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INTRODUCTION OF CRISPR/Cas9 WITH THE TARGET GENES TO IMPROVE AGRONOMIC TRAITS AND LEAF BLIGHT RESISTANCE IN RICE

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SUMMARY

Improving rice (*Oryza sativa* L.) quality is crucial to obtaining local rice with better genetic potential and superiority. The research aimed to construct a CRISPR/Cas9 module cassette and introduce the construct into rice to develop a new non-transgenic superior Mentik Susu variety with early maturity, short stem, high yield, and resistance to bacterial leaf blight. The annealed oligonucleotides of gRNA spacers of the *HD2* gene ligated into pDIRECT-21A vector plasmid used the golden gate reaction to construct a CRISPR/Cas9 module cassette. The recombinant plasmid's verification by digestion engaged a combination of KpnI-HindIII restriction enzymes and Sanger DNA sequencing. The *Agrobacterium*-mediated co-transformation procedure introduced the CRISPR/Cas9 cassettes (four module cassettes with different gRNAs of the genes, i.e., *GA20ox-2*, *OsCKX2*, *OsSWEET11*, and *HD2*) into the rice genome with immature rice embryos as explants. Molecular analysis of the transformed T0 putative lines ensued to identify the insertion of T-DNA fragments (containing the *Cas9* and *hptII* genes) and the occurrence of mutagenesis employing PCR amplification and DNA sequencing. The result showed that the CRISPR/Cas9 cassette vector containing the *Cas9* gene and the targeted gRNA construction succeeded. Rice transformations of Mentik Susu have generated 157 T0 putative lines, with several of the lines harboring the *Cas9* and *hptII* genes detected positively. Sanger DNA sequencing analysis demonstrated that eight rice lines had a mutation occurrence in the target genes, i.e., two mutations in the OsGA20ox line, five mutations in the OsSWEET11 lines, and one mutation in the Gn-1a (OsCKX2) line. Based on these results, it is probable that the mutant lines also have a phenotype change that is beneficial to produce promising rice genotypes with early maturing, short stems, high yield, and bacterial leaf blight resistance.

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Keywords: *Oryza sativa* L., Mentik Susu rice cultivar, genome editing, CRISPR/Cas9, mutagenesis, early maturity, high yield

Key findings: The presented research has successfully constructed the CRISPR/Cas9-gRNA-HD2 vector cassette and introduced the construct into rice cv. 'Mentik Susu' to edit the *HD2* gene for developing an early-maturity rice cultivar. Genome editing of the multiple-gene targets (*GA20ox-2*, *OsCKX2*, *OsSWEET11*, and *HD2* gene) in rice cv. 'Mentik Susu' has resulted in obtaining several rice lines with the CRISPR mutated genes.

INTRODUCTION

Improving aromatic rice productivity and quality is an effort to fulfill food needs in Indonesia and even the world. Rice (*Oryza sativa* L.) cultivar Mentik Susu is an aromatic rice becoming in demand because of its fluffy taste and fragrant aroma. However, farmers rarely cultivate this rice cultivar because of its late maturity, relatively low production, and tall stature, causing plants to lodge easily, which is also susceptible to bacterial leaf blight pathogen. Suryanugraha *et al.* (2017) reported that the cultivar Mentik Susu is taller (117 cm) than nine other local varieties and has the lengthiest harvest age (130 days after planting-DAP), less productive tillers, and more grains per panicle.

Recently, new breeding techniques (NBT) can be applicable in developing a new rice variety with the desired traits. One of the modern technologies emerging as the most widely used tool for creating a new plant variety is the genome editing technique, such as the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (CRISPR-Cas9) method. CRISPR/Cas9 is an alternative to genome editing that can help improve plant yield traits and develop disease resistance. The advantages of CRISPR/Cas9 are more precise, efficient, and low-cost compared with previous editing systems, namely, Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). CRISPR/Cas9 relied on using short sites (gRNA) that gene targets. This gRNA will attach to its complementary partner. With this convenience, the CRISPR/Cas9 system has become widely used for research, one of which is to edit the genome of the Mentik Susu rice plant. Compared with ZFN and TALEN, the CRISPR/Cas system has advantages with its simple design, versatility, cost-effectiveness, high efficiency, and specificity (Brandt and Barrangou, 2019).

