Many rice genes are differentially spliced between roots and shoots but cytokinin hormone application has minimal effect on splicing

Nowlan Freese[1*], April R. Estrada[1*], Ivory C. Blakley[1], Jinjie Duan[2], Ann Loraine[1**]
[1]Department of Bioinformatics and Genomics, University of North Carolina at Charlotte, Charlotte, NC, USA
[2]Bioinformatics Research Center, Aarhus University, C.F. Moellers Alle 8, Aarhus C, Denmark.

[*]Equal contribution.
[**]Corresponding author.

ABSTRACT

Alternatively spliced genes produce multiple spliced isoforms, called transcript variants. In differential alternative splicing, the relative abundance of transcript variants differs across sample types. Differential alternative splicing is common in animal systems and influences cellular development in many processes, but its extent and significance is not as well known in plants. To investigate alternative splicing in plants, we examined rice seedling RNA-Seq data that included approximately 40 million sequence alignments per library, three libraries per sample type, and four sample types: roots and shoots, each treated with exogenous cytokinin delivered hydroponically, plus mock-treated controls. Cytokinin treatment triggered expression changes in thousands of genes, but had minimal effect on alternative splicing. However, there were many splicing differences between roots and shoots. Analysis of a similar (but less deeply sequenced) RNA-Seq data set from *Arabidopsis* showed the same pattern. Quantitative fragment analysis of reverse transcriptase-PCR products made from newly prepared rice samples confirmed nine of ten differential splicing events between rice roots and shoots. In both the *Arabidopsis* and rice RNA-Seq data sets, most genes annotated as alternatively spliced had small minor variant frequencies, i.e., the less frequently observed forms were supported by fewer than 20% of informative RNA-Seq sequences. Of splicing choices where there was more abundant support for minor forms, most alternative splicing events were located within the protein-coding sequence and also maintained the annotated reading frame. A tool for visualizing protein annotations in the context of genomic sequence (ProtAnnot) together with a genome browser (Integrated Genome Browser) were used to visualize and assess effects of differential splicing on gene function. In general, differentially spliced regions coincided with conserved regions in the encoded proteins, indicating that differential alternative splicing is likely to affect protein function between root and shoot tissue in rice.

INTRODUCTION

Differential splicing of pre-mRNA transcripts, called alternative splicing, enables one gene to produce multiple transcript variants encoding different functions. Alternative splicing is an almost universal phenomenon in higher eukaryotes, occurring to varying degrees in every animal and plant genome examined to date [1, 2]. In animals, differential expression of splice variants has been recruited as a regulatory mechanism in multiple processes, such as sex determination in invertebrates and neuronal differentiation in mammals [2-4].

In plants, less is known about the functional significance and patterns of alternative splicing. However, several trends are apparent. Genes involved in circadian regulation are highly alternatively spliced, often producing multiple splice variants that fluctuate in concert with day/night cycling along with overall transcript abundance [5]. The serine and arginine-rich (SR) family of RNA-binding, splicing regulatory proteins is greatly expanded compared to mammals and includes many plant-specific forms [6-9]. SR transcripts themselves are also highly alternatively spliced in plants, with the relative abundance of these alternative transcripts varying according to environmental stresses and hormones [6, 10-13].

A growing body of evidence indicates that cell and tissue specific regulation of alternative splicing occurs in plants, but its significance and extent is not well established [14-16]. We previously found through analysis of RNA-Seq data from *Arabidopsis* pollen that the relative abundance of splice variants

was similar between leaves and pollen, despite the differences between the two tissues [17]. However, this latter analysis was limited by having just one biological replicate for pollen and only two biological replicates for leaves. A more comprehensive analysis of multiple *Arabidopsis* data sets found a high incidence of isoform switching, in which the identity of the most prevalent variant differs between sample types [18]. However, this splicing diversity may have arisen in part from the heterogeneity of the data sets used, which were produced using rapidly changing (and improving) sequencing technologies at different times by different groups.

In this study, we used a well-replicated RNA-Seq data set from rice to re-examine prevalence of alternative splicing between tissues and hormone (cytokinin) treatment. This data set was previously generated to investigate cytokinin regulation of gene expression in roots and shoots from 10-day old rice seedlings [19]. A parallel study produced an analogous data set from *Arabidopsis* for comparison, but was less deeply sequenced [20]. Both the rice and *Arabidopsis* RNA-Seq data sets included three biological replicates per sample type and four sample types – roots and shoots treated with exogenous cytokinin or a mock, vehicle-only treatment. In both data sets, the treatment triggered differential expression of thousands of genes, with roots affected to a greater degree than shoots.

For most alternatively spliced genes, regardless of whether or not they were differentially spliced, the relative abundance of splicing forms was highly skewed, with most alternatively spliced genes producing one major isoform. Nonetheless, there was a large minority of alternatively spliced genes where minor isoforms were more abundant and therefore seemed likely to affect gene function. We found that the relative abundance of splice variants for most alternatively spliced genes was stable, with very few differentially spliced genes between cytokinin treated and control samples. By contrast, many more genes were differentially spliced between roots and shoot, and most differential splicing occurred within the protein-coding sequence. These results provide new evidence that differential alternative splicing likely contributes to gene function diversification between roots and shoots while playing little role in cytokinin signaling.

MATERIALS & METHODS

RNA-Seq library preparation and sequencing

Rice and *Arabidopsis* samples were prepared and sequenced as described in [19, 20]. Rice seedlings (Nipponbare) were grown hydroponically for ten days in a growth chamber set to 14 hours light (28°C) and 8 hours of dark (23°C) with light intensity 700 mmol m^{-2} s^{-1}. Around six to ten seedlings were grown in the same pot, in four pots. On the tenth day, culture media was replaced with new media containing 5 µM of the cytokinin benzyladenine (BA) or 0.05 mN NaOH as a control. After 120 minutes, roots and shoots were harvested separately. Roots and shoots from treatment or control pots were pooled to form three replicates per treatment. RNA was extracted and used to synthesize twelve libraries from BA-treated and mock-treated roots and shoots. Libraries were sequenced on an Illumina HiSeq instrument for 100 cycles, yielding 100 base, single end reads. Sequence data are available from the Sequence Read Archive under accession SRP04905. Aligned, processed data are available from the Oct. 2011 rice genome assembly IgbQuickload directories at http://www.igbquickload.org.

Arabidopsis plants were grown vertically on 1× Murashige and Skoog agar with 1% sucrose for ten days under continuous illumination in a temperature-controlled growth chamber [20]. BA was applied to ten-day-old plants by immersing seedlings in MS solution containing 5 µM BA dissolved using DMSO (or DMSO without BA as a control) and gently shaken for two hours. Plants were harvested, and shoots and roots were collected separately. Libraries were sequenced on an Illumina HiSeq 2000 instrument for 100x2 cycles, yielding 100 base, paired end reads, to a depth of around 10 million sequenced fragments per library. Sequence data are available from the Sequence Read Archive under SRP049054 (rice data) and SRP059384 (*Arabidopsis* data). Aligned, processed data are available from the TAIR10 (June 2009) *Arabidopsis* genome assembly IgbQuickload directories at http://lorainelab-quickload.scidas.org/rnaseq/.

Data processing

Rice sequences were aligned onto the *O. sativa japonica* genome assembly Os-Nipponbare-Reference-IRGSP-1.0 [21] and *Arabidopsis* sequences were aligned onto the TAIR10 June 2009 release of the *Arabidopsis* genome [22] using TopHat [23] and BowTie2 [24] with maximum intron size set to 5,000 bases. A command-line, Java program called "FindJunctions" was used to identify exon-exon junctions from gapped read alignments in the RNA-Seq data. FindJunctions produces BED format files containing junction features, and the score field of the BED file lists the number of read alignments that supported the junction. Only reads that aligned to a unique location in the genome were considered. Source code and compiled versions of FindJunctions are available from https://bitbucket.org/lorainelab/findjunctions.

