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CRISPR/Cas9-mediated sgRNA design targeting UDP-Glycosyltransferases (UGTs) in Indonesian local rice

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Abstract. *Ubaidillah M, Syarif RM, Gusti AIH, Aurela RD, Habibi I, Hartatik S, Harmoko R, Kim KM. 2026. CRISPR/Cas9-mediated sgRNA design targeting UDP-Glycosyltransferases (UGTs) in Indonesian local rice. Biodiversitas 27 (1): d270108. https://doi.org/10.13057/biodiv/d270108.* Sakuranetin serves as a vital phytoalexin in rice and exhibits notable pharmacological properties. Its biosynthesis is catalyzed by the OsNOMT enzyme and regulated by UDP-Glycosyltransferase (UGTs) genes, which divert naringenin into other flavonoids, thereby limiting sakuranetin production. This study aimed to design and construct specific single-guide RNA (sgRNAs) targeting UDP-Glycosyl Transferase (UGTs) genes precisely via CRISPR/Cas9 to establish genetic tools for metabolic engineering. Glucosyltransferases were collected from GenBank and aligned using Clustal Omega 13. The sgRNA oligonucleotides were designed using CHOPCHOP version 3, yielding information related to PAM, sgRNA efficiency, GC content, and others. A total of three sgRNAs were produced, with two showing efficiencies that match the target gene UGTs with predicted highest on-target sites and GC content above 60%. The designed sgRNA was successfully constructed into plasmid pRGEB32 and transformed into *Agrobacterium tumefaciens* LBA4404. Molecular validation confirmed plasmid integrity and sgRNA insertion through PCR amplification and sequencing. Preliminary transformation trials using Indonesian local rice varieties (Merah Wangi, Pandan Wangi, and Pontianak) revealed genotype-dependent variability, with Pandan Wangi showing the highest transformation efficiency and callus survival. These results demonstrate a molecular foundation for precise metabolic pathway engineering to enhance stress-adaptive and disease-resistant traits in local rice cultivars.

Keywords: *Agrobacterium,* genome editing, Indonesian local rice, pRGEB32, sakuranetin

INTRODUCTION

Sakuranetin is a compound of the metoxylated flavanone group and acts as a phytoalexin. The compound exhibits numerous health benefits, including antioxidant, anticancer, anti-inflammatory, and antidiabetic properties. Sakuranetin is the best flavonoid among other flavanones (Stompor 2020). As a phytoalexin, sakuranetin is the defense system of rice plants against attack by pathogenic microorganisms, particularly the fungus *Magnapothe oryzae* and other abiotic stressors. In addition to the immune response, sakuranetin disease attacks have a role in reducing oxidative stress in plant tissues and activating defense enzymes in rice plants (An et al. 2021). This compound is synthesized via the Phenylpropanoid pathway, starting from phenylalanine. The process of forming sakuranetin begins with the alteration of naringenin, which is the main precursor and is catalyzed by the enzyme 7-0-methyltransferase encoded by the OsNOMT gene (Valletta et al. 2023).

Naringenin is a precursor to flavonoid compounds and is not only used to form sakuranetin (Yang et al. 2021). These flavones are often conjugated to sugar groups by UDP-glucosyltransferases (UGTs) to form various flavanone derivatives (Cao et al. 2024). Glycosyltransferase plays a notable role in plants' tolerance (Chen et al. 2024). UGTs mediated the conversion of naringenin to apigenin, thereby limiting the conversion of sakuranetin and resulting in a lower sakuranetin rate in plants (An et al. 2023). Ouyang et al. (2023) reported several UGT genes with activity towards flavonoid, indicating their important roles in conferring abiotic stress. Although CRISPR/Cas9 applications have successfully targeted the UGT gene for metabolic engineering in several crops, including GmUGT knockout in plants that enhance flavonoid-mediated insect resistance (Zhang et al. 2022), direct application of genetic modification of UGT silencing specifically in rice remains absent from current literature. This research gap demonstrates a crucial scope, as targeted UGT silencing in Indonesian local rice could specifically boost sakuranetin biosynthesis for pathogen and biotic resistance.

Case studies on *Oryza sativa* L. have remarked on the successful implementation of CRISPR/Cas9 in optimizing plant characteristics. Production of specific secondary metabolites can be decreased/increased with high accuracy, efficiency, and multiplex targeting by CRISPR/Cas9 technology (Devi et al. 2023), enabling optimal synthesis of

bioactive metabolites (Borah et al. 2024). The production of these valuable compounds can be greatly enhanced by modifying crucial genes in secondary metabolite pathways (Rynjah et al. 2025). For instance, targeting the LOX2 gene in rice led to increased resistance to bacterial blight while simultaneously improving reproductive organ health (Guo et al. 2022). Genome editing in rice offers new opportunities for improving plant traits. The UGTs are a target for the development of elite cultivar engineering studies. Successful genomic engineering of UGTs requires the highly specific single guide RNAs (sgRNAs) that can guide Cas9 cleavage activity with minimal off-target risks.

This research will focus on developing and validating a CRISPR/Cas9 system for genome editing targeting the UGT gene in rice plants. The study aims to design high specifity guides with minimal off-target risks, construct functional pRGEB32::sgRNA vectors, followed by molecular validation to ensure proper sgRNA integration and transformation assessment through *Agrobacterium tumefaciens* strain LBA4404, mediating the recombinant plasmid into plant genomes. The putative transformants were obtained and identified based on hygromycin resistance and preliminary regeneration responses. The present work provides functional information for potential applications in rice improvement programs and enhancing crop productivity and resilience under biotic and abiotic stress. Therefore, we hypothesize that the sgRNAs designed in silico will demonstrate specificity and reproducible transformation efficiency in *A. tumefaciens* LBA4404, and the three Indonesian local rice varieties will differ in responsiveness to *Agrobacterium*-mediated transformation, reflected by the percentage of hygromycin-resistant and early regeneration potential.

MATERIALS AND METHODS

Research materials

The research was conducted at the Agrotechnology Laboratory, Faculty of Agriculture, Universitas Jember, Indonesia. The material utilized in this study included the CRISPR/Cas9 binary vector pRGEB32, *Agrobacterium tumefaciens* strain LBA4404, and three local Indonesian varieties, namely Merah Wangi, Pandan Wangi, and Pontianak.

