRNAs of *Arabidopsis* splicing regulators is regulated in an organ- and stress-specific manner (Schindler et al., 2008). The two isoforms of *Arabidopsis* U1-35K generated by alternative usage of a tandem acceptor differ in the presence or absence of Gln at position 124. The isoform lacking Gln showed altered binding affinity for SR proteins and U11 snRNA (Lorkovic et al., 2005). AS at a tandem acceptor site of ZINC-INDUCED FACILITATOR-LIKE1 produces two mRNA isoforms that differ in two nucleotides. One of these codes for a full-length protein that localizes to the plasma membrane and functions in auxin-regulated processes, whereas the second isoform codes for a truncated protein that localizes to the tonoplast membrane and functions in drought tolerance (Remy et al., 2013). These studies suggest the functional significance of AS as a result of tandem acceptor in plants.

EXPERIMENTAL AND COMPUTATIONAL APPROACHES FOR THE ANALYSIS OF AS

Mechanisms that regulate AS represent a complete black box in plants, as do the roles of the diverse set of RNA binding proteins (RBPs) in regulated AS. Numerous studies with metazoans and

a few studies from plants suggest that multiple regulatory mechanisms determine the splicing outcome. Growing evidence points to elaborate crosstalk among multiple layers of posttranscriptional regulation and between transcription and chromatin. Chromatin landscape changes, such as nucleosome positioning, histone marks, and DNA methylation, together with RNA structural features and splicing regulatory elements (SREs) are important regulators of splicing. How important are RNA secondary and tertiary structural features in regulating CS and AS? There are a few examples in plants, but global studies of pre-mRNA/mRNA structures and correlating the information into AS should shed some light on this. Hence, to understand regulation of AS, it is necessary to integrate the impact of chromatin landscapes changes such as DNA methylation, nucleosome positioning, histone marks, RNA structural features, and SREs with AS. Genome-wide high-throughput tools to address these aspects have been developed recently (Figure 2, Table 1). Application of these tools to plant splicing research should provide novel insights into regulatory mechanisms and contribute to decoding of the plant splicing code. Performing such analyses with a single cell type or tissue would be ideal for such integrative studies. This would also require new computational tools to integrate data from these different sets of experiments.

The consequence of AS is the production of multiple mRNA isoforms from a single gene, which could be regulated by cell and tissue type, various developmental stages, and/or environmental conditions. However, as AS events cannot be easily predicted computationally from the genome sequence, a complete picture of all the possible mRNA isoforms generated from different genes of an organism requires a full exploration of AS in different tissues and diverse conditions and the availability of an accurately annotated genome. Since the first genome-wide analysis of AS, the estimates of AS have increased considerably (e.g., in humans, 5 to 35% [Lander et al., 2001] to now more than 95% [Mortazavi et al., 2008; Pan et al., 2008; Wang et al., 2008; Barbosa-Morais et al., 2012; Merkin et al., 2012]; in *Arabidopsis*, 1.2% [Zhu et al., 2003] to now 61% [Marquez et al., 2012]), indicating that estimates of AS mainly rely on the amount and diversity of the transcriptome data that are available from an organism.

The advent of high-throughput sequencing of RNA (RNA-seq) and large-scale microarray technologies (splice junction arrays and the tiling arrays) have allowed us to obtain a better picture of an organism's transcriptome complexity in tissues and under certain conditions (Johnson et al., 2003; Mortazavi et al., 2008; Pan et al., 2008; Sultan et al., 2008; Wang et al., 2008). The main difference of these two approaches is that with the arrays, it is necessary to have prior knowledge of the alternatively spliced variants, whereas RNA-seq allows de novo identification of transcripts at a single-base resolution (Figure 3). Despite the obvious advantages of these technologies in transcriptome analysis, several obstacles must be overcome, including the storage of the huge amount of data that is being generated (especially in the case of high-throughput sequencing) and, most importantly, a reliable pipeline for the analysis of the data and accurate prediction of AS events and splice variants (Reddy et al., 2012b). Sequencing a plant transcriptome poses another challenge: Just 10 highly expressed genes encoding proteins mainly involved in photosynthesis can generate about a quarter of all transcripts in a cell (Weber, 2007), tremendously compromising the representation of less abundant

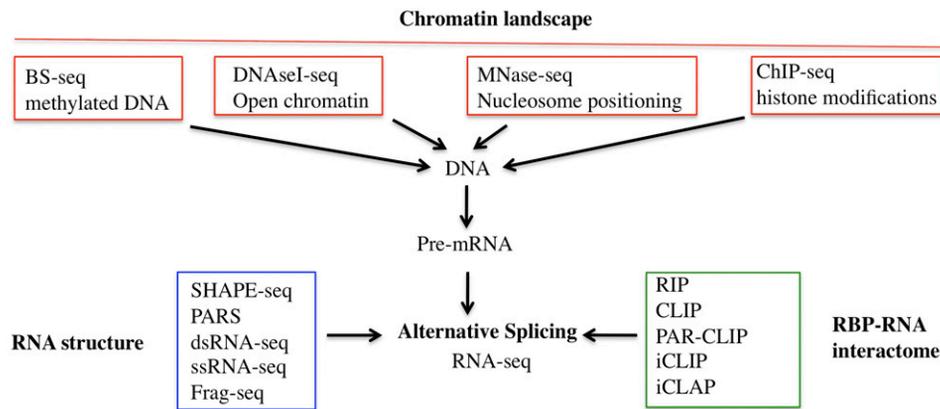


Figure 2. Integration of Chromatin Landscape, RNA Structure, and RBP-RNA Interactome into AS to Obtain a Comprehensive View of AS Regulation in Plants.

Genome-wide high-throughput sequencing tools that can provide insightful information on chromatin landscape, RNA structure, and RBP-RNA interactome are indicated. Integration of these results into AS will allow understanding of crosstalk between AS and other layers of gene regulation and mechanisms of AS. BS-seq, bisulphite sequencing; MNase-seq, micrococcal-nuclease sequencing; ChIP-seq, chromatin-immunoprecipitation sequencing; SHAPE-seq, selective 2'-hydroxyl acylation analyzed by primer-extension sequencing; dsRNA-seq, double-stranded RNA sequencing; ssRNA-seq, single-stranded RNA sequencing; Frag-seq, fragmentation sequencing; RIP-seq, RNA-immunoprecipitation sequencing; PAR-CLIP, photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation; iCLIP, individual-nucleotide resolution cross-linking and immunoprecipitation; iCLAP, individual-nucleotide resolution UV-cross-linking and affinity purification. Table 1 provides a brief description of these tools. [See online article for color version of this figure.]

transcripts in RNA-seq analysis. Thus, deeper sequencing with more reads is required to achieve good gene coverage throughout the genome. Using a normalized *Arabidopsis* cDNA library allowed reducing the abundance of highly expressed transcripts to ~1% and improved gene representation and discovery of AS events by RNA-seq (Marquez et al., 2012).

An RNA-seq experiment starts with the preparation of a cDNA library from mRNA extracted under certain conditions and/or tissue of interest followed by high-throughput sequencing, read alignment, transcript assembly, and transcript quantification (Figure 3). Sequencing results in the generation of millions of reads in the range of 36 to 400 bp, and they can be single-end (only one end of the mRNA fragment is sequenced) or paired-end (both ends of the mRNA fragment are sequenced) reads. The advantage of obtaining hundreds of millions of reads in one run is that it allows a high-resolution view of the transcriptome and the detection of low-abundance transcripts, but the disadvantage lies in the length of the sequenced fragments and the considerable error rates when compared with former sequencing methods. The short length of the sequencing reads is a critical difference in comparison to Sanger sequencing of ESTs or cDNAs that were used previously for predicting AS. The features of high-throughput sequencing (i.e., huge amounts of data and the high error rates and the short reads) created a computational challenge in terms of the alignment and prediction of full-length transcripts, especially when multiple AS events can co-occur in the same transcript or when highly similar paralogs are present in the genome, both of which are common in plant genomes.

Several computational tools have been developed in recent years for the prediction of splice junctions, which perform gapped alignment and detect exon-intron boundaries. These include

QPALMA (Jean et al., 2010), Tophat (Trapnell et al., 2009), Genomic Short-Read Nucleotide Alignment Program (Wu and Nacu, 2010), MapSplice (Wang et al., 2010a), RNA-seq Unified Mapper (Grant et al., 2011), and SpliceMap (Au et al., 2010). Most of the programs available are based on splice junction prediction using a reference genome or gene annotation, and only a few tools are available that allow transcriptome prediction without relying on an annotated genome (e.g., TransABySS [Robertson et al., 2010], Trinity [Haas et al., 2013], and Rnnotator [Martin et al., 2010]). For those programs that use the genome as a scaffold, one of two main strategies is used. One is the exon-first approach, and the second one is called seed-extend approach (Garber et al., 2011). The exon-first approach starts with aligning reads to the genome, and then the remaining unaligned reads are chopped and realigned (e.g., Tophat [Trapnell et al., 2009], MapSplice [Wang et al., 2010a], RNA-seq Unified Mapper [Grant et al., 2011], and SpliceMap [Au et al., 2010]). The seed-extend method starts by splitting the reads followed by their alignment and seed extension (Genomic Short-Read Nucleotide Alignment Program and QPALMA). Both methods assume that most of the gaps that separate the spliced reads when aligned to the genome represent introns. At this point, it is easy to imagine that chopping of the reads into smaller pieces for their alignment represents a challenge in terms of uniqueness of their genomic location, especially when the reads are coming from paralogs with high sequence identity. For this reason, the use of shorter reads when sequencing a very complex transcriptome with a highly repeated genome can result in a very high level of false-positive splice junctions and/or make it impossible to detect splice junctions at certain genomic loci. Recent improvements in obtaining longer reads using high-throughput platforms will improve the spliced alignment reliability. Nevertheless, the use of the

Table 1. Next-Generation Sequencing Tools to Analyze Global Changes in Chromatin Landscape, AS, RNA Structure, and Targets of RBPs

