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Construction of the CRISPR/Cas9 module and genetic transformation of aromatic rice cv. Mentik Wangi for developing bacterial leaf blight resistance

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Abstract. *Rifhani NF, Apriana A, Sisharmini A, Santoso TJ, Trijatmiko KR, Slamet-Loedin IH, Yunus A. 2023. Construction of the CRISPR/Cas9 module and genetic transformation of aromatic rice cv. Mentik Wangi for developing bacterial leaf blight resistance. Biodiversitas 24: 3258-3268.* Rice cv. Mentik Wangi is a local aromatic rice that is susceptible to pests and diseases, such as bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* (Xoo). This bacteria can cause damage to plants thereby reducing crop yields. This study aimed to obtain the CRISPR/Cas9 module construct as well as introducing the construct into rice cv. Mentik Wangi for developing BLB resistance. The manufacture of the CRISPR/Cas9 module was carried out using the Golden Gate method, and the introduction of that construct into rice cv. Mentik Wangi was carried out using *Agrobacterium tumefaciens*. Constructing a CRISPR/Cas9 module with multiple gRNA of OsSWEET11 and OsSWEET14 genes has succeeded, 129 putative transformant lines of T0 generation produced with the regeneration and transformation efficiency values are respectively 9.4% and 9.8%. The results showed that 36 lines were positive for the *hpt* gene, indicating that the CRISPR/Cas9-gRNAOsSWEET module construct was successfully entered into rice cv. Mentik Wangi. Further analysis is needed to identify mutagenesis in the target gene region of TI generation transgenic plants as well as phenotypic tests for BLB resistance.

Keywords: Bacterial leaf blight, CRISPR/Cas9, Mentik Wangi, SWEET gene, transformation

INTRODUCTION

Indonesia has several rice varieties that are quite popular among the people, such as Rojolele, Pandan Wangi, Cianjur, and Mentik Wangi. Local varieties are a priority for farmers in growing rice due to being very popular with consumers. Mentik Wangi is an aromatic local rice variety originating from Central Java which has a distinctive aroma with a very fluffier rice texture (Yulianto 2017). However, rice cv. Mentik Wangi is susceptible to pests and diseases, such as bacterial leaf blight (BLB). BLB is a devastating disease to farmers because it can infect rice at various growth stages. BLB symptoms are marked by spots on the leaves which then developed and the leaves turn pale yellow in color (Khaeruni et al. 2014). BLB in rice is spread in various rice-producing countries in Asia and Africa, including Indonesia. BLB is caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (Xoo) and this disease can affect rice yield loss to 36% (Wahyudi et al. 2011).

Xoo bacteria will induce the SWEET (Sugar Will Eventually be Exported Transporters) gene in the host plant to support its growth. SWEET gene involved in plant growth and development are passive sugar transporters family genes involved in regulating the transport of oligosaccharides, such as glucose or sucrose across cell membranes (Chen et al. 2015). Induction of SWEET gene expression by Xoo is expected to trigger the release of sugar into the apoplast, so that this sugar will be used as a source of nutrition for pathogens for their survival needs and propagation (Chen 2014; Cohn et al. 2014; Chen et al. 2015; Hu et al. 2021). Xoo will produce specific effector molecules called transcription-activator-like effectors (TALes) which will bind to effector binding elements (EBEs) in the promoter section of the SWEET gene in host plants. At least, there are 3 members of the clade III SWEET gene family, namely SWEET11, SWEET13, and SWEET14, that are involved in the interaction between plants and pathogens (Oliva et al. 2019; Hu et al. 2021). Mutations in the EBEs portion of the SWEET gene promoter can increase rice resistance to the pathogenic strain Xoo without experiencing phenotypic changes (Blanvillain-Baufumé et al. 2017; Oliva et al. 2019).

Targeted mutagenesis at the EBEs in the promoter of the SWEET gene to improve rice resistance to Xoo pathogens can be carried out by applying genome editing technology (Gayatonde and Vennela 2016). According to Duy et al. (2021), the Vietnamese rice cultivar TBR225 has improved bacterial leaf blight disease resistance by editing the OsSWEET14 promoter. Genome editing technology that is developing rapidly, especially in rice is the CRISPR/Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeat Associated protein 9 nuclease). In its system, CRISPR/Cas9 has two main components, there are Cas9 which function to cut DNA sequences and sgRNA (single guide RNA) which plays a role in guiding Cas9 to the region of cutting the target DNA sequence correctly (Schaeffer and Nakata 2015).

The CRISPR/Cas9 system has the advantages of specifically cutting target sequences, being efficient in mutation, being able to be used for multiplex genome editing, and being simple in construction. The application of genome editing is carried out by modifying the genome through genetic transformation specifically directed at the target gene (Shan et al. 2013). Genetic transformation method that is quite popular is with the help of the bacterium *Agrobacterium tumefaciens*, because it is known to be more efficient than conventional breeding, easy to handle, and generally produces transformants with a low number of inserted gene copies (Sah et al. 2014b). Therefore, this study aimed to obtain the CRISPR/Cas9 module construct as well as introducing the construct into rice cv. Mentik Wangi for developing BLB resistance.

MATERIALS AND METHODS

Research site

The research was carried out from June 2021 to October 2022 at the Molecular Biology Laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (BB Biogen), Bogor. The pDIRECT_25H vector plasmid was used as the carrier for the CRISPR/Cas9_gRNA_OsSWEET module (OsSWEET11 and OsSWEET14) (Čermák et al. 2017). Immature embryo at the age of 12-14 days after anthesis of rice cv. Mentik Wangi grown in the greenhouse was used as a source of explants for genetic transformation (Slamet-Loedin et al. 2014).

Construction of the CRISPR/Cas9-gRNAOsSWEET module gRNAOsSWEETprimer design

The guide RNA (gRNA) was designed based on the EBEs (Effector-Binding Elements) motif in the promoter region of the SWEET11 and SWEET14 genes. The EBEs sequence used as a reference for designing gRNA primers refers to Oliva et al. (2019) (Table 1).