Identification of alternative splicing events and differential splicing

To date, there have been two major releases of *O. sativa japonica* gene models: the MSU7 gene set [21] and the RAP-Db gene set [25]. The two gene model sets contain mostly the same data, but the MSU7 gene models appear to be the most heavily used and annotated with Gene Ontology terms. For simplicity, and to take advantage of available functional annotations, we used the MSU7 annotations here. For analysis of *Arabidopsis* data, we used TAIR10 [22] and Araport11 [26] gene models.

Annotated alternative splicing events and the number of reads supporting each alternative were identified using the exon-intron overlap method introduced in [27] and further developed here for use with RNA-Seq data. Exons and introns from pairs of gene models from the same locus were compared to identify alternatively spliced regions. Regions where an intron in one model overlapped an exon in another model on the same strand were identified and used to define mutually exclusive splicing choices (Figure 1). Gene models that included an alternatively spliced region were designated the "L" form (for "Long") with respect to the splicing choice. Likewise, models that lacked an alternatively spliced region were designated "S" (for "Short"). Alternatively spliced regions were labeled according to the type of alternative splicing, as follows. Regions flanked by alternative donor sites were designated "DS" for alternative donor site. Regions flanked by alternative acceptor sites were labeled "AS" for alternative acceptor site. AS and DS events that coincided with exon skipping were labeled "AS/ES" and "DS/ES". Alternatively spliced regions arising from introns that the spliceosome sometimes failed to excise were designated "RI" for retained intron.

For each alternatively spliced region representing two mutually exclusive splicing choices, RNA-Seq read alignments that unambiguously supported one or the other splicing choice were counted. For AS and DS events, only gapped reads that aligned across intron junctions were counted. For RI events, gapped reads that aligned across the retained intron were counted as support for the intron-removed (S) form, and un-gapped reads that overlapped at least 20 bases within the intron were counted as support for the intron-retained (L) form.

For each alternatively spliced region in each biological replicate, the number of reads supporting L or S, but not both, were used to calculate percent-spliced-in (PSI) as N/M*100, where N was the number of reads supporting the L form and M was the number of reads that supported S or L but not both. This is the same as the splicing index described in [28]. To identify differentially spliced regions, a two-sided t-test was used to compare PSI between sample types. Because PSI variance was large for events with small M (very few informative reads), only alternatively spliced regions where M was 10 or more in at least three replicate libraries were tested. A false discovery rate (FDR) was calculated for each test using the method of Benjamini and Hochberg [29], as implemented in the R programming language "p.adjust" method. Alternative splicing events with FDR less than or equal to 0.1 were considered differentially alternatively spliced.

Software used to identify and quantify alternative events is available from https://bitbucket.org/lorainelab/altspliceanalysis. Data analysis code used to analyze RNA-Seq data is available from https://bitbucket.org/lorainelab/ricealtsplice. Data analysis code is implemented as R Markdown files designed to be run in the RStudio development environment. Readers interested in experimenting with different analysis parameters can clone the repository, modify the code, and re-run

153 analyses as desired. RNA-Seq alignments, coverage graphs, and junctions data are available for
154 visualization in Integrated Genome Browser [30].
155
156 **RT-PCR and capillary gel electrophoresis analysis of alternative splicing**
157 Differential alternative splicing detected by analysis of RNA-Seq was re-tested using the reverse
158 transcriptase, PCR-based fragment analysis method described in [31]. Differentially spliced regions
159 identified computationally were PCR-amplified using fluorescently labeled primers and quantified using
160 capillary gel electrophoresis. One benefit of the method is that the results are expressed as relative
161 abundances of splice variants within a sample, thus eliminating the need to normalize using reference
162 genes as in traditional qRT-PCR experiments aimed at measuring overall gene expression.
163 For splicing validation, new rice seedlings equivalent to the mock-treated (control) samples from the
164 RNA-Seq experiment were grown and harvested. Seedlings were grown hydroponically in pots
165 containing either liquid media only or calcined clay granules watered with liquid media as recommended
166 in [32]. After twelve days, plants were removed from the pots and roots and shoots were collected
167 separately. Roots and shoots from the same pot were combined to form paired biological replicates.
168 Samples were flash frozen in nitrogen and stored at -80°C prior to RNA extraction.
169 RNA was extracted using the RNeasy Plant Mini Kit from Qiagen following the manufacturer's
170 instructions. First strand cDNA was synthesized using oligo dT primers and 1 µg of total RNA per 20 µL
171 reaction. PCR amplification of cDNA was performed using primers flanking differentially spliced
172 regions, including one primer labeled with 6-carboxyfluorescein (6-FAM) for amplicon detection during
173 fragment analysis. Cycle parameters included denaturation at 94°C for 2 minutes, followed by 24 cycles
174 of 94°C for 15 sec, 50°C for 30 sec and 70°C for 1 min, with a final elongation step of 72°C for 10
175 minutes. This was essentially the same regime described in [31] but with fewer cycles to ensure reactions
176 were stopped before exiting the logarithmic phase. PCR products were combined with size standards and
177 separated on a 3730 Genetic Analyzer (Life Technologies). Amplicons were quantified using
178 manufacturer-provided software by calculating the area under each amplicon peak. The percentage of the
179 variant containing the alternatively spliced region (%L, see above) was calculated by dividing the long
180 form area by the total area for both long and short forms. Spreadsheets with data exported from the
181 instrument, along with PSI calculations, are available in the project git repository
182 (https://bitbucket.org/lorainelab/ricealtsplice) in a subfolder named "Experimental Testing."
183
184 **RESULTS**
185
186 **Most genes annotated as alternatively spliced favored one dominant isoform**
187 Using the exon-intron overlap method described previously [27], alternative splicing events within
188 each gene were identified and annotated as shown in Figure 1. Following annotation of alternative
189 splicing events, RNA-Seq read alignments from the rice and *Arabidopsis* libraries described in [19] were
190 used to assess alternative splicing in four sample types: roots and shoots from seedlings treated with the
191 cytokinin compound benzyladenine (BA) or with a mock, control treatment. For each alternative splicing
192 event, the number of sequence alignments unambiguously supporting each alternative was counted. These
193 counts were used to calculate percent-spliced-in (PSI), the percentage of read alignments supporting the
194 longer (L) isoform.
195 In the combined data from all libraries from the rice data set, 77% of AS events had at least one read
196 supporting each of the two splicing choices, and 19.8% had support for just one splicing choice. Only
197 2.8% of AS events has no reads supporting either form; these corresponded to genes with low or no
198 expression in any of the sample types tested.
199 In the rice RNA-Seq data set, most genes annotated as alternatively spliced had small minor variant
200 frequencies, i.e., the less frequently observed forms were supported by fewer than 20% of informative
201 RNA-Seq sequences (Figure 2). Nevertheless, there was a large minority of alternative splicing events
202 (around one third) where the minor, less-frequently observed form was more abundant and was supported