Method	Description
A. AS	
RNA-seq	cDNA generated from polyadenylated mRNA is fragmented and sequenced by next-generation sequencing. Alignment of reads to the genome allows quantitative analysis of gene expression, AS, and relative quantification of mRNA isoforms (Wang et al., 2008). This tool is also used to sequence other types of RNA.
B. Chromatin landscape	
Bisulphite sequencing	Genomic DNA treated with bisulphite, which converts unmethylated cytosines to uracils, is amplified and sequenced using high-throughput methods. In the sequenced reads, unmethylated cytosines appear as thymines and methylated cytosines appear as cytosines (Cokus et al., 2008). Provides a genome-wide cytosine methylation map at single-base-pair resolution.
DNaseI-seq	Nuclei are treated with DNase I, and short sequence tags are obtained, size selected, and sequenced using next-generation sequencing. The short reads are then mapped to the genome and clustered computationally to identify DNase I hypersensitive sites (Boyle et al., 2008; Hesselberth et al., 2009). Allows genome-wide mapping of open chromatin, which is hypersensitive to DNase I.
Micrococcal-nuclease sequencing	Isolated chromatin is digested with MNase to remove DNA that is not wrapped around nucleosomes, and DNA from the nucleosomes is extracted and subjected to deep sequencing. Alignment of reads to the genome provides global nucleosome occupancy (Barski et al., 2007; Chodavarapu et al., 2010).
Chromatin-immunoprecipitation sequencing	Chromatin is immunoprecipitated with an antibody specific to a histone modification or a DNA binding protein, and the DNA from the precipitate is extracted, amplified, sequenced on a next-generation sequencing platform, and mapped to the genome (Furey, 2012). Used to analyze genome-wide protein–DNA interactions and histone modifications.
C. Pre-mRNA/mRNA structure*	
Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) sequencing	RNA is treated with a SHAPE reagent (e.g., 1-methyl-7-nitroisatoic anhydride, IM) that forms 2'-O-adducts with unpaired nucleotides and blocks the extension of cDNA during reverse transcription, resulting in termination of synthesis. All generated fragments are sequenced and mapped to RNA to determine its structure (Lucks et al., 2011). Allows simultaneous measurement of secondary and tertiary structures of large numbers of RNAs in vitro.
PARS sequencing	Isolated mRNAs are renatured in vitro, treated with structure-specific enzymes (RNAse V1, which preferentially cleaves phosphodiester bonds 3' double-stranded RNA or RNAse S1 that cleaves single-stranded RNA) in parallel, and the resulting RNA fragments are sequenced using next-generation sequencing (Kertesz et al., 2010; Wan et al., 2013). Allows in vitro profiling of the secondary structures of RNA at single-nucleotide resolution globally.
Double-stranded RNA sequencing	RNAs are treated with single-strand-specific RNAases (RNAse ONE) to remove single-stranded regions and select for double-stranded RNA, which is then converted into cDNA for deep sequencing (Zheng et al., 2010). Reads are then used to obtain structural information.
Single-stranded RNA sequencing	RNAs are treated with double-strand-specific RNAases (RNAseV1) to remove double-stranded regions in RNA and select for single-stranded regions of RNA. These are then converted into cDNA for deep sequencing. These reads together with double-stranded RNA sequencing are then used to identify structural elements in RNA (Li et al., 2012).
Fragmentation sequencing	RNA mixture is treated with nuclease P1, which specifically cleaves single-stranded RNA followed by RNA-seq to deduce transcriptome-wide RNA structure (Underwood et al., 2010).
D. Targets of RBPs	
RIP sequencing	RNAs bound to a specific RBP are immunoprecipitated, and the RNA from IP is converted into cDNA and sequenced using a high-throughput sequencing platform (Zhao et al., 2010). The reads are then used to identify direct and indirect targets of that RBP.
HITS-CLIP	RBP–RNA interactions are first covalently cross-linked by exposing cells/tissues to UV (254 nm). An RBP-specific antibody is used to precipitate RNA bound to RBP, which is then treated with RNAse and used for RNA-seq. iCLIP (individual-nucleotide-resolution UV cross-linking and immunoprecipitation) is a variation of HITS-CLIP that exploits premature termination of reverse transcriptase at the cross-link site, thereby providing better resolution of binding sites (König et al., 2010). Allows genome-wide mapping of direct binding sites of an RBP on RNA (Licatalosi et al., 2008).
Individual-nucleotide-resolution UV-cross-linking and affinity purification	Double-tagged RBP is expressed in cells. Following UV cross-linking and RNAse digestion, the RBP-RNA complex is purified by a two-step affinity purification. RNA from these complexes is then deep sequenced (Wang et al., 2010b). Allows purification of RBP-RNA complexes.
Photoactivatable-ribonucleoside-enhanced CLIP	Similar to HITS-CLIP except that RNA is labeled with 4-thiouridine and cross-linking is done using UV light of 365 nm (Hafner et al., 2010b). This allows efficient cross-linking of RBPs with RNA, and identification of nucleotides involved in binding can be identified as the cross-linked regions show T-to-C mutations in the reads. A similar approach (photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation) is also used to identify all RBPs bound to mRNAs and to create binding maps of multiple RBPs. In this case, mRNAs cross-linked with RBPs are isolated using oligo(dT) affinity purification and digested with RNAse followed by identification of proteins by mass spectrometry and RNA by RNA-seq (Baltz et al., 2012).

*These in vitro methods are based on high-throughput RNA-seq and can be applied to all types of RNAs.

pair-end sequencing platform adds additional information about the mRNA fragment, as it also provides the sequence of both ends of a fragment. Based on the fragmentation pattern of the RNA or cDNA prior to sequencing, it is possible to estimate the minimum and maximum distance between the paired reads, which in turn limits the separation of both reads when they are aligned to the genome. This distance restriction improves the accuracy of the read alignment, especially when one of the matched reads is coming from a repetitive sequence.

After aligning the reads, genes that are expressed in the sample and the possible transcripts that can be explained by the RNA-seq data are identified. Initially, few studies attempted to predict novel transcripts and most focused on quantifying the expression of known genes and isoforms and measuring specific types of AS events. Several programs were designed for quantifying only exon-skipping events, as this is the most predominant type of AS in mammalian systems (Keren et al., 2010). In the case of plants, such tools for AS quantification are not ideal because the distribution of AS events is completely different and exon skipping is rare (Figure 1). Nevertheless, using RNA-seq (Marquez et al., 2012) and high-resolution RT-PCR (Kalyna et al., 2012), numerous AS events have been identified and confirmed in *Arabidopsis*. The main problem with the approaches that focus on measuring specific types of AS events is the limitation in quantifying the complete set of AS events under certain condition(s), limiting the biological conclusions of AS regulation.

It is well established that the number of reads that align to a genomic sequence directly correlates to its expression. Three main problems arise when quantifying transcript levels: the existence of reads that do not align uniquely, the presence of alternatively spliced transcripts, and the determination of the functional outcome of the different isoforms. For the first problem, programs such as Cufflinks (Trapnell et al., 2010) and Scripture (Guttman et al., 2010) consider the multiple locations of different isoforms. However, a major difficulty is that most of the features can be shared between different transcripts (e.g., constitutive exons) and often very subtle differences are not shared (e.g., a three-nucleotide difference due to the presence of NAGNAG sites), which limits considerably the assembly of possible transcripts and their quantification. The use of paired end reads can alleviate to some extent the problem of exon connectivity, as there is additional information about the distance between the matched pairs. Moreover, assemblers like Cufflinks (Trapnell et al., 2010) and Scripture have also implemented the prediction of novel isoforms based on known gene models, which considerably improves transcript assembly. Recently, Rogers et al. (2012) developed SpliceGrapher, a method that can combine gene models, EST, and RNA-seq data to predict AS events and visualize splicing graphs in *Arabidopsis*, thereby improving the detection of novel splicing variants. Finally, a reliable determination of transcripts can considerably improve the prediction of isoforms' contribution to proteome diversity and to the regulation of mRNA levels. The combination of RNA-seq together with large-scale proteomic technologies will be valuable in addressing this question.

The ultimate goal is accurate detection and quantification of different mRNA isoforms and the investigation of the impact on the biological system in different conditions or developmental stages. The advent of high-throughput sequencing has provided

the ability to detect novel and lowly expressed transcript isoforms and to explore transcriptome regulation across different samples. In mammals, the use of RNA-seq has been applied multiple times revealing tissue-specific AS (Mortazavi et al., 2008; Pan et al., 2008; Wang et al., 2008; Barbosa-Morais et al., 2012; Merkin et al., 2012), but only a few studies are available for plant systems (Jiao and Meyerowitz, 2010; Loraine et al., 2013). In the near future, the low sequencing costs will not only improve the exploration of plant transcriptomes and AS but also help in understanding the still unknown plant splicing code.

CONSEQUENCES OF AS

AS changes the sequence of an RNA transcript, which can influence gene expression on several levels. It either generates distinct protein isoforms with altered (related, distinct, or opposing) functions or determines the fate of the RNA transcript by regulation of mRNA stability through NMD- and microRNA (miRNA)-mediated mechanisms, or by influencing mRNA transport, localization, and translational efficiency. In plants, there are several examples where protein isoforms from alternatively spliced pre-mRNAs are targeted to different cellular locations (Dixon et al., 2009; Lamberto et al., 2010; Remy et al., 2013). mRNA isoforms may differ in the untranslated regions (UTRs) with functional implications in transcript localization, stability, or translation.

Creating Protein Isoforms

Even though increasing proteome diversity is one of the important postulated roles of AS, the extent of its impact is far from being settled (Severing et al., 2009). The consequences of producing protein isoforms are manifold depending on the AS event. In some instances, AS events can be in frame. It is interesting to note that gene ontology enrichment analysis of alternatively spliced genes with NAGNAG splice sites indicates that members of some functional groups, such as RNA binding splicing regulators and transcriptional factors, are overrepresented (Iida et al., 2008; Severing et al., 2012). Other AS events can introduce frame shifts and result in truncated protein isoforms. These protein isoforms might still have functional modules, might lack certain functional domains, or lack and gain sites of posttranslational modifications. As most transcriptional factors and splicing regulators are modular proteins with multiple functional modules (e.g., nucleic acid binding domain, protein-protein interaction domain, and transcription activation domain), it is likely that mRNA isoforms that produce truncated proteins containing some, but not all, modules may still perform part of their function and hence may act as dominant-negative regulators. Several recent studies provide evidence in support of such a dominant regulatory role for truncated proteins produced by splice variants in plants (Gagete et al., 2009; Penfield et al., 2010; James et al., 2012; Seo et al., 2012; Wang et al., 2012; Liu et al., 2013). For instance, in several cases where proteins form homo- or heteromers, splice variants produce small interfering peptides that participate in dimerization but lack other functional domains, which leads to formation of nonfunctional dimers that can compete with functional dimers resulting in dominant-negative regulation (Staudt and Wenkel, 2011; Seo et al., 2013).

