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Review Article

Decoding co-/post-transcriptional complexities of plant transcriptomes and epitranscriptome using next-generation sequencing technologies

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Next-generation sequencing (NGS) technologies - Illumina RNA-seq, Pacific Biosciences isoform sequencing (PacBio Iso-seq), and Oxford Nanopore direct RNA sequencing (DRS) - have revealed the complexity of plant transcriptomes and their regulation at the co-/post-transcriptional level. Global analysis of mature mRNAs, transcripts from nuclear run-on assays, and nascent chromatin-bound mRNAs using short as well as full-length and single-molecule DRS reads have uncovered potential roles of different forms of RNA polymerase II during the transcription process, and the extent of co-transcriptional pre-mRNA splicing and polyadenylation. These tools have also allowed mapping of transcriptome-wide start sites in cap-containing RNAs, poly(A) site choice, poly(A) tail length, and RNA base modifications. The emerging theme from recent studies is that reprogramming of gene expression in response to developmental cues and stresses at the co-/post-transcriptional level likely plays a crucial role in eliciting appropriate responses for optimal growth and plant survival under adverse conditions. Although the mechanisms by which developmental cues and different stresses regulate co-/post-transcriptional splicing are largely unknown, a few recent studies indicate that the external cues target spliceosomal and splicing regulatory proteins to modulate alternative splicing. In this review, we provide an overview of recent discoveries on the dynamics and complexities of plant transcriptomes, mechanistic insights into splicing regulation, and discuss critical gaps in co-/post-transcriptional research that need to be addressed using diverse genomic and biochemical approaches.

Introduction

Environmental factors such as light (intensity, quality and duration), temperature variation, soil water content, and nutrients are key determinants of all aspects of plant growth, development, and ultimately crop productivity. Suboptimal conditions of one or more of these abiotic stresses or biotic stresses such as bacterial, viral, fungal and oomycete pathogens, and insects severely impair plant growth resulting in significant crop losses annually. Plants perceive variable and adverse environmental conditions through receptors and/or via altering hormonal levels that interface with many integrated signaling networks to rapidly alter their cellular processes in the short-term and growth and developmental patterns in the long-term to adapt and survive under unfavorable conditions [1, 2]. One of the key adaptive changes in response to these cues is the transcriptional reprogramming of gene expression, where the perception of stresses activates signaling networks that converge on transcriptional activation and repression of specific genes [2, 3]. Extensive global transcriptomic studies in plants suggest that co-/post-transcriptional regulation of gene expression in response to stresses and developmental

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cues is a key player in fine-tuning gene expression at the mRNA and protein levels [4, 5]. The key co-/post-transcriptional processes that modulate gene expression at the RNA level are alternative splicing (AS), alternative polyadenylation (APA), and RNA modifications (epitranscriptome), which produce structurally and functionally distinct alternative transcripts. The transcript variants fine-tune gene expression in profound ways by affecting the transport, stability, localization, and/or translatability of mRNAs to produce functionally distinct proteins and increasing proteome complexity [6–10]. Here, we review recent advances in understanding the complexities of plant transcriptomes at the co-/post-transcriptional level using next-generation sequencing (NGS) technologies and discuss the latest studies that are beginning to elucidate the mechanisms through which light and stress signals regulate splicing.

NGS platforms for profiling plant transcriptomes

Accurate reconstruction of all alternative transcripts and estimation of the relative abundance of individual transcript variants are pre-requisites for a comprehensive analysis of transcriptomes, and to decipher the biological functions of individual transcripts. Application of NGS technologies, especially Illumina, PacBio, and Oxford Nanopore Technologies (ONT), has helped immensely in uncovering plant transcriptome complexity.

High-throughput RNA-sequencing (RNA-seq) using Illumina short-read sequencing has been widely used to quantify the steady-state levels of RNAs genome-wide (Figure 1A). This sequencing platform and specialized versions of RNA-seq such as poly(A) tag sequencing have also contributed significantly to transcriptome-wide quantification of AS events (Supplementary Table S1), polyadenylation sites and non-coding RNAs in many plants [4, 11, 12]. However, Illumina-based short-read sequencing has several limitations especially in reconstructing full-length alternative transcripts and in detecting RNA modifications, which are lost during double-stranded cDNA synthesis. Assembly of full-length transcripts from short reads requires complex bioinformatics analyses and the available tools do not accurately reveal full-length splice variants [13, 14]. The PacBio (Figure 1B) and ONT (Figure 1C) platforms that can sequence single molecules of nucleic acids and yield very long reads of cDNAs or RNAs, address these limitations. Hence, these new sequencing technologies are being increasingly used in recent years to analyze plant transcriptomes. During the last four years, in *Arabidopsis*, maize, sorghum, rice, and many other plants, splice isoform sequencing (PacBio Iso-seq) has allowed accurate detection of full-length transcript variants. These studies have uncovered numerous novel transcript isoforms, protein-coding and non-coding genes and alternative polyadenylation sites, and greatly improved annotation of plant transcriptomes [15–18] (see Supplementary Table S1). Unlike all other sequencing technologies, the ONT platform is not dependent on DNA synthesis. Base-calling with the ONT platform relies on the measurement of disruptions in the current intensity as the RNA or DNA molecule passes through the nanopore (Figure 1C). Up to ∼13 kb long sequence of individual plant RNAs could be obtained with ONT-based DRS [19]. The DRS of nascent RNA molecules can be used to interrogate many aspects of co-/post-transcriptional events including alternative transcription start sites, co-transcriptional pre-mRNA splicing, alternative polyadenylation, poly(A) length, the potential interdependency of different co-transcriptional events, and RNA modifications. Recent studies this year in plants have demonstrated the utility of DRS for sequencing plant RNAs in addressing some of these questions [19–21] (see below). DRS technology is currently hindered by the high error rates in base-calling, which is more than the error rate of nanopore DNA sequencing, and the lack of mature tools for identifying different base modifications in RNA [22, 23]. Furthermore, it is expensive to perform DRS. Improvements in tools that can increase the accuracy in base calling and detection of different base modifications in RNA are needed for wider adoption of this technology.

