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CRISPR/Cas9-Mediated Targeting of SD1 Gene in Indonesian Local Varieties: Transformation and Preliminary Phenotypic Assessment of Semi-Dwarf Traits

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ABSTRACT

Indonesia boasts a wealth of local rice germplasm, with approximately 8,500 varieties recorded by IRRI, many of which feature adaptive traits and resistance to biotic and abiotic stresses. Nevertheless, it is likely limited by excessive plant height, which makes it sensitive to lodging. In contrast, the improved yields of modern semi-dwarf rice are twice those of older varieties, but are less resilient to local Indonesian stresses. This study focused on introducing semi-dwarf characteristics into two local varieties, Ketan Hitam and Pandan Wangi, via CRISPR/Cas9-mediated gene editing, which enables specific mutations and encodes the GA20-oxidase enzyme involved in gibberellin biosynthesis. A CRISPR/Cas9 construct (pRGEB32::SD1) carrying two sgRNAs targeting exon 1 and exon 2 was successfully designed and validated. Following *Agrobacterium tumefaciens*-mediated transformation and hygromycin selection. Ketan Hitam exhibited good survival, whereas Pandan Wangi depicted browning and poor regeneration. One regenerated Ketan Hitam T0 plantlet presented a preliminary semi-dwarf phenotype. PCR analysis verified the presence of the CRISPR/Cas9 construct. This study provides an initial demonstration of transformation probability and early phenotypic response in Ketan Hitam. Nevertheless, the phenotypic assay is based on a single T0 specimen, describing a key limitation of this study. The findings provide a basis for further multi-generational evaluation and agronomic improvement of Indonesian local rice.

1. Introduction

Indonesia has abundant rice germplasm, a crucial genetic resource in plant breeding programs. This genetic potential should be harnessed to produce superior varieties that are adaptive and highly competitive. More than 8,500 local and wild Indonesian rice varieties are recorded at the International Rice Research Institute (IRRI) in Los Baños, Philippines (IRRI 2020). Local rice seeds are characterized by their ability to withstand biotic and abiotic stress, owing to their adaptation to the diversity of plant pests (OPT) and the tropical environment of Indonesia (Dwiningsih 2023). Hidayatun *et al.* (2024) demonstrated that 1,540 local rice varieties were resistant to abiotic stress from 2016 to 2021. However, local rice has several weaknesses, including its unusually tall height, which ranges from 120 to 200 cm, and a low number of tillers. A study of more than 100 local rice genotypes in Sumatra reported an average rice plant height of 124 cm (Mulsanti *et al.* 2021), along with an average of only 14.7 panicles per plant (Rahayu *et al.* 2020). This height makes local rice vulnerable to lodging, especially in areas with high rainfall.

On the other hand, modern rice varieties with semi-dwarf characteristics were developed during the Green

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Revolution, focusing on allocating more energy to seed formation, thereby doubling crop yields (Nagai *et al.* 2018). This improvement is highly attributed to a dwarfing allele originating from the 'Dee-geo-woo' gene, which underwent mutation in the SD1 gene that encodes the GA20 oxidase (GA20ox-2), an enzyme that catalyzes late steps of gibberellin biosynthesis (Rana *et al.* 2021). However, modern rice varieties introduced abroad are not well-adapted to tropical environments, making them more susceptible to pest and disease attacks; therefore, their use often requires intensive pesticide applications that negatively impact the agroecosystem (Mercer *et al.* 2012).

Therefore, it is necessary to develop local semi-dwarf rice varieties using a precision breeding approach with genome editing through the CRISPR/Cas9 system, combining the biotic and abiotic resistance of local rice with the superior morphology of modern rice. Consequently, plant height declined due to mutations in the SD1 gene, whereas harvest index and stem strength increased (Asano *et al.* 2007). To obtain these traits, CRISPR/Cas9 has been chosen because it is the most efficient among gene editing techniques, such as ZFN (Zinc-Finger Nucleases) and TALEs (Transcription Activator-like Effectors). Technically, the CRISPR/Cas9 system was inspired by bacterial defense mechanisms against viruses and is the most commonly used system for changing plant DNA sequences (Jinek *et al.* 2012). Based on this technique, the study aimed to construct genome-editing-based mutagenic targets using CRISPR and to conduct a preliminary phenotypic assessment of semi-dwarf rice plants in Indonesia. Notably, previous studies have not reported the SD1 gene mutation using the CRISPR/Cas9 method in Indonesian rice varieties, such as Ketan Hitam and Pandan Wangi. Thus, the results of this study are expected to pool constructs and information on Indonesian rice responses, thereby informing the development of research targeting SD1 to produce adaptive and superior Indonesian semi-dwarf (SD) rice varieties.

2. Materials and Methods

This experimental study was conducted at the Agrobiotechnology Laboratory at the University of Jember. The primary materials required for this study include local rice seeds, specifically Ketan Hitam and Pandan Wangi, obtained from the Balai Besar Penelitian Tanaman Padi (Indonesian Rice Research Institute). This study consisted of five main phases: (1) design and selection of sgRNA targeting the SD1 gene, (2) construction of SD1 gene mutation-targeted sgRNA into pRGEB32, (3) cloning of SD1 in *E.coli* and sequencing analysis, (4) transformation of pRGEB32::SD1 into *Agrobacterium*, and followed by transformation of pRGEB32::SD1 into rice callus via *Agrobacterium*.

2.1. Design and Construction of sgRNA

The sgRNA design process involves determining the conserved domain. Targeting conserved domain areas increases the functional knockout rate, as mutations depend on their location within the protein, and these regions are more likely to disrupt protein structure (Veeneman *et al.* 2020). The sgRNA target domain is generally selected based on 20 nucleotides (nt) adjacent to three nucleotides as the Protospacer Adjacent Motif (PAM). The sgRNA oligonucleotides designed using the CRISPR-based tool CHOPCHOP version 3 (Labun *et al.* 2019) provide information on PAM, sgRNA efficiency, GC content, genomic location, number of mismatches, and location within the coding sequence (CDS). sgRNA targets were selected using the following 5'-NGG-3' rule with GC content between 50-66% to maintain the stability and efficiency, avoiding self-complementary and mismatches. The selected sgRNA is then synthesized, and complementary adapters from pRGEB32 are added to the designed sgRNA oligos. The forward and reverse synthesis results will be treated at 95°C for 5 minutes, then at 25°C for 15 minutes, and finally cooled to 4°C for 10 minutes to produce sgRNA duplexes. This sgRNA will then be used for insertion into the pRGEB32 plasmid.