Procedures

Selection of sgRNA targeting

Glucosyltransferase (accession number: AP005186.4), located on chromosome 7 at locus LOC_Os07g32020 with locus length 2402 bp, was compiled from GenBank and harmonized to determine conserved regions using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sgRNA oligonucleotide was designed using CHOPCHOP's CRISPR version 3 (2025) to generate information related to PAM, sgRNA efficiency, GC content, genome location, number of mismatches, and its location in the Coding Sequence (CDS) (Labun et al. 2019). The selected sgRNA sequence (designed by adding GGCA at the 5' end of a single strand and CAAA at the 5' end of the complementary strand, yielding 23 nt) was synthesized as a single-stranded oligonucleotide, namely sgRNA-Exon1 and sgRNA-Exon2. Candidate sgRNAs were selected based on the absence of off-target predicted sites with less than 2 mismatches in coding regions, followed by a specificity score of GC content, efficiency of more than 50%, and a canonical PAM sequence (5'-NGG-3') sgRNAs were designed using 20 nt-protospacers equipped with a canonical SpCas9 PAM sequence (5'-NGG-3') at the end, because only loci adjacent to this PAM can be recognized and cleaved by SpCas9 (Corsi et al. 2022). Off-target risks were evaluated using *Oryza sativa* var. *japonica* Postiglione reference genome (AP005186.4) mediated by CRISPOR version 5.2 (crispor.gi.ucsc.edu), which generates the similarities between rice genome sequences and sgRNAs designed with PAM sites. Potential off-targets were obtained according to the number of mismatches and linking between PAM recognition and off-target risk prediction.

Construction of sgRNA-targeted glucosyltransferase *gene into pRGEB32 plasmid*

The purified pRGEB32 plasmid was restricted using the Eco31IBsaI (Thermoscientific, Cat. No ER0291) enzyme for vector linearization. Next, 2 µL L^{-1} of Eco31I (BsaI) was added to 38 µL L^{-1} of a mixture of 20 µL L^{-1} of pRGEB32, 4 µL of buffer G (10×), and 14 µL L^{-1} of ddH_2O in Eppendorf tubes and incubated at 37°C overnight, followed by visualising the product in 0.8% agarose electrophoresis. The Eco31I (BsaI) enzyme recognizes the sequence 5'-GGTCTC-3' and produces sticky end cuts with complementary overhangs 5'-GTTT-3' and 3'-CCGT-5'. The pRGEB32 linear plasmid was then extracted and purified from the agarose gel using the Gel Extraction Miniprep Kit (Qiagen, Netherlands). To produce sgRNA inserts of oligo-duplex, single-strand oligonucleotide forward and reverse nucleotides of synthetic sgRNA-Exon1 and sgRNA-Exon2 were mixed in PCR tubes and annealed at 95°C for 3 minutes in a thermal block, followed by placing them at room temperature for 45 minutes (Sambrook and Russel 2001). Each sgRNA oligo-duplex is bound to linear pRGEB32 in a ratio of 2:1. The ligation reaction is a mixture of 1 µL L^{-1} of DNA ligase (Thermoscientific, Cat. No. EL0011), 2 µL L^{-1} of linearized plasmid, and 2 µL L^{-1} of ligation buffer (10×) in a total reaction volume of 20 µL L^{-1} (Promega, USA). The samples were incubated at 4°C overnight before being converted into *Escherichia coli* strain JM109.

Transformation of plasmids into Escherichia coli

The pRGEB32::sgRNA Glucosyltransferase was transformed into the *E. coli* strain JM109 by the heat shock method. Competent cells were prepared with $CaCl_2$ to enhance plasmid uptake (Sambrook and Russel 2001). The samples (pRGEB32::sgRNA Glucosyltransferase) were mixed at 42°C for 90 seconds on a thermal block and immediately placed on ice for 15 minutes to permit plasmid integration into cells. Subsequently, 1 mL L^{-1} of LB Broth was added to aid cell recovery, and the culture was shaken in an incubator at 37°C for 60 minutes. The transformed cells were centrifuged, yielding a pellet, and the supernatant

was removed, leaving approximately 100 µL L^{-1} of cell suspension. The sample was spread on Luria Bertani (LB) agar containing 50 mg L^{-1} kanamycin and incubated at 37°C overnight. Positive colonies exhibiting growth were counted as a parameter of transformation efficiency. Plasmid isolation was performed by harvesting the bacterial cells using the miniprep protocol based on Sambrook and Russel (2001). Followed by Sanger sequencing provided by a company using primer M13 Forward (-21) (5'-TGTAAAACGACGGCCAGT-3') and M13-R (3'-CAGGAAACAGCTATGAC-5') as recommended by Addgene (2024). The expected amplicon sizes were 300 bp to obtain DNA sequences confirming successful transformation and insertion of sgRNA into the pRGEB32::UGTs vector and plasmid integration. Sanger reads approximately 700 bp, providing full coverage of the sgRNA cassette. These positive colonies were selected and transformed into *Agrobacterium* competent cells for further analysis.

Electroporation for transformation of plasmid pRGEB32:UGTs into Agrobacterium tumefaciens

Competent *Agrobacterium* cells from a single colony were cultured in 5 mL LB medium and incubated at 28°C with shaking for 24 hours. The resulting culture was then used for transformation. The incubation result, approximately 1 mL L^{-1}, was transferred into 100 mL L^{-1} LB and grown to an optical density of 0.7, then placed on ice for 15 minutes (Sambrook and Russel 2001). The culture results were then divided into two Falcon tubes and centrifuged at 6000 rpm, 4°C for 10 minutes. The resulting supernatant is discarded, and the pellet is dissolved again with 50 mL L^{-1} of ice-cold water and homogenized with a vortex, then kept on ice for 10 minutes. The bacteria were harvested at 6000 rpm in a 4°C centrifuge for 10 minutes. Then it is dissolved in 25 mL L^{-1} of ice-cold water with a vortex and held for 10 minutes. The cells were harvested by centrifugation at 6000 rpm, 4°C for 10 minutes. Then the samples were dissolved in 1.5 mL L^{-1} of 10% glycerol with pipetting (For 50 mL L^{-1} of the initial culture, 1-1.5 mL L^{-1} of 10% glycerol is dissolved). The cell cultures were transferred to 200 microtubes, each containing a single culture, and frozen in liquid nitrogen. The tube should be stored at -80°C.

Plasmids that were previously frozen in liquid nitrogen were first thawed in ice conditions. Then, 1 µL L^{-1} of vector plasmid is mixed with 200 µL L^{-1} of competent cells and incubated for 20 minutes. The steps are followed by putting the competence cell into the chilled cuvette (gap: 2 mm), then set it into the cuvette holder. The electroporator was used with the following settings: 2.5 kV, 25 µF capacitance, and 400 ohm resistance (Kámán-Tóth et al. 2018). After electroporation, the cuvette was immediately cooled on ice, and 1 mL L^{-1} of liquid YEP was added, then dissolved by pipetting. The resulting culture cells were transferred to an Eppendorf tube and incubated at 28°C on a shaker for 2 hours.

Culture cells that have been incubated in liquid media were then converted into solid LB media with selection media, containing 50 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin to obtain a transformed single colony (Sambrook and Russel 2001). At this stage, the *Agrobacterium* is incubated for 48 hours at 28°C (Safitri et al. 2016). Single colonies that had grown were then counted in number and became one of the parameters in this study. The positive colonies that are counted exhibit the characteristics of being non-pigmented, non-contaminating, and forming colony units.

