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Step-by-Step Cloning of gRNA-Targeted OsCYP71A1 for Gene Editing Study

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A B S T R A C T

Serotonin, a neurotransmitter in mammals, is also synthesized in rice in response to insect infestation. However, this serotonin production enhances susceptibility to two of the most damaging rice pests-planthoppers and stem borers. The cytochrome P450 gene CYP71A1 in rice encodes tryptamine 5-hydroxylase, the enzyme that converts tryptamine into 5-hydroxytryptamine (serotonin). In wild-type rice, herbivory by planthoppers triggers the production of serotonin and salicylic acid. In contrast, CYP71A1 mutants lacking serotonin biosynthesis exhibit elevated levels of salicylic acid and display enhanced resistance to insect attack. To investigate the role of CYP71A1 in pest resistance, we aimed to disrupt this gene using the CRISPR-Cas9 gene editing system. To achieve targeted modification of the OsCYP71A1 gene, we utilized the CRISPR/Cas9 system, specifically designing a 20-bp gRNA to maximize on-target precision while mitigating potential off-target effects. This gRNA was integrated into the pRGEB32 binary vector at the BsaI restriction sites. In this construct, both the codon-optimized Cas9 and the gRNA scaffold are driven by the rice ubiquitin promoter to ensure robust expression. Following ligation, the recombinant plasmids were transformed into *E. coli* TOP10. We confirmed successful transformants through a combination of colony PCR and restriction enzyme digestion, with the final gRNA orientation and sequence integrity being verified via Sanger sequencing

Keywords

Brown Plant Hooper (BPH), CYP71A1, gRNA, Plant Gene Editing

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Introduction

In Southeast Asia, particularly Malaysia, rice remains the primary staple food. However, the industry faces an uphill battle to meet the demands of a growing global population while grappling with land scarcity, climate change, and abiotic stresses (Atabaki *et al.,* 2018). Beyond these environmental hurdles, insect infestations specifically the rice brown planthopper (BPH; Nilaparvata lugens Stål) inflict devastating yield losses. While chemical pesticides are the traditional line of defense, their environmental toxicity and lack of long-

term viability have necessitated more sustainable, biotechnological interventions.

One such intervention involves the precise manipulation of plant defense pathways. Interestingly, recent findings have highlighted the role of the CYP71A1 gene in rice, which encodes tryptamine 5-hydroxylase, an enzyme that converts tryptamine into serotonin. While serotonin is a vital hormone in many organisms, in rice, it appears to act as a "double-edged sword." Lu *et al.,* (2018) demonstrated that BPH infestation actually induces CYP71A1 expression, leading to a serotonin build up that unexpectedly benefits the pest; in fact, serotonin supplementation in artificial diets has been shown to improve BPH survival. Conversely, suppressing serotonin biosynthesis through CYP71A1 mutation has been found to significantly bolster rice resistance, making the silencing of this gene a highly promising strategy for crop protection. While traditional methods like gamma-ray irradiation can induce mutations (Lu *et al.,* 2018), the CRISPR/Cas9 system offers unparalleled precision. This system utilizes a custom-designed guide RNA (gRNA) to direct the Cas9 nuclease to a specific genomic locus, in this case, the OsCYP71A1 gene creating a double-strand break (DSB). The subsequent, often error-prone cellular repair mechanism typically results in site-specific mutations that effectively knock out gene function (Nopitasari *et al.,* 2020).

In this study, we focused on the foundational step of this gene-editing process, the construction of a specialized expression vector. We designed a specific crRNA targeting OsCYP71A1, which was then cloned into the pRGEB32 binary vector. The cloning strategy involved the ligation of the target sequence into the vector backbone, followed by transformation into a bacterial host. Using antibiotic selection markers, we were able to isolate successful recombinant cells, ensuring that only colonies harboring the OsCYP71A1 construct were carried forward for downstream analysis.