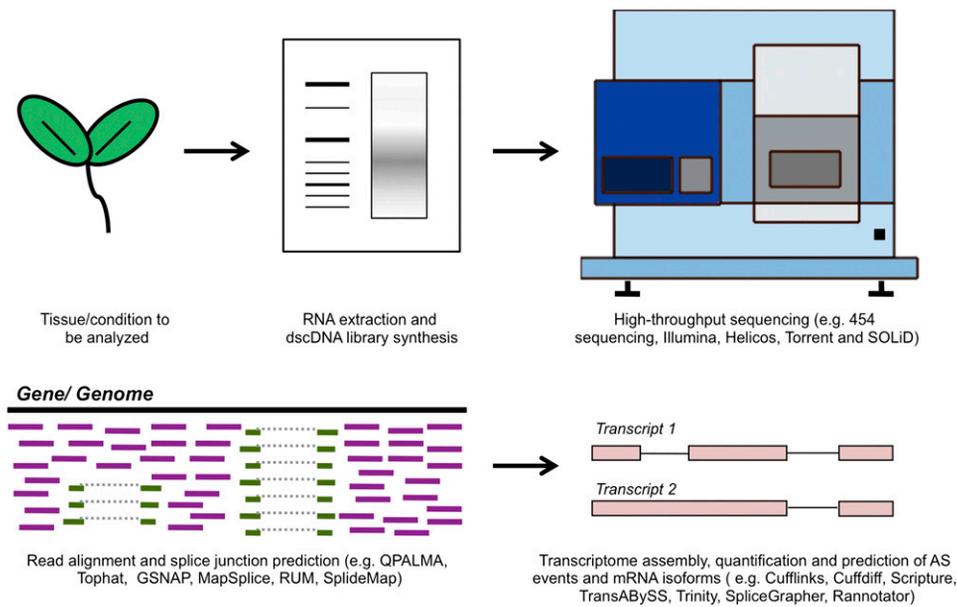


Figure 3. General Pipeline for the Analysis of AS Using High-Throughput Sequencing.

Although in-depth analyses of the consequences of AS isoforms as well as alternative transcription initiation or polyadenylation in plants are lacking, their potential is highlighted by recent global analyses in *S. cerevisiae* and animals. Global transcript isoform profiling in yeast revealed extensive transcriptional heterogeneity primarily due to alternative transcript initiation or polyadenylation with more than 26 major transcript isoforms per protein-coding gene (Pelechano et al., 2013). This study uncovered many short coding RNAs with a cap and poly(A) tail that may encode short peptides (Pelechano et al., 2013). Small bioactive peptides have been shown to perform important functions in morphogenesis by controlling the activity of a transcription factor (Kondo et al., 2010). Global studies in metazoans using computational and experimental methods investigating tissue-specific splice variants suggest that their protein products may rewire protein–protein interaction networks and impact signaling pathways (Buljan et al., 2012; Ellis et al., 2012; Weatheritt et al., 2012; Weatheritt and Gibson, 2012). It was found that alternative exons encode conserved intrinsically disordered regions that generally reside on protein surfaces and participate in protein–protein interaction, leading to rewiring of the protein–protein interaction network and signaling pathways (Buljan et al., 2012; Ellis et al., 2012; Weatheritt and Gibson, 2012). Furthermore, proteins from mRNA isoforms often gain or lose potential phosphorylation sites; hence, AS impacts protein kinase–mediated signaling pathways (Merkin et al., 2012). In *Arabidopsis*, SR and SR-like proteins RS41, CypRS64, and SR45a were found to have phosphorylation sites specific for single splice variants (de la Fuente van Bentem et al., 2006).

Coupling of AS to mRNA Stability through NMD

AS can affect transcript abundance by subjecting it to NMD. NMD is a cytoplasmic RNA degradation system, which occurs on

ribosomes on the first round of translation. According to current models (Figure 4), NMD triggers transcript decay when translation termination is perturbed. UPF1, UPF2, and UPF3 are conserved core proteins of the NMD pathway, and their depletion results in the inactivation of NMD. They recruit mRNA decay enzymes to prematurely terminating mRNAs. For a detailed description and discussion of the NMD pathway, we direct readers to a number of excellent recent reviews (Behm–Ansmant et al., 2007; Isken and Maquat, 2007; Stalder and Mühlemann, 2008; Nicholson et al., 2010; Kervestin and Jacobson, 2012). The NMD machinery is largely conserved in plants, which have orthologs of the key NMD factors, including UPF1, UPF2, UPF3, and SMG-7, but not SMG-1, SMG-5, or SMG-6 (Hori and Watanabe, 2005; Arciga-Reyes et al., 2006; Yoine et al., 2006; Kerényi et al., 2008; Riehs et al., 2008). Though the rules of NMD in plants have been established mostly using artificial constructs and a handful of individual genes (Kertész et al., 2006; Schwartz et al., 2006; Hori and Watanabe, 2007; Kerényi et al., 2008; Nyikó et al., 2009), recent analysis of multiple endogenous NMD sensitive transcripts has shown that the majority comply with existing NMD rules (Kalyna et al., 2012). The classical features of NMD substrates include both long 3'UTRs and introns in 3'UTRs (Kertész et al., 2006; Schwartz et al., 2006; Hori and Watanabe, 2007; Wu et al., 2007), a premature termination codon (PTC) more than 50 to 55 nucleotides upstream of a splice junction (Kerényi et al., 2008), and upstream open reading frames (uORFs) (Nyikó et al., 2009) (Figure 5). All these features can be introduced or changed by AS, thereby altering the stability of a transcript. Therefore, AS transcripts that are subjected to NMD are often barely detectable. However, in mutants affecting NMD or in the presence translation inhibitors, there can be a considerable increase in these transcripts. The existence of PTC-containing transcripts might therefore significantly influence the level of gene expression by reducing fully spliced mRNA levels.

It is not commonly acknowledged that AS in the UTRs of a gene (which does not create a PTC) may trigger NMD. Recent data show that AS of introns in either the 3'UTR or 5'UTR can determine whether transcripts are targets of NMD (Figure 5). Examples of AS-NMD in 3'UTRs include NF-YB1/HAP3A transcription factor and PAUSED/Exportin-t genes, where it has been shown that only the splice variants with a distance >50 to 55 nucleotides from the authentic stop codon to the downstream splice junction were subjected to NMD (Kalyna et al., 2012). AS in 5'UTRs may affect the presence, size, and positions of uORFs, which may then trigger NMD of endogenous splice variants (Kalyna et al., 2012). Generally, stop codons of uORFs might be recognized as PTCs (additionally, a long 3'UTR is created and the downstream splice junctions exist in the case of intron-containing genes) and, thereby, targeting the transcript to the NMD pathway (Figure 5B). A novel feature of uORFs triggering NMD in plants has been identified recently where AS events in 5'UTRs can result in uORFs overlapping the authentic start codon, and such transcripts are NMD sensitive (Kalyna et al., 2012). By contrast, AS transcripts of the same genes with other uORFs are NMD resistant. Since ~20% of *Arabidopsis* genes contain uORFs (Nyikó et al., 2009) and AS frequently occurs in 5'UTRs, it is likely that AS-coupled NMD involving uORFs may emerge as an important mechanism for gene regulation.

A common presumption about NMD is to label an AS variant as an NMD candidate when AS introduces a PTC at a position ≥ 50 to 55 nucleotides upstream of a splice junction. Accordingly, based on EST and RNA-seq analyses of wild-type plants, 36 to 78% of *Arabidopsis* AS transcripts have been estimated to be potential candidates for NMD (Wang and Brendel, 2006; Filichkin et al., 2010). However, tiling and expression microarrays found that only ~1% of plant protein-coding genes were upregulated in NMD-deficient plants (Yoine et al., 2006; Kurihara et al., 2009). These arrays have a limited ability to distinguish AS transcripts, and the usual cutoff (>1.5-fold to twofold) puts a further limitation on the detection of AS-NMD transcripts. An attempt to overcome the limitations of the above methods using a high-resolution AS RT-PCR panel with 950 transcripts of 270 alternatively spliced genes showed that in the NMD-impaired backgrounds 11 to 18% of all transcripts and 16 to 25% of the AS transcripts analyzed were NMD sensitive (Kalyna et al., 2012). Extrapolating from this analysis around 13 to 18% of intron-containing genes in the *Arabidopsis* genome are potentially regulated by AS-NMD. These values were confirmed by a recent comprehensive genome-wide study on AS in NMD mutants in *Arabidopsis* (Drechsel et al., 2013). Taken together, these estimates correlate well with those reported for flies, worms, and human (Rehwinkel et al., 2005; Pan et al., 2006; Ramani et al., 2009; Weischenfeldt et al., 2012). Importantly, Kalyna et al. (2012) identified AS transcripts that contained NMD features but were immune to NMD. Unexpectedly, the majority of IR transcripts were not sensitive to NMD despite containing PTCs, downstream splice junctions, and long 3'UTRs. By contrast, transcripts from the same gene with other types of AS events in the same or nearby intron were NMD sensitive. In line with this, another study has shown that cold-regulated *Arabidopsis* IR transcripts containing PTC and predicted to be the targets of NMD are not affected in UPF3 mutant background (Leviatan et al., 2013). Finding that PTC+ IR transcripts escape the NMD machinery may explain the high frequency of IR events identified in plants.

Interestingly, splicing factors use AS-NMD to autoregulate their expression through a negative feedback loop. Metazoan SR proteins have been shown to activate the inclusion of a PTC+ exon using ultraconserved sequences in their own pre-mRNA, thus resulting in NMD (Morrison et al., 1997; Lareau et al., 2007; Ni et al., 2007; Saltzman et al., 2008). Conservation of AS events to produce PTC-containing transcripts has also been demonstrated for SR protein genes in lower and higher plants (Kalyna et al., 2006), and plant SR proteins are also subjected to AS-NMD regulation (Palusa and Reddy, 2010; Kalyna et al., 2012). An evaluation of all the splice products of 13 alternatively spliced *Arabidopsis* SR genes in a UPF3 mutant revealed that about half of the 53 variants with a PTC are degraded by NMD (Palusa and Reddy, 2010). Interestingly, At-SR34a, an *Arabidopsis* homolog of human SFRS1/ASF/SF2, is regulated by AS-NMD in the 5'UTR, involving a uORF, which overlaps the AUG of the main ORF (Kalyna et al., 2012). Other plant splicing regulators and RBPs have been demonstrated to auto- and cross-regulate their mRNA levels using AS-NMD. An autoregulation feedback loop of *Arabidopsis* RBPs GRP8 and GRP7, components of a slave oscillator coupled to the circadian clock, is tied to NMD of alternatively spliced transcripts (Staiger et al., 2003; Schöning et al., 2008). AS in two splicing regulators, polypyrimidine tract binding protein (PTB)-like homologs, in *Arabidopsis* is also autoregulated; an increase in protein levels of the PTB-like proteins results in an increase of a PTC-containing alternatively spliced transcript (Stauffer et al., 2010; Wachter et al., 2012). A further level of AS-NMD complexity in *Arabidopsis* is evidenced by extensive AS coupled NMD in AFC2, encoding a highly conserved LAMMER kinase, which phosphorylates splicing factors, thus establishing a complex regulatory loop (Marquez et al., 2012). It appears that AS-NMD regulation occurs at multiple levels of RNA biology as multiple RBPs, splicing factors, RNA helicases, spliceosome, and exon junction complex proteins are subjected to this regulation (Kalyna et al., 2012).

Alternative polyadenylation, which results in differing 3'UTRs, is prevalent in plants and is thought to play an important role in regulating gene expression at multiple levels, especially mRNA stability through NMD (Simpson, 2004; Hunt, 2011; Wu et al., 2011). A genome-wide study indicates a link between AS with selection of polyadenylation sites, as over 4000 genes in *Arabidopsis* are predicted to be polyadenylated within introns (Wu et al., 2011). The extent of coupling of AS to alternative polyadenylation globally and its impact on gene regulation needs further investigations.