Molecular analysis

Molecular analysis was performed by mixing several ingredients, including Taq DNA polymerase (10 µL L^{-1}), nuclease-free water (8 µL L^{-1}), and forward and reverse primers (1 µL L^{-1} each) to confirm that *Agrobacterium* has been transformed with pRGEB32 vector plasmids (Safitri et al. 2016). The master mix was then mixed with a bacterial sample by taking colonies that grew on solid media and pipetting them into a PCR tube. Subsequently, the DNA amplification was performed with 36 cycles, including pre-denaturation at 95°C for 10 minutes, denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 1 minute. The last cycle is a final elongation at 72°C for 4 minutes. The denaturation temperature is chosen to disrupt the hydrogen bonds between the complementary base pairs (Khehra et al. 2025). Additionally, an annealing temperature of 60°C provided an optimal condition for the primer pair based on temperature melting for HPTII, while an extension temperature of 72°C is an optimal temperature for generating individual DNA strands (Kulkarni and Goel 2020). The molecular analysis was using HPTII primers with forward sequence 5'-TCGGACGATTGCGTCGCATC-3' and reverse sequence 5'-AGGCTATGGATGCGATCGCTG-3', amplifying a size of 545 bp to confirm the presence of the targeting plasmid in selected transformants. The results of PCR amplification were electrophoresed into a 1% agarose gel, stained using Greenstar, and visualized using a UV transilluminator.

Agrobacterium *and rice callus preparation*

Agrobacterium that has been confirmed to contain pRGEB32::sgRNA UGTs plasmids was inoculated on solid LB media containing 50 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin by the quadrant streak method and followed by incubation for 48 hours at a temperature of 28°C in dark conditions to obtain a single colony (Safitri et al. 2016).

Callus induction was also prepared for genetic transformation, which begins with selecting healthy seeds. The seeds were first sterilized with 30% bleach (NaClO) in a Falcon tube and shaken for 15 minutes. Subsequently, the seeds were dehusked with sterile ddH_2O 4-6 times to remove the bleaching solution. Following these steps, the seeds were dried on sterile filter paper and cultured on N6D callus induction medium (2 mg L^{-1} 2,4-D), incubated at 32°C in the dark for 14 days (Safitri et al. 2016). Characteristics of a good callus for genetic transformations are yellowish-white, more than 3 mm in diameter, and not below 2.7 mm, and crumbly (Ardra et al. 2025; Chitphet et al. 2025).

Infection

Agrobacterium was cultured in 100 mL of LB liquid medium (25 mg L^{-1} LB + 50 mg L^{-1} kanamycin + 50 mg L^{-1} rifampicin) for 25 hours until the OD at 600 nm was 0.5-1 (Sambrook and Russel 2001). The culture was then transferred into a 15 mL L^{-1} falcon tube and centrifuged at 5000 rpm for 10 minutes at 4°C. The resulting pellets were suspended in 100 mL of liquid MS medium, and acetosyringone was added at a concentration of 0.1 mM. Thereafter, callus was added to the MS-Agro solution containing acetosyringone and shaken for 10 minutes. The callus was immediately dried on sterile filter paper. Then, the calli were cultured in N6-AS100 media. The cultures were first incubated at 28°C for 24 hours in the dark and subsequently transferred to 23.5°C for 24 hours under dark conditions (Safitri et al. 2016).

Agrobacterium *and rice callus preparation*

The seeds from co-cultivation were transferred into a falcon tube and washed to remove the remaining *Agrobacterium*. The callus was rinsed with sterile ddH_2O 3-4 times. A 500 mg L^{-1} cefotaxime solution was used in the last wash. These steps are followed by drying the callus with sterile paper. The elimination results were cultured on N6D cef500 medium and incubated at 28°C under bright conditions for seven days (Safitri et al. 2016). Following 7-10 days of incubation, the callus was transferred to a new N6D Cef 500 and then to regeneration medium to obtain a plantlet. A sign that calluses were ready to be transferred to regeneration media was their active development and the presence of green spots. The results of the transformant selection became research parameters in the form of the number and percentage of rice calluses transformed by *Agrobacterium*.

Selection and confirmation of putative transformant plants

Calli have shown green spots were transferred to MS selection media supplemented with cefotaxime and hygromycin for 14 days. The surviving plants were then sub-cultured on the appropriate media until acclimatization was complete. Plantlets resulting from the genetic transformation process were confirmed by detecting T-DNA presence by isolating DNA (Safitri et al. 2016) from putative transformant rice leaves using the CTAB method (Doyle 1991), followed by molecular analysis. Leaf samples will be ground using a cocktail (1 mL CTAB buffer; 0.04 g PVP; 5 µL β-mercaptoethanol) and incubated in a water bath at 65°C for 30 minutes, followed by the addition of PCI, centrifugation, and DNA precipitation by ammonium acetate and isopropanol. The DNA extraction sample that has been added to aquabidest will be subjected to molecular analysis.

The molecular analysis will be performed using HPTII with the forward sequence 5'-CGGACGATTGCGTCGCATC-3' and reverse sequence 5'-AGGCTATGGATGCGATCGCTG-3' primer with a total reaction volume of 41 µL (4 µL HPTII primers, 32 µL water, and 5 µL leaf DNA sample). Molecular amplicon will be electrophoresed using a 1.5% agarose gel with the addition of 1.5 µL ethidium bromide and visualized using a UV transilluminator (BioRad, No. 465 BR).

Data analysis

The observation data included callus induction frequency, the efficiency of plasmid transformation into *A. tumefaciens,* the number of hygromycin-resistant calli, and regeneration response following *A. tumefaciens*-mediated transformation. Data were analyzed in a simple descriptive statistical manner to get an overview of the efficiency of plasmid transformation into *A. tumefaciens*. The transformation assay was performed based on three biological replicates, with separate *Agrobacterium* cultures and callus batches prepared on different days. Results were presented as mean±SD (standard deviation) from three biological replicates.

RESULTS AND DISCUSSION

Design and construction of sgRNA

Uridine Diphosphate (UDP), a dependent Glycosyltransferase (UGTs), is one of the genes that play a role in plant regulation against the presence of stress, both biotic and abiotic, and in rice plants. There are more than 200 UGT genes (Liu et al. 2021). UGTs are enzymes that catalyze the addition of sugars to plant secondary metabolites. The UGTs gene is located on chromosome 7 with the locus name LOC_Os07g32020.1. This gene is known to have 1 Coding Sequence (CDS), which is with coordinates (5'-3') 19045831-19048232 with a length of 1425bp (Figure 1). The sgRNA design process involves determining a conservative domain. This is because the conservative domain can affect the effectiveness of genome editing. Selection of sgRNAs outside the conserved domain area leads to off-target in genome editing. As a result, to determine the specific location of sgRNA, web-based software CHOPCHOP (2025) was used, which produced 388 sgRNA targets and 2 sgRNA candidates with a length of 20 nt and 3 nt PAM were selected. sgRNA length also had an obvious effect on editing efficiency. Liu et al. (2022) reported that lengths of 20 nt sgRNA had the highest editing efficiency and were normally distributed, whereas sgRNA with less than 20 nt reduced editing efficiency in cells and rice.

