Genome-Wide Landscape of Alternative Splicing Events in *Brachypodium distachyon*

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**Abstract**

Recently, *Brachypodium distachyon* has emerged as a model plant for studying monocot grasses and cereal crops. Using assembled expressed transcript sequences and subsequent mapping to the corresponding genome, we identified 1219 alternative splicing (AS) events spanning across 2021 putatively assembled transcripts generated from 941 genes. Approximately, 6.3% of expressed genes are alternatively spliced in *B. distachyon*. We observed that a majority of the identified AS events were related to retained introns (55.5%), followed by alternative acceptor sites (16.7%). We also observed a low percentage of exon skipping (5.0%) and alternative donor site events (8.8%). The ‘complex event’ that consists of a combination of two or more basic splicing events accounted for ~14.0%. Comparative AS transcript analysis revealed 163 and 39 homologous pairs between *B. distachyon* and *Oryza sativa* and between *B. distachyon* and *Arabidopsis thaliana*, respectively. In all, we found 16 AS transcripts to be conserved in all 3 species.

**Key words:** alternative splicing; *Brachypodium distachyon*; expressed sequence tags; functional ontology; intron retention

1. **Introduction**

Recent advances in the next generation sequencing technologies presented us with a wider computational challenge to analyze and correlate the abundance of the protein diversity with the gene content. In eukaryotes particularly, the presence of introns within the protein-coding genes can generate more than one functional mRNA isoforms from one pre-mRNA transcript, through a post-transcriptional event called as alternative splicing (AS), which in turn increases the protein complexity and mRNA abundance without increasing the gene content. It has been proposed that the glycine-rich RNA binding protein AtGRP7 has been suggested as a potential factor to regulate AS in *Arabidopsis thaliana*. In principle, four basic and major type AS events have been reported, including exon skipping (EoxS), alternative donor (AltD) or alternative acceptor (AltA) site, and intron retention (IntronR). However, other types such as alternate terminal exon, retained exon (RE) and skipped exon (SE), initiation within an intron, termination within an intron, and spliced intron have also been...
Alternative Splicing in *Brachypodium distachyon* has been reviewed. RNA-seq has played an important role in the adaptation of plants to their corresponding habitat and environment. It has also served as a model genome to understand the functional protein diversity generated through alternative splice events in cereal crops. ESTs generated from *B. distachyon* were used for gene finding and phylogenetic analysis. In an earlier study, through mapping of 3818 tentative consensus sequences on the assembled genome scaffolds, we have demonstrated the presence of 128 AS events involving 120 genes in *B. distachyon*. In recent years, transcript abundance has been observed in the identification of the ESTs from this model organism, which presents an opportunity to study the AS at a wider level because of the availability of the larger number of transcripts with a higher genomic coverage. RIKEN has recently established a full-length cDNA (FLcDNA) project of *B. distachyon* consisting of the 79 872 ESTs generated using the biotinylated CAP trapper methodology. BrachyFLcDNA DB has been integrated with other available resources to make it an integrated repository. The availability of the FLcDNA in *B. distachyon* will also aid in redefining the earlier gene models and will also aid in the functional analysis of the gene patterns. In the present study, we have reinvestigated the landscape of the AS events and demonstrated the presence of 1219 AS events involving 2204 putatively assembled transcripts generated from 941 genes. We have also classified the AS events according to functional ontology. In addition, we have created an online portal (http://proteomics.ysu.edu/altsplice/plant/) to access the annotation of all ESTs and AS events identified in this study.

### 2. Materials and methods

#### 2.1. Sequence datasets

A total of 30 991 assembled transcript sequences also known as PlantGDB-assembled unique transcripts (PUTs) for *B. distachyon* were downloaded from PlantGDB (http://www.plantgdb.org/). The PUTs sets were generated by assembling 128 098 ESTs and mRNA sequences (version 175a) using the methods described at the site (http://www.plantgdb.org/prj/ESTCluster/PUT_procedure.php). Our previous evaluation confirmed that assemblies generated by CAP3 (parameters –p 95, –o 49, and –t 10 000), which was used by PlantGDB, were suitable for identification of AS isoforms. The genome of *B. distachyon* was recently sequenced, and a total of 25 532 protein-coding genes were predicted as a part of genome analysis pipeline. *B. distachyon* genome exhibits a much higher level of co-linearity and synteny to the genomes of temperate cereal crops and also forage grasses such as *Lolium* and *Festuca* and, thus, it can serve as a model genome to understand the functional protein diversity generated through alternative splice events in cereal crops. ESTs generated from *B. distachyon* were used for gene finding and phylogenetic analysis.

