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Construction of CRISPR/Cas9_gRNA-OsCKX2 module cassette and its introduction into rice cv. Mentik Wangi mediated by *Agrobacterium tumefaciens*

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Abstract. *Ruzyati M, Sisharmini A, Apriana A, Santoso TJ, Purwanto E, Samanhudi, Yunus A. 2022. Construction of CRISPR/Cas9_gRNA-OsCKX2 module cassette and its introduction into rice cv. Mentik Wangi mediated by* Agrobacterium tumefaciens. *Biodiversitas 23: 2679-2689.* Mentik Wangi is an aromatic superior rice variety from the tropical japonica group with high posture and low productivity. The tall plant posture causes Mentik Wangi rice to be prone to lodging, resulting in yield loss. Therefore, improvement of plant height and productivity of Mentik Wangi is still required. The sd-1 (OsGA20ox-2) and CKX2 genes are responsible for the semi-dwarf character and high productivity. The study aimed to construct a CRISPR/Cas9 cassette module carrying a gRNA of OsCKX2 gene and introduce this construct to Mentik Wangi rice mediated by an *Agrobacterium tumefaciens* vector strain LBA4404. The introduction of the CRISPR/Cas9_gRNA-GA20ox-2 cassette plasmid that was constructed in previous research into Mentik Wangi rice was also carried out. The results showed that the CRISPR/Cas9_gRNA-CKX2 cassette module had been successfully constructed using the Golden gate cloning method. The introduction of the CRISPR/Cas9_gRNA-CKX2 and CRISPR/Cas9_gRNA-GA20ox-2 cassette modules into Mentik Wangi rice resulted in 30 putative transformant lines that passed the hygromycin selection. PCR analysis showed that from the 30 transformant lines, 15 lines were positive for the hygromycin resistance gene. Further analysis is necessary to be conducted to identify the occurrence of mutagenesis in the OsCKX2 and GA20ox-2 target genes.

Keywords: CRISPR/Cas9, *OsGA20ox2*, *OsCKX2*, Mentik Wangi, transformation

INTRODUCTION

Rice is an Indonesian food commodity that is a priority in agricultural development because it is included as an important cereal crop in the Poaceae family which acts as a staple food (Marlina et al. 2017). Rice has various varieties, one of which is Mentik Wangi, a superior local variety and is widely cultivated in Central Java Province. Mentik Wangi is very popular with consumers because it has a distinctive fragrance and fluffier rice (Yulianto 2017) but is not favored by farmers because it has a high level of lodging caused by the height of the plant's posture (Yunus et al. 2017). High lodging rates lead to decreased yields (Fitriyanti et al. 2020) due to the reduced flowering ability of plants to increase the number of seeds produced (Supriyanti et al. 2015). Therefore, it is necessary to improve the character of Mentik Wangi rice to obtain rice with low posture and increased crop yields.

Rice with high posture is very susceptible to lodging caused by rain and wind, so farmers prefer to plant rice with short posture (Okuno et al. 2014). Previous research found that low or short posture could be caused by mutations in the *SD1* gene (*semi-dwarf*) (Sasaki et al. 2002) that encodes the *GA20ox-2* enzyme as a key enzyme in the biosynthesis of gibberellins (plant growth hormones). Mutations that occur in the *SD1* gene cause disturbances in the function of the *GA20ox-2* enzyme, resulting in plants with shorter stature (Spielmeyer et al. 2002). The *SD1* mutant gene has been analyzed and shown to control the character of rice plant height (Sasaki et al. 2002). The short posture of the mutation has a pleiotropic effect with a large number of tillers and erect leaf architecture so that it can capture more light energy, the harvest index is higher, and the plant is more responsive to nitrogen fertilization (Hirano et al. 2017). However, it does not reduce the quality, taste, and nutrition of harvested rice (Tomita and Ishii 2018).

Obtaining optimal yields is assisted by mutations in the *OsCKX2* gene as a control character for the number of seeds per panicle. The *OsCKX2* gene encodes cytokinin oxidase dehydrogenase which causes the accumulation of cytokinin in inflorescence meristems to increase the number of reproductive organs and increase the number of seeds. Cytokinin was first discovered as plant hormone that regulates cell division and affects various aspects of plant growth and development, such as germination, apical

dominance, leaf expansion, and the development of reproductive organs (Ashikari et al. 2005). The *OsCKX2* gene has been proven to regulate shoot apical meristem (SAM) activity, which is the main parameter determining seed production. Increased meristem activity causes an increase in panicle production in rice (Li et al. 2013).

Mentik Wangi has a potential that can be maintained, while the weaknesses in Mentik Wangi rice can be improved to increase crop yields (Yunus et al. 2017). Rice improvement was carried out using the latest technology in gene editing, namely Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein9(Cas9) (Zafar et al. 2020). CRISPR/Cas9 is the latest technology in genome editing with a high success rate, more efficient, and easier to perform than other genome editing techniques (Manghwar et al. 2019). CRISPR/Cas9 has a genome editing system that works effectively in directed gene mutations (Jinek et al. 2012). The Cas9 nuclease in the CRISPR/Cas9 system pairs with the single guide RNA (sgRNA) of the target gene to cleave the target sequence assisted by the Protospacer Adjacent Motif (PAM) recognition sequence (Deveau et al. 2008, Mojica et al. 2009; Deltcheva et al. 2011). PAM helps the Cas9 endonuclease distinguish target DNA from foreign DNA so that only the target sequence is truncated or mutated (Jinek et al. 2012).

The aim of this research was to construct a CRISPR/Cas9 plasmid cassette that carried the guiding RNA for the *OsGA20ox2* and *OsCKX2* genes and to obtain the Mentik Wangi mutant which had lower posture and increased yields.