The PAM arrangement is very important in designing sgRNA, because it is at this location of the PAM that the sgRNA will activate the Cas9 nuclease for cutting (Baranova et al. 2022). Furthermore, the sgRNA is located within the conserved domain, namely at the 98th, 968th, and 1327th positions. The selection of the sequence is based on the potential for cutting and the resulting knockout impact. The location of the cut at the beginning and middle can affect indel mutations, Open Reading Frame (ORF) shifts, and genes are not well expressed (Jia et al. 2022). Novel information about PAM, GC content, out-of-frame score, mismatches, cutting frequency determination specificity score, sgRNA's location, and efficiency are presented in Table 1. The three selected sgRNAs have efficiencies above 40% and GC contents above 65%. One of the

sgRNA efficiencies is influenced by the percentage of GC. The optimal GC for sgRNA is in the range of 40-80% with the highest potential at GC more than 50% (Hardiyani et al. 2023). However, Cas9 may induce undesirable off-target mutations because the sgRNA recognizes untargeted DNA sequences via one or more nt mismatches and the nuclease's cleavage activity (Manghwar et al. 2020).

Accurate genome sequence information is essential for predicting off-target effects, which are unexpected, unwanted, or even adverse alterations to the genome (Guo et al. 2023). Generally, the CRISPR/Cas9 system tolerates at least 3 mismatches in a 20-bp target DNA sequence to prevent these undesirable changes. Although the off-target mutation frequencies in many plants are quite low (Wolt et al. 2016), it can lead to undesirable phenotypic changes. Moreover, the CRISPR delivery components, RNPs, have emerged as an efficient strategy to minimize the off-target effects (Leal et al. 2024). The off-target effects of sgRNAs have been investigated across many genetic transformation processes, and conclusions regarding off-target activity vary widely. Doench et al. (2016) presented that the CFD (Cutting Frequency Determination) score will enable avoidance of the majority of high-frequency off-target effects. The specificity score ranges from 0 to 100 and measures the potential of off-target in targeted sgRNA. Higher specificity scores lead to lower off-target effects in the genome. Among the three selected sgRNAs, sgRNA 1 and sgRNA 2 demonstrate the highest on-target efficiency, with the highest efficiency score and the lowest off-target potential score. This comparison illustrates sgRNA 1 and 2 as the most promising candidates for the experiment (Table 1). These features indicate that sgRNA 1 and sgRNA 2 are more likely to produce precision edits targeting UGTs.

The pRGEB32 plasmid contains CRISPR/Cas9 on T-DNA and the hygromycin phosphotransferase (HygR) marker gene with a 35s promoter (CaMV 35s) (Pal et al. 2024). In plasmid pRGEB32, sgRNA is formed and promoted by the U3 promoter and another part of the plasmid is the promoter and terminator (Figure 2). The promoter is a part of the gene system that plays a role in initiating and directing gene expression. The promoter becomes a marker and the beginning for RNA polymerase to start transcription (Dwiyani et al. 2016). Conversely, the terminator is the end of the gene that marks the termination of transcription (Neumann et al. 2020). The pRGEB32 vector, harboring the Cas9 gene under the control of the rice ubiquitin promoter and a BsaI restriction site driven by the rice U3 promoter, was used for sgRNA cloning (Bhavya et al. 2024) (Table 2). In this study, we integrate a hybrid computational and experimental approach, combining the computational results of sgRNA prediction tools with laboratory-based construct assemblage and transformation protocols.

Table 1. sgRNA candidates designed for genome editing mediated by CRISPR/Cas9 in rice plants targeting the UGTs gene

Code	Protospacer (5'- 3')	PAM	GC content (%)	Out of frames score	Mismatches	CFD specificity score	Location	Efficiency
sgRNA 1	CCTCGTGCTGCTGCCAACCT	GGG	65%	73.6	0	98	Seq:59	41.03%
sgRNA 2	CGTGGTTCGTGTACCACGGC	AGG	65%	66.9	0	99	Seq:628	53.79%
sgRNA 3	TGGGTGGGTCGGAGGAAGGG	AGG	70%	63.8	0	83	Seq:1327	49.44%

Note: Seq indicates the protospacer position within the coding sequences of AP005186.4. MM is number of target mismatches tolerated in predicted off-target score, CFD (Cutting Frequency Determination) predicting off-target cleavage site (higher: Greater specificity)

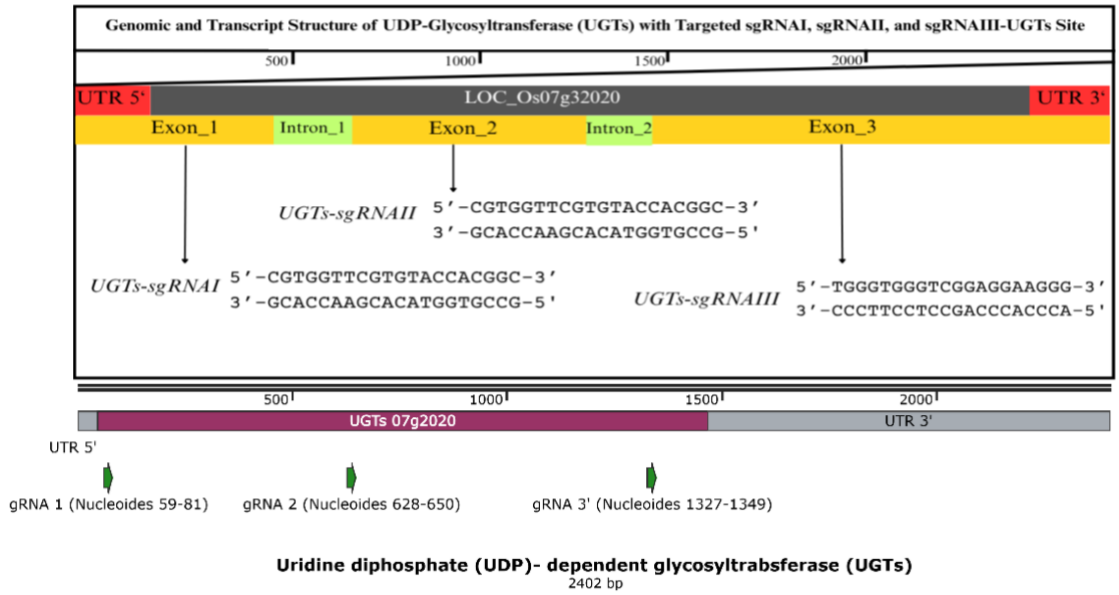

Figure 1. CRISPR/Cas9 design for UGT gene editing in rice plants. The gene structure illustrates the target sites and conserved domains in the UGT gene

