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

Running title: Differential splicing in rice

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ABSTRACT

Alternatively spliced genes produce multiple spliced isoforms, called transcript variants. In differential alternative splicing, transcript variant abundance 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 RNA-Seq data from rice seedlings. The data included three biological replicates per sample type, approximately 30 million sequence alignments per replicate, and four sample types: roots and shoots treated with exogenous cytokinin delivered hydroponically or a mock treatment. Cytokinin treatment triggered expression changes in thousands of genes but had negligible effect on splicing patterns. However, many genes were alternatively spliced between mock-treated roots and shoots, indicating that our methods were sufficiently sensitive to detect differential splicing in a data set. 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. Differential alternative splicing typically changed the relative abundance of splice variants that co-occurred in a data set. Analysis of a similar (but less deeply sequenced) RNA-Seq data set from *Arabidopsis* showed the same pattern. In both the *Arabidopsis* and rice RNA-Seq data sets, most genes annotated as alternatively spliced had small minor variant frequencies. Of splicing choices with abundant support for minor forms, most alternative splicing events were located within the protein-coding sequence and 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.

Key words: rice, alternative splicing, cytokinin, root, shoot, RNA-Seq

Abbreviations: SR - Serine/Arginine, BA - benzyladenine, L - long, S - short, FDR - false discovery rate, PSI - percent spliced in, AS - acceptor site, DS - donor site, ES - exon skipping, RI - retained intron, ABA - abscisic acid, IGB - Integrated Genome Browser, CGE - capillary gel electrophoresis, RPKM - Reads Per Kilobase per Million mapped reads

52 **INTRODUCTION**

53 Differential splicing of pre-mRNA transcripts, called alternative splicing, enables one gene to
54 produce multiple transcript variants encoding different functions. Alternative splicing is an
55 almost universal phenomenon in higher eukaryotes, occurring to varying degrees in every animal
56 and plant genome examined to date (Kalsotra and Cooper, 2011; Reddy *et al.*, 2013). In animals,
57 differential expression of splice variants has been recruited as a regulatory mechanism in
58 multiple processes, such as sex determination in invertebrates and neuronal differentiation in
59 mammals (Kalsotra and Cooper, 2011; Salz, 2011; Barbosa-Morais *et al.*, 2012).

60 In plants, less is known about the functional significance and patterns of alternative splicing.
61 However, several trends are apparent. Genes involved in circadian regulation are highly
62 alternatively spliced, often producing multiple splice variants that fluctuate in concert with
63 day/night cycling along with overall transcript abundance (Filichkin *et al.*, 2015b). The serine
64 and arginine-rich (SR) family of RNA-binding, splicing regulatory proteins is greatly expanded
65 compared to mammals and includes many plant-specific forms (Kalyna and Barta, 2004;
66 Barbosa-Morais *et al.*, 2006; Plass *et al.*, 2008; Filichkin *et al.*, 2015a). SR transcripts
67 themselves are also highly alternatively spliced in plants, with the relative abundance of these
68 alternative transcripts varying according to environmental stresses and hormones (Palusa *et al.*,
69 2007; Gulledge *et al.*, 2012; Filichkin *et al.*, 2015a; Keller *et al.*, 2016; Mei *et al.*, 2017).

70 A growing body of evidence indicates that cell and tissue specific regulation of alternative
71 splicing occurs in plants, but its significance and extent is not well established (Vitulo *et al.*,
72 2014; Li *et al.*, 2016a; Sun *et al.*, 2018). We previously found through analysis of RNA-Seq data
73 from *Arabidopsis* pollen that the relative abundance of splice variants was similar between
74 leaves and pollen, despite the differences between the two tissues (Loraine *et al.*, 2013).
75 However, this latter analysis was limited by having just one biological replicate for pollen and
76 only two biological replicates for leaves. A more comprehensive analysis of multiple
77 *Arabidopsis* data sets found a high incidence of isoform switching, in which the identity of the
78 most prevalent variant differs between sample types (Vaneechoutte *et al.*, 2017). However, this
79 splicing diversity may have arisen in part from the heterogeneity of the data sets used, which
80 were produced using rapidly changing (and improving) sequencing technologies at different
81 times by different groups.

82 In this study, we used a well-replicated RNA-Seq data set from rice to re-examine prevalence
83 of alternative splicing between tissues and hormone (cytokinin) treatment. This data set was
84 previously generated to investigate cytokinin regulation of gene expression in roots and shoots
85 from 10-day old rice seedlings (Raines *et al.*, 2016). A parallel study produced an analogous data
86 set from *Arabidopsis* for comparison, but was less deeply sequenced (Zubo *et al.*, 2017). Both
87 the rice and *Arabidopsis* RNA-Seq data sets included three biological replicates per sample type
88 and four sample types – roots and shoots treated with exogenous cytokinin or a mock, vehicle-
89 only treatment. In both data sets, the treatment triggered differential expression of thousands of
90 genes, with roots affected to a greater degree than shoots.
91 For most alternatively spliced genes, regardless of whether or not they were differentially
92 spliced, the relative abundance of splicing forms was highly skewed, with most alternatively
93 spliced genes producing one major isoform. Nonetheless, there was a large minority of
94 alternatively spliced genes where minor isoforms were more abundant and therefore seemed
95 likely to affect gene function. We found that the relative abundance of splice variants for most
96 alternatively spliced genes was remarkably stable, with very few differentially spliced genes
97 between cytokinin treated and control samples. By contrast, many more genes were differentially
98 spliced between roots and shoot, and most differential splicing occurred within the protein-
99 coding sequence. Moreover, nearly every differential splicing event detected merely change the
100 relative abundance of splice variants that co-occurred in the same sample. These results suggest
101 differential alternative splicing likely contributes to gene function diversification between roots
102 and shoots by moderating the relative abundance of splice co-expressed splice variants, but
103 alternative splicing plays little role in cytokinin signaling.
104
105 **MATERIALS & METHODS**
106
107 **RNA-Seq library preparation and sequencing**
108 Rice and *Arabidopsis* samples were prepared and sequenced as described in (Raines *et al.*,
109 2016; Zubo *et al.*, 2017). Rice seedlings (Nipponbare) were grown hydroponically for ten days in
110 a growth chamber set to 14 hours light (28°C) and 8 hours of dark (23°C) with light intensity 700
111 mmol m^{-2} s^{-1}. Around six to ten seedlings were grown in the same pot, in four pots. On the tenth
112 day, culture media was replaced with new media containing 5 μM of the cytokinin

