THE PERSPECTIVE OF NEW ERA SCIENCES
INTERNATIONAL SCIENTIFIC JOURNAL
www.global-science-review.com
editor@global-science-review.com

CRISPR/CAS9-MEDIATED GENOME EDITING IN RICE (*ORYZA SATIVA* L.): PROSPECTS FOR COMBINED DROUGHT AND SALINITY TOLERANCE

Ismatova Shahzodabonu Rizomat qizi

shahzodabonuismatova@gmail.com

Turayev Ozod Sunnatalievich

ozodturaev@gmail.com

Ziyaev Zafarjon Mashrapovich

zafaruzripi@gmail.com

Research Institute of Plant Genetic Resources under the National Center for Knowledge and Innovation in Agriculture, Tashkent, Uzbekistan

Abstract

Rice (*Oryza sativa* L.) is highly vulnerable to drought and salinity, two major abiotic stresses that increasingly constrain productivity in water-limited and salt-affected environments. CRISPR/Cas genome editing offers a precise strategy to modify stress-responsive genes and generate novel alleles directly in elite genetic backgrounds. This review examines the molecular and practical basis for improving rice adaptation to drought and salinity through targeted genome editing, with particular emphasis on *OsRR22*, *DST*/*OsDST*, and *DEEPER ROOTING 1* (*DRO1*). Loss-of-function editing of *OsRR22* has been experimentally shown to enhance salinity tolerance in rice, while CRISPR/Cas9-mediated modification of DST has generated Cas9-free mutant lines with improved water retention and increased tolerance to osmotic and salt stress. *DRO1*, although not primarily established as a CRISPR-derived tolerance locus, is a well-validated regulator of root growth angle

and represents an important target for engineering drought avoidance through deeper root systems. The review also discusses guide-RNA design, Agrobacterium-mediated transformation, tissue-culture constraints, off-target assessment, and strategies for producing transgene-free edited plants. Current evidence indicates that combining edits affecting stomatal regulation, ion homeostasis, and root architecture could support the development of rice genotypes with broader adaptation to simultaneous or sequential drought and salinity. However, multi-environment validation, yield testing, genotype-specific transformation optimization, and careful regulatory assessment remain essential before deploying such edited materials in breeding programs.

Keywords: *Oryza sativa* L.; CRISPR/Cas9; genome editing; drought tolerance; salinity tolerance; *OsRR22*; *DST*; *DRO1*; transgene-free plants; abiotic stress

Introduction

Ensuring stable rice production under climate change is a major challenge for global food security. Rice is particularly sensitive to water deficit and salinity at several developmental stages, and the expansion of salt-affected soils, together with increasing competition for freshwater, intensifies the need for stress-resilient cultivars. Salinity imposes both osmotic and ionic stress, whereas drought primarily limits plant water status; nevertheless, the two stresses share important physiological responses, including stomatal regulation, reactive oxygen species signaling, osmotic adjustment, and growth inhibition (Munns & Tester, 2008).

Conventional breeding has produced many stress-adapted rice cultivars, but introgression of favorable alleles may require multiple generations and can be complicated by linkage drag. Genome editing offers a complementary approach because it can introduce defined nucleotide changes directly into an elite background. Among available genome-editing platforms, CRISPR/Cas systems

have become dominant in plant research because of their programmable targeting, relatively simple guide-RNA design, capacity for multiplex editing, and compatibility with conventional transformation and regeneration pipelines (Chen et al., 2019; Zafar et al., 2020).

CRISPR/Cas9 has been shown to generate efficient, heritable, and even homozygous edits in rice within the first transformed generation, although editing outcomes depend on target sequence, construct design, genotype, transformation system, and tissue-culture response (Zhang et al., 2014). Importantly, Mendelian segregation can separate edited alleles from integrated CRISPR/Cas9 transgenes, and DNA-free or transient-delivery strategies can further reduce the likelihood of stable foreign-DNA integration (Zafar et al., 2020; Metje-Sprink et al., 2022).

This review critically evaluates how CRISPR/Cas9-mediated editing of stress-related genes can contribute to combined drought and salinity tolerance in rice, with emphasis on *OsRR22*, *DST*/*OsDST*, and *DRO1*, and discusses the technical constraints that must be addressed for practical breeding.

Key Genetic Targets for Drought and Salinity Adaptation in Rice

OsRR22 encodes a type-B response regulator associated with cytokinin signaling and has been identified as a negative regulator of salinity tolerance in rice. Targeted mutagenesis of *OsRR22* using CRISPR/Cas9 produced multiple heritable mutant alleles, and homozygous T2 mutant lines displayed significantly enhanced salinity tolerance at the seedling stage compared with wild-type plants. Importantly, the study did not detect significant penalties in the measured agronomic traits under non-saline conditions (Zhang et al., 2019).

