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

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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) mRNA-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 mRNA-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 mRNA-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 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]. The same study also produced an analogous data set from Arabidopsis for comparison, but was less deeply sequenced. Both the rice and Arabidopsis mRNA-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]. 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 mM 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 1X MS agar with 1% sucrose for ten days under continuous illumination in a temperature-controlled growth chamber (Raines et al., 2016). BA was applied to ten-day-old plants by immersing seedlings in MS solution containing 5 mM BA or vehicle 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 accession SRP059384. 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 [20] and Arabidopsis sequences were aligned onto the TAIR10 June 2009 release of the Arabidopsis genome (the latest) using TopHat and BowTie2 with maximum intron size was 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 [20] and the RAP-Db gene set [21]. 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 and Araport11 gene models.

Annotated alternative splicing events and the number of reads supporting each alternative were identified using the exon-intron overlap method described in [22]. 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 as support. 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 [23]. 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, 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 analyses as desired. RNA-Seq alignments, coverage graphs, and junctions data are available for visualization in Integrated Genome Browser.

154
155 **RT-PCR and capillary gel electrophoresis analysis of alternative splicing**
156 Differential alternative splicing detected by analysis of RNA-Seq was re-tested using the reverse
157 transcriptase, PCR-based fragment analysis method described in [24]. Differentially spliced regions
158 identified computationally were PCR-amplified using fluorescently labeled primers and quantified using
159 capillary gel electrophoresis. One benefit of the method is that the results are expressed as relative
160 abundances of splice variants within a sample, thus eliminating the need to normalize using reference
161 genes as in traditional qRT-PCR experiments aimed at measuring overall gene expression.
162 For splicing validation, new rice seedlings equivalent to the mock-treated (control) samples from the
163 RNA-Seq experiment were grown and harvested. Seedlings were grown hydroponically in pots
164 containing either liquid media only or calcined clay granules watered with liquid media as recommended
165 in [25]. After twelve days, plants were removed from the pots and roots and shoots were collected
166 separately. Roots and shoots from the same pot were combined to form paired biological replicates.
167 Samples were frozen on liquid nitrogen and stored at -80 degrees C prior to RNA extraction.
168 RNA was extracted using the RNeasy Plant Mini Kit from Qiagen following the manufacturer's
169 instructions. First strand cDNA was synthesized using oligo dT primers and 1 μg of total RNA per 20 μL
170 reaction. PCR amplification of cDNA was performed using primers flanking differentially spliced
171 regions, including one primer labeled with 6-carboxyfluorescein (6-FAM) for amplicon detection during
172 fragment analysis. Cycle parameters included denaturation at 94°C for 2 minutes, followed by 24 cycles
173 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
174 minutes. This was essentially the same regime described in [24] but with fewer cycles to ensure reactions
175 were stopped before exiting the logarithmic phase. PCR products were combined with size standards and
176 separated on a 3730 Genetic Analyzer (Life Technologies). Amplicons were quantified using
177 manufacturer-provided software by calculating the area under each amplicon peak. The percentage of the
178 variant containing the alternatively spliced region (%L, see above) was calculated by dividing the long
179 form area by the total area for both long and short forms. Spreadsheets with data exported from the
180 instrument, along with PSI calculations, are available in the project git repository in a subfolder named
181 "Experimental Testing."
182
183 **RESULTS**
184
185 **Most genes annotated as alternatively spliced favored one dominant isoform**
186 Using the exon-intron overlap method described previously [22], alternative splicing events within
187 each gene were identified and annotated as shown in Figure 1. Following annotation of alternative
188 splicing events, RNA-Seq read alignments from the rice and Arabidopsis libraries described in [19] were
189 used to assess alternative splicing in four sample types: roots and shoots from seedlings treated with the
190 cytokinin compound benzyladenine (BA) or with a mock, control treatment. For each alternative splicing
191 event, the number of sequence alignments unambiguously supporting each alternative was counted. These
192 counts were used to calculate percent-spliced-in (PSI), the percentage of read alignments supporting the
193 longer (L) isoform.
194 In the combined data from all libraries from the rice data set, 77% of AS events had at least one read
195 supporting each of the two splicing choices, and 19.8% had support for just one splicing choice. Only
196 2.8% of AS events has no reads supporting either form; these corresponded to genes with low or no
197 expression in any of the sample types tested.
198 In the rice mRNA-Seq data set, most genes annotated as alternatively spliced had small minor variant
199 frequencies, i.e., the less frequently observed forms were supported by fewer than 20% of informative
200 mRNA-Seq sequences (Figure 2). Nevertheless, there was a large minority of alternative splicing events
201 (around one third) where the minor, less-frequently observed form was more abundant and was supported
202 by at least two out of ten informative alignments. These alternatively spliced regions correspond to the
203 middle, trough-like region of Figure 2.
204