MATERIALS AND METHODS

Research materials

The research was conducted at the Molecular Biology Laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development in 2021-2022. The research materials used were primers gRNAs, *Agrobacterium tumefaciens* vector strain LBA4404, plasmid pDIRECT-25H, plasmid pDIRECT-21A, *pBY02-Cas9_GA20ox2*, and immature embryos from rice of Mentik Wangi variety as explants. The regeneration and transformation method used refers to the protocol of Slamet-Loedin et al. (2014) (Figure 1).

Immature embryos were isolated from young rice seeds aged 8-12 days after flowering, the husks removed and sterilized with 70% ethanol for 5-10 seconds and 1% sodium hypochlorite solution for 5 minutes. The immature embryos were then isolated from the seeds using tweezers.

Construction of CRISPR/Cas9_gRNA-OsCKX2 module cassette

CRISPR/Cas9 construction

The CRISPR/Cas9 construct carrying the *gRNA_OsCKX2* was assembled with the pDIRECT-25H backbone plasmid pDIRECT_25H was a gift from Daniel Voytas (Addgene plasmid #91145, http://n2t.net/addgene:91145, RRID: Addgene_91145) (Čermák et al. 2017).

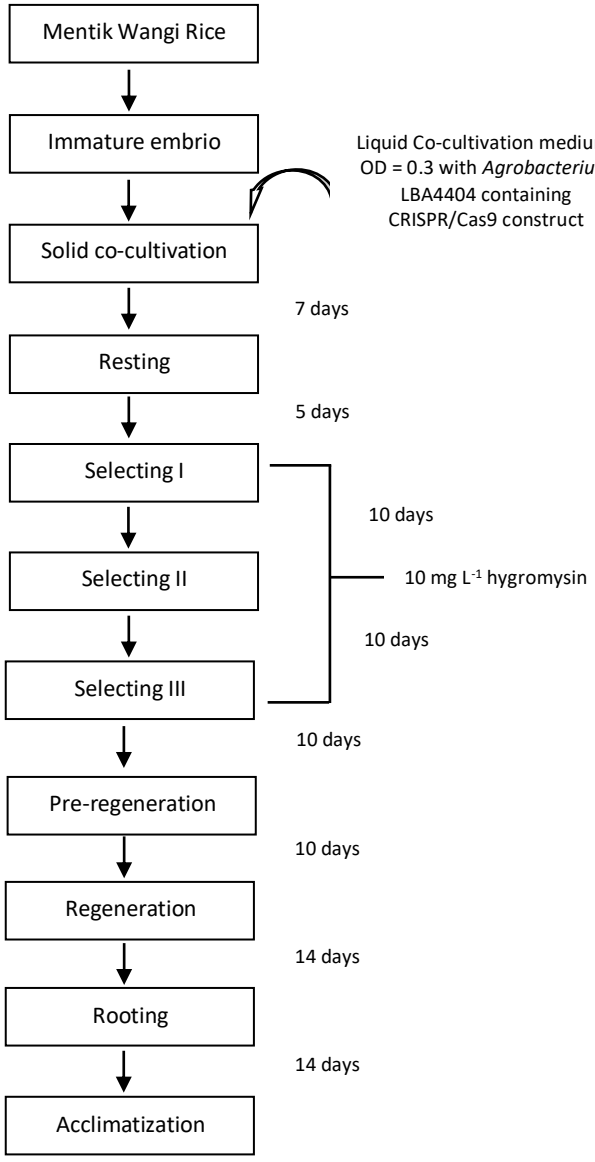


Figure 1. Flow chart describing *Agrobacterium tumefaciens*-mediated transformation using immature embryo of Mentik Wangi

Primer design of OsCKX2 gRNA

The design of the *OsCKX2* gene gRNAs was carried out using the CRISPOR online application on the website http://crispor.tefor.net/ page. Input data as cDNA sequence of the *OsCKX2* gene was entered in the column provided as the target gene for selecting the Protospacer Adjacent Motif (PAM). The next page that appears contains the predicted guide sequences for RNA. The selected predicted gRNA was then checked for secondary structure using the RNAfold Webserver http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi. The spacer sequences selected met the criteria that nucleotides at positions 18-20 (seed region) of the spacer were unpaired and could be accessed freely (Wong 2006) and nucleotides 51-53 of tracrRNA were not paired with the seed region and could be accessed freely (Doench et al. 2014).

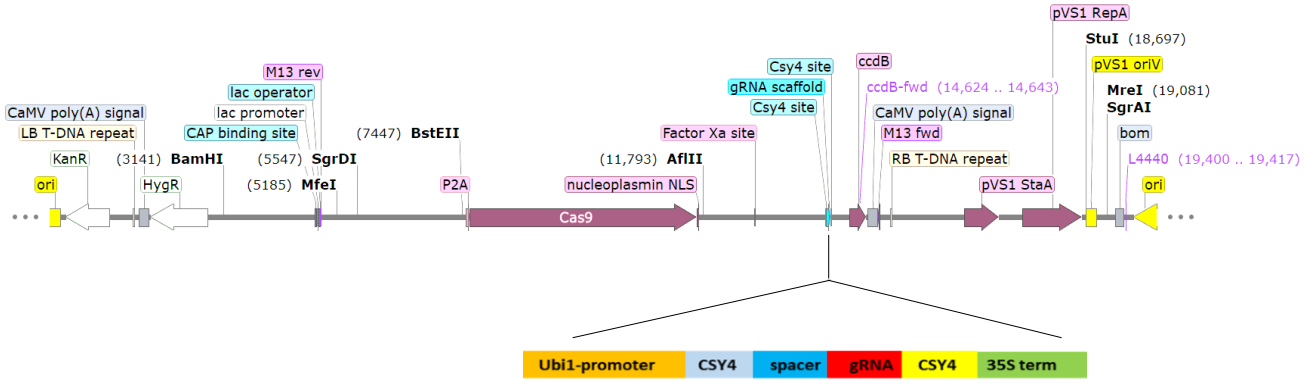
Assembling gRNA into pDIRECT-25H vector backbone

A binary vector pDIRECT-25H plasmid (Čermák et al. 2017) was used as a backbone plasmid. The T-DNA of the vector contains Cas9 gene cassette, guide RNA controlled by the Ubi1 promoter, and HPT gene cassette for plant selection markers. The designed guide RNAs were arranged in an array controlled by the Ubi1 promoter and linked to a terminator (Figure 2.A). The designed gRNA spacer provides the unique sequence used for the Golden Gate junction. The array was arranged in two PCR reactions consisting of primer 1 and primer 2 (Table 1). The PCR product containing the promoter cassette, gRNA spacer, and terminator was assembled in an array in a single golden gate reaction step to generate the pDIRECT-25H expression cassette carrying the array (Figure 2).