Materials and Methods

Plasmid Extraction

Plasmid extraction of pRGEB32 was performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA). Bacterial cultures were first harvested by centrifugation in an Eppendorf centrifuge at 5,000 rpm for 5 minutes at 25°C to collect the cell pellet, discarding the supernatant. The cell pellet was resuspended by adding 250 µL of resuspension solution, followed by the addition of 250 µL of lysis solution and 350 µL of neutralization solution to the tube. The tubes were then centrifuged in a microcentrifuge at 13,000 rpm for 5 minutes. The resulting supernatants were collected and transferred to a Spin Column provided by the kit. The assembly was centrifuged at 13,000 rpm for 1 minute, followed by two successive wash cycles using 500 µL of wash solution to ensure purity. In each step, the flow-through was discarded after a 1-minute spin. To prevent any ethanol or buffer carryover, the empty column was subjected to an additional 1-minute dry spin before being transferred to a clean microcentrifuge tube. For elution, 30 µL of nuclease-free water was applied directly to the membrane and allowed to incubate for 5 minutes at room temperature to maximize yield. The purified plasmids were then recovered via a final 2-minute centrifugation and stored at -20°C for subsequent applications.

Plasmid Digestion

Plasmid digestion was carried out according to the formulations specified in Table 1, with the reaction mixture incubated at 37°C for at least one hour using a MJ Mini Personal Thermal Cycler (BIO RAD, USA). To resolve the digested products, the mixture was subjected to 1% agarose gel electrophoresis, operated at 80V for 30 minutes. The target DNA fragment was subsequently recovered using the QIAquick Gel Extraction Kit (QIAGEN, USA). The purification process involved dissolving the excised gel slice in three volumes of QG Buffer at 50°C for 10 minutes, followed by the addition of one volume of isopropanol to enhance DNA binding. This mixture was then loaded onto a QIAquick spin column and centrifuged for 1 minute, with the flow-through being discarded before proceeding to the subsequent wash steps. This centrifugation step was repeated for samples exceeding 800 µL. To wash the column, 750 µL of Buffer PE was added, and the column was centrifuged for 1 minute. The flow-through was discarded, and the column was centrifuged for an additional minute to ensure the removal of any residual wash buffer. For elution, the QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. The plasmid was eluted by adding 30 µL of water, followed by a 5-minute incubation period before the final centrifugation. The concentration of the purified, digested plasmid was subsequently measured using a Thermo Fisher Scientific Qubit 4 Fluorometer.

Ligation of crRNA OsCYP71A1 into digested pRGEB32

Ligation was performed using a 3:1 insert: vector molar ratio. The required components, detailed in Table 2, were combined in a PCR tube. The insert, crRNA OsCYP71A1, is a 20 bp of forward and reverse primer in primer-dimer form. For ligation into the pRGEB32 vector at the *Eco31L* site (Figure 1), specific overhangs were added: GGCA to the 5' end of the forward crRNA and AAAC to the 5' end of the reverse crRNA. The mixture was then incubated overnight (15 hours) at 16°C in a MJ Mini Personal thermal cycler (BIO RAD, USA) at 16°C. Following incubation, the clone was then proceeded for bacterial transformation using *E. coli* strain TOP10.

Construct Conformation

The ligated gRNAs were transformed into TOP10 competent cells using the heat shock method. Following transformation, the bacteria were plated on LB agar containing Kanamycin (50 mg/L) and incubated overnight at 37°C. Putative positive clones growing on the kanamycin plates were confirmed by colony PCR. This involved using a forward primer specific to the pRGEB32 vector (pRGEB32-F: 5'-GGTGCTACCAG CAAATGCTGGAAGCCG-3') and a reverse primer corresponding to a 20bp sequence of the cloned gRNA (crRNA-R: 5'-GGGATGCGCTGGCGGTTGCC-3'). Positive colonies were subsequently cultured in LB liquid medium supplemented with kanamycin (50mg/mL) for plasmid extraction. Cloned pRGEB32 was extracted from the positive bacterial cultures, and the concentration was measured using a fluorometer. Double digestion (Table. 5) was conducted after extraction for at least an hour at 37°C. Agarose gel electrophoresis was performed using 1% gel for 27 minutes at 80V. The positive cloned pRGEB32 was sequenced to confirm the nucleotide sequences.

