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In silico Identification of Alternatively Spliced Variants from Transcriptome Data of Rice Lines Exhibiting Complete Panicle Exsertion

Anil A Hake[1], Nakul Magar[1], Kalyani M Barbadikar[1*], Suneel Ballichatla[1], Kommana M[1], Amol S Phule[1], Gokulan CG[2], Hitendra K Patel[2], Ramesh V Sonti[3], Sundaram RM[1] and Sheshu Madhav Maganti[1,4*]

[1]ICAR-Indian Institute of Rice Research, Hyderabad, Telangana, India 500030
[2]CSIR-Centre for Cellular and Molecular Biology, Hyderabad, Telangana, India 500007
[3]International Centre for Genetic Engineering and Biotechnology, New Delhi, India 110067
[4]ICAR-Central Tobacco Research Institute, Rajahmundry, Andhra Pradesh, India 533105
*Corresponding author E-mail: sheshu24@gmail.com; kalyaniaau@gmail.com

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Abstract

Alternative splicing is a molecular mechanism governing gene expression, particularly in plants, wherein a single gene can yield multiple mRNA transcripts, thereby diversifying the resulting protein isoforms. Panicle exsertion is an important trait associated with grain yield in rice. We deployed the mRNA sequencing (transcriptome) data from incompletely exserted panicle genotype, BPT-5204 and its ethyl methanesulphonate mutant lines *viz.*, CPE-109 and CPE-110 exhibiting completely exserted panicle for identification of Alternative Splicing events. Our systematic analysis using rMATS package revealed 414 and 368 genes alternatively spliced upon the comparison of CPE-109 with BPT-5204 and CPE-110 with BPT-5204 respectively. We identified alternative 3′ splice site (A3SS) as the predominant AS type upon comparison of CPE-109 with BPT-5204 (51.20% A3SS) and CPE-110 with BPT-5204 (48.91% A3SS) at panicle initiation stage. In total 23 and 19 genes emanated multiple transcripts *via.,* AS. Remarkably, upon comparison of splicing data of CPE-109 with BPT-5204 and CPE-110 with BPT-5204, we found three common genes namely, *Os07g0406600* encoding DDHD domain containing protein, *Os10g0442100* encoding tRNA methyltransferase and *Os04g0675800* encoding H0103C06.10 protein, that generated more than two transcripts *via.,* AS. These genes can be further validated for determining its role in panicle exsertion through gene expression studies, over expression and functional characterization.

Keywords: Alternative splicing, Complete panicle exsertion, Transcriptome, rMATS, DDHD domain

Introduction

Alternative splicing (AS) represents a pivotal post-transcriptional and co-transcriptional mechanism inherent to eukaryotic gene expression. Alternative splicing is a cellular course in which exons from the same gene are joined in different combinations, leading to different, but related, mRNA transcripts. Further, these mRNAs from a single gene can be translated to produce different proteins with distinct structures and functions. This intricate process orchestrates the selective inclusion or exclusion of exonic sequences, as well as variations in noncoding regions within pre-mRNA transcripts. Alternative splicing of pre-mRNAs promotes transcriptome and in turn proteome diversity and plays an important role in a wide range

of biological processes in eukaryotes. The regulation through AS is dynamic and depends on the cell type, tissue, environmental condition etc. The pre-mRNAs and interactions of RNA-binding proteins affects the generation of AS isoforms.

The major types of AS events include intron retention (IR), exon skipping (ES), alternative 5′ splice sites (A5SS; alternative donor site), alternative 3′ splice sites (A3SS; alternative acceptor site), and mutually exclusive exons (MXE). The frequency of AS also varies from species to species for example, in rice and Arabidopsis 33% and 42% of intron-containing genes respectively. It has been reported that 51% of intron-containing genes utilize alternative 5′ or 3′ splice sites or exon skipping events.

