Original Research Article

In Silico Design and Validation of CRISPR/Cas9 gRNAs for Enhancing Drought Tolerance and Yield in Rice

ABSTRACT

Rice is a staple food crop globally, and its yield is significantly impacted by drought, posing a threat to food security. Several genes have been implicated in drought response and yield regulation, which can be targeted using CRISPR/Cas9 genome editing techniques to develop improved rice cultivars. This study aimed to design guide RNAs (gRNAs) for drought-responsive and yield-determining genes in rice, including *flavanone 3-hydroxylase-1* (Os*F3H-1*), *chalcone synthase 31* (Os*CHS31*), *nodulin/SWEET12 (OsSWEET12)*, *MYB47*, and *OsKALA3*, utilizing bioinformatics tools. Genome sequences were retrieved from the Rice Genome Annotation Project (RGAP) and the Rice Annotation Project Database (RAP-DB). Optimal gRNAs were designed using the CRISPR-P v2 tool and evaluated based on parameters such as GC content, off-target score, on-target score, and genome location. The structural stability of the selected gRNAs was validated using the Mathews Lab RNA secondary structure prediction tool. Most gRNAs, except those targeting *OsF3H-1*, exhibited low self-folding probabilities and energy levels within acceptable ranges, indicating favorable structural stability. However, the *OsF3H-1* gRNAs demonstrated higher susceptibility to self-folding, requiring further refinement. The designed gRNAs provide a strong foundation for future CRISPR/Cas9-based genome editing experiments. These findings pave the way for engineering rice to develop stress-resilient and high-yielding varieties, contributing to enhanced agricultural sustainability and global food security.

Keywords: Genome editing, Drought, CRISPR/Cas9, flavanone 3-hydroxylase; OsF3H-1, chalcone synthase 31; OsCHS31, nodulin/SWEET12, MYB47, OsKALA3.

1. INTRODUCTION

Rice (*Oryza sativa*), a staple food for half of the world's population, is a major calorie source, providing over 20 percent of global intake (Fukagawa and Ziska 2019). Over 100 countries produce approximately 715 million tonnes of rice annually, with Asia accounting for around 90

percent of global production (Muthayya *et al.,* 2014). India and China are the leading rice producers, and it is the primary food for 65 percent of the Indian population (Biswas 2018; Muthayya *et al.,* 2014). Given its significance as both a staple food and economic resource, rice is crucial to food security, helping tackle global hunger and supporting millions of livelihoods (Saha *et al.,* 2021). Scientists use genes from various rice varieties to enhance their resilience to environmental stress and diseases, thereby playing a key role in sustaining agricultural stability (Fornasiero, Wing, and Ronald 2022; Saha *et al.,* 2021). Rapid climatic changes significantly impact rice productivity yearly, with drought affecting 50% of its annual yield. Drought, an abiotic stressor characterized by water deficits, impedes root growth, inhibits nutrient uptake, and reduces plant productivity. It also disrupts morpho-physiological, biochemical, and molecular responses, suppressing photosynthesis and inducing oxidative stress, which in turn impairs plant metabolism and cell integrity (Hassan *et al.,* 2023; Sami *et al.,* 2021). Other abiotic stressors, such as salinity and temperature, further contribute to a decline in yield performance (Rezvi *et al.,* 2023). Advancements are crucial for addressing the increasing demand for rice amidst climatic changes. Enhancing the genetic potential of rice varieties through genome editing and molecular breeding promises to improve yield consistency under diverse environmental conditions (Khush 2013; "Genomics-Based Precision Breeding Approaches to Improve Drought Tolerance in Rice" 2013). In the past, traditional breeding methods have contributed to the improvement of crop cultivars with enhanced traits; however, achieving improved yield gains remains challenging, particularly under stress conditions such as drought (Singh *et al.,* 2019). This emphasizes the need for innovative genetic engineering strategies, for example, CRISPR/Cas9, which offer precision, adaptability, and high efficiency, facilitating the rate of crop improvement (Mishra *et al.,* 2018; Vv *et al.,* 2020).