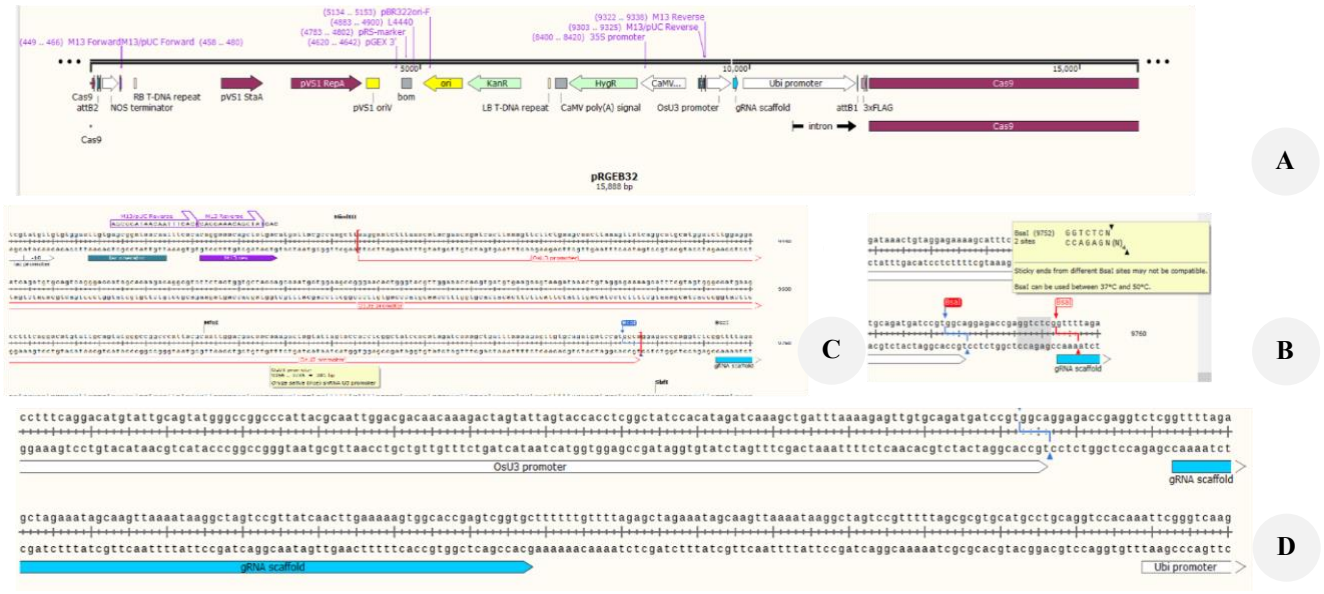

Figure 2. CRISPR/Cas9 system design for UGT gene editing: A. Schematic vector map of UGTs from SnapGene showing promoter regions and Cas9 expression, B. sgRNA promoters in greater detail, C. Insert sites that refer to the target genome location, and D. sgRNA scaffold that provides the detailed sequences

Table 2. General information of vector backbone for UGTs-targeted gene, marking the vector type, promoter, selectable marker, and the growth in bacteria

Backbone	
Vector type	Plant expression, CRISPR
Promoter	Rice snoRNA U3 promoter for sgRNA expression and UBI promoter for Cas9 expression
Selectable marker	Hygromycin
Growth in bacteria	
Bacterial resistance	Kanamycin, 50 µg/mL
Growth temperature	37°C
Growth strain	DH5Alpha
Copy number	High copy

Transformation of plasmids into *Escherichia coli*

The plasmid construction process is carried out using the Bsa1 restriction enzyme (Figure 3). Plasmid cleavage with the Bsa1 Enzyme is only specific to the sgRNA site and causes the presence of sticky ends/asymmetric cuts. Therefore, before the incorporation of sgRNAs, a special nucleotide arrangement is added so that the sgRNA can fuse with the plasmid. The additions are 5'GGCA3' nucleotides and 5'CAAA3' from the complementary nucleotides (Figure 3).

The plasmids that have been designed and arranged are then transformed into *E. coli* by the heat shock method. The presence of colonies on the selection medium indicates that the plasmid carrying the sgRNA target has been successfully introduced and integrated into *E. coli.* The colonies were grown on a medium containing 50 mg L^{-1} kanamycin and showed *E. coli* growth (Figure 4). It can be seen that colonies grow on the selection medium, indicating that the sgRNA has been integrated into the plasmid and successfully inserted into *E. coli*. The use of LB media containing kanamycin is indicated to select for the transformed bacteria. This is because in the pRGEB32 plasmid in the backbone, there is a kanamycin antibiotic resistance gene from the NPTII (neomycin phosphotransferase) derivative, which can destroy antibiotics so that the plasmid can be resistant to kanamycin. When the plasmid is transformed into bacteria, the backbone part will be expressed, and the bacteria will be resistance to kanamycin antibiotics (Hardiyani et al. 2023). Therefore, the growth of colonies on the antibiotic media shows that *E. coli* has been transformed into pRGEB32::UGTs plasmids and directly confirms the success of plasmid construction with sgRNA (Table 3).

Validation of sgRNA-inserted plasmid via Sanger sequencing

Successful cloning of pRGEB32::sgRNA1::sgRNA2 was confirmed via molecular analysis and Sanger sequencing, which detected the presence and correct insertion of the insert. Nine colonies (three per sgRNA) were analyzed and showed positive results for the sgRNA-modified inserted UGTs, with no mutations detected, except for colony 3 of sgRNA II and colony 1 of sgRNA III (Table 4). In addition, the sequenced positive sgRNAs showed identical sequences for each target, confirming that the plasmid was successfully modified with the UGT-targeted sgRNA and that no mutations were present in representative chromatograms (Figure S1).

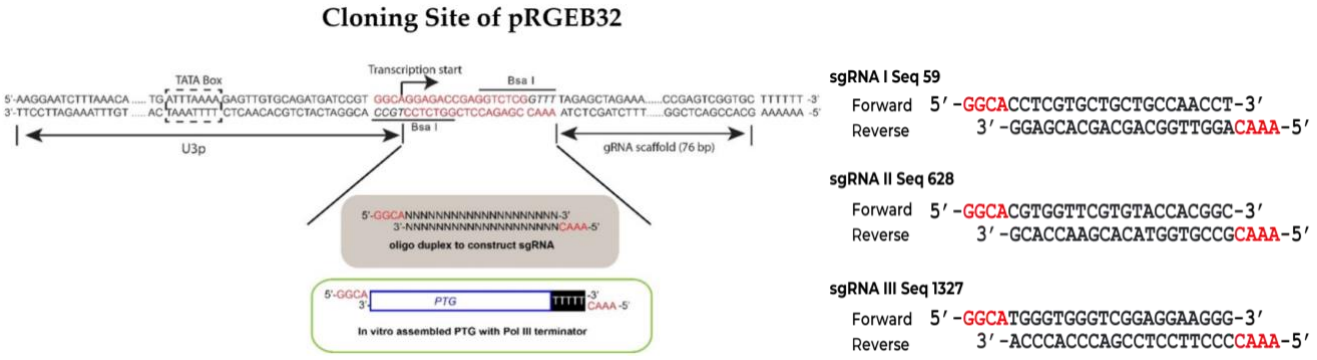

Figure 3. Construction of the pRGEB32 plasmid, which includes the plasmid cutting site by the Bsa-1 enzyme and the forward and reverse sgRNA target sequences that have been added with 4 nucleotides (red letters) to complete the enzyme cutting results of Bsa-1

