



## SEB Main Meeting, Prague 2010

### P9 - Alternative splicing and its impact on gene regulation in plants

#### P9.1

##### Alternative splicing of pre-mRNAs in plants in the post-genomic era

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Alternative splicing (AS) of pre-mRNAs is an important posttranscriptional mechanism in generating protein diversity and in regulating gene expression in multicellular eukaryotic organisms. In plants, over 40% of intron-containing genes are alternatively spliced. Genes encoding proteins with RNA recognition motifs (RRMs), especially serine/arginine-rich (SR) proteins - a conserved family of splicing regulators in eukaryotes, are extensively alternatively spliced. Interestingly, abiotic stresses dramatically alter the AS of genes encoding splicing regulators, suggesting that stresses may affect splicing of many genes globally by producing new splice variants of splicing regulators. A large fraction of splice variants of SR genes contain a premature termination codon (PTC). Analyses of the levels of splice variants for each alternatively spliced SR gene in a mutant lacking UPF3, one of the core components of nonsense-mediated decay (NMD) machinery, have shown that many splice variants with a PTC are the targets of degradation by NMD. These results indicate a widespread coupling of AS with NMD and a strong link between unproductive splicing and the level of functional transcripts. To analyze global changes in the AS events regulated by each SR protein, we generated single and double loss-of-function mutants of SR genes and these are being used for high-throughput RNA sequencing. Experiments are underway for genome-wide mapping of RNA targets of two SR proteins by high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation. Data from such analyses together with mutant phenotypes should provide novel insights into the regulation and physiological consequences of AS at the system level.

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09:10 Saturday 3rd July 2010

#### P9.2

##### The impact of SR proteins on alternative splicing in Arabidopsis

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SR (Ser/ Arg) proteins are a family of splicing regulators with multiple functions in RNA processing. They contribute significantly to splice site selection and are equally important for constitutive and alternative splicing. SR proteins are evolutionary conserved phosphoproteins with one or two N-terminal RNA-recognition motifs and a C-terminal domain rich in arginines and serines. Due to several duplication events, nineteen genes encoding SR proteins are present in the Arabidopsis genome, and their functions are largely unknown. Our aim was to identify RNA targets and to characterize the functions of the Arabidopsis SR proteins. We have studied several SR genes for their expression patterns, post-transcriptional regulation, and over-expression and knockout phenotypes. These analyses have shown that expression patterns of Arabidopsis SR genes are subjected to tight spatiotemporal and environmental control. Accordingly, mis-expression of SR proteins has affected various aspects of plant development and environmental response. To get a global picture about changes of gene expression by SR proteins we have analysed transcriptomes of over-expressing lines by microarray technology. These data were complemented by results of the recently established AS RT-PCR panel, a system of monitoring changes in alternative splicing in multiple genes in Arabidopsis (Simpson et al., 2008). Using this system we have analysed alternative splicing patterns of about 300 genes in SR overexpressor and mutant lines.

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### P9.3

#### Alternative splicing and nonsense mediated decay in Arabidopsis

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To examine the relationship between alternative splicing (AS) and nonsense-mediated decay (NMD), we used an RT-PCR panel of AS events from 285 Arabidopsis genes. Quantitative changes in the relative levels of 951 transcripts were measured in RNA from wild-type plants, NMD protein mutants (*upf1-5* and *upf3-1*), and plants treated with cycloheximide that blocks translation and NMD. A third of transcripts in the *upf* mutants and cycloheximide treated plants showed significant changes compared to wild type. Over 160 transcripts (17%) increased in abundance and were therefore sensitive to NMD. The majority of these naturally-occurring NMD-sensitive transcripts contained PTCs or uORFs allowing an analysis of NMD features in plants. Many of the genes showing a link between AS and NMD were in genes encoding RNA-interacting proteins. In addition, some transcripts containing PTCs were insensitive to NMD in the *upf* mutants. In particular, transcripts with retained introns showed little evidence of NMD suggesting that they avoid mRNA surveillance or are subject to alternative degradative mechanisms.

