Original Research Article

CRISPR/Cas9 construct development for knock-out of the *ROOT ARCHITECTURE ASSOCIATED 1* gene in rice (*Oryza sativa* L.)

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ABSTRACT

Deeper rooting is an important trait for sustained yield under water-limited environments, as drought exhibits serious threat to rice (*Oryza sativa* L.) production. To mitigate the challenge, we aimed to modify the *Oryza sativa ROOT ARCHITECTURE ASSOCIATED 1* (*OsRAA1*) gene, one of the key regulators of the cell cycle that manipulates root development, using CRISPR/Cas9 system. We used CRISPR-P v2.0 tool to select two spacer sequences targeting *OsRAA1* gene. Validation of the guide RNAs (gRNAs) was conducted by analyzing their secondary structure using RNA secondary structure prediction tool. We successfully cloned two spacer sequences targeting *OsRAA1* into a binary vector, pRGEB32. The constructs were transformed into *E. coli* strain DH5α and *Agrobacterium tumefaciens* strain EHA105. The developed gene cassettes can be used for editing of *OsRAA1* gene in rice. By editing, we seek to enhance the root architecture and increase the drought-stress tolerance ability of rice. This research represents a significant step towards developing drought-tolerant rice varieties, a critical solution for ensuring global food security in the face of climate change.

Keywords: Drought; CRISPR/Cas9; Root architecture; OsRAA1; Genome editing

1. INTRODUCTION

Rice is the second most cultivated crop, feeding around two-thirds of the population after wheat worldwide (Pirdashti *et al.,* 2009). There are many hindrances in rice cultivation depending on geography, and environmental changes. Various scientific publications suggest that drought stress is the major obstruction in rice production that affects around 45% of agricultural areas, worldwide (Ambavaram *et al.,* 2014; Todaka *et al.*, 2015; Heinemann *et al.,* 2015). Climate change has resulted in unpredictable, more frequent, and distressing weather patterns, which are likely to continue with increasing global warming (IPCC, 2023).

Drought poses a serious threat to food security for the billions of people who depend on rice as staple food. Though a large number of studies on the development of tolerant rice varieties are in the limelight, most experiments emphasize on modifying the aerial parts of the plant (Lu *et al*., 2015; Yang and Hwa, 2008; Zhao *et al.,* 2015), in comparison, the root structure that plays a significant role in scavenging limited resources and coping up with stressed conditions, remains unexplored. In a study, that involves concurrent assessment of root character and grain yield, Venuprasad *et al*. (2002) conveyed that genotype with deep rooting habits had a better edge towards growth and survival in stressful conditions, and those that had extended their root length before the outbreak of stress period exhibit better productivity.

The plant hormone auxin is pivotal in regulating root architecture, such as promoting lateral roots and root hairs, while inhibiting primary root elongation (Overvoorde *et al.,* 2010). The *OsRAA1* gene expression is comparatively more intense in the roots and spikes of the plants than in other tissues, as elucidated from the quantitative assays (Han *et al.*, 2005). The tissue-specific expression profile presumes that the gene might be involved in root development and reproductive processes (Han *et al.*, 2005). Ge *et al.* (2004) stated that *OsRAA1* gene entails two auxin response element (AuRE) core sequences, which exhibit binding of auxin response factor and regulation of the gene. The study also revealed that Arabidopsis Flowering Promoting Factor 1 (AtFPF1) protein shares 58% homology with the RAA1 amino acid sequence (Ge *et al.,* 2004). The *OsRAA1* gene, influences flowering and hypocotyl growth, when introduced into Arabidopsis (Wang *et al.,* 2009). This finding intensified the study of the *OsRAA1* gene concerning various abiotic stresses. In rice, overexpression of the *OsRAA1* gene led to reduced plant development affecting roots, leaves, and flowers. However, this inhibited the extension of the primary roots, boosted adventitious root production, and delayed response to gravity (Ge *et al.,* 2004; Han *et al.*, 2005).

The biochemical assays performed on OsRAA1 suggest that the gene belongs to the protein family that exhibits GTP-binding activity (Han *et al.*, 2005). Xu *et al.* (2010) provided new insights into a key regulatory mechanism controlling plant cell cycle progression and root development. The results highlight that degradation of RAA1 protein is associated with Anaphase-Promoting Complex (APC), ascertaining a functional association between RAA1 and APC/C complex. RAA1 was established as a cell cycle candidate and an APC/C substrate for proteolysis. Degradation of RAA1 by the ubiquitin-proteosome structure is necessary for the transition of the cell cycle to anaphase during root growth in rice (Xu *et al.*, 2010).

Although a lot of research conveys the biochemical and regulatory role of *OsRAA1*, several gaps exist in comprehending the function of the *OsRAA1* gene under various stress conditions. Genome editing via CRISPR/Cas9 system can be harnessed to stop the functioning of this negative regulatory gene for improved phenotypic plasticity. The information from *OsRAA1* poses ample potential to enhance drought tolerance by better root development.

In this study, we designed gRNAs specific to the *OsRAA1* gene in rice and successfully cloned them into the pRGEB32 binary vector, to precisely target the gene. *E. coli* strain DH5α was transformed with the recombinant vectors, which were further mobilized into *Agrobacterium tumefaciens* strain EHA105.

2. MATERIAL AND METHODS

Bacterial strains used: *E. coli* strain DH5α has been used in the study for the development of the vector-gRNA construct and *Agrobacterium tumefaciens* strain EHA105, for mobilizing the cassette into rice in our further study. The cultures were multiplied on Luria Bertani (LB) agar medium with nalidixic acid (25 mg/L) and rifampicin (25 mg/L), respectively.