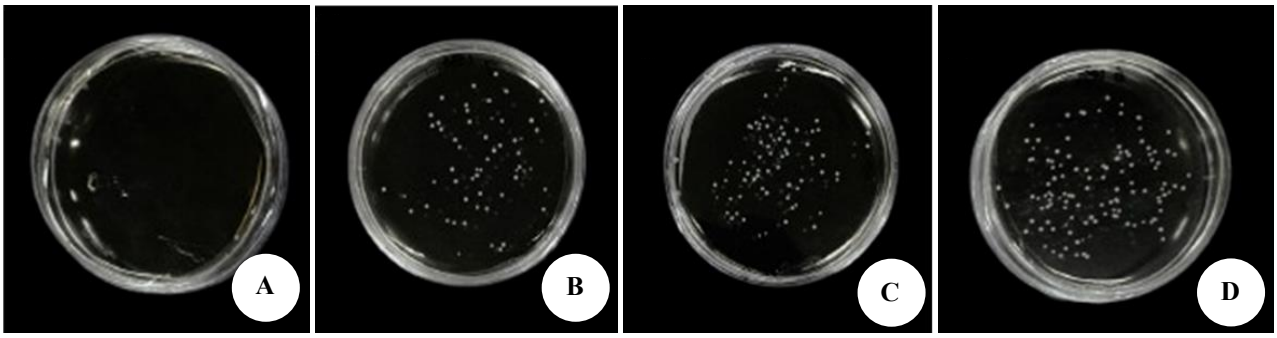

Figure 4. Optimization of plasmid transformation into *Escherichia coli* bacteria. A. Control, B. pRGEB32::sgRNA 1, C. pRGEB32::sgRNA 2, D. pRGEB::sgRNA 3

Table 3. Optimization of plasmid transformation into *Escherichia coli* bacteria was performed by growing *E. coli* on LB agar medium

Plasmid	*E. coli* colony 1	*E. coli* colony 2	Mean
Control (No pRGEB32)	0	0	0
pRGEB32::UGTs.sgRNA I	236	214	225±15.55
pRGEB32:UGTs.sgRNA II	214	191	202.5±16.26
pRGEB32::UGTs.sgRNA III	192	179	185.5±9.19

Transformation of plasmids into *Agrobacterium*

Agrobacterium is the primary vector and is most widely used in genetic transformation (Lacroix and Citovsky 2018). Plasmids are introduced into *Agrobacterium* via electroporation, a method that uses a short electrical pulse to temporarily weaken the cell membrane (Fiedler and Wirth 1988). The transformed *Agrobacterium* was grown on a medium containing 50 mg L^{-1} kanamycin and rifampicin as a selection marker for *Agrobacterium* (Figure 5). Naturally, *Agrobacterium* does not have resistance to kanamycin, so if *Agrobacterium* has been transformed with plasmids, it will have resistance to kanamycin. Unlike *E. coli*, the LBA4404 strain of *Agrobacterium* is rifampicin-resistant, so the antibiotic is given to prevent other bacteria from growing (Tzfira and Citovsky 2006) (Figure 5).

Inoculation on a medium containing an antibiotic as a selective agent reveals the growth of *Agrobacterium* colonies. This result showed the success of the transformation with the electroporation method, and its high effectiveness was characterized by the number of colonies that grew. The *Agrobacterium* colony is yellowish-white and tends to be transparent (van Montagu and Zambryski 2013). In addition, a good colony is solid and does not mix with other colonies. This is because colonies that grow too tightly create a risk of contaminanting bacteria.

The molecular analysis and electrophoresis were carried out using HPTII primer to reconfirm the transformation of the plasmid into *Agrobacterium* because in the pRGEB32 plasmid, there is a selectable marker, hygromycin (Xie et al. 2014) (Figure 6). HPT II primer amplified DNA fragments along 545 bp (Nopitasari et al. 2020). The samples used were pRGEB32::UGT.sgRNA1 and sgRNAII-transformed *Agrobacterium* colonies, with 5 samples each, and controls in the form of pRGEB32 plasmids carrying targeted sgRNAs targeting UGTs. The amplification of DNA with a length of 545 bp in all samples, including the control, so that the plasmid positively transforms the *Agrobacterium* colony (Figure 6). The outcomes of *Agrobacterium*-mediated transformation were subsequently tested across rice varieties, reflecting genotype responses.

Table 4. Confirmation of CRISPR-edited colonies via Sanger sequencing using three sgRNA target constructs

Code	Clone ID	sgRNA targeted	Strand	Sequence mismatch	sgRNA-integrated plasmid
A	WT pRGEB32	-	F	-	-
B	Colony 1	UGTs	F	-	+
C		sgRNA I	R	-	+
D	Colony 2	UGTs	F	-	+
E		sgRNA I	R	-	+
F	Colony 3	UGTs	F	-	+
G		sgRNA I	R	-	+
H	Colony 1	UGTs	F	-	+
I		sgRNA II	R	-	+
J	Colony 2	UGTs	F	-	+
K		sgRNA II	R	-	+
L	Colony 3	UGTs	F	-	-
M		sgRNA II	R	-	-
N	Colony 1	UGTs	F	-	-
O		sgRNA III	R	-	-
P	Colony 2	UGTs	F	-	+
Q		sgRNA III	R	-	+
R	Colony 3	UGTs	F	-	+
S		sgRNA III	R	-	+

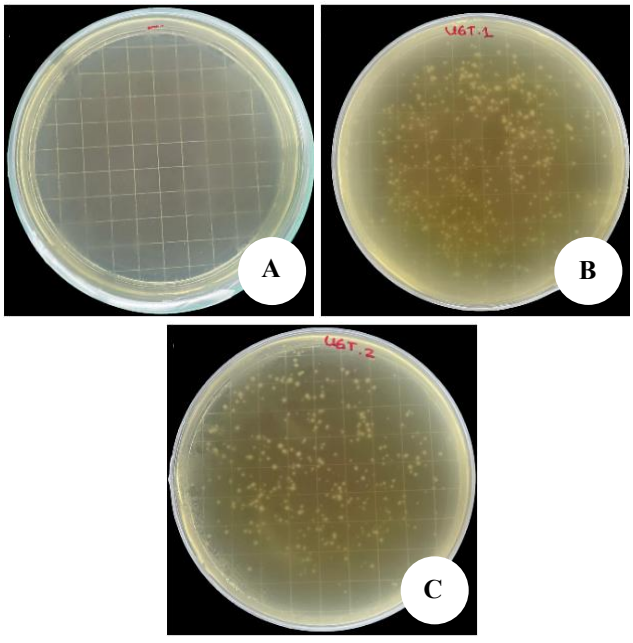

Figure 5. Confirmation of the transformation of *Agrobacterium* strain LBA4404. A. Control, B. pRGEB32::sgRNA 1, C. pRGEB32::sgRNA 2 and showed the presence of colonies in *Agrobacterium* transformed with sgRNA-targeted plasmids

Transformation of rice callus

Prior to transformation into callus rice, *Agrobacterium* was cultured in liquid LB medium supplemented with 50 mg L^{-1} rifampicin and kanamycin. The purpose of suspension culture is to multiply *Agrobacterium* to a specific population density for genetic transformation (Jagadeesh et al. 2022). Colonies of *Agrobacterium* from solid media are taken and suspended in liquid medium for 48 hours. The density used in the *Agrobacterium* suspension is 0.5-1. The density of the *Agrobacterium* can determine the success of the transformation. This is because a too-dense callus can cause stress, while a low-density callus can reduce the rate of *Agrobacterium* infection. *Agrobacterium* in suspension medium for 2 days has an average OD of 0.8 and ready for infection of rice calluses.