Coupling AS to mRNA Stability and Translation through miRNA Regulation

miRNAs, a class of *trans*-acting short (20 to 24 nucleotides long) RNAs, are important regulators of gene expression at the post-transcriptional level. They bind to complementary sequences in mRNA targets and attenuate gene expression either by cleaving the mRNA or repressing its translation (Aukerman and Sakai, 2003; Bartel, 2009; Voinnet, 2009). In plants, transcripts of many transcription factors and other regulatory proteins that perform critical roles in plant growth, development, and stress responses are targets of miRNAs (Rogers and Chen, 2013b). Generation of

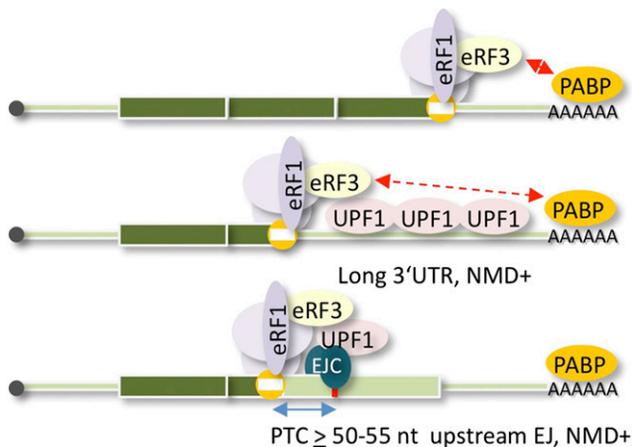


Figure 4. Current NMD Models.

Both authentic and premature stop codons are recognized by translation termination factors eRF1 and eRF3. In the course of canonical termination, interaction of eRF3 with poly(A) binding protein (PABP) leads to the efficient release of a peptide (**top**). According to the long 3' UTR model (middle), this interaction does not occur when the distance between a stop codon (PTC) and poly(A) is extended. eRF3 then interacts with UPF1, which competes with poly(A) binding protein for binding sites. This perturbs translation termination and results in NMD. In the exon junction complex (EJC) model (**bottom**), EJC is deposited 20 to 24 nucleotides (nt) 5' of an exon-exon junction during pre-mRNA splicing (Singh et al., 2012). The EJCs are stripped off from mRNA during the first round of translation. In PTC-containing transcripts, termination of translation more than 50 to 55 nucleotides upstream of an EJC triggers RNA decay through NMD by recruiting UPF3 and its cofactors UPF1 and UPF2 (Schoenberg and Maquat, 2012).

short mature miRNAs from primary transcripts of miRNAs (pri-miRNAs), which are much longer (up to 3000 nucleotides), involves two sequential site-specific endonucleolytic events. The first event crops pri-miRNAs to produce pre-miRNAs with shorter stem-loop structure, and the second event results in release of an miRNA:miRNA* duplex (Rogers and Chen, 2013b, 2013a)

Recent studies suggest that AS can modulate miRNA-mediated regulation of gene expression in multiple ways. These include the generation of mRNA or noncoding RNA isoforms that either contain or lack target sites for miRNA (Figure 6A) and regulation of splicing of pri-miRNAs thereby modulating the levels of miRNAs, which in turn regulates the stability or translation of target mRNAs. Modulation of miRNA levels is also accomplished by regulating the splicing pattern of pri-miRNAs or pre-mRNAs encoding enzymes, such as dicer-like proteins, involved in miRNA biogenesis (Figure 6B). In plants, emerging studies are providing evidence in support of all of these mechanisms.

The presence of an miRNA target in some splice isoforms but not in others from a given gene links regulated AS to miRNA-mediated regulation of gene expression. In plants, miRNA binding sites (MBSs) in mRNAs are found in both the coding as well as UTRs. In *Arabidopsis*, using bioinformatic analysis, it was found that over 12% of identified MBSs (44 out of 354 mRNAs with MBSs) are affected by AS (Yang et al., 2012b), suggesting coupling of AS to miRNA-mediated gene regulation. Removal of

introns in pre-mRNAs of some transacting small interfering RNA (TAS) genes (*TAS1A* and *TAS2*) results in a loss of MBSs and the levels of RNA isoforms of *TAS1A* and *TAS2* that do not contain MBS are predominant, suggesting a role of AS of TAS pre-mRNAs in regulating their transcript isoforms (Yang et al., 2012b). Members of *Squamosa-promoter binding protein* like (*SPL3*, 4, and 5) are key regulators of transition from vegetative to reproductive phase and contain an MBS for miR156 (Wu and Poethig, 2006). Expression of these SPLs is strongly inversely correlated with expression of miR156. *SPL4* generates three mRNA isoforms, but only one isoform (*SPL4-1*) contains an MBS for miR156. Overexpression studies with miR156 have shown that the level of *SPL4-1* transcript but not other isoforms is regulated by this miRNA, suggesting a strong coupling of AS to miRNA-mediated gene regulation. Recently, it was found that introns in rice and *Arabidopsis* are targeted by miRNAs, suggesting that pre-mRNAs or spliced intron-containing transcripts could be cleaved by miRNAs (Meng et al., 2013).

Some studies show direct coupling of AS to miRNA biogenesis at the splicing of pre-miRNA level (Hirsch et al., 2006; Yan et al., 2012). A number of miRNA genes in plants reside within an intron of protein coding genes and are coexpressed and processed with the host protein-coding gene (Brown et al., 2008; Yan et al., 2012; Yang et al., 2012a). AS of primary transcripts due to IR and exon skipping of several plant miRNAs has been reported (Aukerman and Sakai, 2003; Kurihara and Watanabe, 2004; Hirsch et al., 2006; Szarzynska et al., 2009). In some cases, the secondary structure needed for miRNA biogenesis is affected (Hirsch et al., 2006) and hence may affect miRNA biogenesis. *Arabidopsis MIR400* resides within the intron of a protein-coding gene (At1g32853) and is cotranscribed with its host gene. Interestingly, heat regulates AS of this intron, resulting in an increased level of miR400 primary transcripts but a decreased level of mature miR400 (Yan et al., 2012). This coupling of regulated AS to biogenesis of miR400 appears to be important for heat stress tolerance as overexpression of miR400 resulted in decreased germination and seedling growth under heat stress (Yan et al., 2012). Interestingly, two recent studies have shown that introns in plant miRNA genes (*MIR161*, *MIR163*, and *MIR172a*) enhance miRNA production in a position-dependent manner (Bielewicz et al., 2013; Schwab et al., 2013). Removal of introns from these genes reduced the level of miRNAs. In addition to regulated splicing of pri-miRNAs and splicing-independent intron enhancement of miRNA production described above, AS is also coupled to miRNA biogenesis indirectly by regulating the splicing of pre-mRNAs that encode miRNA processing enzymes. *Dicer-like1* (*DCL1*), a key endonuclease in biogenesis of miRNA from pri-miRNA (Rogers and Chen, 2013b), has 20 exons and contains an MBS for miR162 that is interrupted by intron 12. AS in this intron generates four isoforms (*DCL1-1* to *DCL1-4*), and one of these four isoform contains an MBS (Yang et al., 2012a). Hence, regulated AS of *DCL1* pre-mRNAs would affect regulation of *DCL1* protein levels.

How Much of the Observed AS Is Biologically Meaningful?

How much of the observed AS is due to regulated splicing with biological consequences, and how much is due to splicing

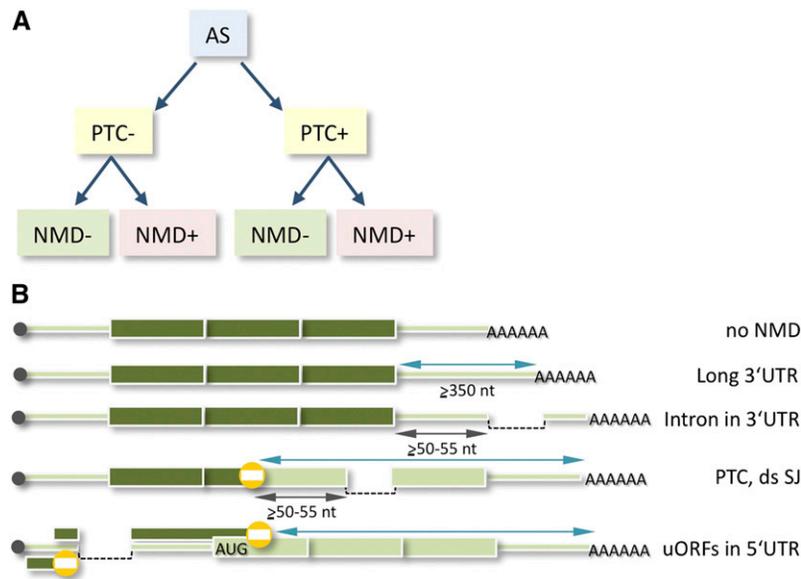


Figure 5. Coupling of AS to NMD.

(A) AS can result in both PTC⁻ and PTC⁺ transcripts. Both PTC⁺ and PTC⁻ transcripts can be subjected to NMD (NMD⁺). Not every PTC⁺ transcript is targeted by NMD (NMD⁻).

(B) Features of NMD sensitive transcripts: 3'UTRs longer than 350 nucleotides; introns in the 3' UTR with a distance >50 to 55 nucleotides (nt) from the authentic stop codon to the downstream splice junction (ds SJ); PTC more than 50 to 55 nucleotides upstream of a splice junction; uORFs in 5'UTR, including uORFs overlapping with authentic start codon (AUG) of the main ORF. PTCs are shown by yellow stop signs. Blue arrows above transcripts indicate long 3'UTRs that can serve as NMD signals.

errors is not known. Some studies argue that much of the AS represents splicing noise and is not physiologically relevant (Melamud and Moulton, 2009; Severing et al., 2009; English et al., 2010; Pickrell et al., 2010). English et al. (2010) analyzed the AS events for genes that have ESTs and found that for most AS events, one major splicing choice was supported by a majority of the ESTs, with <10% of ESTs supporting the minor form. However, there was a subset of genes for which mRNA isoforms appeared about equally, but most (>80%) of these equal forms were affected in UTRs or the changes in the protein were small. An animal study found that a majority of all alternative isoforms resulted in unstable protein conformations, but only a subset of isoforms maintained protein structural integrity (Melamud and Moulton, 2009). These studies suggest that AS represents splicing noise as most of it is not conserved, the level of minor isoforms are low, and many mRNA isoforms are predicted to produce truncated proteins (Melamud and Moulton, 2009; Severing et al., 2009; English et al., 2010). The lack of conservation of an AS event across phylogenetically divergent species is not inconsistent with a biological role for AS, as global studies from a number of vertebrates suggest that lineage-specific AS is important for divergence of their organs' phenotype and functions (Barbosa-Morais et al., 2012; Merkin et al., 2012). Conservation of AS patterns in orthologous genes is not high in animals (Barbosa-Morais et al., 2012). Between human and mouse, only 10% of AS events are conserved in orthologous genes (Pan et al., 2005). However, a recent study in unicellular fungus *Schizosaccharomyces pombe* resulted in a striking finding of identical exon skipping events in

plants, animals, and fungi, species that diverged more than one billion years ago (Awan et al., 2013). Some recent studies suggest that rapid divergence in AS may have contributed to organogenesis and speciation (Barbosa-Morais et al., 2012; Merkin et al., 2012). Unlike conserved AS events, mRNA isoforms generated by lineage-specific AS tend not to code for functional proteins due to disruption of reading frame (Merkin et al., 2012). Hence, these AS events are thought to be important for regulating gene expression. Furthermore, studies suggest that truncated proteins perform functions and a low level of some mRNA isoforms is likely due to AS-coupled regulation of levels of those isoforms. Although it is perhaps likely that most regulated AS has a biological role, future studies on functional significance of splice variants are needed to address this.