CRISPR/Cas9 binary vector: In this study, guide RNA (gRNA) constructs were developed using the pRGEB32 vector. The vector was procured from Addgene (CAT#63142) and was obtained as stab culture as *E. coli* strain DB3.1. Cultures were revived and multiplied on plates containing kanamycin in LB agar.

Retrieval of the *OsRAA1* gene sequence: The sequence of *OsRAA1* was retrieved from the Rice Annotation Project Database (https://rapdb.dna.affrc.go.jp/) and Rice Genome Annotation Project (https://rice.uga.edu/). Additional data related to the gene, like the putative gene function, number of exons, etc., were also collected. The Locus ID was identified from RAP-DB. The gene sequence was downloaded in FASTA format and used for designing gRNA specific to the gene.

Guide RNA (gRNA) designing and synthesis

The spacer sequence or guide sequence for the sgRNAs that target potential protospacers were designed *insilico* using the CRISPR-P v2.0 (http://crispr.hzau.edu.cn/) tool. Various parameters such as on-target score, target site preferably near to start codon, location of on-target sites on the gene, higher GC content (preferably more than 70%), and low off-target score were considered, along with the Protospacer Adjacent Motif (PAM), NGG. The RNA secondary structure prediction tool (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) was also used to predict the secondary structures of the gRNAs. Considering various combinations, two protospacer targets were selected for generating the genome editing cassette. The synthesized oligos were purchased from IDT as high-purity desalted oligos with complementary sticky ends, **GGCA** for the antisense and **AAAC** sense for the strand oligo (Table 1).

Table 1: Selected gRNAs for knock-out of *OsRAA1* gene

Name of gRNA	Strand	Sequence (5' – 3')	On-target Score	PAM
OsRAA1#R1	Antisense	GGCAGGTTTGGGTGTTCAAGAACG	0.8061	GGG
	Sense	AAACCGTTCTTGAACACCCAAACC		
OsRAA1#R2	Antisense	GGCAGCGCTGGTGCACACGCCGAG	0.6207	CGG
	Sense	AAACCTCGGCGTGTGCACCAGCGC		

CRISPR/Cas9-gRNA vector construction: The pRGEB32 plasmid vector was isolated from cultured stock via the alkaline lysis method (Ehrt and Schnappinger, 2003). The *Bsa*I HF-v2 restriction enzyme from New England Biolabs, UK was used to linearize the pRGEB32 plasmid vector. The digestion reaction was set up (Table 2). The digested plasmid was electrophoresed in 1.0% agarose gel to detect linearization. The digested vector was further purified using QIAquick (Qiagen) PCR Purification Kit. The Nanodrop® spectrophotometer (IMPLEN, NP80) reading was taken to analyze the purified plasmid concentration. The digested and undigested plasmids were transformed into competent *E. coli* DH5α cells prepared by calcium chloride ($CaCl_2$) method (Tang *et al.,* 1994; Chang *et al.,* 2017) for re-confirmation of digestion. The transformation was carried out by heat shock method (Chang *et al.*, 2017). The synthesised guide sequence oligos (complementary to each other) were phosphorylated at 5' end using T4 Poly Nucleotide Kinase (ThermoFisher Scientific). The sense and antisense oligos of the spacers were annealed by cooling at a slow ramp rate from 95 to 25°C (Table 3). The annealed gRNAs were diluted with sterile water (1:200) and ligated to the linearized pRGEB32 vector as per the mentioned reaction setup in Table 4. In this study, *E. coli* DH5α and *Agrobacterium* EHA105 transformation was carried out using the two vector constructs— pRGEB32:OsRAA1#R1 and pRGEB32:OsRAA1#R2.