Drought tolerance is a complex quantitative trait involving multiple genes (Sami *et al.,* 2021) that regulate molecular responses of rice to stress, such as signal perception, transduction, and acclimatization. These responses, along with agronomic traits like plant height, panicle length, and grain size— which are also governed by several genes—play a crucial role in determining rice yield (Hassan *et al.,* 2023). The *MYB (Myeloblastosis)* family, which includes 99 R2R3 *MYB* transcription factors, is the largest plant group and plays a major role in abiotic stress response. Kang *et al.,* in 2022 reported an irregular dispersion of these transcription factors across chromosome 12 of rice (Kang *et al.,* 2022). *MYB47* belongs to the VIII-D clade of the R2R3-*MYB* family (Ortiz-García *et al.,* 2022), and the R2R3 protein encoded by *Root Related Stress 1 (RRS1)* gene triggers indole-3-acetic acid 3 (*OsIAA3)* expression, negatively regulating root development. Silencing *RRS1* promotes root growth and enhances drought tolerance (Geng *et al.,* 2024). *Nodulin* genes, vital for Nitrogen fixation in legumes (Verma, Hu, and Zhang 1992), exhibit a structural homology with the early *ENOD40* of rice, including conserved sequences at both 5' and 3' ends (Kouchi *et al.,* 1999). A study reported that osmotic drought stress induces the phosphorylation of *nodulin 26*, affecting its role in water and ammonia transport. This emphasizes *nodulin*'s influence on drought stress, though the exact effects—whether beneficial or detrimental—require further context-specific experimentation (Ding *et al.,* 2018). The *MYB* transcription factor *KALA3*, an R2R3 *MYB* candidate, plays a significant role in anthocyanin biosynthesis in rice pericarp, but its broader functions remain largely unexplored (Zheng *et al.,* 2019). Silencing or reducing the expression of these genes improves drought tolerance and helps to validate the functions of previously uncharacterized genes (Sami *et al.,* 2021; Razzaq *et al.,* 2021). *Flavanone 3-hydroxylase (F3H)* and *chalcone synthase (CHS)* are key enzymes in flavonoid biosynthesis in plants. Studies report that their overexpression enhances agronomic traits and improves drought tolerance by reducing water loss during stress (S.-I. Park *et al.,* 2021; Jan *et al.,* 2021).

This study aims to design optimal CRISPR/Cas9 guide RNAs (gRNAs) for drought-responsive and yield-regulating genes— *OsF3H-1*, *OsCHS31*, *nodulin/SWEET12*, *MYB47*, *and*

OsKALA3—in rice, using various bioinformatics tools. These gRNAs can later be utilized in future studies for editing these genes(Rai *et al.,* 2023). This approach not only aids in crop improvement but also contributes to understanding the functions of these genes, facilitating the development of drought-resistant varieties and achieving higher plant productivity (Babar Hussain, Stuart James Lucas, Hikmet Budak 2018). The genome sequences of these genes were retrieved from the Rice Genome Annotation Project Database (RAP-DB), which provides up-to-date gene annotations for the rice genome (Sakai *et al.,* 2013). Highly specific gRNA spacers for the target genes were designed using the CRISPR Plant V2 tool, known for its precision in evaluating off-target scores (Minkenberg *et al.,* 2018). The secondary structure and base pair probability of the sgRNA were predicted using the Mathews Lab RNA secondary structure online tool (Bandaru *et al.,* 2020). In future studies, gRNAs could be cloned into a suitable binary vector and mobilized into rice using *Agrobacterium tumefaciens* for functional validation (Anjala and Augustine 2022). As drought affects a majority of the annual rice yield and abiotic stressors increasingly threaten global food security, designing CRISPR/Cas9-specific gRNA spacers to edit drought-responsive and yield-determining genes can facilitate the future development of resilient rice varieties, which is critical for sustaining agricultural stability and meeting the growing demand for staple crops (Hassan *et al.,* 2023; Saha *et al.,* 2021). The findings from this research lay the foundation for functional analysis and future genetic engineering strategies aimed at enhancing rice productivity under diverse environmental conditions (Mishra, Joshi, and Zhao 2018; J.-R. Park *et al.,* 2022).

