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REVIEW

Alternative Splicing at the Intersection of Biological Timing, Development, and Stress Responses[OPEN]

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High-throughput sequencing for transcript profiling in plants has revealed that alternative splicing (AS) affects a much higher proportion of the transcriptome than was previously assumed. AS is involved in most plant processes and is particularly prevalent in plants exposed to environmental stress. The identification of mutations in predicted splicing factors and spliceosomal proteins that affect cell fate, the circadian clock, plant defense, and tolerance/sensitivity to abiotic stress all point to a fundamental role of splicing/AS in plant growth, development, and responses to external cues. Splicing factors affect the AS of multiple downstream target genes, thereby transferring signals to alter gene expression via splicing factor/AS networks. The last two to three years have seen an ever-increasing number of examples of functional AS. At a time when the identification of AS in individual genes and at a global level is exploding, this review aims to bring together such examples to illustrate the extent and importance of AS, which are not always obvious from individual publications. It also aims to ensure that plant scientists are aware that AS is likely to occur in the genes that they study and that dynamic changes in AS and its consequences need to be considered routinely.

INTRODUCTION

With the discovery of intervening sequences in eukaryotic genes by Philip Sharp and colleagues, it became apparent that removal of introns through splicing of pre-mRNAs is a key step in eukaryotic gene expression (Berget et al., 1977). Splicing removes intronic sequences defined by short conserved sequence motifs (the 5′ and 3′splice sites) to join the neighboring exons and generate an uninterrupted open reading frame (ORF) for translation. This is accomplished by the spliceosome, a high molecular weight complex that is assembled at every intron. It consists of five small nuclear ribonucleoprotein particles (snRNPs) and over 200 additional proteins (Wahl et al., 2009; Will and Lührmann, 2011; Koncz et al., 2012; Reddy et al., 2013). The five snRNPs contain small nuclear uridine-rich RNAs (U1, U2, U4, U5, and U6 snRNAs). The core particles of the U1, U2, U4, and U5 snRNPs are formed by Sm proteins, whereas the U6 snRNP contains the related Lsm2 (Like Sm2) to Lsm8 proteins (Tharun, 2009). The initial step of splice site recognition comprises U1 snRNP binding to the 5′splice site and U2 auxillary factor (U2AF) binding to the 3′splice site. $U2AF^{35}$, the small subunit of U2AF, binds to the intron/exon border, whereas the large subunit $U2AF^{65}$ binds to a region rich in pyrimidines designated the polypyrimidine tract (Figure 1). Subsequently, U2 snRNP binds to the branch point, and a preformed complex of U4, U5, and U6 snRNPs is recruited to the intron. After major rearrangements and release of the U1 and U4 snRNPs, the splicing reaction takes place.

Alternative splicing (AS) is where alternative splice sites are selected resulting in the generation of more than one mRNA transcript from precursor mRNA (pre-mRNA) transcripts. An extreme example is the *Drosophila melanogaster* Dscam gene with the potential to produce more than 38,000 alternatively spliced variants; this is impressive considering that the *Drosophila* genome contains only 13,000 genes (Graveley, 2005). The decision on which splice sites are selected under particular cellular conditions is determined by the interaction of additional proteins, globally designated as splicing factors (SFs), that guide spliceosomal components and thereby the spliceosome to the respective splice sites (Matlin et al., 2005; Nilsen and Graveley, 2010; Wachter et al., 2012). The main families of these SFs are the Ser/Arg-rich (SR) proteins and heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins. These proteins bind specific sequences in the pre-mRNA called intronic or exonic splicing enhancer or suppressor sequences. Splice site selection will reflect the relative occupation of these sequences and interactions among different proteins on a pre-mRNA (Witten and Ule, 2011). Clearly, differences in the abundance, localization, and activity of proteins in different cells or in cells experiencing different internal or external cues will affect the splicing outcomes. Subtle changes in SF levels or activity can have subtle or profound effects on the expression of downstream target genes (Figure 2). When considering the regulation of AS, it is therefore essential to understand how SFs are regulated and activated. For example, in both animals and plants, many SFs/RNA binding proteins

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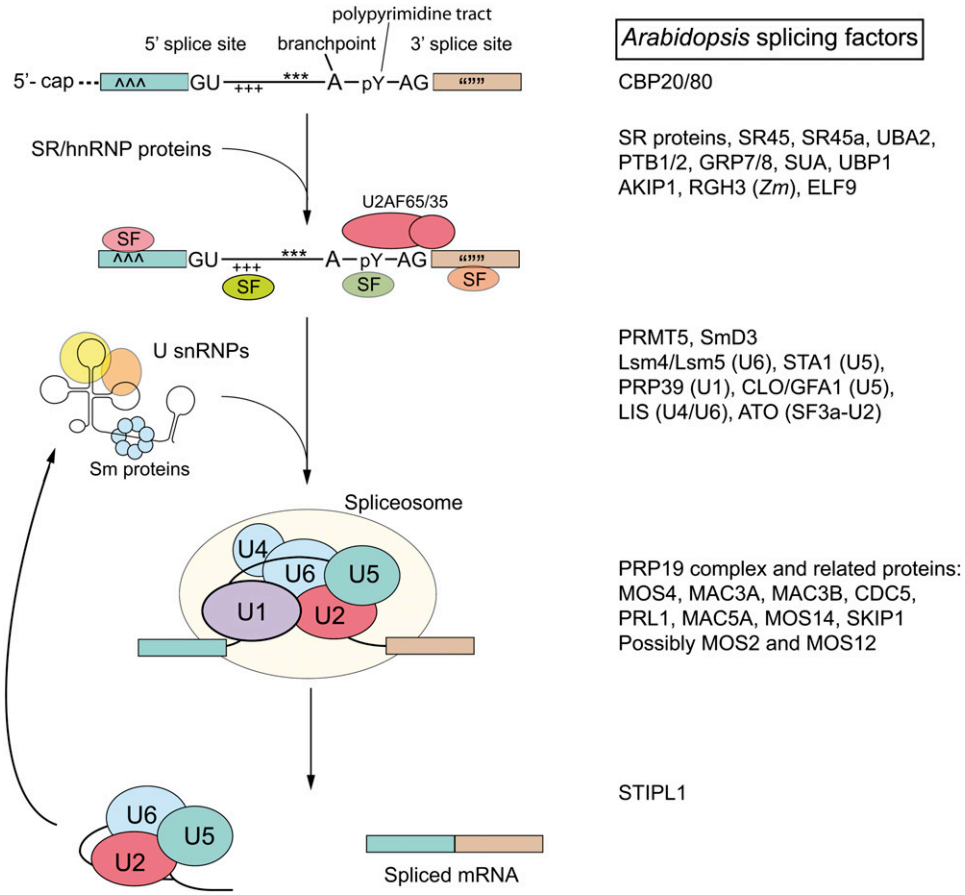

Figure 1. Splicing Signals, SFs, and Spliceosomal Components Involved in Pre-mRNA Splicing.

Pre-mRNAs contain intronic splicing signals (splice sites, polypyrimidine tract, and branch point sequences) as well as exonic and intronic splicing enhancer and suppressor sequences (symbols), which are binding sites for SFs. SFs bind to target sequences and help to recruit spliceosomal factors (e.g., U2AF and U1snRNP) to define splice sites and determine spliceosome assembly. Factors associated with pre-mRNA splicing in *Arabidopsis* are indicated approximately at the steps where they are proposed to act, based on homology to human spliceosome components (Agafonov et al., 2011). Boxes correspond to exons; thin lines correspond to introns.

(RBPs) and some core spliceosomal components themselves undergo AS in response to signals and even control their own levels and those of other SFs via AS (Kalyna et al., 2006; Stauffer et al., 2010; Saltzman et al., 2011; Thomas et al., 2012). In addition, the activity of SFs can be regulated by posttranslational modification in response to environmental cues (Stamm et al., 2005).