These results make *OsRR22* one of the clearest experimentally validated CRISPR targets for improving salt tolerance in rice. The available evidence supports

the conclusion that loss of *OsRR22* function can improve plant performance under saline conditions; however, mechanistic claims about specific changes in Na+ uptake or K^+/Na^+ partitioning should be made cautiously unless directly measured in the edited background. For breeding applications, *OsRR22* editing should therefore be followed by physiological assays of ion accumulation, biomass retention, and yield across multiple salinity levels.

Drought and Salt Tolerance (DST) encodes a zinc-finger transcription factor that regulates stomatal behavior and hydrogen peroxide homeostasis. The original dst mutant showed enhanced drought and salt tolerance linked to altered stomatal regulation, establishing DST as a negative regulator of stress tolerance (Huang et al., 2009).

Direct genome-editing evidence is also available. Santosh Kumar et al. (2020) used two guide RNAs to edit DST in the indica mega-variety MTU1010 and isolated a homozygous mutant carrying a 366-bp in-frame deletion. The resulting $dst^{\Delta}184$–305 plants showed broader leaves, reduced stomatal density, improved leaf water retention under dehydration, moderate tolerance to osmotic stress, and high tolerance to salt stress at the seedling stage. A Cas9-free edited line was recovered, demonstrating that DST can be modified while ultimately removing the editing transgene.

Because drought and salinity both involve water-deficit signaling and stomatal control, DST is particularly relevant for engineering combined stress responses. Nevertheless, stomatal traits influence carbon assimilation as well as water loss; consequently, edited lines should be evaluated for photosynthetic performance, grain filling, and yield stability under both stress and non-stress environments.

DEEPER ROOTING 1 (*DRO1*) is a major determinant of root growth angle in rice. Functional *DRO1* alleles promote steeper root growth and deeper rooting, enabling plants to access water stored in deeper soil layers. Introgression of *DRO1* into a shallow-rooting cultivar improved drought avoidance and maintained higher yield under drought conditions (Uga et al., 2013).

DRO1 differs conceptually from *OsRR22* and DST because its best-established role derives from natural-allele and introgression studies rather than from the specific CRISPR experiments discussed above. Therefore, it should not be described as an already validated CRISPR knockout target for drought tolerance. Instead, *DRO1* is a strong candidate for allele engineering, promoter editing, or targeted regulatory modification to optimize root architecture without compromising other agronomic traits.

Practical Workflow for CRISPR/Cas9 Editing in Rice

A typical rice genome-editing workflow begins with target validation and guide-RNA selection. Candidate guide sequences should combine high predicted on-target activity with minimal similarity to non-target genomic loci. The chosen guide is then assembled into a CRISPR/Cas expression vector, commonly under a rice U3 or U6 promoter for guide-RNA expression and a constitutive or tissue-appropriate promoter for Cas nuclease expression.

Agrobacterium-mediated transformation of embryogenic callus remains one of the most widely used delivery routes in rice. Editing efficiency is strongly affected by cultivar genotype, callus quality, vector design, selection regime, and regeneration capacity. Regenerated T_0 plants should be genotyped by amplicon sequencing or another sequence-resolved method because simple gel-based assays may fail to distinguish small insertions or deletions reliably.

Candidate plants should subsequently be evaluated for zygosity, heritability, and the presence or absence of T-DNA/Cas9 sequences. Where stable transformation is used, selfing and segregation can generate plants that retain the desired edited allele while losing the editing cassette. Alternative transgene-free strategies include transient expression and delivery of RNA or preassembled Cas-gRNA ribonucleoprotein complexes, although their practical efficiency varies by crop and regeneration system (Metje-Sprink et al., 2022).

Evidence Base for Major Targets

Selecting suitable genome-editing targets requires not only evidence of gene involvement in stress responses but also experimental validation of the resulting physiological and agronomic effects. In rice, researchers have characterized several genes associated with salinity tolerance, drought adaptation, stomatal regulation, and root-system architecture using functional genetics, mutant analysis, and, increasingly, CRISPR/Cas-based approaches. However, evidence strength differs among candidate genes. Some targets, such as *OsRR22* and *DST*/OsDST, have been directly validated through CRISPR/Cas9-mediated editing, whereas others, including DRO1, are supported primarily by genetic mapping, natural allelic variation, and introgression studies. Therefore, distinguishing between directly validated genome-editing targets and promising candidate loci is essential for an accurate assessment of their breeding potential. Table 1 summarizes the major genetic targets discussed in this review, the strategies used to modify or characterize them, the experimentally observed phenotypes, and their potential relevance to developing rice with combined drought and salinity tolerance.