113 benzyladenine (BA) or 0.05 mN NaOH as a control. After 120 minutes, roots and shoots were
114 harvested separately. Roots and shoots from treatment or control pots were pooled to form three
115 replicates per treatment. RNA was extracted and used to synthesize twelve libraries from BA-
116 treated and mock-treated roots and shoots. Libraries were sequenced on an Illumina HiSeq
117 instrument for 100 cycles, yielding 100 base, single end reads. Sequence data are available from
118 the Sequence Read Archive under accession SRP04905. Aligned, processed data are available
119 from the Oct. 2011 rice genome assembly IgbQuickload directorie at
120 http://igbquickload.org/quickload/O_sativa_japonica_Oct_2011/.
121 *Arabidopsis* plants were grown vertically on 1× Murashige and Skoog agar with 1% sucrose
122 for ten days under continuous illumination in a temperature-controlled growth chamber (Zubo *et*
123 *al.*, 2017). BA was applied to ten-day-old plants by immersing seedlings in MS solution
124 containing 5 μM BA dissolved using dimethyl sulfoxide (DMSO), or DMSO without BA as a
125 control, and gently shaken for two hours. Plants were harvested, and shoots and roots were
126 collected separately. Libraries were sequenced on an Illumina HiSeq 2000 instrument for 100x2
127 cycles, yielding 100 base, paired end reads, to a depth of around 10 million sequenced fragments
128 per library. Sequence data are available from the Sequence Read Archive under SRP049054 (rice
129 data) and SRP059384 (*Arabidopsis* data). Aligned, processed data are available from the
130 TAIR10 (June 2009) *Arabidopsis* genome assembly IgbQuickload directories at http://lorainelab-
131 quickload.scidas.org/rnaseq/.
132
133 **Data processing**
134 Rice sequences were aligned onto the *O. sativa japonica* genome assembly Os-Nipponbare-
135 Reference-IRGSP-1.0 (Kawahara *et al.*, 2013) and *Arabidopsis* sequences were aligned onto the
136 TAIR10 June 2009 release of the *Arabidopsis* genome (Lamesch *et al.*, 2012) using TopHat
137 (Kim *et al.*, 2013) and BowTie2 (Langmead and Salzberg, 2012) with maximum intron size set
138 to 5,000 bases. A command-line, Java program called "FindJunctions" was used to identify
139 exon-exon junctions from gapped read alignments in the RNA-Seq data. FindJunctions produces
140 BED format files containing junction features, and the score field of the BED file lists the
141 number of read alignments that supported the junction. Only reads that aligned to a unique
142 location in the genome were considered. Source code and compiled versions of FindJunctions are
143 available from https://bitbucket.org/lorainelab/findjunctions.

144

145 **Identification of alternative splicing events and differential splicing**

146 To date, there have been two major releases of *O. sativa japonica* gene models: the MSU7
147 gene set (Kawahara *et al.*, 2013) and the RAP-Db gene set (Sakai *et al.*, 2013). The two gene
148 model sets contain mostly the same data, but the MSU7 gene models appear to be the most
149 heavily used and annotated with Gene Ontology terms. For simplicity, and to take advantage of
150 available functional annotations, we used the MSU7 annotations here. For analysis of
151 *Arabidopsis* data, we used TAIR10 (Lamesch *et al.*, 2012) and Araport11 (Cheng *et al.*, 2017)
152 gene models.

153 Annotated alternative splicing events and the number of reads supporting each alternative
154 were identified using the exon-intron overlap method introduced in (English *et al.*, 2010) and
155 further developed here for use with RNA-Seq data. Exons and introns from pairs of gene models
156 from the same locus were compared to identify alternatively spliced regions. Regions where an
157 intron in one model overlapped an exon in another model on the same strand were identified and
158 used to define mutually exclusive splicing choices (Fig. 1). Gene models that included an
159 alternatively spliced region were designated the "L" form (for "Long") with respect to the
160 splicing choice. Likewise, models that lacked an alternatively spliced region were designated "S"
161 (for "Short"). Alternatively spliced regions were labeled according to the type of alternative
162 splicing, as follows. Regions flanked by alternative donor sites were designated "DS" for
163 alternative donor site. Regions flanked by alternative acceptor sites were labeled "AS" for
164 alternative acceptor site. AS and DS events that coincided with exon skipping were labeled
165 "AS/ES" and "DS/ES". Alternatively spliced regions arising from introns that the spliceosome
166 sometimes failed to excise were designated "RI" for retained intron.