In plants, the functional relevance of certain AS-derived isoforms in responses to biotic and abiotic stresses has been reviewed. RNA-seq has played an important role in revealing the transcript diversity through AS events and revealed the abundance of the alternative isoforms with PTC. Recently, using RNA-seq AS events have also been revealed in plant circadian clock-associated genes in *A. thaliana*. The roles of these genes involved in the processes of photosynthesis, plant disease resistance, flowering, and grain quality in *O. sativa* have been well illustrated (see Reddy for review). Complex networks of regulation of gene expression and variation in AS have played a major role in the adaptation of plants to their corresponding habitat and environment.

*Brachypodium distachyon* is considered to be the model temperate grass for studying the evolutionary genomcs and for the elucidation of the biologic pathways in grass genomics. It has also been suggested to be a model species for studying the evolutionary insights into biofuel crops such as genus *Miscanthus* and *Panicum virgatum*. The genome of *B. distachyon* was recently sequenced, and a total of 25 532 protein-coding genes were predicted as a part of genome analysis pipeline. *B. distachyon* genome exhibits a much higher level of co-linearity and synteny to the genomes of temperate cereal crops and also forage grasses such as *Lolium* and *Festuca* and, thus, it can serve as a model genome to understand the functional protein diversity generated through alternative splice events in cereal crops. ESTs generated from *B. distachyon* were used for gene finding and phylogenetic analysis.
assembly using the maximum available memory, and, thus, it avoids the misassembling of the ESTs and formation of counterfeit longer assemblies. The genome sequence of B. distachyon (diploid inbred line Bd21) was downloaded from Phytozone (v7.0). The assembled genome of B. distachyon measures approximately 272 Mb and spans across 5 chromosomes and 78 unmapped scaffolds.

2.2. Homology mapping, identification, and functional ontology of AS isoforms

Homology mapping was achieved by mapping the PUTs (hereafter referred as ESTs) of B. distachyon to the corresponding genome sequence, and identification of AS isoforms was done using ASFinder (http://proteomics.ysu.edu/tools/ASFinder.html/). ASFinder uses SIM4 program to map ESTs to the corresponding genome. It subsequently identifies those ESTs that are mapped to the same genomic location, but have variable exon–intron boundaries as AS isoforms. For our mapping, we used the following thresholds: a minimum of 97% identity of aligned ESTs with genomic sequences, a minimum of 80 bp of aligned length, and >85% of EST sequence aligned to the genome. The output of ASFinder was subsequently analyzed, and AS events were identified using AStalavista server (http://genome.crg.es/astalavista/). Additional AS isoforms from ESTs that were not mapped to the genome were identified using ASFinder BLASTN method to detect those EST pairs that have two or more segmented alignments. This similar method of using BLAT tool, rather than BLAST, has been previously used to identify AS from ESTs in plants. The functionalities of genes having AS events were identified using rpsBLAST against Pfam and the Conserved Domain Database. Gene ontology (GO) information was retrieved by mapping Pfam to GO (http://www.geneontology.org/external2go/pfam2go) and analyzed using GO SlimViewer with plant-specific GO terms. ESTs were also compared with predicted protein-coding DNA sequences (CDS) in Phytozone using BLASTN, with a cutoff E-value of 1e-10, and a total of 22 591 ESTs had one or more CDS match with >97% identity. The information was available in the database for comparison.

2.3. Conserved alternatively spliced genes in B. distachyon, O. sativa, and Arabidopsis

To identify potentially conserved alternatively spliced genes between B. distachyon, O. sativa, and A. thaliana, we downloaded previously detected mRNA sequences of AS genes in O. sativa and A. thaliana from Plant GDB ASIP portal (http://www.plantgdb.org/ASIP/Download/). Conserved AS genes were identified using O. sativa and A. thaliana AS mRNAs to search against B. distachyon AS ESTs using BLASTN with E-value of 1e-5. The best BLASTN hit with an identity of >70% and a minimum aligned length of >100 bp were identified as conserved AS pairs.

