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SHORT COMMUNICATION

Prolongation of seed viability and grain quality in rice by editing *OsLOX1* using CRISPR/Cas9

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Abstract

Deterioration of rice (*Oryza sativa* L.) affects grain quality and seed viability during storage. Lipoxygenase (LOX), a key enzyme in lipid metabolism, directly affects the rate of ageing. Here, we found that knock-out of lipoxygenase gene *OsLOX*1 by CRISPR/Cas9 delayed loss of seed viability and quality. Transcriptome analysis showed that during storage, *OsLOX1* affected transcription of multiple genes, including genes related to lipid metabolism and antioxidant pathways such as phosphatase and acetaldehyde dehydrogenase, which may regulate the seed storability. The genes significantly down- and up-regulated only in Ningjing 4 after NA for 13 months and 3 days of AA suggesting that *OsLOX1* likely promoted seed viability in rice by balancing ageing and storage related genes, and regulated the seed storability through the amino acid synthesis and metabolic pathways. Moreover, knock-out of *OsLOX1* without CRISPR/Cas9 not only improved the seed viability, but also had little impact on agronomic traits. More importantly, the *OsLOX1* knock-out lines were approved in 2019 (Agricultural Foundation of China Report No. 770). Collectively, our study showed that knock-out of *OsLOX1* is beneficial for prolongation of seed viability and can be directly applied to agricultural production.

Keywords *OsLOX1* · CRISPR/Cas9 · Seed viability · *Oryza sativa* L

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Rice (*Oryza sativa* L.) is a major cereal crop that feeds more than half the world population. Although yield continuously increase, there are numerous hindering factors, the ageing is one of these factors that affects seed viability and quality.

Lipid degradation is one of the factors that affecting the seed deterioration during storage (Takano 1993). LOX catalyzes lipid peroxidation by utilizing phospholipid components from the membrane as substrates (Ebone et al. 2019). The *LOX* gene family is ubiquitous in plants, and various members are reported to affect physiological processes, such as seed germination, fruit ripening, and senescence, defense responses against biotic and abiotic environmental stresses (Viswanath et al. 2020).

Previous studies showed that *OsLOX1* (*Os03g0700700*) plays a defensive role in responding to mechanical damage and insect feeding in rice (Wang et al. 2008). *OsLOX2* (*Os03g0738600*) controls the seed viability during artificially ageing during storage (Huang et al. 2014). *OsLOX3* (*Os03g0700400*) encodes a key enzyme that leading to loss of seed viability (Kenta et al. 2008; Long et al. 2013; Ma et al. 2015). Though these reports above indicate that *OsLOX* genes may participate in insect attack or seed viability, whether *OsLOX1* affects seed ageing is largely unknown.

To test the function of *OsLOX1* in seed ageing, we used CRISPR/Cas9 (CRISPR, clustered regularly interspaced short palindromic repeats/Cas9, CRISPR associated 9) technology which provides an ability to improve crop yield, quality, environmental stress resistance (Bao et al. 2019) to generate *OsLOX1* knock-out lines in the background of *japonica* cultivar Ningjing 4. We finally got three *OsLOX1* knock-out lines, namely *cr-lox1-I*, *cr-lox1-II*, and *cr-lox1-III* (Fig. 1). The *cr-lox1-I*, *cr-lox1-II*, and *cr-lox1-III* had a G deletion, a 16 bp deletion and a 20 bp deletion, respectively, and all changes caused early termination of translation (Fig. S1), leading to truncated proteins without the lipoxygenase domain (*cr-lox1-I* with 29 amino acids, *cr-lox1-II* with 24 amino acids, and *cr-lox1-III* with 164 amino acids) compared to 877 amino acids in the wild type gene (Fig. S2). After series of various time of natural or artificial ageing test, we found that the significant difference between wild type and *OsLOX1* knock-out lines was occurred under natural ageing (stored at room temperature) (NA) for 13 months or artificial ageing (40 °C, RH = 80%) (AA) for 22 days. Thus, we used NA for 13 months or AA for 22 days for further study.

After breaking of dormancy, the germination rates of Ningjing 4 and three *OsLOX1* knock-out lines were all at least 95% and showed no significant differences (Fig. 1a-c). However, after AA for 22 days or NA for 13 months, the germination rates of the lines with *cr-lox1-I*, *cr-lox1-II* and *cr-lox1-III* were significantly higher than that for Ningjing 4 (Fig. 1a-c). These results indicated that knock-out of *OsLOX1* improved the seed viability.

