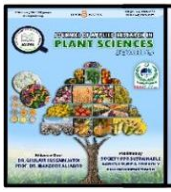
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Optimization of an *Agrobacterium*-Mediated CRISPR/Cas9 Delivery System Targeting the *GS3* Gene in JP-5 *Oryza sativa*

Kashmala Khan[1#], Amna Jamshaid[1#], Safeena Inam[1#], Shahzaib Ahmed[1], Aqdas Vishah Khan[2], Saima Jameel[1], Amber Afroz[3], Nazia Rehman[1], Muhammad Ramzan Khan[1*], Muhammad Uzair[1*]

[1]National Institute for Genomics and Advanced Biotechnology (NIGAB), Park Road, Islamabad.
[2]University of Poonch Rawalakot, AJK.
[3]Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat, Pakistan.
[#]These authors contributed equally.
***Corresponding Author: uzairbreeder@gmail.com (MU); mrkhan@parc.gov.pk (MRK)**
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ABSTRACT

Rice (*Oryza sativa*) is an important food crop for more than half of the world's population. The two main cultivated rice species are *Oryza sativa* and *Oryza glaberrima.* Among the *Oryza sativa* species, JP-5 is a japonica rice variety that is cold tolerant and has short generation time along with other beneficial agronomic characteristics. However, this variety has short grain size, and it is known that the food supply and market value of rice is highly influenced by its grain size and, larger grain size is favored by the consumer. Therefore, the present study is aimed at optimizing the procedure for increasing grain size of rice using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Cas-9 technology. This technique can be used to efficiently mutate the *GS3* (Grain Size 3) gene which is a major QTL (quantitative trait locus) that negatively regulates the grain size in rice. This study has established an optimized and reproducible workflow from rice seed sterilization to callus induction, callus infection with bacteria, selection and then regeneration of the rice plants. For sterilization, 20% Clorox and 75% ethanol have shown best effects, for callus induction, 2mg/L 2,4-D (2,4 dichloro-phenoxy acetic acid) concentration was best showing callus induction rate of 89.17 ± 3.00%, and 35mg/L hygromycin was optimized for selection of transformants. Among the hormonal concentrations for regeneration, 2mg/L BAP (6-Benzylaminopurine), 1mg/L NAA (Naphthalene-1-acetic acid) and 0.5mg/L kinetin gave the best results with a regeneration frequency of 70.59 ± 5.88%. In addition, concentrations of decontaminating antibiotics have also been optimized here which include 300mg/L carbenicillin. PCR analysis confirmed the presence of the CRISPR/Cas9 construct in 12 out of 40 tested plants, demonstrating successful genetic transformation. Further molecular characterization, including sequencing and mutation analysis, is required to validate targeted modifications in the GS3 gene and assess the potential of the developed protocol for future genome-editing applications in rice

KEYWORDS: *Oryza sativa, Agrobacterium*-mediated transformation, CRISPR-Cas9 system, callus induction, regeneration, PCR (Polymerase Chain Reaction)

INTRODUCTION

Rice (*Oryza sativa* L.) is a major staple food crop that feeds more than half of the world's population. More than 3 billion people rely on it as their main source of energy and protein. It is the most widely grown cereal in Asia, which accounts for about 90% of the world's rice production (Muthayya *et al*., 2014). However, it is also grown in Latin America, sub-Saharan Africa, and Europe. In 2023–2024, the world produced 522.62 million metric tonnes of paddy rice, but demand is still higher than supply. This is because the population is expected to reach 9.1 billion by 2050, which will mean a 70% increase in food needs (Bin Rahman & Zhang, 2023). Rice is the second most important food crop in Pakistan after wheat. It makes up 0.6% of GDP (Gross Domestic Product) and more than 3% of the total agricultural value added. Pakistan is one of the world's top rice producers and is usually the third or fourth largest exporter of rice. It exports more than 4 million metric tonnes of rice worth about USD 2 billion every year (Chandio *et al*., 2020).

The size of rice grains is one of the most important economic traits of rice because it affects yield potential, market value, and consumer preference. It is a quantitative trait controlled by a complex network of QTLs that control cell growth, elongation, and grain filling in the spikelet hull (Jiang *et al*., 2022). So far, over 400 QTLs linked to grain size have been found. *GS3* is a major QTL on chromosome 3 that codes for a putative transmembrane protein that works to shorten grain length. Loss-of-function mutations in *GS3* are naturally found in some long-grain varieties, like Minghui 63 and Zenshan 97, and they are enough to make grains that are much longer and heavier (Mao *et al*., 2010). *GS3* competes with positive G-protein

signaling components at the molecular level to stop grain elongation. This makes it a great target for knockout-based methods to make grain size bigger in varieties that have the wild-type growth-limiting allele (Sun *et al*., 2018). The japonica variety JP-5 has an allele that makes it cold-tolerant and has a short generation time, but it also has a short grain phenotype that makes it less valuable on the market.

Genome editing with CRISPR/Cas9 has become the most accurate and effective way to improve specific crops. The CRISPR/Cas9 system uses a short single guide RNA (sgRNA) to direct the Cas9 endonuclease to a specific site in the genome. This creates double-stranded breaks that are fixed by NHEJ or HDR to make targeted mutations. Researchers have shown that CRISPR/Cas9 can be used to edit genes that control the size of rice grains, how well they resist disease, and how well they can handle stress (Malik *et al*., 2026). *Agrobacterium tumefaciens*-mediated transformation is the most effective way for introducing the CRISPR/Cas9 system into rice. It allows for single-copy T-DNA integration and less transgene rearrangement than direct methods. Rice is naturally resistant to *Agrobacterium* infection, and the efficiency of transformation is very sensitive to the conditions at each stage of tissue culture, such as the composition of the media, the balance of hormones, the density of bacteria, and the parameters for co-cultivation (Elakhdar *et al*., 2021). So, to successfully generate genome-edited rice lines, these parameters need to be optimized.

The current study was conducted with the dual objectives of (i) developing a CRISPR/Cas9 expression vector containing two sgRNAs directed at the *GS3* gene, and (ii) formulating an optimized, reproducible protocol for *Agrobacterium*-mediated transformation and regeneration of the JP-5 rice variety.

MATERIALS AND METHODS

Vector Construction: The GS3 sequence (*Os03g0407400*; chromosome 3: 16,729,501–16,735,109, reverse strand) was retrieved from Ensembl Plants (release 56, February 2023; assembly IRGSP-1.0). Two target sites were chosen, with two 20-nucleotide target sites containing NGG protospacer adjacent motifs (PAMs) in exon 5, expressed from the OsU3 promoter (T1: GC content 65%, PAM AGG) and the OsU6a promoter (T2: GC content 75%, PAM CGG). The selection criteria for the targets were whether the site was inside the GS3 coding sequence, the presence of a canonical NGG PAM, and the predicted specificity, while no GC-content threshold was used as a selection criterion; the actual GC content of each target is listed in Table 2. Target design involved prediction of specificity and prediction of potential off-target sites of the designed targets, using CRISPR P-2.0. Neither target had a predicted off-target site with fewer than three mismatches.

The oligonucleotide adaptors containing target sequences (Table1) were ligated into BsaI-digested pYLsgRNA-OsU3 and pYLsgRNA-OsU6a, and the full-length sgRNA expression constructs were synthesized by nested PCR. The purified cassettes were assembled in a single Golden Gate reaction (BsaI and T4 DNA ligase) to assemble the pYLCRISPR/Cas9Pubi-H. The assembly product was electroporated into *E. coli* DH5α and selected on LB agar with kanamycin. They were screened by colony PCR with the flanking primers SP-L1 and SP-R. The positive colonies were then electroporated into *Agrobacterium tumefaciens* LBA4404, selected on LB agar with kanamycin and rifampicin, and screened using the same primers. No sequencing or restriction analysis of the final plasmid was done to verify the construct. Positive strains of *Agrobacterium* were kept at -80 °C in glycerol.