AS has important consequences for the cell. These are mainly at the RNA or protein levels. First, AS can regulate transcript levels by the introduction of premature termination codons (PTCs), which commit the transcript isoform to degradation by the nonsense-mediated decay (NMD) pathway. Linked AS-NMD thus regulates the level of functional mRNA transcripts (which encode protein) via targeted degradation of alternative splice forms (McGlincy and Smith, 2008; Nicholson and Mühlemann, 2010), and in *Arabidopsis thaliana*, at least 13% of genes undergo AS-NMD (Kalyna et al., 2012). The second main consequence of AS is where transcript isoforms give rise to proteins that differ in their sequence and domain arrangement and thus may widely differ in subcellular localization, stability, or function (Syed et al., 2012). Proteins or polypeptides that are truncated as a consequence of AS can act as dominant-negative inhibitors of the authentic proteins (e.g., through unproductive interaction with dimerization partners or nucleic acids) and have been designated micropeptides or small interfering peptides (Seo et al., 2011a).

In humans, the importance of AS is clearly manifested by genetic hereditary diseases caused by defects in splicing/AS due to mutations in, for example, conserved splice site sequences or in SFs. Around 15% of genetic diseases are due to mutations which affect splicing (Kornblihtt et al., 2013). For example, mutations in specific genes that cause aberrant splicing underpin β-thalassemia, cystic fibrosis, myotonic dystrophy, Duchenne/Becker muscular dystrophy, and Hutchinson-Gilford Progeria Syndrome (Figures 3A to 3C), while mutations in SFs or that cause misexpression of SFs underpin spinal muscular atrophy, retinitis pigmentosa, and myotonic dystrophy (Tazi et al., 2009). Interestingly, gene therapies for some of these diseases are

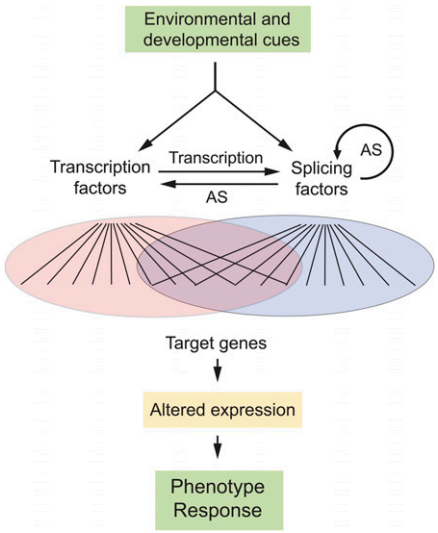

Figure 2. Dynamic Regulation of RNA and Protein Expression by AS.

Environmental and developmental cues impact gene expression at the level of transcription and AS. Signaling cascades impact the transcription, activity, or subcellular localization of transcription factors and/or SFs. SFs themselves often undergo auto- or cross-regulation by AS. An intimate connection between transcription and AS emerges, where transcription can affect expression levels of SFs and AS of genes dependent on the rate of transcription of RNA polymerase II (Luco et al., 2011); AS can influence the level of transcription factor expression via AS/NMD and the domain composition of transcription factors. Downstream genes may be regulated only at the transcriptional level or the AS level or at both levels.

based on modulating AS to ameliorate symptoms (Nlend Nlend et al., 2010). Furthermore, specific alterations in the expression and posttranslational modifications of SFs occur during cancer development, and it was shown that ~50% of AS events in ovarian and breast tissue are altered in tumors (Tazi et al., 2009; Venables et al., 2009).

In plants, natural mutations do not have the prominence of human disease but obviously are the basis of plant evolution and selection in crops. One well-known example is mutation of the *Waxy* (*Wx*) gene of rice (*Oryza sativa*) encoding a granule-bound starch synthase that controls grain amylose content. The *wx* mutant (*wxb*) has a guanosine to uridine mutation at the 5′ splice site of intron 1, activates two cryptic splice sites in exon 1 and reduces splicing efficiency resulting in lower levels of amylose to generate "sticky" rice (Figure 3D) (Cai et al., 1998; Isshiki et al., 1998; Larkin and Park, 1999). However, the generation of mutants in the model plant *Arabidopsis* has advanced plant science massively over the last 25 years. Detailed analyses of many mutants with altered splicing have identified mutations that directly disrupt splice sites or splicing signals, as well as some that affect nearby sequences not predicted to alter splicing, illustrating how subtle sequence changes can determine splicing outcomes (Brown, 1996). Variation affecting splicing/AS outcomes can provide flexibility in the transcriptome and proteome to contribute to the ability of plants to adapt to their environment (Kazan, 2003).

High-throughput RNA-seq has had a major impact on AS research in plant science, as it has allowed the identification of previously unknown transcript isoforms and assessment of dynamic changes in the full complement of transcript isoforms during development or in response to environmental cues (Weber et al., 2007; Filichkin et al., 2010; Marquez et al., 2012; Reddy et al., 2013). The high proportion of genes in *Arabidopsis* that show AS (Filichkin et al., 2010; Marquez et al., 2012) is also being found in other plant species including crop plants (Lu et al., 2010; Zhang et al., 2010; The Potato Genome Sequencing Consortium, 2011; International Barley Genome Sequencing Consortium,

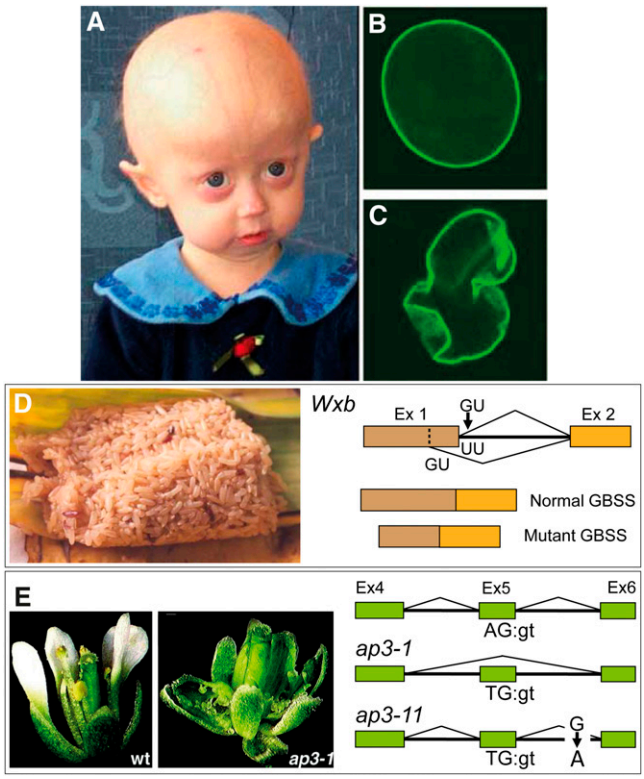

Figure 3. Phenotypic Consequences of Aberrant Splicing.

(A) to **(C)** Human mutations affecting splicing.
(A) Hutchinson-Gilford Progeria Syndrome leads to premature aging. Mutation of nuclear lamin A, which affects splicing, causes aberrant cell nuclei **(C)** compared with the regular shape of nuclei in healthy individuals **(B)**.
(D) and **(E)** Plant mutations affecting splicing.
(D) An SNP at the *wxyb* 5′splice site of intron 1 causes aberrant splicing of granule-bound starch synthase resulting in lower levels of amylose and "sticky" rice (left).
(E) Phenotypes (left) and corresponding transcript structures (right) of *AP3* in *Arabidopsis* wild type (wt) and the *ap3-1* mutant.
The *ap3-1* mutant contains a point mutation at the 5′splice site of intron 5, leading to skipping of exon 5 and a nonfunctional AP3 protein. The suppressor mutant *ap3-11* has a mutation in intron 4 that creates a novel branch point sequence allowing exon 5 to once more be spliced into the mRNA (Yi and Jack, 1998). (***[A]*** *to* ***[C]*** *are reprinted from Scaffidi et al. [2005], Figure 1;* ***[D]*** *and* ***[E]*** *are reprinted from Yi and Jack [1998], Figure 1.*)

2012; Darracq and Adams, 2013; Walters et al., 2013). RNA-seq now provides a means to address the question of splicing networks in a similar way to how transcript profiling with microarrays has provided detailed pictures of transcription factor networks that regulate sets of genes during development and in response to environmental cues (Nakashima et al., 2009; Tsuda et al., 2009; Breeze et al., 2011; Windram et al., 2012). Genome-wide chromatin immunoprecipitation experiments have identified in vivo targets of transcription factors, thus refining our view on the network structure by distinguishing direct from indirect targets (Kaufmann et al., 2010). In addition, we must consider interaction of AS with other posttranscriptional processes, such as the influence of microRNAs (miRNA), and of posttranslational modification, such as phosphorylation, which can affect the characteristics of SFs like SR proteins.