Results and Discussion

Digestion of pRGEB32 vector

The initial phase of this study focused on the extraction and purification of the pRGEB32 plasmid to ensure high-quality starting material for downstream genetic manipulation. To prepare the vector for cloning, the circular pRGEB32 plasmid was linearized through restriction digestion using the Type IIS endonuclease, *Eco31I*. This enzyme was selected for its ability to cleave outside its recognition sequence, generating specific sticky ends that facilitate directional ligation of the target insert (Figure. 4). The efficiency of the digestion was monitored via agarose gel electrophoresis. As shown in Figure 2, the transition from circular to linearized DNA was successful across all samples, with no detectable uncut plasmid remaining. The corresponding bands were then excised and purified from the agarose matrix to remove enzymes, salts, and non-specific fragments. Following purification, the linearized product was quantified, yielding a concentration of 91.0 ng/µL. This concentration provided sufficient high-purity DNA for the subsequent ligation and transformation stages.

Cloning of crRNA OsCYP71A1

To optimize ligation efficiency, the molar ratio between the *OsCYP71A1* crRNA fragment (insert) and the linearized pRGEB32 (vector) was carefully calculated. We employed a 3:1 insert-to-vector ratio, following the optimization guidelines provided by Promega (Figure 3). In our preliminary trials, this 3:1 ratio consistently outperformed other tested ratios, producing a significantly higher yield of recombinant colonies. This increased colony density was vital for streamlining the subsequent transformation and screening processes, ensuring a higher probability of identifying positive clones.

In the ligation reaction, the amount of digested pRGEB32 was standardized at 300 ng, requiring the addition of 3.3 µL of the purified vector stock into the mixture. The *OsCYP71A1* crRNA insert was then ligated into the linearized backbone, utilizing the complementary sticky ends generated during the *Eco31I* digestion. This enzymatic assembly was mediated by T4 DNA ligase to establish the covalent phosphodiester bonds necessary for a stable recombinant construct (Figure 4). The resulting plasmid was subsequently used for transformation into competent cells to allow for further amplification and functional validation of the gene-editing target.

Bacterial Transformation by Electroporation

To achieve high transformation efficiency, electroporation was employed using 2 µL of the cloned pRGEB32 plasmid. This minimal volume was maintained to prevent electrical arcing within the cuvette

a risk often exacerbated by high salt concentrations or air bubbles. We utilized commercially sourced, high-efficiency *E. coli* TOP10 competent cells specifically prepared for electroporation to ensure a low-conductivity environment. While heat-shock transformation remains an alternative, electroporation was selected here for its superior efficiency in handling larger binary vectors like pRGEB32.

Following the pulse, transformants were screened under selective pressure on LB agar supplemented with kanamycin. The appearance of numerous distinct colonies (Figure 5) confirmed the successful uptake of the recombinant plasmid and the expression of the kanamycin resistance marker. These results provided preliminary evidence of the successful integration of the gRNA cassette targeting *OsCYP71A1*, with these colonies being selected for further molecular validation.

Validation of positive pRGEB32 clones through molecular screening

To identify which colonies successfully carried the pRGEB32 vector, we performed a colony PCR by lysing the bacteria to access their genetic material for amplification. Since the target crRNA osCYP71A1 sequence is only 20 base pairs long, making it notoriously difficult to spot on its own, we used a strategic primer pair to confirm its presence. We anchored the forward primer to the vector backbone and designed the reverse primer specifically for the crRNA sequence itself.

This approach produced a distinct, 200-base pair band on the agarose gel (Figure 6); because our primers were so specific to that exact junction, the appearance of this single band served as definitive proof that the crRNA osCYP71A1 had been successfully integrated.