2.2. Construction of SD1 Gene Mutation Targeted sgRNA into pRGEB32

The purified pRGEB32 plasmid was restricted using the BsaI enzyme for vector linearization. The BsaI enzyme will cleave the sequence 5'-GGTCTC-3' and produce compatible overhangs 5'-GTTT-3' and 3'-CCGT-5' for sgRNA insertion. The digestion reaction containing BsaI, pRGEB32, Buffer G (10×), and ddH_2O was incubated overnight at 37°C, and the product was visualized using 0.8% agarose gel electrophoresis. The gel electrophoresis was then extracted and purified using the Gel Extraction Miniprep Kit according to the manufacturer's protocol, yielding linearized pRGEB32. The concentration and purity of linearized pRGEB32 were determined using a Nanodrop.

Construction of sgRNA into pRGEB32 was carried out by ligation reaction by mixing T4 DNA ligase, linear pRGEB32 plasmid, sgRNA duplex (SD1), ligation

buffer (10×), and nuclease-free water. The sample was incubated at 4°C overnight and was ready for transformation into *E. coli* JM109.

2.3. Cloning of SD1 in *E. coli* and Sequencing Analysis

The ligation construct pRGEB32::SD1 was then transformed into *E. coli* JM109 cells using the heat shock method. Competent cells were prepared with $CaCl_2$ to enhance plasmid uptake. This transformation process was carried out by mixing the pRGEB32::SD1 construct with competent JM109 cells and shocking them at 42°C for 90 seconds using a water bath. The cells were then cooled on ice for 15 minutes, followed by the addition of LB broth to recover cells. The cells were subsequently shaken in an incubator at 37°C for 60 minutes. The transformed cells were centrifuged, resulting in a pellet, and the supernatant was removed, leaving approximately 100 µL of cell suspension. Then, a sample of 100 µL was spread on Luria Bertani (LB) media containing 50 mg/L kanamycin and incubated at 37°C overnight. Next, positive *E. coli* were multiplied, plasmids were isolated, and the constructs were sequenced to confirm the successful insertion of the pRGEB32::SD1 construct prior to *Agrobacterium* transformation. The resulting plasmids were sequenced by Sanger sequencing. Plasmids with verified sgRNA insertion were subsequently used for *Agrobacterium*-mediated transformation.

2.4. Transformation of pRGEB32-SD1 into *Agrobacterium*

Plasmid transformation into *Agrobacterium* was performed by electroporation, a technique that uses short-circuit electrical pulses to transiently permeabilize the *Agrobacterium* cell membrane (Fiedler and Wirth 1988). Transformed *Agrobacterium* are grown in media containing 50 ppm of kanamycin and 50 ppm of rifampicin. The use of antibiotics is intended as a selection marker for *Agrobacterium. Agrobacterium* naturally does not carry a kanamycin resistance gene, whereas *Agrobacterium* transformed with plasmids will carry a kanamycin resistance gene. Unlike *E. coli*, the *Agrobacterium* strain LBA4404 is rifampicin-resistant, so it is used to prevent the growth of other bacteria (Tzafira and Citovsky 2006).

Agrobacterium samples were then collected for further confirmation via molecular analysis to ensure the presence of one of the plasmid genes. *Agrobacterium* was inoculated into Luria-Bertani suspension medium containing 50 ppm rifampicin and 50 ppm kanamycin antibiotics to support the transformation of rice callus. The color change in the *Agrobacterium* suspension media from bright yellow to cloudy orange indicates bacterial growth, in contrast to the control suspension, which remained bright yellow, indicating non-transformed *Agrobacterium*.

2.5. Transformation of pRGEB32::SD1 into Rice Cells via *Agrobacterium*

This transformation stage was carried out using *in vitro* callus cell culture on local Ketan Hitam and Pandan Wangi, which were infected with *Agrobacterium* LBA4404 (pRGEB32::SD1). The plants were then selected on media containing hygromycin until putative transformant plants were obtained, and subsequently morphophysiologically characterized.

2.6. Data Analysis

The research data were analyzed using quantitative statistics to determine the percentage of callus response and descriptive qualitative statistics through visual data presentation, including images from the creation of the construct design to the transformation in rice, to obtain information regarding plant transformation efficiency.

3. Results

3.1. sgRNA Sequence Validation

The SD1 coding gene sequence (ID: JN541539.1) was obtained from the National Library of Medicine (NCBI). The FASTA results from NCBI were entered on the Rice Genome Annotation Project (https://rice.uga.edu/) for BLAST, thus obtaining locus information and the position of UTR, Exon, Intron, and CDS of the target locus. The BLAST results indicate that the SD1 gene (OsGA20ox2) is located on chromosome 1, with the locus ID LOC_Os01g66100. The length of the locus is 3,123 bp (comprising exon, intron, and UTR), with a coding sequence length of 1,170 bp. It is also shown that LOC_Os01g66100 has three exons and two introns (Figure 1).

Exon 1 of the SD1 gene was selected as the primary target for sgRNA design. Exon 1 of the SD1 gene encodes critical functional domains for the GA20ox-2 enzyme. Mutations within this exon, such as the G94V (SD1-1 in Jikkoku) and the 2-bp deletion (SD1-3 in Ai-Jiao-Nan-Te), have been shown to effectively disrupt gibberellin biosynthesis, leading to reduced plant height without severe detrimental effects on yield. As shown in Figure 2, the cutting position of sgRNA I is

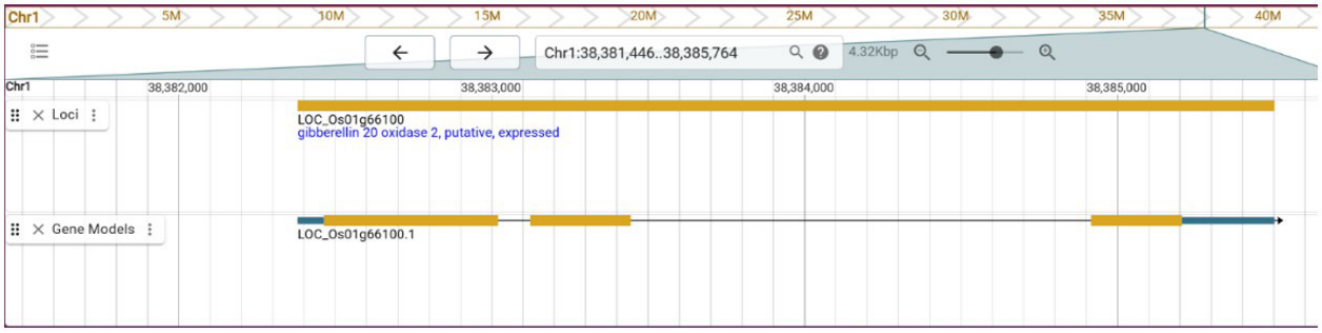


Figure 1. Gene model of SD1 with locus information

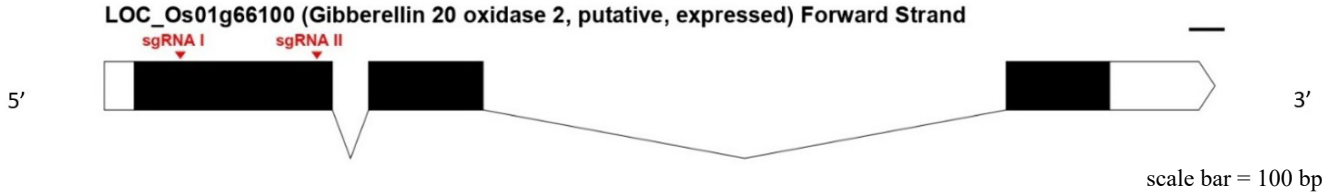


Figure 2. SD1 gene structure with sgRNA I and sgRNA II target sites

located at the sequence of 214 to 237 nucleotide bases, while sgRNA II has a cutting position at 598 to 620 nucleotide bases.