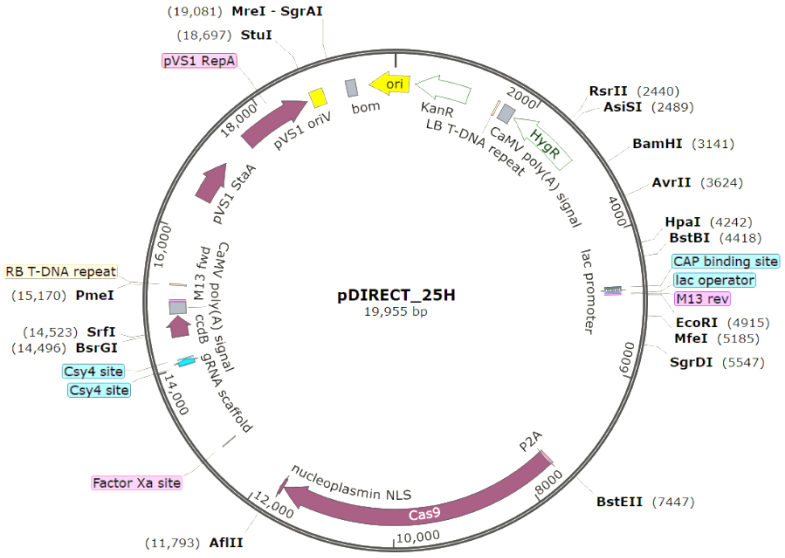
The enzyme used in the assembly of the array is *Sap*I which cleaves pDIRECT_25H in 2 positions flanking the PvUbi1 promoter to ccdb gene (Figure 2.B). This enzyme will facilitate insertion of the array into pDIRECT-25H. *Aar*I enzyme (does not cleave pDIRECT_25H) was used to link the gRNA with promoter and terminator sites so that the splicing site of the *Aar*I enzyme was added to the 5' end of each gRNA.

Table 1. PCR reaction for assembling CRISPR/Cas9 Cassette harboring the gRNA of *OsCKX2* Gene

PCR reaction	Primer 1	Primer 2
Reaction#1	promoter specific primary	CSY_gRNA1_OsCKX2
Reaction#2	REP_gRNA1_OsCKX2	CSY_terminator



A. Scheme of the array in pDIRECT_25H



B. pDIRECT_25H vector

Figure 2. A. Scheme of the array consisting of Ubi1-promoter, *gRNA_OsCKX2* (spacer) and 35S terminator on CRISPR/Cas9 cassette from pDIRECT25H vector, B. pDIRECT_25 vector

The synthesis of the array section consisting of the promoter, gRNA spacer, and terminator was carried out by PCR technique. The synthesis process (reaction #1) was carried out using a PCR machine with a 20 uL total volume of the reaction mix. The DNA polymerase used in this study was Q5 hot start HF 2x master mix with pDIRECT-25H template. Reaction #2 uses a template of pDIRECT_25H which has removed the ccdb gene. The ccdb portion was removed by digestion with the *Ban*I enzyme. The digest result of pDIRECT-25H which is approximately 3500 bp in size is used as the template for reaction #2. The PCR reaction steps were initial denaturation at 98ºC for 1 min, followed by 30 cycles consisting of denaturation at 98ºC for 10 sec, annealing at 60ºC for 15 sec, and elongation at 72ºC for 15 sec. The final elongation stage was at 72ºC for 2 min. The target fragment was then eluted and purified using Zymoclean™ gel DNA recovery kit with eluting in 6-10 uL of nuclease-free water. The eluted DNA concentration was then measured using nanodrop (Unuofin et al. 2019).

Digest and ligation of gRNAs- OsCKX2 to the pDIRECT-25H vector using the Golden Gate (GG) method

The golden gate reaction was carried out using a PCR machine with two stages. In GG stage, the first step is to perform PCR with a template of the purified fragments from the synthesis process of reaction #1 and reaction #2 with the following reaction mix: fragment #1 and fragment #2, T7 DNA ligase buffer 2x, AarI, 50x oligonucleotide 0.025 mM, 10 mM ATP, T7 DNA ligase, and nuclease-free water with a total volume of 20 uL. The reaction mix was proceed in a PCR machine with a 10x cycle (37ºC/5min + 25ºC/10min) + 4ºC hold.

The next step is to cut the PCR product from the first step. Before cutting, a 1:80 dilution fragment was used as a template to check the expected size of fragment. The PCR reaction mixture with a total volume of 25 uL consisted of 2 uL DNA template, 12.5 uL Q5 hot start HF 2x master mix, 2.5 uL promoter-specific primer, 2.5 uL terminator specific primer, and 5.5 uL nuclease-free water. The PCR program was initial denaturation at 98ºC for 1 min, 30 cycles consisting of denaturation at 98ºC for 10 sec, annealing at 60ºC for 15 sec, and elongation at 72ºC for 15 sec. The final elongation stage was at 72ºC for 2 min. The fragment size of amplicon is approximately 2142 bp. The fragments were then eluted and purified using a ZymocleanTM gel DNA recovery kit with eluting in 6-10 uL of nuclease-free water.