205 **Rice genes with abundant support for both alternative splicing choices exhibit a variety of functions**
206 We used Gene Ontology term enrichment to determine if the subset of genes in rice for which
207 alternatively spliced forms were unusually abundant exhibited enrichment with specific functions or
208 processes, e.g., circadian cycling, in which alternative splicing might play a prominent regulatory.
209 Specifically, we asked if some Gene Ontology terms were significantly enriched with genes containing
210 alternative splicing events in which the minor form frequency was between 20 and 50%, corresponding to
211 the central trough region of Figure 2. Interestingly, we found that these genes exhibited a diversity of
212 gene functions, with no significant enrichment of functional categories. Thus, alternative splicing in
213 which minor forms are highly prevalent appears to affect genes with many functions in rice.
214
215 **Many rice genes are differentially spliced between roots and shoots but cytokinin hormone**
216 **application has minimal effect on splicing**
217 In animals, differential splicing between cell or tissue types contributes to cellular differentiation,
218 especially in the nervous system [26]. Less is known about the role of alternative splicing in regulating
219 cellular differentiation and other processes in plants. Rice shoots and roots are profoundly different
220 tissues, but our previous analysis of this same data set found that many of the same genes were expressed
221 in both [19]. This raises the question of how these two different tissues are able to carry out their
222 specialized roles, and suggest the hypothesis that differential splicing could enable differential functions
223 in genes expressed in both tissues, as proposed in [1].
224 Analysis of the effects of cytokinin treatment on this same data set from rice identified many
225 thousands of genes of that were differentially expressed in response to cytokinin [19]. However, little is
226 known about the role alternative splicing during cytokinin response, except for one study in Arabidopsis
227 that reported a shift in splicing of SR protein genes following cytokinin hormone treatment [11].
228 Therefore, we examined differential splicing in the rice RNA-Seq data set comparing root and shoot
229 tissue with or without cytokinin.
230 First, we asked: When an alternatively spliced gene was expressed in two different sample types, was
231 the relative abundance of splice variants the same or different? To address this, we examined correlation
232 of PSI between roots and shoots or between BA-treated versus mock-treated samples (Figure 3). We
233 found that PSI was similar between treated and untreated samples, as revealed by the tighter clustering of
234 scatter plot points in Figures 3A and 3B. This indicated that genes that were alternatively spliced in BA-
235 treated samples were also alternatively spliced in the controls, and that the relative abundance of splice
236 variants was similar. Thus, the cytokinin hormone treatment had minimal effect on splicing. By contrast,
237 there were many genes where the relative abundance of splice variants was different between roots and
238 shoots (Figure 3C). Consistent with Figure 3, statistical testing of PSI differences between sample types
239 identified 90 genes where PSI was significantly different between roots and shoots (FDR ≤ 0.1) but only
240 four and two genes where PSI was different between cytokinin-treated samples and controls in roots and
241 shoots, respectively (See Supplemental Data I). Thus, we observed limited but non-trivial levels of
242 differential alternative splicing between roots and shoots but minimal differential alternative splicing
243 between control and BA-treated samples.
244
245 **Comparison of *Arabidopsis* differential splicing shows similar patterns to rice**
246 To determine whether the observed patterns of differential splicing are similar in other plants, we
247 analyzed splicing in *Arabidopsis* roots and shoots that had also been treated with cytokinin [19]. Due to
248 the *Arabidopsis* libraries not being sequenced to the same depth as the rice libraries, many more splicing
249 events had little or no support. However, PSI was distributed similarly to rice in that most alternatively
250 spliced genes expressed on major isoform (Supplemental Figure 1A). In addition, scatter plots showing
251 average PSI in treated versus untreated samples showed a much tigher cluster of points as compared to
252 scatter ploud comparing roots and shoots (Supplemental Figure 1B). Thus, the cytokinin hormone
253 treatment had minimal effect on splicing in Arabidopsis. Statistical testing of PSI differences confirmed
254 this trend. Using the same FDR threshold as with the rice data set (FDR ≤ 0.1), we identified few
255 differentially spliced regions between shoots and roots (3) and none in the control to treatment