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### P9.5

#### Genome-wide analysis of RNA regulation in plant abiotic stress responses

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Plants respond and adapt to drought, cold and high-salinity stresses in order to survive. Recent studies have demonstrated that RNA regulatory mechanisms such as alternative splicing, RNA degradation and RNA stability have function in the abiotic stress responses. Alternative splicing profiles are changed by the abiotic stresses (Iida et al., 2004; Filichkin et al., 2010) and a high percentage of alternatively spliced mRNA isoforms contain premature termination codons (PTCs) that are targeted by nonsense-mediated mRNA decay (NMD). Recently, we demonstrated that many non-coding RNAs could serve as NMD substrates (Kurihara et al., 2009). Our tiling array analysis under drought, cold, high-salinity and ABA treatment conditions showed that 7,719 non-AGI transcriptional units (TUs) exist in the unannotated "intergenic" regions of Arabidopsis genome (Matsui et al., 2008). Most of the non-AGI TUs are hypothetical non-protein-coding RNAs. About 80% of the non-AGI TUs belong to pairs of the fully overlapping sense-antisense transcripts (fSATs). Significant linear correlation between the expression ratios (treated/untreated) of the sense TUs and the ratios of the antisense TUs was observed in the SATs of AGI code/non-AGI TU. We studied the biogenesis mechanisms of the stress- or ABA-inducible antisense RNAs and found that the expression of sense TUs is necessary for the stress- or ABA-inducible expression of the antisense TUs in the fSATs (AGI code/non-AGI TU).  
References: Iida et al. (2004) NAR 32:5096, Matsui et al. (2008) Plant Cell Physiol. 49: 1135, Kurihara et al. (2009) PNAS 106:2453, Filichkin et al. (2010) Genome Res. 20:45

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### P9.6

#### Mechanisms and logic of alternative splicing in animals

Chris Smith (University of Cambridge)

Alternative splicing is now appreciated to be the rule rather than the exception in many organisms, and is able to bridge the wide gap in complexity between the protein coding genome and the much richer expressed proteomes. The importance of properly regulated programmes of alternative splicing is underscored in humans by a number of diseases which are caused by mis-regulation of alternative splicing. In these cases, perfectly viable protein-coding mRNAs are produced, but in inappropriate locations, quantities or developmental contexts. Much attention has therefore been focused upon understanding the mechanisms and regulatory circuitry of alternative splicing. In recent years, these efforts have been enhanced by the availability of experimental tools that allow examination of alternative

splicing on a global scale. As a result, it is becoming possible to decipher elements of the "RNA code" that underlies different programmes of regulated alternative splicing.

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## P9.7

### Impact of structured mRNA motifs on alternative splicing in plants

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Recent bioinformatical analyses of the rapidly increasing number of available plant transcript sequences have revealed that a significant proportion of all genes generate alternatively spliced messenger RNAs (mRNAs). Despite the wide distribution of alternative splicing (AS), many fundamental questions such as the biological significance and underlying regulatory mechanisms of this process are largely unsolved in plants. In our work, we could demonstrate that complex mRNA structures can play an essential role in regulation of AS and thereby control gene expression. This will be exemplified by two instances of gene regulation, which are based on highly conserved, structured mRNA motifs. First, feedback control of the thiamin biosynthetic gene *THIC* via AS and alternative 3' end processing by a thiamin pyrophosphate-sensing riboswitch will be outlined. In the second example, the role of a 5S ribosomal RNA mimic in AS of transcription factor IIIA and, in consequence, coordination of the synthesis of ribosomal components will be described. These studies provide novel insight into the impact of mRNA structures on AS in plants, but also raise the question how widespread related mechanisms might be. To address this question, future studies need to combine novel bioinformatical approaches with experimental strategies.

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## P9.8

### The Spen Family Protein FPA can control RNA 3' end Formation Within Introns and This Can Explain its Role in Flowering

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The spen family protein, FPA, is required for flowering time control. The mechanism by which FPA carries out this function is unknown. We have now discovered that

FPA can control selection of poly(A) sites within introns and this can explain its role in flowering.