Table 1. Primers and oligonucleotides used for vector construction and clone confirmation

Primer	Purpose	Sequence (5'-3')
T1-F	Target site 1 cloning oligo (pYLsgRNA-OsU3)	GGCACGTTAACGCCGCCCCACATG
T1-R	Target site 1 cloning oligo (pYLsgRNA-OsU3)	AAACCATGTGGGGCGGCGTTAACG
T2-F	Target site 2 cloning oligo (pYLsgRNA-OsU6a)	GCCGCGGCACGAGCATCCCGCGCA
T2-R	Target site 2 cloning oligo (pYLsgRNA-OsU6a)	AAACTGCGCGGGATGCTCGTGCCG
SP-L1	Colony PCR confirmation	GCGGTGTCATCTATGTTACTAG
SP-R	Colony PCR confirmation	CGAAGTTATTGCATCTATGTCGGG

Table 2. Target sites in *GS3* (*Os03g0407400*). Positions refer to the rice assembly IRGSP-1.0 (Ensembl Plants release 56). PAM, protospacer adjacent motif. Both target sites lie on the plus strand of chromosome 3 (antisense to *GS3*) within exon 5. MIT and CFD are specificity scores (maximum 100). Off-target values give the number of other genomic sites with 0, 1, 2, 3, or 4 mismatches

Target	Promoter	Target sequence (5'→3')	PAM	Location (IRGSP-1.0)	Exon	GC (%)	MIT / CFD	Off-targets (0-1-2-3-4 mismatches)
T1	OsU3	CGTTAACGCCGC CCCACATG	AGG	chr 3: 16,729,958–16,729,977 (+)	5 of 5	65	99 / 100	0-0-0-1-6
T2	OsU6a	CGGCACGAGCA TCCCGCGCA	CGG	chr 3: 16,729,815–16,729,834 (+)	5 of 5	75	97 / 98	0-0-0-0-32

Plant material and explant preparation: For the transformation experiment, mature seeds of the JP-5 rice variety were used, which were collected from the National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Center (NARC), Islamabad.

Transformation procedure: Seeds of JP-5 were de-husked and were surface sterilized prior to culturing in the callus induction medium. The sterilization process included treating the seeds with varying concentrations of ethanol (75-100%) for 3-5 minutes and sodium hypochlorite (20-50%) for 10-20 minutes, keeping the sterilization volume at 20ml in a 50ml tube (Table 3). Each step was followed by washing the seeds twice/thrice with sterile distilled water for 1 minute. The seeds were air-dried on sterile filter paper for 10-20 minutes.

Table 3: Seed Sterilization Treatments (ST1-ST8)

No. of Treatments	Sodium Hypochlorite (Clorox) (mins)			Ethanol (mins)		Distilled Water (Washes)	Air Dry Time (mins)	Sterilization Volume (ml)
	20%	30%	50%	75%	100%			
1.	10	-	-	3	-	3	10	20
2.	-	10	-	-	3	3	10	20
3.	-	-	10	5	-	3	10	20
4.	-	-	-	-	5	3	10	20
5.	**20**	**-**	**-**	**5**	**-**	**3**	**10**	**20**
6.	-	20	-	-	5	3	20	20
7.	-	-	20	5	-	3	20	20
8.	20	-	-	-	-	3	20	20

Seed Sterilization: Sterile seeds were placed on callus induction media of varying compositions (Table 3) and kept inside the incubator at 28 ± 2 ℃ to allow callus induction in the dark for 1-2 weeks. Plates can also be placed in growth rooms under light conditions, where callus grows at nearly the same rate as in the dark but with less contamination. After one week, callus growth started from seeds, and on day 12th, the Calli were carefully removed from seeds and were shifted to fresh media for further growth for 2-3 weeks.

Callus Induction and Culture Conditions: Surface-sterilized seeds were inoculated onto Callus Induction Media containing varying concentrations of MS basal salts (2.2–4.3 g/L), sucrose (16–30 g/L), 2,4-D (1.0–10.0 mg/L), and organic supplements including MES (2-(N-morpholino) ethane sulfonic acid), L-Proline, L-Glutamine, and Casein Hydrolysate across ten distinct formulations (C1–C10; Table 4).

Cultures were incubated in a growth chamber at 28 ± 2 °C in complete darkness for 1–2 weeks to induce callogenesis. Following initial induction in the dark, subsequent callus proliferation and maintenance stages were carried out under light conditions to reduce contamination. On day 12, developing calli were carefully excised from the seed explants and transferred onto fresh media of the respective formulations for an additional 2–3 weeks to allow further tissue proliferation and embryogenic differentiation.

The experimental design consisted of three replicate plates per treatment (n = 3), with each plate containing 20 seeds. Each plate was considered an independent experimental unit, and all three plates were prepared during the same experimental run. Evaluated quantitative parameters included:

$$Callus\ Induction\ Rate\ (\%) = \frac{Total\ seeds\ forming\ callus}{Total\ cultured\ seeds} \times 100$$

$$Embryonic\ Callus\ Rate\ (\%) = \frac{Number\ of\ embryonic\ calli}{Total\ calli\ formed} \times 100$$

$$\text{Post} - \text{Agro Regeneration Rate } (\%) = \frac{\text{Calli regenerating shoots post} - \text{infection}}{\text{Total calli infected}} \times 100$$

The media components were varied jointly because the primary objective was to identify the best-performing complete formulation for JP-5 callus induction, rather than to isolate the independent effect of each individual ingredient. The denominators for all rates were defined relative to the total number of explants or calli evaluated in that specific assay.

Table 4. Callus Induction Media Combinations (C1-C10)

No. of Chemicals	MS Salt (g/L)	Sucrose (g/L)	2,4 -D (mg/L)	MES (g/L)	L-Proline (g/L)	L-Glutamine (g/L)	Casein Hydrolysate (g/L)
C1	2.2	16	1	0	0	0	0
C2	2.2	16	2	0	0	0	0
C3	4.3	30	3	0.5	0.5	0.5	0.5
C4	4.3	30	5	0.5	0.5	0.5	0.5
C5	4.3	30	10	0.5	0.5	0.5	0.5
C6	**4.3**	**30**	**2**	**0.5**	**0.5**	**0.5**	**0.5**

Preparation for Infection Culture: Using *Agrobacterium* glycerol stock, *Agrobacterium* solid cultures were prepared by the streaking method on LB plates and were kept at 28°C for 48 hours in the dark. After bacterial colony formation, a colony was picked from the plate with a tip and dropped into a Falcon tube containing LB liquid medium. After the addition of the inoculum, the tubes were kept in the shaker incubator at 28°C for 24-48 hours for growth in the liquid medium. The optical density of the culture was adjusted using a spectrophotometer (Table 5) and was then centrifuged at 5000rpm for 10 minutes to pelletize the bacteria. The pellet was resuspended in inoculation medium (Table 6), and the calli were placed in it for infection. The calli were washed with distilled water, dried, and shifted to co-cultivation media.

Table 5. Combinations of the three parameters used for infection

OD of Agrobacterial suspension	Duration of infection (mins)	Duration of co-cultivation (days)
0.3	5	2
0.4	**10**	**2**
0.5	10	2

Table 6. Inoculation Media Combinations (IM1-IM6)

No. of Chemicals	MS Salt (g/L)	Sucrose (g/L)	Glucose (mg/L)	MES (g/L)	Acetosyringone (μM)	Casein Hydrolysate (g/L)
IM1.	4.3	30	36	0.5	10	0.5
IM2.	4.3	30	36	0.5	50	0.5
IM3.	4.3	30	36	0.5	100	0.5
IM4.	**4.3**	**68**	**36**	**0.5**	**100**	**0.5**
IM5.	4.3	68	36	0.5	150	0.5
IM6.	4.3	68	36	0.5	200	0.5

Media formulations were evaluated in preliminary optimization batches. Final transformation efficiency was assessed on the large-scale pooled batch (n = 1,345 calli), as summarized in Table 17

Calli were shifted to co-cultivation media (Table 6) for 2-3 days to provide the specific contact time so that the bacteria can transfer their T-DNA into the cells, which is a crucial step in transformation. The plates were placed in the dark at 28±2°C as dark conditions are favorable for *Agrobacterium*-mediated transformation.