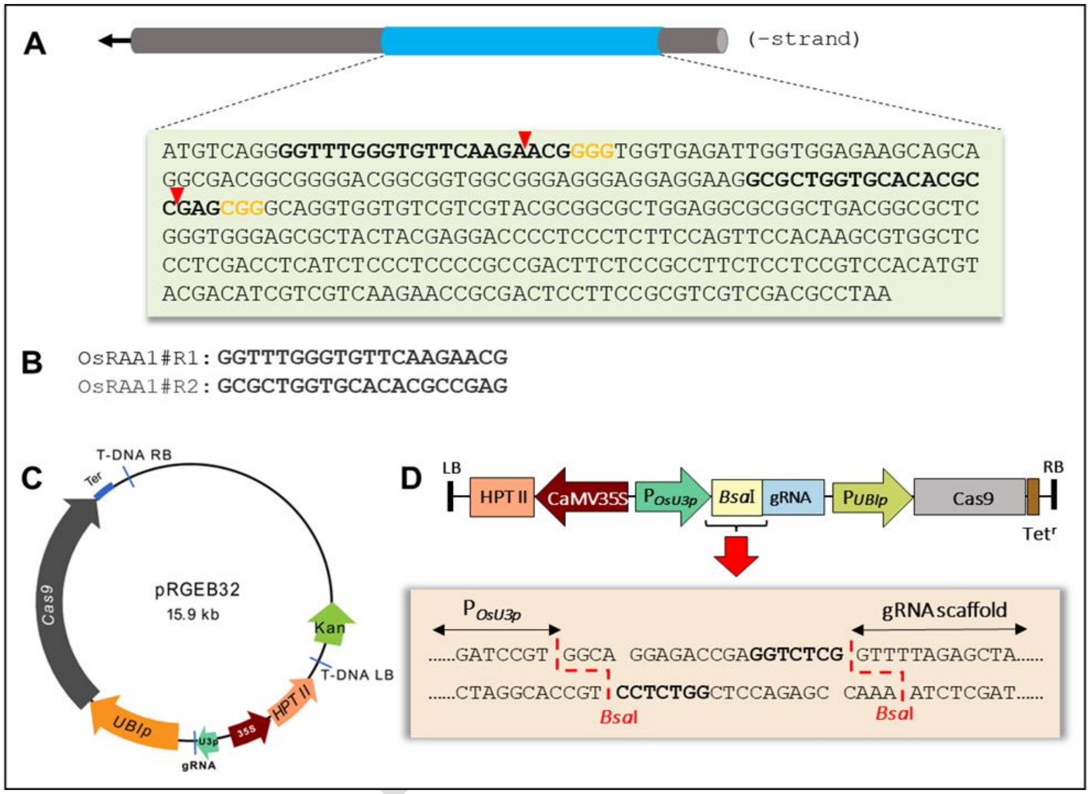


Fig. 1: Illustration of CRISPR/Cas9 target knock-out of *OsRAA1* in the study. (A) Target region (represented in blue) for CRISPR/Cas9-mediated mutagenesis in the rice *OsRAA1* gene. The PAM sequence (NGG) is shown in orange and the 20 bp target sequence is in bold black colour. The red inverted triangle points to the expected cleavage site. (B) Selected spacer sequences in rice *OsRAA1* gene. (C) Vector map of CRISPR/Cas9 binary vector pRGEB32 (Xie *et al.,* 2014). (D) Vector sequence between the left and right border; HPTII: Hygromycin B phosphotransferase II. The gRNA scaffold is flanked by *Bsa*I restriction sites, enabling easy insertion of designed gRNA sequences. The red dotted lines interpret the cleavage points.

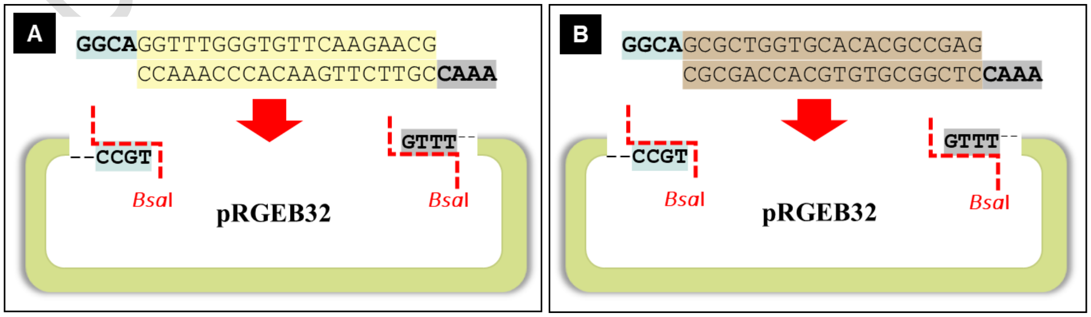


Fig. 2: Illustration of phosphorylated gRNAs ligation to digested pRGEB32 vector. (A) The OsRAA1#R1 gRNA was inserted between the *Bsa*I restriction sites of the pRGEB32 vector, generating the pRGEB32:OsRAA1#R1 construct. Similarly, (B) ligation of the OsRAA1#R2 gRNA resulted in the pRGEB32:OsRAA1#R2 vector construct. In this study, these two constructs were separately used for transformation into *E. coli* and *Agrobacterium.*

Transformation of CRISPR/Cas9-gRNA vector constructs into *E. coli*:

The pRGEB32 binary vector consisting of gRNA sequence was mobilized into the *E. coli* strain DH5α by heat shock method (Chang *et al.,* 2017) with slight modifications. The 10 µL ligated insert-vector mix was added to 100 µL of freshly prepared competent cells and incubated on ice for 15 min. Heat shock was applied by incubating the tubes in 42°C water bath for 90 seconds. The tubes were incubated back in ice for 5 min. LB broth (1 mL) was added to the tubes and incubated at 37°C for 1 h with constant shaking at 180 rpm. The bacterial cells collected by centrifugation at 2000 g for 10 min were resuspended in 100 mL of LB broth. Using L-spreader, the resuspension solution was plated on LB agar plates containing kanamycin and incubated in inverted position at 37°C for 16–18 h.

Confirmation of CRISPR/Cas9-gRNA construct in *E. coli*: Colony PCR was performed to identify the putative positive DH5α clones, using M13 reverse primer as forward primer and the complementary strand of spacer oligo as the reverse. The PCR reaction and program setup were performed as shown in Table 5 and 6, respectively. The PCR reactions conducted in this study were performed using KAPA *Taq* PCR kit (Merck, USA). Two selected colonies were used to subculture for plasmid isolation, followed by confirmation through plasmid PCR. Selected plasmids were sequenced by Sanger method using M13 reverse primer by GeneSpec Pvt. Ltd. Sequence analysis by multiple sequence alignment was performed using BioEdit v7.2 software (Informer Technologies, Inc.).

Mobilization of the CRISPR/Cas9-gRNA construct into EHA105 and confirmation of the construct: Using the freeze-thaw technique by Holsters *et al*., 1978, *Agrobacterium* EHA105 competent cells prepared using the $CaCl_2$ method were transformed. For transformation, recombinant plasmids (~5µg) were mixed with competent cells and thawed in ice for 30 mins. The mixture was frozen in liquid nitrogen for 10 mins, followed by another thawing at 37°C for 5 mins. The tubes were again incubated back in ice for 5 mins. To each tube, 1 mL of LB broth was added and incubated at 28°C with constant shaking at 200 rpm for 6 h. The cells were spreaded onto LB agar plates with kanamycin (50 mg/L) and rifampicin (25 mg/L). The plates were incubated at 28°C for 48 h. Colony and plasmid PCR confirming the presence of the CRISPR/Cas9 construct in EHA105 is carried out as mentioned in Table 5 and 6.