In plants, AS is integral to the regulation of diverse biological processes, encompassing developmental programs and responses to environmental stimuli. The AS events are well characterized in crops like rice, wheat, maize, etc. under various abiotic and biotic stresses and the isoforms differential expression has been linked to tolerance or resistance in plants (Ganie and Reddy *et al.,* 2021). The NGS methods, notably RNA-Seq, offer a high-throughput and cost-effective means to comprehensively analyse the transcriptome. This allows researchers to identify, quantify, and characterize alternative splicing events on a genome-wide basis. The technology's capacity for large-scale data generation has revealed novel insights into the complexity of AS patterns in plants. The methodology and the identification of AS events depends on the technology deployed, sequences / ESTs/ assemblies etc. (Syed *et al.,* 2012). Various tools, pipelines and softwares are available for analyzing alternative splice variations deploying the RNA-seq data sets (Yu *et al.,* 2021).

The rice genotype exhibiting complete exsertion of panicle from flag leaf results in higher grain yield compared to genotype whose panicle is partially choked in flag leaf sheath. Incompletely exerted panicle from flag leaf results in grain yield loss (Guan *et al.,* 2011; Duan *et al.,* 2012). To avoid such loss, complete panicle exsertion (CPE) is desirable in both hybrids and varieties. Recently, quantitative trait loci (QTL)/ genes/ marker underlying complete panicle exsertion have been mapped (Hake *et al.,* 2023); however, role of alternatively spliced genes in complete panicle exsertion in rice is largely unknown. With a hypothesis that AS variations could have role in CPE, in the present study, we have utilized the RNA-seq data of completely exserted panicle lines, CPE-109 and CPE-110, (stabilized mutants of BPT-5204) and their parent BPT-5204 (exhibited incomplete panicle exsertion) to discern the molecular mechanism underlying panicle exsertion.

Materials and Methods

Identification of alternatively spliced (AS) variants

The cleaned reads of RNA-seq data from NCBI Sequence Read Archive (SRA) database (BioProject ID PRJNA687517 and PRJNA772118) of three rice genotypes, namely BPT-5204 (a popular and widely adapted cultivar but incompletely exserted panicle from flag leaf), CPE-109 and CPE-110, stable mutants of BPT-5204, exhibiting complete panicle exsertion from flag leaf were utilized for identification of alternatively spliced variants (Pottupureddi *et al.,* 2021). The reads were mapped on the rice reference genome, R498 using a splice-aware alignment algorithm, HISAT2 (v 2.1.0) (Kim *et al.,* 2019). Splicing events such as skipped exons (SE), intron retention (IR), alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), alternative donor, and acceptor sites were analyzed by utilizing alignment of RNA-Seq data of CPE-109 and CPE-110 with BPT-5204 using the rMATS package 4.1.2, a computational tool to detect differential alternative splicing events from RNA-Seq data (Shen *et al.,* 2014). The graphical representation was executed using the sashimi plot, a tool for RNA-Seq analyses of isoform expression (Kartz *et al.,* 2015).

Results and Discussions

We performed a comprehensive and comparative analysis of AS events using rice transcript (RNA-seq) data from flag leaf at panicle initiation stage of three genotypes namely, BPT-5204 (exhibiting incomplete panicle exsertion), CPE-109 and CPE-110 (both exhibiting complete panicle exsertion). In total, 414 and 368 splicing events were identified from RNA-seq data upon the comparison of CPE-109 with BPT-5204 and CPE-109 with BPT-5204 respectively. Upon comparison of RNA-seq data of CPE-109 with BPT-5204, we found 414 spicing events by alternative 3′ splice site (A3SS; 212 events) followed by alternative 5′ splice site (A5SS; 110 events), retained intron (RI; 64 events) and skipped exons (SE; 28 events) (**Table 1**) observed in 369 genes, off these 22 genes exhibited two or more than two spicing events (**Table 2**). Likewise, upon comparison of RNA-seq data of CPE-110 with BPT-5204, we found 368 splicing events by alternative 3′ splice site (180 events) followed by alternative 5′ splice site (105 events), retained intron (RI; 56 events) and skipped exons (SE; 27 events) (**Table 3**) in 329 genes, off these 19 genes exhibited two or more than two splicing events (**Table 4**)