Table 7. Co-cultivation media combinations (CC1-CC6)

No. of Chemicals	MS Salt (g/L)	Sucrose (g/L)	2,4 -D (mg/L)	MES (g/L)	L-Proline (g/L)	Acetosyringone (μM)	Glucose (g/L)	Casein Hydrolysate (g/L)
CC1.	4.3	30	2	0.5	0.5	10	10	0.3
CC2.	4.3	30	2	0.5	0.5	50	10	0.5
CC3.	4.3	30	2	0.5	0.5	80	10	0.3
CC4.	**4.3**	**30**	**2**	**0.5**	**0.5**	**100**	**10**	**0.3**
CC5.	4.3	30	2	0.5	0.5	150	10	0.5
CC6.	4.3	30	2	0.5	0.5	200	10	0.5

Media formulations were evaluated in preliminary optimization batches. Final transformation efficiency was assessed on the large-scale pooled batch (n = 1,345 calli), as summarized in Table 17

The infected calli were placed on pre-selection media plates (Table 8) and were incubated in light for 1-2 weeks at 25 ± 2ºC. Pre-selection media or callus recovery media allow the calli to recover from stress and help them to proliferate.

Table 8: Pre-selection Media Combinations (PS1-PS4)

No. of Chemicals	MS Salt (g/L)	Sucrose (g/L)	2,4 -D (mg/L)	Carbenicillin (mg/ml)	L-Proline (g/L)	L-Glutamine (g/L)	Casein Hydrolysate (g/L)
PS1.	4.3	30	2	100	0.5	0.5	0.5
PS2.	4.3	30	2	200	0.5	0.5	0.5
PS3.	**4.3**	**30**	**2**	**300**	**0.5**	**0.5**	**0.5**
PS4.	4.3	30	2	400	0.5	0.5	0.5

Selection was performed in two batches; in the first batch, calli from pre-selection media were directly shifted to selection media containing hygromycin to select the transformants. Hygromycin stock was added in different concentrations (Table 9) with a duration of 3-4 weeks to select the edited calli. The hygromycin concentration was first determined using non-infected calli (two plates of 10 calli per concentration) on 10–50 mg/L, which were scored for survival after 10–12 days. Calli were placed on the selection media with increasing concentrations of hygromycin for one week each under light. In the second batch, instead of direct selection, the calli were first regenerated to overcome the stress of infection and to proliferate under favorable conditions in the presence of growth hormones. The regenerated plants were then subjected to selection starting from a minimum dose (10mg/L) of hygromycin to a maximum dose (50mg/L).

Table 9: Selection of Media Combinations (SM1-SM7)

No. of Chemicals	SM1	SM2	SM3	SM4	SM5	SM6	SM7
MS Salt (g/L)	4.3	4.3	4.3	**4.3**	4.3	4.3	4.3
Sucrose (g/L)	30	30	30	**30**	30	30	30
2,4 -D (mg/L)	2	2	2	**2**	2	2	2
MES (mg/L)	0.5	0.5	0.5	**0.5**	0.5	0.5	0.5
Carbenicillin (mg/L)	250	250	250	**250**	300	300	300
L-Proline (g/L)	0.5	0.5	0.5	**0.5**	0.5	0.5	0.5
L-Glutamine (g/L)	0.5	0.5	0.5	**0.5**	0.5	0.5	0.5
Casein Hydrolysate (g/L)	0.5	0.5	0.5	**0.5**	0.5	0.5	0.5
Hygromycin (mg/L)	10	20	30	**35**	40	45	50

For regeneration, the selected calli (calli that survived through all the phases of selection) were shifted to MS media supplemented with sucrose, sorbitol, casein, proline, glutamine, MES, and growth hormones including BAP, kinetin, and NAA in different combinations given in Table 9. Also, carbenicillin was added to control the growth of contaminating bacteria, and hygromycin was used to prevent the regeneration of any non-edited calli. As a solidifying agent, gellan gum was used, and the pH was adjusted at 5.8. After the appearance of green spots on the calli on day 10^{th}, they were subcultured in fresh regeneration media for further growth. After two weeks, when the calli became all green, MS medium supplemented with sucrose and growth hormones (BAP, NAA) along with $CuSO_4$ was used for further elongation of the plants. After two weeks of elongation, the calli were shifted to rooting media shown in Table 10. The grown plantlets were then exposed to air, and tap water was added to the jar to make the roots of the plants stronger. Once mature roots were formed, the plants were shifted to pots containing peat moss for hardening. The plants were kept in intelligent glasshouses at the National Institute for Genomic and Advanced Biotechnology. Conditions were optimized with a 16-hour light period and an 8-hour dark period, with light intensity of 1100 Lux. The temperature was maintained at 25 ± 5 º C, wind speed 3.0m/s and wind direction 185 º. The plants were regularly irrigated to promote optimum growth and stem thickness

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Molecular Screening through PCR: Sampling was done from the plants after 4 weeks of growth (Figure 1). Samples from the fresh leaves were taken in liquid nitrogen to prevent the DNA/RNA or protein from degrading and then, the samples were prepared for PCR Analysis. Around 100mg of sample was weighed for DNA extraction using the Gene Jet Kit. PCR analysis was done using the Thermo Scientific Fisher PCR kit (Thermo Scientific #k0791). After PCR completion, the 10µl PCR product was run on 1% agarose gel, and the band was visualized on the gel documentation machine for confirmation of the transformants

Table 10: Shooting media combinations (SM1- SM8)

No. of Chemicals	MS Salt (g/L)	MES (g/L)	L-proline (g/L)	L-Glutamine (g/L)	Sorbitol (g/L)	Sucrose (g/L)	BAP (mg/L)	NAA (mg/L)	Kin (mg/L)	Carbenicillin (mg/L)	$CuSO_4$ (µl/L)	Casein Hydrolysate (g/L)
SM1.	4.3	0.5	0.5	0.5	0	20	0.5	1.0	0.25	300	0	2
SM2.	4.3	0.5	0.5	0.5	0	20	1.0	2.5	0.5	300	0	2
SM3.	4.3	0.5	0.5	0.5	0	20	1.5	3.75	0.75	300	0	2
SM4.	4.3	0.5	0.5	0.5	30	30	2.0	5.0	1.0	300	0	2
SM5	4.3	0.5	0.5	0.5	30	30	2.0	2.5	0.25	300	80	2
SM6	4.3	0	0	0	30	30	2.0	2.5	0.5	300	80	2
SM7	4.3	0	0	0	30	30	2.0	1.0	0.5	300	80	2
SM8	**4.3**	0	0	0	**30**	**30**	**2.0**	**1.0**	**0.5**	**300**	**80**	**2**

Table 11: Rooting media combinations (RM1-RM6)

No. of Chemicals	MS Salt (g/L)	Sucrose (g/L)	IAA (mg/L)	NAA (mg/L)	Carbenicillin (mg/L)	MES
RM1.	4.3	30	1	2	300	0.5
RM2.	4.3	30	2	2	300	0.5
RM3.	4.3	30	5	2	300	0.5
RM4.	4.3	30	0	0	300	0.5
RM5.	4.3	30	0	0	300	0.5
RM6.	**4.3**	**30**	0	0	**300**	**0.5**



Figure 1: Acclimatization of regenerated rice plants following transfer from in vitro culture vessels to pots containing soil, followed by growth under controlled glasshouse conditions.

Statistical Analysis: Quantitative data were collected from independent experimental replicates. For seed sterilization and callus induction assays, two and three independent biological replicates were used, respectively, with 15-16 seeds and 20 seeds per plate. For shoot regeneration and rooting assays, two independent replicates were evaluated, with 8-10 calli and 2–3 shoots per replicate, respectively. Percentage data were arcsine-transformed (arcsine √p) prior to statistical evaluation to normalize variance. Differences among treatment means were evaluated using One-Way Analysis of Variance (ANOVA), and mean separations were performed using Fisher's Least Significant Difference (LSD) post-hoc test at $p < 0.05$. All statistical analyses were conducted using standard statistical software.

RESULTS

Assembly and cloning of sgRNA Expression Cassettes: Gel analysis of the PCR products validated the expected sizes (Figure 2) of both expression cassettes (U3~564 bp; U6~629 bp), which were previously reported for this vector system (Ma *et al*., 2015). Both expression cassettes were then gel purified and assembled into the binary vector pYLCRISPR/Cas9Pubi-H in a single Golden Gate ligation reaction.