Increasing our knowledge of AS mechanisms, the *trans*-factors and *cis*-sequences involved in determining AS, and the complex networks of SFs is vital for a complete understanding of the interactions between posttranscriptional regulation, transcriptional regulation, and chromatin signatures, as well as the function of AS at the individual gene, whole-plant, and population levels. Here, we demonstrate the extent and importance of AS in plants by summarizing the major conclusions from recent work on AS in response to abiotic and biotic stress, during development, in flowering time control, and in the circadian timing system. In each area, we draw on representative examples of AS in particular genes and which show involvement of SFs; the review is therefore not a comprehensive collection of all AS described to date.

ABIOTIC STRESS

Because of their sessile lifestyle, plants are strongly influenced by environmental factors. Major deviations in ambient light, temperature, or soil characteristics (e.g., water/salt content) from the normal (or optimal) conditions, collectively referred to as abiotic stress, strongly affect plant performance. Responses to abiotic stress include the induction of the major stress hormone abscisic acid (ABA) and a rapid adjustment of the transcriptome, including up-regulation of components that improve plant stress tolerance. Early on, it was observed that both low and high temperature stress not only alter the steady state abundance of many transcripts but also evoke changes in their AS patterns (Christensen et al., 1992; Larkin and Park, 1999; Iida et al., 2004; Filichkin et al., 2010).

Abiotic Stress–Dependent AS

Heat shock transcription factors (Hsfs) are the key players mediating plant responses to strongly elevated temperature, or heat shock, by binding to heat shock promoter elements of, for example, heat shock protein genes (von Koskull-Döring et al., 2007). *Arabidopsis* HsfA2 undergoes posttranscriptional regulation in addition to heat-induced transcriptional upregulation. In plants exposed to 37°C, a 31-bp mini-exon within the conserved intron in the DNA binding domain is spliced into the transcript. This exon introduces a PTC and targets the AS isoform, *HsfA2 II*, to NMD, thus providing a mechanism to adjust the level of active HsfA2 protein (Sugio et al., 2009). At 42°C, another splice variant, *HsfA2 III*, appears that codes for a shorter protein, S-HsfA2, while *HsfA2 II* decreases (Liu et al., 2013). This truncated protein retains the Hsf helix-turn-helix DNA binding motif, localizes to the nucleus, and binds to the *HsfA2* promoter heat shock elements, pointing to a positive autoregulatory loop of *HsfA2* expression through AS. At 45°C, *HsfA2 III* but not *HsfA2 II* is detected, indicating that gradual changes in stressful temperature can change the ratio of these two splice isoforms.

For rice *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2* (*DREB2B*), a temperature- and drought-responsive gene, AS is required for the production of the functional protein (Matsukura et al., 2010). Under normal growth conditions, inclusion of a 53-bp exon 2 introduces a frame shift and a PTC, leading to a nonfunctional transcript isoform (Os-*DREB2B1*). Upon exposure to high temperatures, an AS isoform prevails where exon 2 is skipped giving an mRNA comprising of exons 1 and 3 with the intact ORF (Os-*DREB2B2*), thus allowing a rapid production of DREB2B protein in response to stress independently of transcriptional activation.

Low temperature also induces changes in AS in many genes (Iida et al., 2004). For example, low temperature storage of potato (*Solanum tuberosum*) causes sweetening due to the conversion of starch to Suc and, subsequently, Glc and Fru by vacuolar acid invertase, which is detrimental to processing. Splicing of a mini-exon that forms part of the active site of an invertase gene was modified in cold storage (Bournay et al., 1996), and lines showing resistance to cold-induced sweetening have higher expression of two splice variants (INH2α and INH2β) of the invertase inhibitor gene (*INH2*) (Brummell et al., 2011). Cold also regulates AS of the INDETERMINATE DOMAIN14 (IDD14) transcription factor that activates the expression of the QUA-QUINE STARCH starch-degrading enzyme, leading to inhibition of starch accumulation (Seo et al., 2011b). At low temperatures, intron retention is suggested to lead to a protein variant lacking a functional DNA binding domain that potentially squelches intact IDD14 through unproductive heterodimerization, ultimately leading to starch accumulation. Thus, if AS of IDD14 indeed generates a polypeptide comprising only the C-terminal region, it provides an example of a small interfering peptide (see Introduction). Recently, extensive AS in core circadian clock genes in response to low temperatures has been identified (James et al., 2012a, 2012b) (see section below on the circadian clock). Although many of the above examples of temperature-dependent AS have been analyzed at extremes of temperature, it is important to note that changes in nonstressful ambient temperature as small as 4°C can have a significant effect on a range of AS events (James et al., 2012a; Streitner et al., 2013), suggesting highly dynamic changes in AS are likely to be occurring continually.

Finally, a first example of temperature-induced AS and the interaction with miRNA regulation is expression of miR400. miR400, located in an intron, is downregulated by heat treatment due to a temperature-induced AS event that affects miRNA processing and causes the miRNA to be retained in the transcript (Yan et al., 2012). The altered miR400 level in turn feeds back on the level of its host transcript.

AS of SFs in Response to Abiotic Stress

Temperature change can affect levels of SFs, particularly via AS and AS/NMD, which in turn will impact AS of downstream target genes. Notably, a suite of SR proteins that are key regulators of AS undergo AS themselves in response to extreme temperatures (Lazar and Goodman, 2000; Palusa et al., 2007; Filichkin et al., 2010) and other stresses, such as salt stress (Palusa et al., 2007; Tanabe et al., 2007) and highlight stress (Tanabe et al., 2007; Filichkin et al., 2010). Many of the splice isoforms contain PTCs committing them to the NMD pathway or are predicted to produce protein variants with different combinations of domains that likely affect their function in pre-mRNA splicing. For example, the At-*SR30* AS isoform containing the intact ORF increases at elevated temperatures and high light, whereas an unproductive PTC-containing AS isoform decreases at elevated temperatures, suggesting that AS is a means of dynamically regulating the level of functional protein (Filichkin et al., 2010). Similarly, dehydration stress and heat stress increase the production of transcripts encoding the full-length SR45a protein, an atypical SR-like protein, relative to other splice variants (Gulledge et al., 2012). Furthermore, stress signals affect both the phosphorylation status and subcellular localization of *Arabidopsis* SR and splicing-related proteins (Ali et al., 2003; Tillemans et al., 2005, 2006; de la Fuente van Bentem et al., 2008; Koroleva et al., 2009; Rausin et al., 2010). Phosphorylation may affect the function of SF protein isoforms. The two protein isoforms of SR45, another noncanonical SR-like protein, differ by eight amino acids, which include putative phosphorylation sites and have very different effects on development (Zhang and Mount, 2009; and see below). By analogy to animal systems, phosphorylation provides a means to alter the activity or localization of SR proteins and SFs again without transcriptional activation (Stamm et al., 2005).

The plant *UBA2* genes encode RBPs with similarity to hnRNPs of the A/B and D types in metazoa. Although a role for UBA2 proteins in splicing has not been demonstrated (Lambermon et al., 2002), UBA2a interacts with UBP1, an hnRNP-like protein involved in both mRNA splicing and stability (Lambermon et al., 2000). The *UBA2* transcripts undergo AS in the 3′ untranslated region (UTR), and different splice isoforms respond differentially to wounding (Bove et al., 2008).

Function of SFs in Abiotic Stress Response

An intriguing observation is that a plethora of RNA processing factors have been identified from screens for stress tolerance or sensitivity and thus presumably are associated with responses to different stresses. For example, SR45 negatively regulates Glc signaling during seedling development by downregulating the ABA response pathway (Carvalho et al., 2010). *STABILIZED1* (*STA1*) encodes a nuclear protein similar to the human U5 snRNP–associated 102-kD protein and the SFs Prp1p from *Schizosaccharomyces pompe* and Prp6p from *Saccharomyces cerevisiae* (Lee et al., 2006). *STA1* expression is upregulated by cold stress. The *sta1-1* mutant is cold sensitive and defective in splicing of the cold-induced *COR15A*. Transcripts coding for proteins homologous with U5 snRNP–associated 200-kD protein and the U4/U6 snRNP–associated 90-kD Prp3 protein are upregulated in *sta1-1*, suggesting a mechanism compensating for loss of *STA1*.

