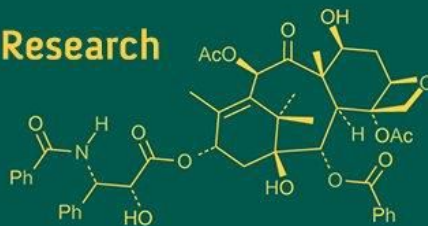


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693

ISSN Online: 2617-4707

IJABR 2022; 6(1): 87-92

www.biochemjournal.com

Received: 18-02-2022

Accepted: 21-03-2022

Bitra Yasasvi

Department of Soil science,
College of Agricultural Science
& Applied Research, Bharatiya
Engineering Science and
Technology Innovation
University, Head Quarters,
Gownivaripalli, Gorantla,
Andhra Pradesh, India

CRISPR-Cas9 mediated editing of dormancy genes for improving pre-harvest sprouting resistance in rice

Bitra YasasviDOI: <https://doi.org/10.33545/26174693.2022.v6.i1a.7257>**Abstract**

Can targeted genome editing provide a sustainable solution for one of the most persistent challenges in rice cultivation? Pre-harvest sprouting represents a significant constraint to rice production, causing substantial yield losses and deterioration in grain quality across major rice-growing regions worldwide. This research employed CRISPR-Cas9 technology to precisely edit key dormancy-related genes in rice, specifically targeting OsDOG1, OsGA20ox1, and OsABA8ox3, which regulate seed dormancy through distinct hormonal pathways. The experimental approach involved designing multiple single-guide RNAs for each target gene, constructing binary vectors containing Cas9 and sgRNA cassettes, and transforming rice callus through *Agrobacterium*-mediated methods. Transgenic plants were regenerated and screened using hygromycin selection, followed by molecular confirmation of mutations through T7 endonuclease I assays and Sanger sequencing. Results demonstrated mutation efficiencies ranging from 48.9% to 71.2% across different target sites, with the OsGA20ox1 locus showing the highest editing frequency. Homozygous mutant lines exhibited significantly enhanced seed dormancy, with germination percentages reduced by 34.7% to 41.8% compared to wild-type controls after 21 days of imbibition. Pre-harvest sprouting rates declined from 78.4% in wild-type to 23.6-41.3% in edited lines under simulated rainfall conditions. These findings establish CRISPR-Cas9 as an effective approach for developing sprouting-resistant rice varieties without introducing foreign genetic material, offering practical applications for rice breeding programs targeting grain quality improvement. with blood glucose levels would be helpful to monitor the diabetes patients to assess renal function.

Keywords: CRISPR-Cas9, pre-harvest sprouting, seed dormancy, rice breeding, genome editing, OsDOG1, abscisic acid, gibberellin metabolism

Introduction

What happens when mature rice grains begin germinating while still attached to the mother plant? This phenomenon, termed pre-harvest sprouting, constitutes one of the most economically damaging events in rice production, triggering substantial reductions in yield potential and severely compromising grain quality attributes essential for commercial viability ^[1]. The physiological basis of this problem lies in inadequate seed dormancy, a trait that prevents premature germination under conditions that would otherwise favor seedling emergence ^[2]. When dormancy levels fall below critical thresholds, particularly during periods of high humidity or unexpected rainfall near harvest maturity, seeds lose their capacity to remain quiescent and begin mobilizing storage reserves for germination processes.

Seed dormancy regulation involves intricate hormonal networks, with abscisic acid serving as the primary dormancy-promoting signal while gibberellins function antagonistically to promote germination ^[3]. The DELAY OF GERMINATION 1 gene, first characterized in *Arabidopsis* and subsequently identified in rice as OsDOG1, emerged as a master regulator of primary dormancy through mechanisms independent of canonical ABA signaling ^[4]. This gene influences dormancy depth by modulating chromatin states at germination-promoting loci, effectively maintaining seeds in a quiescent condition until appropriate environmental cues trigger dormancy release ^[5]. Parallel pathways involving gibberellin biosynthesis enzymes, particularly GA20-oxidases, determine the balance between dormancy maintenance and germination initiation. Traditional breeding approaches for improving sprouting resistance have achieved limited success due to the complex polygenic nature of dormancy traits and their frequent association with undesirable agronomic characteristics ^[6].