Table 1: Study of splicing events for CPE upon comparison of RNA seq data of CPE-109 with BPT-5204

Splicing Type	Chromosome number												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
Alternative 3′ splice site	31	21	28	29	18	12	18	18	9	10	9	9	212
Alternative 5′ splice site	15	15	12	10	7	5	13	5	7	8	9	4	110
Retained Intron	9	9	5	7	5	6	8	3	4	0	5	3	64
Skipped Exon	4	3	5	1	2	1	3	1	3	2	1	2	28
Total	59	48	50	47	32	24	42	27	23	20	24	18	414

Table 2: Multiple transcripts generated by alternate splicing events for CPE upon comparison of RNA seq data of CPE-109 with BPT-5204

Gene (MSU_ID)	Gene (RAP_ID)	Description of gene	Splicing Events			
			A3SS	A5SS	RI	SE
Os01g0720600	LOC_Os01g52250	Starch synthase	✓	✓	×	×
Os08g0451700	LOC_Os08g35050	ARID/BRIGHT DNA-binding domain containing protein	✓	✓	×	×
Os03g0286200	LOC_Os03g17730	P-protein	✓	✓	×	✓
Os01g0113600	LOC_Os01g02334	Expressed protein	✓	✓	×	×
Os10g0436800	LOC_Os10g30054	ENT domain containing protein, expressed	✓	✓	×	×
Os07g0406600	LOC_Os07g22390	DDHD domain containing protein	✓	✓	×	×
Os03g0811900	LOC_Os03g59740	ADP-ribosylation factor	✓	×	✓	×
Os03g0219200	LOC_Os03g11960	Copper/zinc superoxide dismutase, putative, expressed	✓	×	✓	×
Os03g0219900	LOC_Os03g12020	50S ribosomal protein L15, chloroplast precursor, putative, expressed	✓	×	✓	×
Os02g0321000	LOC_Os02g21570; LOC_Os02g21580	PPR repeat containing protein, expressed	✓	×	×	✓
Os03g0156700	LOC_Os03g06090	High-affinity nickel-transport family protein, putative, expressed	✓	×	×	✓
Os09g0513400	None	Hypothetical protein	✓	×	×	✓
Os08g0430300	LOC_Os08g33350	Expressed protein	✓	×	×	✓
Os10g0442100	LOC_Os10g30550	tRNA methyltransferase	✓	×	×	✓
Os04g0675800	LOC_Os04g57920	Similar to H0103C06.10 protein	×	✓	✓	×

Gene (MSU_ID)	Gene (RAP_ID)	Description of gene	Splicing Events			
			A3SS	A5SS	RI	SE
Os05g0519200	LOC_Os05g44290	Protein kinase domain containing protein	×	✓	✓	×
Os06g0102750	LOC_Os06g01304	Spotted leaf 11	×	✓	✓	×
Os11g0434000	LOC_Os11g24630	Magnesium-dependent phosphatase 1	×	✓	✓	×
Os04g0278200	LOC_Os04g20960	Expressed protein	×	✓	×	✓
Os06g0659200	LOC_Os06g44870; LOC_Os06g44880	Type II intron maturase protein	×	✓	×	✓
Os02g0326700	LOC_Os02g22100	OsRhmbd6 - Putative Rhomboid homologue, expressed	×	✓	×	✓
Os07g0139500	LOC_Os07g04700	MYB family transcription factor	×	✓	×	✓

***A3SS**: Alternative 3′ splice site; **A5SS:** Alternative 5′ splice site; **SE:** Skipped Exon; **RI:** Retained Intron*