203 by at least two out of ten informative alignments. These alternatively spliced regions correspond to the
204 middle, trough-like region of Figure 2.
205
206 **Rice genes with abundant support for both alternative splicing choices exhibit a variety of functions**
207 We used Gene Ontology term enrichment to determine if the subset of genes in rice for which
208 alternatively spliced forms were unusually abundant exhibited enrichment with specific functions or
209 processes, e.g., circadian cycling, in which alternative splicing might play a prominent regulatory.
210 Specifically, we asked if some Gene Ontology terms were significantly enriched with genes containing
211 alternative splicing events in which the minor form frequency was between 20 and 50%, corresponding to
212 the central trough region of Figure 2. Interestingly, we found that these genes exhibited a diversity of
213 gene functions, with no significant enrichment of functional categories. Thus, alternative splicing in
214 which minor forms are highly prevalent appears to affect genes with many functions in rice.
215
216 **Many rice genes are differentially spliced between roots and shoots but cytokinin hormone**
217 **application has minimal effect on splicing**
218 In animals, differential splicing between cell or tissue types contributes to cellular differentiation,
219 especially in the nervous system [33]. Less is known about the role of alternative splicing in regulating
220 cellular differentiation and other processes in plants. Rice shoots and roots are profoundly different
221 tissues, but our previous analysis of this same data set found that many of the same genes were expressed
222 in both [19]. This raises the question of how these two different tissues are able to carry out their
223 specialized roles, and suggest the hypothesis that differential splicing could enable differential functions
224 in genes expressed in both tissues, as proposed in [1].
225 Analysis of the effects of cytokinin treatment on this same data set from rice identified many
226 thousands of genes of that were differentially expressed in response to cytokinin [19]. However, little is
227 known about the role alternative splicing during cytokinin response, except for one study in *Arabidopsis*
228 that reported a shift in splicing of SR protein genes following cytokinin hormone treatment [11].
229 Therefore, we examined differential splicing in the rice RNA-Seq data set comparing root and shoot
230 tissue with or without cytokinin.
231 First, we asked: When an alternatively spliced gene was expressed in two different sample types, was
232 the relative abundance of splice variants the same or different? To address this, we examined correlation
233 of PSI between roots and shoots or between BA-treated versus mock-treated samples (Figure 3). We
234 found that PSI was similar between treated and untreated samples, as revealed by the tighter clustering of
235 scatter plot points in Figures 3A and 3B. This indicated that genes that were alternatively spliced in BA-
236 treated samples were also alternatively spliced in the controls, and that the relative abundance of splice
237 variants was similar. Thus, the cytokinin hormone treatment had minimal effect on splicing. By contrast,
238 there were many genes where the relative abundance of splice variants was different between roots and
239 shoots (Figure 3C). Consistent with Figure 3, statistical testing of PSI differences between sample types
240 identified 90 genes where PSI was significantly different between roots and shoots (FDR ≤ 0.1) but only
241 four and two genes where PSI was different between cytokinin-treated samples and controls in roots and
242 shoots, respectively (See Supplemental Data I). Thus, we observed limited but non-trivial levels of
243 differential alternative splicing between roots and shoots but minimal differential alternative splicing
244 between control and BA-treated samples.
245
246 **Comparison of *Arabidopsis* differential splicing shows similar patterns to rice**
247 To determine whether the observed patterns of differential splicing are similar in other plants, we
248 analyzed splicing in *Arabidopsis* roots and shoots that had also been treated with cytokinin [19]. Due to
249 the *Arabidopsis* libraries not being sequenced to the same depth as the rice libraries, many more splicing
250 events had little or no support. Using the same FDR threshold as with the rice data set (FDR ≤ 0.1), we
251 identified few differentially spliced regions between shoots and roots (3) and none in the control to
252 treatment comparisons. However, PSI was distributed similarly to rice in that most alternatively spliced
253 genes expressed one major isoform (Supplemental Figure 1A). In addition, scatter plots showing average

254 PSI in treated versus untreated samples showed a much tighter cluster of points as compared to scatter
255 plots comparing roots and shoots (Supplemental Figure 1B). Statistical testing of PSI differences
256 confirmed the cytokinin hormone treatment had minimal effect on splicing in *Arabidopsis*. Thus, the
257 general pattern of more differential splicing between tissue types as compared to treatment with
258 exogenous cytokinin appears conserved between rice and *Arabidopsis*.
259
260 **Alternative splicing remodeled protein-coding sequence more often than disrupting it in rice**
261 Alternative splicing can occur within the UTR or protein-coding regions of genes. Interestingly, 67%
262 of the differential splicing between roots and shoots occurred within protein-coding regions (Table I and
263 Supplemental Data I), suggesting that differential splicing is likely to affect gene function at the level of
264 the protein product. In nearly every instance, major and minor isoforms were both detected, with
265 differential splicing observed as a change in the relative abundance of the two forms.
266 Because three bases encode one amino acid, the lengths of spliced coding regions are multiples of
267 three. Thus, when alternatively spliced regions occur in the coding regions of and are not multiples of
268 three, then inclusion of these regions in transcripts is likely to introduce a frame shift, resulting in a
269 premature stop codon and a truncated protein product. As shown in Table I, there was an enrichment of
270 alternatively spliced regions in rice that were evenly divisible by three in coding regions versus non-
271 coding in all subsets of the data. These subsets included all annotated alternatively spliced regions,
272 regions where the minor form was unusually prevalent (the trough region of Figure 2), and differentially
273 spliced regions. Thus, alternative splicing within the coding regions of genes was biased against
274 introducing frame shifts and promoted protein remodeling rather than truncation.
275 To further understand the effects of splicing on protein-coding sequences, we visualized differentially
276 spliced regions together with RNA-Seq alignments, coverage graphs, and inferred junctions using
277 genome browsers. Two genome browsers were used to visualize the data - Integrated Genome Browser
278 [30] and ProtAnnot [34]. Integrated Genome Browser (IGB) was used to examine RNA-Seq read
279 alignments and compare alignments to the annotated gene structures. ProtAnnot, an IGB App, was used
280 to search the InterPro database of conserved protein motifs to find out how (or if) splicing inferred from
281 RNA-Seq data was likely to affect gene function through remodeling of protein motifs as detected by the
282 InterProScan Web service [35].
283 Of the 105 differentially spliced regions, 71 overlapped protein-coding sequence regions, suggesting
284 that in these cases, alternative splicing affected protein function. All but one (70/71) of the differentially
285 spliced regions embedded in coding regions overlapped a predicted functional motif (e.g., a predicted
286 transmembrane helix) or a region found by protein classification systems (e.g., Pfam [36] or PANTHER
287 [37]) to be conserved among members of the same protein family (Supplemental Data I and Figure 4).
288
289 **RT-PCR with capillary gel electrophoresis confirmed differential splicing between rice roots and**
290 **shoots for nine of ten genes tested**
291 We used a method based on capillary gel electrophoresis of fluorescently tagged PCR products to
292 assay alternative splicing of ten genes detected as differentially spliced between rice roots and shoots
293 [31]. New rice seedlings were grown under a close-to-identical replication of the RNA-Seq experiment.
294 Primers were designed to amplify differentially spliced regions, including one primer that was conjugated
295 to a fluorescent tag. Following PCR amplification of cDNA prepared from the new rice samples, products
296 were resolved on a capillary-based sequencer and PSI calculated (Table II). In nine out of ten genes,
297 differential alternative splicing was confirmed. In the one case where differential alternative splicing was
298 not confirmed, there were very few RNA-Seq read alignments covering the differentially spliced region,
299 suggesting this was likely a false positive result. The FDR cutoff used to detect differential splicing in the
300 RNA-Seq data was 0.1, corresponding to 1 in 10 false discoveries, in line with results from the
301 microcapillary-based analysis.
302
303 **DISCUSSION**