Assembly of gRNAs into the pDIRECT_25H vector

The assembly of gRNAOsSWEET into the pDIRECT_25H vector was carried out using multiple gRNAs into the pDIRECT_25H expression vector by cloning. The assembly of the CRISPR/Cas9-gRNAOsSWEET module construct was carried out using multiple gRNAs into the pDIRECT_25H expression vector by cloning. The pDIRECT_25H vector used for construct assembly was obtained from Addgene. The T-DNA portion of the pDIRECT_25H vector contains the markers of the hygromycin selection gene (*hpt* gene) which encodes for hygromycin phosphotransferase and the Cas9 gene which encodes the Cas9 endonuclease enzyme. Construction of gRNA into the pDIRECT_25H vector was carried out by PCR technique which consisted of 3 reactions. Before carrying out the PCR, the pDIRECT_25H vector was cut with the restriction enzyme BanI to remove the ccdb gene that was not needed in the construct assembly. The cut vector was used for the PCR template for reaction 1. There were 3 PCR reactions used, i.e., reaction 1 used the promoter primers PvUbi1 and CSY gRNA OsSWEET11, reaction 2 used primer REP gRNA OsSWEET11 and CSY gRNA OsSWEET14, and reaction 3 used primer REP gRNA OsSWEET14 and CSY terminators (Table 2). Each PCR reaction mixture used 12.5 µL of DNA polymerase Q5 Hotstart High Fidelity 2X Master Mix, 2.5 µl primer, 1 µL plasmid pDIRECT_25H, and 6.5 µL NF with a total volume of 25 µL. The PCR program begins with a pre-denaturation stage at 98°C for 1 minute, followed by 30 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 60°C for 15 seconds, an extension stage at 72°C for 15 seconds, and a final extension at 72°C for 2 minutes. Fragments of the desired size 2000 bp of reaction 1 and 136 bp of reaction 2 and reaction 3 were purified using the Zymoclean™ Gel DNA Recovery Kit.

gRNA ligation to expression vectors

The Golden Gate method was carried out in 2 stages which are operated using PCR. In the first stage, the reaction consists of fragments resulting from the purification of reactions 1-3 with 1 µL of reaction 1 each; 1.5 µL reaction 2; and 1 µL reaction 3, 1 µL AarI enzyme, 10 µL T7 DNA ligase buffer 2X, 1 µL 50X oligonucleotide, 1 µL T7 DNA ligase, 1 µL ATP, and 2.5 µL nuclease-free water (NFW) with a total volume of 20 µL. The PCR program at this stage consisted of 10 cycles at 37°C for 5 minutes (the optimum temperature dan time for the AarI enzyme to cutting the fragment), 25°C for 10 minutes (the optimum temperature and time for the T7 DNA ligase to ligate the fragments), and hold 4°C.

The first Golden Gate PCR results were then diluted with a ratio of 1:80 and used as a template for the PCR process to obtain a fragment with a size of 2238 bp. The PCR reaction consisted of 2 µL template (Golden Gate PCR reaction stage 1), 12.5 µL Q5 Hotstart High Fidelity 2X Master Mix, 2.5 µL PvUbi1 promoter, 2.5 µL terminator, and 5.5 µL NFW which had a volume a total of 25 µL. The PCR cycle proceeded with pre-denaturation at 98°C for 1 minute, followed by 30 cycles consisting of denaturation at 98°C for 10 seconds, annealing at 60°C for 15 seconds, elongation at 72°C for 15 seconds with the final elongation at 72°C for 2 minutes.

The PCR amplicon was purified from agarose gel using Zymoclean™ Gel DNA Recovery Kit to obtain a gene module fragment consisting of the PvUbi1_gRNA-

OsSWEET11_gRNA-OsSWEET14_terminator-35SCaMV promoter. Meanwhile, the pDIRECT_25H expression vector was cut using the restriction enzyme SapI to facilitate the attachment of the gene module fragment to the expression vector in the Golden Gate reaction stage 2. The pDIRECT_25H fragment that had been cut was then purified and then connected to the gene module fragment using the PCR technique as a stage 2 Golden Gate reaction. The reaction was made with a total reaction volume of 25.5 µL consisting of 3 µL template (gene module fragment), 7 µl pDIRECT_25H vector (digested using SapI), 12.5 µL T7 DNA ligase 2X, 1 µL T7 DNA ligase, 1 µL of SapI enzyme, and 1 µL of ATP. This reaction was carried out using a PCR machine consisting of 10 cycles at 37°C for 5 minutes (the optimum temperature and time for the SapI enzyme to cut), 25°C for 10 minutes (the optimum temperature and time for the T7 DNA ligase to ligate the fragments) followed by holding at 4°C.

Bacterial transformation of DNA recombinant to *Escherichia coli*

Escherichia coli transformation was carried out using the heat-shock method (Higo et al. 1999; Chang et al. 2017). As much as 10 µL of the Golden Gate reaction was mixed with 100 µL of DH5α competent *E. coli* using a microtube. The reaction mixture was kept on ice for 30 minutes and then incubated at 42°C for 45 seconds. The reaction mixture was kept back on ice for 5 minutes. 500 µL of liquid LB medium was added to the mix, then incubated at 37°C for 3 hours in an incubator shaker. After incubation, the bacterial suspension was centrifuged using a microcentrifuge for 2 minutes at 8000 rpm. The centrifuged supernatant was discarded 450 µL of the total volume. The pellets were resuspended and spread on solid LB medium added with 50 mg/L kanamycin. The bacteria in the spread were then incubated overnight at 37°C. Confirmation of single colony *E. coli* was carried out by PCR technique using specific primers TC306 and M13F (Čermák et al. 2017) (Table 3). A single colony that was confirmed to have the expected construct fragment size of 696 bp was grown in liquid LB media containing the antibiotic kanamycin for further isolation to obtain a recombinant plasmid. Recombinant plasmid verification was carried out by DNA sequencing analysis.

Transformation of the recombinant vector to *Agrobacterium tumefaciens*

Transformation of the recombinant vector into *Agrobacterium tumefaciens* was carried out using an electroporator (www.bio-rad.com). *A. tumefaciens* strain LBA4404 competently mixed with 5 µL of recombinant plasmid. The mixture was transferred to an electroporation cuvette and incubated on ice for 15-20 minutes. Furthermore, the cuvette is placed on the slide chamber on the electroporator machine. After that, 1 mL of liquid YEP medium was added to the cuvette and resuspended. The suspension was transferred to a tube and then incubated at 30°C for 3 hours at 200 rpm. The suspension was centrifuged at 10000 rpm for 1 minute and the resulting supernatant was then discarded as much as 800 µL of the total volume. The remaining supernatant volume was resuspended and spread on solid YEP media (with the addition of 50 mg/L kanamycin) and then incubated at 28°C. Confirmation of *A. tumefaciens* colonies growing on the selection media was carried out using PCR using primer TC306 and primer M13F (Čermák et al. 2017) (Table 3).