With the advent of next-generation sequencing technologies, newer aspects of AS events are unfolded (Barbadikar *et al.,* 2024). The AS events creates new combination of transcripts and thus contributes to expanding of the proteome. The AS isoforms are involved in the regulation of post-transcriptional gene expression (Campbell *et al.,* 2006). Interestingly, during panicle initiation stage, most of the genes transcribed by alternative 3′ splice site mechanism (212 events in CPE-109 vs BPT-5204 and 180 events CPE-109 vs BPT-5204) signifies its role in panicle exsertion. The isoforms of genes resulted due to splicing events represented by the Sashimi plot (**Figure 1**). Upon comparison of the splicing data of CPE-109 with BPT-5204, out of 369 alternatively spliced genes, 21 revealed two types of transcripts while one gene namely, *Os03g0286200* encoding P-protein produced three transcripts *via* alternative spicing by alternative 3′ splice site, alternative 5′ splice site and skipped exon (**Table 2**). Likewise, upon comparison of splicing data of CPE -110 with BPT-5204, out of 329 alternatively spliced genes, 18 revealed two types of transcripts while gene *Os07g0406600*, encoded to DDHD domain containing protein produced three transcripts *via* alternative spicing by alternative 3′ splice site, alternative 5′ splice site and skipped exon.

Table 3: Number of splicing events for CPE upon comparison of RNA seq data of CPE-110 with BPT-5204

Splicing Type	Chromosome number												Total
	1	2	3	4	5	6	7	8	9	10	11	12	
Alternative 3′ splice site	21	26	26	20	9	9	12	14	7	10	14	12	180
Alternative 5′ splice site	15	13	13	9	9	6	11	3	8	6	7	5	105
Retained Intron	11	5	8	5	2	2	5	4	6	2	4	2	56
Skipped Exon	3	1	4	2	1	1	5	3	2	2	3	0	27
Total	50	45	51	36	21	18	33	24	23	20	28	19	368

Table 4: Multiple transcripts generated by alternate splicing events for CPE upon comparison of RNA seq data of CPE-110 with BPT-5204

Gene (MSU_ID)	Gene (RAP_ID)	Description of gene	Splicing Events			
			A3SS	A5SS	RI	SE
Os07g0406600	*LOC_Os07g22390*	DDHD domain containing protein	✓	✓	✓	×
Os01g0178200	*LOC_Os01g08290*	Similar to integral membrane family protein	✓	✓	×	×
Os01g0757800	*LOC_Os01g55300*	DNA polymerase eta domain containing protein	✓	✓	×	×
Os03g0210400	*LOC_Os03g11200*	RNA-processing protein, HAT helix domain containing protein	✓	✓	×	×
Os05g0588800	*LOC_Os05g51119*	Expressed Protein	✓	✓	×	×

Gene (MSU_ID)	Gene (RAP_ID)	Description of gene	Splicing Events			
			A3SS	A5SS	RI	SE
Os06g0691000	*LOC_Os06g47580*	DNA-repair protein, UmuC-like domain containing protein	✓	✓	×	×
Os01g0102900	*LOC_Os01g01340*	Light-induced protein 1-like	✓	×	✓	×
Os02g0740300	*LOC_Os02g50680*	AAA-type ATPase family protein	✓	×	✓	×
Os03g0284100	*LOC_Os03g17570*	Similar to Two-component response regulator-like PRR73	✓	×	✓	×
Os06g0564500	*LOC_Os06g36840*	O-acetylserine (thiol) lyase	✓	×	×	✓
Os11g0112900	*LOC_Os11g02159*	Hypothetical conserved gene	✓	×	×	✓
Os10g0442100	*LOC_Os10g30550*	tRNA methyltransferase	✓	×	×	×
Os11g0526900	*LOC_Os11g32369*	Non-protein coding transcript	✓	×	×	×
Os03g0562000	*LOC_Os03g36419*	Expressed protein	×	×	×	✓
Os07g0203950	*None*	Non-protein coding transcript	×	×	✓	×
Os07g0676200	*None*	Non-protein coding transcript	×	×	✓	✓
Os12g0137200	*LOC_Os12g04260*	Similar to Saccharopine dehydrogenase family protein, expressed	×	×	✓	✓
Os04g0675800	*LOC_Os04g57920*	Similar to H0103C06.10 protein	×	×	✓	✓