304 This study profiled alternative splicing using a high coverage RNA-Seq data from 10-day old,
305 hydroponically-grown rice seedlings treated with a cytokinin hormone. A less-deeply sequenced data set
306 from similarly treated *Arabidopsis* seedlings provided a comparison. We found that cytokinin treatment
307 induced very few splicing changes between treated and untreated controls. However, there were many
308 differences in splicing between untreated roots and shoots, and most of these changed the protein coding
309 region of genes.
310 Palusa et al. found that BA-treatment of *Arabidopsis* seedlings triggered splicing changes in multiple
311 SR genes [11], encoding RNA-binding proteins whose counterparts in metazoans regulated alternative
312 splice site choice. Their study used PCR amplification of cDNA followed by agarose gel electrophoresis
313 to detect changes in splicing and focused on SR protein genes only. Thus, we expected to observe
314 changes in SR gene splicing due to the cytokinin treatment, leading to changes in splicing for many
315 downstream genes. However, no such differential splicing was apparent in either RNA-Seq data set
316 tested. It is possible that the differences in methodology used to measure splicing changes between the
317 two studies (RNA-Seq versus visualization of PCR amplification of cDNA) could account for the
318 differences in observations. However, close examination of SR splicing genes in our dataset revealed no
319 significant differences.
320 One possible explanation of why the cytokinin treatment had minimal effect on splicing was that the
321 treatment itself was ineffective. However, differential expression analysis showed that many genes were
322 up- or down- regulated by the treatment in the two data sets tested – rice and *Arabidopsis* [19]. Fewer
323 genes were detected as differentially expressed in the *Arabidopsis* data set, most likely reflecting higher
324 variability between biological replicates combined with lower sequencing depth as compared to the rice
325 data set. Nevertheless, known cytokinin sensitive genes were regulated in both data sets, showing the
326 cytokinin treatment penetrated plant cells and induced stereotypical cytokinin signaling without also
327 triggering changes in splicing.
328 The relative lack of differential splicing between cytokinin-treated and mock-treated samples suggests
329 that cytokinin signaling does not employ alternative splicing as a regulatory mechanism to the same
330 degree as with other plant hormones, notably abscisic acid (ABA). ABA plays a role in perception and
331 response to stresses, especially desiccation stress [38]. ABA also plays a role in regulating splicing of
332 SR45, an SR-like protein, and SR45 plays a role in regulating downstream splicing of multiple genes
333 [39]. Thus, there is a clear connection between stress signaling and the plant stress hormone ABA.
334 By contrast, cytokinin signaling involves transfer of phosphate groups between successive elements
335 of a phosphorelay signaling pathway culminating in phosophorylation-dependent activation of Myb-type
336 transcription factor proteins called type B ARRs. Cytokinin treatment has no or little effect on
337 transcription of type B ARRs, the key regulators of cytokinin signaling [40, 41]. In addition, type B
338 transcriptional regulators are not highly alternatively spliced. By contrast, a closely related family of
339 similar genes encoding so-called "pseudo-response regulators" have similar sequence to type B ARRs and
340 are highly alternatively spliced [42]. These genes are involved in regulating the circadian clock and have
341 nothing to do with cytokinin signaling.
342 Using the same methods and data set, we identified a relatively large number of genes in rice (90) that
343 were differentially spliced genes between shoots and roots, and we validated nine of ten using fragment
344 analysis and an independent data set. This observation of differential splicing between roots and shoots is
345 important for two key reasons. First, it shows that our data analysis methods can identify differential
346 alternative splicing in a data set. In other words, the roots versus shoots comparison provided a positive
347 control for differential splicing detection. Second, the detection of differential splicing between roots and
348 shoots illuminates the function of alternative splicing in plant cells. For most of the differentially spliced
349 regions, both forms were present, and the difference in relative abundance between forms was often
350 slight, rarely more than 5 or 10% (Supplemental Data I). Our data supports the growing body of evidence
351 that alternative splicing is cell, tissue, and stage specific in plants [14-16, 43], including in roots [44]. It is
352 likely that alternative splicing plays a role in fine-tuning gene function to meet the needs of different plant
353 tissues or cell types where a gene is expressed.

We also examined the prevalence of alternative splicing, independent from differential splicing. That is, we used RNA-Seq read alignments to assess how often annotated alternative splice sites were used in our RNA-Set data sets. For most genes annotated as alternatively spliced, the minor form frequency was typically low, accounting for less than 20% of sequence read alignments across the differentially spliced region. Genes where minor form frequency exceeded 20% exhibited a diversity of functions. Thus, many diverse processes in rice involved alternatively spliced genes in which splice variants were expressed at levels likely to affect gene function.

A major limitation of this study was that we limited our analysis to annotated splice forms and did not attempt to form new transcript models based on the RNA-Seq data. This was done mainly because the libraries used were not strand-specific and attempts to assemble transcripts using transcript assembly tools led to incorrect fusion of neighboring genes and other artifacts (not shown). Future studies will therefore benefit greatly from using better library preparation protocols to simplify and streamline data analysis. Nonetheless, this analysis provides new insight into the role of alternative splicing in plant tissues and hormone response.

CONCLUSION

By analyzing the number of reads that supported different splice variants, we identified examples of differential splicing with confirmation by RT-PCR with capillary gel electrophoresis. There were 90 genes differentially spliced between rice root and shoot tissues, but only four between cytokinin-treated and non-treated samples. For most differential splicing events, the protein-coding regions were affected, strongly suggesting that differential splicing is playing a role in modulating gene function between roots and shoots.

FUNDING AND SUPPORT

This material is based upon work supported by the National Science Foundation through the Postdoctoral Research Fellowship in Biology under Grant No.1523814 awarded to Nowlan Freese and the Plant Genome Research Program award number IOS-1238051. NIH award 2R01GM103463-06A1 to AL supports development of the IGB software.

382 **Table I**. Counts of alternative splicing choices in rice that produce difference regions evenly divisible by
383 three or with remainder of 1 or 2. P-value obtained from a binomial test of the null hypothesis that the
384 true probability of a differentially spliced region having a length divisible by three is 1 in 3 and an
385 alternative hypothesis that the probability is greater than 1 in 3.
386

Alternative Splicing		Divisible by 3	Remainder of 1	Remainder of 2	P-value
Location	Event				
Coding region	Annotated as alternatively spliced	3,248	2,466	2,411	3e-36
UTR	Annotated as alternatively spliced	1,152	1,127	1,113	1
Coding region	Minor form is expressed	173	149	153	8e-6
UTR	Minor form is expressed	34	20	13	0.03
Coding region	Differentially spliced	33	24	18	0.03
UTR	Differentially spliced	6	5	11	0.79

387

388 **Table II.** Differential splicing detected using RNA-Seq and re-tested using capillary gel electrophoresis
389 (CGE) in rice. P is the p-value obtained from comparing roots and shoots PSI from CGE.
390

Gene ID	Description	AS type	Avg. RPKM Expression		RNA-Seq PSI (%L)		CGE PSI (%L)		P-value
			Root	Shoot	Root	Shoot	Root	Shoot	
LOC_Os01g25484	ferrodoxin nitrite reductase	RI	300	131	74.2	31.7	29.9	13	5e-05
LOC_Os01g35580	unknown	AS	55.7	34.9	44.0	66.3	49.9	66.9	2.47e-04
LOC_Os01g45274	carbonic anhydrase	ES	171	1,380	96.8	24.3	97.4	15.4	3e-09
LOC_Os01g51290	protein kinase	RI	49.6	49.9	88.4	95.1	13.3	17.2	0.03466
LOC_Os03g05390	citrate transporter	RI	219	174	86.0	96.3	86	95.5	1.2e-04
LOC_Os12g08260	dehydrogenase E1	RI	8.03	30.3	55.7	2.9	4.1	0.87	3e-05
LOC_Os01g61670	ureidoglycolate hydrolase	DS	59	37.8	78.0	31.0	59	37.8	1.6e-09
LOC_Os05g48040	MATE efflux family protein	DS	7.05	6.72	88.1	100.0	89.4	88.27	0.632
LOC_Os02g05830	ribulose bisphosphate carboxylase	RI	4.79	12.3	88.2	10.1	23.9	3.06	8e-04
LOC_Os06g05110	superoxide dismutase	RI	12.5	39.7	38.5	13.6	22.7	6.5	1.3e-07