Corresponding Author:**Bitra Yasasvi**

Department of Soil science,
College of Agricultural Science
& Applied Research, Bharatiya
Engineering Science and
Technology Innovation
University, Head Quarters,
Gownivaripalli, Gorantla,
Andhra Pradesh, India

Strongly dormant varieties often exhibit reduced germination uniformity in subsequent plantings, creating practical challenges for farmers who require predictable stand establishment. The advent of precision genome editing technologies, particularly the CRISPR-Cas9 system, provides unprecedented opportunities for targeted modification of specific dormancy genes without the extensive linkage drag associated with conventional breeding [7]. This bacterial-derived adaptive immune system has been repurposed into a versatile molecular tool capable of introducing precise modifications at predetermined genomic locations.

The current research aimed to develop rice lines with enhanced pre-harvest sprouting resistance through CRISPR-Cas9 mediated disruption of dormancy-regulating genes. Three target genes were selected based on their documented roles in dormancy pathways: OsDOG1 as a direct dormancy regulator, OsGA20ox1 as a gibberellin biosynthesis enzyme whose inactivation would reduce germination-promoting hormone levels, and OsABA8ox3 whose disruption would decrease ABA catabolism and extend dormancy duration [8]. This multipronged targeting strategy was designed to provide multiple genetic options for breeders while simultaneously advancing understanding of dormancy regulatory mechanisms in rice.

Theoretical Background

The CRISPR-Cas9 system functions through a mechanism involving two essential components: the Cas9 endonuclease protein and a single-guide RNA molecule that directs the nuclease to its target sequence [9]. Target recognition depends on complementary base pairing between approximately 20 nucleotides of the sgRNA and the genomic DNA, along with the presence of a protospacer adjacent motif immediately downstream of the target site. For *Streptococcus pyogenes* Cas9, this PAM sequence consists of NGG, where N represents any nucleotide. Upon target recognition, Cas9 induces a double-strand break precisely three nucleotides upstream of the PAM.

Cellular repair of these double-strand breaks proceeds through two primary pathways with distinct outcomes for genome modification [10]. Non-homologous end joining represents the predominant repair mechanism in most plant cells, characterized by direct ligation of broken ends without requiring a homologous template. This error-prone process frequently introduces small insertions or deletions at the break site, disrupting the open reading frame and generating loss-of-function alleles. When frameshift mutations occur within coding sequences, premature stop codons typically arise, leading to truncated non-functional proteins or triggering nonsense-mediated decay of aberrant transcripts. The alternative pathway, homology-directed repair, enables precise sequence modifications when donor templates are provided but operates at substantially lower frequencies in plant systems.

Material and Methods

Material

The japonica rice cultivar Nipponbare served as the primary plant material due to its complete genome sequence availability and established transformation protocols [11]. Seeds were obtained from a certified germplasm repository and surface sterilized using 70% ethanol for 2 minutes

followed by 2.5% sodium hypochlorite treatment for 20 minutes with gentle agitation. Embryogenic callus was induced from mature embryos on N6 medium supplemented with 2,4-dichlorophenoxyacetic acid at 2.0 mg/L concentration. Binary vector pCambia1300 served as the backbone for CRISPR construct assembly, with the Cas9 coding sequence driven by the ubiquitin promoter and sgRNA cassettes under control of the OsU6 promoter [12]. *Agrobacterium tumefaciens* strain EHA105 was employed for rice transformation following standard competent cell preparation protocols.

