FOCUS ARTICLE

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

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Abstract

Key message Knock out OsABA8ox helps improve pre-harvest spouting resistance and do not affect rice yield. Pre-harvest sprouting (PHS) is a phenomenon that the seeds of crops germinate preharvest, which reduces the yield and quality of rice. Abscisic acid (ABA) is one of the phytohormones that promotes seed dormancy. ABA8′ hydroxylase is the main enzyme that can catabolism ABA in plant. There are three genes that encode ABA8′ hydroxylase in rice, named OsABA8ox1, OsABA8ox2 and OsABA8ox3. In this study, we use CRISPR/Cas9 gene editing technology to target these three genes in Ningjing6 and find that the knockout transgenic lines are all significantly strengthen in seed dormancy and have no effect on the yield. By a series of quantitative experiments, we consider that after knock out OsABA8ox, the high endogenous ABA level will influence the ABA signal which suppress the substantial and energy metabolism in the seeds, and finally led to higher dormancy.

Keywords Abscisic acid · Genome editing · Oryza sativa · Seed dormancy

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 (ABA) is one of the phytohormones that promotes seed dormancy. ABA8ox genes encode abscisic acid 8′ hydroxylase (ABA8OX) which determines the level of ABA content in seed 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.

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′ hydroxy-
lase, 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 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 and 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 T3 generation individuals for

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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 T1 cr-aba8ox lines at 35 days post heading. C Images of germinating seeds from Ningjing6 and T1 cr-aba8ox lines harvested 35 days post heading. D Germination percentage of seeds from Ningjing6 and T1 cr-aba8ox lines after storage for 6 months. E Germination percentages of seeds from Ningjing6 and T1 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 line. 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 line 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

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-aba8ox1-2 (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 days 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%), and 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 °C 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 chose the cr-aba8ox1-2 line representing the group with strongest dormancy to further research. Using seeds harvested 24 days 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 cr-aba8ox1-2 line and the wild type showed that the former (8.7 ± 0.4 ng/g) contain much more endogenous ABA than wild type (7.5 ± 0.1 ng/g) (Fig. 1G). RT-qPCR indicated that the expression of DOG11-3, 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 cr-aba8ox1-2 line compared to Ningjing6; 121 genes were upregulated and 33 genes were downregulated. KEGG pathway classification of these 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 (RSUS2 and RSUS3), and the results matched the transcriptome results (Supplementary Fig. 1C). RSUS2 was reported that its expression increases significantly in seedlings germinated and grown in hypoxia and RSUS3 was reported that its expression is highly specific to the seed at the early phase of grain filling (Huang et al. 1996; Hirose et al. 2008). In cr-aba8ox1-2 line, the expression of RSUS2 and RSUS3 is reduced significantly, which may be the reason of strong dormancy. 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 percentage 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. 1D). 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). We suspect that this may due to the high level ABA as many researches have confirmed that high level ABA will affect plant growth (Yoshida et al. 2019; Liu et al. 2022). 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 the 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.

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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 and QT performed the experiments. KF analyzed the data and wrote the manuscript. XL was responsible field management. All authors read and approved the final manuscript.

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Declarations

Conflict of interest All authors declare no conflict of interest.

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