The best sgRNA sequence was selected based on the effectiveness and GC content of all sgRNAs. Maintaining 40-80% GC content in sgRNAs is critical. Low GC reduces target-binding affinity, whereas high GC increases off-target potential through non-specific interactions. The efficiency score in CHOPCHOP is crucial because a substantial portion of sgRNAs have low or zero cutting rates, and this score predicts the likelihood of successful DNA cleavage. sgRNA candidates were designed using CHOPCHOP with default parameters, without applying additional custom thresholds beyond the tool's built-in filtering. Table 1 summarizes the selection of the top two sgRNAs: sgRNA I, with 60% GC content and 61.16% efficiency, and sgRNA II, with 55% GC content and 54.11% efficiency. Cutting Frequency Determination scoring is also essential for evaluating sgRNA specificity, which helps minimize potential off-target effects. Based on Table 1, sgRNA I and sgRNA II were selected for further experimentation as they demonstrated the highest CFD scores 99% and 99%).

3.2. Plasmid Construction Design

The pRGEB32 plasmid design contained essential elements, including a promoter, sgRNA scaffold, and the Cas9 gene. (Figure 3).

The assembly was performed using the Type IIS restriction enzyme BsaI, which recognizes the 6-base pair sequence 5′-GGTCTC-3′ and catalyzes asymmetric cleavage to generate 4-base 5′ overhangs. The design results indicate a specific location for inserting the sgRNA within plasmids (Figure 2).

3.3. Positive *E. coli* Transformation Colonies

It was demonstrated that the pRGEB32 plasmid successfully integrated into *E. coli* bacteria. This was depicted by the growth of transformant *E. coli* colonies on antibiotic selection media (Figure 4B & C). There was an inconsistency in the control treatment, as indicated by the absence of colony growth of non-transformed *E. coli* in the antibiotic selection media.

The calculation was based on the number of colonies of non-transformant *E. coli* and pRGEB32::SD1 transformant *E. coli* grown on 50 ppm kanamycin antibiotic selection media. Transformation was accomplished using 50 µL of chemically competent *E. coli* cells with 1 µg of plasmid. The calculation results showed no growth of non-transformant *E. coli*, while the pRGEB32::SD1.sgRNA I transformant *E. coli* showed growth of 1229 colonies and the pRGEB32:SD1. sgRNA II transformant *E. coli* showed growth of 792 colonies. Three colonies per sgRNA construct were randomly selected for colony PCR screening. Colonies exhibiting the expected band size were subsequently

Table 1. Recommendation sgRNA target sequence for SD1 gene editing

Rank	Target sequence	GL	St	GC (%)	SC	CFD (%)	Eff.
20	GAGCCATTCGTGTGGCCGAACGG	214	+	60	3	99	61.16
204	GACTACTTCTCCAGCACCCTCGG	598	+	55	1	99	54.11

GL: genomic location, St: self complementarity, Eff: efficiency, CFD: cutting frequency determination score

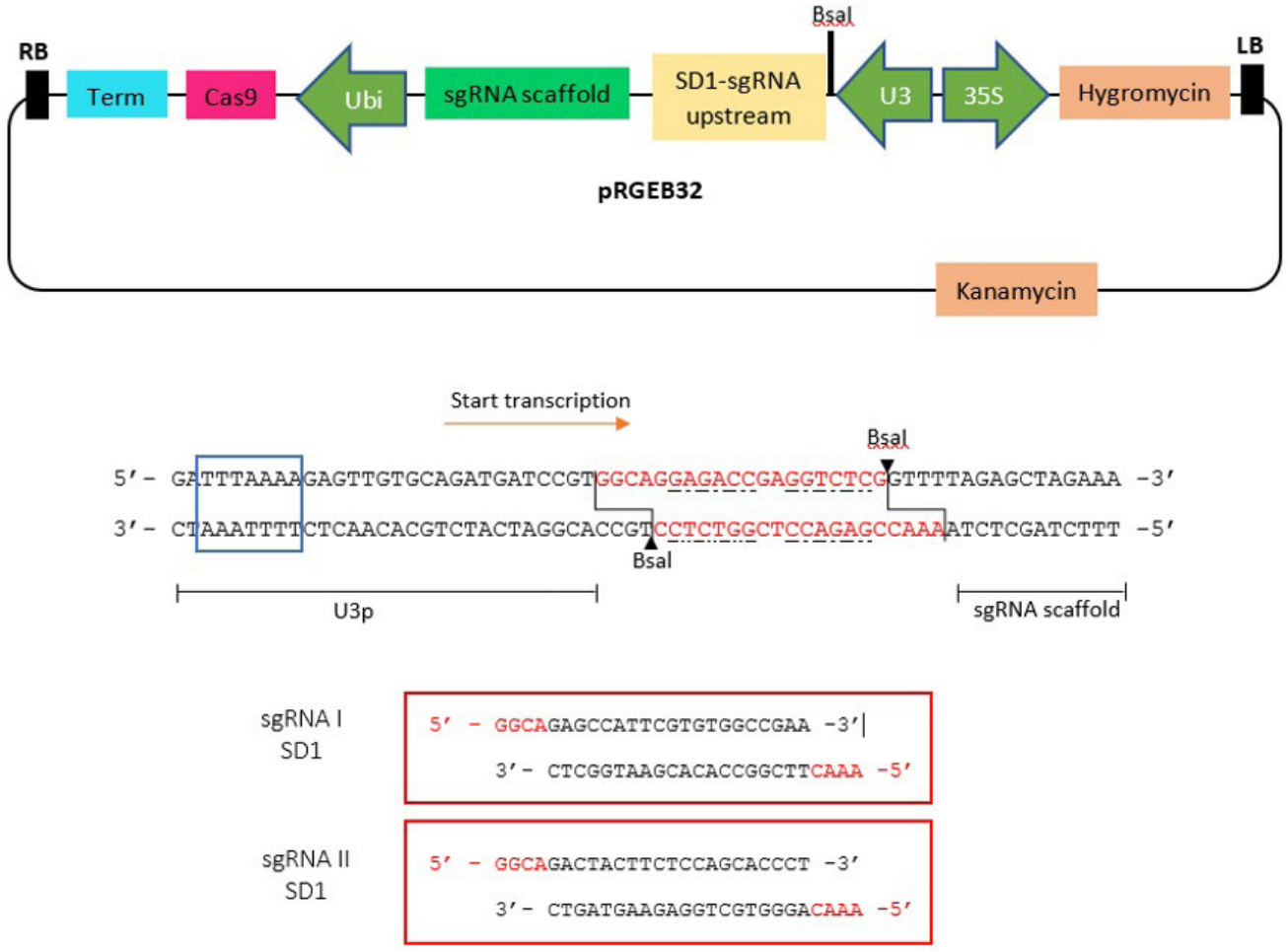


Figure 3. Construction design of plasmid pRGEB32 with sgRNA targeting the SD1 gene