To double-check our results, we performed a double digestion on the suspected positive clones and compared them to a control version of the pRGEB32 vector. On the agarose gel, the difference was clear which the positive clones produced a single, distinct band, while the control yielded two separate bands (Figure 7). The reason for this lies in the restriction sites for Eco31I (BsaI) and HindIII. In its original state, the vector has functional sites for both enzymes. When we digested the control, both enzymes cut the circular plasmid, snapping it into two unequal linear fragments, hence the two bands. However, during the initial preparation for cloning, we used Eco31I to linearize the vector and create the sticky ends needed for our insert. This process effectively "destroys" the Eco31I recognition site.

Therefore, when we ran the double digestion on our successful clones, only the HindIII site remained functional. With only one site available to be cut, the circular plasmid was simply opened into a single linear strand, resulting in the lone band we observed.

The successfully cloned pRGEB32 behaved quite differently under the same conditions. Because the Eco31I site was intentionally destroyed when we prepared the vector for its insert, the "double digestion" actually functioned as a single digest. With only the HindIII site left intact to be cut, the enzymes could only cleave the plasmid once, turning the circular DNA into a single, full-length linear strand.

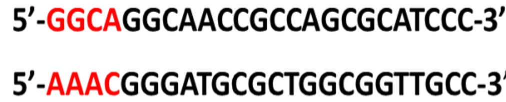
This resulted in the lone, distinct band we observed on the gel (Figure 8). The absence of a second band is a powerful indicator; it unequivocally confirms that the Eco31I site is gone, providing solid molecular evidence that the crRNA osCYP71A1 sequence was successfully integrated exactly where it belongs.

The final and most definitive confirmation of our cloning success came from DNA sequencing of Clones 4 and 5. By analyzing the complete nucleotide sequence, we were able to verify that the crRNA osCYP71A1 was not only precisely integrated into the pRGEB32 vector but also maintained perfect sequence fidelity (Figure 9). We used BioEdit to align and compare the sequences of the original vector, the crRNA, and our recombinant construct (Figure 10).

Seeing a 100% match at the molecular level provided the ultimate validation, giving us the green light to move forward with the next stage of the study.

In conclusion, the success of this experiment confirms that we have successfully cloned the specific guide RNA (crRNA) targeting the osCYP71A1 gene into the pRGEB32 expression vector. This milestone is more than just a technical win, it is the vital first step in establishing a precise genome editing system. By securing this confirmed clone, we have laid the necessary groundwork to develop a reliable and efficient method for targeted mutagenesis, ultimately contributing to the broader fields of rice genomics and crop biotechnology.

Figure.1 A crRNA of OsCYP71A1 in primer-dimer form. Red bases show the added overhangs to be utilized in the cloning process into the pRGEB32.



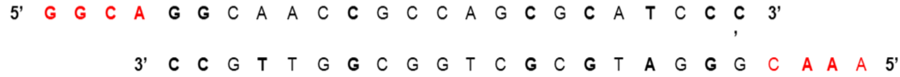


Figure.2 The pRGEB32 vector was successfully linearized using the *Eco31I* restriction enzyme to facilitate gRNA integration

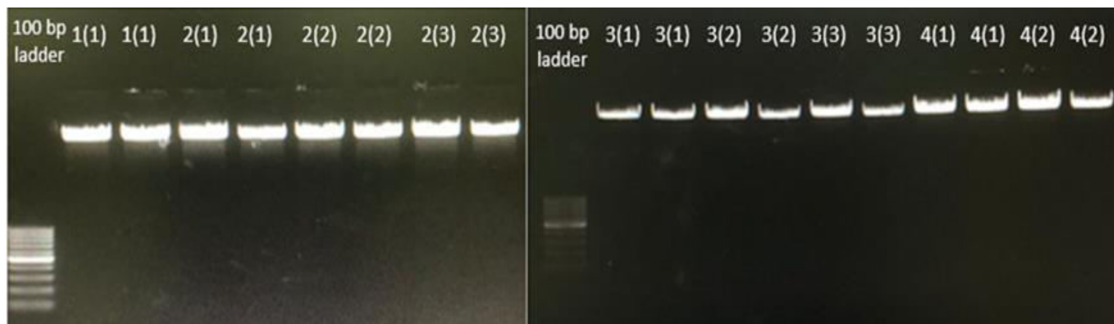


Figure.3 Optimization of the insert: vector molar ratios

$$\left(\frac{\text{ng of vector} \times \text{bp size of insert}}{\text{bp size of vector}}\right) \times \frac{3}{1} = \text{ng of insert}$$