Table 2: Reaction setup for pRGEB32 vector digestion

Reagents	Volume (µL)	PCR Program
Autoclaved distilled water	32	• Incubated at 37°C for 2 h • Heat inactivation at 65°C for 10 min
10x CutSmart buffer	5	
*Bsa*I -HF v2 (20U/µL)	1	
pRGEB32 plasmid vector (~5 µg)	12	
Total volume	**50**	

Table 3: Reaction for annealing of gRNA with T4 PNK

Reagents	**Volume (µL)**	**PCR Program**
Autoclaved distilled water	6.5	• Incubated at 37°C for 3 min • Incubated at 95°C for 5 min • Temperature reduced to 25°C at 0.1°C/s
10X T4 PNK buffer (10U/µL)	1	
100 µM spacer sequence sense strand	1	
100 µM spacer sequence antisense strand	1	
T4 PNK	0.5	
Total volume	**10**	

Table 4: Ligation reaction of phosphorylated gRNAs to the vector

Reagents	**Volume (µL)**	**PCR Program**
10X ligation buffer	1	• Incubated at 25°C for 15 min • Hold at 4°C for shorter time
50ng linearized and purified pRGEB32 vector	7	
T4 DNA ligase	1	
Diluted annealed phosphorylated gRNAs	1	
Total reaction volume	**10**	

Table 5: Colony and plasmid PCR reaction setup

Reagents	**Volume (µL)**	
	Colony PCR	Plasmid PCR
Autoclaved distilled water	12.25	12.05
10X *Taq* buffer with $MgCl_2$	2	2.2
25 mM $MgCl_2$	0.5	0.5
10 mM dNTP mix (2.0 mM each)	1	1
Dimethyl sulfoxide (DMSO)	-	2
10 µM forward primer	1	1
10 µM reverse primer	1	1
Recombinant plasmid	-	2
Taq DNA polymerase (5U/µL)	0.25	0.25
Total reaction volume	**20**	**22**

Table 6: PCR program for colony and plasmid PCR

Initial denaturation	95°C	10 min	
Denaturation	95°C	30 s	35 cycles
Annealing	58°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	10 min	
Final hold	4°C	∞	

3. RESULTS AND DISCUSSION

CRISPR/Cas9 binary vector: The pRGEB32 vector (15.9 kb) is a rice codon optimized to express *sgRNA* driven by the U3 snoRNA promoter (P_{OsU3p}) and a rice ubiquitin promoter plus the complete 5′ UTR (P_{UBIp}) for Cas9 (Xie *et al.,* 2014). The vector also has *Bsa*I restriction site for guide sequence cloning, and kanamycin and hygromycin as markers for bacteria and plant selection, respectively after genetic transformation (Fig. 1, C).

***OsRAA1* gene sequence retrieval:** In rice genome, the *OsRAA1* gene was located on chromosome 1, and the locus ID was identified as Os01g0257300 from RAP-DB. The gene spans about 785 bp and has a coding sequence (CDS) of about 330 bp with only a single exonic region.

Designing and synthesis of guide RNAs: The guide sequences chosen from the CRISPR-P v2.0 outcome window for targeted knock-out of *OsRAA1* gene (Table 1). We used CRISPR-P v2.0 tool which could identify potential target sites for CRISPR/Cas9-mediated editing (Liu *et al.,* 2017). Based on the GC content, on-score value, off-target sites, and location in the genome, two 20 bp length gRNAs were selected. The gRNAs located towards the 5' end of the CDS of the gene with fewer off-target sites and located mostly on the first or initial exons, were considered. The software assigns specificity scores to every expected gRNA, indicating its probability of guiding the targeted DNA sequence without errors, and likely identifies off-target cleavage sites via algorithms to potentially minimize undesirable mutations (Liu *et al.,* 2017).

The sgRNA encoding the chosen spacer sequence should be checked for weaker secondary structure from the RNA secondary structure prediction tool. Also, the 5' end where the spacer sequence is present should be free from any loop.

Construction of CRISPR/Cas9-gRNA and cloning into *E. coli*: The rice codon-optimized binary vector, pRGEB32, used in the study has a very high transformation efficiency. For cloning into the pRGEB32 binary vector, the *Bsa*I sites were added to the 5' end of the spacer oligos (Fig. 1, D). Linearization of the *Bsa*I digested pRGEB32 vector was checked by electrophoresis on 1% agarose gel (Fig. 3, A). The presence of a smaller light fragment in the agarose electrophoresis gel exhibits that restriction would have happened. However, in this case, the fragment generated by *Bsa*I restriction digestion is difficult to visualize by electrophoresis. We observed a slight reduction in the band size compared to the undigested pRGEB32 vector band (Fig. 3, A).

For further confirmation, we transformed *E. coli* DH5α competent cells with *Bsa*I restricted vector. As expected, no colonies were observed in LB agar plates containing with kanamycin compared to undigested pRGEB32 vector transformants (Fig. 3, B; C). The sense and antisense oligos were annealed to each other and were further ligated with the digested vector (Fig. 2; A, B).