Transformation of the CRISPR/Cas9-gRNAOsSWEET construct to rice cv. Mentik Wangi

Rice genetic transformation refers to the method of (Slamet-Loedin et al. 2014) with a few modifications to the composition of the culture media (Table 4) and methods. Construct introduction was carried out with the help of *Agrobacterium tumefaciens*. Bacterial preparation for transformation was carried out by growing *A. tumefaciens* containing the CRISPR/Cas9-gRNAOsSWEET module construct in YEP media added with kanamycin 50 mg/L at 28°C for 2-3 days. The growing bacteria were then harvested and grown in liquid co-cultivation media. The bacterial suspension was measured with 0.3 OD, and then added with 100 µM acetosyringone.

Table 1. EBEs sequences used as a reference for designing gRNA primers

Gene	TALE	EBE sequence (5'-3')
SWEET11	PthXo1	GCATCTCCCCCTACTGTACACCAC
SWEET14	TalC	CATGCATGTCAGCAGCTGGTCAT
	PthXo3	ATATAAACCCCCTCCAACCAGGTGCTAAG
	AvrXa7	ATAAACCCCCTCCAACCAGGTGCTAA
	TalF	AAGCTCATCAAGCCTTCA

Table 2. PCR reactions for assembly of gRNA into the pDIRECT_25H vector

PCR reaction	Primer 1	Primer 2
Reaction 1	Promoter PvUbi1	CSY_gRNA_OsSWEET11
Reaction 2	REP_gRNA_OsSWEET11	CSY_gRNA_OsSWEET14
Reaction 3	REP_gRNA_OsSWEET14	CSY_Terminator35SCaMV

Immature embryos aged 12-14 days after anthesis were used as explants. The rice seeds were harvested and the husks were peeled and then sterilized using 70% alcohol for a few seconds, followed by sterilization using 15% commercial sodium hypochlorite added with 1 drop of tween for 5 minutes. The explants were rinsed with sterile distilled water 6 times for a few seconds.

Immature embryos were isolated from young rice seeds and placed in solid co-cultivation media and co-cultivated with *A. tumefaciens* for 7 days in the dark condition at 25°C. The growing callus was transferred to the resting medium for 5 days under full light conditions. The callus was then cut into 2-4 parts and placed in the selection medium for 10 days with 3-5 subculture periods to the new selection medium. In the selection media, this transformation method uses media with the addition of the antibiotics cefotaxime and vancomycin to prevent bacterial contamination and selection antibiotics in plants, namely hygromycin. The developed callus was then transferred to the pre-regeneration medium. The callus with green spots were transferred to regeneration medium for 14 days. The formed plantlets are placed on the rooting medium for 14-21 days. The plantlets were acclimatized for 3-4 days and then planted in the soil medium.

Data analysis

Variables observed included the number of callus resistant to hygromycin on the selection medium (the number of callus resistant to hygromycin on the third selection medium divided by the number of immature embryos transformed and then multiplied by 100%), the percentage of callus growing on the pre-regeneration medium (calculated by adding callus with green spots divided by the number of callus resistant to hygromycin into three selection media), as well as the transformation efficiency and regeneration efficiency calculated using the following formula described by Sisharmini et al. (2018) as follows:

$$\text{Regeneration Efficiency} = \frac{\text{Number of regenerated calluses}}{\text{Number of callus passed the selection of antibiotics}} \times 100\%$$

$$\text{Transformation Efficiency} = \frac{\text{Number of plants containing the hygromycin gene}}{\text{Number of transformed embryos}} \times 100\%$$

RESULTS AND DISCUSSION

CRISPR/Cas9-gRNA-OsSWEET cassette construction

Gene editing with the CRISPR/Cas9 system consists of 2 basic components, namely gRNA and CRISPR-related endonuclease (Cas protein). gRNA is a synthetic RNA with a short size (about 20 nucleotide bases) to help orient the cutting region at the target site of the gene by Cas9. CRISPR/Cas9-gRNAOsSWEET module construction begins with the design of gRNA primers. The gRNA was designed based on the motif of the EBEs in the promoter of the SWEET11 gene which is the target of TALe PthXo1, while the EBEs motif of the promoter of the SWEET14 gene is the target of TALes AvrXa7, PthXo3, TalC, and TalF.

The primary design of EBEs was based on the EBEs sequences in the promoter regions of the OsSWEET11 and OsSWEET14 genes (Oliva et al. 2019). The positions of the gRNAs designed to direct mutations in the EBEs portion of the OsSWEET11 gene promoter are from -221 to -239 from the start codon to the upstream direction. Whereas the position of the gRNA designed to direct mutations in the EBEs part of the promoter of the OsSWEET14 gene is located at positions -225 to -244 from the start codon position to the upstream (Figure 1).

Table 3. Primers used for colony PCR

Primer	Sequence (5'-3')
TC306	AGCACTACCAATGATGACCT
M13F	GTAAAACGACGGCCAGT

Table 4. The composition of the media used for the genetic transformation of rice cv. Mentik Wangi (Slamet-Loedin et al. 2014)