2. METHODS

2.1 Retrieval of gene sequences

The genome sequence information and functional attributes of the target genes were retrieved from the Rice Genome Annotation Project (RGAP, https://rice.uga.edu/) and Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp/) (Yuan *et al.,* 2005). These resources provided a comprehensive, manually curated dataset of genome annotations for rice (*Oryza sativa japonica* group cv. Nipponbare), supported by literature-based evidence (Sakai *et al.,* 2013). Gene sequences were downloaded in FASTA format, with RGAP utilizing LOCUS IDs for retrieval and RAP-DB allowing direct searches using gene names. These sequences can subsequently be used to design specific gRNAs for the respective genes.

2.2 Designing of CRISPR/Cas9 guide RNAs (gRNAs)

CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/) was a web-based tool used to design optimal gRNAs for target genes (Lei *et al.,* 2014). The input query could either be the FASTA sequence of the gene or LOCUS identifiers. The target genome was selected based on the source of the LOCUS identifiers: *Oryza sativa* (MSU) for sequences retrieved from RGAP and *Oryza sativa* (RAP-DB) for sequences retrieved from RAP-DB. The result page provided detailed information, including the PAM sequence (Protospacer Adjacent Motif, NGG), GC content percentage, on-score values, off-target scores, and genome location. The gRNA sequences with parameter values within the optimal range were chosen for further analysis (Anjala and Augustine 2022).

2.3 Validation of the guide RNAs (gRNAs)

The secondary structure formation probability and stability of the designed guides for all target genes were analyzed using the Mathews Lab RNA secondary structure prediction tool (https://rna.urmc.rochester.edu/RNAstructureWeb/) (Reuter and Mathews 2010). The guide sequences for each gene were entered into the input page alongside the scaffold sequence, which was identical for all guides and retrieved from the CRISPR-P v2.0 tool. The result page

provided a detailed view of the secondary structure, including the formation probability percentage and free energy values for each structure, indicating their stability. Structures with the lowest self-folding probability percentage and minimal free energy were selected (Panda and Ray 2022).

3. RESULTS AND DISCUSSION

3.1 Retrieval of gene sequences

The sequence information of the target genes, including *OsF3H-1*, *OsCHS31*, *Nodulin/SWEET12*, *MYB47*, *and OsKALA3* was retrieved from the 'Rice Genome Annotation Project (RGAP)' in FASTA format. In addition to sequence data, the database provided comprehensive insights into the genetic characteristics of these genes, including chromosomal location, nucleotide length, gene ontology classification, and functional roles. These details, which are crucial for understanding the genomic and functional attributes of the target genes, were summarized in Table 1.

Table 1. Genomic attributes and functional roles of the target genes

Target genes	Locus ID	Genome location	Nucleotide length (Base pairs)	Functions
Flavanone 3-hydroxylase 1 (OsF3H-1)	LOC_Os04g56700	Chr4	1134	Flavonoid biosynthesis
Chalcone synthase 31 (OsCHS31)	LOC_Os12g07690	Chr12	426	Stress response
Nodulin/SWEET12	LOC_Os03g22590	Chr3	903	Abiotic stress response
MYB47	LOC_Os03g04900	Chr3	744	DNA-binding
OsKALA3	LOC_Os03g29614	Chr3	966	Negative regulation of grain length and grain weight

3.2 Designing of CRISPR/Cas9 guide RNAs (gRNAs)

The optimal CRISPR/Cas9 guide sequences for the target genes and their reverse complementary sequences were provided in Table 2. The associated parameter data were summarized in Table 3.