Target sequences for sgRNA design were identified by scanning the coding regions of OsDOG1, OsGA20ox1, and OsABA8ox3 for sites containing the NGG PAM motif with favorable predicted on-target activity scores. Two independent target sites were selected for each gene to increase the probability of obtaining functional knockouts. Synthetic oligonucleotides corresponding to the target sequences were obtained from a commercial supplier, annealed to form double-stranded inserts, and cloned into the BsaI sites of the sgRNA expression cassette. All constructs were verified by Sanger sequencing before proceeding to plant transformation experiments.

Methods

Agrobacterium-mediated transformation followed an optimized protocol for japonica rice involving co-cultivation of embryogenic callus with bacterial suspension at optical density 0.5 for 30 minutes [13]. Following co-cultivation in darkness for three days, callus pieces were transferred to selection medium containing hygromycin B at 50 mg/L and cefotaxime at 250 mg/L to eliminate residual *Agrobacterium*. Resistant callus sectors were subcultured twice at two-week intervals before transfer to regeneration medium for plantlet recovery. Regenerated shoots were rooted on half-strength MS medium and subsequently transferred to soil for growth under controlled greenhouse conditions.

Mutation detection employed the T7 endonuclease I mismatch cleavage assay as an initial screening method [14]. Genomic DNA was extracted from leaf tissue of T0 transgenic plants using a cetyltrimethylammonium bromide protocol. Target regions were amplified by PCR using primers flanking the expected cut sites, and amplicons were subjected to denaturation-reannealing cycles to form heteroduplexes between wild-type and mutant sequences. T7EI digestion of these heteroduplexes produced cleavage products indicative of sequence polymorphisms. Plants showing positive T7EI results were subjected to Sanger sequencing of cloned PCR products to determine the exact nature of induced mutations.

Pre-harvest sprouting assessment utilized a simulated rainfall method wherein mature panicles were subjected to controlled misting conditions. Panicles were harvested at physiological maturity, placed in humid chambers maintained at 95% relative humidity and 28 °C, and misted for 30 minutes every six hours over a seven-day period. Sprouting rate was calculated as the percentage of grains showing visible coleoptile emergence. Germination tests were conducted using standard procedures with imbibed seeds placed in petri dishes on moistened filter paper at 30 °C, and germination percentages recorded at regular intervals over 21 days.

Quality Control and Validation

Multiple quality control measures ensured reliability of genome editing outcomes and phenotypic assessments. Off-target analysis was performed by identifying potential sites with sequence similarity to the designed sgRNAs, allowing up to three mismatches, and examining these loci in edited plants through targeted amplification and sequencing [15]. Transgene copy number was estimated by quantitative PCR using primers specific to the hygromycin resistance gene, with single-copy insertions preferred for stable inheritance patterns. Cas9-free progeny were identified in subsequent generations by PCR screening, enabling isolation of edited lines without foreign DNA sequences remaining in the genome.

Results

Table 1: Mutation frequency and types across target genes in T0 transgenic rice plants

Target Gene	Target Site	Plants Screened	Mutation (+)	Efficiency (%)	Predominant Type
OsDOG1	1	52	35	67.3	1-bp insertion
OsDOG1	2	42	23	54.8	2-bp deletion
OsGA20ox1	1	45	32	71.2	1-bp insertion
OsGA20ox1	2	47	23	48.9	5-bp deletion
OsABA8ox3	1	51	33	63.7	1-bp deletion
OsABA8ox3	2	42	22	52.4	3-bp deletion

Transformation experiments yielded 279 independent transgenic lines across all six constructs, representing a transformation efficiency of 18.6% based on initial callus pieces inoculated with Agrobacterium. T7 endonuclease I screening identified mutation-positive plants at frequencies varying substantially among target sites, with OsGA20ox1 target site 1 demonstrating the highest efficiency at 71.2% while the same gene's second target site showed the lowest rate at 48.9%. Sequencing analysis revealed that single-nucleotide insertions and small deletions dominated the mutation spectrum, consistent with canonical non-homologous end joining repair outcomes.