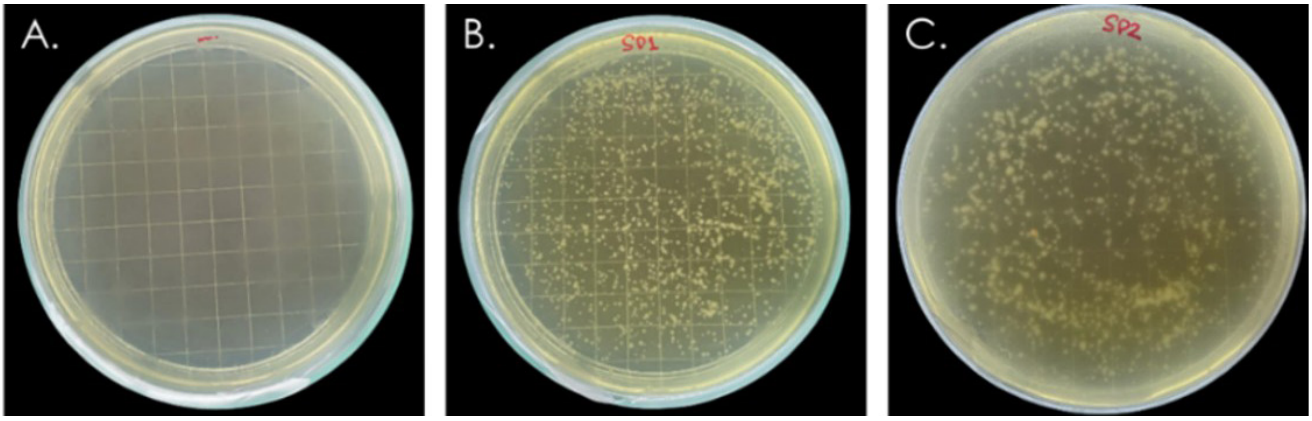


Figure 4. Confirmation of sgRNA integrated with the plasmid inserted into *E. coli* bacteria through LB media agar containing 50 mg L^{-1} Kanamycin. (A) Non-transformant *E. coli*, (B) transformant *E. coli* grown on selection media

subjected to plasmid extraction and Sanger sequencing for verification.

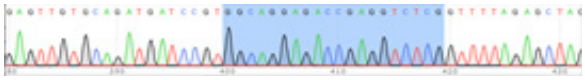
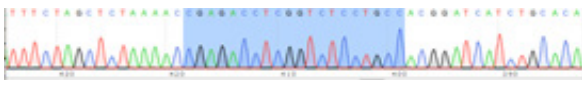
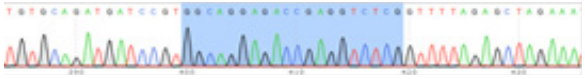
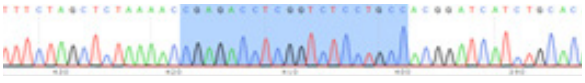
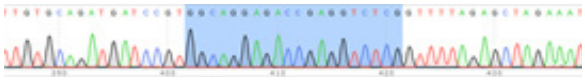
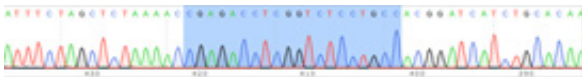
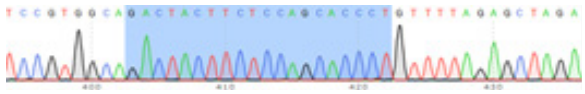
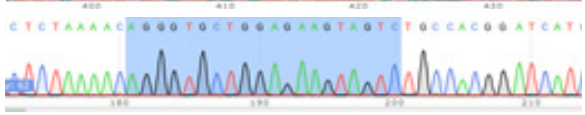
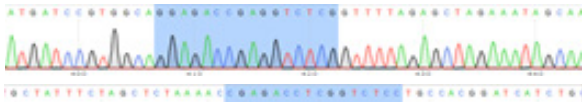
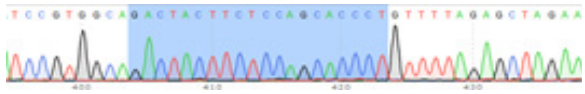
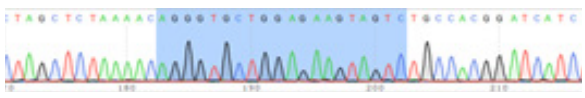
3.4. Confirmation of sgRNA-integrated Plasmid via Sanger Sequencing

According to Table 2, Sanger sequencing showed positive results for the sgRNA II gene SD1. Plasmids from colony 1 and colony 3 showed similarity in nucleotide base sequence at the insert site with the designed SD1-sgRNA II sequence, so it can be ascertained that SD1-targeted sgRNA II has modified plasmids in colonies 1 and 3. In contrast to the second colony from sgRNA II and all colonies from sgRNA I, which did not show the nucleotide base sequence of sgRNA II-SD1 and sgRNA I-SD1 at the insert site, it can be said that plasmids from these colonies were not modified by SD1-targeted sgRNA. This confirmed that sgRNA II has been successfully inserted into the prGEB32 plasmid.

3.5. Positive Colonies and Confirmation of A.tumefaciens integrated pRGEB32::SD1

Agrobacterium tumefaciens was transformed to insert plasmid pRGEB32-SD1 as T-DNA into *A.*

Table 2. Confirmation of plasmid pRGEB32::SD1 integrated sgRNA strand sequence via sanger sequencing

Clone ID	sgRNA targeted	Strand	Chromatogram	Mutation type	sgRNA-integrated plasmid
WT pRGEB32	-	F		-	-
Colony 1	SD1 sgRNA I	F		-	-
		R		-	-
Colony 2	SD1 sgRNA I	F		-	-
		R		-	-
Colony 3	SD1 sgRNA I	F		-	-
		R		-	-
Colony 1	SD1 sgRNA II	F		-	-
		R		-	-
Colony 2	SD1 sgRNA II	F		-	-
		R		-	-
Colony 3	SD1 sgRNA II	F		-	-
		R		-	-

+: confirmed presence, -: confirmed absence

tumefaciens. This transformation process is carried out using electroporation *A. tumefaciens*. Electroporation results can be inoculated into Luria-Bertani media supplemented with rifampicin and kanamycin. Bacteria grow on antibiotic-rich LB media (Figure 5).