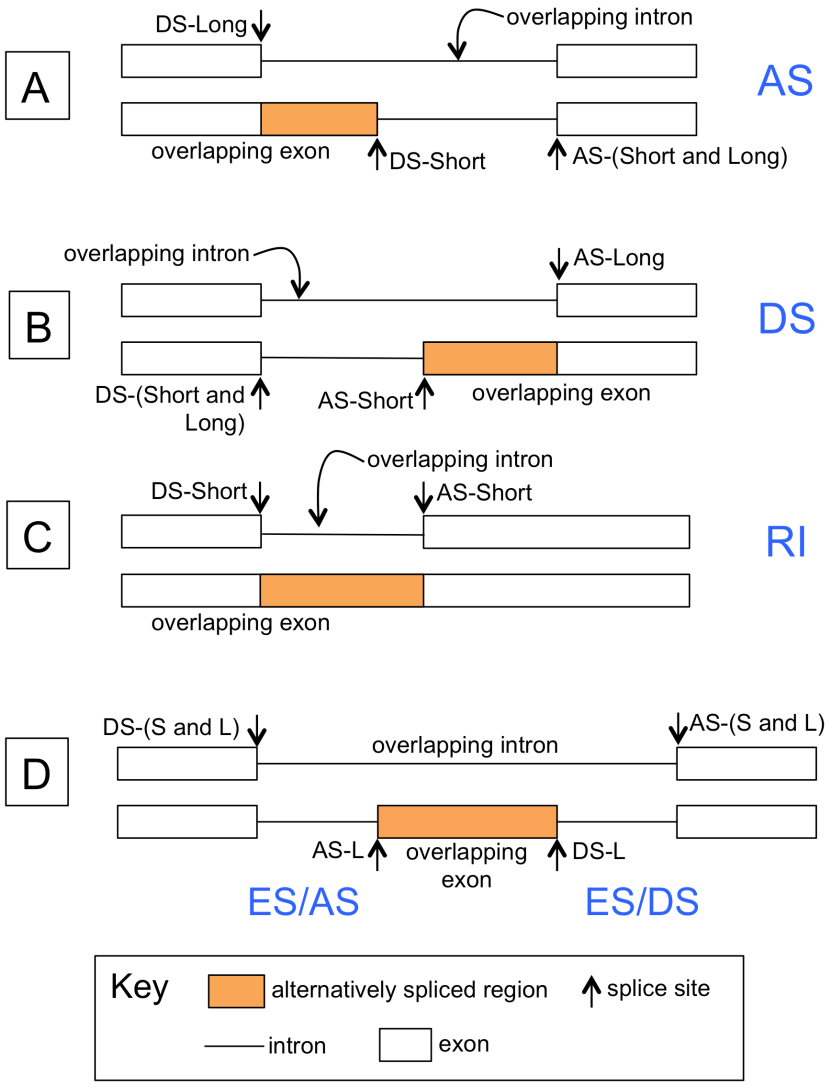


Fig. 1. Alternative splicing annotation. The overlap between an intron in one gene model and an exon in another gene model defines an alternatively spliced region. Arrows indicate splice sites, named AS for acceptor site and DS for donor site. Use of sites named AS-L or DS-L causes inclusion of the differentially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

182 For each alternatively spliced region representing two mutually exclusive splicing choices,
183 RNA-Seq read alignments that unambiguously supported one or the other splicing choice were
184 counted. For AS and DS events, only gapped reads that aligned across intron junctions were
185 counted. For RI events, gapped reads that aligned across the retained intron were counted as
186 support for the intron-removed (S) form, and un-gapped reads that overlapped at least 20 bases
187 within the intron were counted as support for the intron-retained (L) form.
188 For each alternatively spliced region in each biological replicate, the number of reads
189 supporting L or S, but not both, were used to calculate percent-spliced-in (PSI) as N/M*100,
190 where N was the number of reads supporting the L form and M was the number of reads that
191 supported S or L but not both. This is the same as the splicing index described in (Katz *et al.*,
192 2010). To identify differentially spliced regions, a two-sided t-test was used to compare PSI
193 between sample types. Because PSI variance was large for events with small M (very few
194 informative reads), only alternatively spliced regions where M was 10 or more in at least three
195 replicate libraries were tested. A false discovery rate (FDR) was calculated for each test using the
196 method of Benjamini and Hochberg (Benjamini and Hochberg, 1995), as implemented in the R
197 programming language "p.adjust" method. Alternative splicing events with FDR less than or
198 equal to 0.1 were considered differentially alternatively spliced.
199 Software used to identify and quantify alternative events is available from
200 https://bitbucket.org/lorainelab/altspliceanalysis. Data analysis code used to analyze RNA-Seq
201 data is available from https://bitbucket.org/lorainelab/ricealtsplice. Data analysis code is
202 implemented as R Markdown files designed to be run in the RStudio development environment.
203 Readers interested in experimenting with different analysis parameters can clone the repository,
204 modify the code, and re-run analyses as desired. RNA-Seq alignments, coverage graphs, and
205 junctions data are available for visualization in Integrated Genome Browser (Freese *et al.*, 2016).
206
207 **RT-PCR and capillary gel electrophoresis analysis of alternative splicing**
208 Differential alternative splicing detected by analysis of RNA-Seq was re-tested using the
209 reverse transcriptase, PCR-based fragment analysis method described in (Stamm *et al.*, 2012).
210 Differentially spliced regions identified computationally were PCR-amplified using fluorescently
211 labeled primers and quantified using capillary gel electrophoresis. One benefit of the method is
212 that the results are expressed as relative abundances of splice variants within a sample, thus

