Molecular Plant
Letter to the Editor

CellPress
PARTNER JOURNAL

DNA Methylation Affects Gene Alternative Splicing in Plants: An Example from Rice

Dear Editor,

Alternative splicing (AS) of pre-mRNA is an evolutionarily conserved mechanism to increase transcriptomic and proteomic complexity, and hence phenotypic diversity, via the production of multiple mRNA isoforms from a single gene (Reddy et al., 2013). AS is regulated by various *cis*-acting regulatory elements and RNA-binding proteins (Reddy et al., 2013). The finding that a large proportion of pre-mRNA splicing is often co-transcriptional (Gelfman et al., 2013) suggests the involvement of epigenetic mechanisms in splicing regulation. Epigenetic modifications can affect chromatin structure and the elongation rate of RNA polymerase II (Pol II) (Luco et al., 2011), and hence can directly or indirectly recruit spliceosomal proteins (Luco et al., 2011; Maor et al., 2015; Yearim et al., 2015).

DNA methylation at the CG residues (^{m}CG) is a prominent form of epigenetic modification existing in most eukaryotes (Zemach et al., 2010). An established role of cytosine methylation at promoter regions is to transcriptionally repress gene expression (Maor et al., 2015); however, the biological function of this modification within the gene body, which often enhances gene expression, remains contentious (Jones, 2012). Recent studies in animals and human cell lines have shown that gene-body methylation plays a major role in AS regulation (Maor et al., 2015). For example, it was found in honeybee and mouse embryonic stem cells that DNA methylation exerts a global impact on AS, especially on the splicing of alternative exons, and loss of ^{m}CG is causally linked with a significantly changed proportion of AS in either direction (Li-Byarlay et al., 2013; Yearim et al., 2015). Two mechanisms have been proposed to explain how DNA methylation information can be conveyed to splicing regulation: the CCCTC-binding factor (CTCF) and methyl-CG binding protein2 (MeCP2) can modulate the elongation rate of Pol II, and heterochromatin protein1 (HP1) can recruit splicing factors onto transcribed alternative exons (Maor et al., 2015). Except the previous study of potential inhibitory effects of CHG methylation on the gene splicing in maize (Regulski et al., 2013), limited information is currently available regarding whether and to what extent gene-body DNA methylation may affect AS in plants.

We previously characterized a null mutant of a major CG methyltransferase in rice (*OsMet1-2*), which showed genome-wide loss of ^{m}CGs by approximately 76% compared with its isogenic wild-type (WT) (Hu et al., 2014). The mutant also showed reduction of ^{m}CHG and ^{m}CHH, but to a much lesser extent (Hu et al., 2014). Our high-quality transcriptome (RNA-seq) and single-base resolution methylome (BS-seq) data of this rice mutant and its isogenic WT of the same tissue (seedlings) (Hu et al., 2014) provide a robust resource to assay the genome-wide impact of loss of cytosine methylation in gene-body regions on AS changes for the first time in plants.

We quantified the AS events in both WT and mutant based on their RNA-seq data (Hu et al., 2014). Based on the workflow constructed (Supplemental Figure 1), we identified 20 138 and 17 913 AS events in the mutant and WT, respectively, which host 116 065 confident splicing junctions. Diverse categories of AS events were identified including alternative acceptor, alternative donor, alternative first exon, alternative last exons, exon skipping (ES), intron retention, and so forth (Figure 1A). Strikingly, the numbers of AS events of all categories in the mutant were significantly larger than those in WT (binominal exact test, $p < 0.05$; Figure 1A). These results clearly indicate that AS was globally affected by the null mutation of *OsMet1-2*, with a net effect of enhancing AS. A total of 1942 events, distributed in 1446 genes, were identified as differential AS (DAS) events between the two genotypes (Supplemental Table 1). RT–PCR and/or quantitative PCR analysis of 15 selected DAS events validated 14 as *bona fide* ones (Supplemental Figure 2 and Supplemental Table 2), indicating reliability of our RNA-seq data and the workflow. Gene ontology analysis indicated that these 1446 DAS genes were significantly enriched in photosynthesis, generation of precursor metabolites and energy, and some secondary metabolism pathways (Supplemental Table 3).