Media	Composition
Liquid Co-cultivation	AA (Toriyama and Hinata 1985) with the addition of 876 mg L^{-1} L-glutamine, 260 mg L^{-1} aspartic acid, 174 mg L^{-1} arginine, 0.086 g L^{-1} glycine, 500 mg L^{-1} casamino acid, 20 g L^{-1} sucrose and 10 g L^{-1} glucose, pH 5.4
Solid Co-cultivation	N6 (Chu,1978) macro, B5 micro, and vitamins (Gamborg et al. 1968) with the addition of 500 mg L^{-1} casamino acid, 500 mg L^{-1} L-proline, 20 g L^{-1} sucrose, 10 g L^{-1} glucose, 2 mg L^{-1} 2.4-D, 1 mg L^{-1} BAP, 2 mg L^{-1} NAA, 5.5 g L^{-1} Agarose type I, pH 5.4
Resting	N6 macro, B5 micro, and vitamins with the addition of 500 mg L^{-1} casamino acid, 500 mg L^{-1} L-proline, 300 mg L^{-1} glutamine, 36 g L^{-1} mannitol, 20 g L^{-1} maltose, 1 mg L^{-1} 2.4-D, 1 mg L^{-1} NAA, 0.2 mg L^{-1} BAP, 250 mg L^{-1} cefotaxime, 100 mg L^{-1} vancomycin, gelrite 5 g L^{-1}, pH 5.8
Selection	Resting + 10 mg L^{-1} hygromycin
Pre-regeneration	MS (Murashige and Skoog 1962) with the addition of 30 g L^{-1} maltose, 20 g L^{-1} sorbitol, 10 g L^{-1} type I agarose, 2 mg L^{-1} kinetin, 5 mg L^{-1} NAA, pH 5.8
Regeneration	MS with the addition of 30 g L^{-1} sucrose, 2 mg L^{-1} kinetin, 1 mg L^{-1} NAA, 3 g L^{-1} gelrite, pH 5.8
Rooting	MS with the addition of 30 g L^{-1} sucrose, 1 mg L^{-1} IBA, 2 g L^{-1} gelrite, pH 5.8

One of the edited EBEs motifs of the OsSWEET11 gene consists of the cis-acting element CACTFTPPCA1 (CACT) motif (Higo et al. 1999). According to Gowik et al. (2004), the CACT motif at this location is a cis-acting Mem1 element motif that is involved in regulating specific gene expression in the leaf mesophyll. The Xoo pathogen produces TALe secretion which recognizes and attaches to the cis-acting element Mem1 of the OsSWEET11 promoter, and induces the SWEET11 gene in plants to produce sugar in the mesophyll of the leaf. The cis-acting element motifs in the EBEs section of the SWEET14 gene promoter include the cis-acting element motif as the binding domain of the MYB transcription factor which is involved in regulating abiotic tolerance and biotic stress, as well as the EBOXBNNAPA motif which is commonly found in the genes involved in storage-protein (Higo et al. 1999).

The induction of specific expression of a gene can result from several signaling combinations that involve several cis acting elements at once (Jeong and Jung 2015). Binding between TALes from Xoo and EBEs motifs in the promoter section of the OsSWEET11 and OsSWEET14 genes will induce sugars to be utilized by the pathogen as nutrients for survival in the host plant, thereby causing the host plant to become susceptible to the Xoo pathogen. Targeted mutagenesis of the EBEs portion of the promoter of the OsSWEET gene is one of the efforts to develop rice plants resistant to Xoo pathogens. This is possible because if the EBEs in the promoter portion of the OsSWEET gene experience mutations, it is very likely that the TALe produced by the pathogen will not recognize these EBEs. If this happens, induction of the OsSWEET gene will not occur, causing the Xoo pathogens to not get nutrients (sugar) from these plants. With these conditions, plants can avoid pathogen attacks and plants become resistant to HDB disease.

Primer design was continued by designing CSY and REP primers to amplify the Ubiquitin promoter fragments, gRNA for the OsSWEET11 gene, gRNA for the OsSWEET14 gene, and overlapping terminators, so that a gene module with multiple intact gRNAs that connected from the promoter to the terminator would be produced. The designed primer sequences are presented in Table 5.

The promoter fragments of PvUbi1, gRNA OsSWEET11, gRNA OsSWEET14, plasmid pDIRECT_25H, and the terminator were obtained from PDIRECT_25H amplification using pre-designed primers (Table 5), with different reaction mixes. The results of reaction 1 produced an amplicon with a size of 2000 bp which is an amplicon promoter section PvUbi1 + CSY gRNA OsSWEET11 (R1), while PCR reactions 2 and 3 produced amplicons with a size of 136 bp, each of which is an amplicon from the REP gRNA OsSWEET11 + CSY gRNA OsSWEET14 (R2) and REP gRNA OsSWEET14 + CSY terminator (R3) sections (Figure2A). The fragments were then purified from agarose gel. The next process is to ligate the fragments from R1, R2, and R3 to the pDIRECT_25H expression vector using the Golden Gate method. This ligation process produced a product with a size of 2238 bp (Figure 2B) which indicated that the recombinant pDIRECT_25H vector containing the CRISPR/Cas9-gRNAOsSWEET module construct had been successfully assembled.

The results of the Golden Gate ligation were then transformed into competent cells of *E. coli* DH5α using the Heat Shock method. The bacterial transformation produced a single colony on selection media (solid LB containing the antibiotic kanamycin with a concentration of 50 mg/L) (Figure 3A). Verification of single bacterial colonies was carried out by PCR using primer TC306 and primer M13F. The PCR results showed that of the 11 bacterial colonies analyzed, 9 colonies were confirmed to contain the expected recombinant plasmid, which was indicated by an amplicon size of 696 bp (Figure 3B). This size is the result of amplification from the PvUbi1 promoter region, gRNA SWEET11, gRNA SWEET14 to the terminator.

Confirmation of the CRISPR/Cas9-gRNAOsSWEET module construct apart from using PCR was also carried out by sequencing the recombinant vector that had been obtained. The results of recombinant plasmid DNA sequencing showed that the assembly of the CRISPR/Cas9 module construct with multiple gRNAs for the mutation target of the OsSWEET11 and OsSWEET14 genes in the pDIRECT_25H expression vector was successful (Figures 4 and 5).

After it was confirmed that the recombinant plasmid obtained contained the CRISPR/Cas-gRNAOsSWEET module construct, the next step was to insert the recombinant plasmid containing pDIRECT_25H-gRNAOsSWEET into the bacterium *A. tumefaciens* strain LBA4404 using electroporation. The results of this transformation resulted in a single colony of bacteria on solid YEP selection media containing 50 mg/L of the antibiotic kanamycin. Confirmation of the insertion of the construct in *A. tumefaciens* by PCR technique using primer TC306 and primer M13F. PCR results showed that of the 5 single colonies analyzed, all contained recombinant vectors, as indicated by the obtained DNA amplicon with a size of 696 bp. These results indicate that the recombinant vector pDIRECT_25H-gRNAOsSWEET has successfully entered *A. tumefaciens* LBA4404, and the *Agrobacterium* is ready to be used for genetic transformation activities into rice cv. Mentik Wangi (Figure 6B).