Target gene	Editing / genetic strategy	Validated phenotype	Relevance to combined stress	Key evidence
OsRR22	CRISPR/Cas9 loss-of-function mutagenesis	Enhanced seedling salinity tolerance; no significant differences in measured agronomic traits under control conditions	Strong salinity target; combination with drought-related edits is plausible but requires direct testing	Zhang et al., 2019
DST/OsDST	CRISPR/Cas9 deletion / loss-of-function editing	Reduced stomatal density, improved water retention, moderate osmotic-stress tolerance, high	Direct evidence for effects relevant to both drought and salinity	Huang et al., 2009; Santosh Kumar et al., 2020

		salt tolerance at seedling stage; Cas9-free mutant recovered		
DRO1	Natural allele/introgression; candidate for precision regulatory editing	Steeper root angle, deeper rooting, improved drought avoidance and yield maintenance under drought	Complements stomatal and ion-homeostasis mechanisms by modifying water acquisition	Uga et al., 2013

Technical Limitations and Risk Management

Off-target cleavage is a legitimate design consideration, but its frequency in plants depends strongly on guide sequence and experimental system. Whole-genome sequencing studies in rice show that well-designed Cas9 and Cas12a reagents can be highly specific and that many sequence variants detected in regenerated plants originate from tissue culture rather than nuclease activity itself (Tang et al., 2018). Therefore, manage off-target risk through careful *in silico* guide selection, sequencing of predicted off-target loci, and, where justified, whole-genome analysis of advanced lines.

Rice transformation and regeneration are highly genotype-dependent. Elite local cultivars may respond differently from model japonica backgrounds, and

somaclonal variation may arise during prolonged callus culture. For this reason, protocols optimized in one cultivar cannot be assumed to transfer directly to another. Record transformation efficiency, regeneration rate, editing frequency, and fertility independently for each genetic background.

Combined drought and salinity tolerance is polygenic. Multiplex CRISPR systems can edit several loci simultaneously, but stacking edits may also create unexpected pleiotropic effects. For example, changes that reduce stomatal density can conserve water but may also affect CO2 uptake, canopy temperature, and yield potential. Rational multiplex design should therefore combine mechanistically complementary targets and include physiological as well as agronomic validation.

Transgene-free status should be demonstrated analytically rather than assumed. In sexually propagated rice, the CRISPR/Cas9 T-DNA cassette can often be removed by segregation if the edit and transgene are genetically separable. DNA-free editing approaches can avoid stable integration from the outset, but these methods require efficient delivery and regeneration systems (Zafar et al., 2020; Metje-Sprink et al., 2022). Regulatory classification varies among jurisdictions, so scientific characterization and regulatory status should be treated as related but distinct issues.

Future Perspectives

The strongest strategy for developing rice with broad adaptation to water deficit and salinity is unlikely to be a single-gene knockout. Instead, future programs should integrate complementary mechanisms: ion and hormonal signaling (for example, OsRR22), stomatal and oxidative signaling (DST), root-system architecture (DRO1), and additional validated loci involved in ABA responses, transport, and cellular protection.

Recent advances in base editing, prime editing, CRISPR/Cas12a, promoter editing, and multiplex editing expand the range of alleles that can be created without necessarily eliminating gene function. This is especially important for genes with pleiotropic effects, where fine-tuning expression may be preferable to knockout (Chen et al., 2019; Rengasamy et al., 2024).

Future studies should also prioritize combined-stress experiments rather than testing drought and salinity only in isolation. Edited plants should be evaluated at seedling and reproductive stages using replicated pot, controlled-environment, and field experiments. Measurements should include survival, biomass, leaf water status, Na+/K+ accumulation, stomatal conductance, photosynthesis, root depth, flowering, grain number, thousand-grain weight, and final yield.

Conclusion

CRISPR/Cas9 has moved rice stress-biology research from gene discovery toward direct allele engineering. Among the targets considered in this review, OsRR22 has robust CRISPR-based evidence for enhanced salinity tolerance, while DST has direct genome-editing evidence linking altered stomatal traits with improved responses to both osmotic and salt stress. DRO1 provides a complementary, well-established route for improving drought avoidance through deeper rooting, although its role should be presented primarily as a validated root-architecture locus and a candidate for future precision editing rather than as an already proven CRISPR drought-tolerance knockout.