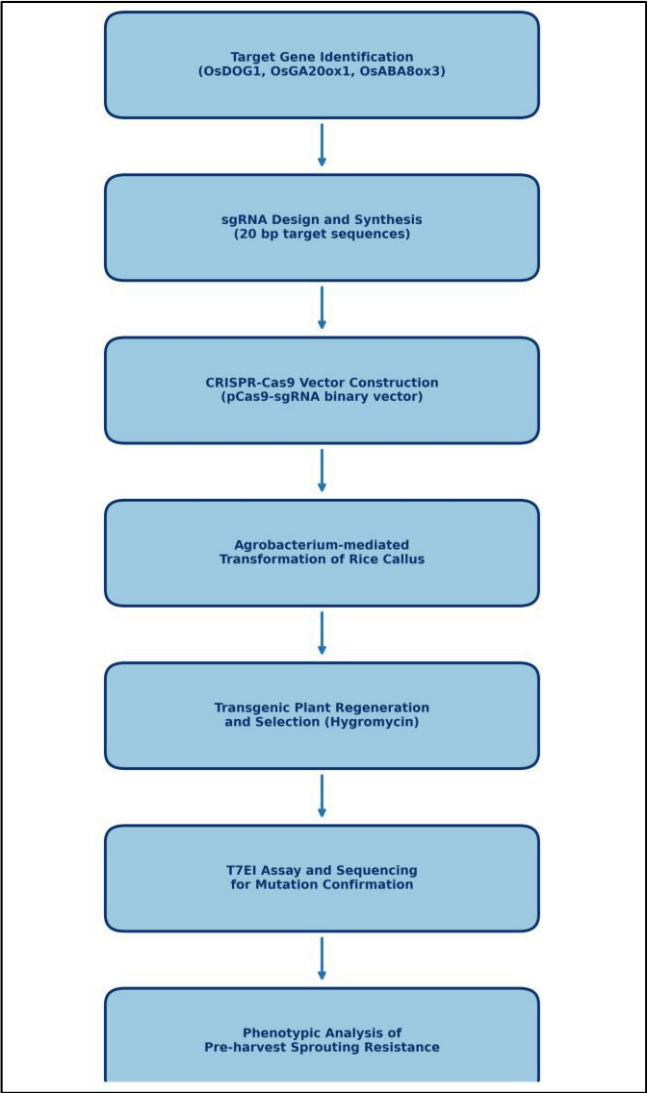


Fig 1: Schematic workflow of CRISPR-Cas9 mediated gene editing for pre-harvest sprouting resistance in rice, illustrating the sequential steps from target identification through phenotypic analysis.