A3SS: Alternative 3′ splice site; **A5SS:** Alternative 5′ splice site; **SE:** Skipped Exon; **RI:** Retained Intron

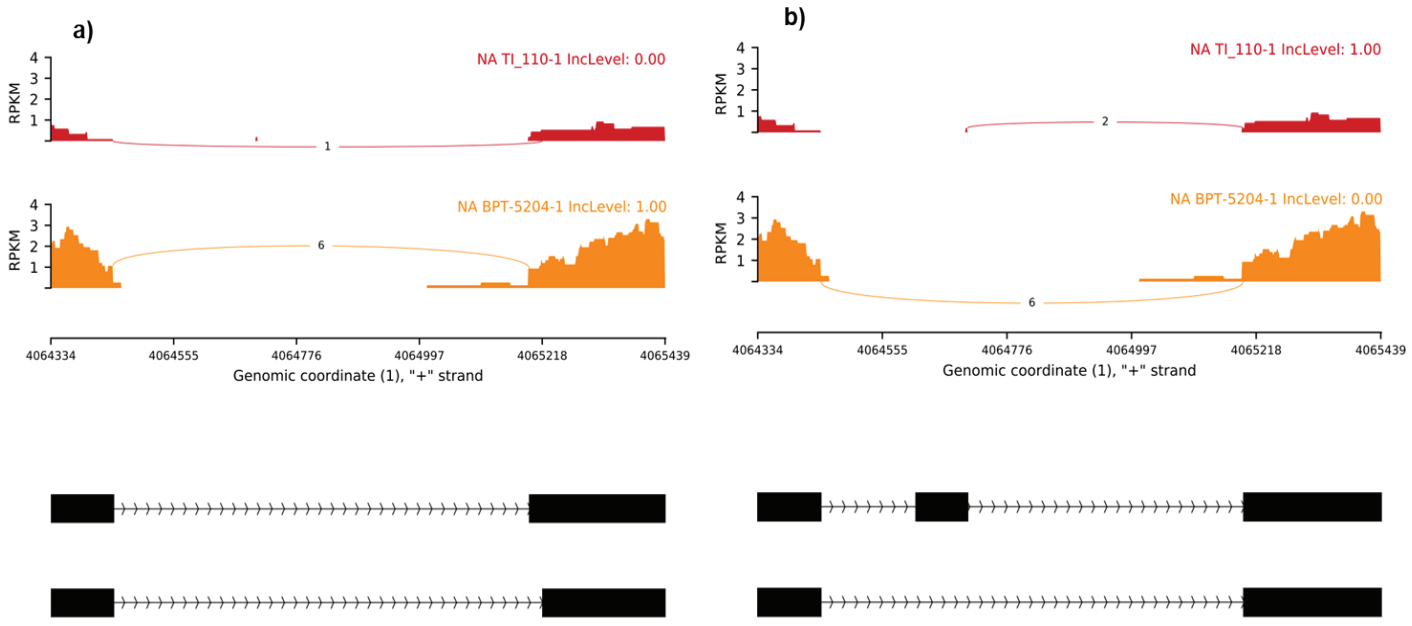

Figure 1: Two transcripts of gene, *Os01g0178200* produced by alternative splicing a) by alternative 3′ splice site and b) by skipped exon mechanism