The second GG stage is ligation of the target fragment (harboring the promoter, *gRNA_OsCKX2*, and the 35S terminator) and the SapI digested- pDIRECT-25H vector using PCR technique. The PCR reaction with a total volume of 20 uL consisted of a mixture of 220 ng the pDIRECT_25H, 175 ng the purified fragment array 2142 bp, T7 DNA ligase buffer 2X, SapI, 10 mM ATP, T7 DNA ligase, and nuclease-free water. The PCR machine was run with 10x cycles (37ºC/5min + 25ºC/10min) + 4ºC hold.

Bacterial transformation of CRISPR/Cas9_gRNA-OsCKX2 into Escherichia coli *and* Agrobacterium tumefaciens

The transformation of the *CRISPR/Cas9-gRNAs_OsCKX2* constructs to *E. coli* strain DH5α was carried out using the heat shock method (Chang et al. 2017). A total of 5 uL of the golden gate reaction was mixed into 50 uL of competent *E. coli* DH5α cells and put on ice for 30 minutes, then put in a heating block at 42ºC for 15 seconds, and transferred back to the ice for 5 minutes. A total of 500 uL of liquid LB medium was added to the mixture, and then incubated at 37ºC for 3 hours with shaking. The tube containing the transformed *E. coli* was centrifuged at 1200 rpm for 1 minute. The supernatant was discarded and left to the final volume of 450 uL. The pellet was resuspended in the tube and spread on solid LB medium + 50 mg/L kanamycin, then incubated at 37ºC overnight. The 10 selected single colonies were verified using direct PCR using primer pairs TC306 (5'-AGCACTACCAATGATGACCT-3') and M13F (5'-GTAAAACGACGGCAGT-3'). The DNA sequencing analysis was conducted to confirm the presence or absence of array insertion into pDIRECT-25H vector. The correct recombinant plasmid was then transformed into *Agrobacterium tumefaciens* LBA4404 using an electroporator.

A total of 5 uL of plasmid DNA was added to 50 uL of *A. tumefaciens* competent cells. The plasmid and *A. tumefaciens* bacterial competent were transferred to the electroporation cuvette and tapped until the suspension was at the bottom of the cuvette. The suspension was incubated on ice for 15-20 minutes to allow the plasmid to adhere to the wall of *A. tumefaciens*. The electroporator is turned on and the cuvette is placed on the chamber slide. The slide is pushed into the chamber until the cuvette sits between the box at the bottom of the chamber, then pulse once is pressed 1 mL of liquid YEP was added to the cuvette and then resuspended. The suspension was then transferred to a 1.5 mL tube and incubated for 3 hours at 30ºC at 200 rpm with shaking at 200 rpm, after which it was centrifuged at 10,000 rpm for 1 minute. 800 uL of supernatant was discarded and resuspended again, after that it was spread on solid YEP media + 50 mg/L kanamycin and incubated at 28ºC for 2 days. Single colonies that grew were taken and checked using PCR. Positive colonies were grown on liquid YEP + 50 mg/L kanamycin media and incubated at 28ºC for 2 days.

Regeneration study of rice cv. Mentik Wangi

The regeneration medium used refers to Slamet Loedin et al (2014). Immature rice embryos were grown on a callus induction medium for 7 days. The callus induction medium used was macro N6 (Chu et al. 1975) with the addition of micro B5 and vitamin B5 (Gamborg et al. 1968), 500 mg L-1 casamino acid, 500 mg L^{-1} L¬-proline, 30 g L-1 sucrose, 2 mg $^{L-1}$ 2.4-D, 1 mg $^{L-1}$ BAP, dan 2 mg $^{L-1}$ NAA (Table 2).

Tabel 2. Media composition for the transformation of immature embryo (Slamet-Loedin et al. 2014)

Media	Composition
Liquid co-cultivation	AA with 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 dan 10 g L^{-1} glucose, pH 5.4
Solid co-cultivation	NB with 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	NB with 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 + 30 mg L^{-1} hygromycin
Pre-regeneration	MS with 30 g L-1 maltose, 20 g L-1 sorbitol, 10 g L-1 agarose type I, 2 mg L-1 kinetin, 5 mg L^{-1} NAA, 250 mg L^{-1} cefotaxime, 100 mg L^{-1} vancomycin, 30 mg L^{-1} hygromycin, pH 5.8
Regeneration	MS with 30 g L-1 sucrose, 2 mg L^{-1} kinetin, 1 mg L^{-1} NAA, 250 mg L^{-1} cefotaxime, 100 mg L^{-1} vancomycin, 30 mg L^{-1} hygromycin, 3 g L^{-1} gelrite, pH 5.8
Rooting	MS with 30 g L^{-1} sucrose, 1 mg L^{-1} IBA, 2 g L^{-1} gelrite, 30 mg L^{-1} hygromycin, pH 5.8

On day 7, sprouts growing on the media were cut and subcultured on fresh media. After the callus grew larger, the callus was cut into 2-4 parts and subcultured on the selection media. Callus on the selection medium was subcultured several times until the callus gave rise to a green spot. Callus with green spots was transferred to pre-regenerated media to form root hairs. The developed calli were transferred to regeneration medium to grow larger and the root hairs developed into roots.