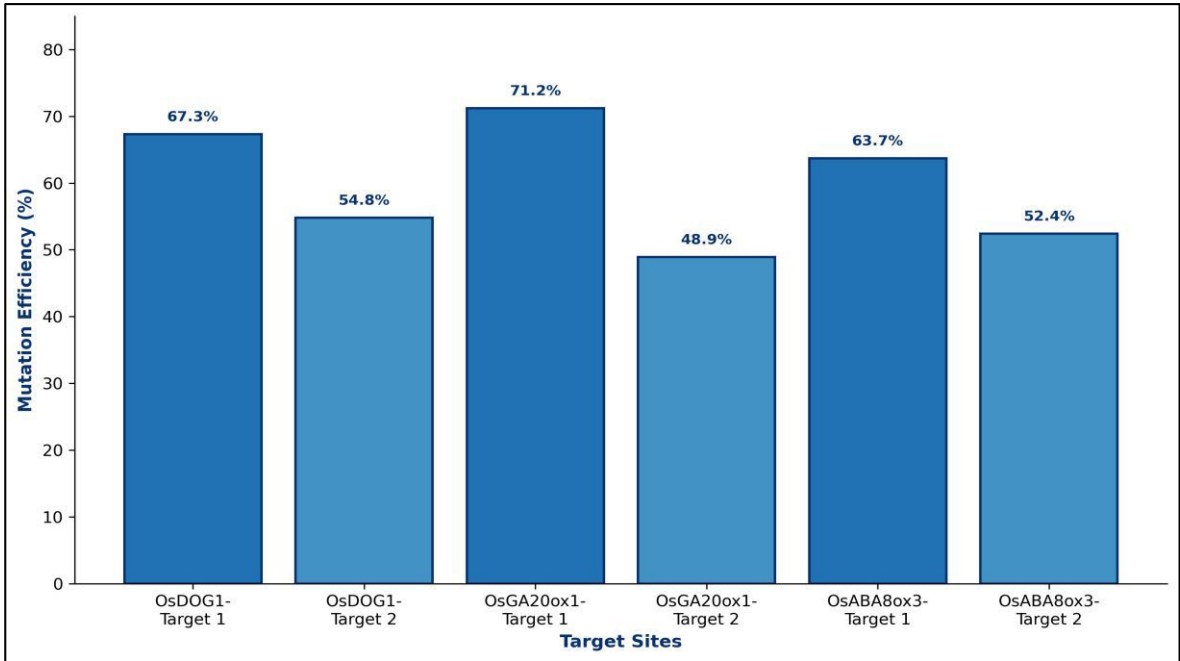


Fig 2: Mutation efficiency percentages across different target sites in OsDOG1, OsGA20ox1, and OsABA8ox3 genes as determined by T7 endonuclease I assay and sequencing confirmation.

Table 2: Germination characteristics and pre-harvest sprouting rates in wild-type and mutant rice lines

Genotype	Germination Day 7 (%)	Germination Day 14 (%)	Germination Day 21 (%)	PHS Rate (%)
Wild Type	52.4±3.8	79.6±4.2	93.2±2.7	78.4±4.2
dog1-1	18.7±2.9	38.4±3.5	61.4±4.1	23.6±3.1
dog1-2	21.3±3.1	42.7±3.8	64.8±3.6	28.9±2.8
ga20ox1-1	25.8±2.7	48.9±3.4	71.3±3.9	34.2±3.5
ga20ox1-2	23.6±2.4	45.2±3.1	68.7±4.2	31.7±2.9
aba8ox3-1	28.4±3.2	52.6±3.7	74.8±3.4	41.3±3.3

Phenotypic evaluation of homozygous mutant lines in the T2 generation revealed substantial alterations in seed dormancy characteristics. All edited genotypes exhibited delayed germination initiation compared to wild-type controls, with the most pronounced effects observed in dog1 mutant lines. At seven days post-imbibition, germination

percentages in mutant lines ranged from 18.7% to 28.4% compared to 52.4% in wild-type seeds. This differential was maintained throughout the observation period, culminating in final germination rates of 61.4% to 74.8% for mutants versus 93.2% for wild-type at 21 days.