Three Indonesian local rice varieties are used to transform into rice calluses, namely Merah Wangi, Pandan Wangi, and Pontianak (Figure 7). The use of local varieties is based on the regenerative power of calluses, which can form plantlets. Different genotypes also play a crucial role in callus induction and plant regeneration (Mostafiz and Wagiran 2018). Nabilah et al. (2022) reported that Pandan Wangi variety has a callus induction percentage of 90% and regeneration of 80%, whereas Dikari (2024) presented a callus induction and regeneration percentage of 100% (Dikari 2024). In Pontianak variety, the callus induction percentage is 98.67% and regeneration is 68.75% (Choirunnisa 2024). In contrast, Merah Wangi consistently showed difficulty in callus formation and lower regeneration capacities (Arisandi et al. 2020). A good callus for transformation is yellowish-white and crumbly texture, indicating embryogenic and healthy potential (Jagadeesh et al. 2022). The process of callus infection begins by inserting calluses into an *Agrobacterium* suspension containing acetosyringone, which grown in a co-cultivation medium.

The co-cultivation phase is the *Agrobacterium* optimization phase for infecting the callus explant. In this phase, N6-D media is used, supplemented with acetosyringone. Co-cultivation is carried out for 2 days in dark conditions. 2 days of incubation is the optimal time for *Agrobacterium* to carry out infection. An incubation period that is too short results in suboptimal *Agrobacterium*-mediated transformation, whereas excessive incubation leads to *Agrobacterium* overgrowth, which can negatively affect explant regeneration (Tzfira and Hohn 2013). Incubation is carried out in a dark environment, because the process of infection by *Agrobacterium* naturally occurs under dark conditions. Acetosyringone serves as an activation signal for *Agrobacterium*, enabling it to carry out infection (Chauhan et al. 2021). The compound can also promote rapid growth of *Agrobacterium*, leading to overgrowth in explants (Sutradhar and Mandal 2023).

The elimination stage is performed to remove *Agrobacterium* after the co-cultivation stage. Subcultures at the elimination stage were performed using N6-D media supplemented with 500 mg L^{-1} cefotaxime. Adding antibiotics to the media is intended to prevent *Agrobacterium* growth in explants. The elimination phase lasts 7 days and is incubated in a bright place. In this phase, determine the transformed sterile explant and remove the *Agrobacterium*. Additionally, placing induction in a bright location is intended to suppress *Agrobacterium* growth.

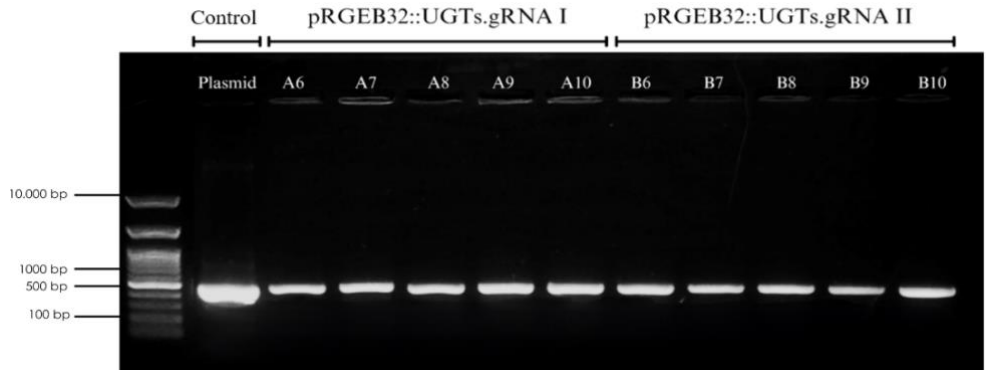

Figure 6. Molecular analysis was carried out using HPTII and confirm the plasmid integration. The entire bacterial colony showed a transformed positive result as indicated by amplification of 545 bp. Lane 1: 1 kb DNA ladder was used as a molecular size marker, Lane 2: Negative control, Lane 3-7: pRGEB32 inserted UGTs.gRNA 1, Lane 8-12: pRGEB32 inserted UGTs.gRNA 2

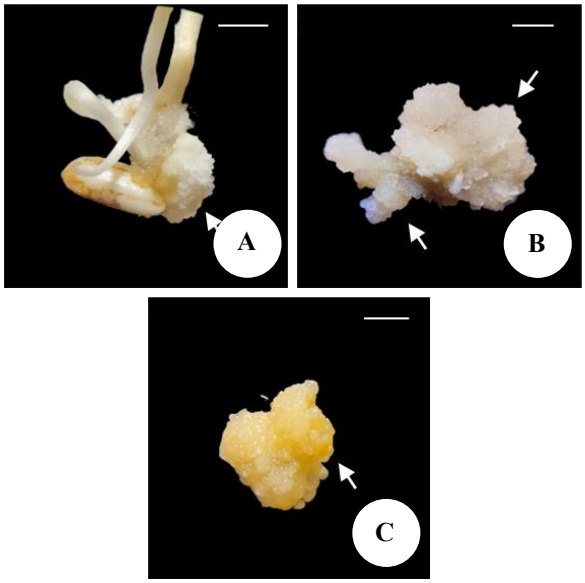

Figure 7. Morphology of callus induction. A. Merah Wangi, B. Pandan Wangi, C. Pontianak on callus induction medium. Scale bar: 1 mm

Table 5. Percentage of explant mortality during the selection and regeneration phase 1

Varieties	Explant quantity	Number of dead explants	Explant mortality percentage (%)
Merah Wangi	124±3.61	77±3	62.1±3.22
Pandan Wangi	37±3	1±0.25	2.7±1.08
Pontianak	43±3.6	14±2.65	32.6±2.24

Note: Values are presented as mean±SD (n=3 biological replicates). Explant mortality was calculated as dead explants × 100% per replicate

The explant elimination phase then enters the selection and regeneration stage after 7 days (Figure 8). In the regeneration phase, it forms a new individual. During the selection and regeneration phase, MS media is used, supplemented with the hormones NAA and kinetin, along with cefotaxime and hygromycin. The use of cefotaxime aims to prevent the growth of *Agrobacterium*, which can be a source of contamination and interfere with the explant regeneration process. Additionally, hygromycin is added to select the transformant callus. This is because the pRGEB32 plasmid contains a hygromycin resistance selection marker. Thus, calli transformed with *Agrobacterium* carrying the plasmid acquire resistance to hygromycin (Xie and Yang 2013). Most explants are browned to death. Explant mortality was assessed two weeks after regeneration, and the variety with the lowest mortality rate and the highest percentage of dead explants was identified. The highest percentage was found in the Merah Wangi variety, with an explant mortality of 62.1%, followed by the Pontianak variety, with 32.6% (Table 5). The death of explants can be caused by a suboptimal *Agrobacterium* infection, leading to non-transformation and susceptibility to hygromycin (Priyadarshani et al. 2019). Explants that have a positive response to selection and regeneration 1 is the Pandan Wangi variety, which continues to grow well.