Co-/post-transcriptional processing of pre-mRNAs

Quantification and characterization of nascent transcripts, and accurate mapping of transcription start sites (TSS) were achieved by employing global nuclear run-on sequencing (GRO-seq) and 5′GRO-seq (capped RNA sequencing), respectively [24]. In this study, 3′ pausing of RNA polymerase was found to be unique to plant transcription. Zhu et al. [25] have analyzed the elongation dynamics of RNA polymerase II (Pol II) *in planta* during the transcription process. They have used native elongating transcript sequencing (NET-seq) of differentially phosphorylated Pol II-nascent RNA complexes followed by the high-throughput sequencing of the associated nascent RNA, and GRO-seq to characterize nascent RNAs genome-wide [25]. This study has revealed that Pol II promoter-proximal pausing is a common feature in *Arabidopsis*. Unlike in animals, *Arabidopsis* Pol II showed a broad peak in the promoter-proximal region, suggesting that Pol II pausing and release in plants is not tightly regulated. However, this needs to be verified by other approaches. In non-plant systems, the

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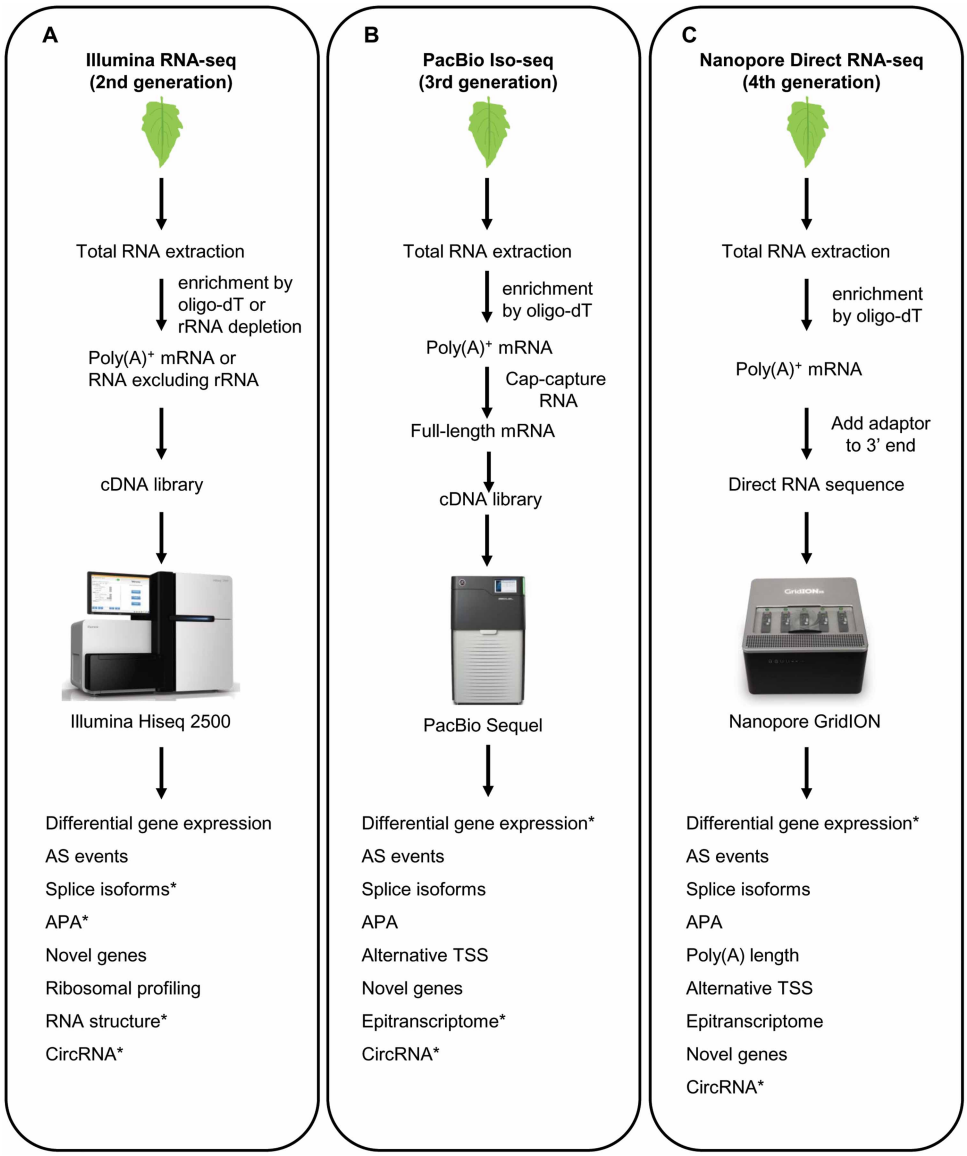

Figure 1. Schematic illustration of widely used NGS platforms used to study co-/post-transcriptional gene regulation.
(**A**) Schematic illustration of Illumina RNA-seq. Poly(A)$^+$ mRNA or RNA excluding rRNA is used to make cDNA libraries, which are then sequenced using the Illumina HiSeq platform. The RNA-seq reads are then analyzed for differential gene expression, AS events, splice isoforms, alternative polyadenylation (APA) sites, translating RNAs (Ribo-seq), circular RNAs, novel genes and non-coding RNAs. The picture of Illumina Hiseq 2500 is from https://www.illumina.com/systems/sequencing-platforms/hiseq-2500.html. (**B**) Schematic illustration of PacBio Iso-seq. Full-length mRNAs enriched by isolating Poly(A)$^+$ RNA using oligo-dT followed by cap-capture are used for preparing cDNA libraries, which are then sequenced using the PacBio platform. The RNA-seq reads are then used to analyze full-length splice isoforms, APA, alternative TSS, circular RNAs, and novel genes. The picture of PacBio Sequel is from https://www.dnalinkseqlab.com/pacbio-sequel-rsii/. (**C**) Schematic illustration of Nanopore direct RNA-seq (DRS). Poly(A)$^+$ mRNA from total RNA is isolated, then a sequencing adaptor is added to the 3′ end of poly(A)$^+$ mRNA and sequenced using the nanopore platform. The RNA-seq reads are then used to analyze differential gene expression, AS events, splice isoforms, APA, Poly(A)$^+$ length, alternative TSS, epitranscriptome, and novel chimeric genes. The picture of Nanopore GridION is downloaded from https://nanoporetech.com/products/gridion#. The asterisks indicate that the corresponding platform is either not widely used for that purpose or requires some additional steps or variations in library preparations.

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carboxyl-terminal domain (CTD) of the largest Pol II subunit regulates different steps of transcription depending on its phosphorylation status of heptapeptide (Tyr 1-Ser 2-Pro 3-Thr 4-Ser 5-Pro 6-Ser 7) repeats. In plants, Pol II with an unphosphorylated CTD mainly accumulates downstream of the TSS, while Pol II with a Ser 5P CTD associates with spliceosomes, and Pol II with a Ser 2P CTD pauses immediately downstream of the polyadenylation site (PAS), suggesting different phosphorylated forms of Pol II regulate initiation, splicing/elongation, and transcription termination and polyadenylation, respectively [25]. Differential elongation speed across exons and introns and accumulation of Pol II with a Ser 5P at 5′ splice sites (5′SS) appear to play a role in splice site recognition and 5′SS cleavage during elongation [25]. Analysis of mRNA stability using nascent 5-ethynyl uridine (5-EU)-labeled RNA sequencing (Neu-seq) and 5-EU immunoprecipitation chase-seq (ERIC-seq) has shown that plant RNAs have shorter half-lives than previous estimates based on inhibitors of transcription and that polyadenylated RNAs exhibit low stability [26].