The CRISPR/Cas9 technique's employment in this study targets genes for mutagenesis, namely, *HD2*, *Gn-1a* (*OsCKX2*), *sd1* (*GA20ox-2*), and *SWEET11* genes. Genome editing is a genetic engineering method that can delete, insert, and transfer DNA sequences from the genome of one organism to another by using nuclease enzymes that are useful as molecular scissors (Schinkel and Schillberg, 2016). Furthermore, Sprink *et al.* (2015) stated that Cas9, as an enzyme, cuts target DNA located in sequences positioned close to the protospacer adjacent motif (PAM).

The *HD2* gene plays a vital role in the flowering process of plants. Modifying this gene may alter the plant to become early maturing. Meanwhile, according to research findings of Tsago *et al.* (2020), rice cytokinin mutants named cytokinin oxidase/dehydrogenase Osckx2-2 and the *OsCKX2-2* gene are beneficial in increasing rice yield. Another study by Mubarok *et al*. (2019) revealed that the *GA20ox-2* mutant gene in the Kitaake rice lines showed shorter plant height and leaf length compared with the wild type in genotypic and phenotypic observations. The *SWEET11* gene helps prevent excessive sugar utilization by proliferating fungal hyphae and limiting sugar availability by downregulating the *SWEET11* sucrose transporter gene to retain sugar in cells (Rouina *et al.*, 2021). Based on the above discussion, developing the Mentik Susu rice cultivar with improved traits of early maturity, short stem, high yield, and bacterial leaf blight resistance through DNA editing technology with the CRISPR/Cas9

system is a must to conduct. The research aimed to construct a CRISPR/Cas9 module cassette and introduce the construct into rice for developing a new non-transgenic superior Mentik Susu variety with early maturity, short stem, high yield, and resistance to bacterial leaf blight.

MATERIALS AND METHODS

Experimental site and procedure

Construction of CRISPR/Cas9 HD2 cassette, gene transformation of *HD2*, *Gn-1a* (*OsCKX2*), *sd1* (*GA20*), and *SWEET11*, molecular analysis, planting rice (*Oryza sativa* L.) cultivar Mentik Susu for preparing explants and sowing mutant rice plants proceeded in the laboratory and greenhouse of the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Ministry of Agriculture, Bogor, Indonesia. Explants used for genetic transformation were 14–16 day-old immature embryos of rice plants, cv. Mentik Susu. The pDIRECT_21A plasmid vector harboring the *Cas9* and *hptII* gene served as the material in the study for constructing the CRISPR/Cas9 cassette vector. pDIRECT_21A was a gift from Daniel Voytas (Addgene plasmid # 91128; http://n2t.net/addgene: 91128; RRID: Addgene_91128) (Čermák *et al.,* 2017).

CRISPR/Cas9 constructs; digest and ligation of gRNA in pDIRECT-21A plasmid vector using golden gate reaction

The designed sequence of the gRNA spacer for the *HD2* gene engaged the software program CRISPOR online tool (http://crispor.tefor.net/) (Concordet and Haeussler, 2018) with the *HD2* gene sequence input from rice. The oligonucleotide annealing procedure had the following order: gRNA primers dissolved with ddH_2O to reach a stock solution concentration of 100 pmol/µl; then the primers' stock diluted to 10 pmol/µl, with the primers added to a reaction mix containing the 10x buffer, ATP, and T4 Kinase; the reaction gained incubation at 37 °C for 30 min; and afterward, the annealed gRNA was ready for the ligation process into the pDIRECT-21A expression vector plasmid by using the golden gate reaction. The reaction mix consisted of 50 ng of the pDIRECT_21A, 5 µl annealed oligonucleotides, 0.4 µl AarI oligonucleotide, 0.5 µl AarI, 2 µl 10X T4 DNA ligase buffer, 1 µl T4 DNA ligase, and ddH_2O, with a final total volume of 20 µl.

