An RT-PCR Panel to Monitor Changes in Alternative Splicing in Multiple Genes

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Introduction

Regulation of splice site selection occurs through combinatorial control as a result of the levels and activities of a range of *trans*-acting factors in different cells that act on positive and negative *cis*-sequences on the pre-mRNA.

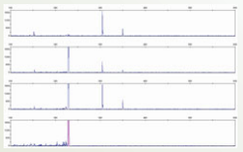
To monitor changes in multiple alternative splicing (AS) events simultaneously, we have established an RT-PCR panel that accurately and reproducibly measures the ratio of alternatively spliced products under different conditions or in different plant organs. The utility of this approach was shown by detecting significant changes in splicing in plants grown under different light conditions, in different plant organs and in plants over-expressing plant SR proteins.

Establishing the RT-PCR panel

AS events were selected from annotated events found in different databases
(Riken Genomic Science Centre, TIGR and more recently Alternative Splicing in Plants - ASIP).
31 5' and 59 3' AS events with over 11nt between the alternative splice sites were selected.
Selected events are found in 5' and 3'UTRs and coding sequence.
Events on the pilot panel were not selected on the basis of biological function.
RT-PCR was carried out on extracted RNA using 96 pairs of gene specific primers that span the AS event.
One of the primer pair was fluorescently labelled.
AS events were separated according to their transcript length by an ABI 3730 sequencer.
AS products were identified according to size and the ratio of the alternatively spliced products was calculated.
A minimum of three biological reps were performed to determine statistically significant changes in AS in the conditions tested.

Extract RNA

RT-PCR

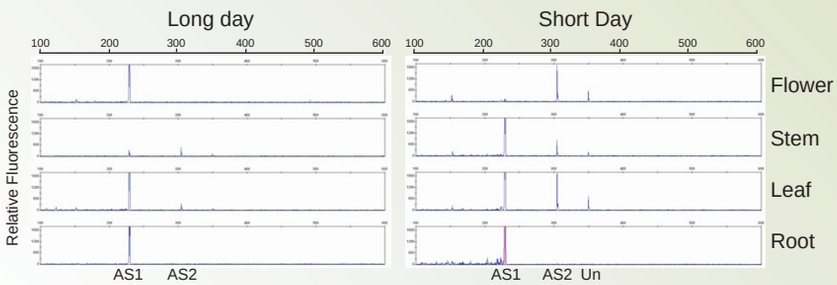


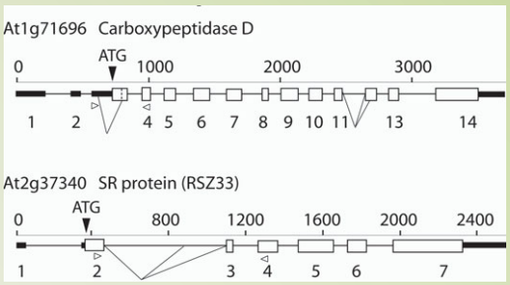
Separation on ABI 3730

Alternative splicing in reponse to light

The panel was tested using RNA extracted from *Arabidopsis* grown under different light regimes. In long day and short day grown plant tissue, significant changes (p=<0.1) in the ratios of AS transcripts were observed.

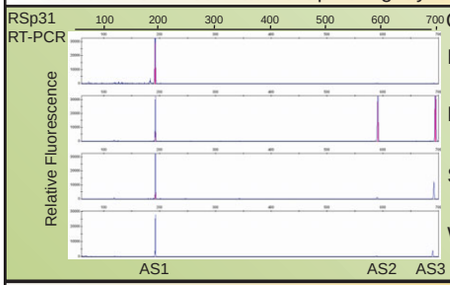
In light and dark grown seedlings 10 out of 90 AS events showed a significant change (p=<0.1)





Consequences of AS are altered N- or C-terminal sequences, inclusion or exclusion of amino acid sequences or introduction of premature stop codons (PTC).
In carboxypeptidase D (At1g71696), the alternative intron in exon 3 leads to a variable start codon and the presence or absence of an N-terminal signal sequence.
Alternative selection of a 3' splice site in intron 2 of SR protein RSZ33 (At2g37340) leads to the presence of a PTC which may lead to the formation of truncated protein or activate nonsense mediated decay.

Induction of Alternative splicing by SR proteins in over-expressed lines

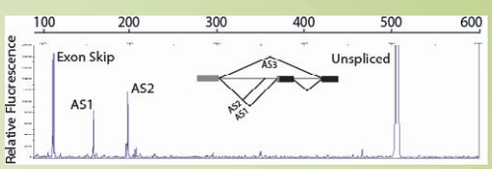


Changes in AS were monitored in transgenic plants over-expressing SRp30, RSZ33 and RSp31 SR protein regulators of splicing. Analysis of variance identified 16 AS events that changed significantly (p=<0.1) between seedlings of the wild type and the three over-expressing lines.
For example, two transcription factors (At5g65050 and At3g14230) showed significant changes in alternative splicing in the RSp31 line. Over-expressing lines of SR protteins also significantly altered splicing of other RNA binding proteins including an hnRNP like protein (At5g66010) and putative RNA binding protein (At5g04430).
Over-expression of SR proteins also significantly altered splicing of other SR proteins. The example shows over-expression of RSZ33 which leads to activation of the proximal alternative 3' splice site of RSp31.

Identification of Novel Alternative Splicing Events

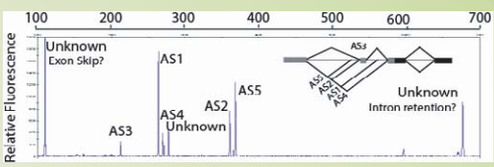
RT-PCR identified novel bands which would represent new alternatively spliced events.

We have characterised novel events by sequencing and by examining the most recent versions of TAIR which includes a number of new annotated AS events.
The transcriptional regulator Sir2 (At5g09230) shows a significant exon skipping event that was confirmed by sequencing.



An unknown protein (At3g53270) shows a complex pattern of alternative splicing in the 5'UTR. Five of these AS events are annotated in TAIR, but three significant alternative splicing events remain to be characterised.

These additional AS events increase the complexity of the panel and will lead to a more accurate representation of variable splicing in these genes.



Summary and Future Directions

We have established a procedure to analyse 90 alternative splicing events simultaneously from different conditions. Over a third of the AS events showed changes in AS under the different conditions tested. We are currently increasing the number of AS events studied to 384, following AS splicing events in transcription factors, RNA interacting proteins and stress response proteins. We will study changes in splicing in over-expressing and knock-out lines of various *trans*-acting factors and we have already begun to analyse the effect of CBP20 and CBP80 knockouts on splicing of these genes (with Jarmowlowski lab, AMU, Poznan, Poland). We will further investigate pathway-specific AS in well characterised genetic or biochemical pathways. This will allow us to address questions of co-ordinated regulation of AS in plants.