391
392

Supplemental Data I. Excel spreadsheet file (SuppTable_PSI.xlsx) is provided as a supplement containing the rice mock root vs mock shoot, mock root vs treated root, and mock shoot vs treated shoot splicing data. It is available from the "Manuscript" folder in the project source code repository (https://bitbucket.org/lorainelab/ricealtsplice). The spreadsheet contains output of R script SplicingAnalysis.Rmd run with "coverage_threshold" parameter set to 10, where coverage_threshold is the number of reads overlapping a differentially spliced region which supports one or the other splicing choice when comparing mutually exclusive splicing choices. The R script resides in the project source code repository in the "AltSplice" subfolder. Data files produced by the script reside in subfolder "AltSplice/results". The spreadsheet SuppTable_PSI.xlsx contains worksheets copied from script output files rootsVshoots_MSU7_10.xlsx, roots_MSU7_10.xlsx, and shoots_MSU7_10.xlsx. The worksheet comparing roots and shoots alternative splicing (from rootsVshoots_MSU7_10.xlsx) was edited by hand to include information obtained from manual examination of differentially spliced genes and regions using Integrated Genome Browser and ProtAnnot. A key describing each column and its meaning is also included. The first column of each data worksheet contains hyperlinked alternatively spliced region identifiers. To use the hyperlinks, users should first download and launch Integrated Genome Browser from http://bioviz.org. Clicking the links sends a message to Integrated Genome Browser instructing it to zoom and scroll to the alternatively spliced region. Users can then open and view RNA-Seq data sets from the study by selecting the RNA-Seq folder in the **Available Data Sets** section of the IGB **Data Access** tab.

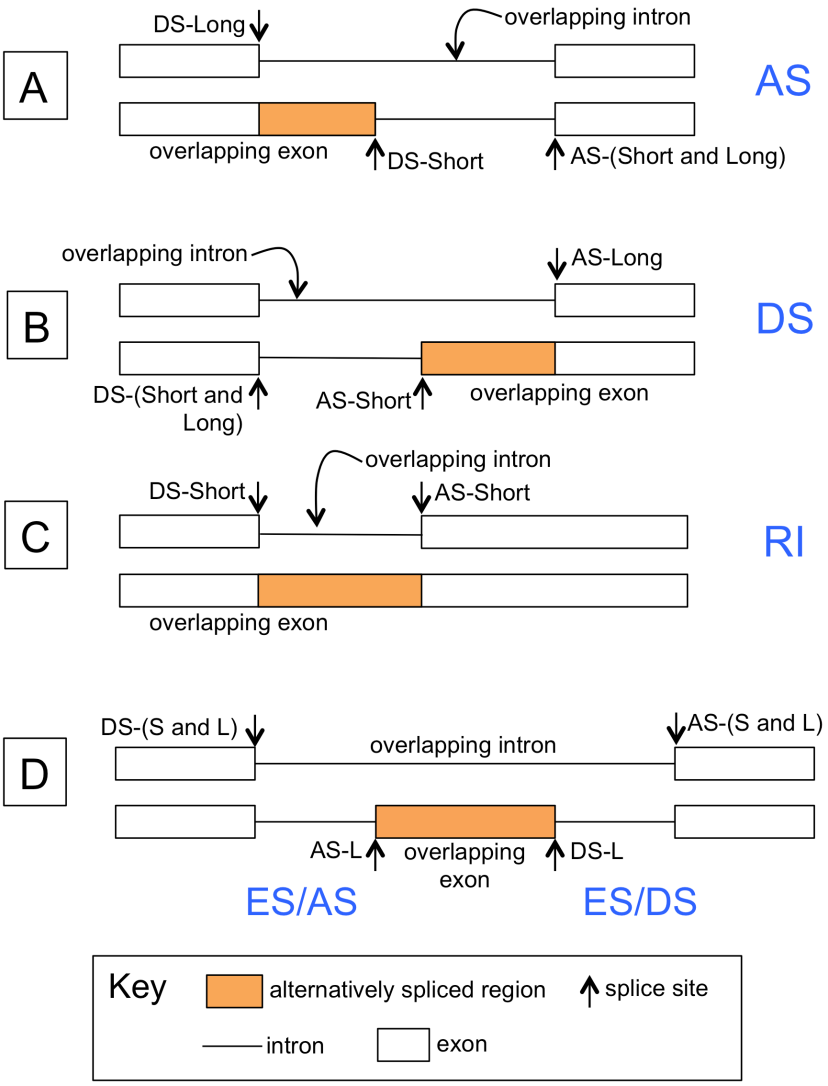
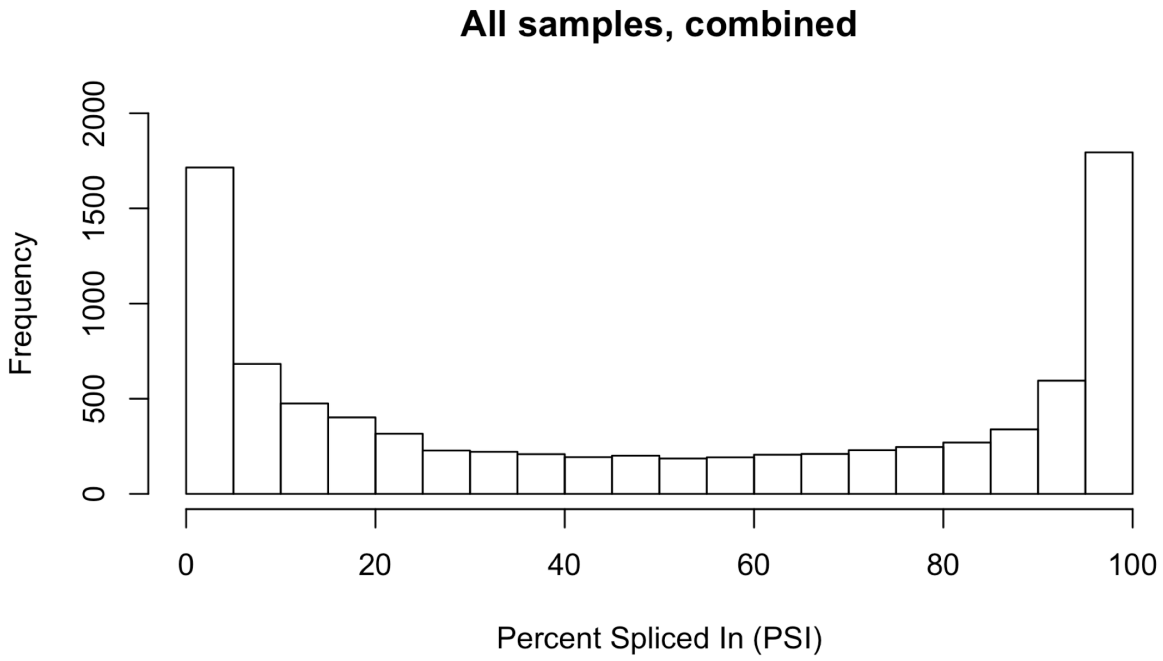


Figure 1. Alternative splicing annotation. The overlap between an intron in one gene model and an exon in another gene model defines an alternatively spliced region. Arrows indicate splice sites, named AS for acceptor site and DS for donor site. Use of sites named AS-L or DS-L causes inclusion of the differentially spliced region, generating the longer (L) isoform. Similarly, DS-S and AS-S refer to sites that exclude the differentially spliced region and generate the shorter (S) isoform. (**A**) Alternative donor sites, in which the U2 snRNP complex forms at alternative locations on the 5' end of introns. (**B**) Alternative acceptor sites, in which the U1 snRNP complex forms at alternative sites near the 3' end of alternatively spliced introns. (**C**) Alternatively spliced intron, in which a donor/acceptor site pairing can either be used or not used, forming a retained intron (RI). (**D**) Alternatively spliced, skipped exon. In exon skipping, alternative splicing involves four sites, indicated by DS-S/L, AS-L, DS-L, and SD-S/L. Exon inclusion requires assembly of two spliceosome complexes linking DS-S/L with AS-L and DS-L with AS-S/L, while exon skipping requires linking DS-S/L and AS-S/L only.