213 eliminating the need to normalize using reference genes as in traditional qRT-PCR experiments
214 aimed at measuring overall gene expression.
215 For splicing validation, new rice seedlings equivalent to the mock-treated (control) samples
216 from the RNA-Seq experiment were grown and harvested. Seedlings were grown hydroponically
217 in pots containing either liquid media only or calcined clay granules watered with liquid media
218 as recommended in (Eddy *et al.*, 2012). After twelve days, plants were removed from the pots
219 and roots and shoots were collected separately. Roots and shoots from the same pot were
220 combined to form paired biological replicates. Samples were flash frozen in nitrogen and stored
221 at -80°C prior to RNA extraction.
222 RNA was extracted using the RNeasy Plant Mini Kit from Qiagen following the
223 manufacturer's instructions. First strand cDNA was synthesized using oligo dT primers and 1 μg
224 of total RNA per 20 μL reaction. PCR amplification of cDNA was performed using primers
225 flanking differentially spliced regions, including one primer labeled with 6-carboxyfluorescein
226 (6-FAM) for amplicon detection during fragment analysis. Cycle parameters included
227 denaturation at 94°C for 2 minutes, followed by 24 cycles of 94°C for 15 sec, 50°C for 30 sec
228 and 70°C for 1 min, with a final elongation step of 72°C for 10 minutes. This was essentially the
229 same regime described in (Stamm *et al.*, 2012) but with fewer cycles to ensure reactions were
230 stopped before exiting the logarithmic phase. PCR products were combined with size standards
231 and separated on a 3730 Genetic Analyzer (Life Technologies). Amplicons were quantified using
232 manufacturer-provided software by calculating the area under each amplicon peak. The
233 percentage of the variant containing the alternatively spliced region (%L, see above) was
234 calculated by dividing the long form area by the total area for both long and short forms.
235 Spreadsheets with data exported from the instrument, along with PSI calculations, are available
236 in the project git repository (https://bitbucket.org/lorainelab/ricealtsplice) in a subfolder named
237 "Experimental Testing."
238
239 **RESULTS**
240
241 **Most genes annotated as alternatively spliced favored one dominant isoform**
242 Using the exon-intron overlap method described previously (English *et al.*, 2010) and Fig. 1,
243 alternative splicing events within each gene were identified and annotated. Following annotation

244 of alternative splicing events, RNA-Seq read alignments from the rice and *Arabidopsis* libraries
245 described in (Raines *et al.*, 2016; Zubo *et al.*, 2017) were used to assess alternative splicing in
246 four sample types: roots and shoots from seedlings treated with the cytokinin compound
247 benzyladenine (BA) or with a mock, control treatment. For each alternative splicing event, the
248 number of sequence alignments unambiguously supporting each alternative was counted. These
249 counts were used to calculate percent-spliced-in (PSI), the percentage of read alignments
250 supporting the longer (L) isoform.

251 In the combined data from all libraries from the rice data set, 77% of AS events had at least
252 one read supporting each of the two splicing choices, and 19.8% had support for just one splicing
253 choice. Only 2.8% of AS events has no reads supporting either form; these corresponded to
254 genes with low or no expression in any of the sample types tested. Most genes annotated as
255 alternatively spliced had small minor variant frequencies, i.e., the less frequently observed forms
256 were supported by fewer than 20% of informative RNA-Seq sequences (Fig. 2). Nevertheless,
257 there was a large minority of alternative splicing events (around one third) where the minor, less-
258 frequently observed form was more abundant and was supported by at least two out of ten
259 informative alignments. These alternatively spliced regions correspond to the middle, trough-like
260 region of Fig. 2.

261

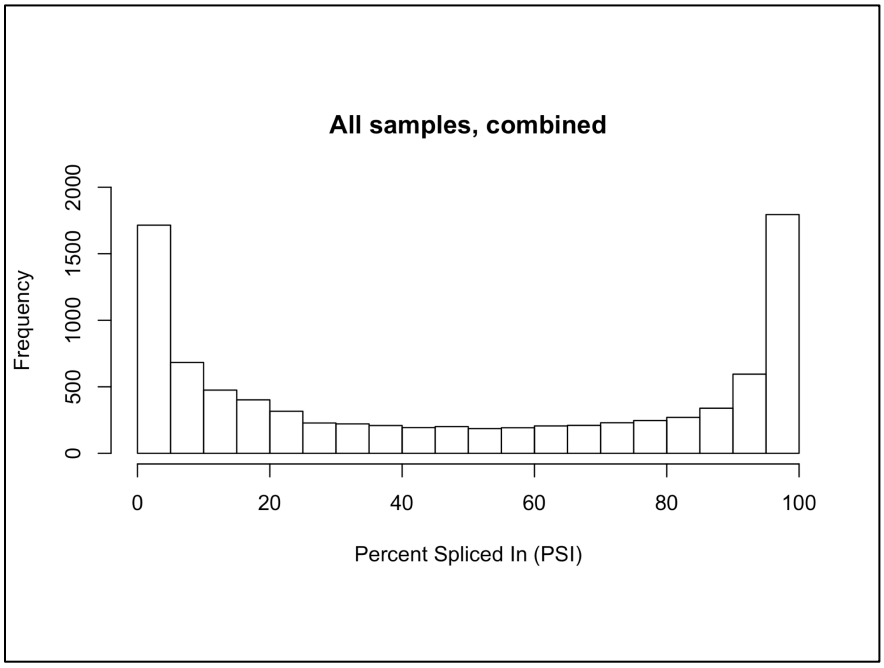
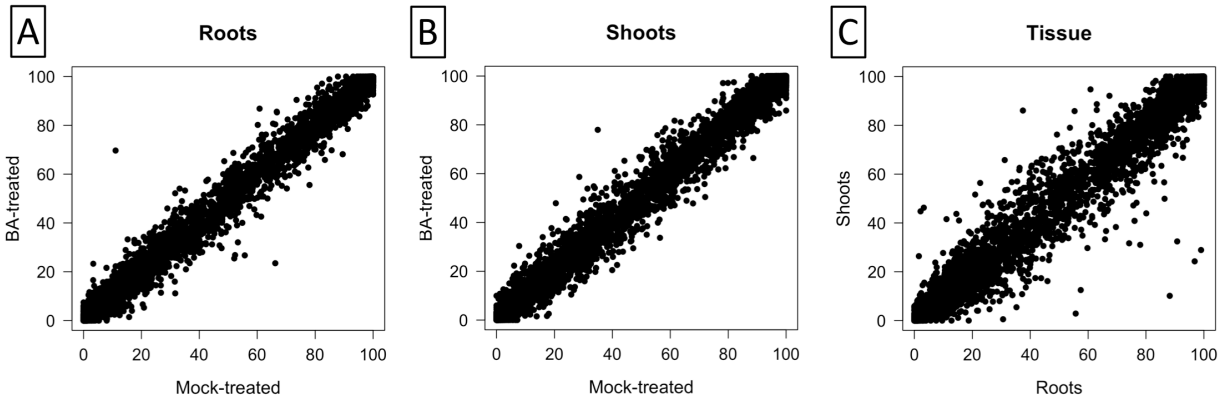


