Regulation of plant gene expression by alternative splicing

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Abstract

AS (alternative splicing) is a post-transcriptional process which regulates gene expression through increasing protein complexity and modulating mRNA transcript levels. Regulation of AS depends on interactions between *trans*-acting protein factors and *cis*-acting signals in the pre-mRNA (precursor mRNA) transcripts, termed 'combinatorial' control. Dynamic changes in AS patterns reflect changes in abundance, composition and activity of splicing factors in different cell types and in response to cellular or environmental cues. Whereas the SR protein family of splicing factors is well-studied in plants, relatively little is known about other factors influencing the regulation of AS or the consequences of AS on mRNA levels and protein function. To address fundamental questions on AS in plants, we are exploiting a high-resolution RT (reverse transcription)–PCR system to analyse multiple AS events simultaneously. In the present paper, we describe the current applications and development of the AS RT–PCR panel in investigating the roles of splicing factors, cap-binding proteins and nonsense-mediated decay proteins on AS, and examining the extent of AS in genes involved in the same developmental pathway or process.

Introduction

AS (alternative splicing) generates more than one spliced mRNA isoform from the same gene through the selection of alternative splice sites. Different types of AS event include alternative 5′ and 3′ splice site selection, intron retention, exon skipping and mutually exclusive exon splicing, resulting in the inclusion or exclusion of intronic or exonic sequences [1–3]. The consequences of such changes in transcript sequence are altered protein sequence and functionality (activity, protein–protein or protein–substrate interactions, localization, protein modification, etc.) or the introduction of premature termination codons giving truncated proteins or leading to degradation of AS isoforms by NMD (nonsense-mediated decay) [1–5]. In humans, 95 % of multi-exon genes undergo AS [6], whereas, in plants, current estimates are that approx. 35 % of genes (in *Arabidopsis* and rice) undergo AS [7–11]. This level of AS is likely to be underestimated owing to the relatively lower level of ESTs (expressed sequence tags) available in plants when compared with, for example, humans or mice, and because many AS events are not represented or are under-represented in EST collections as they occur only in specific cells and tissues, growth conditions or stages of development [10–13]. AS is found in genes involved in a wide range of cellular processes, including transcription, splicing, development, signal transduction, and responses to biotic and abiotic stress contributing to, for example, seed development and quality, germination, disease resistance, flowering time and the circadian clock [10,11,14–20].

Although more and more examples of AS in plant genes are being discovered, very little is known about (i) the dynamic changes in AS of the majority of annotated alternatively spliced genes, (ii) how such changes are regulated, or (iii) the functions of protein isoforms produced from the AS transcripts. In order to begin to address the aspects of dynamic modulation and regulation of AS, we developed a high-resolution RT (reverse transcription)–PCR system to monitor changes in AS isoform abundance of approx. 300 *Arabidopsis* AS events [21]. The AS events represent different types of AS and are mainly in transcription factor, RNA-binding protein or stress-related genes. By using overexpression lines and knockout mutants of factors involved in splicing regulation [e.g. SR (serine/arginine-rich) proteins [21] and PTB (polypyrimidine tract-binding protein)-like proteins] and mRNA biogenesis [e.g. CBPs (cap-binding proteins)] [22], the AS RT–PCR system detects whether the factors influence AS and which events and types of AS event are affected. Similarly, by analysing mutants impaired in NMD, it is possible to identify AS isoforms which are subject to degradation by NMD. Although the AS RT–PCR system does not report on

Key words: alternative splicing, *Arabidopsis*, precursor mRNA, splicing factor.

Abbreviations used: AS, alternative splicing; AtGRP, *Arabidopsis thaliana* glycine-rich protein; CBC, cap-binding complex; CBP, cap-binding protein; EST, expressed sequence tag; NMD, nonsense-mediated decay; PTC, premature termination codon; RT, reverse transcription; snRNP, small nuclear ribonucleoprotein; SR, serine/arginine-rich; UPF, up-frameshift; UTR, untranslated region.

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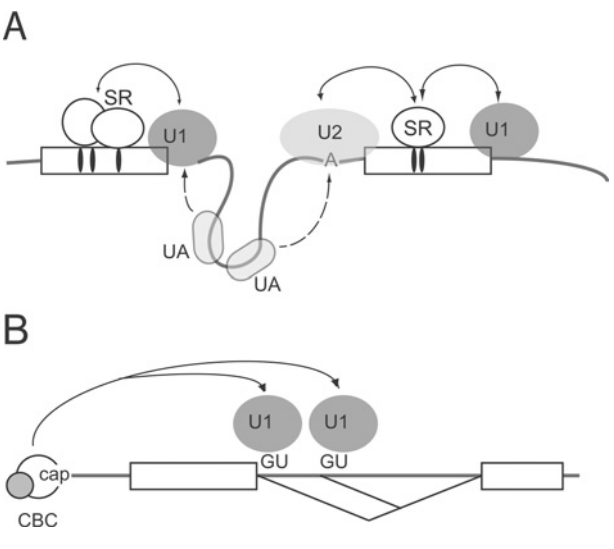
Figure 1 | Regulation by AS

(**A**) Splice site selection depends on multiple interactions between splicing factors and components and splicing signals in the transcript. SR proteins interact with other splicing factors and U1 and U2 snRNPs early in spliceosome assembly to select splice sites. In plants, this process may further involve UA-binding proteins interacting with the UA-rich introns. Differential interactions of different SR proteins with exon splicing enhancers promote alternative splice site selection. (**B**) In plants, the CBC can influence AS and does this preferentially at the 5′ splice site of the first intron, but can promote splicing at either the proximal or distal 5′ splice site [22]. Boxes represent exons; lines represent introns; vertical lines in exons represent exon splicing enhancer sequences; U1, U1 snRNP at the 5′ splice site; U2, U2 snRNP at the branchpoint (A); UA, UA-binding protein.