Rice cv. Mentik Wangi transformation

Transformation of rice cv. Mentik Wangi was carried out using the CRISPR/Cas9-gRNAOsSWEET construct with multiple gRNAs mediated by the bacterium *A. tumefaciens* LBA4004 (Figure 7A). The explants used in this study were immature embryos taken from rice seeds aged 12-14 days after anthesis (Figure 7B). Direct use of healthy immature embryo explants is one of the keys to the successful genetic transformation of recalcitrant plants (Hiei and Komari 2008). In addition, rice immature embryos contain a scutellum that is still meristematic. Meristematic tissue is a tissue with cells that are actively dividing and more responsive to culture media.

Immature embryos were isolated from rice seeds and infected with *A. tumefaciens* carrying the CRISPR/Cas9-gRNAOsSWEET module construct (Figure 7C). The infection media used was added with acetosyringone which

is a phenolic compound, which aims to induce the vir gene present in *Agrobacterium* to transfer T-DNA to plant cells (Nakano 2017). According to Sisharmini et al. (2018), the use of 100 µM acetosyringone in infection and co-cultivation media resulted in higher regeneration and transformation efficiency compared to media without acetosyringone or acetosyringone with a concentration of 200 µM. The use of acetosyringone concentrations that are too high can cause necrosis in plant tissue, which can reduce the efficiency of transformation (Sah et al. 2014b).

Immature embryos that have been co-cultivated for 7 days will germinate, and the growing shoots are then removed (cut) (Figure 7D). The embryos that had been cut off were then blotted and grown on resting media for 5 days. At this stage the culture media does not contain the antibiotic hygromycin which is used to select plant cells, in the media only the antibiotics cefotaxime and vancomycin are added to eliminate *Agrobacterium*. Calluses that developed in resting media were then grown in selection media containing the antibiotic hygromycin to select plant callus. Callus selection media contains the hormone 2,4 D which can stimulate cell proliferation in the callus, especially in the wound area (Figure 7E) (Waryastuti 2017). The use of the right concentration of 2.4 D hormone in the media can affect the quality of the callus that is formed. The expected callus is an embryogenic callus, where the callus is characterized by a callus that is round, variable in texture, and has a yellowish color. Early detection of callus carrying the CRISPR/Cas9-gRNAOsSWEET construct was seen in callus that was resistant in selection media containing the antibiotic hygromycin and able to proliferate into embryogenic callus (Figure 7F).

The addition of the antibiotic hygromycin to the selection culture medium can reduce callus regeneration, thereby eliminating non-transformant callus during transformation (Htwe et al. 2014; Tran and Sanan-Mishra 2015). Embryogenic calluses that can grow and develop in the selection medium are then transferred to pre-regenerated media. On the pre-regeneration media, callus can be seen growing and forming green spots (Figure 7G). The callus that had produced shoots was then transferred to the regeneration medium to form plantlets (Figure 7H). The plantlets were transferred to the rooting medium to stimulate root formation (Figure 7I). The plantlets were ready to be acclimatized with water (Figure 7J) then acclimatized in the greenhouse, and also planted on soil media (Figure 7K).

The growth and development of untransformed callus is relatively faster compared to the transformed callus. The regeneration efficiency value of immature embryos that were not transformed and those that were transformed with *Agrobacterium* containing the CRISPR/Cas9-gRNAOsSWEET module construct decreased. The callus regeneration efficiency which was not transformed was higher (14%) compared to that which was transformed (9.4%) (Table 6). This is probably due to the transformed callus, besides experiencing pressure due to *Agrobacterium* infection, it is also due to exposure to hygromycin antibiotics. Exposure of the antibiotic hygromycin to regeneration media can reduce callus growth and callus regeneration, and can eliminate non-transformant callus during transformation (Htwe et al. 2014; Tran and Sanan-Mishra 2015).

The total number of immature embryos used for the genetic transformation of rice cv. Mentik Wangi were 1306 immature embryos. The 145 calluses were resistant to selection media containing hygromycin. The number of transformants produced from the transformation process was 129 putative plants of Mentik Wangi generation T0 with a transformation efficiency value of 9.8% (Table 7). This result is higher than the results of a study by Ruzyati et al. (2022), where the transformation efficiency value obtained only reached 3.8% using the same variety. Modification of the protocol during the transformation process is something that needs attention to get more Mentik Wangi transformants. Thakur et al. (2022) stated that the composition of the regeneration medium is also important for increasing the efficiency of plant transformation from transformed callus. Regeneration is the most important final stage in plant genetic transformation activities to obtain transformants. The regeneration process consists of 2 stages, namely pre-regeneration (pre-differentiation) and regeneration (differentiation) (Chen et al. 1998). Plant regeneration depends on the media used, especially growth hormone. The ratio between the auxin and cytokinin hormones applied in the culture medium will determine the direction of shoot and root formation (Cheng et al. 2013). Cytokinin hormones play a role in inducing cell division, proliferation and budding, while auxins play a role in cell growth, apical dominance of callus formation, and organogenesis. In addition, transformation efficiency is also influenced by the selection of *A. tumefaciens* strains and the use of immature rice embryos (Hiei and Komari 2008). In this study, a modification of the transformation method was also carried out, namely by sub-culture of callus 3-5 times in pre-regeneration media. Sub-culture at this stage is to obtain embryogenic callus with more green spots, so it is hoped that more shoots will be obtained in the regeneration medium. Callus with green spots will differentiate into shoots in the regeneration medium.