Fig. 2. Distribution of percent-spliced-in (PSI) for annotated splicing events in rice where each choice was supported by at least one RNA-Seq alignment. PSI was calculated as 100*L/(S+L), where L and S were the number of reads that supported the splicing choice that included (L) or excluded (S) the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

262

263 **Rice genes with abundant support for both alternative splicing choices perform many**
264 **diverse functions**
265 We used Gene Ontology term enrichment to determine if the subset of genes in rice for
266 which alternatively spliced forms were unusually abundant exhibited enrichment with specific
267 functions or processes, e.g., circadian cycling, in which alternative splicing might play a
268 prominent regulatory role. We asked if some Gene Ontology terms were significantly enriched
269 with genes containing alternative splicing events in which the minor form frequency was
270 between 20 and 50%, corresponding to the central trough region of Fig. 2. Interestingly, we
271 found that these genes exhibited a diversity of gene functions, with no significant enrichment of
272 functional categories. Thus, alternative splicing in which minor forms are highly prevalent
273 appears to affect genes with many functions in rice.
274
275 **Many rice genes are differentially spliced between roots and shoots but cytokinin hormone**
276 **application has minimal effect on splicing**
277 In animals, differential splicing between cell or tissue types contributes to cellular
278 differentiation, especially in the nervous system (Naftelberg *et al.*, 2015). Less is known about
279 the role of alternative splicing in regulating cellular differentiation and other processes in plants.
280 Rice shoots and roots are profoundly different tissues, but our previous analysis of this same data
281 set found that many of the same genes were expressed in both (Raines *et al.*, 2016). This raises
282 the question of how these two different tissues are able to carry out their specialized roles, and
283 suggest the hypothesis that differential splicing could enable differential functions in genes
284 expressed in both tissues, as proposed in (Reddy *et al.*, 2013).
285 Analysis of the effects of cytokinin treatment on this same data set from rice identified many
286 thousands of genes that were differentially expressed in response to cytokinin (Raines *et al.*,
287 2016). However, little is known about the role of alternative splicing during cytokinin response,
288 except for one study in *Arabidopsis* that reported a shift in splicing of SR protein genes
289 following cytokinin hormone treatment (Palusa *et al.*, 2007). Therefore, we examined differential
290 splicing in the rice RNA-Seq data set comparing root and shoot tissue with or without cytokinin.
291 First, we asked: When an alternatively spliced gene was expressed in two different sample
292 types, was the relative abundance of splice variants the same or different? To address this, we
293 examined correlation of PSI between roots and shoots or between BA-treated versus mock-

294 treated samples (Fig. 3). We found that PSI was similar between treated and untreated samples,
295 as revealed by the tighter clustering of scatter plot points (Fig. 3A-B). This indicated that genes
296 that were alternatively spliced in BA-treated samples were also alternatively spliced in the
297 controls, and that the relative abundance of splice variants was similar. Thus, the cytokinin
298 hormone treatment had minimal effect on splicing. By contrast, there were many genes where the
299 relative abundance of splice variants was different between roots and shoots (Fig. 3C).
300 Consistent with Fig. 3, statistical testing of PSI differences between sample types identified 90
301 genes where PSI was significantly different between roots and shoots (FDR ≤ 0.1) but only four
302 and two genes where PSI was different between cytokinin-treated samples and controls in roots
303 and shoots, respectively (Supplementary Table S1). Thus, we observed limited but non-trivial
304 levels of differential alternative splicing between roots and shoots but minimal differential
305 alternative splicing between control and BA-treated samples.
306



307 **Fig. 3.** Scatter plots comparing percent- spliced-in (PSI) between sample types in rice for annotated splicing events.
308 PSI was calculated from RNA-Seq reads obtained from sequencing rice seedling shoots and roots grown
309 hydroponically and subjected to a two-hour treatment with BA, a cytokinin analog, or a mock-treatment (control).
310 PSI is the average of three biological replicates. Only events with at least 15 informative read alignments in all six
311 samples being compared were included. (A) BA-treated rice roots (y axis) compared to mock roots (x axis). (B) BA-
312 treated rice shoots (y axis) compared to mock shoots (x axis). (C) Mock shoots (y axis) compared to mock roots (x
313 axis).
314