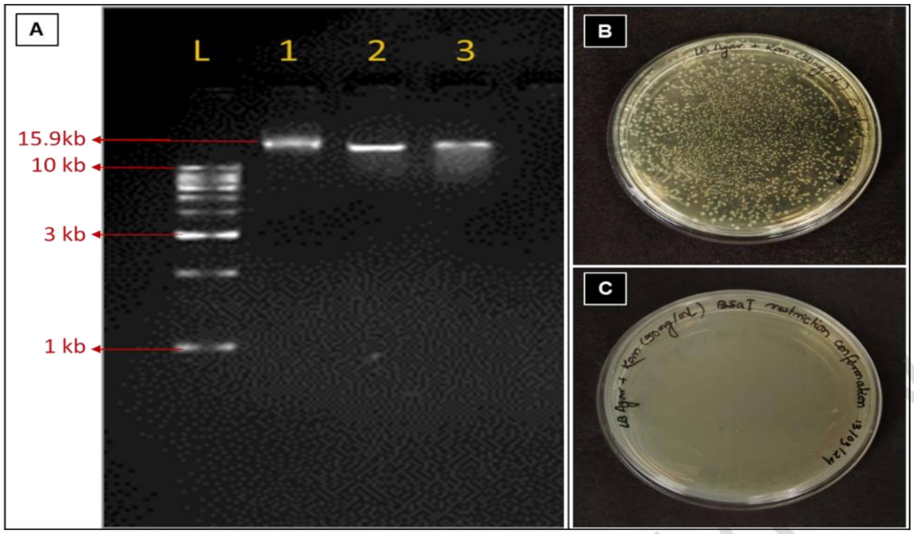


Fig. 3: Restriction digestion confirmation of the vector plasmid pRGEB32. (A) *Bsa*I restriction digestion profile; L- 1 kb ladder; 1- Undigested pRGEB32 vector (control); 2,3- Vector digested with restriction enzyme *Bsa*I. (B) *E. coli* DH5α colonies transformed with an undigested vector; (C) No colonies were obtained when *E. coli* cells were transformed with the *Bsa*I digested vector.

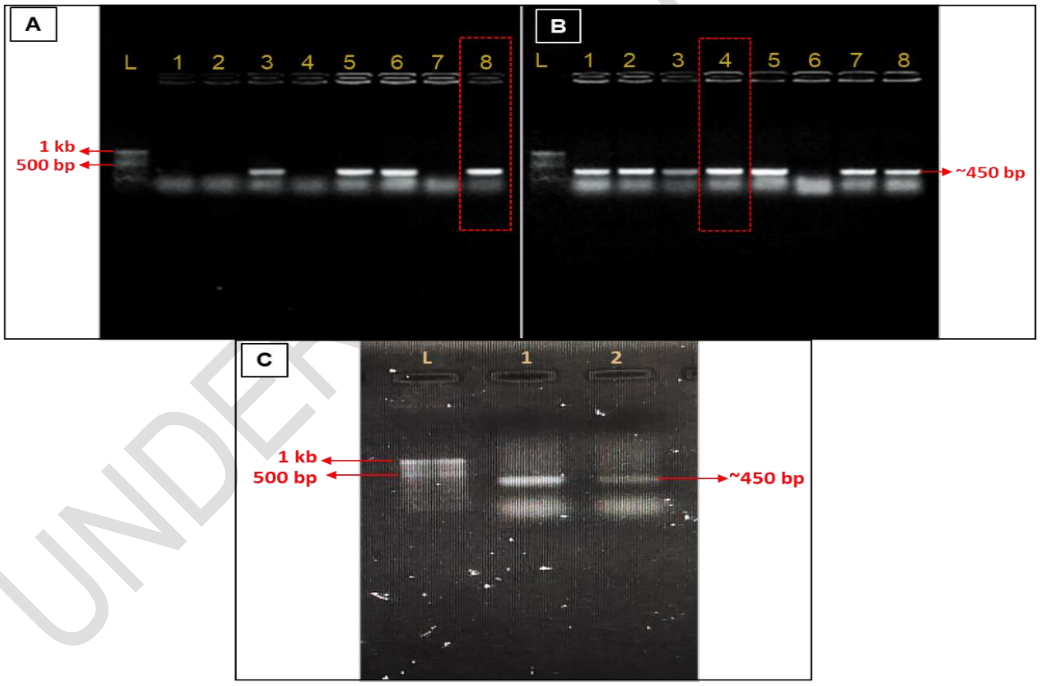
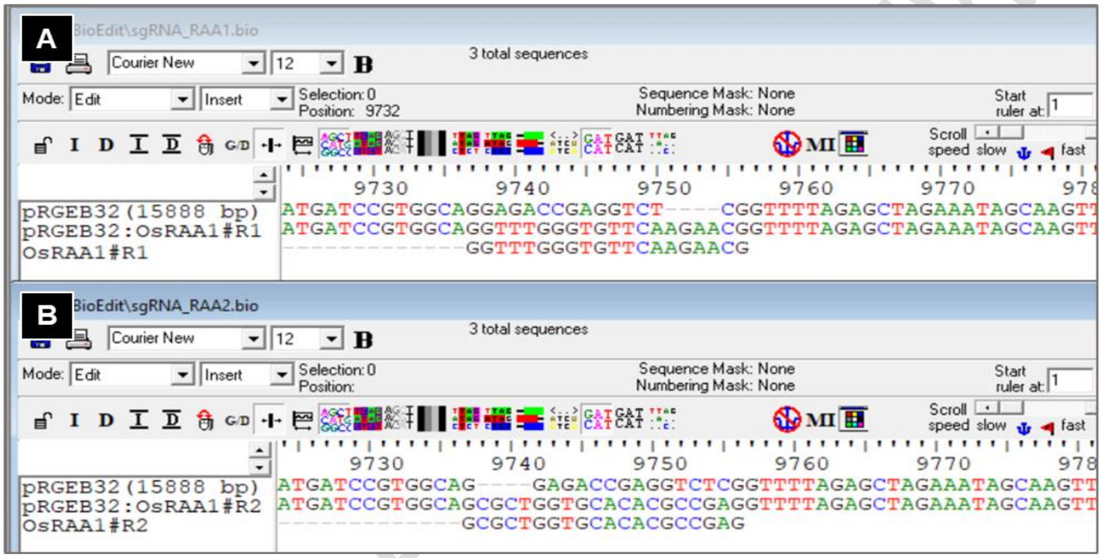