Table 5. Primers used for construction of CRISPR/Cas9 cassettes

Primer	Sequence (5'-3')
Promoter PvUbi1	TGCTCTTCGCGCCACGTCAGTGTTTGGTTTCC
CSY gRNA OsSWEET 11	TTCCACCTGCACACCACCACCAAAACCTGCCTATACGGCAGTGAAC
REP gRNA OsSWEET 11	GCTCACCTGCGTCGGGTGTACAGTAGGTTTTAGAGCTAGAAATAGC
CSY gRNA OsSWEET 14	TTCCACCTGCACACCTAAGCTCATCACTGCCTATACGGCAGTGAAC
REP gRNA OsSWEET 14	GCTCACCTGCGTCGTTAGCACCTGGTGTTTTAGAGCTAGAAATAGC
CSY Terminator-35SCaMV	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC

Table 6. Percentage efficiency of rice cv. Mentik Wangi regeneration

Treatment	No. of immature embryo	No. of callus on selection media	No. of callus with green spots on pre-regeneration media	No. of callus on regeneration media	Percentage of regeneration efficiency (%)
Not transformed	198	128	30	18	14
Transformed	438	127	12	12	9.4

Table 7. Percentage efficiency of the transformation of the CRISPR/Cas9 construct in rice cv. Mentik Wangi

Transformation	No. of immature embryo	No. of callus on selection media	No. of callus with green spots on pre-regeneration media	No. of callus on regeneration media	No. of plantlets in rooting media	No. of plants	Percentage of transformation efficiency (%)
I	438	127	12	12	12	12	9.8
II	461	220	94	91	91	91	
III	407	180	39	26	26	26	
Total	1.306	527	145	129	129	129	

A. gRNA OsSWEET11

AAAAGCAAAGGTTAAATATGCATCTCCCCCTACTGTACACCACCAAAAGTGGAGGGTCTCCAACTA**TATAAA**CACTGAGCCA
TGGCCAAGGCCAAACCACACATGCAGTTGTAGTAGCACTTAAGCCTTCCTCTCTAGCTAGCATCTCTTGTGTCAGGAAGTTG
GAAGGGATTTCTGGCTAGTTTCTAGCTGGTGTCTCCTCTCCTCTTCCTAACCTTCTCACTGATTAACACCTTAAAGTTAGTT
AATAACCTTCATCACCAGTAGCA**ATG**

B. gRNA OsSWEET14

CTTTTCATTCCCTTCTTCCTTCCTAGCAC**TATATAAA**CCCCCTCCAACCAGGTGCTAAGCTCATCAAGCCTTCAAGCAAAGC
AAACTCAAGTAGTAGCTGATTACCAGCTCTTCTCTCTTCTCATTGAGAAGAGGGAATTAAGTTTTGATCTCTGCTTTATTGC
CTGATCATCCTCTTGTTACTTGCAAGCAAGAACAGTAGTGTACTGTGCCTCATTGATCTCCTCCCACCAAACTCTCTCTCTC
TCTCTCATATTCCGAGCTAGCTAGTTAATCAAGATCTTGCTGCA**ATG**

Figure 1. Position of gRNA. A. EBEs promoter region of the OsSWEET11 gene (-239 to -221 from the start codon position). B. EBEs promoter region of the OsSWEET14 gene (-244 to -225 from the start codon position). Letters in green: gRNA sequence, ATG: start codon, TAAAA: TATA box sequence, TGG, CCA, and GCC: PAM motif (Protospacer Adjacent Motif)

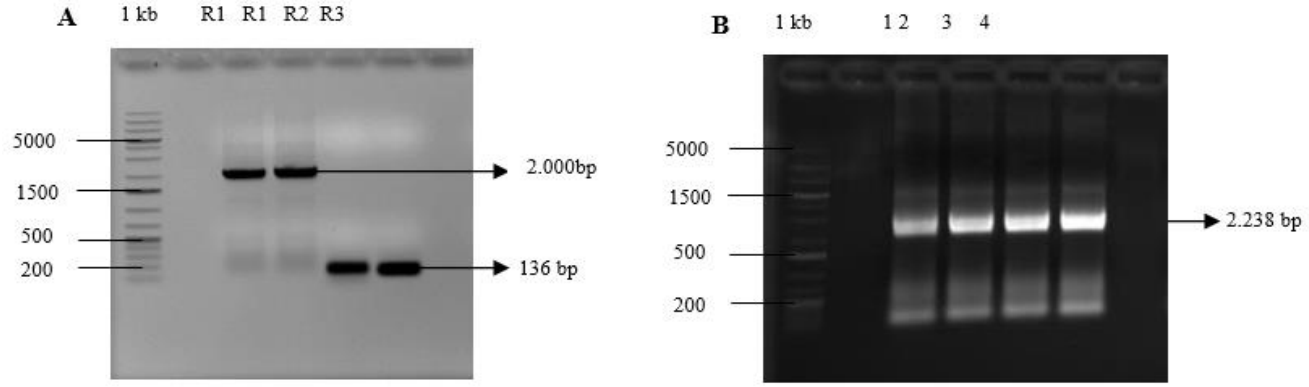

Figure 2. Assembly of gRNA PCR result into the pDIRECT_25H vector. A. PCR results for reaction 1 (R1: promoter PvUbi1 + CSY gRNA OsSWEET11) produced an amplicon with a size of 2000 bp, reaction 2 (R2: REP gRNA OsSWEET11 + CSY gRNA OsSWEET14) produced an amplicon with a size of 136 bp, and reaction 3 (R3: REP gRNA OsSWEET14 + CSY terminator) produced an amplicon with a size of 136 bp. B. The result of ligation of the pDIRECT_25H vector with fragments R1, R2, and R3 using the Golden Gate method, produced an amplicon with a size of 2238 bp (Note of dilution number: 1=1:80, 2=1:40, 3=1:20, 4=undiluted)

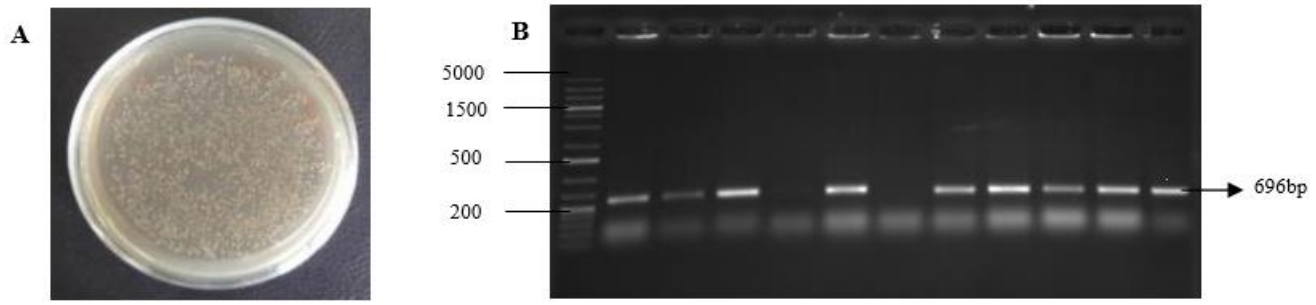

Figure 3. *Escherichia coli* transformation result. A. Single colony of *E. coli* bacteria that grows from the transformation by the Golden Gate ligation reaction. B. Visualization of the results of a single colony PCR confirmation of *E. coli* containing a recombinant vector, which produces an amplicon with a size of 696 bp