RNA helicases use the energy of ATP to unwind local RNA duplex structures. The cold-inducible RNA helicase REGULATOR OF C-REPEAT BINDING FACTOR GENE EXPRESSION1 is essential for splicing and is important for cold-responsive gene expression and cold tolerance in *Arabidopsis* (Guan et al., 2013). PRMT5, a type II protein Arg methyltransferase that symmetrically dimethylates Arg side chains, also impacts splicing/AS in *Arabidopsis*. In mammals, PRMT5 is part of a complex that modifies Sm proteins and subsequently helps to load them onto U snRNAs, forming U snRNPs. Among the substrates of *Arabidopsis* PRMT5 are RBPs and U snRNP proteins, and PRMT5 has been shown to affect splicing globally (Hong et al., 2010; Sanchez et al., 2010). The *prmt5* mutant, also known as *shk1 kinase binding protein1* (*skb1*), is sensitive to salt (Zhang et al., 2011). It was proposed that PRMT5/SKB1 affects plant development and the salt response by altering the methylation status of H4R3sme2 (for symmetric dimethylation of histone H4 arginine 3) and LSm4 and thus linking transcription to pre-mRNA splicing (Zhang et al., 2011).

A number of RNA processing factors are associated with responses to ABA. The *Arabidopsis supersensitive to ABA and drought1* (*sad1*) mutant shows an increased sensitivity to drought and ABA (Xiong et al., 2001). It encodes the homolog of LSm5 protein, a component of the U6 snRNP core (Perea-Resa et al., 2012). Recently, reduced levels of U6 snRNA and accumulation of unspliced pre-mRNAs have been observed in the *sad1*/*lsm5* mutant, suggesting that it has a role in pre-mRNA splicing by contributing to U6 stability (Golisz et al., 2013). Similarly, the *lsm4* mutant is hypersensitive to salt and ABA and shows mis-splicing (Zhang et al., 2011). The *abh1* and *cbp20* mutants are impaired in ABH1/CBP80 and CBP20, the subunits of the CAP binding complex, and are hypersensitive to ABA (Hugouvieux et al., 2001; Papp et al., 2004). ABH1/CBP80 and CBP20 contribute to the regulation of AS and preferentially affect AS of the first intron, particularly at the 5′ splice site (Laubinger et al., 2008; Raczynska et al., 2010). The hnRNP-like At-GRP7 (for glycine-rich RNA binding protein 7) that is upregulated by cold and oxidative stress has also been associated with ABA responses (Carpenter et al., 1994; Cao et al., 2006; Kim et al., 2008; Schöning et al., 2008; Schmidt et al., 2010; Streitner et al., 2010). GRP7 regulates a number of AS events, in particular those involving alternative 5′ splice sites. A number of the target transcripts coimmunoprecipitate with GRP7 in plant extracts, suggesting that GRP7 regulates AS of these transcripts by direct binding in vivo (Streitner et al., 2012). The *sr45-1* mutant defective in the noncanonical SR45 protein (see above) is also hypersensitive to ABA (Carvalho et al., 2010). Collectively, this points to a prominent role for RNA processing including AS in ABA signal transduction.

AAPK-INTERACTING PROTEIN1 (AKIP1) is an RBP with homology to hnRNP A/B. Upon phosphorylation by ABA-activated protein kinase, AKIP binds to mRNAs encoding dehydrins (involved in stress response) and relocalizes to speckle-like domains (Li et al., 2002). Thus, AKIP may sequester mRNAs under stress. This illustrates how phosphorylation of the AKIP1 RBP may determine its specificity of binding to particular mRNAs and location in the cell in response to stress.

An interaction between AS and miRNA function has been observed for ABA-related regulation of miRNA846. miRNA846, together with miRNA842, originates from a pre-mRNA that undergoes AS (Jia and Rock, 2013). ABA reduces miR846 levels by changing the ratio of AS isoforms that either produce or do not produce miR846. Concomitantly, a predicted mRNA target of miR846, an ABA-inducible jacalin, accumulates.

The above examples show that many SFs and AS of numerous mRNAs can be controlled by different abiotic stress factors. Combinations of stresses, such as drought and heat, impact plant development more severely than a single cue. Thus, it will be important to investigate how combinatorial control at the level of AS serves to integrate the impact of different stress factors.

BIOTIC STRESS

AS of *Resistance* Genes

Resistance (R) proteins are crucial for plant defense against pathogens. They serve to survey virulence factors produced by the pathogens or their action in the plant cell and accordingly trigger defense responses. Aberrant high *R* gene expression is associated with fitness costs for the plant, and regulation at the RNA level via AS and small interfering RNAs appears to control *R* transcript levels (Figure 4) (Zhang et al., 2003; Mastrangelo et al., 2012; Staiger et al., 2013).

The tobacco (*Nicotiana tabacum*) *N* gene is a member of the Toll-Interleukin1 receptor homology region (TIR)–nucleotide binding site–leucine-rich repeat region (LRR) class of *R* genes and confers resistance to *Tobacco mosaic virus* (TMV) (Whitham et al., 1994). AS gives rise to two transcripts: the short N_S transcript encoding the functional N protein and the long N_L transcript (Figure 4A). N_L contains an alternative 70-nucleotide exon within the third intron that leads to a frame shift and a PTC (Dinesh-Kumar and Baker, 2000). Thus, N_L can encode a truncated protein that lacks most of the LRRs. N_S is prevalent before infection, whereas N_L takes over 4 to 8 h after TMV infection and is about 60-fold higher than N_S. When transgenic plants are engineered to express only N_S, they show little resistance against TMV, as do plants that express N_S and N_L equally (Dinesh-Kumar and Baker, 2000). Thus, the alternative exon in intron 3 is required for full resistance against TMV.

Similarly, *Arabidopsis* RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) that activates defense responses to avirulent pathogens expressing the cognate avirulence protein avrRps4 exists in several AS forms (Figure 4B) (Zhang and Gassmann, 2003, 2007). Upon inoculation with avirulent *Pseudomonas syringae* pv *tomato* (hereafter *Pto*) DC3000 (*avrRps4*), an AS form retaining intron 3 with a PTC strongly increases (Zhang and Gassmann, 2007). Transgenes that cannot produce this AS form do not complement the *rps4* mutant, showing that AS is required for RPS4 function. Also for RPS6, AS produces transcript isoforms that can encode protein variants containing only the TIR domain or a combination of the TIR and nucleotide binding site domain (Figure 4C) (Marone et al., 2013). AS of all of the above genes generates isoforms with PTCs that are potential substrates

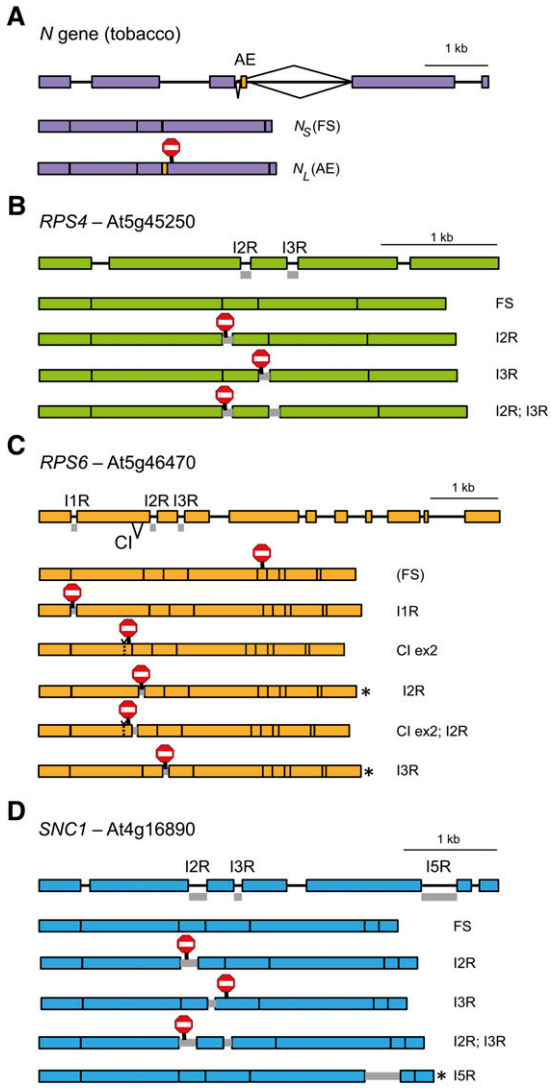

Figure 4. AS of *R* Genes during Pathogen Infection.