2.4. Data access and visualization of AS

We annotated all ESTs according to function and structure, which involves the prediction of the protein-coding regions in the ESTs using OrfPredictor (http://proteomics.ysu.edu/tools/OrfPredictor.html), mapping to genomic sequences, BLASTN matching ESTs to predicted protein CDS (>97% identity used) of this species downloaded from Phytozone, and Pfam and conserved domain search. We further developed an online web portal for the systematic display of the mapped ESTs and associated AS events (http://proteomics.ysu.edu/altsplice/plant/). The database can be queried using EST ID, keywords, or AS event types. The AS events can be systematically browsed using GBrowse, and the CDS annotations were added using the GFF file of the annotated genome downloaded from Phytozone. We provide a functionality to query for all ESTs and AS events using BLASTN.

3. Results and discussion

3.1. Detection and classification of AS events

In our study, a total of 23 693 ESTs were successively mapped to 14 841 genomic loci of B. distachyon genome sequence. We identified a total of 1 219 pairs of ESTs (2021 unique ESTs), which were mapped to 941 genomic loci having AS, suggesting that ~6.3% of expressed genes in B. distachyon are under the potential influence of AS (Supplementary Table S1). The observed AS percentage is relatively low in comparison to the percentage of the genes observed in A. thaliana (21.8%) and O. sativa (21.2%). This potential difference in the distribution of the AS genes versus non-AS genes may be due to the fact that relatively more transcript resources are available for A. thaliana and O. sativa in comparison to B. distachyon. Classification of the AS events showed that the majority of the observed events (676) corresponded to IntronR (55.5%), followed by Alta (204, 16.7%), Altd (107, 8.8%), and ExonS (61, 5.0%). We observed 171 ‘complex events’ that correspond to AS events having more than 1 basic event (14.0%) (Fig. 1). Inspite of the difference in the transcript abundance, the observed AS events, particularly IntronR, showed conservation across the plant lineage, which may be an indication of dominance of the splice site recognition by intron definition as previously suggested in A. thaliana and O. sativa.
There was a whole genome duplication event that occurred before the diversification of the grasses, and also grass-specific tandem gene expansion in *B. distachyon* was observed. In an earlier study, the relation between gene family size and AS was studied by comparing the distribution of AS frequency in single-copy genes versus gene families, and, interestingly, a higher percentage of AS was found in gene families than in single-copy genes in *A. thaliana* and *O. sativa*. In our analysis, we clustered paralogs using the blastclust tool (–S 50 and –L 0.7) in BLAST package and identified 3407 gene families and 15 169 single genes. Then, mapping AS transcripts to them revealed 7.7 and 5.9% of AS in gene families and in single-copy genes, respectively. Recent analysis showed that gene duplication and AS evolved independently, which is further exemplified with the confirmation of the independent evolution of these two processes using isochorismate synthase in *Arabidopsis* and *Populus*. AS divergence between duplicated genes may have contributed to gene functional evolution and led to preservation of some duplicated genes. Our results are in accordance with the recent finding which suggests that the moderately duplicated gene families have potentially higher AS isoforms when compared with the singleton genes. AS, along with gene duplication, represents an evolutionary strategy that rapidly increases genome plasticity to deal with various plant stresses.

In addition to the AS genes and events identified by mapping ESTs to the genome, using the BLASTN method implemented in ASFinder, we also identified 56 genes (involving 129 ESTs) from 7298 ESTs, which were not mapped to the genome. However, specific category of AS events in this subset of genes cannot be identified because of the lack of the genomic information. All ESTs and spliced variants can be queried, visualized, explored, and browsed at the Plant Alternative Splicing Database (http://proteomics.ysu.edu/altsplice/plant/) using several query patterns such as EST IDs, gene, and AS event type. One similar resource for the visualization of AS events, which has been developed for *A. thaliana*, is available at ArabiTAG; http://transvar.org/arabitag/.