Recently, two groups [27, 28] have sequenced chromatin-bound nascent RNAs in *Arabidopsis* using the Illumina platform to assess the extent of co-transcriptional splicing. Both studies have revealed that splicing of most pre-mRNAs occurs co-transcriptionally. Furthermore, a large fraction of alternatively spliced transcripts was also spliced co-transcriptionally, indicating that the decisions about alternative splice site choice are mostly made co-transcriptionally [27]. The number and position of introns were found to be the key determinants of co-transcriptional splicing. Genes with many introns were efficiently spliced whereas those with fewer introns, introns at the 3′end, and alternative introns were less efficiently spliced co-transcriptionally [28]. The efficiency of co-transcriptional splicing was positively associated with gene expression level [27] whereas non-coding RNAs splicing was not as efficient as protein-coding RNAs [28]. The MOS4-Associated Complex (MAC) in *Arabidopsis*, a counterpart of human NineTeen Complex (NTC) involved in splicing, contains MAC3A, MAC3B, MAC7, PLEIOTROPIC REGULATORY LOCUS1 (PRL1), PRL2 and other factors, and promotes intron splicing [29]. Analysis of co-transcriptional splicing in two double mutants (*mac3a mac3b* and *prl1prl2*) of MAC components and a double mutant, *rz-1b rz-1c*, of heterogeneous nuclear ribonucleoprotein [hnRNP]-like proteins, RZ-1B and RZ-1C revealed impaired splicing of nascent RNAs, suggesting that these proteins promote splicing at the chromatin level [27, 28]. The direct association of RZ-1C with nascent RNAs further supports that it promotes co-transcriptional splicing [27]. In another study, chromatin-bound RNA from *Arabidopsis* was converted into double-stranded cDNA and sequenced using ONT to analyze nascent RNAs splicing status, Pol-II position, and polyadenylation globally [30]. Unlike the previous two studies that used Illumina short-read sequencing, in this study the nanopore method was used to sequence cDNAs [30], which has the advantage of detecting poly(A)-tail length and the splicing order of introns. This study also confirmed co-transcriptional splicing of pre-mRNAs and shown that splicing of introns generally follows their ordering in the gene whereas in humans intron splicing does not always follow the order of transcription [30, 31]. Interestingly, ∼30% of chromatin-bound full-length RNA molecules in *Arabidopsis* are polyadenylated with unspliced introns at specific positions, suggesting that some incompletely spliced transcripts are retained on the chromatin and full-splicing of these may be regulated, which could be an important regulatory step in the maturation of certain mRNAs [30]. Whether developmental cues and/or stresses regulate the splicing of these chromatin-bound intron-containing RNAs remains to be seen. The observed chromatin-bound intron-containing poly(A) RNAs could be still in the process of polyadenylation. Alternatively, removal of one or more remaining introns may be coupled to completion of the polyadenylation process or release of mRNA may depend on the splicing of retained introns [30]. Whether there is any causal relationship between splicing and polyadenylation in plants needs further investigation. With nanopore-based DRS and improved methods of data analysis that can determine the correlation between specific AS events with polyadenylation events, it should be possible to elucidate the relationship between APA and AS in plants.

More recently, DRS of mature RNAs [19, 20] from *Arabidopsis* using the ONT has been used to investigate transcriptome-wide start sites in cap-containing RNAs, splicing events, poly(A) site choice, and poly(A) tail length in RNAs. Parker et al. [19] performed DRS with mature RNAs from two-week-old seedlings. The DRS studies have revealed a much greater complexity of splicing isoforms as compared with annotations based on short-read data. Although *Arabidopsis* transcripts are well annotated as compared to other plants, with DRS about 8700 unique splicing variants were observed that were not present in either TAIR10 or AtRTD2 [19]. DRS has revealed that the median length of the poly(A) in *Arabidopsis* is 68 nts with most (95%) are in the range of 13–200 nts and the poly(A) length negatively correlates with gene expression. A previous report using poly(A)-tail profiling by sequencing (PAL-seq) reported a median length of 51 nucleotides in *Arabidopsis* leaves [32]. The difference in the median length in these two reports could be due to the technologies used or

variation associated with the developmental stages of tissues. The DRS using mature RNA from 14-day-old seedlings and unopened flower buds [20] has uncovered over 38 500 potential novel transcript isoforms including many fusion transcripts generated from two adjacent genes that are not currently annotated in the Araport11 database. The vast number of the putative novel transcripts identified in this work need to be further validated using other methods to confirm the authenticity of these isoforms.

Chromatin architecture regulates co-/post-transcriptional processing

As described above, several studies this year have established co-transcriptional processing of mRNAs, including splicing, AS, and polyadenylation in *Arabidopsis*. Emerging studies are providing evidence in support of multiple regulatory mechanisms including the chromatin state (open vs closed chromatin, epigenetic modifications including histone modifications and DNA methylation), and the speed of transcription as key regulators that determine the outcome of AS in plants [4]. In rice, analysis of AS in wild type and a mutant (*OsMet1-2*) with impaired DNA methylation influenced all types of AS events [33]. Overall, about 7% of alternative splice junctions were affected due to reduced methylation in the mutant, suggesting that DNA methylation controls AS of some pre-mRNAs [33]. A mutant (knockdown of *SDG725*) with reduced histone H3 lysine 36 (H3K36)-specific methyltransferase in rice showed increased intron retention in the 5′ region and promoted intron excision in the 3′ region of transcripts [34]. In *Arabidopsis* and rice, open chromatin, which is impacted by the epigenetic state of chromatin, was found to be associated with intron retention [35]. This could be due to either faster transcription that does not allow the spliceosome to recognize and excise introns and/or differential recruitment of proteins to open chromatin that inhibit splicing [35].

Several studies have shown that light regulates AS of numerous plant genes including those that are involved in pre-mRNA splicing, chloroplast retrograde signaling, and phytochrome-mediated light responses [36, 37]. It was recently shown that light-regulated AS of pre-mRNAs encoding two splicing factors is controlled by the rate of pol II elongation during transcription [38, 39]. Slower transcription elongation in the dark allowed recognition of weaker splice sites whereas faster elongation resulted in skipping of weaker sites, resulting in changes in the ratio of splice isoforms in response to light [38]. However, how light regulates Pol II elongation is not known. Interestingly, in *Arabidopsis* a point mutation in Pol II that is expected to slow its elongation speed is lethal whereas another point mutation in Pol II that increased its elongation speed resulted in read-through transcription of genes, increased splicing, indicating the importance of Pol II elongation dynamics in splicing regulation [39]. In another study, high levels of epigenetic modifications (H3K4me3 or H3K9ac) that cause higher transcription elongation rate resulted in lower co-transcriptional splicing efficiency, while levels of H3K27me3 did not affect co-transcriptional splicing, suggesting that epigenetic state regulates splicing [27]. Collectively, these studies indicate that the epigenetic state of chromatin and the dynamics of transcription modulate AS in plants. However, it remains to be seen what the optimum elongation is speed to promote constitutive splicing and how chromatin context and environmental factors modulate the splicing process.