3.6. Confirmation of *A. tumefaciens* integrated pRGEB32::SD1

Molecular analysis was performed to confirm the successful transformation of the pRGEB32::SD1 plasmid into *A. tumefaciens* (Figure 6). Five *A. tumefaciens* SD1 colony samples were used for molecular analysis. This molecular analysis used HPTII (Hygromycine-resistant) primers with forward sequence 5'-TCGGACGATTGCGTCGCATC-3' and reverse sequence 5'-AGGCTATGGATGCGATCGCTG-3', amplifying a size of 545 bp. The presence of the targeting plasmid in selected transformants was visualized using a UV transilluminator.

The molecular analysis revealed that all samples amplified a 545-bp product (Figure 6), indicating that the pRGEB32::SD1 plasmid was successfully transformed into 5 *A. tumefaciens* samples.

3.7. Rice Callus Transformation

Transformation into rice plants begins with the callus induction stage. Observations of explant response include callus morphology, texture, color, diameter, fresh weight, and percentage of callus induction (Table 3).

Callus induction was performed using 1,500 seeds of Ketan Hitam and Pandan Wangi. Good callus characteristics for the transformation process include compactness and yellowish-white or yellow coloration,

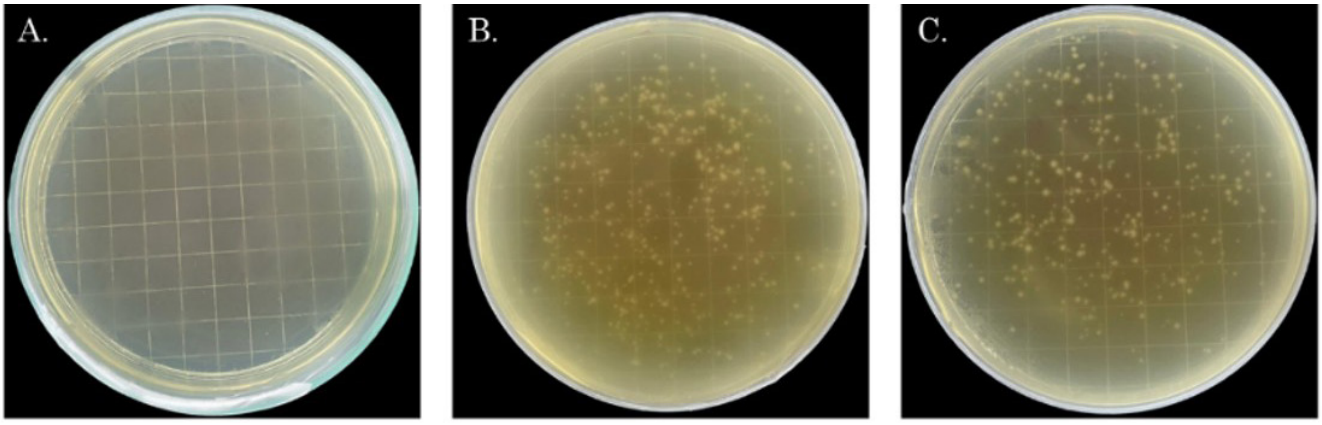


Figure 5. Confirmation of sgRNA integrated with the plasmid inserted into *Agrobacterium tumefaciens* through media LB containing 50 mg L^{-1} Rifampicin and 50 mg L^{-1} Kanamycin. (A) Non-transformant *A. tumefaciens*, (B and C) transformant *A. tumefaciens*

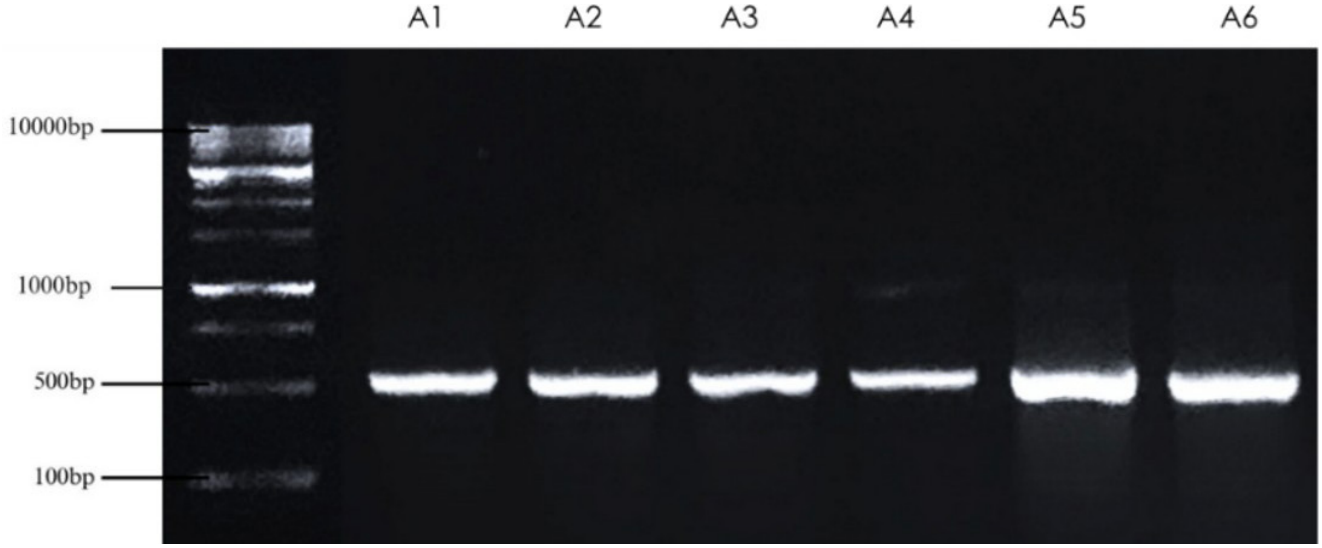


Figure 6. Visualization of molecular analysis results of *A. tumefaciens* strain LBA4404 pRGEB32::SD1 sgRNA II colonies using HPTII primers

Table 3. Callus induction response and morphology of rice explant after 2 weeks on N^ 2,4-D medium

Variety	Color	Texture	Diameter (mm)	Wet weight (g)	Callus percentage (%)
Ketan hitam	White yellowish	Compact	6.3991	0.0359	73.1
Pandan wangi	White yellowish	Compact	7.5549	0.02935	83.26

GL: genomic location, St: self complementarity, Eff: efficiency, CFD: cutting frequency determination score

as demonstrated by Ketan Hitam (Figure 7A) and Pandan Wangi (Figure 7B). The Pandan Wangi rice variety showed a high percentage of callus growth, 83.26%, as shown in Table 3.