The golden gate reaction process continued on a PCR machine with the following program: 37 °C for 5 minutes, 16 °C for 10 min, 37 °C for 15 min, 80 °C for 5 min, and 20 °C for 5-10 min. A total of 5 µl of the results of the golden gate reaction attained transforming into *E. coli* DH5α competent cells, then spread on solid LB agar medium, adding 50 mg/L kanamycin. The recombinant plasmid's confirmation continued by amplifying the plasmid using a specific primer pair, i.e., M13(-21)-F (5'-TGTAAAACGACGGCCAGT-3') and gRNA_21A-R (5'-GATAAGGAGCTCCGGTGACG-3'). The positive recombinant plasmid, after PCR confirmation, became the material for DNA sequencing. DNA sequencing analysis helped ensure that the target gRNA has succeeded in ligation to the p-DIRECT_21A vector. The correct recombinant plasmid verification progressed by digestion with a combination of KpnI and HindIII restriction enzymes. The CRISPR/Cas9 vector cassette carrying the target gene *gRNA* continued transformation into *A. tumefaciens* competent cells using the electroporator technique.

Introduction of CRISPR/Cas9-gRNA constructs to rice cv. Mentik Susu

For plant genetic transformation, using four CRISPR/Cas9 constructs with different gRNA from these genes included the *HD2* (this study), *Gn-1a* (*OsCKX2*) (Ruzyati *et al*., 2022), *sd1* (*GA20ox2*) (Santoso *et al.,* 2016), and *SWEET11* (Rifhani *et al.,* 2023). According to Slamet-Loedin *et al.* (2014), the stages of rice transformation comprised the following: Isolation of immature embryos and sterilization of the immature embryo using 70% ethanol and 30% of clorox (bleaching solution); The co-cultivation stage, *A. tumefaciens* vector

containing the CRISPR/Cas9 cassettes put on liquid medium proceeded measuring to reach the optical density (OD) of 0.1-0.3, adding the 100 µl/L of acetosyringone; The resting step, cutting the coleoptiles for the developed callus sub-culture consisted of cleaning the callus and incubating in the rest medium; The selection stage, dividing calluses into several parts continued their growing into the selection medium, with the developed callus then transferred to the pre-regeneration medium; Regeneration stage, transferring the well-developed callus characterized by the appearance of the green spot to the regeneration of medium till they develop to form a plantlet; and Rooting stage, the normal plantlets then transferred to the rooting medium, and finally, the plantlets transfer to the soil medium to obtain T0 plants.

Molecular analysis of T0 putative rice lines cv. Mentik Susu

Isolating T0 plant DNA used the CTAB miniprep method (Doyle and Doyle, 1987). Performing PCR utilized hptII and Cas9 primers to detect T0 plants containing *hptII* and *Cas9* genes. Primary nucleotide sequences are Cas9-314-F (5'-GGTGGAATACCACGAGAAGT- 3') and Cas9-314-R (5'-GAACCTCATTGCTCAGCTCC-3'). As a control, PCR analysis used binary vector plasmid vectors pDIRECT-25H, pDIRECT-21A, and pCAMBIA1300, which contained the *hptII* gene as a marker for selecting transgenic plants and the *Cas9* gene in the T-DNA region. The PCR composition for *hptII* and *Cas9* gene amplification consisted of 0.25 µl or 25 pmol primer, 3.5 µl nuclease-free water (NFW), 5 µl Kapa 2G, and 1 µl template DNA at a concentration of 50 ng. The PCR conditions for both genes were the same, namely, pre-PCR DNA denaturation at 95 °C for 4 min, followed by 32 cycles consisting of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, followed by post-PCR at 72 °C for 5 min, and cooling at 10 °C for 10 min. PCR amplification ensued using electrophoresis with 1% agarose gel, then staining with the ethidium bromide solution and visualized under UV light to determine the presence of the target amplicon.