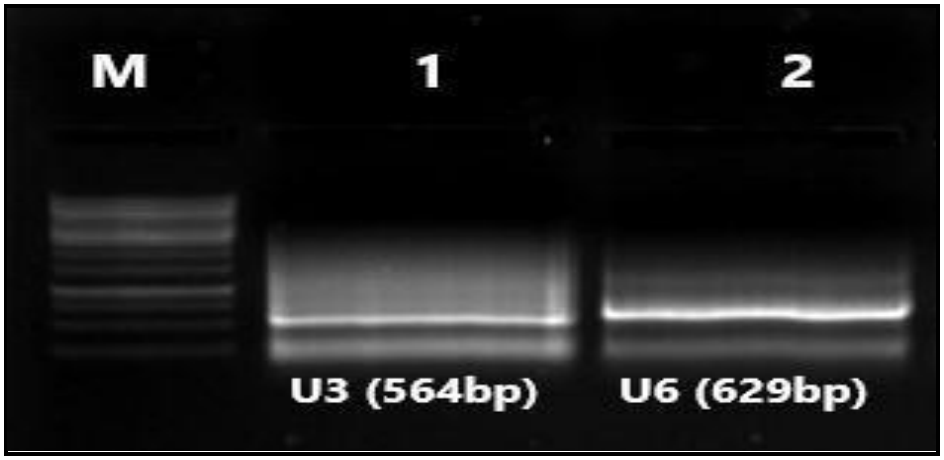


Figure 2: The PCR products of both sgRNA-expression cassettes, OsU3 cassette carrying T1 gave band size of approximately 564 bp and OsU6 carrying T2 gave band size of 629 bp, as described previously for this vector system. M= 1 kb DNA ladder.

Confirmation of Construct in *E. coli* and *Agrobacterium tumefaciens:* The ligation products of the assembly reaction were then transformed into *E. coli* DH5α cells via electroporation to provide selectable transformants on LB agar plates containing kanamycin. Colony PCR using flanking primers, SP-L1 and SP-R, yielded a PCR amplicon of approximately 900 base pairs in all positive clones, indicating the insertion of both sgRNA cassettes into the binary vector successfully (Figure 3A). Confirmed *E. coli* plasmid DNA clones were electrically transformed into *Agrobacterium tumefaciens* strain LBA4404. Agrobacterium colony PCR confirmed insertion of the construct by producing an amplicon of approximately 1,175 base pairs for each positive clone (Figure 3B). Only verified by colony PCR, no sequencing of the plasmid was performed. The rice callus was transformed by these recombinant Agrobacterium strains, which carry the CRISPR/Cas9 construct targeting GS3 gene. Only verified by colony PCR, no sequencing of the plasmid was performed. The rice callus was transformed by these recombinant Agrobacterium strains, which carry the CRISPR/Cas9 construct targeting GS3.

Surface Sterilization of Seeds: Surface sterilization treatments significantly affected seed contamination ($F_{(7,8)} = 31.42$, $p < 0.0001$) and callus induction ($F_{(7,8)} = 184.60$, $p < 0.0001$). Treatment ST5 (20% Clorox for 20 min + 75% ethanol for 5 min) showed 0.00% contamination and the highest callus induction rate (90.00 ± 5.00%), which was statistically superior to all other treatments (Table 12).

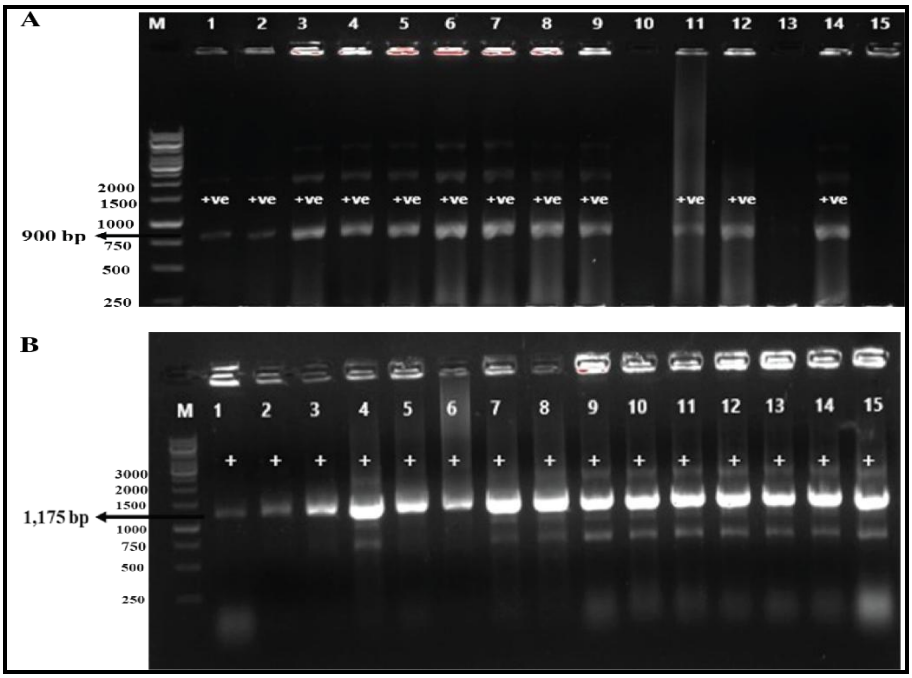


Figure 3. Detection of ligated cloning product. A fragment of 900 bp shows successful cloning of expression cassettes into the binary vector (A). M = 1 kb marker. Positive transformed colonies in *Agrobacterium tumefaciens* (B). Fragments of 1,175 bp are positive. M = 1kb ladder.

Table 12: Effect of different surface sterilization treatments on seed contamination and callus induction. Values represent the mean ± SE from two independent replicates (20 plates per replicate, 15–16 seeds per plate).

Treatment	Contamination Rate (%)	Uncontaminated / Clean Rate (%)	Callus Induction Rate (%)	Biological Outcome
ST1	100.00 ± 0.00^{a}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	Severe fungal/bacterial growth
ST2	40.00 ± 2.50^{c}	60.00 ± 2.50^{c}	42.50 ± 0.00^{b}	Partial contamination
ST3	0.00 ± 0.00^{d}	100.00 ± 0.00^{a}	11.25 ± 3.75^{cd}	Low tissue viability
ST4	100.00 ± 0.00^{a}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	Severe fungal/bacterial growth
ST5 (Optimal)	**0.00 ± 0.00^{d}**	**100.00 ± 0.00^{a}**	**90.00 ± 5.00^{a}**	**Optimal sterilization & viability**
ST6	0.00 ± 0.00^{d}	100.00 ± 0.00^{a}	18.75 ± 1.25^{c}	Reduced explant viability
ST7	0.00 ± 0.00^{d}	100.00 ± 0.00^{a}	0.00 ± 0.00^{e}	Over-sterilized / Tissue necrosis
ST8	55.00 ± 20.00^{b}	45.00 ± 20.00^{d}	7.50 ± 0.00^{de}	High variation / Ineffective

Means sharing different superscript letters within a column differ significantly according to Fisher's Least Significant Difference (LSD) test ($p < 0.05$). Data were arcsine-transformed before analysis.

Evaluation of Callus Induction Media: Callus induction, embryogenic callus formation, and post-infection regeneration differed significantly among the six media (one-way ANOVA on arcsine-transformed data: callus induction $F(5,12) = 35.43$, embryogenic callus $F(5,12) = 81.25$, regeneration $F(5,12) = 81.96$; all $p < 0.0001$). Media C1 and C2 produced minimal callus (5.00%) with no embryogenic callus or regeneration (Figure. 5).