427
428 **Figure 2. Distribution of percent-spliced-in (PSI) for annotated splicing events in rice where each**
429 **choice was supported by at least one RNA-Seq alignment.** PSI was calculated as 100*L/(S+L), where
430 L and S were the number of reads that supported the splicing choice that included (L) or excluded (S) the
431 differentially spliced region. Read alignment counts from all twelve libraries were combined to obtain a
432 global view of alternative splicing occurrence in rice seedlings. The U-shaped character of the distribution
433 persisted whether lower or higher thresholds of informative reads were used.
434

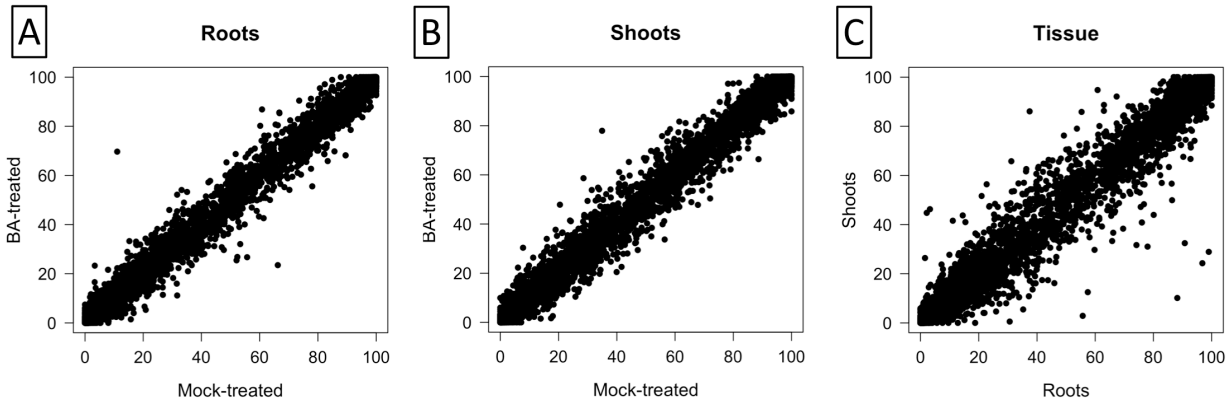


Figure 3. Scatter plots comparing percent- spliced-in (PSI) between sample types in rice for annotated splicing events. PSI was calculated from RNA-Seq reads obtained from sequencing rice seedling shoots and roots grown hydroponically and subjected to a two-hour treatment with BA, a cytokinin analog, or a mock-treatment (control). PSI is the average of three biological replicates. Only events with at least 15 informative read alignments in all six samples being compared were included. (A) BA-treated rice roots (y axis) compared to mock roots (x axis). (B) BA-treated rice shoots (y axis) compared to mock shoots (x axis). (C) Mock shoots (y axis) compared to mock roots (x axis)

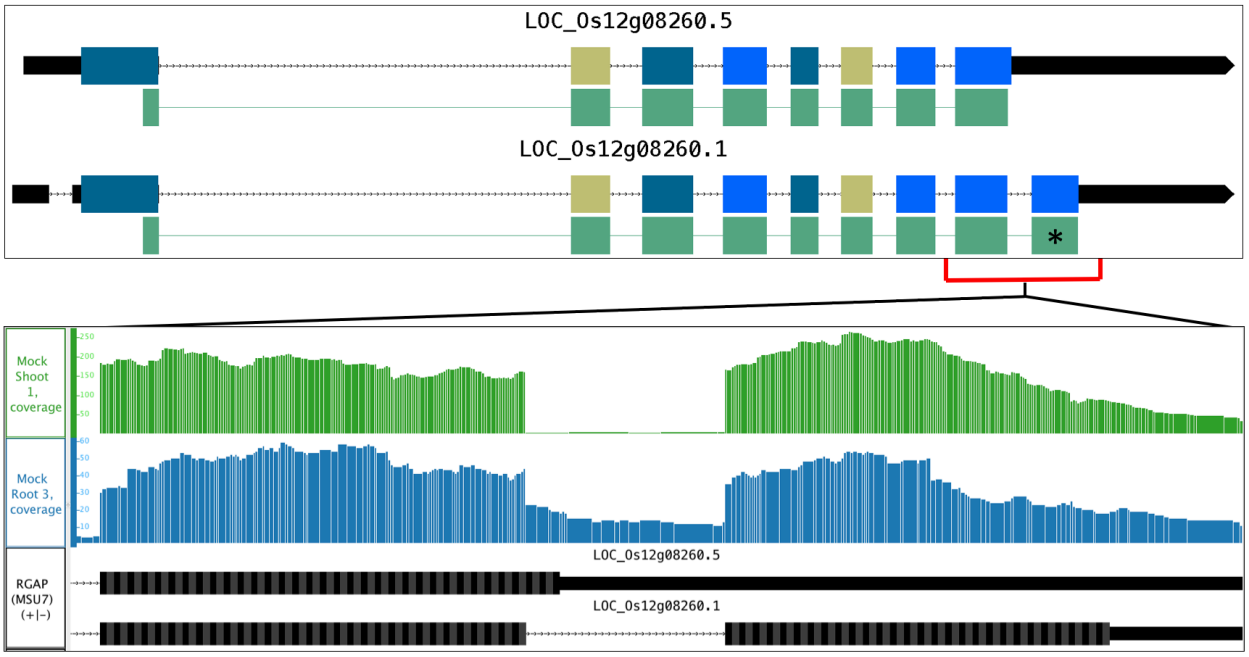
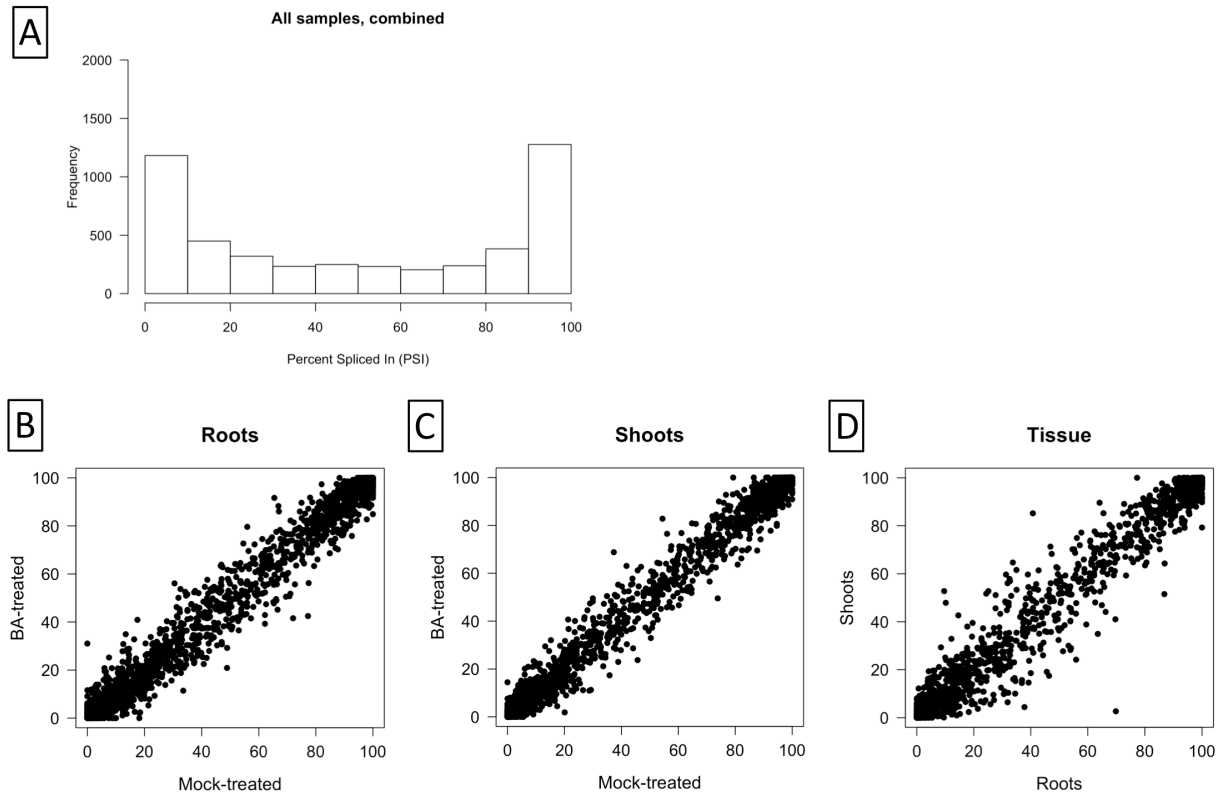