3.8. Post-Transformation Response of Rice Callus

The browning response was observed in the callus of both varieties, characterized by a change in color to brownish black (Figure 8A and D). The highest browning percentage was shown in the callus of Pandan Wangi variety, at 63.23% (Table 4). Another problem at this stage was overgrowth of the callus. The highest overgrowth percentage was shown in the callus of the Ketan Hitam variety, at 13.30%.

The browning response indicates that the calli of both varieties (Figures 9A and E) did not withstand stress after transformation. As shown in Table 5, the Pandan Wangi rice variety had the highest percentage of browning at the regeneration stage, at 67.58%. Cytokine staining using acetocarmine and Evans blue displayed the presence of embryogenic cells and the level of cell death in Ketan Hitam (Figure 9B) and Pandan Wangi (Figure 9F) calli.

The appearance of greenspots is shown in the callus of the Ketan Hitam variety (Figure 9C) and Pandan Wangi (Figure 9G). The selection results showed that the Ketan Hitam plantlets could survive the selection stage (Figure 9D), while the callus from the Pandan Wangi rice variety experienced browning on the entire callus surface (Figure 9H).

3.9. Early Observation of SD-1 Putative Transformant via Molecular Analysis

One regenerated planlet was selected on MS medium containing 50 ppm hygromycin for 14 days. The putative transformant plantlets grew normally on the selection media because pRGEB32::SD1 had successfully integrated into the plant genome, conferring hygromycin resistance. Planlets that successfully passed the selection period were acclimatized for 18 days before being transferred to the greenhouse. Genomic DNA was extracted from leaves and subjected to PCR analysis. The control sample showed no amplification at the target position (545 bp), suggesting the absence of transgene integration (Figure 10). In contrast, lane 2, a putative transformant, showed a 500-bp band, indicating T-DNA integration.

3.9.1. Preliminary Phenotypic Assessment of Putative SD1-Edited Lines

Phenotypic assessment of putative SD-1-edited and wild-type Ketan Hitam was conducted at 12 weeks post-transplanting under controlled greenhouse conditions. Visual assessment revealed a difference in the semi-dwarf phenotype between transgenic lines and wild-type controls, which underwent an identical regeneration phase but without *Agrobacterium* infection or transformation (Figure 11). Morphological analysis revealed that the transformed SD1 edited plants exhibited a plant height of approximately 81 cm, representing a 29.56% reduction compared to the wild type, which reached 115 cm under identical growing conditions. Despite the significant height reduction, the putative edited lines exhibited enhanced vegetative characteristics, including increased tiller capacity, typical leaf morphology, and natural green coloration. However, molecular characterization through sequencing is required to confirm the specific mutations introduced by CRISPR/Cas9 editing.

4. Discussion

4.1. sgRNA Design and Efficiency

The design of the pRGEB32::SD1 plasmid construct was achieved by targeting an sgRNA to the correct target DNA sequence (Zhang *et al.* 2023). The sgRNA construction involves selecting the appropriate cutting site for the SD1 gene (OsGA20ox2). The cutting site at the SD1 locus can be selected between exon 1 and exon 2 to yield a rice plant phenotype with shorter stems and improved lodging resistance (Tomita and Ishii 2018). The amount of Guanine and Cytosine in sgRNA plays an important role in the CRISPR/Cas9 system. The optimal GC content for sgRNA is 50-60% (Fu *et al.* 2013; Gagnon *et al.* 2014). Efficiency assessment by CHOPCHOP can be based on GC content, specificity, and potential for self-complementarity (Labun *et*

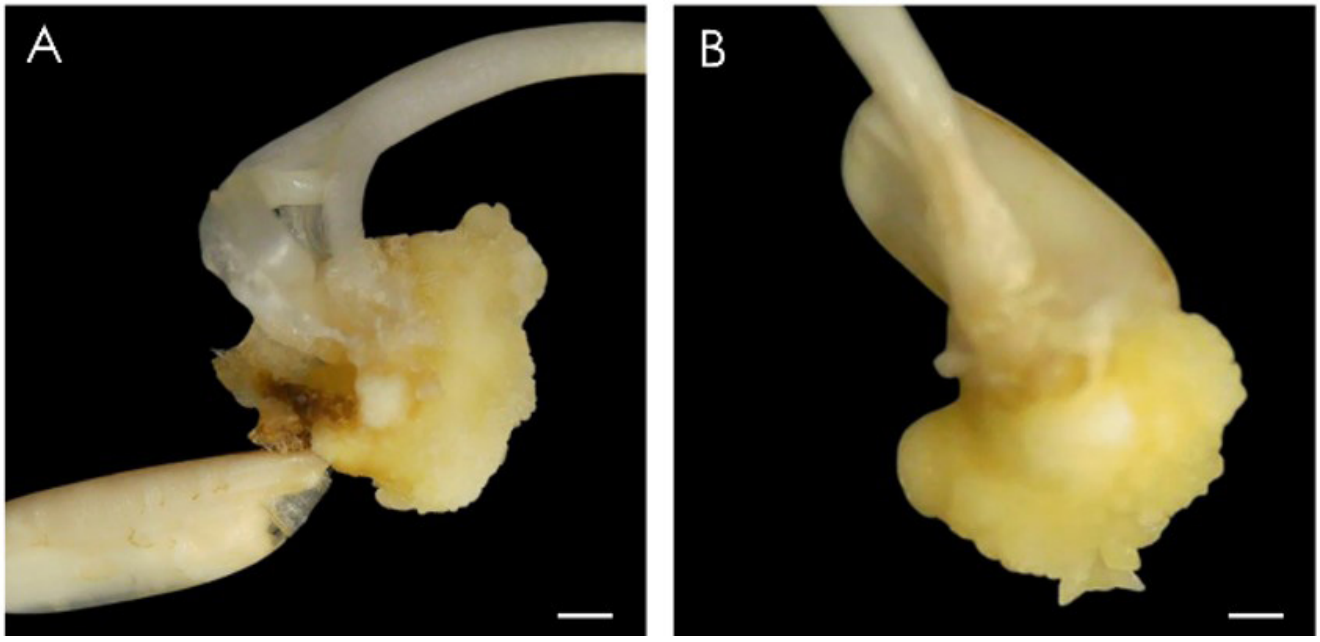


Figure 7. Morphology of callus of rice varieties (A) Ketan Hitam and (B) Pandan Wangi on MS 2,4-D media with a concentration of 2 mg/L