Remarkably, upon comparison of splicing data of CPE-109 with BPT-5204 and CPE-110 with BPT-5204, we found three common genes namely, *Os07g0406600* encoded to DDHD domain containing protein, *Os10g0442100* encoded to tRNA methyltransferase and *Os04g0675800* encoded to similar to H0103C06.10 proteins (**Table 2 and 4**), generated more than two transcripts *via* alternative splicing, determining its crucial role in complete panicle exsertion. Further, evaluating the data of these three differentially genes, we found DDHD domain containing protein (*Os07g0406600*) upregulated in both CPE-109

(log_2FC=+1.69) and CPE-110 (log_2FC=+1.79) while similar to H0103C06.10 protein (*Os04g0675800*) was down regulated in both CPE-109 (log_2FC=-0.80) and CPE-110 (log_2FC=-0.64). The gene *Os10g0442100* revealed genotype dependent expression response, it was downregulated in CPE-109 (log_2FC=-0.24) while upregulated in CPE-110 (log_2FC=+0.89). In rice, Dong *et al.,* (2012) reported alternatively spliced genes encoding to SR proteins (critical regulators of Zn, Mn, and P nutrition) regulates P uptake and remobilization between leaves and shoots of rice. Thus, in our study, alternatively spliced genes, *Os07g0406600* (DDHD domain containing protein), and *Os04g0675800* (similar to H0103C06.10 proteins) determining its crucial role in complete panicle exsertion.

Alternative splicing has been thoroughly studied in rice for various traits. Yu *et al.,* 2018 studied grain size related parameters and executed characterization of a QTL for grain length *OsLG3b*, encoding a MADS-box transcription factor 1 (*OsMADS1*). Candidate gene association revealed six SNPs in *OsLG3b* region responsible for AS and associated with the levels of gene expression during panicle and seed development. Lui *et al.,* 2022 identified that AS is involved in grain size 3 *GS3* isoforms. *GS3.1* accounting for 50% total transcripts encodes the full-length protein and *GS3.2*, 40% of total transcripts, generate truncated proteins due to a 14 bp intronic sequence retention. Grain size is observed to be decreased in overexpressed lines for *GS3.1* but in *GS3.2*, no significant effect was observed. Also, due to the competitive binding to intermediate gene, *GS3.2* disrupts *GS3.1* signaling. So, it is evident that AS has regulatory role for maintaining the transcripts spatiotemporally. Deep rooting, a crucial parameter for nutrient use efficiency for climate resilience in rice has been studied for AS regulation through RNA-seq. The Intron Retention (IR) in *OsPIN1* contributes to increased root depth in response to drought stress by altering the polar transport of auxin (Wei *et al.,* 2020). Additionally, AS in the 3' untranslated region (UTR) of Rice Nutrition Response and Root Growth (NRR) pre-mRNA modifies gene expression in roots during macronutrient deficiency, thereby influencing root architecture (Zhang *et al.,* 2012). The variations in pre-mRNA splicing has been recently analyzed genome-wide in rice for salinity tolerance. Under the salt stress conditions, two candidate genes with splice variants, *OsNUC1* and *OsRAD23* exhibited differences between the variants for shoot growth in rice (Yu *et al.,* 2021).

In our study, first time we have reported the role of AS in panicle for CPE. We report highly differential expressed alternatively spliced variants commonly expressed in two mutant lines exhibiting complete panicle exertion as compared to BPT-5204. These genes may be involved in the regulation or molecular mechanism of CPE. The results can be further confirmed through gene expression studies. Accordingly, the differentially expressed AS isoforms can be associated with CPE by using functional characterization techniques like overexpression or genome editing. Additionally, functional characterization efforts can be complemented by downstream analyses, including transcriptomic and proteomic profiling, to unravel the molecular pathways and networks influenced by the identified AS isoforms. These comprehensive analyses will provide a holistic understanding of the regulatory mechanisms underlying complete panicle exertion and the intricate interplay between alternative splicing and gene expression in this context.

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