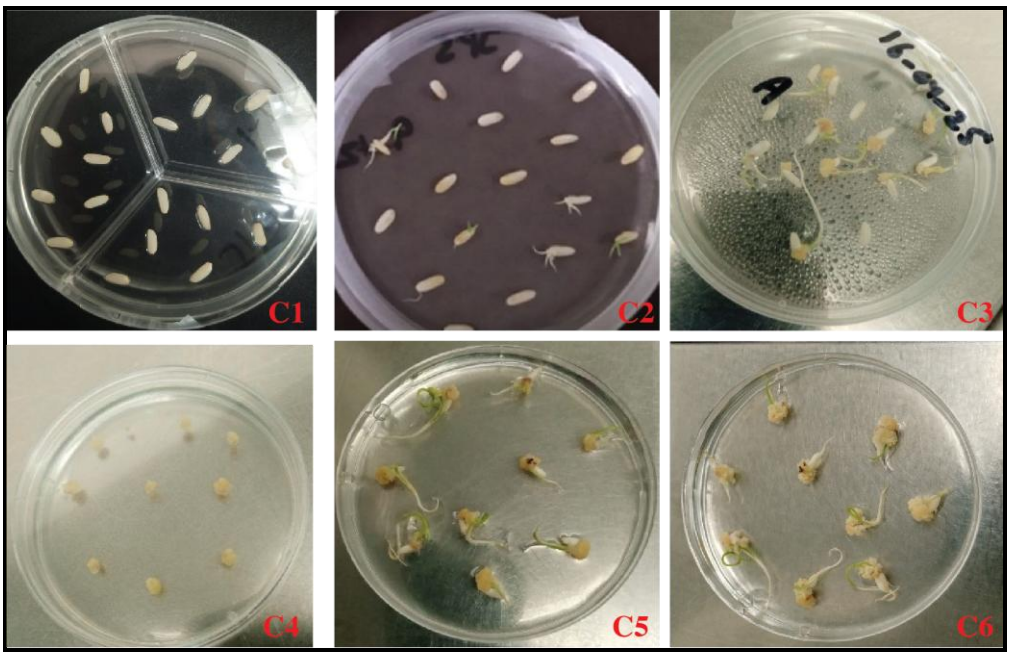


Figure 4. Callus induction response of rice seeds cultured on different callus induction media compositions (C1–C6). Callus development was assessed across the tested media formulations, with C6 exhibiting the highest callus growth (89.17 ± 3.00%), compared with the other treatments.

Callus induction improved with full-strength MS salts (4.3 g/L) and 30 g/L sucrose combined with organic supplements (MES, L-proline, L-glutamine and casein hydrolysate). C6 (4.3 g/L MS, 30 g/L sucrose, 2.0 mg/L 2,4-D and 0.5 g/L each of MES, L-proline, L-glutamine and casein hydrolysate) gave the highest callus induction (89.17 ± 3.00%), embryogenic callus (68.33 ± 3.33%) and post-infection regeneration (70.83 ± 2.20%) (Table 13). Because MS strength, sucrose and organic supplements differed together between C1-C2 and C3-C6, their individual effects could not be separated. The effect of 2,4-D could be compared only among C3-C6, which differed only in 2,4-D concentration.

Table 13: Callus induction, embryogenic callus and post-infection regeneration on media C1–C6.

Medium	Callus induction (%)	Embryogenic callus (%)	Post-infection regeneration (%)
C1	5.00 ± 5.00 ^{d}	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{e}
C2	5.00 ± 2.50 ^{d}	0.00 ± 0.00 ^{d}	0.00 ± 0.00 ^{e}
C3	30.00 ± 2.89 ^{c}	5.00 ± 2.50 ^{c}	5.00 ± 2.50 ^{d}
C4	42.50 ± 2.50 ^{c}	10.00 ± 2.50 ^{c}	21.67 ± 3.33 ^{c}
C5	65.83 ± 3.00 ^{b}	43.33 ± 1.67 ^{b}	41.67 ± 3.33 ^{b}
C6	89.17 ± 3.00 ^{a}	68.33 ± 3.33 ^{a}	70.83 ± 2.20 ^{a}

Values represent mean ± SE (n = 3 replicates per treatment; each replicate consisted of 20 plates with 20 seeds per plate). Percentages are the number of seeds forming callus, embryogenic callus or regenerating, divided by the seeds plated. Means sharing the same letter within a column do not differ significantly (Fisher's LSD, α = 0.05, on arcsine-transformed data)

Evaluation of Inoculation Medium: Six inoculation media combinations (IM1–IM6) were evaluated to optimize *Agrobacterium*-mediated transformation. Among these, IM4, containing 100 µM acetosyringone and 36 g/L glucose, yielded the most consistent callus survival and highest transformation efficiency. A total of 1,345 high-quality calli were inoculated using the optimized IM4 formulation, resulting in 735 surviving calli post-infection (54.6% survival rate). Increasing sucrose and acetosyringone concentrations beyond IM4 (IM5 and IM6) did not improve survival and was associated with increased tissue browning. Therefore, IM4 was selected for all subsequent transformation experiments.

Evaluating Co-cultivation Media: Six co-cultivation media formulations (CC1–CC6) were tested to determine the optimal conditions for T-DNA transfer. CC4, supplemented with 100 µM acetosyringone, provided the best balance between transformation efficiency and bacterial overgrowth control. Using CC4 with a 2-day co-cultivation period, the contamination rate was successfully reduced to approximately 5%, which was significantly lower than the other formulations. This optimized condition was therefore adopted for the large-scale transformation pipeline.

Evaluating Pre-selection Media: Four pre-selection media formulations (PS1–PS4) were evaluated for their ability to support callus recovery and proliferation following *Agrobacterium* infection. Among these, PS3 (supplemented with 300 mg/L carbenicillin) showed the best recovery rate, with the majority of infected calli surviving and proliferating successfully. A recovery period of one week on PS3 was sufficient before transferring the calli to selection media. Media with lower carbenicillin concentrations (PS1 and PS2) showed higher bacterial overgrowth, while higher concentrations (PS4) did not provide any additional benefit. Therefore, PS3 was selected as the optimal pre-selection medium.

Effect of Plant Growth Regulators on Shoot Organogenesis: Shoot regeneration capability varied significantly across the evaluated media formulations (One-way ANOVA: $F_{7,\ 8} = 32.80$, $p < 0.0001$; Table 14). Media formulation SM8 demonstrated superior organogenic efficiency, achieving a maximum regeneration frequency of 70.59 ± 5.88%, which was significantly higher than all other treatments ($p < 0.05$). Furthermore, SM8 produced the highest shoot proliferation density (2.50 ± 0.25 shoots per callus). Formulation SM3 ranked as the second most effective medium (47.06 ± 5.88%), whereas SM1 and SM6 yielded poor organogenic responses (11.76% and 17.65%, respectively). Inclusion of BAP alone in SM5 induced moderate shoot formation (32.35 ± 2.94%), confirming its primary role in shoot initiation, though synergistic supplementation in SM8 was essential for maximum organogenic yield (Tables 14 and 15).

Table 14. Shoot regeneration frequency and mean shoots per callus on media SM1–SM8. Values are mean ± SE (n = 2 replicates, 8–10 calli per replicate).

Media Formulation	Regeneration Frequency (%) (Mean ± SE)	Mean Shoots per Callus (Mean ± SE)	Fisher's LSD Grouping (p≤0.05)
SM1	11.76 ± 2.94^{e}	1.00 ± 0.00^{c}	e
SM2	32.35 ± 2.94^{cd}	1.00 ± 0.00^{c}	cd
SM3	47.06 ± 5.88^{b}	1.50 ± 0.25^{b}	b
SM4	38.24 ± 2.94^{bc}	1.50 ± 0.25^{b}	bc
SM5	32.35 ± 2.94^{cd}	1.25 ± 0.25^{bc}	cd
SM6	17.65 ± 0.00^{de}	1.00 ± 0.00^{c}	de
SM7	32.35 ± 2.94^{cd}	1.00 ± 0.00^{c}	cd
SM8	70.59 ± 5.88^{a}	2.50 ± 0.25^{a}	**a**

Values with different superscript letters within a column are significantly different at $p \leq 0.05$ according to Fisher's LSD post-hoc test.

Table 15: One-way ANOVA for shoot regeneration frequency (%) across media SM1–SM8 (n = 2 replicates per treatment).

Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Square (MS)	F-Value	p-Value
Between Treatments	7	4584.78	654.97	**32.80**	< 0.0001
Within Treatments (Error)	8	159.78	19.97	—	—
Total	15	4744.56	—	—	—

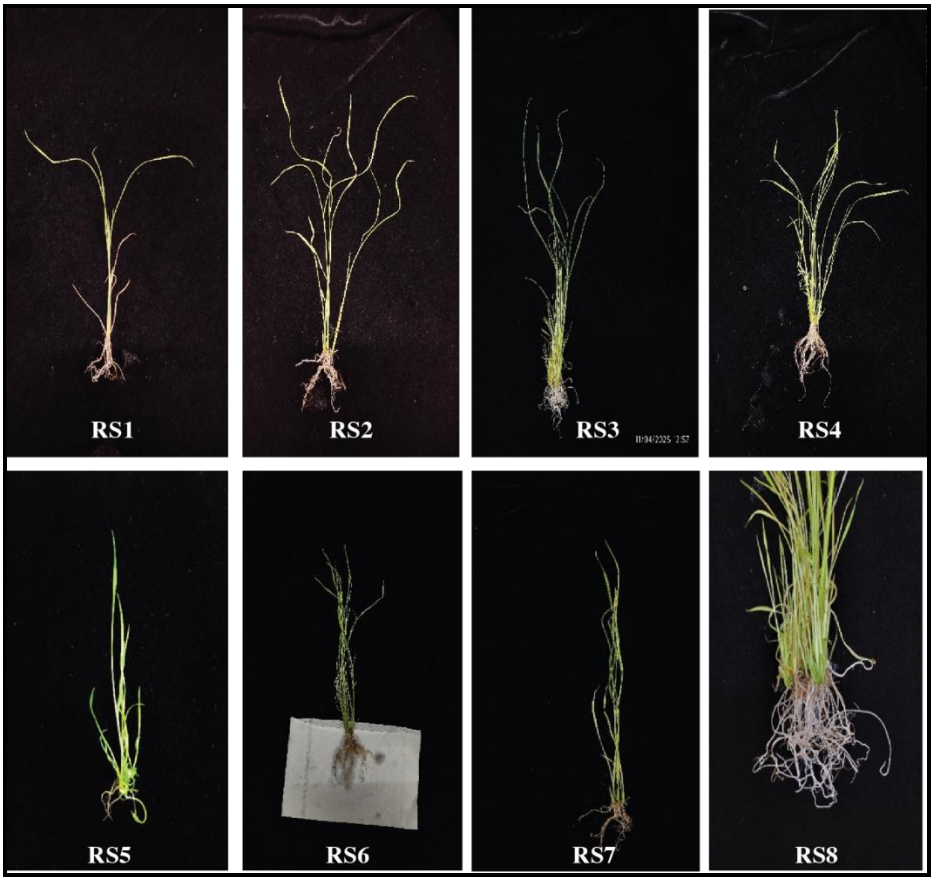


Figure 5: Shoot regeneration of rice Calli infected with Agrobacterium tumefaciens strain LBA4404 on eight regeneration media formulations (SM1–SM8), with the best regeneration response observed on RM8 (70.59 ± 5.88%).

Assessment of Selection Media: In the first batch, only 3 of the infected calli survived all the phases of selection, and because of this, in the second batch the plants were first regenerated and then subjected to selection by hygromycin. Around 735 *Agrobacterium*-infected calli were subjected to regeneration using various media combinations. Around 609 calli were fully regenerated and the plantlets were then subjected to hygromycin selection in the elongation medium starting from 10 mg/L to 35 mg/L (Figures 8 and 9).

Non-transformed control calli showed 100% survival at 10 and 20 mg/L hygromycin and 60–70% survival at 30 mg/L. At 35 mg/L, 20–30% of control calli survived at day 10, but none survived by day 12, and no control calli survived at 40 or 50 mg/L (Table 15). In contrast, infected material continued to grow at 40 and 50 mg/L (Table 16, Figure. 6).

Table 16. Survival of non-transformed control calli and infected material on increasing hygromycin concentrations.

Hygromycin (mg/L)	Control calli survival (%)	Infected material survival (%)
10	100	90
20	100	70
30	60-70	50
35	20-30 (day 10); 0 (day 12)	not recorded
40	0	20
50	0	1–2 plants

Controls were two plates of 10 calli per concentration, scored for survival on day 10 (35 mg/L was also scored at day 12). Infected material survival refers to regenerated plants.

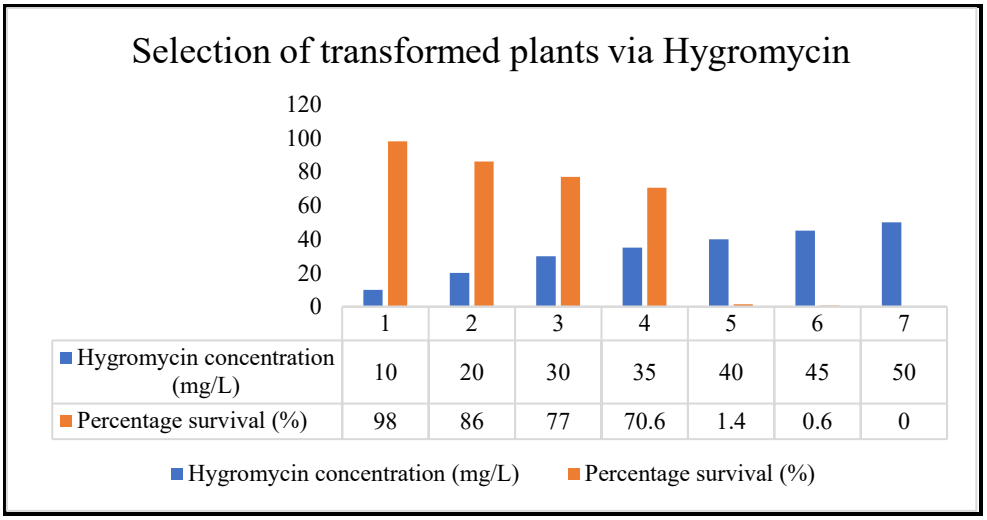


Figure 6: Effect of increasing hygromycin concentrations (10–50 mg L^{-1}) on the survival percentage of transformed rice plants during selection 35mg/L was optimized for selection whereas 50mg/L being the lethal dose.

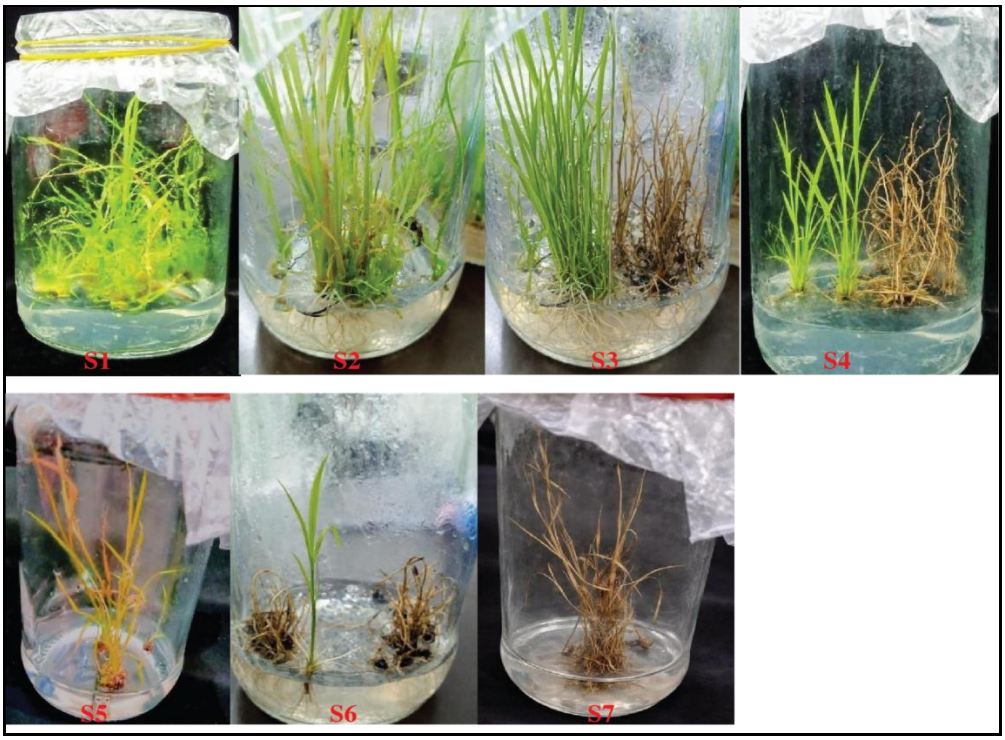


Figure 7: Growth and selection of *Agrobacterium tumefaciens* infected and regenerated rice plants on selection media supplemented with increasing concentrations of hygromycin.