To determine why knock-out of *OsLOX1* led to higher germination than in the wild type, we first investigated the expression of *OsLOX1* after breaking dormancy and before aging treatment. Expression of *OsLOX1* in the knock-out lines caused no significant change compared with that in Ningjing 4 (Fig. 1d), whereas the LOX activity of knockout lines was significantly decreased (Fig. 1e). Based on these results, we concluded that knock out of *OsLOX1* may abolish lipoxygenase activity and reduced the rate of deterioration of seeds during storage. As the linoleic acid is the main fatty acid and substrate in the LOX pathway in rice (Ebone et al. 2019),

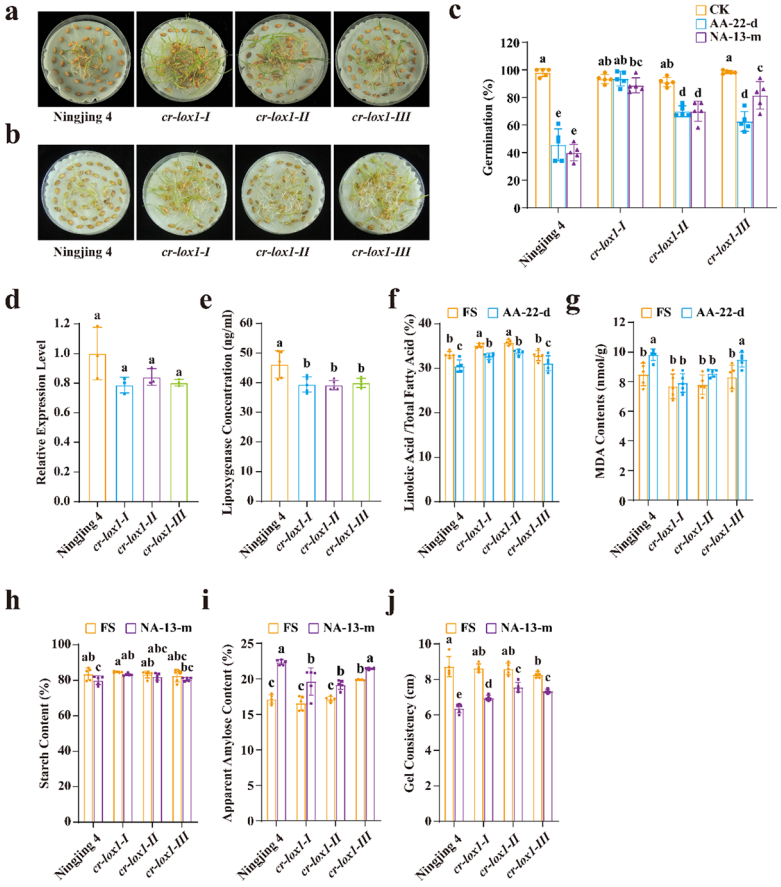

Fig. 1 The germination and the physicochemical properties of wild type and *OsLOX1* knock-out lines. **a**-**b** Germination of Ningjing 4 and *cr-lox1* lines after AA of 22 days (**a**) and NA of 13 months (**b**), respectively. **c** Germination rates of Ningjing 4 and *cr-lox1* lines with dormancy breaking followed by AA and NA. CK, fresh, untreated seeds; 'AA-22-d', seeds treated by AA; 'NA-13-m', seeds treated by NA; $n=5$. Samples with different letters were significantly different ($P<0.05$, One-way ANOVA with Tukey's post-test). **d** Relative expression of *OsLOX1* in Ningjing 4 and *OsLOX1* knock-out lines after breaking dormancy and before aging. $n=3$; the data were normalized with the level for Ningjing 4 as 1. The ubiquitin gene (*Os03g0234200*) was used as the internal control. **e** Comparison of lipoxygenase activities among Ningjing 4 and *OsLOX1* knock-out lines after breaking dormancy and before aging. $n=5$. **c**-**e** Samples with different letters were significantly different ($P<0.05$, One-way ANOVA with Tukey's post-test). Proportions of linoleic acid (**f**) and contents of MDA (**g**) among Ningjing 4, and *cr-lox1-I*, *cr-lox1-II*, and *cr-lox1-III* lines before and after AA for 22 days, $n=5$. starch (**h**), amylose (**i**) and gel consistency (**j**) among Ningjing 4, and *cr-lox1-I*, *cr-lox1-II*, and *cr-lox1-III* lines before and after NA for 13 months, $n=5$. **f**-**j** Samples with different letters were significantly different ($P<0.05$, One-way ANOVA with Dunnett's test). FS, AA-22-d and NA-13-m represent fresh seed, seeds treated by AA for 22 days and seeds treated by NA for 13 months, respectively

