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Improving pre-harvest sprouting resistance in rice by editing OsABA8ox using CRISPR/Cas9

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Abstract

Pre-harvest sprouting (PHS) in cereal crops, is a universal phenomenon that affects grain yield and quality (Tai et al. 2021). Breeders use different methods to strengthen seed dormancy to prevent PHS including genome editing. Abscisic acid is one of the phytohormones that promote seed dormancy. *ABA8ox* genes encode abscisic acid 8' hydroxylase (ABA8OX) which determines the level of ABA content by catabolizing ABA (Vallabhaneni and Wurtzel 2010). Three genes in rice encode ABA8' hydroxylases, namely *OsABA8ox1* (*LOC_Os02g47470*), *OsABA8ox2* (*LOC_Os08g36860*) and *OsABA8ox3* (*LOC_Os09g28390*) (Kushiro et al. 2004; Cai et al. 2015; Zhang et al. 2020). Each gene is expressed in different tissues of rice.

Key Message

Knock out *OsABA8ox* helps improve pre-harvest spouting resistance and do not effect rice yield.

Full Text

Previous studies showed that ABA content in seed is positively correlated with the level of seed dormancy and that ABA content is controlled by its biosynthesis and catabolism. Therefore, genes that encode abscisic acid 8' hydroxylase, which catabolizes ABA, play a crucial role in seed dormancy. However, few *OsABA8ox* knock out alleles have been generated to strengthen seed dormancy. Here, to improve rice pre-harvest sprouting resistance we developed CRISPR/Cas9 editing strategies to generate new *OsABA8ox*s mutant lines with increased levels of seed dormancy in the background of elite, high yielding *japonica* variety Ningjing6, which often sprouts before harvest under high temperature, rainy conditions.

We used online tools CRISPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR) to design sgRNAs targeting regions close to the respective start codon of each of *OsABA8ox1, OsABA8ox2* and *OsABA8ox3*. The three sgRNAs were separately cloned into vector pCAMBIA1305.1 and the resulting plasmids were individually introduced into Ningjing6 by *Agrobacterium*-mediated transformation. We selected T-DNA-free homozygous T_3 generation individuals for further research and analysis (Fig. 1A). After editing the *OsABA8ox1* gene we obtained three different lines named *cr-aba8ox1-1* (carrying a G insert), *cr-aba8ox1-2* (carrying a T insert and *cr-aba8ox1-3* (carrying an A insert). All three mutations caused a change in amino acid sequence from the same position. After editing *OsABA8ox2* gene we obtained line *cr-aba8ox2-1* that carried a T insert causing a frame shift. After editing *OsABA8ox3* we obtained lines *cr-aba8ox3-1* (carrying an A insert causing a frame shift) and *cr-aba8ox3-2* (carrying an ACGA deletion leading to a frame shift). All these transgenic lines excluded the transgene construct.

To investigate the effect of the different knock out mutations on seed dormancy we harvested mature seeds at 35 d post anthesis (DPA) and performed germination experiments using Ningjing6 as the control. All the knock out lines exhibited lower germination percentages than Ningjing6 (33.3±2.1%).

Among the knock out lines, the dormancy of *cr-aba8ox1-1* (4.4±0.5%), *cr-aba8ox1-2* (4.3±0.7%), *cr-aba8ox1-3* (6.6±0.8%) were the strongest; germination of the *cr-aba8ox2-1* line (15.1±1.2%) suggested moderate dormancy; and the *cr-aba8ox3-1* (20.7±1.3%) and *cr-aba8ox3-2* (21.3±2.0%) lines had the weakest dormancy (Fig. 1B-C). To exclude the effect of gene editing we compared the seed viability of Ningjing6 and the transgenic lines using freshly harvested seeds held at 50℃ for 5 days and naturally aged seed after 6, 12 and 18 months. We found that the seed germination and seedling vigor was not affected by the gene editing (Fig. 1D-E, Supplementary Fig. 1A-B).

Next, we choose the *cr-aba8ox1-2* line Representing the group with strongest dormancy to further research. Using seeds harvested 24 d post heading we found that after knock out of *OsABA8ox1*, the ABA8' hydroxylase content in the *cr-aba8ox1-2* line (140.1±10.9 U/L) was significantly lower than that in the wild type (204.1±13.2 U/L) (Fig. 1F). Quantification of the endogenous ABA level in line *cr-aba8ox1-X* and the wild type showed that the former (8.7±0.4 ng/g) contain much more endogenous ABA than the latter (7.5±0.1 ng/g) (Fig. 1G). RT-qPCR indicated that the expression of *DOG1L-3*, a well-known dormancy gene, in the *cr-aba8ox1-2* line was significantly higher than in the wild type. In addition, *ABI5*, a positively regulated ABA signaling gene was induced and negatively regulated ABA signaling genes *ABI1* and *ABI2* were suppressed after knocking out *OsABA8ox1* (Fig. 1H). An RNA sequencing (RNA-seq) experiment on seeds 24 DPA revealed 154 differentially expressed genes (DEGs; P <0.05) in the mutant line compared to Ningjing6; 121 genes were upregulated and 33 genes were downregulated. KEGG pathway classification of thee 154 DEGs indicated that most of the genes were associated with metabolism of carbohydrates and amino acids, especially 'starch and sucrose metabolism' which is highly associated with seed germination (Fig. 1I). We used RT-qPCR to verify the expression levels of the most significant DEGs, including two genes encoding sucrose synthase, and the results matched the transcriptome results (Supplementary Fig. 1C). These results demonstrate that the *cr-aba8ox1-2* line with an edited *OsABA8ox1* gene had reduced seed ABA8OX content and an increased level of endogenous ABA that enhanced ABA signaling, finally leading to stronger seed dormancy.