Although stresses profoundly change splicing patterns (Supplementary Table S1) it is not known how they regulate AS. Numerous studies have demonstrated the involvement of epigenetic changes in transcriptional regulation in response to abiotic stresses [40]. Since AS occurs mostly co-transcriptionally, it is likely that stresses alter chromatin architecture through epigenetic changes and/or dynamics of the transcription process to modulate AS. Accumulating evidence lends support to this hypothesis. For example, an increase in temperature from 16°C to 25°C promotes flowering in *Arabidopsis*. By analyzing AS in plants moved from 16°C to 25°C, Pajoro et. al. [41] have shown that this shift in temperature promotes changes in AS and histone 3 lysine 36 trimethylation (H3K36me3) is a key regulator of temperature-induced AS. Analysis of dynamics of nascent RNA Pol II transcription in plants subjected to cold revealed that low temperature influences RNA Pol II elongation kinetics and reduces co-transcriptional splicing [42]. The silencing of a chromatin modifier in maize resulted in changes in nucleosome occupancy, Pol II elongation speed and altered splicing patterns of a set of genes in response to osmotic stress [43]. In *Arabidopsis*, jasmonate (JA)-induced production of splice isoforms of JASMONOATE ZIM-DOMAIN (JAZ) transcriptional repressors is regulated by MED25, a subunit of mediator complex that is essential for Pol II-dependent transcription, through its interaction with two subunits of U1 small nuclear ribonucleoprotein particle (U1SnRNP). This suggests that AS of JA-induced JAZ transcripts is regulated co-transcriptionally where MED25 couples transcription and splicing [44].

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PP4R3, a regulatory subunit of protein phosphatase 4 complex, is a highly conserved Ser/Thr-specific phosphoprotein phosphatase in eukaryotes. It associates with chromatin and is required for Pol II occupancy on some promoters. Global splicing defects are observed in *pp4r3a-1* and *pp4r3a-2* mutants of *Arabidopsis*. PP4R3 appears to promote intron excision in some pre-mRNAs, as loss of this regulatory subunit increases IR [45]. The phosphorylation status of many spliceosomal proteins and regulatory splicing factors is known to play an important role in pre-mRNA splicing [46]. Hence, this phosphatase could be one of the components of stress-mediated signaling pathways that modulate co-transcriptional AS. Future studies aimed at comparison of co-transcriptional splicing changes using DRS of chromatin-bound RNA under normal and stress conditions should provide insights into the extent of stress-regulated splicing at the co-transcriptional level. Similar studies with chromatin-bound mRNAs that are associated with different epigenetic and epitranscriptomic modifications in plants subjected to different stress conditions should help elucidate the role of individual epigenetic changes in co-transcriptional splicing, AS and stress-modulated co-transcriptional processes.

The epitranscriptome of linear transcripts and circular RNAs

Co-/post-transcriptional reversible chemical modifications of bases in mRNAs (epitranscriptome) play a crucial role in regulating many fundamental processes including AS of pre-mRNAs, polyadenylation, nuclear export, mRNA stability and localization, RNA secondary structure, and translation efficiency, as well as many aspects of plant and animal growth and development [47–55]. Methods for detecting base modifications globally use RNA immunoprecipitation (RIP) with antibodies specific to base modifications such as N6-methyladenosine (m^6A) and 5-methylcytosine (m^5C), N1-methyladenosine (m^1A) followed by high-throughput sequencing (meRIP-seq/m6A-seq/m5C-seq/m^1A-seq) [47, 56–58]. However, antibody-based methods for detecting modified bases have several limitations [59] including the need for highly specific antibodies for each modified base, laborious RNA immunoprecipitation protocols, and in most cases, this method does not provide the exact location of modified bases [14, 47]. m^6A occurs in the consensus RRACH (R = purine, H = A/C/U) motif in the RNA sequence. An antibody-independent method called m^6A-REF-seq [60] or MAZTER-seq [61, 62], which involves digestion of m^6A sites in the ACA motif, has been used to profile m^6A modification in an ACA context at single-nucleotide resolution in animals. In addition to these, other antibody free methods such as DART-seq [63] in animals and m^6A-SEAL in animals and plants [64] have been used.

Recently, single-molecule sequencing is being increasingly used to detect base modifications. The ONT-based DRS has the potential to identify any base modifications, eliminates the need for antibodies and removes inherent biases in PCR amplification in preparing me-RIP libraries and allows identification of modified bases at a single-base resolution [14]. However, there are only a few base-calling methods available to accurately detect modified bases. Currently, *EpiNano* [22], MINES (m^6A Identification using Nanopore Sequencing) [65], ELIGOs (epitranscriptional landscape inferring from glitches of ONT signals) [66], and Nanocompore [67] can profile m^6A sites based on DRS reads. In *EpiNano*, which was developed using support vector machines (SVM), the detection of modified bases relies on base-calling errors around the modified RNA base due to decreased base-calling qualities [22]. This tool can identify m^6A bases in DRS reads with about 90% accuracy. MINES, a random forest classifier developed on empirical methylation data within the DRACH (D = A/G/T, R = purine, H = A/C/U) motif, had about 80% accuracy in predicting m^6A in this motif [65]. A workflow called 'MasterOfPores' that incorporated *EpiNano* was recently developed using the NextFlow framework to detect m^6A base modifications and poly(A) tail length from DRS reads reproducibly [68]. ELIGOS uses the percent error of specific bases (%ESB) to predict modified bases from DRS reads [66]. The Nanocompore [67] method uses a model-free approach that involves a comparison of experimental DRS reads with reads that have fewer or no modifications, which limits the utility of this method. These methods do not perform a quantitative comparison of m^6A (i.e. fraction of transcripts from a gene with modification) in different samples, which is important to gain functional insights about this modification. Furthermore, none of these methods predict modifications directly from the sequencing signal. A recent paper describes a new computational method called xPore, which uses the signal information and allows quantification of differential modifications across samples [69]. xPore extended the two-Gaussian distributions model using wild type cells (modified RNAs) and *Mettl3* knockout cells (unmodified RNAs) as the reference points to estimate modification rates and differential modification rate (DMR). The DMRs from this study can be regarded as differential m^6A sites since these sites are inferred by comparing wild-type and *Mettl3*-knockout cells. However, other

types of RNA modifications, such as m^1A might hinder the identification of DMR in m^6A among different samples since both m^1A and m^6A can induce signal shift for each k-mer. Thus, the current version of xPore does not estimate the type of RNA modification without the aid of data from METTL3 knockout cells.