(A) Scheme of the TMV resistance gene *N* and the two AS isoforms N_S encoding the functional protein and N_L. Inclusion of a 70-nucleotide exon introduces a PTC, leading to a truncated protein variant, based on Dinesh-Kumar and Baker (2000).

(B) Scheme of *Arabidopsis RPS4* and AS isoforms, based on Zhang and Gassmann (2007). The *RPS4* transcript isoform, retaining both introns 2 and 3, was identified in a global transcriptome analysis by Marquez et al. (2012).

(C) Scheme of *Arabidopsis RPS6* and AS isoforms, based on Kim et al. (2009). The *RPS6* transcript isoform retaining intron 3 was identified by Marquez et al. (2012). Note also that The Arabidopsis Information Resource gene model has five exons in the 3′UTR, suggesting that this model (FS) is not in fact the fully spliced model.

(D) Scheme of *Arabidopsis SNC1* and AS isoforms, based on Xu et al. (2012b).

Note that all *SNC* transcript variants identified by Marquez et al. (2012) retain intron 5.

of NMD or could be translated into truncated proteins. Exactly how AS of *R* genes functions in enhanced disease resistance is still unknown, but it has been suggested that the truncated proteins may promote *R* gene function by alleviating self-inhibition of the intact protein (Zhang and Gassmann, 2003). Alternatively, the truncated proteins may interfere with downstream signaling. This has been observed in the mammalian innate immune system where truncated forms of NOD2 (for nucleotide binding oligomerization domain2), which senses the presence of components derived from bacterial peptidoglycan and activates NF-κB signaling, affect downstream signaling of intact NOD2 (Kramer et al., 2010). One AS isoform encodes a short NOD2 variant that interacts with intact NOD2 and attenuates the activation of the NF-κB transcription factor. Another AS isoform encodes a truncated protein variant that activates NF-κB signaling independent of the ligand but also competes with intact NOD2.

Factors involved in AS of *R* Genes and Plant Defense

Numerous mutants in predicted splicing components show an immunity-related phenotype, underscoring the importance of correct splicing for plant defense. A screen in *Arabidopsis* for suppressors of the *suppressor of npr1-1, constitutive1* (*snc1*) mutation that leads to constitutive activation of the TIR-nucleotide binding-LRR–type R protein SNC1, and resistance in the absence of pathogens identified several subunits of a splicing-associated protein complex. These included MODIFIER OF SNC1 4 (MOS4) that shows homology to human Breast Cancer-Amplified Sequence 2, the *Arabidopsis* CELL DIVISION CYCLE5 (CDC5) Myb-transcription factor, and the WD-40 repeat PLEIOTROPIC REGULATORY LOCUS1 (Palma et al., 2007). Notably, their counterparts in humans and yeast interact with one another and with Prp19 (for Precursor RNA Processing 19) in the core of the Nineteen Complex (so named for Prp19) that is essential for catalytic activation of the spliceosome (Hogg et al., 2010). A proteomics approach for proteins interacting with MOS4 subsequently identified two closely related proteins with sequence homology to Prp19, termed MAC3A (for MOS4-associated complex 3A) and MAC3B (Monaghan et al., 2009). Changes in AS of *SNC1* in the *mos4*, *cdc5*, and *mac3a mac3b* mutants provide compelling evidence that MAC mediates AS of a subset of *R* genes (Xu et al., 2012a). Another predicted RBP copurifying with MOS4, MAC5A, is also involved in pathogen defense (Monaghan et al., 2010). The human MAC5A counterpart is RBM22 (for RNA Binding Motif Protein22) that interacts with U6 snRNA and pre-mRNAs and participates in splicing. This suggests that MAC5A may also be involved in pre-mRNA splicing in *Arabidopsis* (Koncz et al., 2012; Rasche et al., 2012).

Another *Arabidopsis* protein associated with the MAC is MOS12, which shows homology to cyclin L in humans and harbors an atypical SR-rich domain presumably interacting with other SFs. In the *mos12-1* mutant, the *SNC1* and *RPS4* AS pattern and immune responses are impaired (Xu et al., 2012a). Furthermore, *mos12-1* decreases the intron retention splice isoforms of *SR1/SRp34* and *JAZ2* (see below), suggesting a specificity of MOS12 for splicing of distinct transcripts (Xu et al., 2012a).

Impaired AS of *SNC1* and *RPS4* as well as enhanced pathogen susceptibility is also observed in *mos14* mutants (Xu et al., 2012b). MOS14 shows homology to transportin-SR that mediates nuclear import of SR proteins in metazoa. Indeed, MOS14 interacts with RAN1 and SR proteins, suggesting that it may accomplish nuclear import of SR proteins that in turn contribute to AS of *SNC1* and *RPS4*. Interestingly, *mac3a3b*, *mos4*, *mos12*, and *mos14* mutants also show defects in RNA-directed DNA methylation and transcriptional silencing (Zhang et al., 2013).

AS is often linked to NMD of PTC-containing AS isoforms, resulting in changes of transcript levels (McGlincy and Smith, 2008; Kalyna et al., 2012). In *Arabidopsis*, mutants in the homologs of the NMD components UP FRAMESHIFT1 (UPF1), UPF2, UPF3, and SMG7 show a higher resistance to *P. syringae* infection (Jeong et al., 2012; Rayson et al., 2012; Riehs-Kearnan et al., 2012; Shi et al., 2012). Among genes misexpressed in the *upf1-5* and *upf3-1* mutants, genes connected to pathogen response are enriched. *UPF1* and *UPF3* mRNAs are themselves downregulated in response to *Pto* DC3000 (Jeong et al., 2012). One may envisage that PTC-containing transcripts encoding putative truncated *R* gene products are stabilized for defense by downregulation of the NMD pathway. Clearly, a more detailed analysis is required to disentangle causes and consequences of the phenotypes: whether the increased level of defense-associated transcripts is due to an inhibition of mRNA degradation or an indirect consequence of elevated salicylic acid (SA) level in the mutants (Jeong et al., 2012; Rayson et al., 2012; Riehs-Kearnan et al., 2012).

Among SFs with a role in biotic stress are At-GRP7 and the SR protein Ad-RSZ21 from *Arachis diogoi.* The RBP and SF At-GRP7, discussed above, is also involved in plant immunity, and ADP ribosylation of a conserved RNA binding Arg residue by a bacterial effector protein interferes with plant defense (Jeong et al., 2011; Nicaise et al., 2013). At-GRP7 has been shown to upregulate *PATHOGENESIS RELATED* transcripts associated with SA-dependent defense and downregulate transcripts associated with jasmonic acid (JA)/ethylene dependent defense, but a role in AS of defense-associated transcripts has not yet been described (Hackmann et al., 2013). Ad-RSZ21 is related to At-RSZ22 in *Arabidopsis* and has been implicated in hypersensitive response–like cell death and upregulation of defense-related transcripts (Kumar and Kirti, 2012).

Attenuation of JA signaling that is generally associated with defense against herbivores and necrotrophic pathogens is another aspect of plant defense that involves AS. In the absence of JA, JAZ (for JASMONATE ZIM-domain) proteins inhibit JA-responsive gene expression by sequestering MYC2 and other MYC2-related transcription factors. In most members of the *Arabidopsis* JAZ family, retention of an intron generates truncated protein variants that show reduced interaction with the JA receptor CORONATINE INSENSITIVE1 in the presence of the active JA-Ile conjugate and thus are resistant to proteosomal degradation (Chung et al., 2010; Moreno et al., 2013). The production of these dominant JAZ repressors is another example of an AS/small interfering peptide strategy and may reduce the negative consequences associated with inappropriate activation of the JA response pathway.

Finally, a comparative analysis of isochorismate synthase (ICS) converting chorismate into isochorismate in the shikimate pathway unveiled differential regulation of *ICS* in poplar (*Populus* spp.)

versus *Arabidopsis* (Yuan et al., 2009). The single-copy *ICS* gene in poplar is not responsive to stress but undergoes extensive AS. By contrast, *Arabidopsis* contains two highly similar *ICS* genes of which only *ICS1* is pathogen inducible for SA-mediated defense. Whereas AS is prevalent for the single-copy *ICS* genes in *Populus* and other species, *Arabidopsis* homologs appear to have lost this property following the duplication of the *ICS* genes. Thus, AS and gene duplication followed by differential regulation appear to represent different strategies to achieve the same regulatory potential (Yuan et al., 2009).