RESULTS AND DISCUSSION

Establishment of the HD2 CRISPR/Cas9 construct

The designed gRNA spacer sequence used the CRISPOR online tool. The result of gRNA designing indicated that there are two different gRNAs selected from the tool, i.e., the HD2-g1-gRNA located at the nucleotide sequence 90-112 and HD2-g2gRNA at the nucleotide sequence 207-229 of the locus LOC_Os07g49460. The primers designed for HD2-gRNA acquired the Aar1 restriction enzyme site to facilitate the insertion of the gRNA into the plasmid vector pDIRECT_21A (Table 1). For ligation purposes, DNA fragments added with restriction enzyme sites will be suitable for golden gate assembly with the vector for use (Marillonnet and Grützner, 2020). The sequences selected in the design of the gRNA process are those of the target gene, which has a close location to the PAM. According to Ren *et al*. (2021), the target sequence DNA that the Cas nuclease will break has the PAM restricting it. Besides that, Hassan *et al*. (2021) stated that the target gRNA design selected should be in an exon of the target gene because it could produce more NHEJ-induced gene editing that causes gene function losses.

The ligation process of gRNA_HD_g1 and gRNA_HD_g2 oligonucleotides into pDIRECT_21A occurred using the PCR-based golden gate reaction. In this reaction, the digestion process using a restriction enzyme and the ligation process transpired in one reaction mediated by the PCR machine. The digestion-ligation reaction produces high cloning competency, allowing up to 24 fragments to join in one cloning reaction (Potapov *et al.*, 2018). The next stage is introducing the ligands into E. coli competent cells to check the precise recombinant plasmids, and the E. coli transformation produced several single colonies (Figure 1). Verifying the colonies used the PCR with primer pairs M13(-21)F (5'-TGTAAAACGACGGCCAGT-3') and gRNA_21A-R (5'-GATAAGGAGCTCCGGTGACG-3'), resulting in 12 positive colonies carrying the target gRNA

Table 1. gRNA primer design of the gene *HD2*.

Primer	Oligonucleotides (Forward)	Oligonucleotides (Reverse)
gRNA_HD2_g1	GATTGGGTGGGGGCTACAGCGACC	AAACGGTCGCTGTAGCCCCCACCC
gRNA_HD2_g2	GATTCGATGACTCCACCAGGCAGG	AAACCCTGCCTGGTGGAGTCATCG

Description: The underlined sequence is the Aar1 restriction enzyme sequence to facilitate the insertion of gRNA into the plasmid vector pDIRECT_21A.

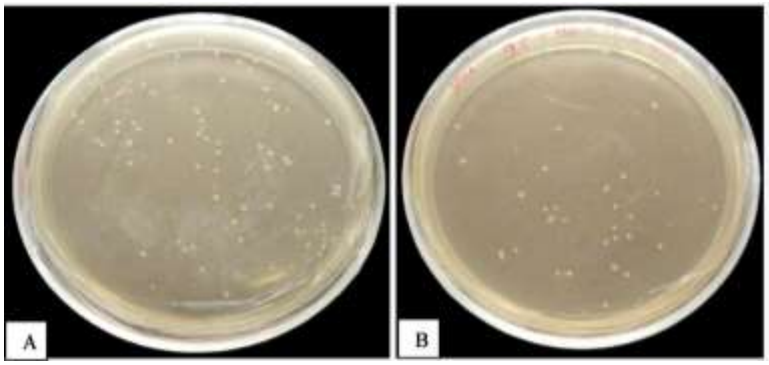

Figure 1. Colonies of pDIRECT_21A transformed *E. coli* carrying gRNA_HD2_g1 (A) and gRNA_HD2_g2 (B).

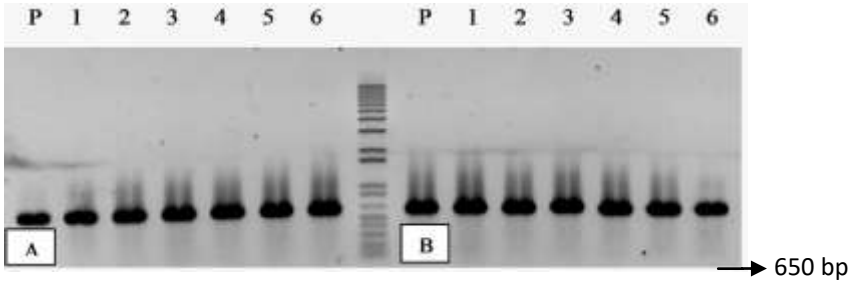

Figure 2. Amplification results of plasmids carrying gRNA_HD2_g1 (A) and gRNA_HD2_g2 (B). Notes: P = pDIRECT_21A, Number 1-6 = Colony number.

indicated by the presence of a 650 bp DNA band (Figure 2).