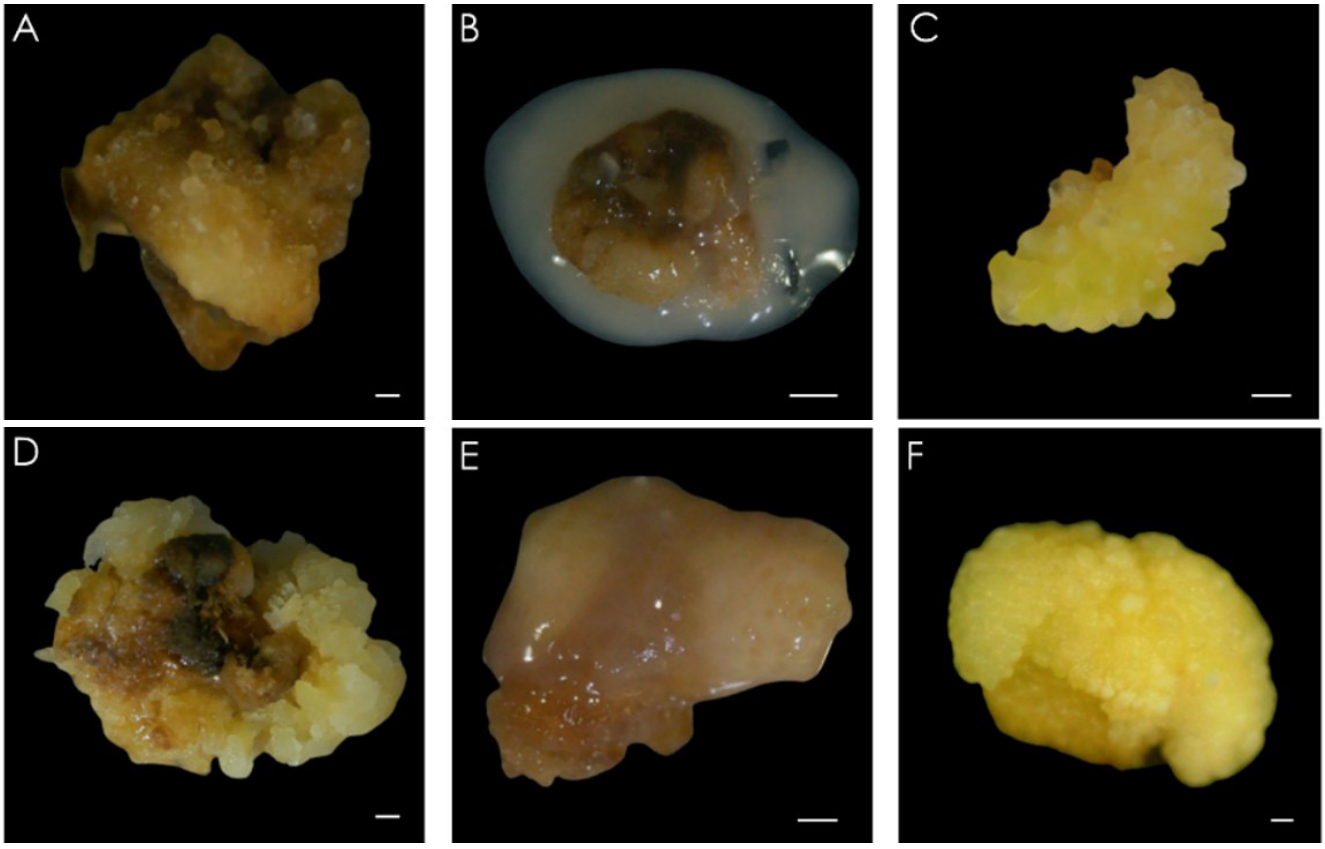


Figure 8. Response of rice callus at the cocultivation-elimination stage of the Ketan Hitam, indicating (A) browning, (B) overgrowth, (C) surviving callus, and Pandan Wangi varieties showed (D) browning, (E) overgrowth, (F) surviving callus

Table 4. Response of rice callus to co-cultivation-elimination stage on murashige and skoog medium with cefotaxime antibiotic

Variety	Overgrowth (%)	Browning (%)
Ketan hitam	13.30±6.7	56.99±23.7
Pandan wangi	10.56±7.8	63.23±20.4

Measurement of overgrowth and browning percentage using ImageJ software

Table 5. Response of rice callus to regeneration and selection stage on murashige and skoog medium with hygromycin antibiotic

Variety	Browning (%)	Embriogenic (%)	Greenspot (%)	Number of Planlets
Ketan hitam	41.64±24.3	20.84	12.71±7.1	1
Pandan wangi	67.58±18.8	0.87	0.83	0

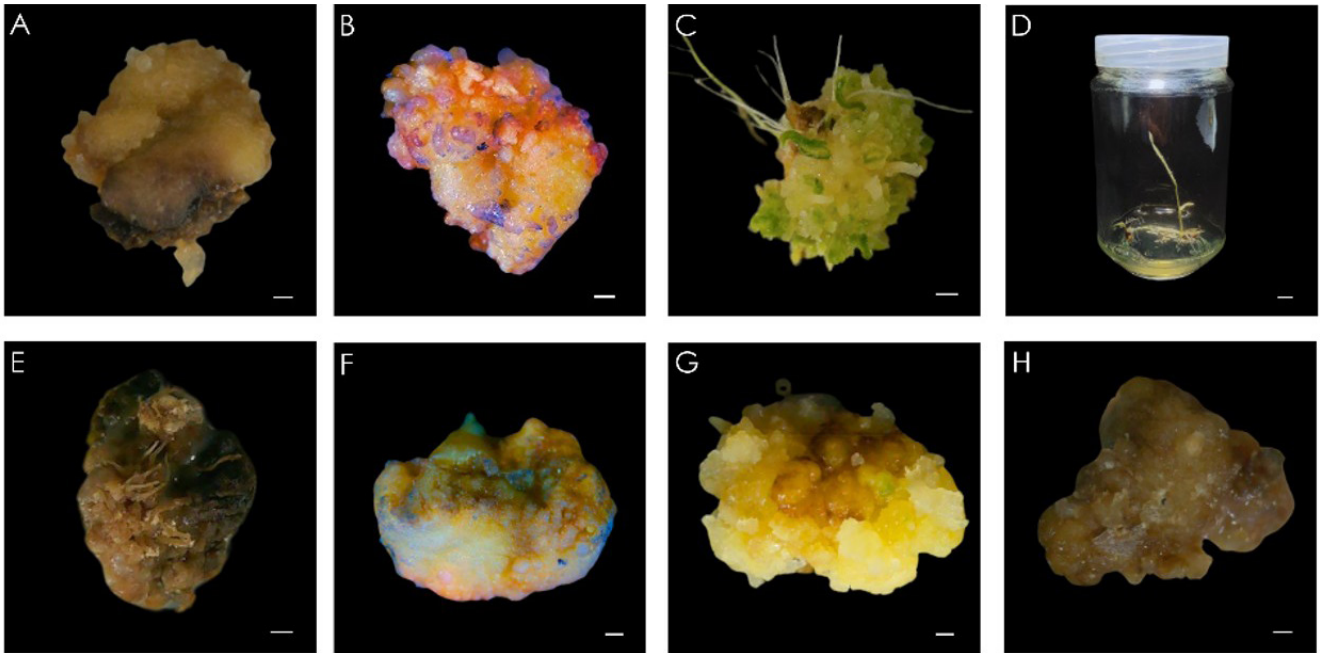


Figure 9. Response of callus of Ketan Hitam variety in regeneration-selection phase, (A) browning, (B) embryogenic, (C) greenspot, (D) selected plantlets, and response of Pandan Wangi variety in regeneration-selection phase, (E) browning, (F) embryogenic, (G) greenspot, (H) dead callus after selection