ENDOGENOUS DEVELOPMENTAL CUES

Organ-Specific AS Events

The AS pattern of many transcripts changes with developmental stages (Iida et al., 2004). Recently, tissue-specific AS was demonstrated for the auxin biosynthetic enzyme gene, *YUCCA4*, where AS leads to altered subcellular localization. A ubiquitously expressed *YUCCA4* transcript isoform encodes a protein that localizes to the cytoplasm, whereas a second flower-specific transcript isoform codes for a protein localized at the endoplasmic reticulum (ER) (Kriechbaumer et al., 2012). Subcellular relocalization regulated at the mRNA level has also been observed for *Arabidopsis* bZIP60, a transcription factor associated with the ER. In response to ER stress that is elicited by misfolded proteins, a 23-nucleotide intron is removed, leading to a frameshift, introduction of a PTC, and loss of the membrane anchoring domain (Nagashima et al., 2011). The smaller protein variant relocalizes to the nucleus and activates ER stress–inducible genes. However, the splicing event is unconventional, as it involves two cleavage reactions in two highly conserved loop regions of two adjacent stem-loop structures, removal of 23 nucleotides, and religation of the cleavage products.

An interesting study has shown that AS of a Major Facilitator Superfamily transporter, ZINC-INDUCED FACILITATOR-LIKE1 (ZIFL1) leads to two protein isoforms, both of which function through modulating H^+-coupled K^+ transport, but differ in tissue distribution and subcellular localization (Remy et al., 2013). The full-length ZIFL1.1 isoform is targeted to the vacuolar membrane of root cells. The ZIFL1.3 transcript arises from selection of an alternative 3′splice site located two nucleotides downstream of the authentic 3′splice site, causing a frameshift mutation and introduction of a PTC that leads to the loss of the two last C-terminal membrane-spanning domains and localization of the truncated protein to the plasma membrane of leaf stomatal guard cells. Differential complementation of the *zifl1* drought sensitivity and auxin-related defects shows that the full-length ZIFL1.1 protein influences cellular auxin efflux and polar auxin transport in roots, whereas the truncated ZIFL1.3 isoform regulates stomatal movement (Remy et al., 2013).

The maize (*Zea mays*) Viviparous1 (Vp1) transcription factor also is a major regulator of seed development through simultaneously activating embryo maturation and repressing germination. Hexaploid wheat (*Triticum aestivum*) varieties show weak embryo dormancy and are susceptible to preharvest sprouting, similar to maize *vp1* mutants. This has been attributed to misplicing of Vp-1 homoeologs (McKibbin et al., 2002).

Function of SFs in Development

Mutants defective in SR proteins or transgenic plants with ectopic SR expression show a range of morphological phenotypes (Lopato et al., 1999; Kalyna et al., 2003), underscoring the importance of AS for the correct realization of genetic information during development. A mutant defective in the atypical SR protein SR45 exhibits developmental abnormalities, including narrow leaves and petals, altered number of petals and stamens, and short roots (Ali et al., 2007). The use of alternative 3′splice sites generates two AS isoforms that differ by eight amino acids, and expression of individual isoforms differentially complement the defects of the *sr45-1* mutant in petal development or root growth (Zhang and Mount, 2009).

A number of mutants with altered gametic cell specification in the embryo sac, *lachesis* (*lis*), *clotho* (*clo*), and *atropos* (*ato*), encode splicing-associated proteins. Mutation in *LIS* that shows homology to the *S. cerevisiae* SF PRP4, an integral part of the U4/U6 complex, resulted in supernumerary egg cells implicating splicing in cell fate decisions (Gross-Hardt et al., 2007). CLO/GAMETOPHYTIC FACTOR1 (GFA1) encodes a homolog of Snu114p/U5-116kDa protein and physically interacts with At-Brr2 and At-Prp8, the putative U5 snRNP components of *Arabidopsis* (Moll et al., 2008; Liu et al., 2009). This suggests that CLO/GFA1 is involved in mRNA biogenesis through interaction with Brr2 and Prp8. ATO is the homolog of human SF3a60 and *S. cerevisiae* PRP9, which are required for the formation of the spliceosome (Moll et al., 2008). It will be revealing to define the splicing substrates affected by these mutations as a general splicing defect appears less likely, given that the mutants are viable.

In maize, ROUGH ENDPSPERM3 (RGH3) encodes a $U2AF^{35}$-related protein with a role in differentiation of cell types in the endosperm, endosperm–embryo interactions, and in embryo and seedling development. The RGH3 protein localizes to the nucleolus and to speckles in the nucleoplasm and colocalizes with $U2AF^{65}$. The *rgh3* mutant did not have a general splicing defect but affected AS of some transcripts and appeared to induce the use of noncanonical splice sites (Fouquet et al., 2011).

SUPPRESSOR OF *abi3-5* (SUA) is a novel SF in *Arabidopsis* that affects seed maturation by controlling AS of *ABSCISIC ACID INSENSITIVE3* (*ABI3*). ABI3 generates two AS isoforms: *ABI3*-α and *ABI3*-β, which encode full-length and truncated proteins, respectively (Sugliani et al., 2010). At the end of seed maturation, the *ABI3*-β transcript that lacks a 77-bp cryptic intron accumulates and probably contributes to a fast down-regulation of full-length ABI3 in ripe seeds (Sugliani et al., 2010). This AS event is repressed by SUA, a homolog of the human splicing regulator RBM5 that interacts with $U2AF^{65}$. SUA interacts with At-$U2AF^{65}$ and thus may be involved in spliceosome formation.

At-*SmD3-b* mutants defective in the D3 Sm core protein of snRNPs show pleiotropic phenotypes, including delayed flowering time, reduced root growth, partially defective leaf venation, abnormal numbers of trichome branches, and changed numbers of floral organs. Splicing of selected genes was impaired in the *smd3-b* mutant (Swaraz et al., 2011). The At-*smu2* mutant shows developmental phenotypes, including abnormal cotyledon numbers and higher seed weight, and is defective in a homolog of the

Caenorhabditis elegans splicing regulator SUPPRESSORS OF MEC-8 AND UNC-52 (SMU-2) and the human spliceosomal component RED (Spartz et al., 2004; Chung et al., 2009). Like *C. elegans smu-2* mutants, At-*smu2* mutants shows altered splicing of pre-mRNAs.

FLOWERING TIME

The switch from vegetative to reproductive development is a crucial decision for plants. Accordingly, the timing of floral transition is regulated by endogenous developmental cues and environmental signals, including daylength, ambient temperature, and vernalization (an extended cold period like winter) (Andrés and Coupland, 2012). Key transcriptional regulators in the flowering time network and their target transcripts are well described in *Arabidopsis*, and regulated protein stability and protein trafficking have been found to be crucial in flowering time control. Several lines of evidence now point to an important role of AS in the floral network.

Misexpression of several splicing regulators alters flowering time (Lopato et al., 1999; Ali et al., 2007; Streitner et al., 2010; Zhang et al., 2011). Similarly, when transcriptome changes were monitored in *Arabidopsis* plants transferred from 16 to 25°C, leading to accelerated flowering via the ambient temperature pathway, RNA processing–related factors were enriched (Balasubramanian et al., 2006; Balasubramanian and Weigel, 2006). Alteration of the AS profile under these conditions was found for MADS ASSOCIATED FLOWERING1 (MAF1)/FLOWERING LOCUS M (FLM) and MAF2, paralogs of the key floral repressor FLOWERING LOCUS C (FLC). *FLC* is downregulated by vernalization to allow flowering in spring. MAF2 has been implicated in distinguishing short cold periods to prevent precocious flowering under these conditions (Rosloski et al., 2013). Upon shorter cold treatment the accumulation of the AS *MAF2* var1, the transcript isoform predicted to generate the full-length protein (a repressor of flowering) is maintained, and the abundance of *MAF2* var2 transcript isoform (predicted to encode a truncated MAF2 protein) decreases. The functional significance of the PTC-containing MAF2 var2 isoform is not yet clear.