we then detected the proportions of linoleic acid in freshly harvested seeds. The result showed that proportions of linoleic acid in the *cr-lox1-I* and *cr-lox1-II* knock-out lines were higher than that in Ningjing 4 (Fig. 1f). Whereas the proportions of

linoleic acid were all lower in Ningjing 4 and *OsLOX1* knock-out lines after AA than freshly harvested seeds (Fig. 1f). In addition, MDA is an important indicator in measuring lipid peroxidation of rice during storage (Ebone et al. 2019). The MDA contents in Ningjing 4 and knockout lines showed no significant difference (Fig. 1g) in freshly harvested seeds. After AA for 22 days, however, the MDA contents of *cr-lox1-I* and *cr-lox1-II* were unchanged (Fig. 1g). On the contrary, the contents of MDA began to accumulate after AA in Ningjing 4 and *cr-lox1-III* (Fig. 1g). The changes on linoleic acid and MDA contents in Ningjing 4 and *OsLOX1* knock-out lines before and after AA, indicated that knockout of *OsLOX1* reduced the rate of lipid peroxidation in seeds during storage.

Starch, made up of amylose and amylopectin and the main seed component determining rice quality undergoes significant changes during storage. Amylose has the greater impact on quality. Gel consistency reflects the colloidal characteristics of rice flour and is an important indicator in evaluating eating quality and storage quality. We thus assessed the total starch content, amylose content and gel consistency of seeds in the *cr-lox1* lines compared with Ningjing 4 before and after NA for 13 months. There were no significant changes in total starch content, apparent amylose (reflects the proportion of amylose in starch) or gel consistency in fresh seeds of the *cr-lox1* lines compared with that in fresh seeds of Ningjing 4, but not for *cr-lox1-III*. Following NA, the total starch content in seeds was lower in Ningjing 4, while unchanged in *OsLOX1* knock-out lines compared with fresh seeds (Fig. 1h). The amylose content was higher, and gel consistency was lower in the *cr-lox1* mutants and Ningjing 4 after AA (Fig. 1i, j). However, the range of variation in the *cr-lox1* lines was lower than that in Ningjing 4 (Fig. 1h-j). Thus, knock-out of *OsLOX1* may slowed down the rate of loss in quality during seed storage.

We also used the seeds (treated by NA for 13 months) of Ningjing 4 and *cr-lox1-I* for the RNA sequencing to gain a deeper understanding of *OsLOX1* in regulating seed viability. As a result, we found that there were 1,650 up-regulated and 1,408 down-regulated genes in the *cr-lox1-I* line compared with Ningjing 4 after NA (Supplemental Table S4).

We defined the downregulated genes (*cr-lox1-I* mutant compared with Ningjing 4) as the genes that positively regulate seed ageing. After KEGG enrichment, these ageing-related genes with lower expression levels in the *cr-lox1-I* line than that in Ningjing 4 after NA, including multiple genes encoding phospholipases (Fig. S3a): *OsPLDζ1* (*Os05g0358700*), *OsPLDζ2* (*Os01g0310100*), *OsPLDβ1* (*Os10g0524400*) (Li et al. 2007), the expression of which were confirmed by qRT-PCR (Fig. S3c). Phospholipase D (PLD) is involved in lipid signal transduction, metabolism and degradation by converting hydrated phospholipid to non-hydrated phospholipid during seed storage and processing (Deepika and Singh 2022). Thus, our results suggested that these phospholipase genes may be induced and catalyzed hydrolysis of phospholipids on the cell membranes to fatty acids which were then catalyzed to harmful peroxides by *OsLOX1*, leading to loss of viability during storage.