3.2. Features of exons and introns in protein-coding genes

Following the mapping of the ESTs to the corresponding genomic sequence, we extracted the lengths of 34 087 internal exons and 48 000 introns. We observed size variation with internal exons that had a size ranging from 15 to 1461 bp with an average size of 130 ± 87 bp. We further observed that approximately 96% of exons had a size below 300 bp and 58% lied between 60 and
140 bp (Fig. 2). The observed exon length is in line with the earlier observed mean internal exon length in *A. thaliana* (172 bp) and *O. sativa* (193 bp). It has been previously shown that the shorter exons have high potential for AS events by exon shuffling events coupled with exon duplication and may promote the genome and transcriptomic complexity. It has been demonstrated previously that organisms having the shorter exons have higher gene content ratio. However, when compared with exon size, the distributions of intron size were more variable, ranging from 10 bp to >10 kb. There were 32 introns (0.07%) having a size >10 kb. However, in considering possible errors in EST assembly and genome assembly, these long introns identified in this study need to be further examined experimentally. Excluding those long introns with >10 kb in size, the remaining introns had an average size of 420 bp with a standard deviation of 622 bp, ~63% had a size below 300 bp, and ~47% of introns lied in 60–140 bp (Fig. 3). The average intron size in *B. distachyon* was very close to rice intron size (433 bp), and both of them were much longer than the average intron size (173 bp) in *Arabidopsis*, calculated using a similar method.

### 3.3. Features of retained introns, SEs, and other DNA fragments involved in AS events

We examined the size distribution of DNA fragments involved in AS events, including retained introns, SEs, and fragments in AltD or AltA sites (Table 1). The retained introns had a range from 8 to 1142 bp with an average size of 184 bp, which is quite small in comparison to the average size of the introns in the species. Although the overall frequency distribution of retained intron size was similar to the distribution of all introns, there were no introns longer than 1150 bp in the retained intron set (Fig. 4). The SEs (ExonS) had a range of 23–376 bp.

### Table 1. Summary of DNA fragment sizes involved in AS events

| Fragment Type | Size Range (bp) | Mean | Standard Deviation |
|---------------|-----------------|------|-------------------|
| IntronR       | 8–1142          | 184  | 152               |
| AltA          | 3–468           | 49   | 82                |
| AltD          | 3–553           | 67   | 103               |
| ExonS         | 23–376          | 111  | 68                |

### Figure 2. Distribution of internal exon size: The x-axis indicates the size of internal exons. Bin sizes are right inclusive (e.g. bin 100 comprises sequences of lengths 1–100 bp). The y-axis indicates the frequency of internal exons. The inset shows a detailed distribution of smaller internal exons.

### Figure 3. Distribution of intron size: The x-axis indicates the size of introns. Bin sizes are right inclusive (e.g. bin 100 comprises sequences of lengths 1–100 bp). The y-axis indicates the frequency of introns. The inset shows a detailed distribution of smaller introns.

### Figure 4. Distribution of retained intron size: The x-axis indicates the size of retained introns with bin sizes that are right inclusive (e.g. bin 100 comprises sequences of lengths 1–100 bp). The y-axis indicates the frequency of retained intron size.
with an average size of 111 bp. The fragments involved in AltD or AltA sites ranged from 3 to 468 bp (average size 49 bp) in AltA and from 3 to 553 bp (average size 67 bp) in AltD. Overall, the average size of the fragments involved in AS was relatively short. It has been previously suggested that the presence of the retained intron within the coding sequence may give rise to the nonsense-mediated decay (NMD). However, recently it has been proposed in Arabidopsis that splice isoform with the retained introns is not sensitive to NMD. Although the high proportion of retained introns in AS events in plants supports the intron definition model in splicing, the evidence that the size of the retained introns was more similar to typical exons, lack of long introns, makes the exon definition model an alternative reasonable explanation.

3.4. Functional ontology of AS genes

Functional annotation of the alternative spliced transcripts and their association with certain domains can give the mechanistic overview of the functional impact of the AS on the domain and also on domain-mediated regulation of AS. Recently, it has been predicted that AS events have potential effects on the protein domain function. In a recent study, it was demonstrated that MIKC MADS-box genes are under the strong influence of the AS events, and conserved AS events were observed across the borders or with in the K-box domain. In a recent study, it has been demonstrated that two spliceosomal proteins, U1-70K and U2AFb are known to function in 5' and 3' splice site selection and regulate the AS in SR45 that is a serine/arginine-rich (SR)-like protein with two arginine/serine-rich (RS) domains.