m^6A, a prevalent modification in plants, occurs predominantly in the 3′UTR of many protein-coding transcripts [47, 53]. Transcriptome-wide m^6A modifications were mapped in *Arabidopsis* using a differential error rate approach [19] and 2/3 of the modifications detected with this method were also found with m^6A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP), suggesting that this error-based method can detect true m^6A sites but misses some. Analysis of m^6A modifications in wild type, a *VIRILIZER* mutant (*vir-1*) in which m^6A modification is impaired as *VIR* encodes a component of m^6A writer complex, and a *VIR* complemented line provided the evidence that m^6A modifications in mRNA enhance RNA stability probably by protecting from endonucleolytic cleavage [19, 57]. In *vir-1*, circadian rhythms are impaired and transcripts encoding key proteins in the circadian clock and flowering are methylated, suggesting a role of methylated RNAs in regulating flowering time and circadian rhythms. In an m^6A impaired mutant (*vir-1*) proximal poly(A) site selection is increased, indicating the importance of mRNA modification in poly (A) site choice. Dynamic changes in m^6A modifications and RNA secondary structures were observed in response to salt stress [49]. Specific transcripts encoding stress response proteins either gained or lost m^6A in salt-treated tissues. Transcripts that lost m^6A in response to salt stress are located predominantly in CDS whereas those that gained new m^6A are in UTRs [49]. An inverse relationship between m^6A and the secondary structure was observed. Based on the results from this study on m^6A dynamics in response to salt stress, it is proposed that salt increases m^6A modification in 3′UTRs of specific transcripts encoding proteins involved in stress responses, which relieves secondary structure in mRNAs and stabilizes them for translation to produce stress response proteins [49]. However, the mechanism by which m^6A regulates mRNA stability in plants is not known.

In maize, m^6A is positively correlated with APA and hypermethylation of transcripts resulted in reduced translation. Furthermore, intraspecific variations in m^6A appear to regulate alternative splicing [53]. In rice, a mutant (*Osnsun2*) that lacks an RNA 5-methylcytosine (m^5C) methyltransferase enzyme showed hypersensitivity to heat stress [55]. This mutant has reduced m^5C in many RNAs that are known to function in abiotic stresses, suggesting the importance of this modification in plant stress responses.

Circular RNAs, generated from back-splicing of intron-containing pre-mRNAs, are ubiquitous in plants and are implicated in regulating many cellular processes including splicing [21, 70, 71]. DRS of circular RNAs to detect m^6A is not as straightforward as these RNAs are covalent closed-loop structures without a poly(A) tail. Recently, a novel method used for DRS of circular RNAs includes multiple steps to enrich circular RNAs first, followed by fragmentation, synthesis of first-strand cDNA using custom primers. These RNA-DNA hybrids are then ligated to a 1D sequencing adapter and subjected to DRS to obtain RNA strand sequence [21]. Application of this method to plant circular RNAs and detection of m^6A using the EpiNano tool [22] revealed that about 11% (out of a total of 470) circular RNAs in bamboo are methylated primarily near the donor or acceptor splice sites [21], suggesting that this modification may regulate back-splicing. A recent study in male germ cells also showed that most of the back-splicing events occur in m^6A enriched regions [72]. Further analysis of DRS reads of bamboo circular RNAs with other tools to detect base modifications should help validate these results. In bamboo, about 240 open reading frames (ORFs) were found in circular RNAs and over 50% of the predicted proteins from these ORFs are similar to proteins in the non-redundant protein database at the NCBI, indicating that many of these circular RNAs may encode proteins. Overall, this study opens new avenues to profiles RNA modifications in plant circular RNAs and non-coding RNAs that lack poly(A) tail under normal and stress conditions to investigate their biological roles.

Environmental cues and stresses target specific splicing factors to modulate alternative splicing

Alternative splicing using Illumina short-read sequencing has been widely studied in response to developmental cues and diverse stresses. In plants, many AS events are altered in response to abiotic and biotic stresses (summarized in Supplementary Table S1). Some stress-induced changes in AS are associated with changes in gene expression whereas others are not, indicating that AS can function as an independent layer of gene regulation in stress responses [73–77]. In a genome-wide analysis of splicing quantitative trait loci (sQTLs) in over 600

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geographically distributed diverse ecotypes of *Arabidopsis thaliana,* many stress-responsive genes associated with significant sQTLs were identified, supporting the role of AS in plant stress responses [78].

Light is one of the most essential environmental cues for plant growth and development. Several studies demonstrated that light and circadian clock induce extensive changes in AS [73, 79–82]. For example, RNA-seq analysis showed that 1505 genes (6.9%) in *Arabidopsis* undergo AS changes within 1 h of exposure to red light; 8.4% and 8.9% of AS events in *Physcomitrella patens* rapidly respond to red and blue light, respectively [79]. Red pulse triggers AS changes in pre-mRNAs encoding some splicing factors, light-signaling components, and germination regulators during seed germination in *Arabidopsis* [83]. Recent studies have uncovered mechanisms of light-regulated AS regulation (Figure 2A). For example, phytochromes PpPHY4 targets the splicing regulator PphnRNP-H1 to regulate pre-mRNA splicing and photomorphogenic responses in *Physcomitrella patens* [84], PpPHY4 also interacts with another splicing regulator PphnRNP-F1 to regulate light-responsive AS via an exonic splicing silencer [37]. Forward genetic studies also revealed that *Arabidopsis* phytochrome B regulates AS of many light-signaling genes by affecting the activity of splicing factors such as SPLICING FACTOR FOR PHYTOCHROME SIGNALING (SFPS) [85]. Further investigations revealed that SFPS strongly interacts with REDUCED RED-LIGHT RESPONSES IN CRY1CRY2 BACKGROUND1 (RRC1) and forms a complex to control pre-mRNA splicing of light signaling and circadian clock genes to fine-tune the light-regulated developmental processes [82].

Abiotic stresses, such as salt, nutrient limitation, cold, and drought also alter AS of many pre-mRNAs in plants [74, 88–90] (see Supplementary Table S1). However, biochemical mechanisms of most abiotic stress-regulated AS are yet to be elucidated. A recent study using genetic, RNA-seq and cell biological studies have revealed a key role for the Highly ABA-Induced 1 (HAI1), a member of the protein phosphatase 2C family, and it's interacting protein partner (HAI interactor 1, HIN1), in drought acclimation [74]. The HAI1 through HIN1, a plant-specific non-canonical RNA binding protein, and its interacting proteins of serine/arginine-rich (SR) family of splicing factors regulate splicing, especially intron retention (Figure 2B). Interestingly, the sequence motif enriched in light- and drought-regulated alternatively spliced transcripts is a GAA repeat of 9 to 12 nts long [37, 74]. Previous studies have shown that several splicing regulators (SCL33, SCL30, SR45) bind or likely to bind to this element, suggesting that stress signaling pathways could converge on these splicing