Evolution of AS Complexity

Soon after the discovery that eukaryotic genes are split, it was speculated that split gene organization could accelerate evolution (Gilbert, 1978). Recent AS studies in phylogenetically divergent organisms are lending support to this hypothesis. Analysis of AS in seven organs from 10 vertebrates ranging from frog to human and spanning ~350 million years of evolution revealed the highest AS complexity in human, and decreasing complexity as the evolutionary distance increased from primates (Barbosa-Morais et al., 2012). Based on this study, it was proposed that divergence in AS may have contributed to speciation. In another study, comparing gene expression in several tissues from mammals and

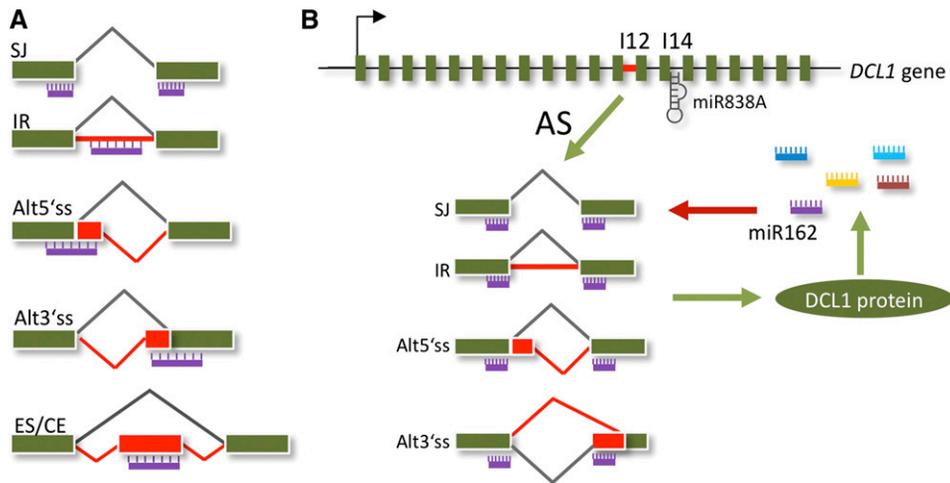


Figure 6. AS Can Modulate miRNA-Mediated Regulation of Gene Expression.

(A) Generation of RNA splice variants that either contain or lack target sites for miRNA. SJ, splice junction; ES/CE, exon skipping/cassette exon. **(B)** Modulation of miRNA levels by regulating the splicing pattern of pre-mRNAs encoding enzymes, such as DCL1, involved in miRNA biogenesis. MBS for miR162 (shown in violet) is split between E12 and E13 of *DCL1* pre-mRNA. Only one of the four isoforms generated by AS contains a miRNA target site. In addition, miR838A is encoded in the intron 14 of *DCL1*. AS events and regions are marked red. *DCL1* gene structure is not to scale.

a bird it was found that tissue-specific gene expression is mostly conserved, whereas AS is highly diverged with many lineage-specific differences (Merkin et al., 2012). Phenotypic divergence in organs in different vertebrates is attributed to lineage-specific AS (Barbosa-Morais et al., 2012). Such in-depth comprehensive AS studies in land plants that diverged about ~450 million years ago should provide information on conservation of AS and evolution of AS over time.

AS REGULATION: DECODING THE SPLICING CODE IN PLANTS

Cis-Regulatory Elements

The basis for CS and regulated AS is differential usage of splice sites. Elucidating the mechanisms of CS and AS is of central importance to understanding how various signals influence AS (Chasin, 2007; Chen and Manley, 2009; Roca et al., 2013). Decoding of the splicing code requires comprehensive understanding of rules that dictate splice site choices to combine separate parts of a transcript and generate different genetic messages. The canonical splice site consensus sequences in introns are very short and degenerate; hence, they do not provide enough information for distinguishing correct pairs of splice sites from alternative splice sites. Research using various strategies has identified *cis*-acting sequence elements in exons and introns that are important for the correct recognition of pairs of splice sites (Blencowe, 2006; Chasin, 2007; Chen and Manley, 2009). These SREs are referred to as exonic splicing enhancers (ESEs)/silencers and intronic splicing enhancers/silencers (Chen and Manley, 2009; Huelga et al., 2012). The mechanisms that control the differential usage of splice sites have not been elucidated in plants. Studies on AS regulation in animals have uncovered that

features in RNA sequences (predominantly *cis*-elements and in some cases RNA structures) and the RBPs (*trans*-factors that bind *cis*-elements), including SR proteins and hnRNPs, are important regulators of splice site choice and AS. This regulatory code of splicing is complex and involves numerous loosely conserved *cis*-elements, a plethora of RBPs, and an elaborate network of interactions among them.

SREs

SREs are short (5 to 10 nucleotides) conserved sequences that often occur in clusters (Chen and Manley, 2009; McManus and Graveley, 2011). Interactions between these *cis*-elements in pre-mRNA and RBPs either promote or suppress the use of a splice site. Studies with individual genes and several transcriptome-wide maps for *cis*-elements for different RNA binding splicing regulators in animals have identified numerous *cis*-elements and their effect on AS (Licatalosi et al., 2008; Chen and Manley, 2009; Barash et al., 2010; Hafner et al., 2010a). In fact, extensive analysis of such elements involved in exon skipping in animals has led to deciphering of the splicing code that is now used to predict exon skipping in animals (Barash et al., 2010). The fact that sequence motifs can be used to predict splicing outcomes in some cases suggest that they are the predominant *cis*-elements in regulated splicing (Barash et al., 2010; Zhang et al., 2010a). Splicing regulatory proteins bind to SREs and regulate CS and AS by recruiting/stabilizing spliceosomal components and/or antagonizing other splicing inhibitory proteins. Protein-protein interactions between SR proteins and proteins involved in both 5'SS and 3'SS aid in finding correct splice sites. Although numerous binding proteins have been identified in plants based on the presence of different types of RBPs (Lorković, 2009), the sequences that bind to these proteins are not known in most cases (see *trans*-factors below).

In animals, selective evolution of ligands through exponential enrichment (SELEX) (Coulter et al., 1997) and its variations (functional SELEX and genomic SELEX), RNA immunoprecipitation (RIP) (Barkan, 2009; Zhao et al., 2010), cross-linking and immunoprecipitation (CLIP) (Wang et al., 2009b) and its variations, high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP) (Licatalosi and Darnell, 2010) and photoactivatable-ribonucleoside-enhanced CLIP (Hafner et al., 2010a) have been used to identify targets of RBPs (Figure 2, Table 1). The binding regions of many tissue-specific RNA binding splicing regulators in animals have been identified using these tools. The wealth of experimentally obtained information on sequences in RNA that bind to splicing regulators and the vast repertoire of mRNA isoforms with various AS events has paved the way to develop computational tools to predict *cis*-elements and AS events, especially exon skipping, in animals (Barash et al., 2010; Zhang et al., 2013a). In animals, at least 70 bases in introns flanking some of the alternative exons are conserved across organisms, suggesting intronic splice regulatory elements. Furthermore, analysis of intronic sequences downstream of broadly conserved alternative exons for 5mers identified sequences that match perfectly with consensus sequences of many tissue-specific splicing regulators (Merkin et al., 2012), suggesting the importance of intronic splicing regulatory elements in AS of broadly conserved exons.

Highly conserved intronic sequences flanking alternative splice sites have been found in plant-specific SR genes in mosses, monocots, and dicots, taxa separated by more than 400 million years of evolution implicating an ancient regulatory function (Kalyna et al., 2006). In plants, very little is known about the SREs that regulate AS. Genomic SELEX has been used to identify the sequences that bind to AtCyp59, which is known to interact with components of transcription and splicing (Bannikova et al., 2013). Using computational tools, Perteau et al. (2007) have identified over 80 ESEs in *Arabidopsis*, some of which are similar to human ESEs. Several of these were experimentally validated. In addition to these, only few other SREs have been identified experimentally in plants (Yoshimura et al., 2002; Lopato et al., 2006; Schöning et al., 2007, 2008; Reddy et al., 2012b; Thomas et al., 2012). The lack of information on experimentally identified SREs in plants is a major limitation in developing computational tools to predict RNA binding elements, AS, and the plant splicing code. However, accumulating data on various types of AS events and empirical identification of *cis*-elements in plants, as in animals, should pave the way for the development of computational tools to predict conserved patterns (motifs) associated with an AS event.

Regulation of AS by Secondary Structures

Regulatory information for splicing in RNAs can also be present in its secondary and tertiary structures that involve base pairing and structural conformations. Structural elements within the pre-mRNA can suppress splicing by masking splice sites or promote splicing either by unmasking splice sites or by bringing sequences that bind to splicing regulatory proteins to close proximity (Donahue et al., 2006; Warf and Berglund, 2010; Jin et al., 2011). In the Down Syndrome Cell Adhesion Molecule gene in *Drosophila melanogaster*, an extreme example of AS with 95 alternatively

spliced exons, competing RNA structures were found to be required for mutual exclusion exons (May et al., 2011). In nonplant systems, there are well-documented examples demonstrating the roles RNA structures play in regulated splicing (Donahue et al., 2006; Warf and Berglund, 2010; Jin et al., 2011; Wan et al., 2011). In plants, it has been shown that insertion of an artificial secondary structure (stem-loop) in an intron strongly inhibits splicing *in vivo* in tobacco (*Nicotiana tabacum*) protoplasts (Goodall and Filipowicz, 1991). Furthermore, the inhibitory effect was observed only when the stem-loop was placed at the 5' splice site or in the middle of an intron but not at the 3' splice site (Liu et al., 1995), suggesting a regulatory role for secondary structures in pre-mRNA splicing in plants. So far, there are at least two well-characterized secondary structures (a riboswitch and a 5S rRNA mimic) in plants that are known to regulate splicing *in vivo*.