comparisons. Thus, the general pattern of more differential splicing between tissue types as compared to treatment with exogenous cytokinin appeared conserved between rice and *Arabidopsis*.

Alternative splicing remodeled protein-coding sequence more often than disrupting it in rice

Alternative splicing can occur within the UTR or protein-coding regions of genes. Interestingly, 67% of the differential splicing between roots and shoots occurred within protein-coding regions (Table I and Supplemental Data I), suggesting that differential splicing is likely to affect gene function at the level of the protein product. In nearly every instance, major and minor isoforms were both detected, with differential splicing observed as a change in the relative abundance of the two forms.

In general, when alternatively spliced regions overlap the coding region of genes and the lengths of these regions are not multiples of three, then inclusion of these differential regions in transcripts is likely to introduce a frame shift, resulting in a premature stop codon and a truncated protein product. As shown in Table I, there was an enrichment of alternatively spliced regions in rice that were evenly divisible by three in coding regions versus non-coding in all subsets of the data. These subsets included all annotated alternatively spliced regions, regions where the minor form was unusually prevalent (the trough region of Figure 2), and differentially spliced regions. Thus, alternative splicing within the coding regions of genes was biased against introducing frame shifts and promoted protein remodeling rather than truncation.

To further understand the effects of splicing on protein-coding sequences, we visualized differentially spliced regions together with RNA-Seq alignments, coverage graphs, and inferred junctions using genome browsers. Two genome browsers were used to visualize the data - Integrated Genome Browser [27] and ProtAnnot [28]. Integrated Genome Browser (IGB) was used to examine RNA-Seq read alignments and compare alignments to the annotated gene structures. ProtAnnot, an IGB App, was used to search the InterPro database of conserved protein motifs to find out how (or if) splicing inferred from RNA-Seq data was likely to affect gene function through remodeling of protein motifs as detected by the InterProScan Web service [29].

Of the 105 differentially spliced regions, 71 overlapped protein-coding sequence regions, suggesting that in these cases, alternative splicing affected protein function. All but one (70/71) of the differentially spliced regions embedded in coding regions overlapped a predicted functional motif (e.g., a predicted transmembrane helix) or a region found by protein classification systems (e.g., Pfam [30] or PANTHER [31]) to be conserved among members of the same protein family (Supplemental Data I and Figure 4).

RT-PCR with capillary gel electrophoresis confirmed differential splicing between rice roots and shoots for nine of ten genes tested

We used a method based on capillary gel electrophoresis of fluorescently tagged PCR products to assay alternative splicing of ten genes detected as differentially spliced between rice roots and shoots [24]. New rice seedlings were grown under a close-to-identical replication of the RNA-Seq experiment. Primers were designed to amplify differentially spliced regions, including one primer that was conjugated to a fluorescent tag. Following PCR amplification of cDNA prepared from the new rice samples, products were resolved on a capillary-based sequencer and PSI calculated (Table II). In nine out of ten genes, differential alternative splicing was confirmed. In the one case where differential alternative splicing was not confirmed, there were very few RNA-Seq read alignments covering the differentially spliced region, suggesting this was likely a false positive result. The FDR cutoff used to detect differential splicing in the RNA-Seq data was 0.1, corresponding to 1 in 10 false discoveries, in line with results from the microcapillary-based analysis.