The experimental design carried out was a completely randomized design. The treatment given was the elimination of the antibiotic components in the pre-regenerated, regenerating, and rooting media. The percentage of regenerated callus was calculated by the following formula:

$$\textit{Percentage of regenerated callus} : \frac{\textit{total callus on regeneration media}}{\textit{total tested callus}} \; x\; 100\%$$

Introduction of CRISPR/Cas9 cassette into rice cv. Mentik Wangi via *A. tumefaciens*

Transformation of Mentik Wangi rice was carried out by dripping immature embryo explants on solid co-cultivation media with *A. tumefacient* suspension harboring CRISPR/Cas9-gRNA constructs i.e. *pDIRECT25H-gRNAOsCKX2* and *pBY02-gRNAOsGA20ox2*. TheCRISPR/Cas9-*pBY02-gRNA-OsGA20ox2* cassette used in this study was previously constructed by (Santoso et al. 2020). Before transformation, *A. tumefacient* was grown for 3 days on solid YEP media with the addition of 50 mgL^{-1} kanamycin at 28ºC. Bacterial cultures in YEP media were harvested and grown on liquid co-cultivation media and then used to infect young rice embryos. The co-cultivated embryos were then incubated in the dark at 25ºC for 7 days and then cultured on resting media for 5 days. Explants were transferred to selection media for 10 days with 5 sub-culture periods. The selected callus is transferred to pre-regenerated media for 14 days with 2 sub-culture periods. Callus that proliferated into planlet with small sprouts and root hairs were transferred to a regeneration medium for approximately 14 days. The planlets with strong roots were transferred to the root media to make the roots stronger and more numerous and the acclimatized to water media and transferred to soil media in a greenhouse. The transformation efficiency was calculated using the following formula:

$$\textit{Transformation efficiency} : \frac{\textit{planlet on solid media}}{\textit{total tasted callus}} \; x\; 100\%$$

Data analysis

Molecular analysis of the putative transformant T0 rice lines cv. Mentik Wangi was carried out by direct PCR to confirm the presence of CRISPR/Cas9 construct. Amplification of hpt gene was performed using a KAPA2G Fast ready mix kit and a pair of oligonucleotides 5'TCCGACCTCATGCAGCTCTC'3 (Forward) and 5'GATTCCTTGCGGTCCGAATG'3 (Reverse). Direct PCR analysis was performed with a total PCR reaction of 20 uL consisting of 10 uL of Kappa 2G, 0.6 uL of primer hpt forward and reverse, 1 uL of DNA template, and 7.8 uL of nuclease-free water. The PCR mixture was run using the DNA Engine Tetrad® Peltier Thermal Cycler (Bio-Rad). The PCR products were electrophoresed using 0.8% TAE (Tris-acetate-EDTA 1x buffer) agarose for 40 mins with 100 bp ladder plusmarker. DNA bands were visualized using a UV Transilluminator. Water, wild-type plants, and plasmid DNA were used as positive and negative controls. Putative mutant T0 rice cv. Mentik Wangi which showed positive for HPT gene was indicated by obtaining an amplicon product of 500 bp.

RESULTS AND DISCUSSION

Construction of CRISPR/Cas9-gRNAOsCKX2

RNA that guides Cas9 protein to edit the target gene was selected with the size of 20bp at 960 to 980 positions of the 2nd exon part of *OsCKX2* gene through CRISPR/Cas9 webserver (Figure 3). Primers for array-forming fragments of the CRISPR/Cas9 cassette construct with pDIRECT_25H backbone were designed (Table 3). The primer consists of primer to amplify a promoter Ubi1, gRNA, and terminator. In the promoter and terminator, a *Sap*I site is added which facilitates to insert the array into pDIRECT_25H vector.

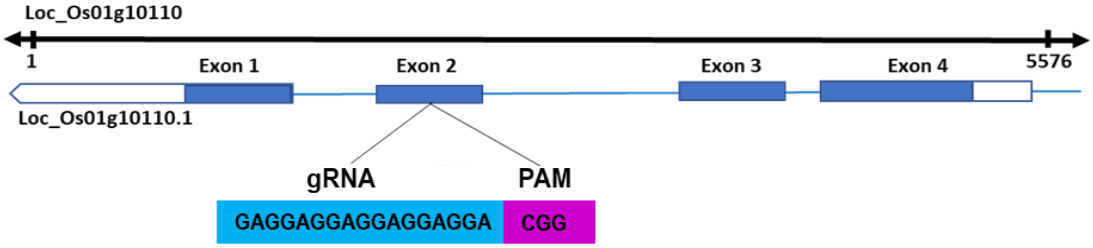


Figure 3. The OsCKX2 gene (loc_Os01g10110) on chromosome 1 and the position of the exon (loc_Os01g10110.1) and the position of the PAM and gRNA sites on exon 2

Table 3. The primer design for CRISPR/Cas9 cassette construction with pDIRECT25H backbone

Primer	Sequence
Promoter Ubi1	TGCTCTTCGCGCCACGTCAGTGTTTGGTTTCC
CSY_gRNA1_OsCKX2	TTCCACCTGCACACCTCCTCCTCATCCTGCCTATACGGCAGTGAAC
REP_gRNA1_OsCKX2	GCTCACCTGCGTCGGGAGGAGGAGGAGTTTTAGAGCTAGAAATAGC
CSY_terminator	TGCTCTTCTGACCTGCCTATACGGCAGTGAAC

The promoter, gRNA, and terminator region amplicon fragments were obtained using the designed primer (Figure 4.A). The fragments were ligated through the golden gate reaction and the succeeded ligation was confirmed by PCR analysis using the promoter and terminator primers (Figure 4.B). The accurate amplicon size obtained from the golden gate reaction is about 2.142 bp. The fragment was ligated to the pDIRECT_25H backbone plasmid. The pDIRECT_25H ligation product with array fragments (promoter, gRNA, and terminator) was transformed into *E. coli*. The result of *E. coli* transformation and kanamycin antibiotics selection was obtained from several of the bacterial single colonies. The bacterial colonies were then selected by PCR analysis using a primer pair TC306 and M13.