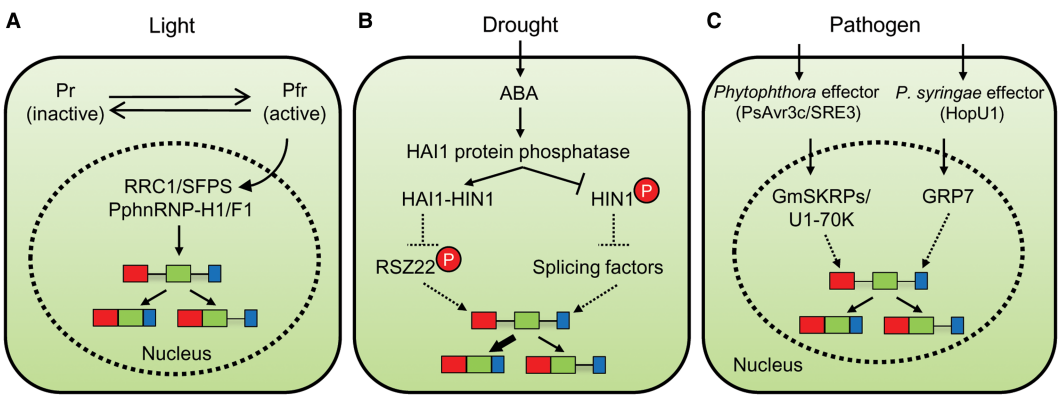

Figure 2. Mechanisms of light-, drought-, and pathogen-regulated alternative splicing.

(**A**) Light modulation of AS through light receptors, phytochromes, in regulating photomorphogenesis. Activated phytochromes (Pfrs) translocate to the nucleus and interact with splicing factors (RRC1 and SFPS) or heterogeneous nuclear ribonucleoproteins (PphnRNP-H1 and PphnRNP-F1) to modulate AS and photomorphogenic/light-regulated responses [82, 84, 85]. (**B**) Proposed mechanisms by which HAI1-HIN1 regulate drought-induced changes in splicing. Drought stress stimulates ABA accumulation and induces HAI1 (Highly ABA-Induced 1) protein phosphatase. Dephosphorylation of HIN1 (HAI1 interactor 1) by HAI1 may affect HIN1 interaction with other splicing factors, or HIN1 may recruit HAI1 to facilitate the dephosphorylation of splicing regulators (e.g. RSZ22). One or both of these will increase the splicing efficiency of specific IR prone introns [74]. (**C**) A model of pathogen-induced splicing regulation. *Phytophthora* species secrete RXLR effector proteins such as PsAvr3c and SRE3 (Pi06094) into host cells, which interact with core spliceosomal and/or splicing regulatory proteins, GmSKRPs and U1-70K, to modulate AS of host pre-mRNAs and weaken plant immunity [75, 86]. *P. syringae* type III effector HopU1 targets plant GRP7 to suppress AS-mediated plant immunity [87].

regulators [91–93]. Also, a loss-of-function mutant of SR45 showed altered responses to abiotic and biotic stresses [94, 95]. Taken together, these studies provide new insights into the biochemical mechanisms of abiotic stresses-regulated AS in plant systems.

Numerous studies using high-throughput RNA sequencing have also shown that a large number of plant genes undergo AS in response to diverse biotic stresses (viral, bacterial, fungal, and oomycete infection, and insect attack) (see Supplementary Table S1). For example, genome-wide analysis of the AS landscape in *Brachypodium distachyon* during plant-virus interaction has shown that about 669 genes were specifically alternatively spliced in response to *Panicum mosaic virus* infection [96]. Likewise, potato spindle tuber viroid and tobacco hornworm also trigger genome-wide AS changes of host protein-coding genes [97, 98]. More recently, global analysis of AS in tomato leaves infected with *Phytophthora infestans* revealed significant changes in splice isoforms ratios of many genes without altering the overall expression of the corresponding genes [86]. Although it is now well established that biotic stresses profoundly change splicing patterns from many genes, the precise molecular mechanism of how pathogens regulate AS remains largely unknown. Interestingly, several recent studies have found that pathogens secrete effectors into the host cell to regulate host pre-mRNA splicing and suppress plant immunity (Figure 2C). One example is that *Phytophthora sojae* RXLR effectors PsAvr3c could bind to the soybean serine/lysine/arginine-rich proteins (GmSKRPs) and modulate host pre-mRNA splicing to promote disease [86]. Further analysis of PsAvr3c family members in other *Phytophthora* species showed that *P. cinnamomi var. robiniae* ProbiAvh89 modulate plant AS in a manner similar to PsAvr3c [99]. Recently several RXLR effectors from *P. infestans* were found to modulate AS. One of these (SRE3, gene ID *Pi06094*) interacts directly with spliceosomal (U1-70K) and splicing regulatory proteins (e.g. SR30 and SR45) to manipulate AS of host pre-mRNAs to subvert plant immunity [75]. Another example is that *Pseudomonas syringe* type III effector HopU1 targets several plant RBPs such as glycine-rich RNA-binding protein 7 (GRP7), to suppress plant immunity. GRP7 is known to affect the choice of alternative 5′ splice sites and impacts AS of certain transcripts via direct interaction with their mRNAs [87]. Recently, it was demonstrated that the rapid alkalinization factor 1 (RALF1) peptide through its receptor FERONIA, a receptor-like kinase, phosphorylates GRP7 resulting in its increased nuclear accumulation and AS modulation. The observed interaction of GRP7 with U1 snRNP 70K protein is thought to modulate AS [100]. Collectively, these studies indicate that plant AS regulation is important during plant-microbe interaction and pathogens have evolved effectors that target host splicing components and subvert plant immunity.

Strategies to identify splicing regulatory proteins

In *Arabidopsis*, there are over 500 RNA-binding proteins (RBPs) and the precise roles of most of these proteins in co-/post-transcriptional processes are unknown [101]. For an in-depth understanding of mechanisms that regulate splicing it is critical to identify proteins that play a key role in splice site choice. Many approaches to address these have been developed and are briefly described here. Recently, several of these have been applied to plants to identify splicing regulatory proteins. In non-plant systems, *in vitro* splicing assay has been used extensively to study various aspects of splicing and identification of proteins involved in AS regulation. In plants, there is no robust *in vitro* splicing system. However, an *in vitro* splicing assay system with plant nuclear extract was recently reported [102]. Further refinement of this system with constitutive and alternative spliced pre-mRNA substrates offers an opportunity to identify splicing regulatory proteins by complementing splicing deficient extracts (Figure 3A). Genetic screening is a powerful tool to identify splicing regulatory proteins. A forward genetic screen with *Arabidopsis* plants expressing an alternatively spliced intron-containing GFP reporter gene (Figure 3B, i) has led to the identification of 16 splicing regulatory proteins such as RBM25, RBP45d, PRP39a, PRP18a, SMU1, RTF2, CDKG2, CBP80 and SMFa that function in splicing regulation [103]. Recently, a luciferase-based novel reporter system (Figure 3B, ii) containing an alternatively spliced region of a tomato gene (*RLPK*) has been used to identify nine *Phytophthora infestans* effectors as modulators AS in plants [75]. Several groups have used yeast two-hybrid screening and co-immunoprecipitation methods to search for interacting proteins of a protein of interest in identifying splicing regulatory proteins (Figure 3C,D). For example, Shih-Long Tu group has used yeast two-hybrid screening with *Physcomitrella patens* phytochromes PpPHY4 and showed that it interacts with heterogeneous nuclear ribonucleoprotein PphnRNP-H1 and PphnRNP-F1 to regulate light-mediated AS [37, 84], Likewise, Huang et al. also demonstrated that *Phytophthora sojae* RXLR effector PsAvr3c binds to the soybean novel splicing factors, GmSKRPs, and reprograms host pre-mRNA splicing to promote disease [86]. By performing co-immunoprecipitation with SPLICING FACTOR FOR PHYTOCHROME SIGNALING (SFPS) followed by mass spectrometry, Xin et al.