To annotate the AS transcripts and to identify their possible association with functional domains, we performed a BLASTX search of all ESTs of B. distachyon against UniProt database. The protein-coding sequences [open reading frames (ORFs)] of ESTs were identified using OrfPredictor webserver. The functional domains of the AS genes, using the longest ORF of each AS gene, were predicted using rpsBLAST searching Pfam and CDD database. Among a total of 941 proteins encoded by AS genes, 651 were predicted having functional domains (Supplementary Table S1). We have further classified the genes according to the gene functional ontology (GO) categories, i.e. cellular component, molecular functions, and biological processes (Table 2, Supplementary Table S2). It was observed that the overall distribution of molecular functions in genes having AS and non-AS genes was similar (Table 2). Most of the functionally annotated transcripts were found to be associated with binding activity or catalytic activity, and some of which were found to be associated with DNA and RNA binding, respectively (Table 2). These transcripts with functional annotation may be of functional importance in revealing the genes in various biological pathways. It has been suggested that the binding of the proteins to cis-regulatory sequences in exons and introns and associated splicing regulators may regulate the loading of the splicing machinery to splice site.

Earlier, it has been shown that the AS also plays an important role in promotion of the floral transitions, which gives a layout of the involvement of the AS events in the biological pathways. In a study, it has been shown that the AS of the FCA transcript, which encodes a RNA-binding domain and a putative PPI domain, promotes floral transition in A. thaliana fca mutants. Recently, it has been demonstrated that a splice variant (IDD14) of Arabidopsis INDETERMINATE DOMAIN14 (IDD14) transcription factor regulates the function of the IDD14a in starch metabolism by acting as an inhibitor forming heterodimers, which potentially explains the role of the AS in regulation of the transcription factor activity. Recent reports suggest that AS also plays a potential role in the regulation of the freezing tolerance through the splicing of the clock compounds (CIRCADIAN CLOCK-ASSOCIATED1; CCA1). We observed GO categories enriched in carbohydrate metabolic process (GO:0005975) and in response to stress (GO:0006950) in the AS transcripts (Supplementary Table S2), which gives a strong indication of the modulation of the biosynthetic pathway and increases the

| Molecular function       | AS genes | Non-AS genes |
|--------------------------|----------|--------------|
| GO term                  | count    | %            | GO term   | %            |
| Binding                  | 100      | 21.0         | 1936      | 22.3         |
| Catalytic activity       | 76       | 16.0         | 1253      | 14.4         |
| Nucleotide binding       | 54       | 11.3         | 1059      | 12.2         |
| Transferase activity     | 52       | 10.9         | 1049      | 12.1         |
| Hydrolase activity       | 44       | 9.2          | 843       | 9.7          |
| Protein binding          | 32       | 6.7          | 539       | 6.2          |
| Kinase activity          | 31       | 6.5          | 546       | 6.3          |
| DNA binding              | 25       | 5.3          | 396       | 4.6          |
| Nucleic acid binding     | 13       | 2.7          | 220       | 2.5          |
| Structural molecule activity | 13    | 2.7          | 251       | 2.9          |
| Transporter activity     | 13       | 2.7          | 308       | 3.5          |
| RNA binding              | 11       | 2.3          | 141       | 1.6          |
| Others                   | 12       | 2.5          | 145       | 1.7          |
| Total                    | 476      | 8686         |           |              |
protein diversity for a subset of genes using AS events. Recently, using a systematic investigation of an intronic miR400 under heat stress in A. thaliana, it has been shown that the AS acts as a regulatory link and possibly regulates the interactions of miRNAs and environmental stress. Several recent reports postulate the association of the AS events with biosynthesis in a couple of genes involved in auxin biosynthesis shows the patterns of tissue-specific splicing.50,51 However, all these functions still remain uncovered in B. distachyon that is a model organism for grass monocot lineage. Further experimental verification of the AS events with functional annotation, identified in the present study, may highlight some important biological phenomenon in detail, which are specific to grass lineage.