Confirming UGTs putative transformant via molecular analysis

The regeneration response indicates the presence of putative UGT-transformants among regenerated plantlets derived from the Pandan Wangi, Merah Wangi, and Pontianak varieties. However, the overall regeneration efficiency was low, as most explants failed to regenerate into Plantlets. This variation highlights the strong species- and genotype-dependent (Maren et al. 2022). During the transformation process, three plantlets were regenerated from Pandan Wangi callus (Figure 9). Out of three regenerated plantlets subjected to the hygromycin selection phase, only one plantlet demonstrated resistance and survived on the selection medium, exhibiting a normal green phenotype (Figure 9.A). The two remaining plantlets displayed hygromycin sensitivity symptoms, including chlorosis and growth inhibition, indicating a lack of transgene integration (Figures 9.B and 9.C). Notably, the limited number of suggested transformants (n=3) demonstrated a limited but observable response to the transformation process. Molecular analysis via PCR was subsequently conducted to confirm the presence of the transgene in the surviving hygromycin-resistant plantlet, showing its classification as a putative transformant.

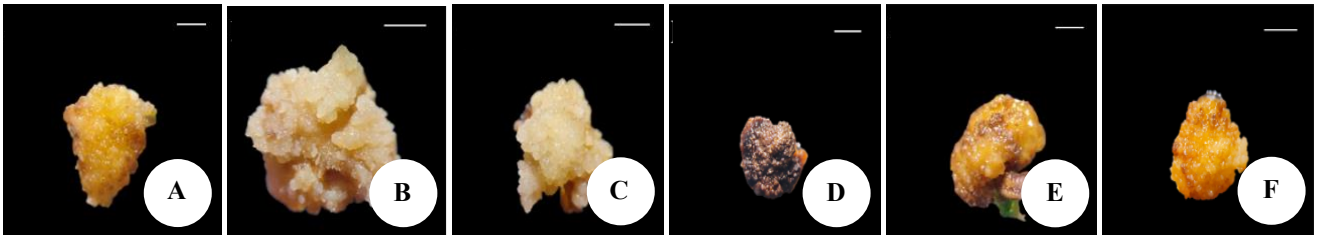

Figure 8. Explant characters in the selection and regeneration phase 1 in the selection medium: A. Merah Wangi, B. Pandan Wangi, C. Pontianak, and Explant mortality in varieties: D. Merah Wangi, E. Pandan Wangi, F. Pontianak

Figure 9. Putative transformant of Pandan Wangi variety obtained from *Agrobacterium*-mediated transformation. A. Hygromicin-resistant putative transformant demonstrated normal green phenotype, B. and C. Non-resistant exhibiting chlorosis, and tissue browning

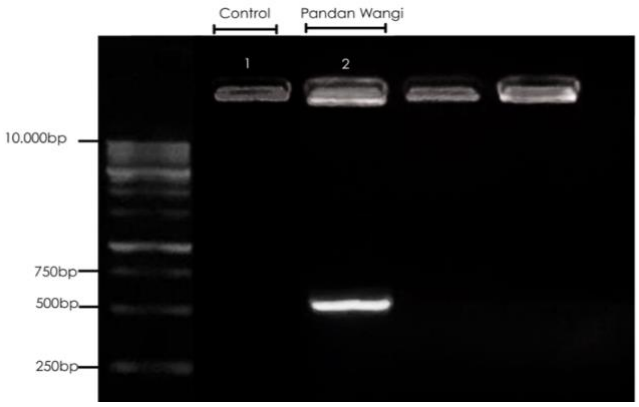

Figure 10. Molecular verification of transgene integration in putative transformant of transgene integration in putative transformant in Pandan Wangi rice varieties. Lane 1: 1 kb DNA ladder was used as a molecular size marker, Lane 2: Negative control, Lane 3: Positive transformant

Regenerated plantlets were cultured on MS medium containing 50 mg L^{-1} hygromycin for 14 days. The putative transformant grew normally on selection media, indicating pRGEB32::UGTs had successfully integrated into the plant genome, and showed resistance to hygromycin. Plantlets 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 depicted no amplification at the target position (545 bp), confirming the absence of transgene integration (Figure 10). In contrast, Pandan Wangi, a putative transformant, showed a 500 bp band, indicating successful T-DNA integration.

In conclusion, this study developed and validated the constructed CRISPR/Cas9 plasmid pRGEB32, which carries three UGT-targeting sgRNAs and was validated in *E. coli* and the *A. tumefaciens* strain LBA4404. The plasmid transformation produced positive *Agrobacterium* colonies, and preliminary trials across three rice callus varieties revealed genotypic variation in survival and selection, with Pandan Wangi showing the highest transformation efficiency. Putative transformants were obtained via hygromycin selection, and transformation responses varied among rice genotypes. These findings highlight a beneficial platform for future studies on rice defense responses and stress-associated secondary metabolism. Future work may verify UGT gene edits in regenerated plants, assess potential off-target effects, and quantify sakuranetin and related flavonoids. Expanding transformation trials across rice genotypes and integrating CRISPR/Cas9 with transcriptomic and metabolomic analyses will enhance understanding of *UGT* function in secondary metabolite biosynthesis and stress response.

From a regulatory and biosafety viewpoint, the future scope of CRISPR/Cas9-edited rice lines is reasonable and needs careful consideration. Although genome editing enables precise genetic modification, the classification of edited lines beyond laboratory and greenhouse settings depends on extensive molecular characterization, evaluation of off-target effects, and on national and international regulatory frameworks. Accurate assessment will be essential to support the responsible development and broader approval of gene-edited rice varieties.

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SUPPLEMENTARY FIGURE

Figure S1. Sanger sequencing validation of CRISPR/Cas9 targeting UGTs gene illustrates high peak at target region. Representative chromatogram: A. Wild-type control that showing target sequence, B. UGTs sgRNA I colony 1 forward strand, C. UGTs sgRNA I colony 1 reverse strand, and D. UGTs sgRNA I colony 2 forward strand