315 **Comparison of *Arabidopsis* differential splicing shows similar patterns to rice**

316 To determine whether the observed patterns of differential splicing are similar in other plants,
317 we analyzed splicing in *Arabidopsis* roots and shoots that had also been treated with cytokinin
318 (Zubo *et al.*, 2017). Due to the *Arabidopsis* libraries not being sequenced to the same depth as
319 the rice libraries, many more splicing events had little or no support. Using the same FDR

320 threshold as with the rice data set (FDR ≤ 0.1), we identified few differentially spliced regions
321 between shoots and roots (3) and none in the control to treatment comparisons (Supplementary
322 Table S2). However, PSI was distributed similarly to rice in that most alternatively spliced genes
323 expressed one major isoform (Supplementary Fig. S1A). In addition, scatter plots showing
324 average PSI in treated versus untreated samples showed a much tighter clustering of points as
325 compared to scatter plots comparing roots and shoots (Supplementary Fig. S1B-D). Statistical
326 testing of PSI differences confirmed the cytokinin hormone treatment had minimal effect on
327 splicing in *Arabidopsis*. Thus, the general pattern of more differential splicing between tissue
328 types as compared to treatment with exogenous cytokinin appears conserved between rice and
329 *Arabidopsis*.
330

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

333 Alternative splicing can occur anywhere in a gene, including UTR and protein-coding
334 regions. Most differential splicing between roots and shoots (67%) occurred within protein-
335 coding regions (Table 1 and Supplementary Table S1), suggesting that differential splicing is
336 likely to affect gene function at the level of the protein product. In every instance of differential
337 alternative splicing, major and minor isoforms were both detected, with differential splicing
338 observed as a change in the relative abundance of the two forms.

339 Because three bases encode one amino acid, the lengths of spliced coding regions in a
340 transcript are multiples of three. Thus, when alternatively spliced regions occur in coding regions
341 and are not multiples of three, then inclusion of these regions in transcripts is likely to introduce
342 a frame shift, resulting in a premature stop codon and a truncated protein product. As shown in
343 Table 1, there was an enrichment of alternatively spliced regions in rice that were evenly
344 divisible by three in coding regions versus non-coding in all subsets of the data. These subsets
345 included all annotated alternatively spliced regions, regions where the minor form was unusually
346 prevalent (the trough region of Fig. 2), and differentially spliced regions. Thus, alternative
347 splicing within the coding regions of genes was biased against introducing frame shifts and
348 promoted protein remodeling rather than truncation.

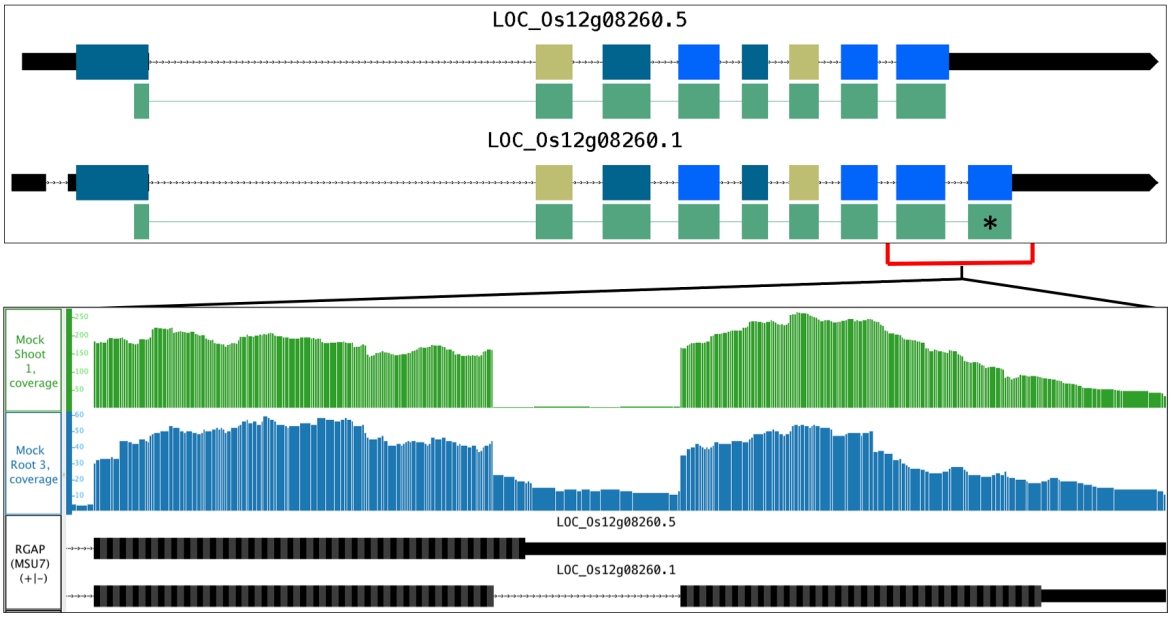
Table 1. *Counts of alternative splicing choices in rice that produce difference regions evenly divisible by three or with remainder of 1 or 2.*

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

*P-value obtained from binomial test of the null hypothesis that the true probability of a differentially spliced region having a length divisible by three is 1 in 3 and an alternative hypothesis that the probability is greater than 1 in 3.