DISCUSSION

This study profiled alternative splicing using a high coverage RNA-Seq data from 10-day old, hydroponically-grown rice seedlings treated with a cytokinin hormone. A less-deeply sequenced data set from similarly treated Arabidopsis seedlings provided a comparison. We found that cytokinin treatment induced very few splicing changes between treated and untreated controls. However, there were many

306 differences in splicing between untreated roots and shoots, and most of these changed the protein coding
307 region of genes.
308 Palusa et al. found that BA-treatment of Arabidopsis seedlings triggered splicing changes in multiple
309 SR genes [11], encoding RNA-binding proteins whose counterparts in metazoans regulated alternative
310 splice site choice. Their study used PCR amplification of cDNA followed by agarose gel electrophoresis
311 to detect changes in splicing and focused on SR protein genes only. Thus, we expected to observe
312 changes in SR gene splicing due to the cytokinin treatment, leading to changes in splicing for many
313 downstream genes. However, no such differential splicing was apparent in either RNA-Seq data set
314 tested. It is possible that the differences in methodology used to measure splicing changes between the
315 two studies (RNA-Seq versus visualization of PCR amplification of cDNA) could account for the
316 differences in observations. However, close examination of SR splicing genes in our dataset revealed no
317 significant differences.
318 One possible explanation of why the cytokinin treatment had minimal effect on splicing was that the
319 treatment itself was ineffective. However, differential expression analysis showed that many genes were
320 up- or down- regulated by the treatment in the two data sets tested – rice and *Arabidopsis* [19]. Fewer
321 genes were detected as differentially expressed in the *Arabidopsis* data set, most likely reflecting higher
322 variability between biological replicates combined with lower sequencing depth as compared to the rice
323 data set. Nevertheless, known cytokinin sensitive genes were regulated in both data sets, showing the
324 cytokinin treatment penetrated plant cells and induced stereotypical cytokinin signaling without also
325 triggering changes in splicing.
326 The relative lack of differential splicing between cytokinin-treated and mock-treated samples suggests
327 that cytokinin signaling does not employ alternative splicing as a regulatory mechanism to the same
328 degree as with other plant hormones, notably abscisic acid (ABA). ABA plays a role in perception and
329 response to stresses, especially desiccation stress [32]. ABA also plays a role in regulating splicing of
330 SR45, an SR-like protein, and SR45 plays a role in regulating downstream splicing of multiple genes
331 [33]. Thus, there is a clear connection between stress signaling and the plant stress hormone ABA.
332 By contrast, cytokinin signaling involves transfer of phosphate groups between successive elements
333 of a phosphorelay signaling pathway culminating in phosophorylation-dependent activation of Myb-type
334 transcription factor proteins called type B ARRs. Cytokinin treatment has no or little effect on
335 transcription of type B ARRs, the key regulators of cytokinin signaling [34,35]. In addition, type B
336 transcriptional regulators are not highly alternatively spliced. By contrast, a closely related family of
337 similar genes encoding so-called "pseudo-response regulators" have similar sequence to type B ARRs and
338 are highly alternatively spliced [36]. These genes are involved in regulating the circadian clock and have
339 nothing to do with cytokinin signaling.
340 Using the same methods and data set, we identified a relatively large number of genes in rice (90) that
341 were differentially spliced genes between shoots and roots, and we validated nine of ten using fragment
342 analysis and an independent data set. This observation of differential splicing between roots and shoots is
343 important for two key reasons. First, it shows that our data analysis methods can identify differential
344 alternative splicing in a data set. In other words, the roots versus shoots comparison provided a positive
345 control for differential splicing detection. Second, the detection of differential splicing between roots and
346 shoots illuminates the function of alternative splicing in plant cells. For most of the differentially spliced
347 regions, both forms were present, and the difference in relative abundance between forms was often
348 slight, rarely more than 5 or 10% (Supplemental Data I). Our data supports the growing body of evidence
349 that alternative splicing is cell, tissue, and stage specific in plants [14-16,37], including in roots [38]. It is
350 likely that alternative splicing plays a role in fine-tuning gene function to meet the needs of different plant
351 tissues or cell types where a gene is expressed.
352 We also examined the prevalence of alternative splicing, independent from differential splicing. That
353 is, we used RNA-Seq read alignments to assess how often annotated alternative splice sites were used in
354 our RNA-Set data sets. For most genes annotated as alternatively spliced, the minor form frequency was
355 typically low, accounting for less than 20% of sequence read alignments across the differentially spliced
356 region. Genes where minor form frequency exceeded 20% exhibited a diversity of functions. Thus, many