CCTTGATGATGTTTCTGTTTGTTTGAGGCTTGTGTTACTGCTAGTTACTTACCCTGTTGCCTGGCTAATCTTCTGCAGATCCTGCAGGTTC
ACTGCCGTATAGGCAGCTTTTGGTGGTGTACAGTAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA
AAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGTGATGAGCTTAGCACCTGGTGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCGTTCACTGCCGTATAGGCAGGTCGATCGACAAGCTCGAGTTTCTC
CATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTAT
GTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTACTAAAATCCAGATCCCCCGAATTACCG
GTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCA
GCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGCTAGAGCAGCTTGAGCTTGGA
TCAGATTGTCGTTTCCCGCCTTCAGTTTAAACTATCAGTGTTTGACAGGATATATTGGCGGGTAAACCTAAGAGAAAAGAGCGTTTATTAG
AATAATCGGATATTTAAAAGGGCGTGAAAAGGTTTATCCGTTCGTCCATTTGTATGTGCATGCCAACCACAGGGTTCCCCTCGGGATCAAA
GTACTTTGATCCAACCCCTCCGCTGCTATAGTGCAGTCGGCTTCTGACGTTCAGTGCAGCCGTCTTCTGAAAACGACATGTCGCACAAGTC
CTAAGTTACGCGACAGGCTGCCGCCCTGCCCTTTTCCTGGCGTTTTCTTGTCGCGTGTTTAGTCGCATAAAGTAGAATACTTGCGACTAGA
ACCGGAGACATTACTCCATGAACAAGACCGCCGCGCTGCCTGCTGGGCTATGCCGCGTCACCCCGACAACAGGACTTGACAACCAACGGGC
CAAATGCACCGCCGGCTTCCCCAACTGTTTTCCAAAA

Figure 4. Results of sequencing analysis of the pDIRECT_25H-gRNAOsSWEET construct, note: gray: PvUbi1 promoter, green: CSY, turquoise: gRNA OsSWEET11, yellow: REP, purple: gRNA OsSWEET14, blue: terminator, dark green: primer M13F

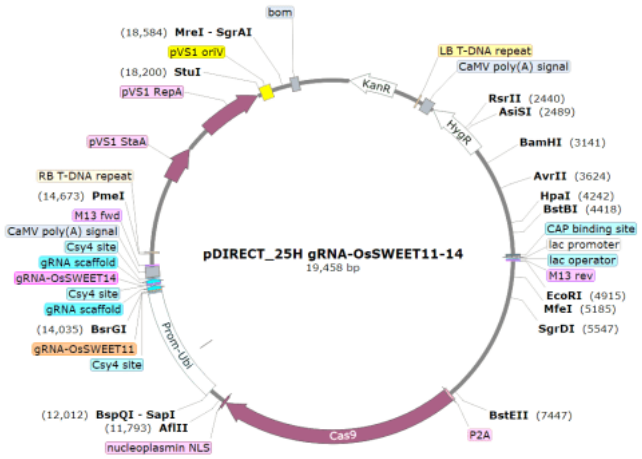

Figure 5. Schematic of the recombinant pDIRECT_25H vector carrying multiple gRNAs of the OsSWEET11 and OsSWEET14 genes controlled by the Ubiquitin promoter (Anon n.d.)

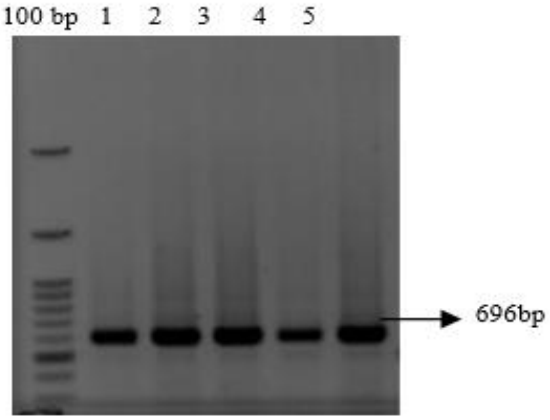

Figure 6. PCR results of 5 single colonies of *A. tumefaciens* carrying the CRISPR/Cas9-gRNAOsSWEET module constructed with primers TC306 and M13F to produce an amplicon with a size of 696 bp

Molecular analysis of putative T0 mutant of rice cv. Mentik Wangi using PCR

A total of 129 rice lines were produced and plant DNA was isolated for molecular analysis by PCR using specific primers to detect the presence of the *hpt* gene. This molecular analysis is to determine the transformants of rice cv. Mentik Wangi containing the CRISPR/Cas9-gRNAOsSWEET construct. The results of molecular analysis showed that of the 129 putative mutant rice lines, not all contained the *hpt* gene, only 36 rice lines contained the *hpt* gene, with a percentage value of 27.9% of the total putative mutant lines. Transformants that do not contain the

hpt gene may escape from the media containing the antibiotic hygromycin. The hpt positive rice lines were marked by the *hpt* gene amplicon with a size of 500 bp (Figure 8). The rice lines that are positive for the *hpt* gene are expected in the transformed tissue to contain the CRISPR/Cas9-gRNAOsSWEET construct in their genomes.

