Brief Communication

CRISPR/Cas9 induces exon skipping that facilitates development of fragrant rice

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Received 9 September 2020; revised 24 October 2020; accepted 11 November 2020.
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Keywords: CRISPR, Cas9, exon skipping, alternative splicing, premature termination codon, mis-splicing, BADH2, fragrant rice.

Splicing of precursor mRNAs (pre-mRNAs) is a critical biological process of gene expression. Normally, pre-mRNAs are processed by spliceosomes to produce mature mRNAs by removing introns at 5′-(donor) and 3′-(acceptor) splice sites based on the canonical GU-AG rule (Reddy et al., 2013). Mutation at either the intron donor or acceptor sites could cause mRNA mis-splicing. Recently, an exon skipping method has been developed to generate loss of gene function in mammalian cells using base editors to mutate nucleotides at the acceptor sites (Gapinski et al., 2018). Moreover, it has been approved that CRISPR/Cas9-mediated exon skipping in rabbits depends on non-sense-associated altered splicing induced by premature termination codon (PTC) mutation (Sui et al., 2018). In contrast, triggering exon skipping by genome editing in plants is still controversial as disruption of the intron donor or acceptor sites in plant genes did not trigger exon skipping but allowed either intron retention or aberrant splicing (Li et al., 2019), following the fact that intron retention is the most frequent mode of alternative splicing in plants (Ner-gaon et al., 2004).

Fragrance is one of the most important rice quality traits (Abdelrahman and Zhao, 2020). The natural aroma substance 2-acetyl-1-pyrroline (2AP) is the major contributor to the aroma flavor of fragrant rice. The rice gene OsBADH2 encodes a betaine aldehyde dehydrogenase (BADH) that inhibits 2AP biosynthesis. Mutations in OsBADH2 could result in 2AP accumulation and rice fragrance (Chen et al., 2008). To check the alteration of the OsBADH2 mRNA in the edited plants, RT-PCR with a forward and a reverse primer in exons 1 and 6, respectively, was conducted to amplify the cDNAs from the homozygous mutants (Rbadh2AG and Rbadh2AAG) and the wild type (WT). A 528bp-cDNA fragment was amplified from the mutant plants, while the WT generated a 671bp-fragment as expected (Figure 1d). Sanger sequencing revealed that the cDNA fragment from the WT is the expected size of exons of OsBADH2, indicating that the spliceosome recognizes the 5′-splice sites and cleaves the sites following normal splicing process. To our surprise, not only the introns but also the exon 2 were absent in the 528bp-cDNA from the edited plants (Figure 1e, f), indicating that the deletion of nucleotides (ΔG or ΔAAG) caused an exon skipping splicing pattern (Figure 1f). Apparently, the alteration (ΔG or ΔAAG) at the donor site of intron 2 prevented the spliceosome from normal splice site recognition. The Osbadh2 mRNA missing exon 2 from Rbadh2AG and Rbadh2AAG proves that the exonic nucleotide (G) immediately upstream of the exon–intron junction is critical for the normal splicing process. Deletion of this exonic nucleotide caused the entire exon skipping. Recently, it has been shown that base editing-mediated disruption of either the native intron donor site or acceptor site in plant genes caused intron retention or mis-spliced segments, but no exon skipping mutation (Li et al., 2019). Our present study showed that exon skipping could happen to occur in plants by CRISPR/Cas9-mediated mutations at the exon end immediately upstream of the exon–intron junction. However, it is unknown that whether the deletion of exonic nucleotide upstream of the intron donor site can always lead to exon skipping in plants.
It has been reported that base editing-mediated exon skipping depends on PTC mutations in rabbits (Sui et al., 2018). However, our results showed that exon skipping occurred due to the CRISPR/Cas9-mediated alterations at the splice site of a plant gene. Moreover, we found the exon 2 removal during the splicing process in the Rbadh2D and Rbadh2DAAAG mutants caused shifting of reading frame in the processed mRNA, resulting in a PTC in the exon 3 (Figure 1g). The existence of the PTC did not cause any further exon skipping in Rbadh2D and Rbadh2DAAAG, which was in line with the results obtained by Lee et al. (2020). The transcripts containing a PTC will be degraded by non-sense-mediated decay (Capito et al., 2018), as indicated by the reduced expression level of the mutated Osbadh2 compared with the WT (Figure 1d, h).

Since loss of OsBADH2 function promotes accumulation of 2AP, we explored the consequence of Osbadh2 exon 2 skipping on the 2AP content in grains of the homozygous transgene-free plants of Rbadh2D and Rbadh2DAAAG. Error bars represent standard deviation (n = 4). P values were calculated by t-test. **, P < 0.01. (i) 2AP content in R317, DHK2, Rbadh2D and Rbadh2DAAAG. Error bars represent standard deviation (n = 3).
control. The GC-MS internal standard for 2AP measurement was 2,4,6-trimethyl pyridine (TMP) (Laohakunjit and Kerdchoechuen, 2007), as it has similar chemical properties to 2AP. Results showed that the 2AP content in the mutants (Rbadh2<sup>DG</sup> and Rbadh2<sup>DAAAG</sup>) is as high as that in the positive control (about 0.08 mg/kg), while the 2AP in the WT was null (Figure 1i). These results indicate that the Osbadh2 in the mutants is not functional to hinder the production of 2AP, resulting in the grain fragrance.

We further investigated the effect of the OsBADH2 exon 2 skipping on the phenotypic characteristics of the mutants. Data showed that there were no significant differences between the edited plants and WT for vegetative and yield characteristics, indicating that the edited gene has no effect on the agronomic traits except the 2AP content or grain fragrance.

In conclusion, this study provides the first evidence that CRISPR/Cas9-mediated deletion of the exonic nucleotide at the exon–intron junction of a plant gene could cause exon skipping during pre-mRNA splicing. Furthermore, the OsBADH2 exon 2 skipping caused shifting in the reading frame, resulting in a downstream PTC in exon 3. Moreover, our results highlighted that CRISPR/Cas9-mediated exon skipping could facilitate improvement of agronomically important traits of plants.

**Acknowledgements**

The authors thank Professor Yaoguang Liu, College of Life Sciences of South China Agricultural University, for providing the CRISPR/Cas9 system.

**Conflict of interests**

The authors declare that they have no competing interests.

**Funding**

This research was supported by grants from the Major Science and Technology Project to Create New Crop Cultivars Using Gene Transfer Technology (2016ZX08001002), the Innovation Program of Chinese Academy of Agricultural Sciences (to K.Z.) and the Talented Young Scientist Program of China (to M.A.).

**Authors’ contributions**

K.Z., C.W. and F.W. designed experiments. Y.T., J.L., Z.J. and C.W. performed experiments. H.Q. provided the rice seeds. Y.T., M.A. and K.Z. analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

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