Figure.4 Single-stranded overhangs produced after restriction digestion, ensuring precise and directional integration of the crRNA insert into the pRGEB32 backbone.

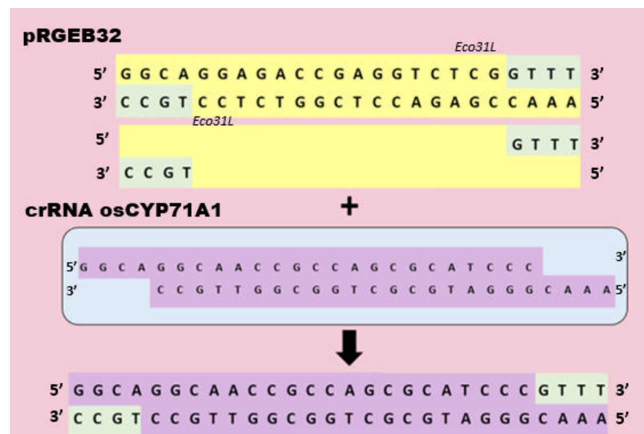


Figure.5 Representative plate of *E. coli* TOP10 transformants on LB agar supplemented with kanamycin following electroporation

Figure.6 Colony PCR analysis to conform the resulting colonies were carrying the correct pRGEB32 constructs

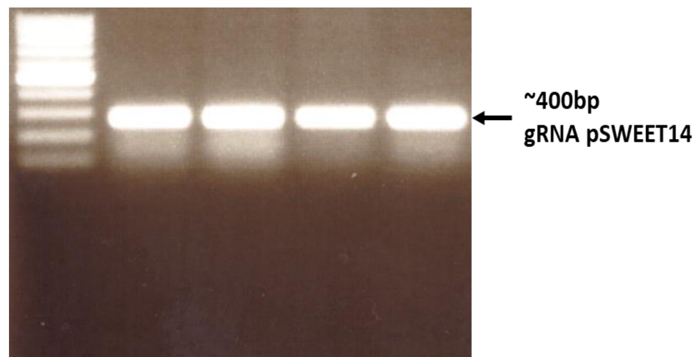


Figure.7 Restriction enzyme analysis to verify that the gRNA had been successfully integrated into the vector.

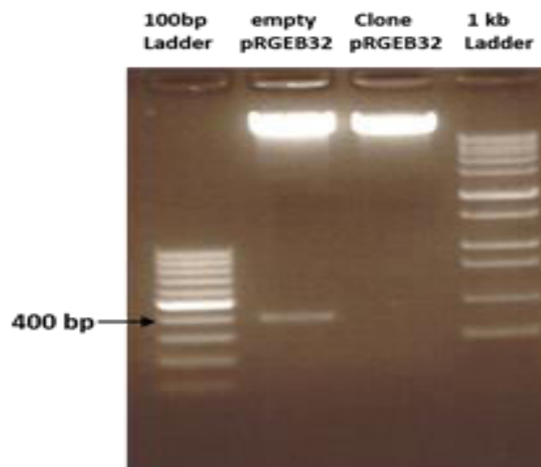


Figure.8 Observation of a single DNA band following double digestion indicating successful gRNA integration

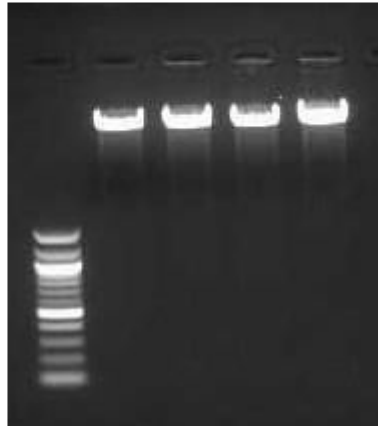


Figure.9 DNA sequencing for Clones 4 and 5, confirming the seamless insertion of the crRNA osCYP71A1 into the pRGEB32 vector.