AS across the whole genome, the number of different events (approx. 300) is large enough to indicate the extent to which particular proteins affect AS. Finally, in addition to exploiting the genetic resources of *Arabidopsis* (in terms of mutant lines and transgenic overexpression lines), the AS RT–PCR system is being used to assess splicing efficiency and AS in different plant organs, developmental stages and under different growth and stress conditions (e.g. temperature). For example, a subset of intron-retention events have been used to examine splicing efficiency in plants under anoxic conditions which cause a core exon junction complex protein to relocalize to the nucleolus [23].

Regulation of AS

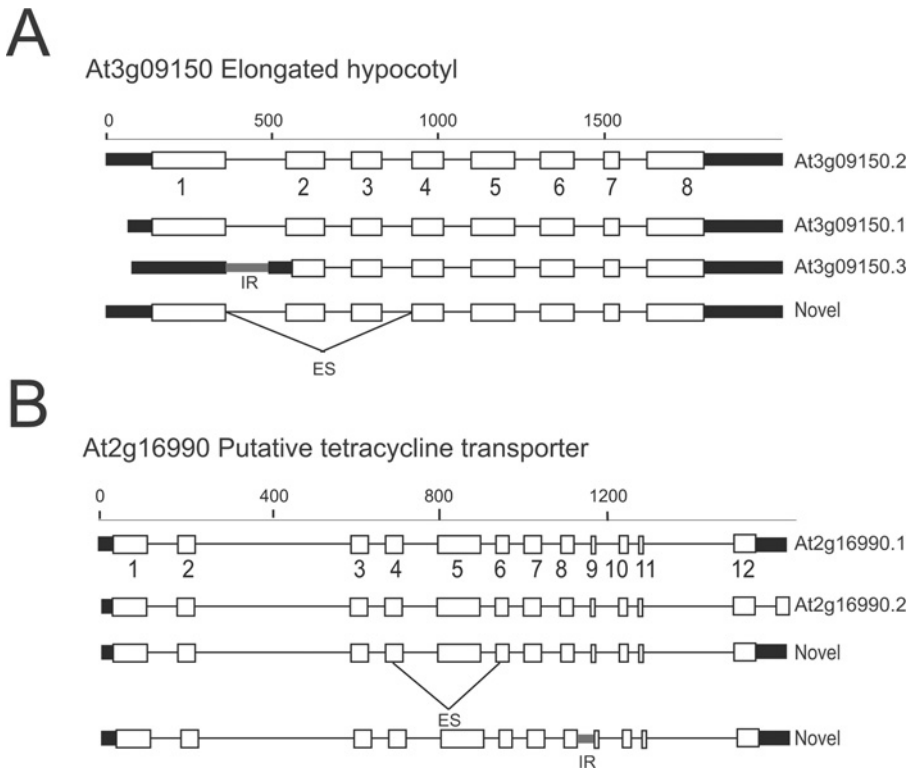
Regulation of AS is determined by the interactions of multiple *trans*-acting factors with intronic and exonic signals in the transcript giving rise to the concept of combinatorial control and a splicing code [24,25] (Figure 1A). Most progress in terms of *trans*-acting factors involved in plant splicing regulation has been made in the characterization of *Arabidopsis* SR proteins [26,27]. SR proteins are involved in both constitutive splicing and AS and generally promote splice site selection by binding to splicing enhancer sequences in the pre-mRNA and through protein–protein interactions (Figure 1A). In *Arabidopsis*, the essential role of these proteins is reflected in the range of developmental changes in plants where SR proteins are overexpressed or mutated [27–30]. SR protein genes are also of particular interest because the majority are themselves alternatively spliced and some of the AS events are evolutionarily conserved, suggesting that their regulation by AS is important for plant development [31]. Moreover, protein isoforms, produced from different AS transcripts of the *Arabidopsis* SR protein gene, *SR45*, are able to complement different developmental phenotypes of an *sr45* mutant demonstrating functional differences for the AS products [32]. Changes in expression of SR proteins are, not surprisingly, associated with changes in AS of many genes, including other SR protein genes themselves [20,28,29], and the AS RT–PCR panel is currently being used to analyse the subsets of AS events which are affected by particular SR proteins.