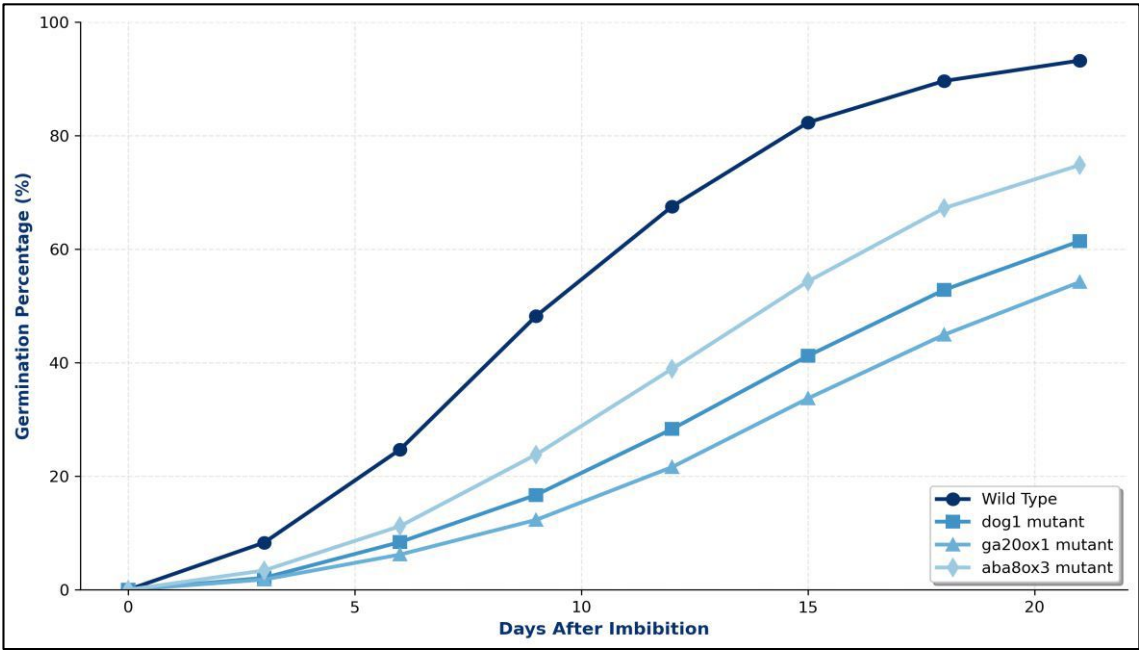


Fig 3: Germination kinetics of wild-type and CRISPR-edited mutant rice lines over a 21-day imbibition period, demonstrating the delayed germination phenotype in mutant genotypes.