FPA pre-mRNA is alternatively polyadenylated at promoter distal sites downstream of the stop codon and at promoter proximal sites within FPA intron 1. We have discovered that FPA negatively autoregulates its expression by promoting the selection of this proximal poly(A) site.

We next asked whether such an activity was relevant to the function of FPA in flowering time control. FPA enables flowering by limiting the expression of the floral repressor, FLC. We show that FPA controls poly(A) site selection of antisense RNAs expressed at the FLC locus. These antisense RNAs are capped, alternatively spliced and alternatively polyadenylated. FPA promotes the selection of a proximal poly(A) site located within intron 2 of FLC antisense RNAs and this is associated with weak FLC expression. In *fpa* mutants, increased read-through to a distal poly(A) site antisense to the FLC promoter is detected and this is associated with high levels of sense strand transcription, leading to late flowering.

Our findings suggest that FPA can promote polyadenylation in situations where splicing and polyadenylation reactions are in competition. At FLC, the alternative processing choices of antisense RNAs appears to influence sense strand transcription. We will speculate on the mechanism and generality of this form of gene regulation.

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## P9.9

### Auto- and cross-regulation of polypyrimidine tract-binding protein homologues from Arabidopsis

Eva Stauffer (University of Tübingen)

One in vertebrates well-studied member of the splicing regulatory hnRNP family is the polypyrimidine tract-binding protein (PTB), which binds to CU-rich sequence elements in target precursor mRNAs and thereby influences splice site selection. In plants, several PTB homologues exist, but many questions about their function and putative role as splicing regulators remain to be addressed. We analyzed three PTB homologues from *Arabidopsis thaliana* and provide evidence for extensive auto- and cross-regulation of their expression. Particularly, our results reveal that PTB-mediated expression control is based on alternative splicing as well as downstream, splicing-independent regulatory mechanisms. Furthermore, these multiple modes of action are reflected by their localization in distinct cellular compartments. Our findings highlight the gene regulatory potential of *Arabidopsis* PTB homologues which provides a basis for identification of additional

target mRNAs and, ultimately, aims at gaining novel insight into the functional implications of PTBs in plants.

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15:40 Saturday 3rd July 2010

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## P9.10

### Alternative Splicing mediates the adaptation of the Arabidopsis circadian Clock to Temperature Changes

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Most organisms, including *Arabidopsis thaliana*, have evolved endogenous circadian clocks that harmonise the physiology of the organism to predictable external cues, such as light and temperature cycles. In contrast to the standardised light and temperature growth regimes often applied to laboratory studies of circadian systems, the natural environment in a constant state of flux - and for sessile organisms such as plants there is a constant requirement to adapt to rapid changes in the environment, ranging from modest fluctuations in light intensity and temperature to more severe extremes of, for example, temperature, water availability and salinity. Our fundamental question is - how do plants maintain a regular pace of time keeping in a world of constantly fluctuating environmental conditions? Here, we demonstrate that temperature transitions are associated with alternative splicing of components of the central oscillator of the clock. In particular, splicing of the genes encoding the single Myb-domain transcription factors, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) - components of the central oscillator in Arabidopsis - is modulated by temperature. Abundance of the 'correct' or canonical splice variant falls in low temperature for LHY, but rises for CCA1. Furthermore, a novel exon containing an in-frame stop codon is found in LHY transcripts only in the cold. Overall, alternative splicing appears to modulate the amplitude of oscillations in these clock genes and allows the clock to adapt rapidly to temperature transients.