Taken together, these genes illustrate how ion regulation, water-loss control, and root water acquisition can be combined conceptually within a multi-trait breeding strategy. The most scientifically defensible path toward drought- and salinity-resilient rice is to couple precise editing with rigorous molecular verification, transgene-free line selection where required, and multi-environment physiological and yield validation. Such an approach can accelerate the development

of climate-resilient rice while avoiding unsupported claims about the performance of edited lines.

References

1. Chen, K., Wang, Y., Zhang, R., Zhang, H., & Gao, C. 2019. CRISPR/Cas genome editing and precision plant breeding in agriculture. Annual Review of Plant Biology, 70, 667–697. https://doi.org/10.1146/annurev-arplant-050718-100049

2. Huang, X.-Y., Chao, D.-Y., Gao, J.-P., Zhu, M.-Z., Shi, M., & Lin, H.-X. 2009. A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. Genes & Development, 23(15), 1805–1817. https://doi.org/10.1101/gad.1812409

3. Metje-Sprink, J., Menz, J., Modrzejewski, D., & Sprink, T. (2022). Transgene-free genome editing in plants. Frontiers in Plant Science, 13, 818293. https://doi.org/10.3389/fpls.2022.818293

4. Munns, R., & Tester, M. 2008. Mechanisms of salinity tolerance. Annual Review of Plant Biology, 59, 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

5. Osakabe, Y., Watanabe, T., Sugano, S. S., Ueta, R., Ishihara, R., Shinozaki, K., & Osakabe, K. 2016. Optimization of CRISPR/Cas9 genome editing to modify abiotic stress responses in plants. Scientific Reports, 6, 26685. https://doi.org/10.1038/srep26685

6. Rengasamy, B., Manna, M., & Thajuddin, N. B. 2024. Breeding rice for yield improvement through CRISPR/Cas9 genome editing method: current technologies and examples. Physiology and Molecular Biology of Plants, 30(2), 185–198. https://doi.org/10.1007/s12298-024-01423-y

7. Santosh Kumar, V. V., Verma, R. K., Yadav, S. K., Yadav, P., Watts, A., Rao, M. V., & Chinnusamy, V. 2020. CRISPR-Cas9 mediated genome editing of drought and salt tolerance (OsDST) gene in indica mega rice cultivar MTU1010. Physiology and Molecular Biology of Plants, 26(6), 1099–1110. https://doi.org/10.1007/s12298-020-00819-w

8. Tang, X., Liu, G., Zhou, J., Ren, Q., You, Q., Tian, L., Xin, X., Zhong, Z., Liu, B., Zheng, X., Zhang, D., Malzahn, A., Gong, Z., Qi, Y., Zhang, T., & Zhang, Y. 2018. A large-scale whole-genome sequencing analysis reveals highly specific genome editing by both Cas9 and Cpf1 (Cas12a) nucleases in rice. Genome Biology, 19, 84. https://doi.org/10.1186/s13059-018-1458-5

9. Uga, Y., Sugimoto, K., Ogawa, S., Rane, J., Ishitani, M., Hara, N., Kitomi, Y., Inukai, Y., Ono, K., Kanno, N., Inoue, H., Takehisa, H., Motoyama, R., Nagamura, Y., Wu, J., Matsumoto, T., Takai, T., Okuno, K., & Yano, M. 2013. Control of root system architecture by DEEPER ROOTING 1 increases rice yield under drought conditions. Nature Genetics, 45, 1097–1102. https://doi.org/10.1038/ng.2725

10. Zafar, K., Sedeek, K. E. M., Rao, G. S., Khan, M. Z., Amin, I., Kamel, R., Mukhtar, Z., Zafar, M., Mansoor, S., & Mahfouz, M. M. 2020. Genome editing technologies for rice improvement: Progress, prospects, and safety concerns. Frontiers in Genome Editing, 2, 5. https://doi.org/10.3389/fgeed.2020.00005

11. Zhang, A., Liu, Y., Wang, F., Li, T., Chen, Z., Kong, D., Bi, J., Zhang, F., Luo, X., Wang, J., Tang, J., Yu, X., Liu, G., & Luo, L. 2019. Enhanced rice salinity tolerance via CRISPR/Cas9-targeted mutagenesis of the

OsRR22 gene. Molecular Breeding, 39, 47. https://doi.org/10.1007/s11032-019-0954-y

12. Zhang, H., Zhang, J., Wei, P., Zhang, B., Gou, F., Feng, Z., Mao, Y., Yang, L., Zhang, H., Xu, N., & Zhu, J.-K. 2014. The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. Plant Biotechnology Journal, 12(6), 797–807. https://doi.org/10.1111/pbi.12200