As ABA not only influences seed dormancy, but also plays a role in abiotic stress resistance. We hypothesized that the high level of endogenous ABA in the *cr-aba8ox1* line might also lead to stronger salt tolerance in rice seedlings. For verification we performed a salt tolerance assay by treating seedlings of knock out lines and the wild type with 150 mM NaCl. We found that the survival rate of the *cr-aba8ox1-2* line (78.9±4.6%) was much higher than that of the wild type (34.9±2.9%) (Fig. 1J).

An investigation of several agronomic traits indicated that the knock out transgenic lines in Ningjing6 had no significant changes except for plant height that was reduced by about 10 cm (Fig.1K, Supplementary Fig.1 D). We knocked out *OsABA8ox1* in other widely grown varieties, Ningjing4 and Ningjing8. Assessments of seed dormancy and agronomic traits in these new transgenic knock out lines compared to the respective parental lines indicated similar results to those obtained for Ningjing6; that is, knock out of the *OsABA8ox1* gene strengthened seed dormancy with no significant effects on other agronomic traits apart from reduced plant height (Fig. 1L, Supplementary Fig. 1E). Therefore, genetic engineering of *OsABA8ox1* involved in ABA metabolism has potential application in breeding.

In summary, we used CRISPR/Cas9 gene editing to target *OsABA8ox1*, *OsABA8ox2* and *OsABA8ox3* in Ningjing6 and obtained a total of six *cr-aba8ox1*, *cr-aba8ox2* and *cr-aba8ox3* transgenic mutant lines. The knockout lines were assessed for 3 years through molecular identification of target genes and detection of main agronomic traits, physiological and biochemical indicators. We found that knock out *OsABA8ox* genes, especially *OsABA8ox1*, significantly strengthened seed dormancy and improved pre-harvest spouting resistance. RT-PCR and RNA-seq analyses suggested that enhanced ABA signaling caused stronger dormancy phenotypes. Knock out of the same gene in additional varieties led to similar results and suggested that genetic modification of the *OsABA8ox1* gene has potential for application in breeding.

Declarations

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Author contribution statement All authors contribute to the study conception and design. LJ and JM provided the idea and designed the experiments. KF, WS, CC, CM, YH, FZ, QH, PW, TM, YC, ZZ, MZ and QT performed the experiments. KF analyzed the data and wrote the manuscript. XL was responsible for field management. All authors read and approved the final manuscript.

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Conflict of interest All authors declare no conflict of interest.

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Figures

Figure 1

Improvement of pre-harvest sprouting resistance in rice using CRISPR/Cas9 gene editing to target *OsABA8ox* genes. (A) Molecular structures of transgenic lines. Black arrows indicate the start and stop codons. Numbers in brackets indicate the distance from the ATG start codon. Black box denotes exons, line denotes introns, and white boxes are the untranslated regions. (B) Germination percentage for seeds harvested from Ningjing6 and T_3 *cr-aba8ox* lines at 35 days post heading. (C) Images of germinating seeds from Ningjing6 and T_3 *cr-aba8ox* lines harvested 35 days post heading. (D) Germination percentages of seeds from Ningjing6 and T_3 *cr-aba8ox* lines after storage for 6 months. (E) Germination percentages of seeds from Ningjing6 and T_3 *cr-aba8ox* lines after artificial breaking of dormancy by heat treatment. (F) ABA8'hydroxylase contents in Ningjing6 and the *cr-aba8ox1-2* line. (G) Endogenous ABA levels in Ningjing6 and the *cr-aba8ox1-2* line. (H) Expression levels of dormancy and ABA signaling-related genes in Ningjing6 and *cr-aba8ox1-2* lines. (I) Transcriptome analysis indicating the most enriched DEGs in seeds of Ningjing6 and the *cr-aba8ox1-2* line. The x-axis is the enrichment ratio, the y-axis is the KEGG pathway; bubble size represents the number of genes annotated to a specific KEGG pathway, and the color represents the enrichment significance value. (J) Survival percentages of Ningjing6 and *cr-aba8ox1-2* lines after treatment with 150 mM NaCl. (K) Plant heights of Ningjing6 and *cr-aba8ox* lines. (L) Dormancy phenotypes of *cr-aba8ox1* mutant lines in Ningjing4 and Ningjing8.

Supplementary Files

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- SupplementaryFigure1.docx