Based on a novel set of gene annotations with complete gene models considered, the null mutation of *OsMet1-2* caused altered expression of 9409 genes from its isogenic WT in seedling. Of these differentially expressed (DE) genes, more up- than down-regulated genes (6672 versus 2737) were detected in the mutant, consistent with the major role of DNA methylation in repressing gene expression (Hu et al., 2014). Only 350 (19%) DAS genes overlapped with DE genes containing AS (3092), suggesting that DAS and DE genes are regulated independently (binomial test, $p < 0.001$), consistent with earlier observations (Li et al., 2013). We next tested the possibility that loss of ^{m}CG may have affected expression of AS factor-coding genes, which in turn caused changes of AS in the mutant. We identified a total of 169 AS factor-coding genes, of which only 32 were differentially expressed between mutant and WT, but 113 contained AS events (Supplemental Table 4, binomial test, $p < 0.05$). These observations agree with earlier findings that AS factor-coding genes are little regulated at the transcriptional level but frequently alternatively splice themselves (Reddy et al., 2013), which can be affected by altered DNA methylation. Indeed, we found a substantial proportion of AS factor genes (159, 94.1%) that showed altered gene-body methylation in the mutant relative to WT (Supplemental Table 4). For example, one gene (LOC_Os02g12850) encoding a RNA recognition

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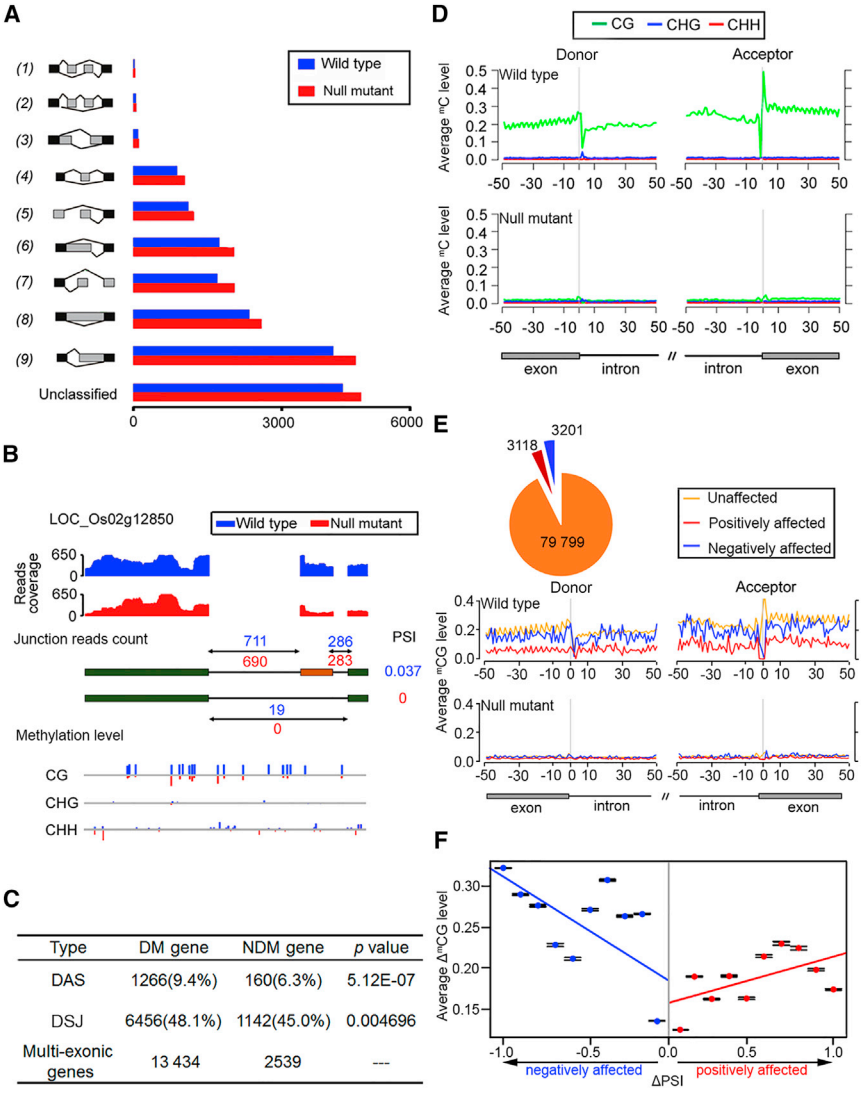