Verifying the recombinant plasmid using a combination of KpnI and HindIII restriction enzymes also confirmed the PCR-positive colonies. The correct recombinant plasmids' digestion resulted in two DNA fragments, while the control plasmid formed three fragments (Figure 3). The occurrence of inserting the gRNA sequences into the pDIRECT_21A vector plasmid led to the correct recombinant plasmid having only one KpnI site. It proves the insertion of the target gRNA-HD2_g1 and gRNA-HD2_g2 have succeeded into the vector pDIRECT_21A. The Sanger DNA sequencing analysis also confirmed the precise recombinant plasmids' insertion of the target gRNA into the vector plasmid (Figure 4). The T-DNA part of the pDIRECT21A-gRNA_HD2_g1 (Figure 5A) and pDIRECT21A gRNA_HD2_g2 (Figure 5B) constructs carry the *HygR* (*hptII)* gene, *Cas9* gene, and the scaffold gRNA, respectively (Figure 6). The correct recombinant plasmids carrying the insertion of gRNA_HD2_g1 and gRNA_HD2_g2 proceeded for transformation into *A. tumefaciens* vector before their use for genetic transformation into rice cv. Mentik Susu.

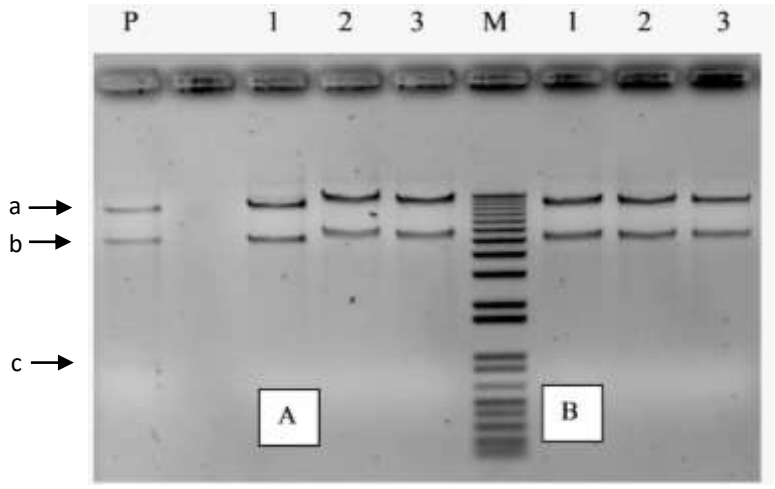

Figure 3. Digest of gRNA-HD2_g1 (A) formed 3 fragments (a-c) and gRNA-HD2_g2 (B) 2 fragments formed. Notes: P = plasmid pDIRECT_21A, M = Marker 1kb, Number 1-3 = Colony number.

Introduction of CRISPR/Cas9-gRNA constructs of target genes into Mentik Susu rice

Genetic transformation of rice cv. Mentik Susu progressed by the co-transformation approach combining two/four *A. tumefaciens* bacterial suspensions that contain CRISPR/Cas9 constructs for editing the genes, i.e., *GA20ox2, OsCKX2*, *OsSWEET*, and *HD2*. The transformation of Mentik Susu rice with constructs for target genes *GA20ox2* and *OsSWEET* produced 53 T0 putative lines (Table 2). The transformation with a construct for editing the *OsCKX2* and *HD2* genes developed 24 T0 putative lines. Meanwhile, the transformation for editing target genes (*GA20ox2, OsSWEET, OsCKX2,* and *HD2*) produced 157 lines. The conducted transformation process basis for reference appears in Figure 7A-7H. Further analysis will continue to determine the hptII and Cas9 insertion.