The spacer sequence or gRNA, was 18–20 nucleotides long to ensure optimal target efficiency (Liu *et al.,* 2017), followed by the NGG PAM sequence for Cas9 recognition and cleavage (Hiranniramol *et al.,* 2020). gRNA optimization necessitated balancing key factors, including off-target activity, on-target efficiency, GC content, and location in the genome (Uniyal *et al.,* 2019; Konstantakos *et al.,* 2022). An ideal gRNA had a high on-target score (≥0.50) to guarantee efficient binding and cleavage at the target site (Konstantakos *et al*., 2022). In this study, the gRNAs for the target genes were selected based on an optimal on-score value

greater than 0.50, which ranged from 0.53 to 0.81, as shown in Table 3. The gRNA for MYB47 (GATACCTGTGAGCTTGGCCACGG) was identified as having a high on-target score of 0.8114, indicating a strong binding affinity to its target site. In contrast, the gRNA for OsCHS31 (CCACATGGTGTCCAATCGAATGG) had a lower on-target score, which may reduce the likelihood of precise target binding and potentially impact genome editing efficiency. However, despite this, both guides may still be functional within a suboptimal range (Konstantakos *et al.,* 2022). Another important consideration was the genome location, with gRNAs typically being selected from the 5' end of the coding sequence (CDS). Therefore, all gRNAs targeted the protein-coding region of the gene (Anjala and Augustine 2022).

The number of off-target sites was carefully assessed and minimized when designing an optimal CRISPR/Cas9 gRNA, as these sequences have shared high homology with on-target regions (Anjala and Augustine 2022; Manghwar *et al.,* 2020). Cas9 binding and cleavage at off-target sites could lead to genomic instability, potentially disrupting the target gene's function (Lin and Wong 2018). Based on the data from Table 3, the number of off-target sites for the genes *OsF3H-1*, *OsCHS31*, *MYB47*, *nodulin/SWEET12*, and *OsKALA3* was found to be below 50, with guide 1 of *OsCHS31* showing the fewest off-target sites (=6). Despite having a small number of off-target sites, the off-score values for these guides were below 0.50, suggesting minimal off-target effects and a reduced likelihood of significant Cas9 binding to non-target regions (Naeem *et al.,* 2020). The efficiency of gRNAs was also influenced by their GC content (Naeem *et al.,* 2020; Uniyal *et al.,* 2019), with an optimal range between 40-65% as suggested by several studies. Guides with GC content outside this range tended to be ineffective (Naeem and Alkhnbashi 2023). The presence of GC pairs, with their additional hydrogen bond compared to AT pairs, enhanced the thermodynamic stability of gRNA-target binding (Konstantakos *et al.,* 2022). In the present research, the selected gRNAs for the target genes fell within the optimal GC content range, although guide 1 of OsKALA3 exhibited the lowest percentage, at 45%. However, the relatively high GC content of these guides, above 65%, may have facilitated secondary structure formation (Konstantakos *et al.,* 2022; Chan *et al.,* 2009), potentially leading to self-looping, which could hinder the efficiency of the CRISPR system (Jung *et al.,* 2024). Overall, the selection of gRNAs prioritized a proportion between a high on-target score, optimal GC content, and low off-target sites to maximize editing efficiency while maintaining specificity (Anjala and Augustine 2022).

Table 2. CRISPR/Cas9 gRNA sequences and reverse complements for target genes

Target Genes	Guide	gRNA sequences (5'-3') and corresponding reverse complement (3'-5')
Flavanone 3-hydroxylase 1 (OsF3H-1)	1	5'-GTCCATGTCGACGCAGGCAT-3' 3'-CAGGTACAGCTGCGTCCGTA-5'
	2	5'-TCCTCGTCGCGCACGAACGA-3' 3'-AGGAGCAGCGCGTGCTTGCT-5'
Chalcone synthase 31 (OsCHS31)	1	5'-CCACATGGTGTCCAATCGAA-3' 3'-GGTGTACCACAGGTTAGCTT-3'
Nodulin/SWEET12	1	5'-GCTTACGATGATGGTGAGCG-3' 3'-CGAATGCTACTACCACTCGC-5'
MYB47	1	5'-GATACCTGTGAGCTTGGCCA-3' 3'-CTATGGACACTCGAACCGGT-5'

OsKALA3	1	5'-ATTTCATTGTCTGTTCGCCC-3' 3'-TAAAGTAACAGACAAGCGGG-5'