Figure 1. Global Loss of ^{m}CG due to *met1-2* Null Mutation Affects Alternative Splicing in Rice.

(A) Quantification of categorized alternative splicing (AS) events in the *met1-2* null mutant and its isogenic wild-type (WT). AS events were classified into 10 categories: (1) mutually exclusive exons; (2) two exons skipping; (3) alternative donor and acceptor; (4) exon skipping; (5) alternative first exon; (6) alternative donor; (7) alternative last exon; (8) intron retention; (9) alternative acceptor; and (10) unclassified including all other types of events.

(B) An exemplary case of exon skipping in an AS factor-coding gene (LOC_Os02g12850) in the mutant and WT. Upper panel: Mapped RNA-seq reads in each exon of the gene in WT and mutant. Middle panel: Number of RNA-seq reads covering each arrow-denoted splicing junction of the transcript isoforms in WT and mutant. Bottom panel: ^{m}CG, ^{m}CHG, and ^{m}CHH levels per cytosine site within the compared region of LOC_Os02g12850 in WT and mutant.

(C) Tabulation of differential AS events (DAS), differential AS junctions (DSJ), and multi-exonic genes in the differential methylation (DM) and non-differential methylation (NDM) genes in WT and mutant. For DAS and DSJ, the *p* values (Fisher's exact test) of comparisons between DM and NDM group are listed in the last column.

(D) Average methylated cytosine (^{m}C) per cytosine site in each context (^{m}CG, ^{m}CHG, or ^{m}CHH) around the donor (exon-and-intron) and acceptor (intron-and-exon) regions at both strands of all splicing junctions in WT and mutant.

(E) Changes of average ^{m}CG level of AS junctions in WT and mutant. Upper panel: Pie chart illustrating the numbers of AS junctions that are unaffected, positively affected, and negatively affected by cytosine methylation. Middle and bottom panels: Changes of average ^{m}CG level around the donor and acceptor regions of the three types of AS junctions before (middle panel) and after null mutation of *OsMet1-2* (bottom panel).

(F) Bi-directional effects of reduced ^{m}CG level on AS junctions. ΔPSI values ($PSI_{null} - PSI_{wild}$, on *x* axis) of positively and negatively affected AS junctions were grouped into 10 bins, respectively. The average ± SD of Δ^{m}CG value in each bin are illustrated on the *y* axis. Regression lines of Δ^{m}CG versus ΔPSI in positively and negatively affected AS junctions, respectively, were constructed (Pearson's correlation: $R_{negative} = -0.68$, $p_{negative} = 0.02 < 0.05$; $R_{positive} = 0.56$, $p_{positive} = 0.09$).

motif-containing-protein showed significant loss of ^{m}CG and concomitant reduction of the ES events (Figure 1B). Thus, although we cannot rule out the possibility that the changed expression of these 32 AS factor-coding genes due to null mutation of the major CG methyltransferase plays a role in the globally altered AS, we are emphasizing the corollary that loss of ^{m}CG might have directly affected AS based on the following lines of evidence.