Fig. 4: Colony PCR of transformed DH5α colonies and plasmid PCR profile of plasmid vectors isolated from transformed DH5α. (A, B) The colony PCR profile of pRGEB32:OsRAA1#R1 and pRGEB32:OsRAA1#R2 transformed DH5α, respectively. L- 100 bp ladder; 1 to 8- Eight randomly selected colonies from transformed DH5α plates. Colony 8 from OsRAA1#R1 and 4 from OsRAA1#R2 (mentioned in the red dotted box) were selected for plasmid isolation; (C) Plasmid PCR of isolated plasmid from OsRAA1#R1 and OsRAA1#R2. L- 100 bp ladder; 1- pRGEB32:OsRAA1#R1(8); 2- pRGEB32: OsRAA1#R2(4).

This CRISPR/Cas9-gRNA vector construct was then mobilized into the *E. coli* cells. Following transformation, bacterial colonies were obtained on the LB agar plates consisting of kanamycin, after overnight incubation. Despite of large size of pRGEB32, we observed a significant number of colonies (more than 100) for each pRGEB32:gRNA construct. This might be due to the highly competent *E. coli* DH5α cells used for transformation or the optimum transformation conditions provided. Eight colonies were randomly selected to screen for inserts by colony PCR with gRNA and universal M13 (vector-specific) primer. Expected bands of size ~450 bp were observed on 1% agarose gel (Fig. 4, A; B). Colony 8 of OsRAA1#R1 (pRGEB32:OsRAA1#R1) and 4 of OsRAA1#R2 (pRGEB32:OsRAA1#R2) were selected for plasmid isolation and sequencing. Universal M13 reverse primer was used for sequencing of isolated plasmids, and the results were analyzed using BioEdit v7.2 software by multiple sequence alignment (Fig. 5).



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pRGEB32(15888 bp)  ATGATCCGTGGCAGGAGACCGAGGTCT----CGGTTTTAGAGCTAGAAATAGCAAGTT
pRGEB32:OsRAA1#R1  ATGATCCGTGGCAGGTTTGGGTGTTCAAGAACGGTTTTAGAGCTAGAAATAGCAAGTT
OsRAA1#R1          -------------GGTTTGGGTGTTCAAGAACG
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pRGEB32(15888 bp)  ATGATCCGTGGCAG----GAGACCGAGGTCTCGGTTTTAGAGCTAGAAATAGCAAGTT
pRGEB32:OsRAA1#R2  ATGATCCGTGGCAGCGCTGGTGCACACGCCGAGGTTTTAGAGCTAGAAATAGCAAGTT
OsRAA1#R2          -------------GCGCTGGTGCACACGCCGAG
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Fig. 5: Multiple sequence alignment profile representing the gRNA cloned region in the pRGEB32 vector. The clone sequences were aligned with the pRGEB32 vector sequence in BioEdit v7.2 software. (A) pRGEB32:OsRAA1#R1; (B) pRGEB32:OsRAA1#R2.

Mobilization of the vector-guide construct into *A. tumefaciens*: The CRISPR/Cas9-gRNA construct, pRGEB32:OsRAA1#R1 and pRGEB32: OsRAA1#R2 were confirmed positive for cloning after sequence analysis and were further mobilized into *Agrobacterium* by freeze-thaw method (Holsters *et al.,* 1978). Subsequently, the transformed cells were spreaded on plates containing kanamycin and rifampicin in LB agar and were incubated for around 2 days at 28˚C. A total of 17 and 15 colonies respectively were observed on pRGEB32:OsRAA1#R1 and pRGEB32: OsRAA1#R2 transformed plates, respectively. The number of *Agrobacterium* EHA105 colonies observed was relatively low compared to *E. coli* DH5α. The presence of even a minimal quantity of contaminants along with pRGEB32 could affect transformation efficiency of *Agrobacterium*.

Confirmation of vector-guide construct in *Agrobacterium*: Eight colonies were randomly selected to screen for inserts by colony PCR with gRNA and universal M13 (vector-specific) primer. Expected bands of size ~450 bp were observed on 1% agarose gel (Fig. 6, A; B). Isolation of the plasmid was performed on two randomly selected *Agrobacterium* colonies from each gRNA construct for validation using plasmid PCR (Table 5 and 6). Universal M13 reverse primer was used as forward primer and primers specific to respective gRNAs were used as reverse primers. Plasmids from colonies 2, 4 of the OsRAA1#R1 construct and 3, 6 of the OsRAA1#R2 construct were isolated. An expected amplicon size of ~450 bp was detected in all the selected colonies on 1% agarose gel after plasmid PCR (Fig. 6, C).