We defined the upregulated genes (*cr-lox1-I* mutant compared with Ningjing 4) as the genes positively regulating seed storability. After KEGG enrichment, these storage-related genes with higher expression levels in the *cr-lox1-I* line

than that in Ningjing 4 after NA contained multiple acetaldehyde dehydrogenases (Fig. S3b). Aldehyde dehydrogenase (ALDH) detoxifies cytotoxic compounds in plants, and can oxidize aldehydes to corresponding carboxylic acids, which are important in maintaining seed longevity (Niranjan et al. 2021). In this study, the ALDH genes with higher expression levels in *cr-lox1-I* than that in Ningjing 4 included *OsALDH2B1* (*Os06g0270900*) and *OsALDH6B2* (*Os07g0188800*), the expression of which were confirmed by qRT-PCR (Fig. S3c). Thus, after knock-out of *OsLOX1*, it was likely that the level of toxic substances in the seeds were much lower whereas detoxification ability remained at a high level effectively maintaining seed viability. These results and analysis of RNA-seq indicated that *OsLOX1* may promote the seed viability by balancing ageing and storage related genes in rice.

Interestingly, we detected the expression of *OsLOX1* following different days of AA in Ningjing 4 and *cr-lox1-I* seeds after NA for 13 months. As a result, the largest difference in seed germination rate was at 3rd day and then declined to zero at 9 days between Ningjing 4 and *cr-lox1-I* (Fig. S4). Thus, we selected the seeds after NA for 13 months and subsequent 3 days of AA for RNA-seq analysis. With AA for 3 days after NA (13 months), there were 380 up-regulated and 304 down-regulated genes in *cr-lox1-I* line compared with Ningjing 4 (Supplemental Table S4). In addition, after NA for 13 months and subsequent 3 days of AA, there were 438 genes up-regulated and 401 genes down-regulated in Ningjing 4, whereas only 16 genes were up-regulated and 23 genes were down-regulated in *cr-lox1-I* (Supplemental Table S4). After NA of 13 months and additional 3 days of AA, the expression pattern of *OsPLDζ1* (*Os05g0358700*), *OsPLDζ2* (*Os01g0310100*), *OsPLDβ1* (*Os10g0524400*), *OsALDH2B1* (*Os06g0270900*) and *OsALDH6B2* (*Os07g0188800*) were similar to that of NA for 13 months only (Fig. S3c).

The next question was the changes in DEGs in seeds after NA and subsequent AA for 3 days. The 272 genes (Fig. S5a) significantly down-regulated only in Ningjing 4 were mainly enriched in the endocytosis pathway, linolenic acid metabolism, lipid metabolism and other pathways (Fig. S5c), suggesting that Ningjing 4 seeds may be on the verge of inviability. In addition, the 266 genes (Fig. S5b) up-regulated only in Ningjing 4 were mainly enriched in the biosynthesis and metabolic pathways of amino acids such as phenylalanine, tyrosine, tryptophan, cysteine and methionine, indicating a role of amino acid synthesis and metabolic pathways in regulating seed storability (Fig. S5d). These results indicated that *OsLOX1* likely promoted seed viability in rice by indirectly balancing ageing and storage related genes, and regulated the seed storability through the amino acid synthesis and metabolic pathways.

In order to evaluate the application of *OsLOX1* in agricultural production, we further examined the agronomic traits of *OsLOX1* knockout lines without the Cas9 vector. Investigations of the plant agronomic traits showed that there was no significant difference of agronomic traits between the *OsLOX1* knock-out lines and Ningjing 4 (Fig. S6). Overall, *OsLOX1* knock-out not only had no significant impact on agronomic traits but also improved seed viability. In addition, the *OsLOX1* knock-out lines were approved in 2019 (Agricultural Foundation of China Report No. 770), and will be used in breeding to improve the vitality of rice seeds.

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Authors' contributions JMW and LJ supervised this research; YPC performed the experiments. YPC and CLM wrote the manuscript; LJ revised the paper; PZ, QKT, ZYZ, TFM, PW and KF participated in the experiments; CC, YSH, FLZ, QXH and MZ were involved in the data discussions; SJL were involved in the generation of the transgenic plant. all authors read and approved the final manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The author(s) declare that they have no conflict of interest.

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