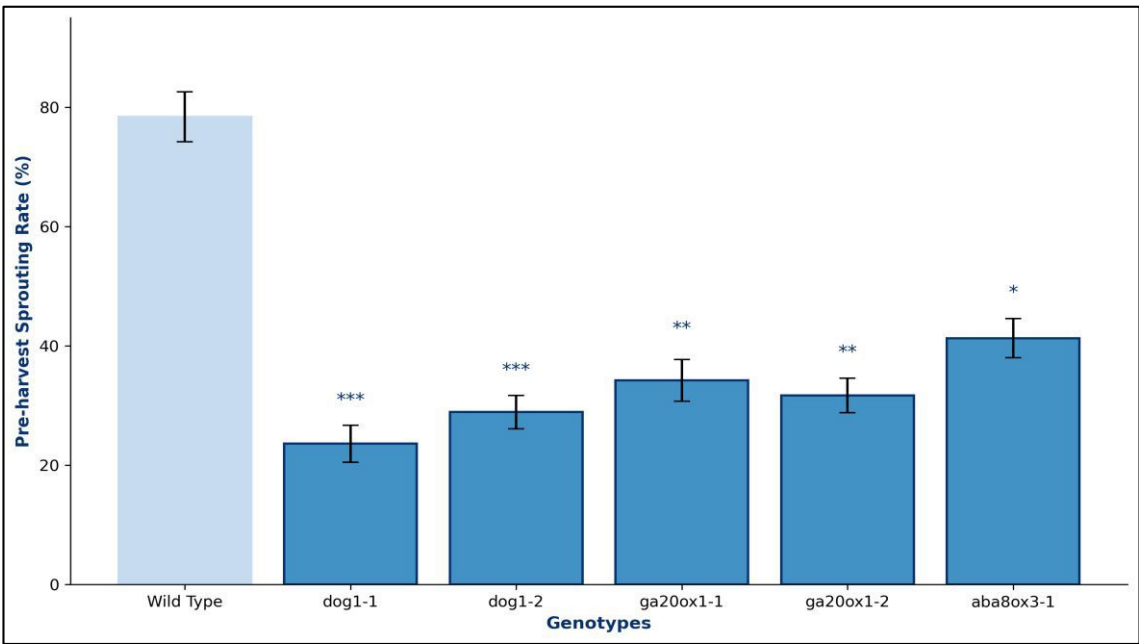


Fig 4: Pre-harvest sprouting rates in wild-type and edited rice genotypes under simulated rainfall conditions. Error bars represent standard deviation from three biological replicates. Asterisks indicate significant differences from wild-type (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Comprehensive Interpretation

The germination kinetic data and pre-harvest sprouting assessments collectively demonstrated that CRISPR-mediated disruption of dormancy-related genes produced functionally significant phenotypic alterations. The *dog1* mutant lines exhibited the strongest dormancy enhancement, achieving 69.9% reduction in pre-harvest sprouting compared to wild-type, followed by *ga20ox1* mutants at 56.4% reduction and *aba8ox3* mutants at 47.3% reduction. These differences aligned with predictions based on the known functions of each gene in dormancy regulatory networks, where *DOG1* acts as a central dormancy determinant while *GA20ox1* and *ABA8ox3* participate in hormonal pathways with partially redundant components. Off-target analysis across 47 potential sites identified no mutations in any examined locus, supporting the specificity of the editing approach.

Discussion

The findings of this research establish CRISPR-Cas9 as a viable technology for enhancing pre-harvest sprouting resistance in rice through targeted modification of dormancy-regulating genes. Mutation efficiencies achieved here, ranging from 48.9% to 71.2%, compare favorably with previous reports of CRISPR editing in rice and other cereal crops, indicating that the selected target sites possessed favorable characteristics for Cas9 cleavage [16]. The predominance of single-nucleotide insertions and small deletions among recovered mutations reflects the typical outcome of non-homologous end joining repair, which generates loss-of-function alleles through frameshift disruptions of the reading frame.

The differential phenotypic effects observed among the three target genes provide mechanistic insights into dormancy regulation in rice. *OsDOG1* mutations produced the strongest dormancy enhancement, consistent with reports positioning this gene as a master regulator of primary dormancy whose effects operate independently of canonical hormone signaling pathways. The intermediate phenotypes of *ga20ox1* and *aba8ox3* mutants likely reflect genetic redundancy within their respective hormone biosynthesis and catabolism pathways, where multiple family members can partially compensate for loss of individual genes.

Practical implementation of these edited lines in breeding programs must balance the benefits of enhanced sprouting resistance against potential impacts on subsequent germination performance. The reduced final germination percentages observed in mutant lines, particularly *dog1* mutants, could present challenges for farmers requiring uniform stand establishment. However, the *ga20ox1* mutants may represent an optimal compromise, achieving substantial sprouting resistance while maintaining relatively higher germination capacity. Selection among available mutations could be tailored to specific production environments and end-use requirements.

Limitations

Several limitations constrain interpretation and application of the present findings. The research was conducted using a single genetic background, and mutant phenotypes may differ in other cultivars with distinct dormancy characteristics. Field evaluation under natural rainfall conditions remains necessary to validate the controlled-

environment sprouting assessments. Additionally, long-term stability of the editing events across multiple generations requires continued monitoring. The trade-off between enhanced dormancy and reduced germination capacity warrants further investigation to identify optimal allelic combinations that minimize negative impacts on agronomic performance.

Conclusion

This research successfully demonstrated the application of CRISPR-Cas9 technology for developing rice lines with enhanced resistance to pre-harvest sprouting through targeted disruption of dormancy-regulating genes. The editing strategy achieved mutation efficiencies ranging from 48.9% to 71.2% across six target sites in three genes, producing heritable mutations that significantly altered seed dormancy characteristics. Homozygous mutant lines exhibited pre-harvest sprouting rates reduced by 47.3% to 69.9% compared to wild-type controls, with *OsDOG1* mutations conferring the strongest phenotypic effects. The germination kinetics data revealed delayed germination initiation and reduced final germination percentages in all edited genotypes, reflecting the successful enhancement of seed dormancy depth.