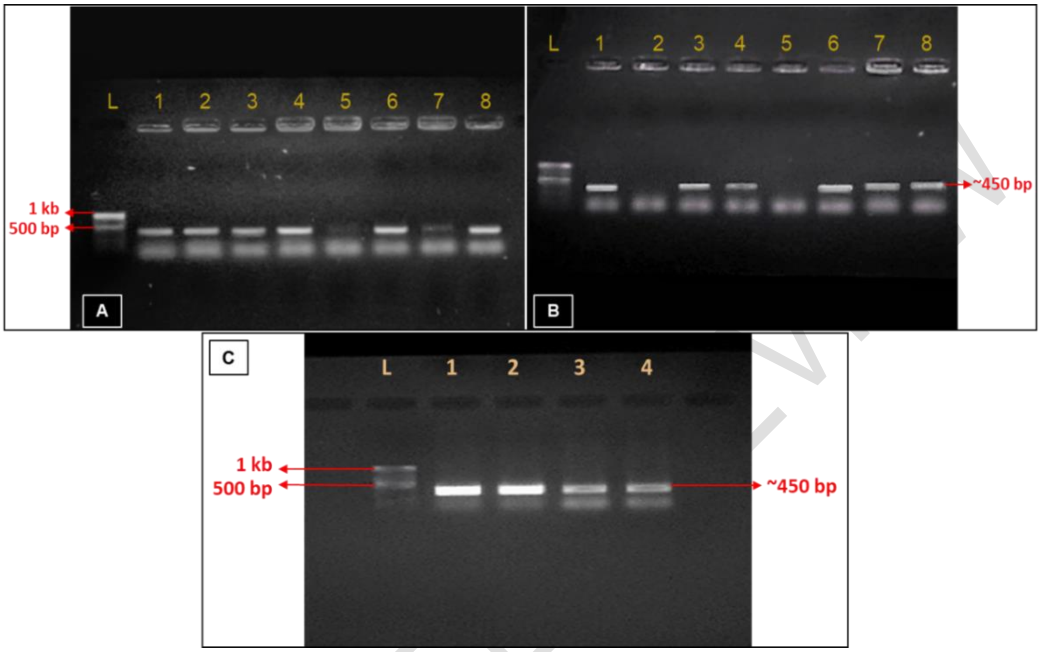

Fig. 6: Colony PCR of transformed EHA105 colonies and plasmid PCR profile of recombinant vectors isolated from transformed EHA105. (A, B) The colony PCR profile of OsRAA1#R1 and OsRAA1#R2 transformed DH5α, respectively. L- 100 bp ladder; 1 to 8- Eight randomly selected colonies from transformed DH5α plates. Colonies 2, 4 from OsRAA1#R1 and 3, 6 from OsRAA1#R2 clones were selected for plasmid isolation; (C) Plasmid PCR of isolated plasmid from OsRAA1#R1 and OsRAA1#R2. L- 100 bp ladder; 1- pRGEB32:OsRAA1#R1(2); 2- pRGEB32:OsRAA1#R1(4); 3- pRGEB32:OsRAA1#R2(3); 4- pRGEB32:OsRAA1#R2(6).

Several approaches for rice transformation have been successfully deployed including electroporation, PEG-mediated, biolistic bombardment, and *Agrobacterium*-mediated DNA delivery methods (Hayashimoto *et al.,* 1990; Tada *et al.,* 1990; Li *et al.,* 1993; Ayres and Park, 1994). However, introducing foreign DNA into rice calli through *Agrobacterium*-mediated transformation remains a popular approach. The major reason could be the efficient single-copy integration of foreign DNA, resulting in stable inheritance, and the fact that whole plants from rice calli are often regenerated more efficiently than those transformed by other methods. As a line of future work, the constructs generated in this study can be used for *Agrobacterium*-mediated rice genetic transformation with improved root system architecture.

Developing drought-tolerant rice cultivars in areas with water scarcity is vital for ensuring food security. Researchers have potentially elucidated the association of the *OsRAA1* gene with drought stress responses (Han *et al.,* 2005). Genetic engineering through CRISPR/Cas9 can eliminate unfavorable traits and generate new species characteristics by knocking out undesirable genes. This study aims to design sgRNA targeting the *OsRAA1* gene in rice and develop CRISPR/Cas9 constructs using the vector pRGEB32. We have cloned the pRGEB32:gRNA vector construct into the *Agrobacterium* EHA105 strain, which will be further used to target the *OsRAA1* gene in rice and explore its potential for improving drought tolerance.

The expression pattern of *OsRAA1* gene in the root was similar to that of auxin-responsive gene families, such as the *AUX/IAA* and the *SAUR* (*small auxin up-regulated RNA*) family (Hagen *et al.,* 1984; Abel and Theologis, 1996; McClure and Guilfoyle, 1989). Xu *et al.* (2010) stated that the OsRAA1 protein is a negative controller of drought tolerance, whose accumulation halts the transition of the cell cycle to anaphase for root development in rice. This leads to an increase in lateral and adventitious root growth, while a decrease in primary root growth (Ge *et al.,* 2004; Han *et al.*, 2005). Similar phenotypic expressions were also observed in case of certain other auxin-responsive genes like *NAC1* and *TIR1* in Arabidopsis (Gray *et al.,* 1999; Xie *et al.,* 2000).