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 (Freese *et al.*, 2016) and ProtAnnot (Mall *et al.*, 2016). 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 (Finn *et al.*, 2017).

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 (Finn *et al.*, 2016) or PANTHER (Thomas *et al.*, 2003)) to be conserved among members of the same protein family (Supplementary Table S1 and Fig. 4).



365 **Fig. 4.** ProtAnnot and IGB images showing difference in splicing between rice shoot and root. ProtAnnot
366 (upper panel) shows coding region exons color-coded by frame, with regions matching InterPro profiles
367 indicated by green, linked rectangles. Asterisk highlights difference in the PANTHER InterPro profile
368 PTHR11516 between isoforms 1 and 5 of the LOC_Os12g08260 gene. Integrated Genome Browser
369 (lower panels) shows a zoomed-in view of RNA-Seq coverage graphs from rice root (blue) and shoot
370 (green). Y-axis is the number of RNA-Seq aligned sequences with MSU7 gene models in black below.
371

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

374 We used a method based on capillary gel electrophoresis of fluorescently tagged PCR
375 products to assay alternative splicing of ten genes detected as differentially spliced between rice
376 roots and shoots (Stamm *et al.*, 2012). New rice seedlings were grown under a close-to-identical
377 replication of the RNA-Seq experiment. Primers were designed to amplify differentially spliced
378 regions, including one primer that was conjugated to a fluorescent tag. Following PCR
379 amplification of cDNA prepared from the new rice samples, products were resolved on a
380 capillary-based sequencer and PSI calculated (Table 2). In nine out of ten genes, differential
381 alternative splicing was confirmed. In the one case where differential alternative splicing was not
382 confirmed, there were very few RNA-Seq read alignments covering the differentially spliced
383 region, suggesting this was likely a false positive result. The FDR cutoff used to detect
384 differential splicing in the RNA-Seq data was 0.1, corresponding to one in ten false discoveries,
385 in line with results from the microcapillary-based analysis.
386

Table 2. *Differential splicing detected using RNA-Seq and re-tested using capillary gel electrophoresis (CGE) in rice.*

Gene	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

*P-value obtained from comparing roots and shoots PSI from CGE.

387

388

389 **DISCUSSION**

390 This study profiled alternative splicing using a high coverage RNA-Seq data from 10-day
391 old, hydroponically-grown rice seedlings treated with a cytokinin hormone. A less-deeply
392 sequenced data set from similarly treated *Arabidopsis* seedlings provided comparison with
393 another plant species. We found that cytokinin treatment induced very few splicing changes
394 between treated and untreated controls. However, there were many differences in splicing
395 between untreated roots and shoots, and most of these changed the protein coding region of
396 genes.

397 Palusa et al. found that BA-treatment of *Arabidopsis* seedlings triggered splicing changes in
398 multiple SR genes (Palusa *et al.*, 2007), encoding RNA-binding proteins whose counterparts in
399 metazoans regulated alternative splice site choice. Their study used PCR amplification of cDNA
400 followed by agarose gel electrophoresis to detect changes in splicing and focused on SR protein
401 genes only. Thus, we expected to observe changes in SR gene splicing due to the cytokinin
402 treatment, leading to changes in splicing for many downstream genes. However, no such
403 differential splicing was apparent in either RNA-Seq data set tested. It is possible that the
404 differences in methodology used to measure splicing changes between the two studies (RNA-Seq
405 versus visualization of PCR amplification of cDNA) could account for the differences in
406 observations. However, close examination of SR splicing genes in our dataset revealed no
407 significant differences.

408 One possible explanation for why the cytokinin treatment had minimal effect on splicing was
409 that the treatment itself was ineffective. However, differential expression analysis showed that
410 many genes were up- or down- regulated by the treatment in the two data sets tested – rice and
411 *Arabidopsis* (Raines *et al.*, 2016). Fewer genes were detected as differentially expressed in the
412 *Arabidopsis* data set, most likely reflecting higher variability between biological replicates
413 combined with lower sequencing depth as compared to the rice data set. Nevertheless, known
414 cytokinin response genes were differentially regulated in both data sets, showing the cytokinin
415 treatment penetrated plant cells and induced stereotypical cytokinin signaling without also
416 triggering changes in splicing.

417 The relative lack of differential splicing between cytokinin-treated and mock-treated samples
418 suggests that cytokinin signaling does not employ alternative splicing as a regulatory mechanism
419 to the same degree as with other plant hormones, notably abscisic acid (ABA). ABA plays a role