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11.10 Saturday 3rd July 2010

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## P9.11

### Complex processing of *Arabidopsis thaliana* pri-miRNAs

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Using quantitative real-time PCR platform for parallel quantification of *A. thaliana* pri-miRNAs we have shown strong accumulation of 57 individual miRNA precursors in the *hyl1-2* mutant what suggests that numerous miRNA primary transcripts require the HYL1 (DRB1) protein for their efficient maturation. Accumulation of pri-miRNAs in the *hyl1-2* mutant allowed us to identify the structure of 33 arbitrarily chosen HYL1 - dependent pri-miRNAs and their genes. We found that plant miRNA genes are unexpectedly long and their transcripts undergo complex maturation events. The longest *MIR* gene (*MIR472*) consists of 4975 bp and contains 3 introns. The biggest intron found in *MIR472* gene is 3649 bp long. Analysis of obtained micro RNA precursor sequences revealed the presence of classical U2 introns in 18 out of 33 *MIR* genes. Five of the characterized intron-containing pri-miRNAs undergo alternative splicing events such as: exon skipping, intron retention, 5' and 3' alternative splice sites usage, suggesting that this process may be involved in the regulation of miRNA biogenesis. We also observed that *A. thaliana* pri-miRNAs are polyadenylated in alternative sites and possess putative alternative transcription start sites. Our recent observations show that additional small RNAs (19 - 24 nt long) are processed from 5' regions of some pri-miRNAs. We speculate that these molecules may be involved in the regulation of individual *MIR* gene expression.

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## P9.12

### Does Exon Junction Complex protein eIF4A-III in animal heart cells show the same dynamics in response to hypoxia as in plant cells?

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The Exon Junction Complex (EJC) links mRNA biogenesis (transcription, splicing, export, surveillance) and translation. In *Arabidopsis* cells, the RNA helicase protein eIF4A-III, a component of the EJC, rapidly relocates under hypoxic stress conditions from the nucleoplasm to the nucleolus and nuclear splicing speckles [1, 2]. The nucleoplasmic fraction is highly mobile, while the speckles are the least mobile, and the nucleolar fraction has an intermediate mobility [1]. Sequestration of eIF4A-III into nuclear pools with different mobility is likely to affect the mRNA processing in the cell. The relocation of the EJC proteins and associated mRNAs into the nucleolus and splicing speckles might prevent translation of particular mRNAs into protein with major consequences for cell metabolism and adaptation to environmental changes. This protein is well conserved across most eukaryotes indicating that it plays an important role. The mammalian heart adapts to changes in physiological conditions by altering its gene expression. The heart responds to changes in oxygen and ATP availability as well as to mechanical stress. The cellular mechanisms for oxygen sensing, hypoxia and mechanical stress are poorly understood. We are examining whether eIF4A-III plays a role as a stress sensor in cardiac myocytes and fibroblasts.

#### References:

1. Koroleva et al (2009) *Plant Cell*, 21:1592
2. Koroleva et al (2009) *Plant Signaling & Behavior*, 4:1148

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### P9.17

#### Massive sequencing reveals an unexpected high level of alternative splicing in *Arabidopsis*

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Alternative splicing (AS) is one of the mechanisms involved in the regulation of gene expression. Despite its role in important biological processes like development and stress response, it has remained largely unexplored. AS is the differential processing of exons from a single gene resulting in distinct transcripts that can diverge in structure, function, localization and other aspects contributing in some extent to phenotypic variation. In human, ~92-95% of the multi-exonic genes undergo AS and that the production of the different isoforms in distinct tissues is highly regulated<sup>1</sup>.

Transcriptome studies in plants at genome level are restricted to cDNA/ESTs sequences<sup>2</sup> and few high-throughput sequencing analysis<sup>3,4</sup> which might have resulted in an underestimation of genes that undergone AS in plants. The current estimate is that ~42% of multi-exonic genes in *Arabidopsis* undergo AS<sup>3</sup>, which is much less than estimates for animals. With the aim to discover AS events in *Arabidopsis thaliana* and determine the impact of this process in plant systems, we used a normalized cDNA library from WT plants and subjected it to paired-end read sequencing Solexa/Illumina technology. The preliminary results show that ~27% of the exon-junctions that we retrieved are not annotated, suggesting that data and technological limitation may have driven to an overlook of the extension of AS in plants.