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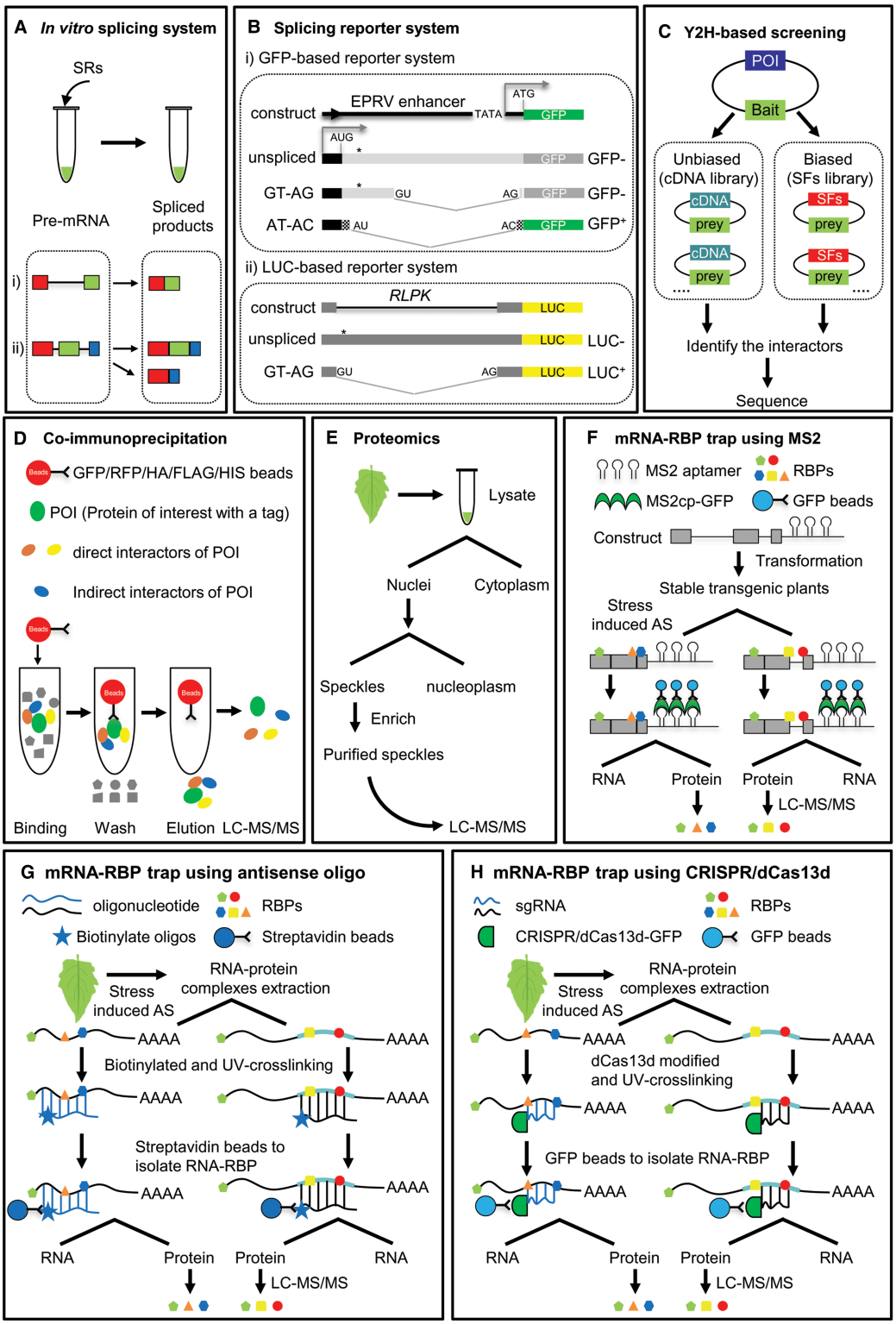

Figure 3. Approaches to identify splicing regulatory proteins. Part 1 of 2

(**A**) *In vitro* splicing system. Left. Splicing regulatory proteins (e.g. SR proteins and other RNA binding proteins) are added to *in vitro* splicing assays with a pre-mRNA substrate that is either constitutively or alternatively spliced. Right, spliced products after *in vitro* splicing of pre-mRNA substrate. The schematic representation of constitutive (i) and alternative splicing (ii) with different pre-mRNA substrates are shown in the bottom. (**B**) Splicing reporter system. (i) GFP-based reporter system. *GFP* gene is under

Figure 3. Approaches to identify splicing regulatory proteins. Part 2 of 2

the transcriptional control of viral regulatory elements, features a GT–AG splice site and a noncanonical AT–AC splice site. Unspliced and GT-AG transcript has a premature termination codon and does not produce GFP, while AT-AC transcript will produce a functional GFP protein [103]. (ii) LUC-based reporter system. The genomic fragment containing an alternatively spliced region of the tomato *RLPK* gene is cloned and ligated with the *luciferase (LUC)* gene to yield *RLPK-LUC*. GT-AG transcript will produce a functional luciferase that can be quantified, while the unspliced transcript with retained intron has a premature termination codon and does not produce luciferase [75]. (**C**) Y2H (yeast two-hybrid)-based screening. POI (Protein of interest) that affects splicing is cloned into bait plasmid. For an unbiased screen, a plant cDNA library is used for screening (left). For a biased screening, known/putative splicing factors are cloned into the prey vector to generate splicing factors library and used for screening (right). All the interactors will be identified by sequencing. (**D**) Co-Immunoprecipitation (Co-IP). The POI is expressed as a fusion to a tag (GFP/RFP/HA/FLAG/HIS). Plant total proteins are extracted and incubated with GFP/RFP/HA/FLAG/HIS-antibody beads, after wash and elution steps, the direct or indirect interactors of POI are identified using LC-MS/MS. (**E**) Proteomics method to identify splicing regulatory proteins. Plant total proteins are extracted and fractionated into nuclei and cytoplasm, then nuclei are separated into speckles and nucleoplasm, finally, proteins in the purified speckles are identified using LC-MS/MS. (**F**) mRNA-RBP trap using MS2-MS2cp. The MS2 aptamer is added to the 3′ end of a gene of interest that undergoes stress-induced AS, the construct is then stably expressed in plants and transgenic plants are challenged with stress. The RBPs associated with the different splicing isoforms are captured by MS2cp-GFP and GFP-antibody beads. Proteins bound to mRNA are then identified by LC-MS/MS. (**G**) mRNA-RBP trap using antisense oligo. Plants used for RNA extraction are challenged with a stress that is known to alter AS. The biotinylated antisense oligonucleotides that are isoform-specific are hybridized to RNA and cross-linked with UV light. The RBPs associated with the different isoforms of endogenous RNAs of interest are captured by streptavidin beads and identified using LC-MS/MS. (**H**) mRNA-RBP trap using CRISPR/deadCas13d. Plants used for RNA extraction are challenged with the desired stress that induces AS. The CRISPR/dCas13d-GFP and sgRNAs targeted to different isoforms of interested RNA and crosslinked with UV light. The RBPs associated with the different isoforms are captured by GFP-antibody beads and analyzed using LC-MS/MS.