The transformation of the *pDIRECT_25H-gRNA-OsCKX2* cassette constructs into *A. tumefaciens* bacteria produce a single colony (Figure 5.A). Direct PCR analysis of ten single colonies of bacteria was conducted with specific primers M13F and TC306. The PCR results showed that two out of ten single colonies of bacteria produced a DNA band with a size of 600 bp as expected size of the target DNA (Figure 5.B). The final construct validation was carried out by DNA sequencing analysis and the result indicated that the array consisting of the pUbi1, CSY1 promoter, spacer, *gRNA_OsCKX2*, CSY2, and 35S terminator (Figure 5.C) was successfully inserted into the pDIRECT_25H plasmid (Figure 5.D). DNA Sequencing analysis verified that the *pDIRECT_25H-gRNA OsCKX2* construct cassette had been transformed into *A. tumefaciens* and was ready for genetic transformation into Mentik Wangi (Kámán-Tóth et al. 2018).

Regeneration study of Mentik Wangi

Regeneration study of Mentik Wangi was conducted to find out the ability of callus to regenerate into plantlets. The ability of Mentik Wangi's callus regeneration revealed that 80% of callus were able to regenerate on media without the addition of antibiotics and only 2.2% of callus were able to regenerate on media with addition of 10 mg/L of hygromycin antibiotic (Table 4). According to this, concentration of hygromycin used for the selection step in the genetic transformation of Mentik Wangi was 10 mg/L which is the minimum lethal concentration. The use of a 10 mg/L hygromycin concentration indicated inhibition of callus growth and only about 2.2% of callus were able to survive and then regenerate to form plantlets (Figure 6). The level of callus sensitivity to antibiotic selection differs between plant genotypes and depends on the plant species or cultivar used (Tran and Sanan-Mishra 2015). Hygromycin can destroy transformant and non-transformant cells so that it affects the regeneration of callus (Tran and Sanan-Mishra 2015). Zhu and Wu (2008) also stated that hygromycin added to the media was capable to inhibit callus growth and development so that it causes the death of the callus. Besides the addition of hygromycin, other antibiotics in the regeneration medium such as cefotaxime and vancomycin are functioning to eliminate *Agrobacterium* after the transformation process (Zhu and Wu 2008). The addition of these antibiotics turned out to have an inhibitory effect on shoot and root induction, causing a loss of phytohormonal balance and affecting callus regeneration ability (Ogawa and Mii 2007) (Figure 7). Extra antibiotics added to the culture media can cause inhibition of callus growth and development (Tran and Sanan-Mishra 2015).

Table 4. Percentage of efficiency callus on regeneration media

Culture media	Repetition	Total number of calli	Number of calli on selection media	Number of embriogenic calli	Calli on regeneration media	Percentage of regeneration efficiency
Free antibiotic	I II III	90	72	72	72	80%
Addition antibiotic	I II III	90	80	19	2	2.2%

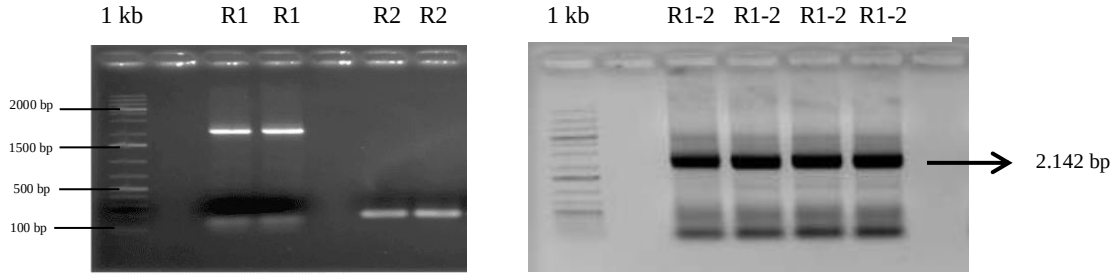
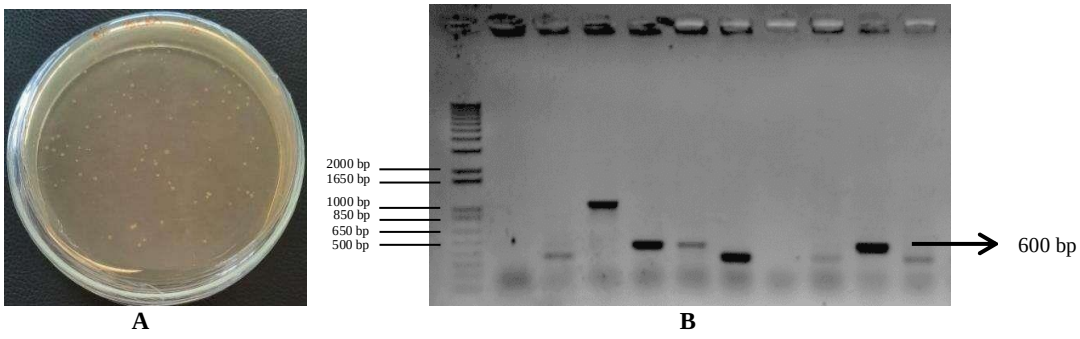


Figure 4. A. R1: amplicon fragment from reaction 1 (promoter and *CSY_gRNA1_OsCKX2*), R2: amplicon fragment from reaction 2 (*REP_gRNA1_OsCKX and CSY-term*). B. R1-2: amplification fragment of the golden gate reaction between the R1 and R2 amplicons