Statistical Analysis of Media Combinations of Root Development: A statistically significant variation in root formation was observed across the tested media formulations ($F_{5,6} = 51.33$, $p < 0.0001$). Formulations RM1 and RM2 failed to induce root initiation (0%), indicating complete suppression of rhizogenesis under these chemical treatments. In contrast, media formulation RM6 demonstrated superior rooting performance (Table 16), achieving (100.00 ± 0.00%) root conversion efficiency, which was significantly higher than all other formulations ($p < 0.05$). RM6 also exhibited the rapid onset of root primordia (7.00 ± 0.00 days), maximum root proliferation (12.00 ± 0.00 roots/shoots), and greatest primary root elongation (17.00 ± 0.00 cm). Formulation RM5 ranked as the second-best treatment (70.00 ± 10.00% efficiency), while RM3 and RM4 showed moderate responses (50.00%).

Table 16: Analysis of variance

Source of Variation	Degrees of Freedom (df)	Sum of Squares (SS)	Mean Square (MS)	F-Value	p-Value
Between Treatments	5	15400.00	3080.00	**51.33**	< 0.0001
Within Treatments (Error)	6	360.00	60.00	—	—
Total	11	15760.00	—	—	—

Table 17: Medium combinations for root generation

Media Formulation	Rooting Frequency (%) ± SE	Mean Roots per Shoot ± SE	Root Length (cm) ± SE	Days to Root Induction ± SE
RM1	0.00 ± 0.00 d	0.00 ± 0.00 c	0.00 ± 0.00 d	N/A d
RM2	0.00 ± 0.00 d	0.00 ± 0.00 c	0.00 ± 0.00 d	N/A d
RM3	50.00 ± 10.00 bc	7.50 ± 0.50 b	10.50 ± 1.50 b	8.50 ± 0.50 a
RM4	50.00 ± 10.00 bc	10.50 ± 0.50 ab	6.50 ± 1.50 c	10.50 ± 0.50 b
RM5	70.00 ± 10.00 b	12.00 ± 1.00 a	14.50 ± 0.50 ab	8.00 ± 0.00 a
RM6	100.00 ± 0.00 a	12.00 ± 0.00 a	17.00 ± 0.00 a	7.00 ± 0.00 a

Values with different superscript letters within a column are significantly different at $p \leq 0.05$ *according to Fisher's LSD test.*

Molecular Screening of Putatively Transformed Plants: From the hygromycin-resistant plants, PCR was performed to screen the transformed plants for the construct. Multiple PCR reactions were carried out on the hygromycin-resistant plants, and the results showed a total of 12/40 positive PCR results, indicating successful transformation rate of 1.632 % of the *Agrobacterium*-treated explants (Figure 10).

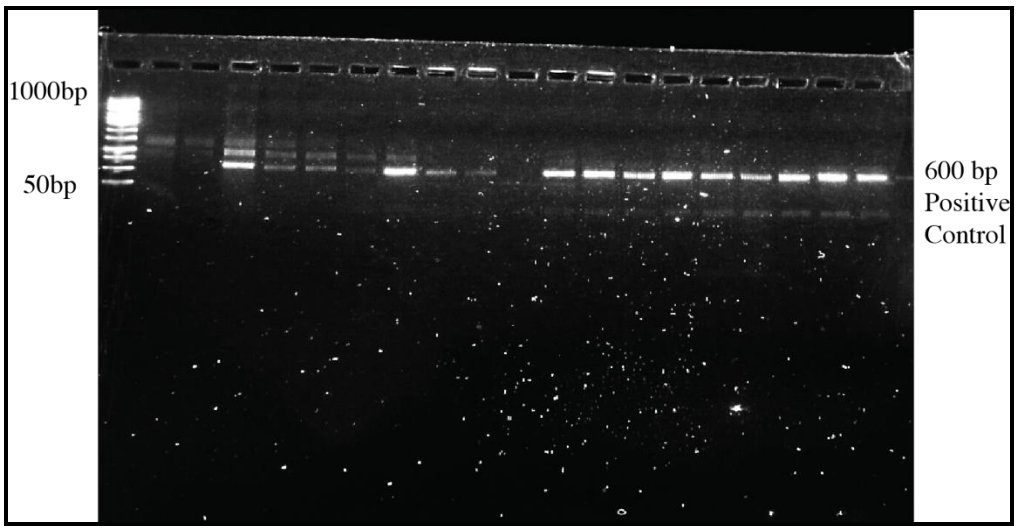


Fig 8: PCR verification of Cas9 gene integration in T_0 rice plants. DNA fragments amplified using Cas9-specific primers and resolved on a 1% agarose gel using a 1 kb ladder (left) and positive control (right) The bands indicate the expected 601bp amplicon confirming the presence of Cas9 transgene.

Overall Pipeline, Efficiency and Transgenic Validation: To evaluate the overall scalability and efficiency of the optimized transformation workflow, 2,500 mature seeds were processed from culture initiation through acclimatization (Table 18). Primary callus induction yielded 1,600 calli, representing an overall induction efficiency of 64%. Of these, 1,345 high-quality calli were inoculated using optimized Agrobacterium parameters (OD = 0.4, 10 min infection, 2 days co-cultivation), yielding 735 surviving calli post-infection (54.6% survival rate). Tissue differentiation produced 609 shoots (70.83% regeneration efficiency), which were subjected to hygromycin selection, yielding 430 resistant plantlets (70.6% selection efficiency). A total of 215 plants were successfully hardened and acclimatized in soil pots (50.0% survival rate). Molecular screening of 40 randomly selected hardened plants via PCR confirmed target gene integration in 12 lines, establishing an overall transformation efficiency of 30.0% among the tested plants. Notably, the maximum callus induction rate (89.17%) was observed for the optimized C6 medium (Table 13) under controlled optimization conditions. In contrast, the overall pipeline induction of 64% reflects the cumulative rate across the large-scale batch of 2,500 seeds, which includes multiple media batches and non-optimal conditions. Therefore, these two values represent different experimental scales and denominators.

Table 18: Summary of the experiment

Total No. of Seeds	Total Callus Produced	Total Callus Treated	Calli Surviving After Infection	Regenerated plants	Hygromycin Resistant Plants	Shifted To pots	PCR positive plants
2500	1600	1345	735	609	430	215	12/40

DISCUSSION

Rice is an annual grass that belongs to the Gramineae family and is largely cultivated in the tropical and subtropical regions of the world (Jamal *et al*., 2009). Due to various climatic conditions in Pakistan, different types of rice are cultivated there, including aromatic long grain, medium grain and round grain varieties (Sagar *et al*., 1988). JP-5 is a short-medium grained variety of rice with grain length of 0.33cm, grain width of 0.69 cm, and grain thickness of 0.20cm (Ilhamuddin *et al*., 1988). The plant height is 125cm and usually takes 110-115 days for maturation (Jamal *et al*., 2009).

Golden Gate assembly was used in our experiments to allow two sgRNA cassettes to be incorporated into the binary vector at the same time. It facilitated the elimination of sequential ligation reactions and the carrying out of multiple cloning procedures. The positive clones, as demonstrated by a PCR product of approximately 900 bp in E. coli, indicate the validity of the method, consistent with earlier work utilizing the same pYL vector system (Ma *et al*., 2015).

The two distinct sgRNAs (directed against two separate sites in the *GS3* coding region) were transcribed by two different promoters, U3 and U6, respectively. Using two sgRNAs raises the probability of disrupting gene function either by mutation at one of the two sites or by deletion of the intervening sequence. Dual sgRNA systems have been reported to yield much larger interstitial deletions than single cut approaches and offer significantly more reliable means of disrupting gene function. The validity of this strategy for *GS3* editing has previously been established by (Usman *et al*., 2021) as they demonstrated editing efficiencies of 73% and 80% for their respective first and second targets using the same vector backbone employed here; therefore, establishing an excellent foundation for the design of the current experiment. The ability of the assembled system to function must be demonstrated by detecting edits at the GS3 target sites.

The binary vector pYLCRISPR/Cas9Pubi- H was selected for its plant- optimized Cas9, the maize ubiquitin promoter driving expression in monocots, and the hygromycin- resistance gene used for selection in rice (Komori *et al*., 2007). Agrobacterium tumefaciens LBA4404, which carries the disarmed helper plasmid pAL4404, is widely used for rice transformation and supports efficient T- DNA delivery (Khanna & Raina, 1999). Colony PCR products of the expected size in Agrobacterium indicate that the strain carried the construct; because the plasmid was not sequenced, correct assembly is supported by PCR evidence alone.