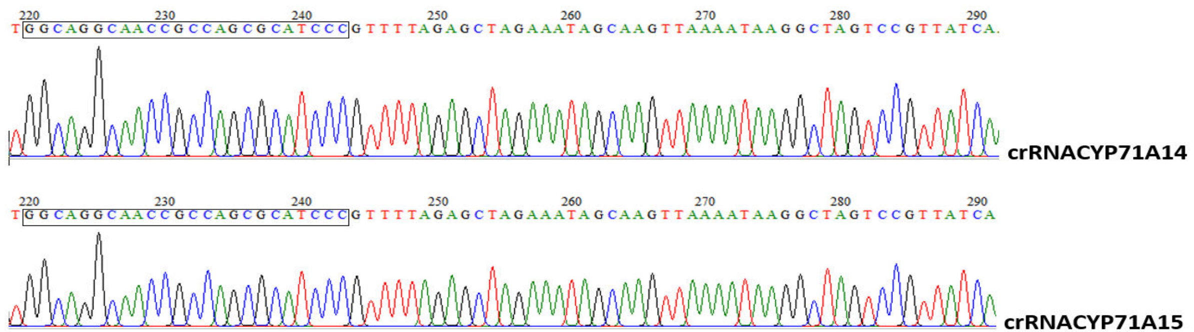


Figure.10 Sequence alignment showing the successful integration of the crRNA into the pRGEB32 backbone, verified against the empty vector control

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pRGEB32       TGATCCGTGGCA:GGAG:ACCGA::GGTCTCGGTTTTAGAGCTAGAAATAGCAAGTTAAA
gRNA:CYP71A1  TGATCCGTGGCAGGCAACCGCCAGCGCATCCCGTTTTAGAGCTAGAAATAGCAAGTTAAA
gRNA          ------------GGCAACCGCCAGCGCATCCC----------------------------
```

Table.1 Plasmid digestion of pRGEB32 vector

Components	Volume (µL)	Reaction Conditon
10x Fast Digest Green Buffer	5	
pRGEB32	4	
Fast Digest *Eco31L*	5	• 37°C Incubation for 2 h
Water	36	
Total	50	

Table.2 Ligation Reaction of crRNA into digested pRGEB32 at a molar ratio of 3:1

Components	Volume	Reaction Condition
Vector (pRGEB32)	3.3 µl (300 ng)	
Insert (crRNA osCYP71A1)	0.8 µl (1.2 ng)	
Buffer	2 µl	• 16°C Incubation for overnight
T4 DNA Ligase	1 µl	
Water (topup to 20 µL)	12.9 µl	
Total	20 µl	

Table.3 PCR Components for Construct Conformation Analysis

Components	Volume (µL)
1.5 mM $MgCl_2$	0.6
Green Buffer	2
dNTP mix	1
Forward primer (10 µM ospRGEB32)	0.2
Reverse primer (10 µM crRNA osCYP71A1)	0.2
Taq polymerase	0.25
Water	5.75
Total	10

Table.4 Components of Restriction Enzyme for Double Digestion

Components	Volume (µL)
10x Fast Digest Green Buffer	2
Cloned pRGEB32/pRGEB32	2
Eco31L	1
HindIII	1
Water	14
Total	20

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Author Contributions

Nur Suhadah Abdul Kahar: Conducting research and analyzed the gene. Ng Jing Xuan: Conducting research and analyzed the gene. Fatin Athirah Mustaffa: Conducting research and analyzed the gene. Zuraida Ab Rahman: Providing ideas and assisting in writing

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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