A differential impact of AS isoforms on flowering time has been shown for the MADS (for MCM1, AGAMOUS, DEFICIENS, SRF) box transcription factor SHORT VEGETATIVE PHASE (SVP). In silico analysis of MADS box MIKC-type transcription factors in *Arabidopsis* predicted protein isoforms that affect dimerization properties or higher order protein complex formation (Severing et al., 2012). The potential for AS to influence function was shown by the differential effects on flowering time and floral development of overexpression of isoforms of SVP, consistent with their different protein–protein interactions (Severing et al., 2012). Overexpression of the *SVP1* splice isoform leads to late flowering, as expected for a floral repressor, whereas overexpression of the *SVP3* splice isoform, which lacks a protein–protein interaction domain, did not.

Recently, a differential function of antagonistic splice isoforms in temperature-dependent flowering time control has been directly shown (Posé et al., 2013). At lower temperatures SVP interacts with the protein splice variant MAF1/FLM-β containing exon 2 of the MIKC protein interaction domain to repress flowering. At elevated temperatures, another isoform, FLM-δ, containing the alternative exon 3 instead takes over. The resulting SVP-FLM-δ complex acts as a dominant-negative inhibitor due to reduced DNA binding activity, ultimately resulting in accelerated flowering.

The floral integrator SUPPRESSOR OF CONSTANS OVEREXPRESSION1 (SOC1) undergoes AS, and *SOC1* AS isoforms are targeted to NMD by EARLY FLOWERING9 (ELF9), a protein with two RRMs most similar to the RRMs of *S. cerevisiae* CUS2 (Song et al., 2009). CUS2 is reported to be a SF that aids the assembly of U2 snRNPs. ELF9 has been implicated in NMD, as other NMD substrates besides SOC1 AS isoforms increase in abundance in the *elf9* mutant (Song et al., 2009).

sr45-1 delays flowering under both long days and short days and is rescued by vernalization. FLC, a key flowering repressor, is upregulated in *sr45-1*, demonstrating that SR45 influences the autonomous pathway (Ali et al., 2007). The *prmt5* mutant is also early flowering. PRMT5/SKB1 dissociates from the *FLC* promoter after high-salt and ABA treatment, and H4R3sme2 levels at the *FLC* promoter decrease correspondingly. This increases *FLC* expression and results in late flowering in the wild type upon salt stress (Zhang et al., 2011). Furthermore, the *FLOWERING LOCUS K* transcript, encoding an RBP of the autonomous pathway that promotes flowering by downregulating *FLC*, is mis-spliced in *prmt5* (Deng et al., 2010). Another late-flowering mutant is affected in AtPRP-39-1, a homolog of the *S. cerevisiae* U1 snRNP component PRP9 (Wang et al., 2007).

THE CIRCADIAN CLOCK

Plants use an endogenous timekeeper, the circadian clock (from Latin circa diem, about a day), to direct physiological processes to the appropriate time of the day (McClung, 2006). The clock regulates >30% of the transcriptome. The core clockwork consists of proteins that generate self-sustained oscillations by feedback on transcription of their own genes. These negative feedback loops are controlled by several different mechanisms, including protein phosphorylation, protein turnover, gene expression, and chromatin remodeling to maintain a 24-h period (Schöning and Staiger, 2005; Más, 2008; Herrero and Davis, 2012).

AS Events in the Circadian System

A comprehensive analysis on the ATH1 microarray unraveled clock regulation of large parts of the transcriptome (Harmer et al., 2000). An initial analysis of coding and noncoding regions on whole-genome tiling arrays detected rhythmically expressed introns (Hazen et al., 2009). In many cases, they were in phase with adjacent rhythmic exons, indicating production of a transcript isoform with a retained intron that would most likely lead to a truncated protein variant. On the other hand, some genes displayed rhythmic introns where exon expression was arrhythmic, suggesting circadian control of AS.

For the core clock gene encoding the Myb-type transcription factor CCA1, transcript isoforms retaining intron 4 were detected that increased upon exposure of the plants to high light and decreased at low temperatures (Filichkin et al., 2010). This AS

event is conserved in the monocot grasses *Brachypodium* and *Oryza*, and the dicot tree *Populus*, pointing to functional importance. Subsequently, extensive AS in the majority of the core clock genes in *Arabidopsis* was found, and dynamic changes in AS profiles were observed in response to changes in temperature (James et al., 2012a, 2012b). AS events were either induced or increased in abundance to 10 to 50% of the total transcripts at low temperatures. The majority of these events were nonproductive, resulting in a reduction of functional mRNAs and, thus, potentially impacting protein levels. For example, transcripts that retained the first intron in the LHY 5′UTR and/or included an alternative exon (E5a) upon cold treatment intron are turned over by NMD, thus reducing the level of functional LHY protein (James et al., 2012b). Furthermore, the partially redundant gene pairs *LHY* and *CCA1*, and *PRR7* and *PRR9* behaved differently with respect to AS, implying functional differences between them. Upon exposure to cold, AS of *LHY* and *PRR7* generated unproductive isoforms, while AS has little effect on *CCA1* and *PRR9*. Unproductive splice variants were also generated from *PRR5* and *TOC1* in the cold (James et al., 2012b).

It has recently been predicted that the *CCA1* transcript isoform retaining intron 4 can produce a protein that consists of the C-terminal dimerization domain without the N-terminal DNA binding MYB domain, designated CCA1β (Seo et al., 2012b). In a transgenic approach, CCA1β interferes with the formation of CCA1 and LHY dimers that are necessary for their repressive effect on transcription. Indeed, overexpression of CCA1β leads to a short period phenotype, as observed in *cca1 lhy* mutants, consistent with CCA1β acting as a dominant-negative inhibitor. While this is an interesting scenario, it remains to be demonstrated whether the CCA1β protein is made in planta as it would require ribosomes to ignore multiple translation start and stop codons before initiating translation at an AUG downstream of intron 4.

The importance of AS for correct clock function was underscored by the *prmt5* mutant showing long-period leaf movement and gene expression rhythms (Hong et al., 2010; Sanchez et al., 2010). AS of the clock gene *PRR9* is affected by loss of PRMT5. Wild-type plants have two readily detectable *PRR9* splice forms that oscillate slightly out of phase: a mature mRNA and an AS form with eight additional nucleotides at the end of exon 2 that is an NMD substrate but potentially encodes an N-terminally truncated PRR9 protein. In the *prmt5* mutant, transcripts that retain intron 3 predominate. The mRNA encoding the full-length protein is barely detectable, suggesting that the circadian defect in the *prmt5* mutants is caused by changes in *PRR9* splicing. Similarly, a mutation within the putative RBP SPLICEOSOMAL TIMEKEEPER LOCUS1 (STIPL1) induces a long period (Jones et al., 2012). STIPL1 is a homolog of the spliceosomal proteins TFP11 in humans and Ntr1p in *S. cerevisiae* involved in spliceosome disassembly. The *stipl1* mutation reduces the splicing efficiency of numerous introns and alters the accumulation of circadian transcripts including increased levels of the intron 3 retained variant of *PRR9* (Jones et al., 2012).

A mutation of the SNW/Ski-interacting protein (SKIP) domain protein SKIP has also been shown to lengthen the circadian period in a temperature-sensitive manner and affect light input to the clock (Wang et al., 2012). SKIP physically interacts with SR45 and associates with *PRR7* and *PRR9* pre-mRNAs. In the *skip-1* mutant, unproductive AS variants of *PRR7* and to a lesser extent of *PRR9* increase at the expense of fully spliced mRNAs, which partly accounts for the long period phenotype. Human SKIP interacts with $U2AF^{65}$, whereas the *S. cerevisiae* and *S. pompe* SKIP homolog Prp45 is a component of the Nineteen Complex, and At-SKIP has similarly been shown to be involved in AS of many genes (Wang et al., 2012). Previously, At-*SKIP* expression was found to increase in response to salt, mannitol, and ABA treatment, and At-SKIP overexpression or antisense lines show altered tolerance to a suite of abiotic stress factors (Lim et al., 2010), and it is likely that a role in AS contributes to these phenotypes.

AS of Clock Output Genes

The transcript encoding RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE ACTIVASE undergoes circadian oscillations in steady state abundance and AS (Sanchez et al., 2010). A long transcript isoform codes for a protein whose activity is regulated by light intensity, whereas the activity of the protein encoded by the short transcript isoform is light independent (Zhang et al., 2002). AS of the mRNA isoform that encodes the light-regulated protein increases during the day (Sanchez et al., 2010).