CCTTGATGATGTTTCTGTTTGTTTGAGGCTTGTGTTACTGCTAGTTACTTACCCTGTTGCCTGGCTAATCTTCTGCAGATCCTGCAGGTTCACTGCCGTAT
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CGTTCACTGCCGTATAGGCAGGTCGATCGACAAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTT
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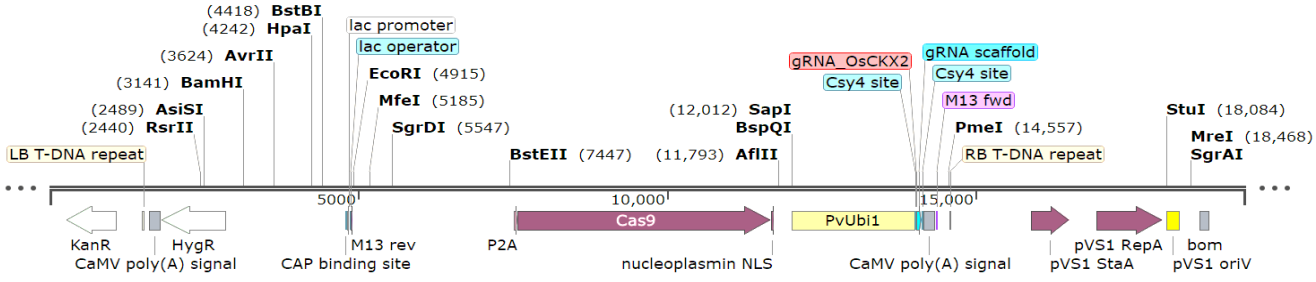
TAAAATCCAGATCCCCCGAATTACCGGTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCC

C



D

Figure 5. A. Colonies of *A. tumefaciens* carrying recombinant plasmid *pDIRECT_25H_gRNA_OsCKX2*, B. PCR amplification results for verification *A. tumefaciens* colonies harboring CRISPR/Cas9 construct, C. Results of DNA sequencing analysis of positive recombinant plasmids carrying *gRNA_OsCKX2*, Note. color: Gray: pUbi1 promoter, green: CSY, blue: *gRNA_osCKX2*, yellow: gRNA scaffold, purple: terminator, D. Schematic of *pDIRECT_25H_gRNA-OsCKX2* recombinant plasmid carrying the pUbi-CSY-*gRNA_OsCKX2*-gRNA scaffold-terminator array

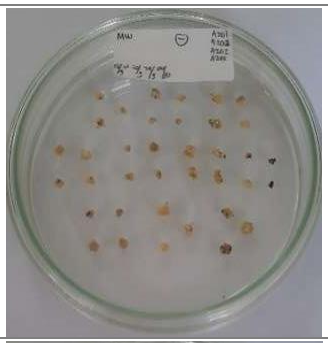
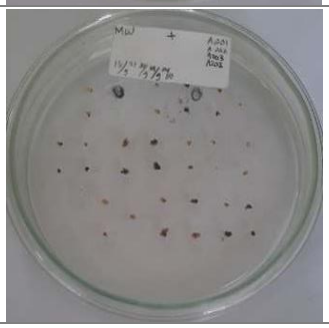
Culture media	Tested callus	Callus on selection media	Callus on regeneration media
Free antibiotic			
Addition antibiotic			

Figure 6. Comparison of callus regeneration on media with and without antibiotics addition

A **B**

Figure 7. Callus performance on culture medium. A. good callus is yellowish-white (free antibiotic), B. abnormal callus is blackish

Transformation study of Mentik Wangi

Transformation study of Mentik Wangi was carried out by using mixtur of *A. tumefaciens* carrying CRISPR/ Cas9_gRNA-*OsGA20ox* and CRISPR/Cas9_gRNA-*OsCKX2 constructs*. The transformation stages include preparation of *Agrobacterium* culture, preparation of explants in the form of young rice seeds, infection of immature embryos with *Agrobacterium*, co-cultivation, callus selection process, regeneration and rooting processes, acclimatization in water and soil media (Figure 8). The development of the scutellum in Mentik Wangi embryos was followed by the appearance of coleoptiles after seven days in co-cultivation media. At this stage, the coleoptiles were removed and the callus was transferred to resting media for five days. The well-developed callus was cut into two to four parts and transferred to a selection medium with 10 mgL^{-1} hygromycin added as a selection marker for ten days with a duration of 3-5 times sub-culture. At the selection stage, it will be seen the difference between calluses that are resistant and sensitive to hygromycin (Figure 7). Callus resistance will have the ability to proliferate (Figure 5.F). A resistant and embryogenic callus will form green spots which are a sign of the start of the regeneration process. The callus was transferred to pre-regenerated media for 14 days with two sub-cultures until they regenerated into shoots (Figure 8.G). For shoot maturation, the callus that has shot and rooted is transferred to a regeneration medium for 14 days to form plantlets. Plantlets were transferred to the root medium to stimulate the growth of new roots and to strengthen the roots (Figure 8H). Plantlets with strong roots were acclimatized in aqueous media for three to five days and then transferred to soil media (Figure 8.I, 8.J).

A total of 783 immature embryos were transformed with two CRISPR/Cas9 cassette constructs. The callus that showed resistance to hygromycin were 57 calli (7.3%). The transformant callus, after being transferred to the regeneration medium, only about 30 calli (3.8%) were able to regenerate into transformant shoots with a regeneration efficiency of 56.1%. Transformant shoots will develop into plantlets and are maintained until the planlets entered the acclimatization stage and are transferred to soil media. A total of 30 putative transformant rice lines T0 cv. Mentik Wangi has been obtained in the process of transformation. The transformation efficiency of Mentik Wangi rice displayed an efficiency of 3.8% (Table 5). The transformation efficiency is highly depend on the genotype and the ability of plant regeneration in culture media (Hiei and Komari 2008; Low 2018). To increase the efficiency of the transformation, several modifications can be made to the transformation method to optimize the transformation system.

Molecular analysis of putative mutant rice t0 cv. Mentik Wangi

Molecular analysis was carried out on the putative mutant rice lines T0 cv. Mentik Wangi to confirm the existence of the CRISPR/Cas9 cassette construct. Out of the 30 putative mutant rice lines T0 cv. Mentik Wangi only 15 lines tested are positively harboring the *CRISPR/Cas9-gRNA-GA20ox2* and *CKX2* construct cassettes, which were characterized by the formation of a 500 bp DNA band (Figure 9). To ensure the occurrence of mutations in the target gene, it is required to conduct DNA sequencing analysis on 2 target genes, namely *GA20ox2* and *CKX2*.