In addition to splicing factors, other proteins influence splicing. For example, the CBC (cap-binding complex) has a number of functions in mRNA biogenesis, including splicing, mRNA export, protection of the transcripts from nuclease degradation and 3′-end formation by stabilizing the interaction of the 3′-end processing machinery. In mammals and yeast, the CBC at the 5′ end of the pre-mRNA transcript promotes the initial interaction between U1 snRNP (small nuclear ribonucleoprotein) and the 5′ splice site of the first intron in the transcript and enhances the formation of spliced mRNAs [33]. However, the CBC has not been shown to affect AS to date. The single-knockout mutants of CBP20 and CBP80 and the double mutant are viable, but have slow growth, have serrated leaf margins, are late-flowering, are hypersensitive to abscisic acid and have enhanced drought tolerance [34–37]. Effects on splicing by knocking out CBC proteins have been observed in flowering time genes and in the increased occurrence of unspliced introns detected on microarrays [36–38]. To investigate effects on AS, the AS RT–PCR panel has been used in conjunction with single and double mutants of CBP20 and CBP80 [22]. Of ~250 AS events, 101 showed significant changes in AS in at least one mutant, and 41 of these showed changes in all three mutant lines. In the latter group, the number of events involving the first intron in a transcript and alternative 5′ splice site selection was significantly increased, suggesting that the *Arabidopsis* CBC preferentially (but not exclusively) affected AS of the first intron and particularly at the 5′ splice site [22] (Figure 1B). Moreover, we observed that CBP80 exerts a stronger influence on splicing than CBP20.

The NMD pathway targets mRNAs containing PTCs (premature termination codons) for degradation. In both mammals and plants, mRNAs with an increased distance between the PTC and the 3′ end of the mRNA [long 3′ UTRs (untranslated regions)] and/or with an exon junction complex downstream of a PTC are targeted for degradation [4,39,40]. NMD is thought to reduce the consequences of mutations or mistakes in expression, to reduce genomic noise

Figure 2 | Identification of novel AS events for process-specific RT–PCR panels

Cloning and sequencing of RT–PCRs covering the gene sequences has identified novel AS events of flowering time genes (an example is shown in **A**) and cold-stress induced genes (an example is shown in **B**). AS events in TAIR (The *Arabidopsis* Information Resource) are shown below the exon–intron structure of the gene, and novel events are labelled. White boxes represent exons; black boxes represent 5′ and 3′ UTRs; horizontal lines represent introns; lines below gene structures represent splicing events. IR, intron retention; ES, exon skip.



and prevent production of potentially detrimental truncated proteins. In addition, NMD modulates the levels of AS isoforms which contain PTCs. Approx. 10 % of the human and yeast transcriptome is turned over by NMD [41,42] and 30 % of human AS transcripts contain PTCs and are degraded by NMD [5]. In *Arabidopsis*, examples of regulation by AS and NMD include the genes encoding the circadian clock proteins, AtGRP (*Arabidopsis thaliana* glycine-rich protein) 7 and AtGRP8 [43,44]. By analysing mutants of the NMD proteins, UPF (up-frameshift) 1 and UPF3 (*upf3-1* and *upf1-5*), the AS RT–PCR system readily detects increases in the abundance of specific AS isoforms in a significant number of AS events. These potentially represent transcripts which are turned over by the NMD degradation pathway and are currently being characterized.

Process-specific AS

Ultimately, we wish to understand how transcriptional and post-transcriptional control systems are integrated in the regulation of gene expression. Current models suggest that post-transcriptional control networks such as AS are super-imposed on transcriptional networks and that regulation of AS must be co-ordinated with regulation of transcription [45]. For example, the fine-tuning of developmental or metabolic pathways will reflect the integrated outputs of both transcription and AS networks such that changes in expression of one gene at the transcriptional or post-transcriptional levels could feed back on its own expression and the expression of other genes in the pathway. An excellent example of this type of control is the auto- and cross-regulation of the clock proteins AtGRP7 and AtGRP8 mentioned above.

Our current knowledge of AS events in plants is incomplete, making global approaches to studying AS (such as the use of exon arrays or splice junction arrays) presently unfeasible. Indeed, one of the outcomes of the use of the AS RT–PCR panel has been the unexpected number of novel RT–PCR products, some of which have been characterized and shown to be novel unannotated AS events. The number of novel RT–PCR products which we detect in our amplicons suggests that the level of AS is much higher in plants than currently estimated. Therefore, to be able to address co-ordination of post-transcriptional control by AS in genes in the same pathway, it is necessary to identify all the possible AS events in these genes. To this end, we are systematically cloning and sequencing AS events for all genes in particular pathways (e.g. the flowering time pathway) (Figure 2). AS

events are assembled from existing transcript information available in databases and from cloning and sequencing of RT–PCR products covering the coding sequence and UTRs of each gene. Novel AS events have been observed and characterized (Figure 2) and, combining this information, the feasibility of generating process- or pathway-specific AS RT–PCR panels is currently being tested. In the future, next-generation sequencing is expected to provide a platform for the global identification and quantification of AS isoforms in different plant species, but robust methods of data analysis for this particular function are still under development. The detailed characterization of AS events by the AS RT–PCR system will be invaluable both upstream in developing the next-generation sequencing data-analysis systems and downstream in validating changes in AS.

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