357 diverse processes in rice involved alternatively spliced genes in which splice variants were expressed at
358 levels likely to affect gene function.
359 A major limitation of this study was that we limited our analysis to annotated splice forms and did not
360 attempt to form new transcript models based on the RNA-Seq data. This was done mainly because the
361 libraries used were not strand-specific and attempts to assemble transcripts using transcript assembly tools
362 led to incorrect fusion of neighboring genes and other artifacts (not shown). Future studies will therefore
363 benefit greatly from using better library preparation protocols to simplify and streamline data analysis.
364 Nonetheless, this analysis provides new insight into the role of alternative splicing in plant tissues and
365 hormone response.
366
367 **CONCLUSION**
368 By analyzing the number of reads that supported different splice variants, we identified examples of
369 differential splicing with confirmation by RT-PCR with capillary gel electrophoresis. There were 90
370 genes differentially spliced between rice root and shoot tissues, but only four between cytokinin-treated
371 and non-treated samples. For most differential splicing events, the protein-coding regions were affected,
372 strongly suggesting that differential splicing is playing a role in modulating gene function between roots
373 and shoots.
374
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379 supports development of the IGB software.

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

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

385

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

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

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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.

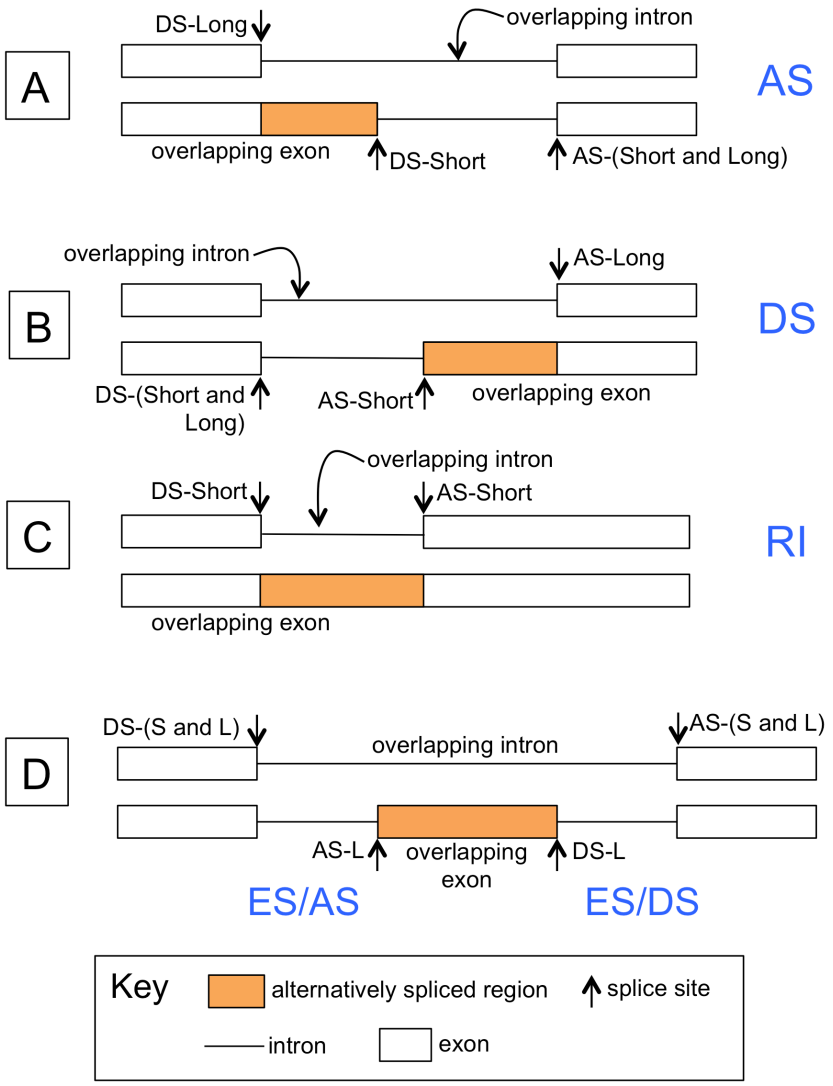
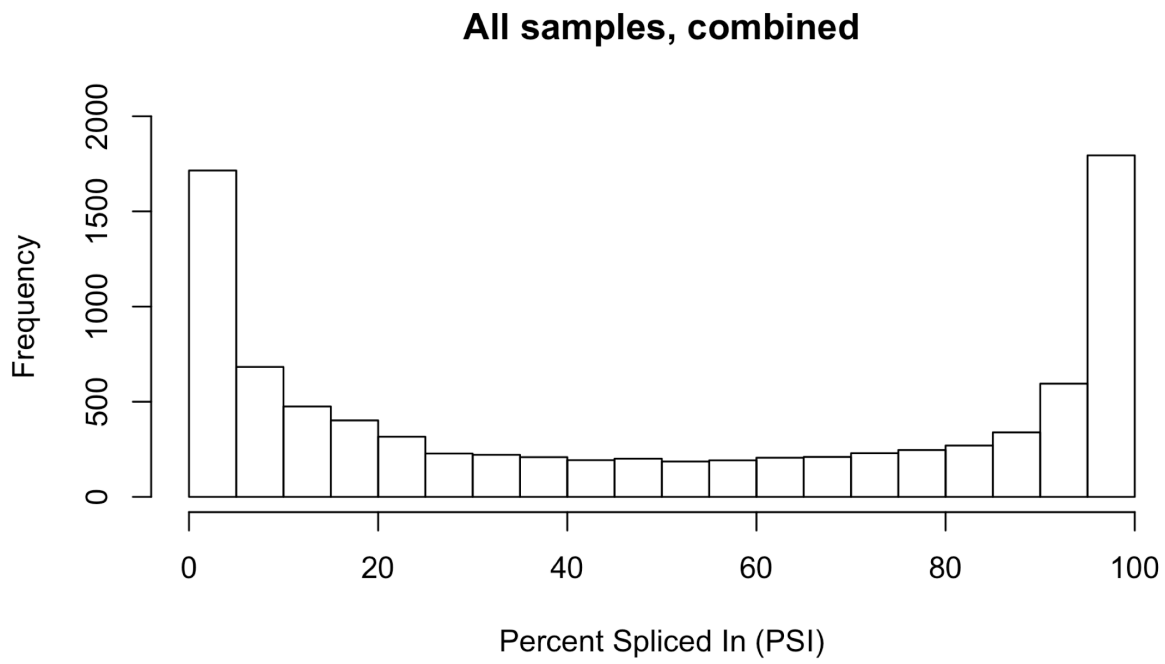