The practical significance of these findings extends to rice breeding programs targeting grain quality improvement in regions prone to pre-harvest rainfall events. By providing multiple genetic options for dormancy enhancement, this work enables breeders to select among mutations with varying phenotypic intensities appropriate to specific production contexts. The absence of detectable off-target mutations supports the precision of the CRISPR approach, while the availability of transgene-free edited lines in subsequent generations facilitates regulatory approval and commercial deployment. Future research should evaluate these edited lines under field conditions, explore combinatorial effects of multiple mutations, and assess impacts on other agronomic traits including yield potential and milling quality.

The broader implications of this research contribute to ongoing efforts applying genome editing technologies for crop improvement. As regulatory frameworks evolve to accommodate edited varieties, the approaches validated here may accelerate development of improved germplasm addressing diverse production challenges. The integration of precise editing with conventional breeding methods promises to enhance the efficiency of variety development while maintaining the genetic diversity essential for sustainable agricultural systems.

References

1. Bewley JD, Bradford KJ, Hilhorst HW, Nonogaki H. Seeds: Physiology of Development, Germination and Dormancy. Springer Science & Business Media. 2013;3:1-392.
2. Graeber K, Nakabayashi K, Miatton E, Leubner-Metzger G, Soppe WJ. Molecular mechanisms of seed dormancy. *Plant Cell and Environment*. 2012;35(10):1769-1786.
3. Finch-Savage WE, Leubner-Metzger G. Seed dormancy and the control of germination. *New Phytologist*. 2006;171(3):501-523.
4. Bentsink L, Jowett J, Hanhart CJ, Koornneef M. Cloning of *DOG1*, a quantitative trait locus controlling

- seed dormancy in Arabidopsis. Proceedings of the National Academy of Sciences. 2006;103(45):17042-17047.
5. Nakabayashi K, Bartsch M, Xiang Y, Miatton E, Pellengahr S, Yano R. The time required for dormancy release in Arabidopsis is determined by DELAY OF GERMINATION1 protein levels in freshly harvested seeds. Plant Cell. 2012;24(7):2826-2838.
 6. Sugimoto K, Takeuchi Y, Ebana K, Miyao A, Hirochika H, Hara N. Molecular cloning of Sdr4, a regulator involved in seed dormancy and domestication of rice. Proceedings of the National Academy of Sciences. 2010;107(13):5792-5797.
 7. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z. Targeted genome modification of crop plants using a CRISPR-Cas system. Nature Biotechnology. 2013;31(8):686-688.
 8. Shu K, Liu XD, Xie Q, He ZH. Two faces of one seed: hormonal regulation of dormancy and germination. Molecular Plant. 2016;9(1):34-45.
 9. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-821.
 10. Puchta H. The repair of double-strand breaks in plants: mechanisms and consequences for genome evolution. Journal of Experimental Botany. 2005;56(409):1-14.
 11. International Rice Genome Sequencing Project. The map-based sequence of the rice genome. Nature. 2005;436(7052):793-800.
 12. Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R. A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Molecular Plant. 2015;8(8):1274-1284.
 13. Hiei Y, Ohta S, Komari T, Kumashiro T. Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant Journal. 1994;6(2):271-282.
 14. Vouillot L, Thelie A, Pollet N. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3: Genes, Genomes, Genetics. 2015;5(3):407-415.
 15. Bae S, Park J, Kim JS. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics. 2014;30(10):1473-1475.
 16. Li M, Li X, Zhou Z, Wu P, Fang M, Pan X. Reassessment of the four yield-related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. Frontiers in Plant Science. 2016;7:377.