1. Wang *et al.*; (2008); *Nat.* 456:470-476; 2. Iida *et al.*; (2004); *Nucl. Acid. Res.* 32: 5096-5103; 3. Jones-Rhoades *et al.*; (2007); *PLOS Gen.* 3:1848-1861; 4. Filichkin *et al.*; (2010); *Gen. Res.* 20:45-58.

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### P9.13

#### Alternative Splicing in the *Arabidopsis* Core Clock Genes

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Circadian rhythms regulate many aspects of metabolism, physiology and response to various environmental stresses. In plants, the circadian clock regulates about 5% of the genome (>1000 genes in *Arabidopsis*). The rhythmic function of these genes control many processes, including leaf and petal movements, the opening and closing of stomata and many metabolic activities, especially those associated with photosynthesis. Alternative splicing is an important mechanism by which multiple transcripts are made from the same pre-mRNA increasing proteome diversity or regulating expression. Recently, alternative splicing of CCA1 and LHY has been shown to be important in regulating the levels of these proteins (Allan James and Hugh Nimmo, unpublished data). To discover the extent of AS in the core clock genes, we used a pool of RNAs derived from *Arabidopsis* seedlings grown in a range of temperature conditions and light-dark cycles. Cloning and sequencing of RT-PCR products amplified with gene-

specific primers identified 14 novel AS events for 7 of the core clock genes. Many events were intron retentions but examples of exon skipping and the use of alternative 3' or 5' sites were also found. Ten of these novel events introduced a PTC and could be substrates for NMD. Two events in *TOC1* and *CCA1* change the C-terminus of the protein. Fluorescently labelled primers have been made to determine how functional and non-functional isoforms of the core genes changes during the clock cycle and under different light and temperature conditions.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

### P9.14

#### **Arabidopsis snoR146 affects alternative splicing and expression of multiple mRNAs**

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Small nucleolar RNAs are a class of small RNAs which guide nucleotide modifications (2'-O-methyl ribose; pseudouridylation) of ribosomal RNA through base pairing of the snoRNA with rRNA targets. Several 'orphan' snoRNAs, which lack identifiable rRNA targets, have been identified in different organisms. Some orphan snoRNAs have targets within messenger RNA sequences and either affect alternative splicing of those mRNAs or degrade the mRNA via microRNA-like properties. We have identified a novel class of snoRNAs in *Arabidopsis* which contain a number of atypical sequence features as well as a tri-methyl-G cap. These snoRNAs show similarity to the U13 snoRNA of metazoans and also contain regions of complementarity to a number of mRNAs in *Arabidopsis*. Using a bioinformatic approach, we have identified putative mRNA targets with varying degrees of complementarity to the U13-like snoRNAs. In particular, 126 potential mRNA targets were identified for the U13-like RNA, snoR146. We have characterized two *snoR146* mutants and screened putative mRNA targets for changes in splicing patterns or expression level. At least 4 genes are subject to changes in splicing pattern in the *snoR146* mutants, undergoing either alternative splice site selection or intron inclusion events. In addition, around two thirds of the putative targets tested so far showed changes in abundance between wild-type and mutant plants. Together, these data suggest that snoR146 has a non-classical role in regulating mRNA expression. The observation of multiple mRNA targets of snoR146 provides us with a basis for further investigation of this mechanism.

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### P9.15

#### **The SR45 plant-specific splicing factor is a negative regulator of sugar signaling during early growth in *Arabidopsis***

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Serine/arginine-rich (SR) proteins play key roles in pre-mRNA splicing and other aspects of RNA metabolism. SR45 is the sole member of an SR protein *Arabidopsis thaliana* subfamily with no orthologs in animals. A loss-of-function mutant for this gene, *sr45-1*, displays pleiotropic developmental changes under standard growth conditions<sup>1</sup>. Our further characterization of this mutant has shown that SR45 is a novel player in sugar signaling during early seedling development. The *sr45-1* mutation confers hypersensitivity to glucose, with mutant seedlings grown in the presence of the sugar displaying impaired cotyledon development and hypocotyl elongation, as well as altered glucose-responsive gene expression. The *sr45-1* mutant shows enhanced ability to accumulate ABA in response to glucose, and an ABA biosynthesis inhibitor is able to partly rescue the glucose-mediated growth arrest. In accordance, several ABA biosynthesis and signaling genes are over induced in *sr45-1*. Taken together, these results indicate that SR45 negatively regulates glucose signaling by repressing the ABA pathway. Transcriptional regulation of the conserved glucose sensor, *hexokinase 1 (HXK1)*, is unaffected in *sr45-1*, but a sugar-responsive transcription factor, *bZIP63*, appears over-repressed by glucose in the mutant. In order to investigate the genetic interaction between SR45 and these two glucose signaling components, we have generated the corresponding double mutants and their characterization will be discussed.