Figure 1. Alternative splicing annotation. The overlap between an intron in one gene model and an exon in another gene model defines an alternatively spliced region. Arrows indicate splice sites, named AS for acceptor site and DS for donor site. Use of sites named AS-L or DS-L causes inclusion of the 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.



425
426 **Figure 2. Distribution of percent-spliced-in (PSI) for annotated splicing events in rice where each**
427 **choice was supported by at least one RNA-Seq alignment.** PSI was calculated as 100*L/(S+L), where
428 L and S were the number of reads that supported the splicing choice that included (L) or excluded (S) the
429 differentially spliced region. Read alignment counts from all twelve libraries were combined to obtain a
430 global view of alternative splicing occurrence in rice seedlings. The U-shaped character of the distribution
431 persisted whether lower or higher thresholds of informative reads were used.
432

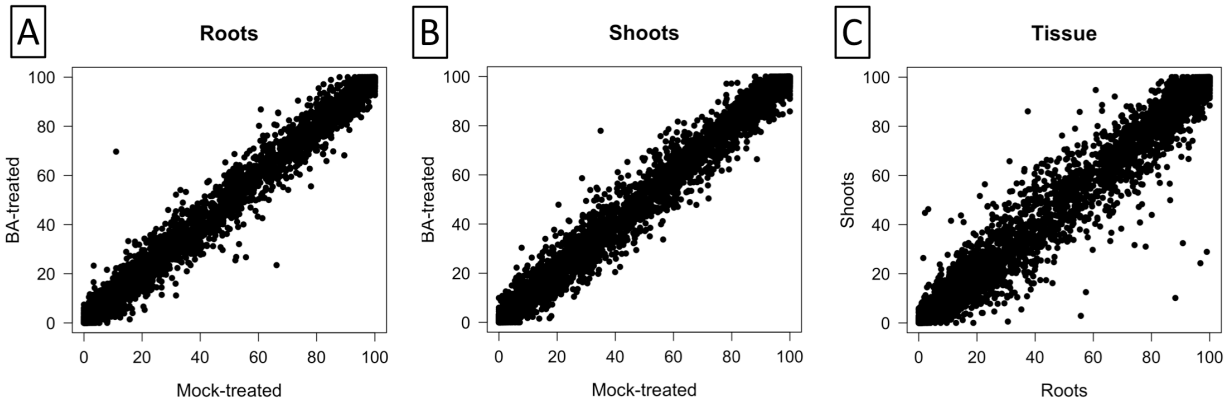


Figure 3. Scatter plots comparing percent- spliced-in (PSI) between sample types in rice for annotated splicing events. PSI was calculated from RNA-Seq reads obtained from sequencing rice seedling shoots and roots grown hydroponically and subjected to a two-hour treatment with BA, a cytokinin analog, 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)