In addition to roles discussed above, the RBP At-GRP7 is part of a negative feedback loop controlled by the circadian clock (Schmal et al., 2013). At-GRP7 negatively autoregulates via AS: Elevated levels promote the use of a cryptic 5′splice site in the intron, leading to a switch to a PTC-containing transcript isoform that rapidly decays via NMD. At-GRP7 also affects AS of numerous downstream targets some of which are rhythmic themselves (Streitner et al., 2012).

NATURAL VARIATION

A major factor contributing to specialization of ecotypes in their growth habitats is the ability to cope with environmental conditions. Given the widespread nature of AS and its pervasive effect on plant stress responses and performance, one may expect global alterations in AS profiles in different plant ecotypes. Sequencing the genomes and transcriptomes of geographically and phenotypically diverse *Arabidopsis* ecotypes and association with phenotypes (Gan et al., 2011) provides a basis for examining the diversity in AS of specific genes and pathways and its effects on adaptation. Single nucleotide polymorphisms (SNPs) are found once every 200 bp for different *Arabidopsis* accessions (Ossowski et al., 2008), and small variations in sequence can influence splicing efficiency and splice site choice. Although not a natural mutant, *apetala3* (*ap3*) and suppressor mutants illustrate the effects of single-nucleotide changes on splicing, which reflect the interplay between strengths of different splicing signals. In the weak *ap3-1* mutant, petals and stamens are partially converted to sepals or carpels, respectively. *ap3-1* contains a point mutation near the 3′end of exon 5 at position −2 relative to the 5′splice site of intron 5, leading to skipping of exon 5 and a nonfunctional AP3 protein (Yi and Jack, 1998). Skipping of exon 5 is corrected in a suppressor mutant with wild-type-like flowers, *ap3-11*, which has a mutation in intron 4. This creates

a novel branch point sequence allowing exon 5 to once more be spliced into the mRNA (Figure 3E).

In the recent comprehensive study based on 18 *Arabidopsis* accessions, nearly 50% of the expressed genes varied between ecotypes (Gan et al., 2011). Extensive SNP and indel variation was found among the genotypes, and when compared with Columbia-0 (Col-0;TAIR10), one-third of protein-coding genes were disrupted/altered in at least one accession. Sequence variation affected translation start and stop sites, introduced PTCs or changed the frame of the coding sequence, or potentially generated protein isoforms in different accessions. Of 2572 genes with disrupted splice sites when compared with TAIR10, nearly two-thirds had new splice sites, and in a quarter, these sites were close to the splice sites in Col-0, showing mutations able to correct splicing defects caused by another mutation (Gan et al., 2011). This analysis concentrated on splice site mutations. Other mutations could affect branch point sequences, polypyrimidine tracts, or UA-rich sequences as well as splicing enhancer and suppressor sequences and binding sites for the range of SFs. Generation of robust quantitative data on AS among ecotypes may aid the identification of key splicing regulatory elements and the position of binding sites. Quantitative differences in AS between Col and C24 using a limited number of genes/AS events revealed that 28% of the AS events showed significant changes between the two ecotypes, whereas more than 70% were not affected (Streitner et al., 2012).

Natural variation in Pro accumulation among accessions of *Arabidopsis* have been associated with variation in expression of Δ1-pyrroline-5-carboxylate synthetase1 gene due to relative levels of functional and nonfunctional AS isoforms (Kesari et al., 2012). The AS phenotypes reflect small sequence variation in the introns flanking a skipped exon. Similarly, variation in the relative levels of functional and nonfunctional AS variants of a polygalacturonase gene underlie differences in fruit ripening among strawberry cultivars (Figure 5) (Villarreal et al., 2008). Finally, in *Arabidopsis*, the single C-function floral organ identity gene, *AGAMOUS*, specifies male and female organ development. Snapdragon (*Antirrhinum majus*) contains two C-function genes: *PLENA* and *FARINELLI* (*FAR*), and following duplication, *FAR* has generated a NAGNAG sequence (containing two potential 3′ splice sites) at the 3′splice site of intron 5 resulting in inclusion of a single Glu that affects protein–protein interactions such that *FAR* only specifies male organs (Airoldi and Davies, 2012). Thus, subtle qualitative and quantitative variation in splicing of genes in different plant cultivars and species can contribute to major developmental and physiological differences.

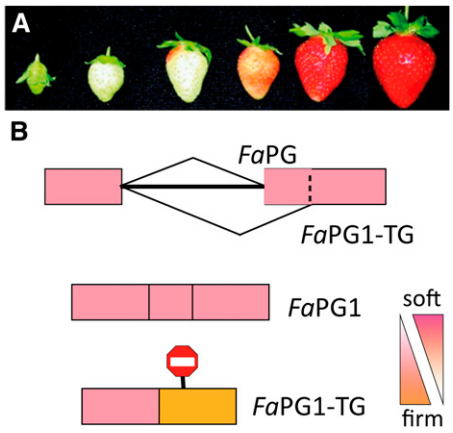

Figure 5. Natural Variation in AS.

(A) Fruit ripening of strawberry (*Fragaria* × *ananassa*).
(B) AS of *Fragaria* × *ananassa* polygalacturonidase (*Fa*PG) causes a frame shift and a PTC, leading to a nonfunctional protein variant. In soft varieties, the AS isoform Fa-*PG1* encoding functional polygalacturonidase predominates, whereas in firm varieties, the AS isoform Fa-*PG1-TG* corresponding to the truncated, nonfunctional protein predominates (Villarreal et al., 2008).

CONCLUSIONS AND PERSPECTIVES

The number of genes known to undergo AS continues to increase such that the majority of intron-containing genes are likely to be alternatively spliced. Here, we illustrate the increasing evidence for AS of plant genes with functional relevance during stress responses and development and in circadian timekeeping, illustrating the importance of identifying more factors involved in AS decisions. Clearly, gene expression reflects the balance of transcription, AS, and transcript stability (e.g., AS/NMD), and it is therefore necessary that AS information is integrated with transcriptional data. For example, microarray expression analyses for the most part only report on transcript levels and do not distinguish between functional and nonfunctional AS isoforms. The latter can represent significant portions of the transcripts of a gene giving misleading information when extrapolated to protein expression and missing important components of a cell's regulatory potential. An important consideration is the response of AS to various stimuli and that AS of different genes show distinct dynamic behavior (James et al., 2012a and b). Deep sequencing by RNA-seq of time courses of development or stress response can now monitor the dynamic AS changes in detail and integrate these with transcriptional responses.

Studies on the impact of a small number of SFs on the transcriptome have mainly been performed under normal growth conditions with little variation in conditions (Simpson et al., 2008; Raczynska et al., 2010; Rühl et al., 2012; Streitner et al., 2012). In the future, it will be important to identify SFs and their gene targets systematically and to understand how dynamic changes in AS during stress contribute to both short-term responses and long-term adjustment or acclimation. Furthermore, plants often, or even always, experience different stress conditions at the same time: How does the plant integrate different signals to generate a survival expression profile?

The emerging evidence of the interrelation between chromatin status, transcriptional regulation, and AS (Sen and Fugmann, 2012) is an area that is also attracting attention in plants. The histone code is elucidated at a genome level and must be correlated with expression/AS levels during stress responses. The challenge will be to address how epigenetic regulation determines AS (Luco et al., 2011).

We are beginning to gain a better idea of the potential of AS to control transcript and expression levels, such as through NMD,

but we still know virtually nothing about what happens at the protein level. For example, transcripts that contain PTCs have the potential to be translated into truncated proteins, but how many actually produce such proteins? Parallel RNA-seq and proteomic approaches will begin to address the consequences of AS at the protein level. On a practical note, increasing identification of AS forms by RNA-seq will expand the current in silico peptide mass databases and thereby improve protein detection.

Finally, the subtlety of AS regulation to fine-tune expression must have contributed in a major way to plant adaptation and evolution (Villarreal et al., 2008; Zhang and Mount, 2009; Airoldi and Davies, 2012; Kesari et al., 2012). The wealth of information on mutations in *Arabidopsis* genes illustrates how small sequence changes can have a major impact on splicing and expression, and the same principles will apply to natural sequence variation. Sequence changes in splicing signals including binding sites of SFs can generate new AS events or quantitative changes in functional mRNAs on which selection can operate. Correlation of transcriptomic profiles (transcript levels and AS) with the distribution of SNPs and the phenotypes and fitness of different accessions will provide an integrated view of expression variation and help to determine how AS contributes to plant performance.

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