<sup>1</sup> Ali, G. S. et al., Regulation of plant developmental processes by a novel splicing factor. *PLoS ONE* 2, e471 (2007).

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### P9.16

#### **Study of the subcellular localization and shuttling properties of RSZp22, an *Arabidopsis* SR splicing factor, and role of its RNA-binding domains**

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SR proteins are essential splicing regulators that are known to be involved in both constitutive and alternative splicing. They are characterized by a modular structure consisting of at least one RNA Recognition Motif (RRM domain) and one C-terminal domain rich in Ser and Arg residues (RS domain). Herein, we have investigated the subcellular distribution and dynamics of the *Arabidopsis* RSZp22 SR protein fused to GFP after *Agrobacterium*-mediated transient expression and in *Arabidopsis* transgenics. We provided a detailed analysis of the RSZp22 expression profile and observed the nuclear speckle-like pattern of this protein which surprisingly concentrates in the nucleolus under certain cellular conditions. We have used confocal methods such as Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss In Photobleaching (FLIP) combined to nuclear export inhibition by using leptomycin B and showed that RSZp22 is highly dynamic and shuttles between the nucleus and the cytoplasm through the CRM1/Xpo1 export pathway. RSZp22 is characterized by a particular Zn-knuckle motif which is involved in RNA binding. The role of the RNA-binding motifs in RSZp22 nucleolar targeting, dynamics and nucleocytoplasmic shuttling was analyzed by introducing site-directed point mutations in the Zn-knuckle motif and RRM domain. We showed that these mutant proteins are defective for their export through the CRM1/Xpo1 pathway though they retain their nuclear speckled-pattern localization and shuttling properties. Finally, we demonstrated by Fluorescence Resonance Energy Transfer (FRET) imaging that the RNA-binding motifs are involved in RSZp22 interactions.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

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Using the AS RT-PCR panel we analyzed the alternative splicing (AS) profiles of 435 *Arabidopsis* gene transcripts mainly from transcription factor, splicing factor and stress-related protein genes. The splicing events included alternative 5' and 3' splice sites, exon skipping and intron retention. AS profiles were determined in wild type plants and the cap-binding protein complex mutants: *cbp20*, *cbp80(abh1)* and *cbp20/80*. We found significant changes in the ratios of AS isoforms in 101 genes. Of these, 41% were common to all three CBC mutants and 15% were observed only in the double mutant. We conclude that the CBC is therefore directly involved in AS of some genes. In most cases the CBC influenced AS of the first intron, particularly at the 5' splice site. Moreover, the *cbp80(abh1)* and *cbp20/80* mutants had many more changes in AS in common than did *cbp20* and *cbp20/80*, suggesting that CBP80 plays a more significant role in alternative splicing than CBP20, probably being a platform for interactions with other splicing factors. An influence on AS was also shown for miRNA processing proteins: HYL1, SERRATE and DCL1. We found significant changes in the ratios of splicing isoforms of 26 genes in the three analyzed mutants in comparison to wild type plants. Using *in silico* approaches we are searching plant miRNAs databases and deep-sequencing results to find putative sRNAs that could bind specifically to only one alternatively spliced isoforms. One known microRNA and many 21-24 nt RNAs are potential molecules that can target alternative spliced isoforms.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

## P9.18

### Regulation of alternative splicing in *Arabidopsis thaliana*

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