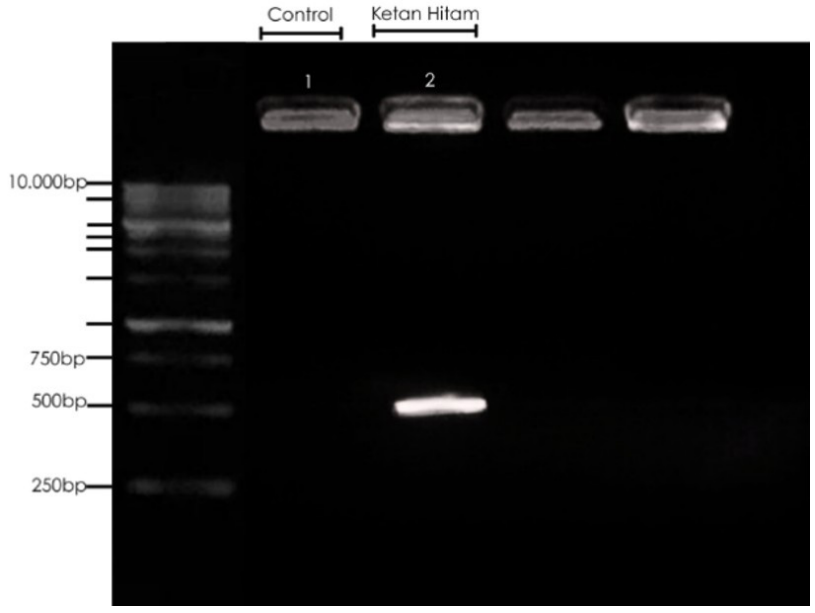


Figure 10. Preliminary indication of putative-transformant carrying pRGEB32:sgRNAI carrying construct via molecular analysis

Figure 11. Phenotypic comparison of putative-SD1 edited and wild-type Ketan Hitam at post-transplantation

al. 2016), so the most efficient figures must also be considered when optimizing sgRNA. There are two selected sgRNA candidates: the first has a GC content of 60% and an efficiency of 61.16%, and the second has a GC content of 55% and an efficiency of 54.11%. The PAM motif is necessary for Cas9-sgRNA complex recognition at the target site (Kumar *et al.* 2024). The selected sgRNA candidates are shown to have their respective PAM motifs with a CGG sequence downstream of their 3' target site.

4.2. Transformation and Validation of pRGEB32::SD1—sgRNAI to *E.coli*

The assembled plasmid was confirmed to have successfully transformed into *E. coli*. The growth of *E. coli* on LB kanamycin media was due to the presence of the NPTII selection marker gene from the pRGEB32 plasmid. The presence of the NPT-II (neomycin phosphotransferase) gene can confer kanamycin degradation, thereby rendering unicellular or NPT-II-producing organisms resistant to kanamycin (Katta *et al.* 2021). This was in contrast to bacteria in the control treatment (non-transformants), which showed no colony growth on LB kanamycin media. Confirmation of the presence of sgRNA in the plasmid was carried out by sequencing, which showed the fidelity of sgRNA sequences in the target region without unexpected base nucleotide substitutions in colonies 1 and 3 of the pRGEB32::SD1—sgRNAII plasmid. Negative results in plasmid sequencing from the sgRNA I colony sample may be due to the high potential for self-complementarity of the sgRNA. High self-complementarity may form hairpin structures that inhibit sgRNA function (Labun *et al.* 2016).

4.3. Transformation and Validation of pRGEB32::SD1 to *Agrobacterium tumefaciens*

The cloned plasmid pRGEB32::SD1 was then transformed into *A. tumefaciens*. *A. tumefaciens* is a frequently used vector due to its efficiency in genetic transformation, especially in indica and japonica rice varieties (Majumder *et al.* 2021). Confirmation via PCR also showed that all *A. tumefaciens* samples amplified a 545 bp product (Primer HPTII). This indicates that the pRGEB32::SD1 plasmid was successfully transformed into *A. tumefaciens* strain LBA4404.

4.4. Callus and Regeneration Response

The *Agrobacterium*-mediated transformation process can stress rice callus, leading to overgrowth and browning as a callus response. The emergence of overgrowth can be caused by the explant drying stage occurring too quickly after washing, which can leave the callus wet, thereby increasing the potential

for excessive growth of *A. tumefaciens*. The browning response in callus indicates decreased regeneration capacity or even cell death. The appearance of browning can be caused by high activity of polyphenol oxidase (PPO) in response to stress (Su *et al.* 2023). PPO catalyzes the oxidation of phenolic compounds into quinones, which then polymerize to form brown pigments (Zhang *et al.* 2020). This response in Indica rice transformation has been widely reported in indica rice and remains a major challenge in tissue culture-based transformation systems (Lou *et al.* 2024).

Callus staining with acetocarmine was performed to determine the number of embryogenic cells in the explants. The presence of embryogenic cells is crucial because it increases the potential for callus regeneration (Visarada *et al.* 2002). The genotype of each variety may influence the induction of embryogenic callus, as indicated by the appearance of brighter callus color (Khan *et al.* 2021). The appearance of green spots can indicate the onset of successful callus regeneration. It has been widely reported that the formation of green spots in callus is the part that will develop into shoots (Yan *et al.* 2021).

4.5. Limitations of the Study and Future Research

The regeneration of a single Ketan Hitam plantlet represents preliminary evidence of transformation success. However, the overall regeneration frequency remains low, and the absence of regenerated Pandan Wangi lines indicates that further refinement is required. At the same time, the Pandan Wangi variety showed death in all selected calli. Different genotypes also play a crucial role in callus induction and plant regeneration (Mostafiz and Wagiran 2018). This study was limited to T0 plant generation, and functional validation at the molecular and phenotypic assessment remains to be performed. Further research is needed regarding the edits of SD1, which comprises molecular-level assays, and developing the mutant lines to T2 plants to test genetic stability. Then, abiotic and biotic tests should be performed on SD1 mutant lines to evaluate yield potential and productivity.

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