Secondary and tertiary structures in RNAs serve as a binding site for a metabolite (in the case of riboswitches) or an RBP (5S rRNA mimic) and regulate AS. Riboswitches, which are widely used in prokaryotes, are metabolite binding structures in mRNA that function as genetic switches to turn on or off gene expression at the transcription or translation level in response to metabolites (Breaker, 2011; Serganov and Nudler, 2013). In bacteria, riboswitches regulate the biosynthesis of essential metabolites, including vitamins and amino acids. Riboswitches that regulate pre-mRNA splicing have recently been identified in land plants, algae, and fungi where a small molecule regulates splicing by modulating RNA structure. In these organisms, AS of pre-mRNAs encoding proteins involved in thiamine metabolism is modulated by a metabolite (thiamin-pyrophosphate [TPP]) binding riboswitch (Bocobza and Aharoni, 2008; Wachter, 2010). Three TPP switches were identified in the fungus *Neurospora crassa* (Cheah et al., 2007). Interestingly, it was found that one activates and two repress gene expression by controlling pre-mRNA splicing. The location of a TPP binding aptamer within the pre-mRNA can be in the 5' UTR, 3' UTR, or coding region (Wachter, 2010). In *Arabidopsis* and other plants, conserved TPP riboswitches in the 3' UTR of the *THIAMINE C SYNTHASE (THIC)* gene regulate thiamine metabolism (Bocobza et al., 2007; Wachter et al., 2007). The binding of TPP to an aptamer changes the splicing pattern of pre-mRNA by masking a splice site resulting in a splice variant with a retained intron, which is less stable. Therefore, an increase in TPP would lead to its binding to the aptamer, resulting in production of the less stable intron-retained variant of *THIC* and in a decrease in TPP production. This negative feedback regulation of thiamine biosynthesis at the AS level ensures a high level of transcripts under low cellular TPP concentrations. Plants expressing *THIC* with a disrupted TPP riboswitch in a *thic* mutant showed growth retardation, chlorosis, and delayed flowering, indicating the physiological significance of riboswitch-mediated regulation of *THIC* (Bocobza et al., 2013). It is not known to what extent riboswitches regulate AS of other pre-mRNAs in plants. There are speculations that riboswitch-mediated AS regulation may exist for other genes (Grojean and Downes, 2010). Synthesis of 5S rRNA (5S rRNA) by RNA polymerase III requires transcription factor IIIA (TFIIIA). In the case of plant TFIIIA pre-mRNA, a structural element that mimics 5S rRNA in its third exon regulates AS (Fu et al., 2009; Hammond et al., 2009). This exon is highly conserved in land plants. Skipping of this exon produces a functional

transcript, whereas its inclusion results in a nonfunctional PTC-containing transcript that is targeted for degradation; hence, it is referred to as a suicide exon. Interestingly, skipping of this exon is induced by ribosomal protein L5 that binds to the 5S rRNA mimic. These studies indicate that ribosomal protein L5 controls the synthesis of 5S rRNA by regulating AS of TFIIIA pre-mRNA via an exonic mimic of 5S rRNA binding site. It is likely that more of these will be discovered as global RNA secondary structure analyses are performed and those structures are integrated with AS data.

Recently, several novel *in vitro* high-throughput sequencing approaches to obtain genome-wide maps of mRNA secondary structures, referred to as the RNA structurome or RNA foldome have been developed (Wan et al., 2013). These include selective 2'-hydroxyl acylation analyzed by primer extension sequencing, fragmentation sequencing, parallel analysis of RNA structures (PARS) (Kertesz et al., 2010), double-stranded RNA-seq, and single-stranded RNA-seq (Table 1, Figure 2). These tools have been applied to yeast and animals systems (Kertesz et al., 2010; Warf and Berglund, 2010; Wan et al., 2011, 2013), while only two such global RNA structure analyses have been reported recently in plants (Zheng et al., 2010; Li et al., 2012). A recent *in vivo* genome-wide RNA structure study revealed secondary structure patterns at sites of AS (Ding et al., 2013). All of these are based on high-throughput sequencing of cDNAs prepared from RNAs that are either modified with specific chemicals or digested with specific types of nucleases. The data can be integrated into programs like SeqFold, which predicts structures, to obtain accurate RNA structure (Wan et al., 2013). Correlating the structural information obtained with these methods with AS in plants will likely uncover additional RNA structural elements involved in regulated splicing in plants (Figure 2). Understanding the roles of regulatory *cis*-elements in mRNA translation and identification of these in splice variants may also provide clues to roles of AS in translational regulation. Coupling of RIP methods with PARS should provide insights into the roles of RNA secondary structures in regulated splicing.

Modulation of splicing by secondary (e.g., hairpins and stem loops) and tertiary structural features in RNA that are responsive to ligands or altered cellular environments (e.g., osmolytes, ion concentration, and temperature) is appealing as it is rapid and may or may not require RBPs. RNA structures are highly sensitive to concentration and types of ions and osmolytes (Draper, 2004; Lambert and Draper, 2007; Mullen et al., 2012). Osmolytes have both stabilizing and destabilizing effects on RNA tertiary structure (Lambert and Draper, 2007). It is known that many abiotic stresses, such as high salinity, heat, and drought, impact cellular concentrations of ions and osmolytes in plants (Seki et al., 2007; Mullen et al., 2012; Chan et al., 2013), which might impact RNA secondary structure and regulate splicing and other aspects of posttranscriptional gene regulation. Hence, structural elements in pre-mRNA might function as sensors of abiotic stresses, such as temperature, drought, and salinity. Castiglioni et al. (2008) have shown that bacterial RNA chaperones (CspA or CspB) in maize (*Zea mays*) promote stress tolerance, indicating the importance of RNA secondary structures during stress responses. Recruitment of SR proteins by RNA secondary structure has also been reported (Buratti and Baralle, 2004; Buratti et al., 2004; Shepard and Hertel, 2008; McManus and Graveley, 2011). In prokaryotes,

structures in mRNAs often referred to as RNA thermometers function as temperatures sensors, which occlude the ribosome binding site at normal temperature. High temperature unfolds these structures to enable translation (Kortmann and Narberhaus, 2012; Wan et al., 2012). In theory, temperature-sensitive structures in pre-mRNAs in eukaryotes may also mask or unmask binding sites for splicing factors and modulate splicing rapidly in response to temperature changes. Forty percent of introns in budding yeast (*S. cerevisiae*) contain a predicted secondary structure between the branch site and 3'SS (Meyer et al., 2011). Furthermore, a secondary structure at the 3'SS in an intron of *APE2* pre-mRNA was shown to function as an RNA thermosensor by detecting changes in temperature and modulating AS (Meyer et al., 2011). Since pre-mRNA splicing occurs cotranscriptionally, the rate of transcription can also have a significant effect on RNA structure-mediated splicing as slow folding structures may form only if the rate of transcription is low.

Trans-Acting Factors

There are over 800 proteins with different types of RNA binding domains in *Arabidopsis* (Silverman et al., 2013), and a vast majority of these contain one or more, or a combination of, RNA binding domains, such as an RNA recognition motif (RRM), a K homology domain, or a pentatricopeptide repeat domain that bind RNA. Many plant RRM-type RBPs have no orthologs in animals (Lorković, 2009). Of the non-snRNP proteins, there are many SR proteins or hnRNPs that regulate splice site selection by binding to RNA motifs (see Supplemental Table 1 online). The SR family of splicing regulators contains one or two RRMs in the N terminus and a Arg/Ser-rich (RS) domain at the C terminus and function in CS and AS (Barta et al., 2010). Pre-mRNAs of most SR proteins and several hnRNPs are alternatively spliced (Lopato et al., 1999b; Lazar and Goodman, 2000; Kalyna et al., 2003; Kalyna and Barta, 2004; Isshiki et al., 2006; Palusa et al., 2007; Schöning et al., 2007; Wachter et al., 2012). In plants, SR proteins have been investigated for their function in splicing (Lopato et al., 1999a, 1999b). In animal systems, SR proteins, apart from their role in pre-mRNA splicing, function in diverse processes associated with RNA metabolism and gene regulation, including mRNA export, stability and translation, chromatin binding, transcription elongation, subcellular localization of transcripts, genome stability and formation of cellular RNA granules, and miRNA processing structures (stress granules and processing bodies) (Long and Caceres, 2009; Shepard and Hertel, 2009; Risso et al., 2012; Yoon et al., 2013). Whether plant SR proteins, like their animal counterparts, have wide-ranging roles in other aspects of RNA metabolism remains to be seen.

The SR proteins interact with RNA through their RRM, which provides RNA binding specificity, and with other spliceosomal proteins through their RS domain, which aids in spliceosome assembly. In some instances, the RS domain interacts directly with pre-mRNA either at the BP or 5'SS (Shen et al., 2004). Some plant SR and SR-like proteins have been shown to bind alternatively spliced introns (Day et al., 2012; Thomas et al., 2012). Complex interaction networks among SR proteins and interaction with many other snRNP and non-snRNP proteins (Golovkin and Reddy, 1998; Golovkin and Reddy, 1999; Lopato et al., 2002;

Gullerova et al., 2006; Reddy, 2007; Barta et al., 2008) suggest an important role for this family of proteins in regulated splicing. Expression levels and AS patterns of SR proteins in plants vary in different tissues, implying their target specificity (Lopato et al., 1996, 1999a, 1999b, 2002; Golovkin and Reddy, 1998, 1999; Lazar and Goodman, 2000; Kalyna et al., 2003; Kalyna and Barta, 2004). The fact that some SR proteins have been shown to interact with components of the U12-dependent minor spliceosome suggests a role in splicing catalyzed by the minor spliceosome (Reddy, 2007; Barta et al., 2008).

The hnRNPs are a heterogeneous group of proteins involved in diverse functions in mRNA metabolism, mainly characterized by their ability to bind RNA due to the presence of RRM domains or other domains like K homology domains and additional Gly-rich domains that add versatility to binding to RNA and/or that can extend their functions (Martinez-Contreras et al., 2007; Han et al., 2010; Wachter et al., 2012). Many of the hnRNPs are located in the nucleus and nuclear compartments, but some of them exhibit nucleus-cytoplasm shuttling that is regulated by posttranslational modifications, indicating a broader set of functions of these proteins besides nuclear mRNA processing (Wachter et al., 2012). The most extensively studied hnRNPs are the PTB proteins where their role in AS was first described in mammals (Mulligan et al., 1992; Wagner and Garcia-Blanco, 2001; Izquierdo et al., 2005). They act through their binding to pyrimidine-rich sequences in the pre-mRNA by competition and/or interaction with other hnRNPs and splicing factors exerting a positive or negative effect in the selection of specific splice sites that define the AS outcome in the transcript. In *Arabidopsis*, three genes have been identified (*PTB1*, *PTB2*, and *PTB3*; see Supplemental Table 1 online), all of them generating two alternatively spliced variants where in all the cases one of the two transcripts is targeted by NMD (Stauffer et al., 2010). The AS variants in *PTB1* and *PTB2* are derived from a mode of negative auto- and cross-regulation by these PTB proteins, a regulatory mechanism that is consistent with the human PTB orthologs (Grabowski, 2007; Stauffer et al., 2010). The analogies found in the mode of action of the PTBs in plants and human suggested that the plant proteins might also influence the AS of a considerable number of genes. A recent genome-wide study by Rühl et al. (2012) using overexpression and artificial miRNA lines of the different PTB proteins showed that mainly *PTB1* and *PTB2*, but not *PTB3*, influence the AS outcome of at least 300 genes that comprise important factors involved in diverse developmental processes, including flowering and seed germination. Interestingly, in contrast with what has been found in mammals where principally exon skipping events are altered, diverse types of AS were affected by *PTB1* and *PTB2*, including 5' alternative splice site, IR, and exon inclusion events. Nevertheless, the mechanisms of action and the evidence for binding of *PTB1* and *PTB2* to generate the observed AS patterns remain to be elucidated.