Figure 4. ProtAnnot and IGB images showing difference in splicing between rice shoot and root. ProtAnnot (upper panel) shows coding region exons color-coded by frame, with regions matching InterPro profiles indicated by green, linked rectangles. Asterisk highlights difference in the PANTHER InterPro profile PTHR11516 between isoforms 1 and 5 of the LOC_Os12g08260 gene. Integrated Genome Browser (lower panels) shows a zoomed-in view of RNA-Seq coverage graphs from rice root (blue) and shoot (green). Y-axis is the number of RNA-Seq aligned sequences with MSU7 gene models in black below.



Supplemental Figure 1. Percent-spliced-in (PSI) in *Arabidopsis* RNA-Seq data set between sample types for annotated splicing events. (A) Counts of PSI events in all samples combined. PSI was calculated from RNA-Seq reads obtained from sequencing *Arabidopsis* seedling shoots and roots grown with or without BA, a cytokinin analog. PSI is the average of three biological replicates. Only events with at least 10 informative read alignments in all six samples being compared were included. (B) BA-treated *Arabidopsis* roots (y axis) compared to mock roots (x axis). (C) BA-treated *Arabidopsis* shoots (y axis) compared to mock shoots (x axis). (D) Mock shoots (y axis) compared to mock roots (x axis).

465 **REFERENCES CITED**

466

467 1. Reddy ASN, Marquez Y, Kalyna M, Barta A. Complexity of the Alternative Splicing
468 Landscape in Plants. The Plant Cell. 2013;25(10):3657-83. doi: 10.1105/tpc.113.117523.
469 PubMed PMID: PMC3877793.
470 2. Kalsotra A, Cooper TA. Functional consequences of developmentally regulated
471 alternative splicing. Nature Reviews Genetics. 2011;12(10):715-29. doi: 10.1038/nrg3052.
472 PubMed PMID: PMC3321218.
473 3. Barbosa-Morais NL, Irimia M, Pan Q, Xiong HY, Gueroussov S, Lee LJ, et al. The
474 evolutionary landscape of alternative splicing in vertebrate species. Science (New York, NY).
475 2012;338(6114):1587-93. Epub 2012/12/22. doi: 10.1126/science.1230612. PubMed PMID:
476 23258890.
477 4. Salz HK. Sex Determination in Insects: a binary decision based on alternative splicing.
478 Current opinion in genetics & development. 2011;21(4):395-400. doi:
479 10.1016/j.gde.2011.03.001. PubMed PMID: PMC3134629.
480 5. Filichkin Sergei A, Cumbie Jason S, Dharmawardhana P, Jaiswal P, Chang Jeff H, Palusa
481 Saiprasad G, et al. Environmental Stresses Modulate Abundance and Timing of Alternatively
482 Spliced Circadian Transcripts in Arabidopsis. Molecular Plant. 2015;8(2):207-27. doi:
483 http://dx.doi.org/10.1016/j.molp.2014.10.011.
484 6. Filichkin S, Priest HD, Megraw M, Mockler TC. Alternative splicing in plants: directing
485 traffic at the crossroads of adaptation and environmental stress. Current opinion in plant biology.
486 2015;24:125-35. doi: http://dx.doi.org/10.1016/j.pbi.2015.02.008.
487 7. Plass M, Agirre E, Reyes D, Camara F, Eyras E. Co-evolution of the branch site and SR
488 proteins in eukaryotes. Trends in Genetics. 2008;24(12):590-4. doi:
489 http://dx.doi.org/10.1016/j.tig.2008.10.004.
490 8. Kalyna M, Barta A. A plethora of plant serine/arginine-rich proteins: redundancy or
491 evolution of novel gene functions? Biochemical Society transactions. 2004;32(Pt 4):561-4. doi:
492 10.1042/BST0320561. PubMed PMID: PMC5362061.
493 9. Barbosa-Morais NL, Carmo-Fonseca M, Aparício S. Systematic genome-wide annotation
494 of spliceosomal proteins reveals differential gene family expansion. Genome Research.
495 2006;16(1):66-77. doi: 10.1101/gr.3936206. PubMed PMID: PMC1356130.
496 10. Keller M, Hu Y, Mesihovic A, Fragkostefanakis S, Schleiff E, Simm S. Alternative
497 splicing in tomato pollen in response to heat stress. DNA Research. 2016:dsw051.
498 11. Palusa SG, Ali GS, Reddy ASN. Alternative splicing of pre-mRNAs of Arabidopsis
499 serine/arginine-rich proteins: regulation by hormones and stresses. The Plant Journal.
500 2007;49(6):1091-107. doi: doi:10.1111/j.1365-313X.2006.03020.x.
501 12. Mei W, Boatwright L, Feng G, Schnable JC, Barbazuk WB. Evolutionarily Conserved
502 Alternative Splicing Across Monocots. Genetics. 2017;207(2):465-80. Epub 2017/08/26. doi:
503 10.1534/genetics.117.300189. PubMed PMID: 28839042; PubMed Central PMCID:
504 PMCPMC5629316.
505 13. Gulledge AA, Roberts AD, Vora H, Patel K, Loraine AE. Mining Arabidopsis thaliana
506 RNA-seq data with Integrated Genome Browser reveals stress-induced alternative splicing of the
507 putative splicing regulator SR45a. American journal of botany. 2012;99(2):219-31.
508 14. Sun Y, Hou H, Song H, Lin K, Zhang Z, Hu J, et al. The comparison of alternative
509 splicing among the multiple tissues in cucumber. BMC Plant Biology. 2018;18:5. doi:
510 10.1186/s12870-017-1217-x. PubMed PMID: PMC5755334.

15. Vitulo N, Forcato C, Carpinelli EC, Telatin A, Campagna D, D'Angelo M, et al. A deep survey of alternative splicing in grape reveals changes in the splicing machinery related to tissue, stress condition and genotype. BMC Plant Biology. 2014;14:99-. doi: 10.1186/1471-2229-14-99. PubMed PMID: PMC4108029.

16. Li S, Yamada M, Han X, Ohler U, Benfey PN. High resolution expression map of the Arabidopsis root reveals alternative splicing and lincRNA regulation. Developmental cell. 2016;39(4):508-22. doi: 10.1016/j.devcel.2016.10.012. PubMed PMID: PMC5125536.