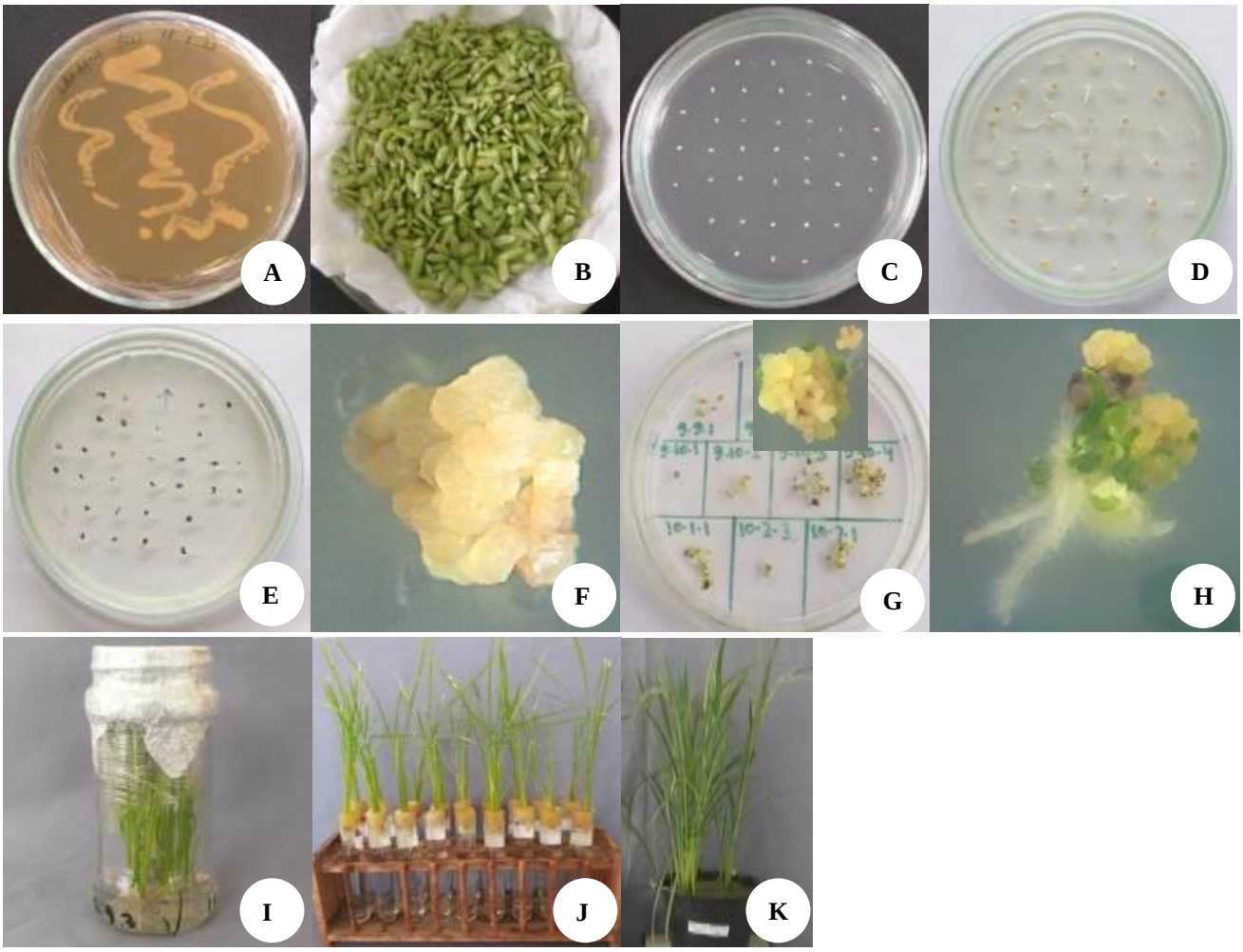

Figure 7. Stages of transformation through *A. tumefaciens* in immature embryo of rice cv. Mentik Wangi. A. *A. tumefaciens* culture. B. Mentik Wangi young rice seeds. C. Immature embryo infection with *A. tumefaciens*. D. Explants 7 days after co-cultivation. E. Callus on hygromycin selection media. F. Proliferation of hygromycin-resistant callus. G. Callus which forms green spots on pre-regeneration media. H. Formation of callus into plantlets on regeneration media. I. Plantlets on root media. J. Acclimatization, K. Plants on soil media

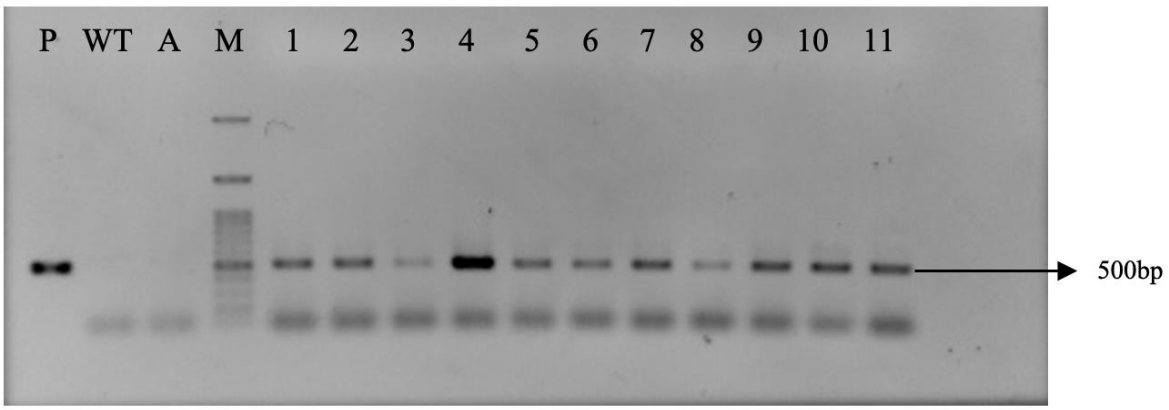

Figure 8. Results of the *hpt* gene amplification in the putative mutant rice cv. Mentik Wangi line T0. P: plasmid control pDIRECT_25H, WT: wild type control, A: water control, M: 100 bp ladder marker, 1-11: representative samples

In conclusion, the CRISPR/Cas9 module construct was successfully assembled with several gRNAs targeted to mutate the promoter region of the OsSWEET11 and OsSWEET14 gene promoters using the Golden Gate method. The introduction of the CRISPR/Cas9-gRNAOsSWEET construct to rice cv. Mentik Wangi through *Agrobacterium* produced 129 transformant lines, with a regeneration efficiency value of 9.4%, a transformation efficiency of 9.8%, and the percentage of transformants containing the CRISPR/Cas9-gRNAOsSWEET construct of 27. 9%.

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