Detection of the *Cas9* gene in putative T0 strains of Mentik Susu rice by the PCR technique

Confirming transformant lines also ensued using specific primers for the *Cas9* gene. The transformant strains for the target genome editing genes *GA20ox-2* and *OsSWEET11* produced 36 strains that positively carried the

Detection of the *hptII* gene of T0 putative lines of Mentik Susu rice

Detecting the *hygromycin* gene used PCR employing a specific primer, and the result showed that almost all the putative transformant lines are positive PCR, as indicated by the DNA fragment with an expected size of 500 bp (Figure 8). The hygromycin phosphotransferase is a protein synthesis inhibitor antibiotic. The PCR-positive lines meant that the CRISPR/Cas9-gRNAs cassettes succeeded in integrating them into the Mentik Susu rice genome. The selected marker gene encodes a protein that provides resistance to antibiotics. It is essential in the genetic transformation because the transformants must be distinct from non-transformant lines (Becker *et al*., 2021).

Cas9 gene, indicated by the amplification of DNA bands measuring 850 and 500 bp, respectively. The PCR amplification of OsCKX/HD2-transformant strains produced 15 strains positively carrying the *Cas9* gene with amplicon sizes of 500 and 314 bp, respectively. These results confirmed that the 64 transformant lines contained the *Cas9* gene (Figures 9 and 10).

HD2_G1_1_M13F:

CCGGAAACCGACTCGGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGG
TCGCTGTAGCCCCCACCCAATCACTACTTCGACTCTAGCTGTATATAAACTCAGCTTCGTTTTCTTATCTAAGCGATGTGGGA
CTTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATTGTTCTTTCCCATATAAATGGGCCTGCTTCTCTT
CTTTCAGATTCCCAGGGGCCTTTTGAAGATTATCTTCATATCTTAAGAATGAAGATGTTTTATTCAATCAAATTCTTGAAGGT
TCGATGCCTAATCATTCTAATCCTGGGACAAACTATGAAACAAGATACAAAAACTCCGAATGGAAAGTTAAAAAGAAGAAAAC
GAAAGCTACGGTTCAAGAAAATGTAAGCTGATAAACAAAAAAAAACTGTATGAACGAAGAAGAAGAAAAAAAGCTAAGAAGAA
ATGATGTATTGTGCGGAAGGCAAGTCGAGTTTCCGTTGTTCAACGAAGCCATGGGTCCGTCACCGGAGCTCCTTATCTTTAAT
CATATTCCATAGTCCATACCATAGCACATACAGTAGTTATATGCTGCAGAAGAGATCCAACAAAACATTCACAATGGATTATA
GAAACATTTGTTTATTCATTATAATGTGATCTTACATTCATTTAATATTAGAAAAAGCCACAAATTCATAACACAACAAGCCA
AGAAAAAAACACAAACTTAAGCACACAAGCTTTTTATTTGACACACCAAATATTTCATCTTCATCTTCATATCTCGAGTCAAA
CCTTCCTCTTCTTCTTAGGATCAGCCCTTGAATCACCACCGAGCTGTGAGAGATCGATCCTAGTCTCGTAGAGTCCAGTGATA
GACTGATGGATGAGGGTAGCATCGAGCACTTCTTTGGTAGAGGTGTATCTCTTCCTATCGATGGTTGTATCGAAGTACTTGAA
AGCAGCAGGAGCACCGAGGTTGGTAAGGGTGAAGAGAATGGATGATGTTCTCTGCCTGTTCCCTGATAGGCTTATCTCTGTGC
TTGTTGTAAGCAGACAACACCTTATCGAGGTTTGCATCAGCGAGGATCACCNTTTTAGAGAACTCAGAGATCTGCTCGATGAT
CTCATCCAAGTAGTGCTTGTGCTGCTCAACGAAAAGTTGCTTCTGCTCGTTNTCTTCGGGAGATCCCTTCACTTTCCGAAGGN
NNAGGGGGGGAAAGAAAGTTAACG

HD2_G2_6_M13F:

CCGGAANCCGACTCGGGTGCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCCT
GCCTGGTGGAGTCATCGAATCACTACTTCGACTCTAGCTGTATATAAACTCAGCTTCGTTTTCTTATCTAAGCGATGTGGGAC
TTTTGAAGATTGTTTTCAACTTAAATGGGCCTATATAAGAAATACTATTGTTCTTTCCCATATAAATGGGCCTGCTTCTCTTC
TTTCAGATTCCCAGGGGCCTTTTGAAGATTATCTTCATATCTTAAGAATGAAGATGTTTTATTCAATCAAATTCTTGAAGGTT
CGATGCCTAATCATTCTAATCCTGGGACAAACTATGAAACAAGATACAAAAACTCCGAATGGAAAGTTAAAAAGAAGAAAACG
AAAGCTACGGTTCAAGAAAATGTAAGCTGATAAACAAAAAAAAACTGTATGAACGAAGAAGAAGAAAAAAAGCTAAGAAGAAA
TGATGTATTGTGCGGAAGGCAAGTCGAGTTTCCGTTGTTCAACGAAGCCATGGGTCCGTCACCGGAGCTCCTTATCTTTAATC
ATATTCCATAGTCCATACCATAGCACATACAGTAGTTATATGCTGCAGAAGAGATCCAACAAAACATTCACAATGGATTATAG
AAACATTTGTTTATTCATTATAATGTGATCTTACATTCATTTAATATTAGAAAAAGCCACAAATTCATAACACAACAAGCCAA
GAAAAAAACACAAACTTAAGCACACAAGCTTTTTATTTGACACACCAAATATTTCATCTTCATCTTCATATCTCGAGTCAAAC
CTTCCTCTTCTTCTTAGGATCAGCCCTTGAATCACCACCGAGCTGTGAGAGATCGATCCTAGTCTCGTAGAGTCCAGTGATAG
ACTGATGGATGAGGGTAGCATCGAGCACTTCTTTGGTAGAGGTGTATCTCTTCCTATCGATGGTTGTATCGAAGTACTTGAAA
GCAGCAGGAGCACCGAGGTTGGTAAGGGTGAAGAGATGGATGATGTTCTCTGCCTGTTCCCTGATAGGCTTATCTCTGTGCTT
GTTGTAAGCAGACAACACCTTATCGAGGTTTGCATCAGCGAGGATCACCCTTTTAGAGAACTCAGAGATCTGCTCGATGATCT
CATCCAAGTAGTGCTGTGCTGCTCAACGAAAAGTTGCTTCGGCTCGTTACTTCTGGAAATCCCTTCACTTCTCGAAGGGGAAA
GCGAGGTAAANAAAGTTACGTACTTTAATGGG

Figure 4. Sequencing results of recombinant plasmids pDIRECT_21A-gRNA_HD2_g1 and pDIRECT_21A-gRNA_HD2_g1. Notes: purple = scaffold RNA, yellow = gRNA_HD2_g1, blue = gRNA_HD2_g1, and grey = part of the *Cas9* gene.

The *GA20ox2* gene involves a gibberellin biosynthesis pathway, and the increased GA content can considerably promote flowering (Mao *et al.*, 2022). High *HD* gene expression can also produce dwarfed plants (Halim *et al.*, 2022). The HD-ZIP VI gene *Roc 8* was visible with high photosynthetic efficiency and low lignin content (Sun *et al*., 2020). Hd1 has a double function, which increases *florigen* gene expression in short-day conditions; however, it also inhibits *florigen* gene expression in long-day conditions (Zhang *et al*., 2019). The Gn1a/OsCKX2 controls the meristem activity during the influence of cytokinins' accretion loss of function results in increased cytokinin production, thereby increasing cell propagation (Ashikari *et al.*, 2005; Li *et al.*, 2013). SWEET is the encoding genes' family for sugar transfer proteins that are often concerned with the *Xanthomonas oryzae (Xoo*) infection mechanism by releasing sugars into the bacterial growth apoplast (Singh *et al*., 2016; Ngoc *et al.,* 2022; Putri *et al*., 2023).