Small Gly-rich hnRNPs-like GRP7 and GRP8 in *Arabidopsis* have also been shown to have a role in AS. These two proteins are controlled by the circadian clock, and their protein levels are regulated similarly to PTBs by AS and the production of NMD-sensitive transcripts (Carpenter et al., 1994; Heintzen et al., 1994; Staiger, 2001; Staiger et al., 2003). Using a high-resolution RT-PCR panel, it was shown that GRP7 affects 59 events from a total

of 288 AS events tested (Streitner et al., 2012). RIP experiments confirmed binding of GRP7 to seven targets that was dependent on the RRM domain. Interestingly, some of the splicing events affected by GRP7 are also influenced by other RBPs, like SR proteins and the cap binding complex proteins (CBC), suggesting that the splicing profiles observed in *Arabidopsis* are the result of a complex interplay between RBPs and/or splicing factors (Streitner et al., 2012).

Despite the evident expansion in plants of plant-specific members of hnRNPs and hnRNP-like proteins, little is known about their actual role in AS. Few studies have identified functional features of plant specific hnRNPs-like proteins. The oligouridylylate binding protein (UBP1) in *Nicotiana plumbaginifolia* was found to have binding affinity to uridine-rich sequences and increase the efficiency of poorly spliced introns (Lambermon et al., 2000). Nevertheless, the UBP1-related proteins RBP45 and RBP47 did not affect splicing efficiency (Lorković et al., 2000). Similar results were found for *Arabidopsis* UBA1a and UBA2a where no stimulation of splicing was detected, suggesting a role of UBP1 and their associated proteins in mRNA stabilization (Lambermon et al., 2002). The prevalence of AS in plants (Syed et al., 2012) implies that the presence of multiple factors involved in splicing play important roles in the production of alternative spliced transcripts and that crosstalk and tight regulation between these factors exist to produce, in a very orchestrated manner, transcripts relevant in important biological aspects like development and response to the environmental cues.

In addition to SR and hnRNP proteins, the involvement of nuclear CBC in AS regulation has been shown by analyzing the AS events in single/double *Arabidopsis* mutants of the CBC components, *cbp20* and *cbp80/abh1*. Changes in AS isoforms were shown for 101 genes, with 41% of them common to all mutants and 15% arise only in double mutants. The authors propose a direct involvement of CBC in plant AS of the first intron, particularly at the 5'SS (Raczynska et al., 2010).

Subcellular Localization and Dynamics of Trans-Acting Factors

Speckles (also called interchromatin granule clusters) are a type of nonmembranous structure enriched in snRNPs, SR proteins, and other splicing factors (Spector and Lamond, 2011). Using SR proteins tagged to fluorescent reporters, it was shown that plant SR proteins localize to the nucleoplasm and nuclear speckles (reviewed in Reddy et al., 2012a). The number, size, and shape of speckles vary depending on the cell type and in response to external signals. Using fluorescence recovery after photobleaching and fluorescence loss in photobleaching, it was shown that plant SR proteins show high mobility within the nucleus. SR proteins shuttle between the nucleoplasm and speckles, and this movement is regulated by protein phosphorylation and dephosphorylation. Localization and dynamics of plant SR proteins has been recently reviewed and hence is not discussed in detail here (Reddy et al., 2012a). Until recently, speckles were thought to be storage sites of splicing factors (Spector and Lamond, 2011). However, a recent demonstration of the presence of a protein that is part of catalytically active spliceosomes in speckles and the presence of spliceosomes near or within the speckles indicate that posttranscriptional splicing

may occur in speckles (Girard et al., 2012). Little is known about the biogenesis of nonmembranous bodies containing RNA and proteins. Recent studies show that lowcomplex-disordered regions present in many of the splicing and RNA processing proteins are necessary and sufficient for formation of these bodies. Furthermore, phosphorylation of these regions contributes to the dynamic nature of these bodies (Han et al., 2012; Kato et al., 2012).

AS REGULATION BY EPIGENETIC MARKS ON DNA AND HISTONES

Splicing occurs mostly cotranscriptionally, but removal of some introns, especially alternatively spliced introns, takes place posttranscriptionally (Khodor et al., 2011; Braunschweig et al., 2013). Evidence is accumulating that chromatin modifications, transcription, and splicing are highly integrated, each influencing the other (Braunschweig et al., 2013; Dujardin et al., 2013; Gómez Acuña et al., 2013). Multiple aspects associated with transcription, such as promoter strength, transcription factor occupancy, rate of transcription, speed of RNA polymerase, and chromatin modifications, are known to change AS (Braunschweig et al., 2013). Although the means by which chromatin modifications affect splicing remain to be elucidated, there are indications that these modifications may directly or indirectly recruit spliceosomal proteins (Luco et al., 2010; Pradeepa et al., 2012; Schor et al., 2012; Tolstorukov et al., 2012; Braunschweig et al., 2013). Phosphorylation of the catalytic subunit of polymerase II at its C-terminal domain recruits many splicing factors, such as SRs and hnRNPs, that function in AS (Hsin and Manley, 2012). Some studies show that spliceosomal proteins bound to nascent transcripts may impact chromatin architecture and polymerase II elongation (Braunschweig et al., 2013). An emerging theme is that polymerase II elongation rate affects AS through kinetic coupling (Luco et al., 2011). Fast elongation rates favor exon skipping, whereas slow elongation favors spliceosome assembly and exon inclusion.

Recent studies in animals indicate that chromatin remodeling, which in turn affects the rate of transcription as well as nucleosome positioning, can alter the AS pattern (Alló et al., 2009; Kolasinska-Zwierz et al., 2009; Schwartz et al., 2009; Tilgner et al., 2009). Global studies in plants and animals indicate that DNA modifications, such as cytosine methylation, and chromatin changes, such as histone modifications and nucleosome occupancy, mark exon-intron boundaries (Braunschweig et al., 2013). Increased methylation is found in exons compared with introns. Further, exon-intron boundaries showed sharp transitions in methylation (Chodavarapu et al., 2010; Laurent et al., 2010). In *Arabidopsis* and human, using MNase-seq, it was found that nucleosomes are enriched in exons, especially at the boundaries (Schwartz et al., 2009; Chodavarapu et al., 2010). Furthermore, specific posttranslational modifications of histones are also enriched in exons (Schwartz et al., 2009). Differential nucleosome occupancy on exons that differ in splice site strength and a correlation between nucleosome occupancy and exon inclusion in metazoans suggest a role for chromatin architecture and regulated splicing (Tilgner et al., 2009; Braunschweig et al., 2013).

In plants, environmental signals are known to alter chromatin modifications in a gene-specific manner (Kim et al., 2010; Downen

et al., 2012; Gutzat and Mittelsten Scheid, 2012). Since stresses have a dramatic effect on pre-mRNA splicing, including those that are stress induced (Ali and Reddy, 2008b; Duque, 2011), it is likely that some of these changes in AS are regulated through stress-induced modification of chromatin. Nucleotide composition is different between exons and introns, with high GC content in exons and high AU in plant introns (Goodall and Filipowicz, 1989). Experimental evidence supports a role for differential nucleotide composition in pre-mRNA splicing. RNA polymerase II elongation rates are impacted by nucleotide sequence composition with a slow rate in adenine-thymine-rich regions.

A report in *S. pombe* implicates a role for splicing factors in RNA-induced chromatin silencing that is uncoupled from splicing (Bayne et al., 2008). In plants, recent reports indicate a role of splicing machinery and splicing factors in regulating transcription. Introduction of an intron into an intronless gene was shown to suppress RNA silencing that is dependent on efficient splicing of the intron, suggesting a role for splicing and transcriptional regulation (Christie et al., 2011). Some studies suggest a role for splicing machinery in RNA-directed methylation (RdDM). A mutant screen aimed at identifying proteins involved in transcriptional silencing through RdDM identified a splicing factor (ZINC FINGER AND OCRE DOMAIN-CONTAINING PROTEIN1) as one of the key regulators of RdDM (Zhang et al., 2013b). SR45, an SR-like splicing regulator, was isolated in a screen for mutants that affect the establishment of RNA-directed DNA methylation (Ausin et al., 2012). The loss-of-function mutant of *SR45* displayed reduced methylation, suggesting a role of this splicing factor in RdDM, but an indirect role through splicing regulation of pre-mRNAs of genes involved in RdDM is not excluded (Ausin et al., 2012).

ELUCIDATING PATHWAYS THAT CONNECT SIGNALS TO AS REGULATION

Rapid alteration of transcription complexity in response to stresses is a simple mechanism for rapid reprogramming of the transcriptome and alteration of proteome complexity. Several studies in plants indicate that splicing of pre-mRNAs is regulated by external signals (Ali and Reddy, 2008b; Duque, 2011; Mastrangelo et al., 2012; Leviatan et al., 2013). The mechanisms by which signals regulate pre-mRNA splicing are not known in plants. Figure 7 shows potential mechanisms through which signals regulate AS. Posttranslational modification of splicing factors and spliceosomal components, especially phosphorylation, are known to regulate splicing in animals. Several protein kinases that phosphorylate splicing regulators have been identified in plants (reviewed in Ali and Reddy, 2008a), and phosphoproteomic analysis revealed that several plant SR proteins and other splicing factors are phosphoproteins (de la Fuente van Bentem et al., 2006). It is possible that signals through second messengers, such as calcium, and reactive oxygen species modulate the activity of kinases and phosphatases that regulate the phosphorylation status of splicing regulators (de la Fuente van Bentem et al., 2006). Although second messengers are known to regulate transcription of many genes by modulating the activity of transcription factors (Reddy et al., 2011; Mittler et al., 2012; Smékalová et al., 2013), little is known about their role in posttranscriptional gene regulation.