First, we divided all expressed genes (15 973) that contain multiple exons into two groups: differential methylation (DM) genes (13 434) and non-differential methylation (NDM) genes (2539) (Figure 1C). There were 1266 and 160 DAS genes in the DM group and NDM group, respectively. Likewise, there were 6456 and 1142 genes containing differential spliced junctions (DSJs) in the DM group and NDM group, respectively. Thus, both DAS and DSJ genes were significantly enriched in the DM group as comparison with the NDM group (Fisher's exact test, $p < 0.05$, Figure 1C), strongly suggesting that DNA methylation affects AS in rice.

Second, it has been established in animal cells that a generally higher ^{m}CG level in exon than intron of a given exon–intron junction may serve as a marker of exons to facilitate splicing (Gelfman et al., 2013; Yearim et al., 2015). In light of this, we compared ^{m}CG levels around the donor (exon-and-intron) and acceptor (intron-and-exon) regions of all splicing junctions in WT and mutant. We found that the ^{m}CG level in WT was markedly higher in exons than in their adjacent introns (Figure 1D, upper panel), consistent with previous findings in human and mouse cells (Gelfman et al., 2013; Yearim et al., 2015). We also confirmed that the higher exon-versus-intron ^{m}CG levels is not due to their higher CG content in the former, because the same pattern was seen after leveling the CG contents (Supplemental Figure 3).

The fact that this exon-versus-intron methylation difference was fully abolished in the mutant (due to 91% ^{m}CG loss in the latter) (Figure 1D, lower panel) would thus explicitly implicate that AS was directly affected in the mutant due to massive loss of ^{m}CG. Because the mutant did not show substantial reduction of methylation levels of ^{m}CHG and ^{m}CHH both globally (Hu et al., 2014) and at the exon–intron junctions (Figure 1D), their impacts on splicing efficiency cannot be assessed using this mutant.

Third, given the previous documentation that DNA methylation can affect AS in both directions, either positive or negative (Yearim et al., 2015), we also addressed this issue with our data. We found that at least 7% (6319) of AS junctions showed differential splicing efficiency between WT and mutant (DSJ, Supplemental Table 1), with 3201 (3.7%) and 3118 (3.3%) showing enhanced and reduced splicing in the mutant, respectively (Figure 1E, upper panel). This suggests that the splicing efficiency of different AS junctions had distinct responses to the null mutation of *OsMet1-2* in rice. Interestingly, we found that the three groups of AS junctions (unaffected, positively affected, and negatively affected) showed different ^{m}CG levels in WT, yet they all were reduced to similar low levels in the mutant (Figure 1E, middle and bottom panels). This suggests that intrinsic splicing signals are the prevailing factors deciding AS, while levels of ^{m}CG can only affect AS efficiencies of certain junctions, i.e., junctions in the positively and negatively affected groups. We thus further tested the correlations between alternations of splicing efficiency (ΔPSI) and changes of the average ^{m}CG levels (Δ^{m}CG) in each of the two groups independently (Figure 1F). We found that enhanced and reduced splicing efficiencies were significantly affected by loss of ^{m}CG in the corresponding group (Figure 1F). Overall, this analysis indicates that ^{m}CG level indeed plays a role in the splicing efficiency of a subset of AS junctions in rice.

In conclusion, we document here that DNA methylation could affect gene alternative splicing in plants in ways similar to that in animals and human cells, suggesting evolutionary conservation of the mechanism. However, we found that only approximately 7% (6319) of the alternative junctions were influenced by global loss of ^{m}CG. Our results are in line with the suggestion that DNA methylation is not essential for splicing, but could play a ''fine-tuning'' regulatory role on its efficacy (Maor et al., 2015; Yearim et al., 2015).

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

This work was supported by the State Key Basic Research and Development Plan of China (2013CBA01404) and the Program for Introducing Talents to Universities (B07017).

ACKNOWLEDGMENTS

We thank Dr. Blake Mayer for constructive suggestions on this work. No conflict of interest declared.

Received: August 17, 2015
Revised: September 29, 2015
Accepted: September 29, 2015
Published: October 5, 2015

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http://dx.doi.org/10.1016/j.molp.2015.09.016

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