17. Loraine AE, McCormick S, Estrada A, Patel K, Qin P. RNA-seq of Arabidopsis pollen uncovers novel transcription and alternative splicing. Plant Physiol. 2013;162(2):1092-109. Epub 2013/04/18. doi: 10.1104/pp.112.211441. PubMed PMID: 23590974; PubMed Central PMCID: PMC3668042.

18. Vaneechoutte D, Estrada AR, Lin YC, Loraine AE, Vandepoele K. Genome-wide characterization of differential transcript usage in Arabidopsis thaliana. The Plant journal : for cell and molecular biology. 2017;92(6):1218-31. Epub 2017/10/17. doi: 10.1111/tpj.13746. PubMed PMID: 29031026.

19. Raines T, Blakley IC, Tsai Y-C, Worthen JM, Franco-Zorrilla JM, Solano R, et al. Characterization of the cytokinin-responsive transcriptome in rice. BMC Plant Biology. 2016;16:260. doi: 10.1186/s12870-016-0932-z. PubMed PMID: PMC5146874.

20. Zubo YO, Blakley IC, Yamburenko MV, Worthen JM, Street IH, Franco-Zorrilla JM, et al. Cytokinin induces genome-wide binding of the type-B response regulator ARR10 to regulate growth and development in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America. 2017;114(29):E5995-E6004. doi: 10.1073/pnas.1620749114. PubMed PMID: PMC5530654.

21. Kawahara Y, de la Bastide M, Hamilton JP, Kanamori H, McCombie WR, Ouyang S, et al. Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. Rice. 2013;6(1):1-10. doi: 10.1186/1939-8433-6-4.

22. Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, et al. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic Acids Research. 2012;40(D1):D1202-D10. doi: 10.1093/nar/gkr1090.

23. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome biology. 2013;14(4):R36. doi: 10.1186/gb-2013-14-4-r36.

24. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature methods. 2012;9:357. doi: 10.1038/nmeth.1923 https://www.nature.com/articles/nmeth.1923#supplementary-information.

25. Sakai H, Lee SS, Tanaka T, Numa H, Kim J, Kawahara Y, et al. Rice Annotation Project Database (RAP-DB): an integrative and interactive database for rice genomics. Plant & cell physiology. 2013;54(2):e6. Epub 2013/01/10. doi: 10.1093/pcp/pcs183. PubMed PMID: 23299411; PubMed Central PMCID: PMC3583025.

26. Cheng CY, Krishnakumar V, Chan AP, Thibaud-Nissen F, Schobel S, Town CD. Araport11: a complete reannotation of the Arabidopsis thaliana reference genome. The Plant journal : for cell and molecular biology. 2017;89(4):789-804. Epub 2016/11/20. doi: 10.1111/tpj.13415. PubMed PMID: 27862469.

27. English AC, Patel KS, Loraine AE. Prevalence of alternative splicing choices in Arabidopsis thaliana. BMC Plant Biol. 2010;10:102. Epub 2010/06/08. doi: 10.1186/1471-2229-10-102. PubMed PMID: 20525311; PubMed Central PMCID: PMC3017808.

28. Katz Y, Wang ET, Airoldi EM, Burge CB. Analysis and design of RNA sequencing experiments for identifying isoform regulation. Nature methods. 2010;7(12):1009-15. Epub 2010/11/09. doi: 10.1038/nmeth.1528. PubMed PMID: 21057496; PubMed Central PMCID: PMCPMC3037023.
29. Benjamini Y, Hochberg Y. Controlling The False Discovery Rate - A Practical And Powerful Approach To Multiple Testing1995. 289-300 p.
30. Freese NH, Norris DC, Loraine AE. Integrated genome browser: visual analytics platform for genomics. Bioinformatics. 2016;32(14):2089-95. Epub 2016/05/07. doi: 10.1093/bioinformatics/btw069. PubMed PMID: 27153568; PubMed Central PMCID: PMC4937187.
31. Stamm S, Smith C, Lührmann R. Alternative pre-mRNA Splicing: Theory and Protocols: Wiley-Blackwell; 2012.
32. Eddy R, Hahn DT, Moulton T. Optimizing Greenhouse Rice Production: What Is the Best Root Medium? Purdue Methods for Rice Growth. 2012.
33. Naftelberg S, Schor IE, Ast G, Kornblihtt AR. Regulation of alternative splicing through coupling with transcription and chromatin structure. Annual review of biochemistry. 2015;84:165-98.
34. Mall T, Eckstein J, Norris D, Vora H, Freese NH, Loraine AE. ProtAnnot: an App for Integrated Genome Browser to display how alternative splicing and transcription affect proteins. Bioinformatics. 2016;32(16):2499-501. Epub 2016/05/07. doi: 10.1093/bioinformatics/btw068. PubMed PMID: 27153567; PubMed Central PMCID: PMC4978921.
35. Finn RD, Attwood TK, Babbitt PC, Bateman A, Bork P, Bridge AJ, et al. InterPro in 2017—beyond protein family and domain annotations. Nucleic Acids Research. 2017;45(Database issue):D190-D9. doi: 10.1093/nar/gkw1107. PubMed PMID: PMC5210578.
36. Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, et al. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Research. 2016;44(D1):D279-D85. doi: 10.1093/nar/gkv1344.
37. Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, et al. PANTHER: A Library of Protein Families and Subfamilies Indexed by Function. Genome Research. 2003;13(9):2129-41. doi: 10.1101/gr.772403.
38. Maia J, Dekkers BJ, Dolle MJ, Ligterink W, Hilhorst HW. Abscisic acid (ABA) sensitivity regulates desiccation tolerance in germinated Arabidopsis seeds. The New phytologist. 2014;203(1):81-93. Epub 2014/04/05. doi: 10.1111/nph.12785. PubMed PMID: 24697728.
39. Cruz TM, Carvalho RF, Richardson DN, Duque P. Abscisic acid (ABA) regulation of Arabidopsis SR protein gene expression. International journal of molecular sciences. 2014;15(10):17541-64. Epub 2014/10/01. doi: 10.3390/ijms151017541. PubMed PMID: 25268622; PubMed Central PMCID: PMCPMC4227177.
40. Argyros RD, Mathews DE, Chiang Y-H, Palmer CM, Thibault DM, Etheridge N, et al. Type B Response Regulators of Arabidopsis Play Key Roles in Cytokinin Signaling and Plant Development. The Plant Cell. 2008;20(8):2102-16. doi: 10.1105/tpc.108.059584.
41. Kieber JJ, Schaller GE. Cytokinin signaling in plant development. Development. 2018;145(4). doi: 10.1242/dev.149344.
42. James AB, Syed NH, Bordage S, Marshall J, Nimmo GA, Jenkins GI, et al. Alternative splicing mediates responses of the Arabidopsis circadian clock to temperature changes. Plant

602 Cell. 2012;24(3):961-81. Epub 2012/03/13. doi: 10.1105/tpc.111.093948. PubMed PMID:
603 22408072; PubMed Central PMCID: PMCPMC3336117.
604 43. Gupta V, Estrada AD, Blakley I, Reid R, Patel K, Meyer MD, et al. RNA-Seq analysis
605 and annotation of a draft blueberry genome assembly identifies candidate genes involved in fruit
606 ripening, biosynthesis of bioactive compounds, and stage-specific alternative splicing.
607 GigaScience. 2015;4:5. Epub 2015/04/02. doi: 10.1186/s13742-015-0046-9. PubMed PMID:
608 25830017; PubMed Central PMCID: PMCPMC4379747.
609 44. Li S, Yamada M, Han X, Ohler U, Benfey PN. High-Resolution Expression Map of the
610 Arabidopsis Root Reveals Alternative Splicing and lincRNA Regulation. Developmental cell.
611 2016;39(4):508-22. Epub 2016/11/15. doi: 10.1016/j.devcel.2016.10.012. PubMed PMID:
612 27840108; PubMed Central PMCID: PMC5125536.
613