Complex regulatory feedback loops linking AS, NMD, and kinase signaling may exist as exemplified by AFC2, a highly conserved LAMMER kinase, which phosphorylates splicing factors (Marquez et al., 2012). Other posttranslational modifications of splicing regulators, such as sumoylation, may also impact AS, as several RBPs including splicing regulators are found to be sumoylated in global proteomic studies (Miller et al., 2013). It is also likely that stresses may change the cellular environment (change in ion concentration and other molecules) that might have an impact on RNA structure leading to changes in splicing patterns. It has been documented that RNA structures change in response to concentration and types of ions and osmolytes (Draper, 2004; Lambert and Draper, 2007; Mullen et al., 2012) and temperature (Meyer et al., 2011). Stress signals in plants are known to alter cellular concentration of ions and osmolytes (Seki et al., 2007; Mullen et al., 2012; Chan et al., 2013). Hence, RNA structures may serve as sensors to detect changes in cellular environment or temperatures. The expression and AS of splicing regulators may also be regulated by these signals thereby impacting splicing. AS of master regulators of splicing, such as SRs and hnRNPs, is changed in response to stresses (Lazar and Goodman, 2000; Palusa et al., 2007; Reddy and Ali, 2011). Such changes will have global impacts on AS of other pre-mRNAs leading to reprogramming of gene expression.

GENOME DUPLICATION AND AS

Genome duplication and AS are two major mechanisms that lead to expansion and diversification of the proteome. Three models have been proposed to explain the relationship between genome duplication and AS. The independent model predicts no relationship between gene family size and the amount of AS, and the function-sharing model predicts a reverse correlation between gene family size and the extent of AS (Su et al., 2006; Jin et al., 2008). A study of genome duplication and AS events in mouse and humans by Jin et al. (2008) led to a third model called accelerated AS. They found that as predicted with the function-sharing model, AS decreased in large families but that AS increased in small families compared with singletons. The accelerated AS model suggests that singletons are likely to be duplicated, relaxing functional constraints allowing for more AS. Jin et al. (2008) argue that large families preclude the possibility for new useful functions to be created by AS, as different members in a large gene family could acquire new functions through sub- or neofunctionalization. In this respect, it is interesting that the family of 18 *Arabidopsis* SR genes, which partially originated from several genome duplication events (Kalyna and Barta, 2004), shows a remarkably high level of AS where 14 SR genes create at least 95 splice isoforms (Palusa et al., 2007).

Some plant studies have looked at duplication and AS in individual genes. Zhang et al. (2010c) used RT-PCR to look at 104 whole-genome gene duplicates in six organ types and in plants grown under abiotic stress. In a large majority of the duplicated pairs, they found differences in splicing patterns between the genes in one or more organs or under stress conditions, indicating AS divergence after genome duplication. Some plant genomes are polyploids as a result of hybridization rather than genome duplication. AS patterns in the wheat (*Triticum aestivum*) DREB2 homolog, WDREB2, were compared for polyploid wheat and for

their diploid progenitors (Terashima and Takumi, 2009). Three variants of WDREB2 (α , β , and γ) result from AS, and the β -form is nonfunctional. In response to stresses, the WDREB2 α and γ increase, but WDREB2 γ remains constant in hexaploid wheat, while in the diploid progenitors the WDREB2 γ decreased rapidly. The functional forms were not produced as rapidly in the hexaploids as in the diploid progenitors, suggesting that allopolyploidization inhibits the efficient splicing of the WDREB2 gene.

POTENTIAL BIOTECHNOLOGICAL APPLICATIONS OF AS

Transcriptional activation of transgenes has been widely used in plants to manipulate gene expression and to introduce desired traits into plants. Understanding the mechanisms involved in regulation of AS and the functions of splice variants will lead to novel ways to regulate gene expression and to develop crop plants with novel traits. For instance, expression of individual splice variants may have a different outcome compared with expression of all isoforms from a gene. Insertions of the first intron from the *UBQ10* gene into intronless genes markedly increased mRNA accumulation over 150-fold (Emami et al., 2013). Recently, plant 5S RNA mimic (discussed above), a naturally occurring alternatively spliced suicide exon (Hammond et al., 2009; Barbazuk, 2010), has been used to regulate gene expression at the pre-mRNA splicing level (Hickey et al., 2012). In this case, skipping of a suicide exon produces a functional transcript, whereas inclusion of this exon generates a transcript that is a target for NMD. With the optimized version of this suicide exon, \sim 100-fold gene activation was achieved by regulating AS. This work opens new avenues for the conditional expression of multiple genes at the splicing level by incorporating the same suicide exon into the transgenes. In another case, addition of the TPP riboswitch in *THIC* to a reporter construct resulted in metabolite-dependent regulation of reporter gene expression, with decreased expression under high TPP levels (Bocobza et al., 2007), suggesting a potential use of riboswitches for conditional expression of transgenes. Expression of a bacterial RNA chaperone that modulates RNA structure has also been shown to confer abiotic stress tolerance in maize under field conditions and increase yield (Castiglioni et al., 2008). A thorough understanding of AS regulatory elements and proteins that bind to these elements will offer novel ways to manipulate splicing in vivo and manipulate gene expression.

KEY FUTURE CHALLENGES

From limited transcriptome-wide studies in plants, it is clear that AS is much more prevalent in plants than previously thought. The true extent of AS from multiexon genes in plants is expected to be greater than now known when more information is gained from in-depth AS studies with different tissues/cell types and in response to developmental cues and diverse environmental and stress signals. Indeed, from recent studies, it is now becoming clear that AS is regulated developmentally and in response to diverse stress signals. These studies underscore the importance of understanding the functions of splice variants and mechanisms that regulate AS. Some important unanswered questions that need to be addressed are discussed below.

It is hypothesized that splice variants generated by AS have the potential to increase the number of proteins produced. However, currently, it is not known, both in plants and animals, what fraction of splice variants contributes to proteome diversity with functional consequences. In addition to contributing to proteome diversity, AS can be an important factor in fine-tuning gene expression at the transcript level by regulating the amount of stable and unstable transcripts. The percentage of mRNA isoforms that contributes to gene regulation at the mRNA stability through NMD, small noncoding RNAs, and other mechanisms (e.g., translation efficiency) is not known. Although a few recent studies point to an important role for AS in plant development and stress responses, functional analysis of individual mRNA isoforms is necessary to understand the importance of AS in plant growth, development, and, especially, stress responses. As there are thousands of mRNA isoforms, it is a daunting task to study the functions of each and every isoform. Initial focus should be on AS events that are conserved among all land plants, as such events are likely to have functional consequences as shown for the plant-specific SR protein families (Iida and Go, 2006; Kalyna et al., 2006). There is evidence that AS remodels protein–protein interactions in regulating cellular processes (Buljan et al., 2012; Ellis et al., 2012). Pairwise interaction of proteins coded by all AS forms using yeast two-hybrid could shed light on the functions of mRNA isoforms. It has been shown that tissue-specific regulated AS of exons

modifies surface loops and unstructured binding regions of encoded proteins and rewires the protein–protein interaction networks (Buljan et al., 2012; Ellis et al., 2012).

The regulation of AS and the roles of the diverse set of RBPs (SRs and hnRNPs) are almost completely unknown. The availability of an *in vitro* splicing system in animals has contributed to enormous progress in understanding steps in spliceosome assembly, spliceosome composition, and *cis*-elements in RNA that regulate constitutive and regulated splicing. The Holy Grail in plant splicing research is a plant-derived *in vitro* splicing system. With new technological advances to isolate pure nuclei using tagged nuclei from plant cells (Deal and Henikoff, 2010, 2011) it is worth revisiting this. The accumulating data on AS in different plants across the phylogenetic spectrum will allow the development of computational tools to identify conserved, lineage-specific AS and predict regulatory elements (enhancers and suppressors) that determine the outcomes of splicing. The ultimate lofty goal in using computational tools to analyze AS is to develop algorithms that can predict if a pre-mRNA is alternatively spliced in a cell-specific manner and/or under certain cellular environments based on nucleotide sequence. To develop such algorithms requires a vast amount of empirical data on sequence elements that bind to splicing regulators and on the complexity of AS and splicing factor expression in diverse plants.

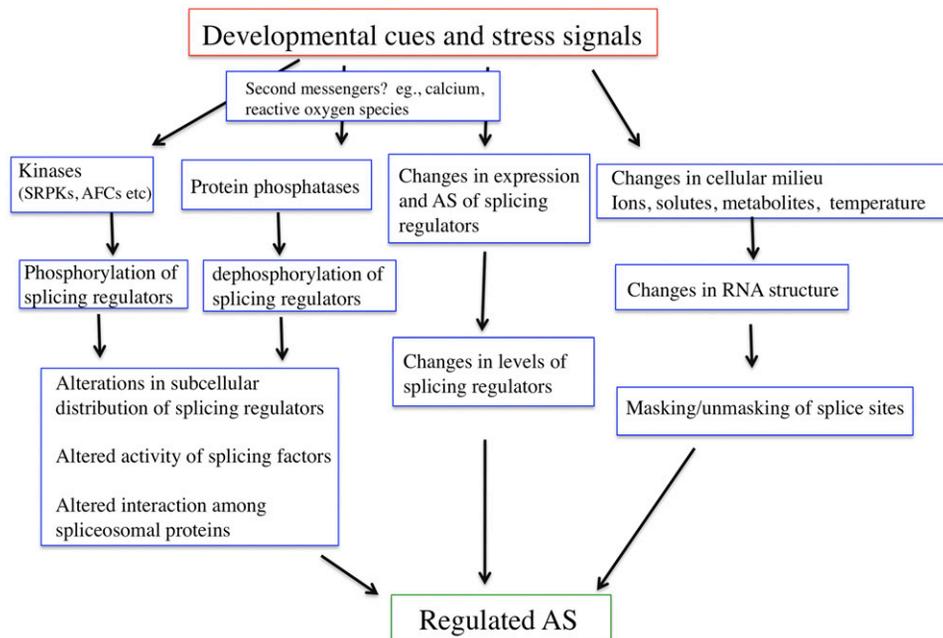


Figure 7. Potential Mechanisms through Which Developmental Cues and Environmental Signals Regulate AS.

Signal-induced changes in messengers such as calcium and reactive oxygen species, which are known regulate transcription of numerous genes (Reddy et al., 2011; Mittler et al., 2012; Smékalová et al., 2013), may also regulate AS through protein kinases and protein phosphatases as the activity of many splicing regulators is modulated by protein phosphorylation and dephosphorylation. In addition, the impact of signal-induced changes in cellular milieu, which are known to occur in plants (Seki et al., 2007; Mullen et al., 2012; Chan et al., 2013), on RNA structure and AS is unexplored in plants. See text under Elucidating Pathways That Connect Signals to AS Regulation for details pertinent to this figure. SRPK, Serine/arginine-rich protein-specific kinase; AFC2, *Arabidopsis fus3*-complementing cDNA 1 (AFC1) homolog (a LAMMER kinase).

[See online article for color version of this figure.]

Understanding how developmental and environmental signals are transduced into AS regulation and elucidating the role of AS in reprogramming gene expression in response to biotic and abiotic stresses will have implications in crop improvement. This will open new avenues to enhance and/or fine-tune gene regulation for biotechnological applications.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Spliceosomal Proteins in Human and *Arabidopsis thaliana*.

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AUTHOR CONTRIBUTIONS

All authors contributed to writing the article.

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