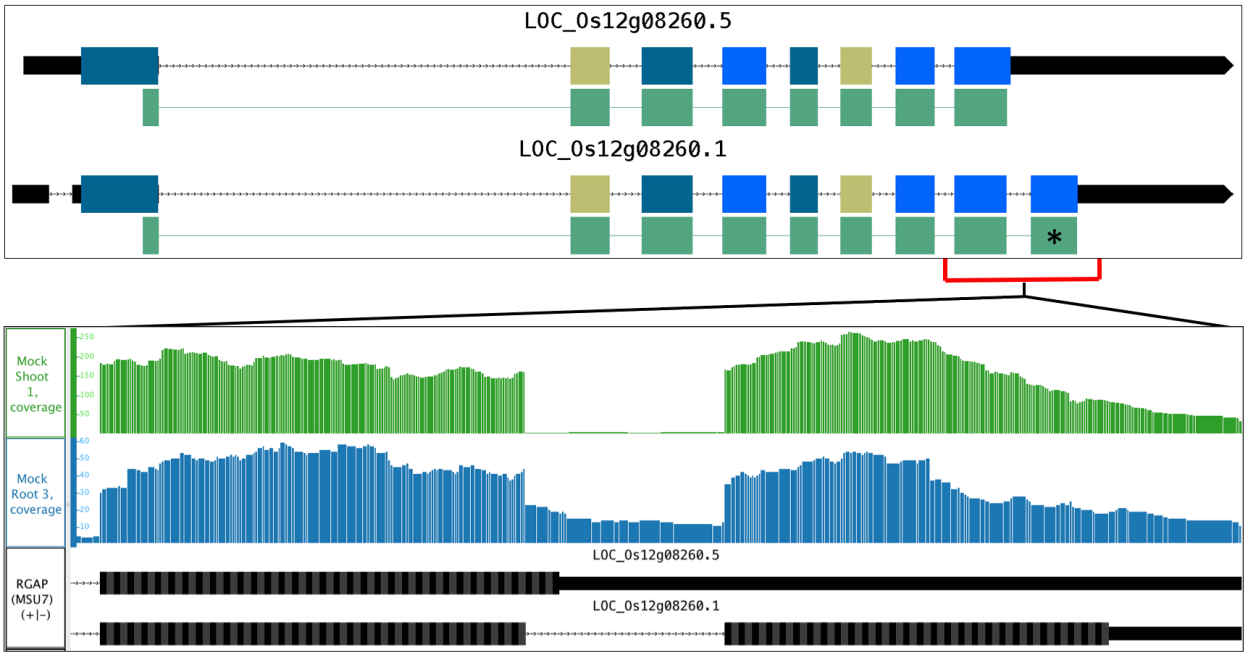
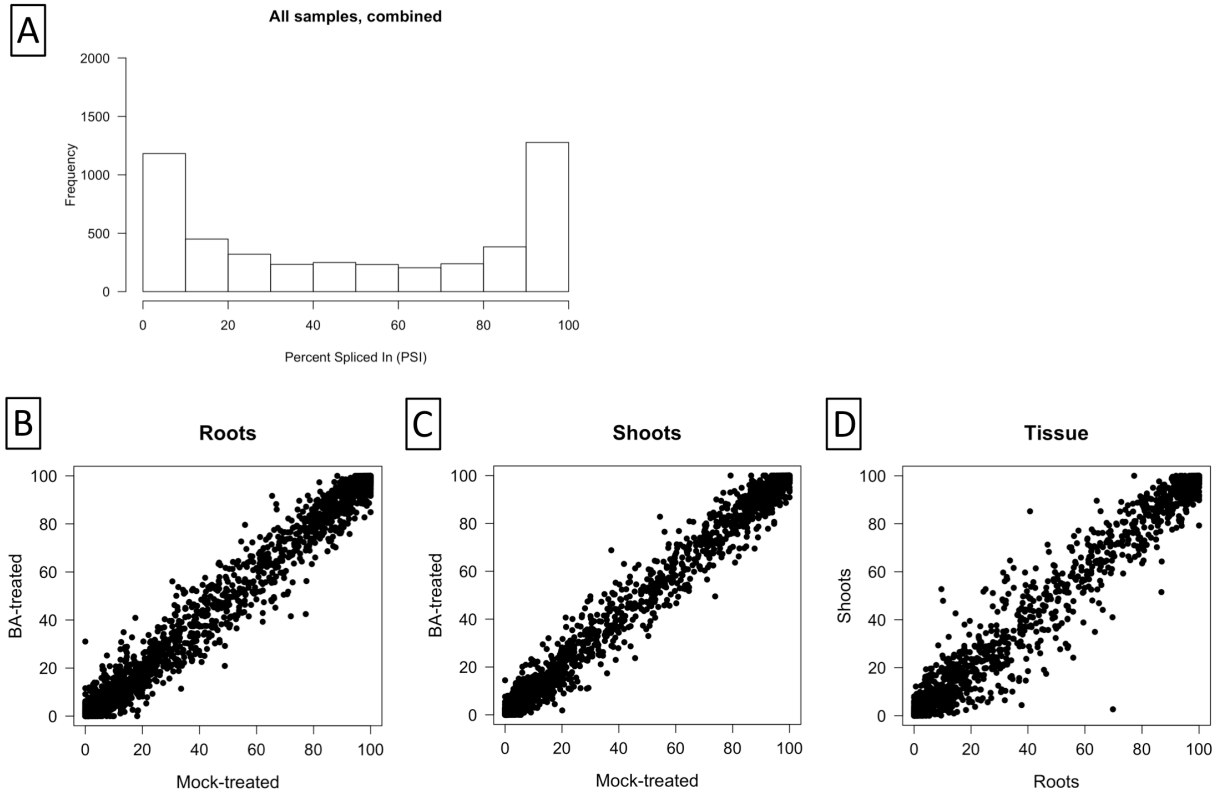


Figure 4. ProtAnnot and IGB images showing difference in splicing between rice shoot and root. ProtAnnot (upper panel) shows coding region exons color-coded by frame, with regions matching InterPro profiles indicated by green, linked rectangles. Asterisk highlights difference in the PANTHER InterPro 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.



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453
454 **Supplemental Figure 1. Percent-spliced-in (PSI) in Arabidopsis RNA-Seq data set between sample**
455 **types for annotated splicing events in *Arabidopsis*.** PSI was calculated from RNA-Seq reads obtained
456 from sequencing *Arabidopsis* seedling shoots and roots grown hydroponically and subjected to a two-
457 hour treatment with BA, a cytokinin analog, or a mock-treatment (control). PSI is the average of three
458 biological replicates. Only events with at least 10 informative read alignments in all six samples being
459 compared were included. (B) BA-treated *Arabidopsis* roots (y axis) compared to mock roots (x axis). (C)
460 BA-treated *Arabidopsis* shoots (y axis) compared to mock shoots (x axis). (C) Mock shoots (y axis)
461 compared to mock roots (x axis).
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