4. CONCLUSION

Two spacer sequences were selected and gRNAs were validated using the CRISPR-P v2.0 and RNA secondary structure prediction tool respectively. The spacer sequences were cloned into *E. coli* strain DH5α using pRGEB32 vector to generate pRGEB32:OsRAA1#R1 and pRGEB32:OsRAA1#R2 constructs. Further, the CRISPR/Cas9 constructs were confirmed by Sangar sequencing and transferred into *Agrobacterium* strain EHA105. In our future research, these constructs will be utilized to perform *Agrobacterium*-mediated genetic transformation of rice calli to develop deeper rooting plants with water-deficit stress. For a deeper understanding of the underlying molecular mechanisms, gene expression, RAA1 protein interactions, and various physiological parameters including root growth, chlorophyll stability, and root auxin levels can be studied.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that generative AI technologies, Quillbot and ChatGPT have been used during the writing and editing of this manuscript.

REFERENCES

Abel, S. and Theologis, A. (1996). Early genes and auxin action. Plant physiology, 111(1),9.

Ambavaram, M. M., Basu, S., Krishnan, A., Ramegowda, V., Batlang, U., Rahman, L., Baisakh, N. and Pereira, A. (2014). Coordinated regulation of photosynthesis in rice increases yield and tolerance to environmental stress. Nature Communications, 5(1),5302.

Ayres, N.M. and Park, W.D. (1994). Genetic transformation of rice. Critical Reviews in Plant Sciences, 13(3),219-239.

Chang, A.Y., Chau, V., Landas, J.A. and Pang, Y. (2017). Preparation of calcium competent *Escherichia coli* and heat-shock transformation. JEMI methods, 1,22-25.

Ehrt, S. and Schnappinger, D. (2003). Isolation of plasmids from E. coli by alkaline lysis. *E. coli* Plasmid Vectors, Methods and Applications, 75-78.

Ge, L., Chen, H., Jiang, J.F., Zhao, Y., Xu, M.L., Xu, Y.Y., Tan, K.H., Xu, Z.H. and Chong, K. (2004). Overexpression of *OsRAA1* causes pleiotropic phenotypes in transgenic rice plants, including altered leaf, flower, and root development and root response to gravity. Plant Physiology, 135(3),1502-1513.

Gray, W.M., Del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H. and Estelle, M. (1999). Identification of an SCF ubiquitin–ligase complex required for auxin response in *Arabidopsis thaliana*. Genes & development, 13(13),1678-1691.

Hagen, G., Kleinschmidt, A. and Guilfoyle, T. 1(984). Auxin-regulated gene expression in intact soybean hypocotyl and excised hypocotyl sections. Planta, 162,147-153.

Han, Y., Wang, X., Jiang, J., Xu, Y., Xu, Z. and Chong, K. (2005). Biochemical character of the purified *OsRAA1*, a novel rice protein with GTP-binding activity, and its expression pattern in *Oryza sativa*. Journal of plant physiology*,* 162(9),1057-1063.

Hayashimoto, A., Li, Z. and Murai, N. (1990). A polyethylene glycol-mediated protoplast transformation system for production of fertile transgenic rice plants. Plant physiology, 93(3),857-863.

Heinemann, A. B., Barrios-Perez, C., Ramirez-Villegas, J., Arango Londoño, D., Bonilla-Findji, O., Medeiros, J. C. and Jarvis, A. (2015). Variation and impact of drought-stress patterns across upland rice target population of environments in Brazil. Journal of Experimental Botany, 66(12),3625-3638.

Holsters, M., De Waele, D., Depicker, A., Messens, E., Van Montagu, M. and Schell, J. (1978). Transfection and transformation of *Agrobacterium tumefaciens*. Molecular and General Genetics MGG, 163,181-187.

IPCC, 2023, Sections. In, *Climate Change 2023, Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* [Core Writing Team, H. Lee and J. Romero (eds.)]. IPCC, Geneva, Switzerland, 35-115. Available from, 10.59327/IPCC/AR6-9789291691647

Li, L., Qu, R., de Kochko, A., Fauquet, C. and Beachy, R.N. (1993). An improved rice transformation system using the biolistic method. Plant Cell Reports, 12,250-255.

Liu, H., Ding, Y., Zhou, Y., Jin, W., Xie, K. and Chen, L.L. (2017). CRISPR-P 2.0, an improved CRISPR-Cas9 tool for genome editing in plants. Molecular plant, 10(3),530-532.

Lu, G., Coneva, V., Casaretto, J.A., Ying, S., Mahmood, K., Liu, F., Nambara, E., Bi, Y.M. and Rothstein, S.J. (2015). *OsPIN5b* modulates rice (*Oryza sativa*) plant architecture and yield by changing auxin homeostasis, transport, and distribution. The Plant Journal, 83(5),913-925.

McClure, B.A. and Guilfoyle, T. (1989). Rapid Redistribution of Auxin-Rregulated RNAs during Gravitropism. Science, 243(4887),91-93.

Overvoorde, P., H. Fukaki, and T. Beeckman. (2010). Auxin control of root development. Cold Spring Harbor perspectives in biology, 2(6),p.a001537. Available from, 10.1101/cshperspect.a001537

Pirdashti, H., Sarvestani, Z.T. and Bahmanyar, M.A. (2009). Comparison of physiological responses among four contrast rice cultivars under drought stress conditions. World Academy of Science, Engineering and Technology, 49,52-54.

Tada, Y., Sakamoto, M. and Fujimura, T. (1990). Efficient gene introduction into rice by electroporation and analysis of transgenic plants, use of electroporation buffer lacking chloride ions. Theoretical and Applied Genetics, **80**,475-480.

Tang, X., Nakata, Y., Li, H.O., Zhang, M., Gao, H., Fujita, A., Sakatsume, O., Ohta, T. and Yokoyama, K. (1994). The optimization of preparations of competent cells for transformation *of E. coli.* Nucleic Acids Research*,* 22(14),2857.