[82] revealed that SFPS directly interacts with REDUCED RED-LIGHT RESPONSES IN CRY1CRY2 BACKGROUND1 (RRC1) and forms a complex to regulate light-mediated AS in *Arabidopsis*. Since most splicing regulatory proteins are present in nuclear speckles, which are highly dynamic and change in response to stresses, proteomics analysis of nuclear speckles [104] under different conditions should provide new insights into proteins involved in stress-induced splicing (Figure 3E). Although animal speckles have been purified for proteomics studies [104], no such studies have been performed in plants. Recently, RNA capture strategies have been used to identify splicing regulatory proteins that interact with specific transcripts [105]. Splice isoforms are expressed as a fusion to MS2 aptamer, and nascent/mature RNA bound to proteins are subjected to proteomics analysis (Figure 3F). Alternatively, biotinylated oligos specific to a given isoform can be used to capture RNA-protein complexes for liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis (Figure 3G). Also, synthetic guide RNAs can be targeted to specific splice isoform using GFP-tagged CRISPR/deadCas13d system [106] and the RNA-RBP complexes are isolated for proteomic analysis using GFP antibody beads (Figure 3H). By using plants that are exposed to different stresses for these analyses, it should be possible to identify RNA binding proteins that interact with specific isoforms only under certain conditions. As discussed above, the approaches presented in Figure 3B,C and 3D have been successfully applied to plant splicing research whereas other approaches (Figure 3A,E–H) have not yet been applied to identify splicing regulatory proteins in plants.

RNA immunoprecipitation (RIP) followed by high-throughput sequencing (RIP-seq) [92] and high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP, also known as CLIP-seq) and individual nucleotide resolution crosslinking and immunoprecipitation (iCLIP) have led to the identification of global targets of plant RBPs [107, 108]. Application of enhanced CLIP (eCLIP), a modified iCLIP method that was successfully used to identify targets of 150 human RBPs [109], to plant systems should expedite the progress in this area. Recent methods such as TRIBE (targets of RNA-binding proteins identified by editing) and hyper-TRIBE to identify RNA targets of RBPs are based on modifying RNA bases *in vivo* (e.g, adenosine to inosine) with an RNA-binding domain of RBP fused to a catalytic domain of a base-modifying enzyme (adenosine deaminase) and identifying the modified bases using short reads [110]. Identification of edited RNAs in plants expressing TRIBE/HyperTRIBE constructs by DRS should help define targets of individual RBPs rapidly and overcome the limitations associated with other methods.

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Conclusions

NGS tools have significantly contributed to our understanding of the complexities associated with plant transcriptomes and the impact of developmental cues and stresses on reshaping the landscape of plant transcriptomes. It is now well established that most plant genes produce multiple transcripts with varying lengths due to alternative transcription start and polyadenylation sites and due to alternative splicing. Several studies this year have established that most of the mRNA processing steps (splicing, polyadenylation and decisions about alternative splice site choice) occur co-transcriptionally before the release of nascent transcripts. Gene features that determine co-transcriptional splicing efficiency have been elucidated. Also, it is becoming increasingly evident that the different steps in gene expression - transcription, processing of mRNAs and translation - are intimately coupled. However, the functions of most splice isoforms and APA variants are largely unknown. This is an interesting but challenging area that needs a shift from descriptive studies to focused functional analyses of alternative transcripts. Single-cell and single-nucleus RNA sequencing (sc/snRNA-seq) in humans has provided unprecedented insights into the complexity of the human transcriptome in heterogeneous tissues [111]. Application of sc/snRNA-seq in plants under different conditions should provide cell-specific co-/post-transcriptional changes in gene regulation under different conditions and further our understanding of stress responses in plants.

Perspectives

- Analysis of chromatin-bound RNA (nascent RNA) for base modifications using DRS is a powerful approach to uncover the level of co-transcriptional epitranscriptomic modifications and their impact on splicing.
- Future studies should focus on unraveling the mechanisms through which mRNA processing events are regulated co-/post-transcriptionally and elucidating how stresses modulate splicing.
- The use of RNA *in situ* conformational sequence **(**RIC-seq) [112] in plants should lead to the mapping of global intra- and inter-molecular RNA-RNA interactions mediated by RNA binding proteins and uncover regulatory roles of RNAs and RBPs. For a comprehensive analysis of the interplay among various steps in gene expression integrated multi-omics approaches [113] need to be used with each sample.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

5′GRO-seq, Global nuclear run-on sequencing of RNAs with 5′ cap; APA, alternative polyadenylation; AS, alternative splicing; CB-RNA-seq, chromatin-RNA sequencing; DRS, direct RNA sequencing; ELIGOs, epitranscriptional landscape inferring from glitches of ONT signals; ERIC-seq, 5-EU immunoprecipitation chase-seq; GRO-seq, Global nuclear run-on sequencing; HITS-CLIP, High-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation (also known as CLIP-seq); miCLIP, m^6A individual-nucleotide-resolution cross-linking and immunoprecipitation; MINES, m^6A Identification using Nanopore Sequencing; Neu-seq, nascent 5-ethynyl uridine–labeled RNA sequencing; NGS, next-generation sequencing; ONT, Oxford Nanopore Technology; PacBio Iso-seq, Pacific Biosciences isoform sequencing; pNET-seq, plant native elongating transcript sequencing; RBPs, RNA-binding proteins; RIC-seq, RNA *in situ* conformational sequence; RIP-seq, RNA immunoprecipitation followed by sequencing; RNA-seq, RNA-sequencing; sc/snRNA-seq, Single-cell and single-nucleus RNA sequencing.

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