Table 3. CRISPR/Cas9 guide RNA parameters for target genes

Target genes	Guides	On-score value	PAM sequence	GC content (%)	No.of off-target sites
Flavanone 3-hydroxylase (OsF3H-1)	1	0.6753	CGG	60	23
	2	0.6654	CGG	65	20
Chalcone synthase 31 (OsCHS31)	1	0.5322	TGG	50	6
Nodulin/SWEET12	1	0.7183	GGG	55	28
MYB47	1	0.8114	CGG	55	15
OsKALA3	1	0.6892	CGG	45	43

3.3 Validation of guide RNAs (gRNAs)

The probability percentage of secondary structure formation (self-folding) and the associated free energy values of the formed structures were presented in Table 4.

The secondary structure prediction of gRNAs, performed using the Mathews Lab tool (Bandaru *et al.,* 2020), was critical for assessing their efficacy. gRNAs could form secondary structures, such as hairpins, due to self-folding, where lower (more negative) free energy indicated greater thermodynamic stability. However, excessive folding could have obstructed the spacer region, hindering target recognition and cleavage efficiency (Motoche-Monar *et al.,* 2023). The gRNAs designed for the target genes—*OsF3H-1*, *OsCHS31*, *nodulin/SWEET12*, *and OsKALA3*—presented varying probabilities and energy values in this study, reflecting differences in structural stability and genome editing potential. Based on the data presented in Table 4, the guides for genes such as *OsCHS31*, *nodulin/SWEET12*, *MYB47* and *OsKALA3* exhibited self-loop formation probabilities below 50%, with the associated secondary structures showing lower stability, as indicated by their energy values being less than -15.5 kcal/mol, except for the *OsCHS31* guide, which had an energy of -21.2 kcal/mol. These results suggested that these guides possessed better target accessibility due to their more open, less stable structures (Motoche-Monar *et al.,* 2023; Corsi *et al.,* 2022).

Conversely, the guides for *OsF3H-1* exhibited a higher self-folding probability, ranging from 80% to 95%, and formed highly stable structures with free energy values above -25.0 kcal/mol (Table 4). The high folding probabilities and stability of these guides may negatively influence genome editing efficiency (Jensen *et al.,* 2017), as the formation of stable secondary

structures could reduce the accessibility of the target gene and increase the risk of off-target effects (Naeem *et al.,* 2020).

Table 4. Probability and free energy values of gRNAs for target genes

Target genes	Guides	Probability (%)	Free Energy (kcal/mol)
Flavanone 3-hydroxylase 1 (OsF3H-1)	1	≥80 - ≥95	-25.1
	2	≥90	-26.7
Chalcone synthase 31 (OsCHS31)	1	<50	-21.2
Nodulin/SWEET12	1	<50	-15.0
MYB47	1	<50	-14.7
OsKALA3	1	<50	-14.0

4. CONCLUSION

The findings of this study establish a solid foundation for leveraging CRISPR/Cas9 genome editing to enhance drought tolerance and yield performance in rice. By targeting critical genes such as *OsF3H-1*, *OsCHS31*, *nodulin/SWEET12*, *MYB47, and OsKALA3*, this research highlights the potential of gene editing to address the challenges posed by drought stress—a major threat to global food security. This research identifies potential guides for precise genome editing by validating gRNA design based on parameters such as GC content, off-target effects, genomic location, and on-score values. It highlights those with higher editing specificity and stability. Notably, gRNAs for *OsF3H-1* exhibited higher self-folding probabilities, suggesting greater stability and a need for careful handling during molecular cloning. Additionally, *OsKALA3* gRNAs demonstrated a relatively higher number of off-target sites and lower GC content compared to the other target genes. While these factors require careful consideration, they do not diminish the potential utility of the proposed gRNAs but instead suggest opportunities for further refinement to improve their efficiency and applicability.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that generative AI technologies such as Large Language Models, etc. have been used during the writing or editing of manuscripts. This explanation will include the name, version, model, and source of the generative AI technology and as well as all input prompts provided to the generative AI technology

Details of the AI usage are given below:

1. Grammarly

2. ChatGPT

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