420 in perception and response to stresses, especially desiccation stress (Maia *et al.*, 2014). ABA also
421 plays a role in regulating splicing of SR45, an SR-like protein, and SR45 plays a role in
422 regulating downstream splicing of multiple genes (Cruz *et al.*, 2014). Thus, there is a clear
423 connection between stress signaling and the plant stress hormone ABA.
424 By contrast, cytokinin signaling involves transfer of phosphate groups between successive
425 elements of a phosphorelay signaling pathway culminating in phosophorylation-dependent
426 activation of Myb-type transcription factor proteins called type B ARRs. Cytokinin treatment has
427 no or little effect on transcription of type B ARRs, the key regulators of cytokinin signaling
428 (Argyros *et al.*, 2008; Kieber and Schaller, 2018). In addition, type B transcriptional regulators
429 are not highly alternatively spliced. By contrast, a closely related family of similar genes
430 encoding so-called "pseudo-response regulators" have similar sequence to type B ARRs and are
431 highly alternatively spliced (James *et al.*, 2012). These genes are involved in regulating the
432 circadian clock and have nothing to do with cytokinin signaling.
433 Using the same methods and data set, we identified a relatively large number of genes in rice
434 (90) that were differentially spliced genes between shoots and roots, and we validated nine of ten
435 using fragment analysis of independently produced rice samples. This observation of differential
436 splicing between roots and shoots is important for two key reasons. First, it shows that our data
437 analysis methods can identify differential alternative splicing in a data set. In other words, the
438 roots versus shoots comparison provided a positive control for differential splicing detection.
439 Second, the detection of differential splicing between roots and shoots illuminates the function of
440 alternative splicing in plant cells. For most of the differentially spliced regions, both forms were
441 present, and the difference in relative abundance between forms was often slight, rarely more
442 than five or ten percent (Supplementary Table S1). Our data supports the growing body of
443 evidence that alternative splicing is cell, tissue, and stage specific in plants (Vitulo *et al.*, 2014;
444 Gupta *et al.*, 2015; Li *et al.*, 2016a; Sun *et al.*, 2018), including in roots (Li *et al.*, 2016b). It is
445 likely that alternative splicing plays a role in fine-tuning gene function to meet the needs of
446 different plant tissues or cell types where a gene is expressed.
447 We also examined the prevalence of alternative splicing, independent from differential
448 splicing. That is, we used RNA-Seq read alignments to assess how often annotated alternative
449 splice sites were used in our RNA-Set data sets. For most genes annotated as alternatively
450 spliced, the minor form frequency was typically low, accounting for less than 20% of sequence

451 read alignments across the differentially spliced region. Genes where minor form frequency
452 exceeded 20% exhibited a diversity of functions. Thus, many diverse processes in rice involved
453 alternatively spliced genes in which splice variants were expressed at levels likely to affect gene
454 function in different ways.

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

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

468

469 **Supplementary Table S1**. Spreadsheet containing the rice mock root vs mock shoot, mock root
470 vs treated root, and mock shoot vs treated shoot splicing data.

471

472 **Supplementary Table S2**. Spreadsheet containing the *Arabidopsis* mock root vs mock shoot
473 splicing data.

474

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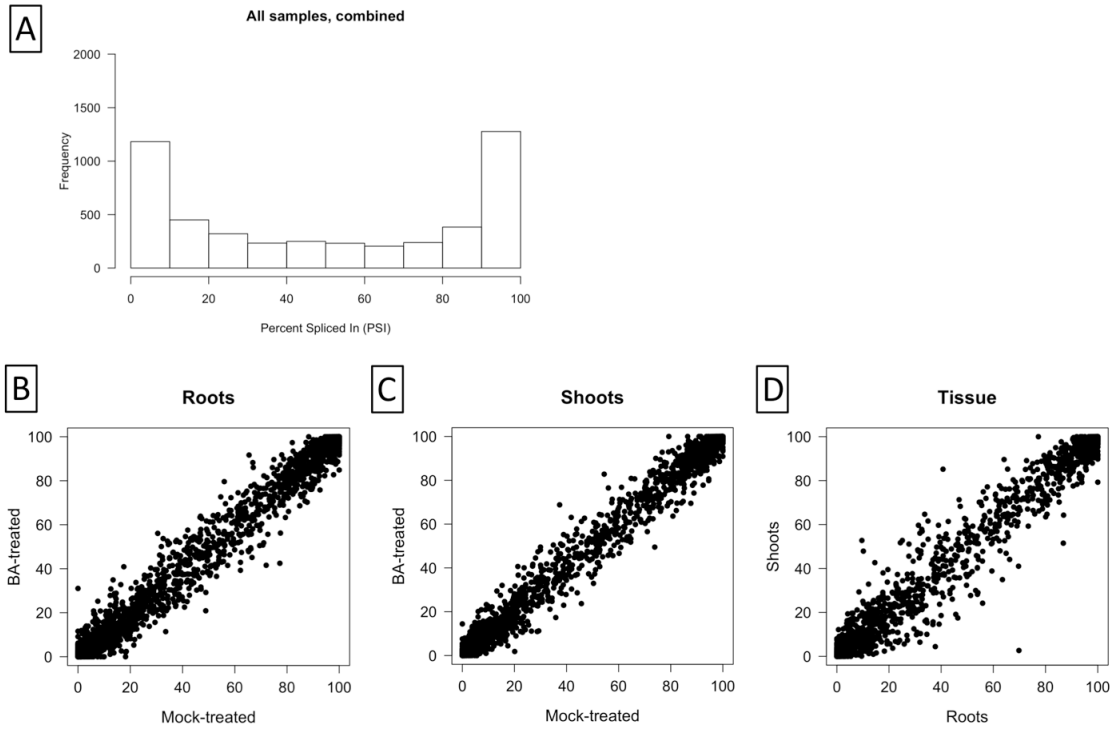
480



481 **Fig. S1. Percent-spliced-in (PSI) in *Arabidopsis* RNA-Seq data set between sample types for**
482 **annotated splicing events.** (A) Counts of PSI events in all samples combined. PSI was calculated from
483 RNA-Seq reads obtained from sequencing *Arabidopsis* seedling shoots and roots grown with or without
484 BA, a cytokinin analog. PSI is the average of three biological replicates. Only events with at least 10
485 informative read alignments in all six samples being compared were included. (B) BA-treated
486 *Arabidopsis* roots (y axis) compared to mock roots (x axis). (C) BA-treated *Arabidopsis* shoots (y axis)
487 compared to mock shoots (x axis). (D) Mock shoots (y axis) compared to mock roots (x axis).
488

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