Table 5. Percentage of transformation efficiency of Mentik Wangi

Repetition	Number of immature embryos	Number of calli on selection media	Number of calli on pre-regeneration media	Number of calli on regeneration media	Number of shoots on rooting media	Number of plantlets	Number of plants	Percentage of transformation efficiency
I	135	59	4	3	2	2	2	3.8%
II	288	145	36	17	17	17	16	
III	360	199	17	12	12	12	12	
Mean	783	403	57	32	31	31	30	

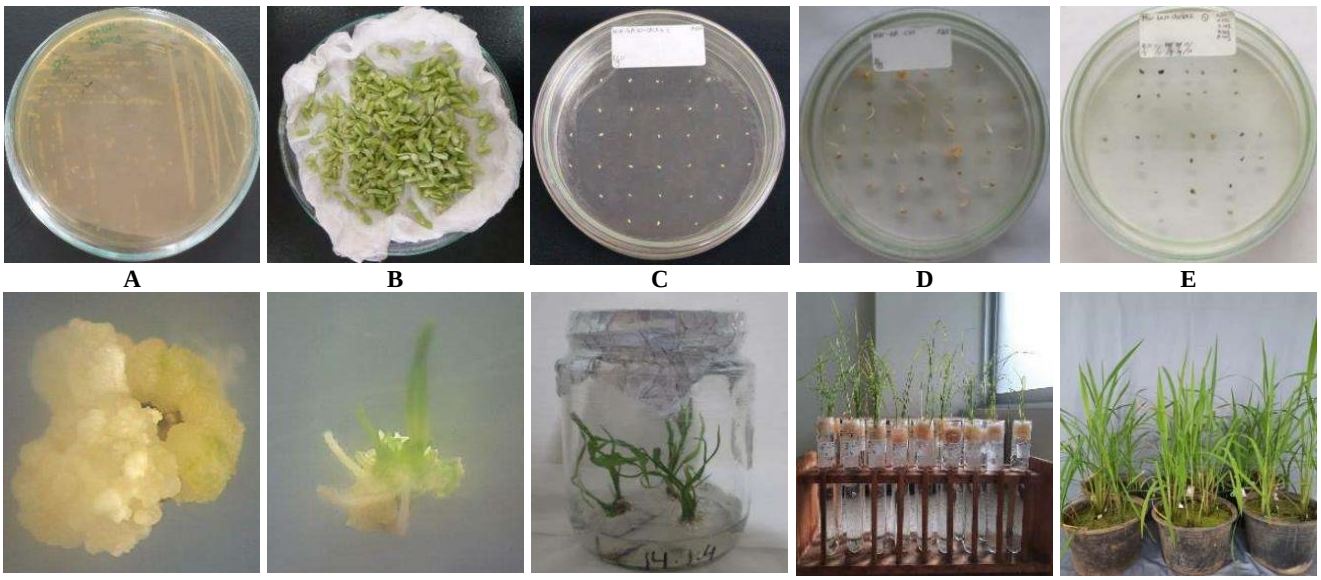


Figure 8. Step in *Agrobacterium*-mediated transformation of immature embryos from Mentik Wangi. A. *Agrobacterium* culture, B. Isolation phase, C. *Agrobacterium* infection, D. 7 days after co-cultivation, E. Callus on selection medium, F. Resistant calli on pre-regeneration medium, G. Shoot transformant on regeneration medium, H. Planlet on rooting medium, I. Acclimatization, J. Plant on soil

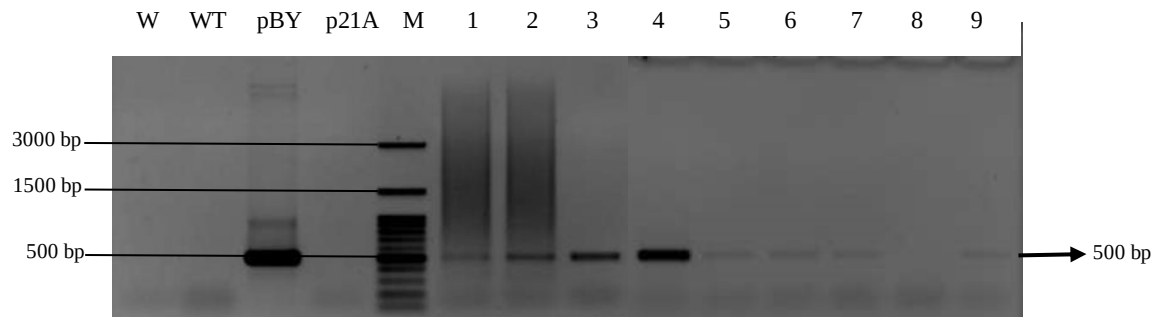


Figure 9. HPT gene amplification in the putative mutant rice plant T0 cv. Mentik Wangi. W: water, WT: wild type, p21A dan pBY: plasmid, M: 100bp ladder, 1-9: sample

Based on this study, it can be concluded that the *CRISPR/Cas9-gRNA-OsCKX2* cassette with the pDIRECT-25H backbone has been successfully constructed. Preliminary study on the regeneration of Mentik Wangi has also been carried out with an efficiency of 80%. Modification of regeneration media parameters was carried out to optimize plantlet formation in the transformation process. A total of 30 putative transformant lines of Mentik Wangi were successfully obtained from the transformation process and 15 lines after PCR testing showed positively carrying the *CRISPR/Cas9-gRNA-GA20ox2* and *CRISPR/Cas9-gRNA-CKX2* construct cassettes.

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