3.5. Conserved alternatively spliced genes

Identification of the conserved AS events can also assist us in understanding the evolution of the functional genes and their regulation at the transcriptional and at the translational level, which may shed light on the understanding of the trade-off going on between the plant machinery and the environmental adaptation. The identification of the AS isoforms in plants may explain and postulate plausible answers to adaptation to environmental stress, domestication, and also may likely explain the trait selection.51 It has been stressed that the detection of the conserved AS events may potentially help in the identification of the AS isoforms containing PTC.52 Recent analysis further revealed that AS underlies the exonization of the 5S rRNA, which regulates the TFIIA.53 The serine/arginine-rich (SR) protein family plays an important role in constitutive AS and regulates AS in a tissue-specific and stress-responsive manner.54 Three sets of AS events were conserved between A. thaliana and rice in the plant-novel-SR protein, SC35-like and two Zn-knuckles-type 9G8 sub-families.54 It was reported that 41.7% of AS genes in A. thaliana were found to have close homologs in O. sativa that were also identified to be alternatively spliced, and 30% of the intron-retained transcripts also showed conservation between A. thaliana and O. sativa.2

In our study, we identified 163 homologous pairs of AS genes between B. distachyon and O. sativa, ~17.3% of all AS genes identified in B. distachyon and 39 homologous pairs of AS genes between B. distachyon and A. thaliana (Supplementary Table S3). Among the functionally annotated AS events, 16 AS genes were found be conserved among all the species (Table 3). Our study postulates the potential list of 16 conserved genes that may act as potential marker genes/ transcripts for further, detailed experimental examination of their biological significance of AS in plant growth, development, and tracing the evolution of certain biological pathways.

In summary, B. distachyon is currently a model plant for studying grass system and evolutionary biology. Identification of alternatively spliced genes and AS events is the first step for understanding which

Table 3. Conserved alternatively spliced genes in B. distachyon, O. sativa, and A. thaliana

| BD_PUT_ID | Rice_gi    | Arabidopsis_gi | Functional domain                                                   |
|-----------|------------|----------------|-------------------------------------------------------------------|
| PUT_Bd26530 | 7212633   | 315633         | pfam13 893, RRM_5, RNA recognition motif                         |
| PUT_Bd2017 | 4878380   | 14532699       | pfam04928, PAP_central, poly(A) polymerase central domain        |
| PUT_Bd87  | 428653    | 935609         | pfam03949, Malic_M, malic enzyme, NAD-binding domain             |
| PUT_Bd26130 | 702742, 2280709 | 2763737 | pfam02991, Atg8, autophagy protein Atg8 ubiquitin like            |
| PUT_Bd28467 | 428194   | 937533         | pfam01717, Meth_synt_2, cobalamin-independent synthase, catalytic|
| PUT_Bd27829 | 702009    | 496827         | pfam01370, Epimerase, NAD-dependent epimerase/dehydratase family |
| PUT_Bd1551 | 571572    | 17474          | pfam01287, elf-5a, eukaryotic elongation factor 5A hypusine       |
| PUT_Bd31189 | 701361    | 166645         | pfam00504, Chloroa_b-bind, chlorophyll A–B-binding protein      |
| PUT_Bd940  | 431832, 3762355 | 16406        | pfam00230, MIP, major intrinsic protein                          |
| PUT_Bd3432 | 572134, 2310784 | 16869        | pfam00179, UQ_con, ubiquitin-conjugating enzyme                  |
| PUT_Bd4338 | 701921    | 935078         | pfam00085, thioredoxin, thioredoxin                              |
| PUT_Bd2952 | 427824, 2310078, 2434385 | 937253 | pfam00071, Ras, Ras family                                       |
| PUT_Bd2364 | 428011, 2311034 | 398603     | Function unknown                                                 |
| PUT_Bd7431 | 2312070   | 1045041        | Function unknown                                                 |
| PUT_Bd4686 | 287194, 571706, 1631641 | 1053979     | Function unknown                                                 |
| PUT_Bd10494 | 286390    | 1217256        | Function unknown                                                 |
categories of genes are regulated post-transcriptionally and to identify the potential mechanisms for transcriptomic complexity. Our systematically analyzed data provide a visualization portal to *B. distachyon* community researchers who aim to identify potential mechanisms of transcriptomic diversity and adaption to stress and to expand the functional relevance of the identified AS events through experimental approaches.

**Supplementary data:** Supplementary Data are available at www.dnaresearch.oxfordjournals.org.

**Authors’ contribution**

B.W. and G.L. contributed to the database construction, G.S. and X.J.M. contributed to the experiment design, data analysis, and preparation of the manuscript. All authors have read and approved the final version of the manuscript.

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