Different sterilizing agents have been used for the surface sterilization of seeds, such as mercuric chloride, hydrogen peroxide, calcium hypochlorite, etc. In our experiment, for surface sterilization of the seeds of the given variety, Clorox and ethanol were used in different combinations, and it was found that 20% Clorox and 75% ethanol were the best combination for sterilization. Similar results have been reported by (Poeaim *et al*., 2016), where 20% Clorox and 70% ethanol have been suggested as the best combination for decontamination of rice seeds. Lower concentrations of Clorox and ethanol have also been used for other rice seeds, as reported by (Oyebanji *et al*., 2009), and even higher concentrations, i.e., 70% Clorox, have also been reported to be optimum for some rice varieties (Naqvi *et al*., 2005). However, at higher concentrations, the ability of the seeds to form callus is reduced; thus, this study suggests 20% Clorox and 75% ethanol to be optimum for seeds sterilization of JP-5 rice. After seed sterilization, callus induction is the first step in most of the research that involves tissue culturing and plant regeneration (Visarada *et al*., 2002). In this study, different concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid) were used for callus induction. Induction of callus in rice using 2,4-D has also been reported by (Sundram *et al*., 2012). In this experiment, 2mg/L concentration of 2,4-D was observed to be the best for callus induction in JP-5. However, other researchers have reported different concentrations of 2,4-D as shows 3mg/L to be optimum, while reported 3.5mg/L concentration to be the best for callus induction in the same variety. Growing the calli under light conditions resulted in a lower contamination rate and effective callus growth as compared to dark conditions in incubators. It has also been reported that light conditions are favorable for callus growth from rice seeds (Chitphet *et al*., 2025). Once the calli were formed, 18- 20-day-old calli were used for *Agrobacterium* infection. (Sahoo *et al*., 2011) also used 18-day-old calli for transformation; some other researchers (Ali *et al*., 2023; Garg *et al*., 2002; Hiei & Komari, 2008) have used several weeks older calli, while others used only 11- 13-day-old calli for transformation (Rengasamy *et al*., 2024).

Using *Agrobacterium* for vector delivery, it was crucial to infect the explants at a certain density of the bacteria because a higher density would end up causing contamination, while a lower concentration of up to 0.2 unit/ml at OD600 would show no transformation efficiency at all (Mayavan *et al*., 2015) reported 0.4-0.6 OD and (Kumar *et al*., 2005) reported 0.5 OD ideal

for transformation. Similar results have been experienced in this study, that bacterial concentration of about 0.4-0.5 showed higher transformation efficiency.

Addition of Acetosyringone to inoculation and co-cultivation media helps to activate the bacterial Vir genes and so, is helpful in the transformation process (Yaqoob *et al*., 2017). In the present study, 100µM Acetosyringone was found to be effective in the transformation of the calli. These results are compatible with the findings of (Balasubramanian *et al*., 2018; Karthikeyan *et al*., 2012; Yaqoob *et al*., 2017). Although some researchers have reported that the presence of Acetosyringone is not necessary for transformation and transformant rice plants can be achieved even in the absence of Acetosyringone (Rao & Rao, 2007). In addition to other factors, the time and temperature of co-cultivation also affect the transformation efficiency because, if the co-cultivation time increases, overgrowth of bacterial colonies occurs, while at shorter times, there are lower chances of transformation. In this research, co-cultivation at 25°C for 2 days was more effective compared to 28°C for 3 days and the results are like those reported by (Yaqoob *et al*., 2017).

A pre-selection media was used for the infected calli before hygromycin selection. A similar pre-selection step has been used by (Sutradhar *et al*., 2025). For selection, among the various concentrations of hygromycin, we compared non-transformed control calli with infected material across 10–50 mg/L hygromycin. Control calli survived at 10–30 mg/L and were gradually killed at 35 mg/L, with zero percent survival rate at 40 and 50 mg/L, whereas some infected calli kept growing at these higher concentrations. We therefore used 35 mg/L, the lowest concentration at which controls were completely inhibited by day 12. Even so, resistance to hygromycin alone does not prove transformation, and only 12 of 40 resistant plants tested were PCR-positive for Cas9, so some of the resistant plants may be escapes. Similar trends in selection have been reported by where they used 30mg/L hygromycin concentration for the selection of IR64 calli (Rengasamy *et al*., 2024). (Wang *et al*., 2010) also used 30mg/l hygromycin for selection of IR64 and IR67 transformants in their research. Some researchers, i.e., (Mukhtar & Hasnain, 2018; Ramesh & Gupta, 2006), even selected their transformed rice plants on 50mg/L hygromycin. The surviving calli were subjected to regeneration using different concentrations of growth hormones. Among the combinations of BAP and NAA, a 2:1 ratio along with 0.5mg/L kinetin was best for the regeneration of JP-5 plants, and these results are comparable to those of (Zahid Hussain *et al*., 2010). (Ali *et al*., 2023) also reported the same BAP to NAA ratio, i.e., 2:1 and 0.5mg/L kinetin for the regeneration of IRRI-6 plants. Different combinations of growth hormones are used for different rice varieties as in the same research, 3mg/L BAP and 1mg/L NAA have also been reported for the regeneration of KSK-282 and IRRI-9 (Izhar Muhammad *et al*., 2014). In another study, (Tarmizi *et al*., 2023) reported that the optimum combination of the two hormones for rice plant regeneration is 1mg/l BAP and 0.1mg/L NAA, and (Sah *et al*., 2014) used 3mg/L BAP and 0.2mg/L NAA. It means that hormonal combinations for the regeneration of rice plants depend on the variety of the rice used and for JP-5, the above-mentioned concentrations are optimum for regeneration of a complete plant.

Regarding callus induction, the maximum rate of 89.17% was achieved on the optimized C6 medium under controlled conditions. However, the cumulative induction efficiency for the entire large-scale pipeline was 64% (1,600 calli obtained from 2,500 seeds). This reduction reflects the inclusion of non-optimal media formulations (such as C1 and C2) and batch-to-batch variations during the scale-up process.

In the present study, 430 of the 735 calli that survived infection was hygromycin-resistant (58.5%), giving a selection-based efficiency of 32.0% relative to the 1,345 infected calli. PCR screening detected the Cas9 transgene in 12 of 40 plants tested (30%), corresponding to an estimated PCR-based transformation efficiency of about 9.6%, with a minimum of 1.6% based on confirmed plants alone. The selection-based value is comparable to the 35% and 41% hygromycin-resistant calli reported for the indica cultivars HKR126 and Pusa Basmati 1 (Kant *et al*., 2007) and to the 20–50.9% reported for IR 64 (Nguyen *et al*., 2017), although the latter used seeds as the denominator. The PCR-based estimate is similar to the PCR-confirmed efficiencies of 12.5% and 22.0% in HKR126 and Pusa Basmati 1 and the 22% reported for Basmati 370 (Rashid *et al*., 1996), and higher than the 3.6% reported for the indica restorer line JK1044R and the 0.9–5.2% reported for four indica genotypes (Nishimura *et al*., 2006). For japonica rice, higher efficiencies of 30–50% have been reported for high-regeneration varieties such as Nipponbare and Taichung 65. These differences reflect variation in genotype, selection system, and how efficiency is defined (Nishimura *et al*., 2006). Since only a subset of plants was screened by PCR and editing at the GS3 target sites was not examined, the present values indicate transgene recovery and not genome editing.

CONCLUSION

This study established an optimized protocol for callus induction, *Agrobacterium*-mediated transformation, and plant regeneration, with PCR-based confirmation of the presence of the Cas9 construct, providing a foundation for subsequent development and validation of gene-edited plants. The protocol provides a reliable and reproducible framework which can be used for genome editing and plant regeneration in rice that can accelerate functional genomics studies and facilitate the development of improved rice varieties with desirable agronomic traits. This optimized protocol can also be used as a reference

for applying genome editing technologies in other crop species with similar tissue culture and transformation requirements. A limitation of this study is that the final CRISPR/Cas9 construct was verified by colony PCR only and was not confirmed by sequencing.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTION

K.K., A.J., S.I., and S.A. contributed to the conceptualization, methodology, and investigation. A.V.K. and S.J. contributed to data collection and analysis. A.A. and N.R. assisted with laboratory investigations and data interpretation. M.R.K. and M.U. supervised the study, contributed to project administration, and critically revised the manuscript. All authors reviewed and approved the final version of the manuscript

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