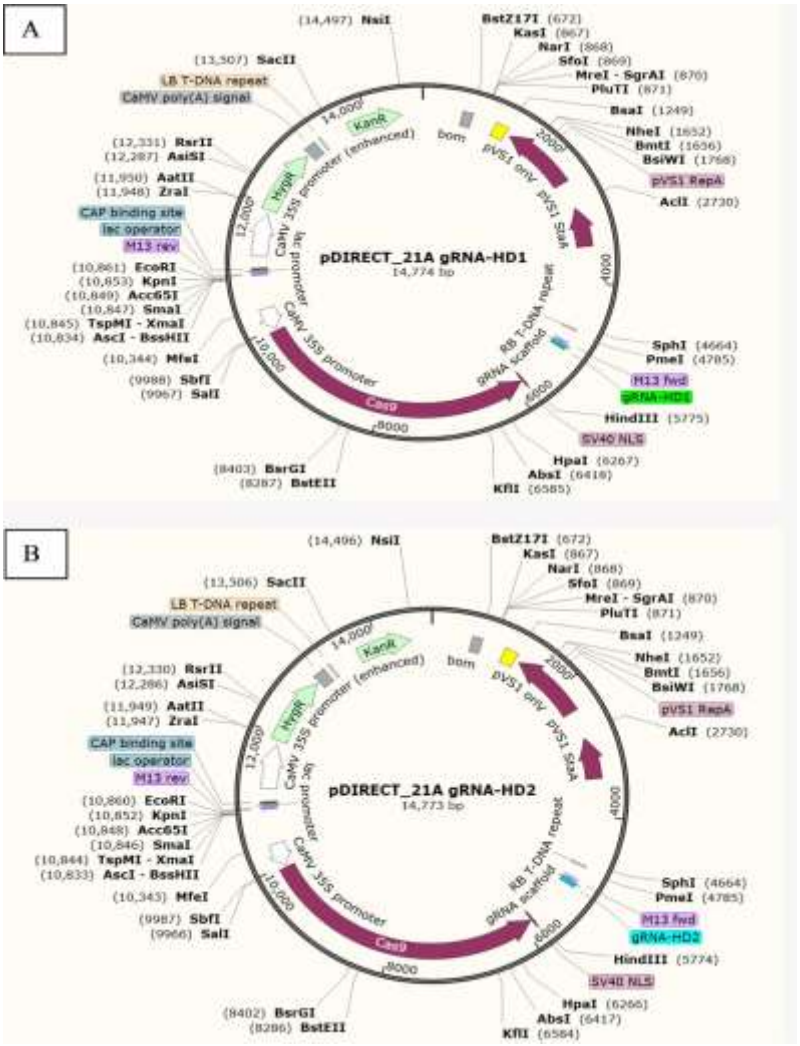

Figure 5. Map of plasmids pDIRECT21A-gRNA_HD2_g1 (A) and pDIRECT21A-gRNA_HD2_g2 (B).

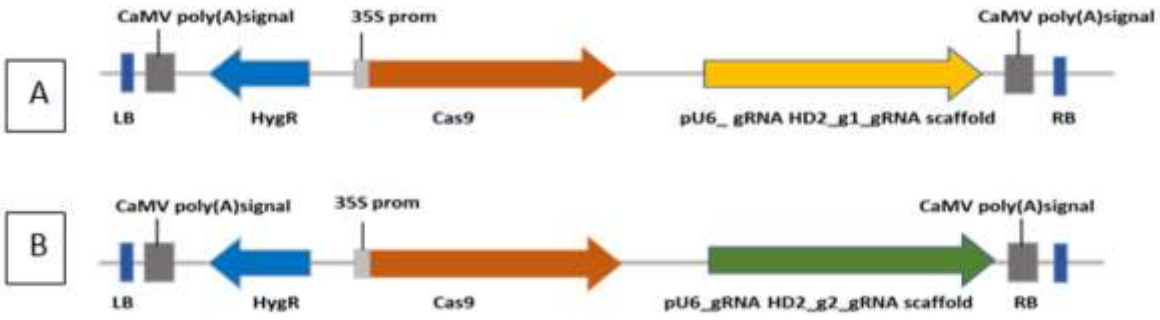

Figure 6. Schematic of the T-DNA sections of pDIRECT21A-gRNA_HD2_g1 (A) and pDIRECT21A-gRNA_HD2_g2 (B).

Table 2. Transformation results of the Mentik Susu rice with CRISPR/Cas9 constructs for gene targets GA20ox2-OsSWEET, OsCKX2-HD2, and GA20ox2-OsSWEET-OsCKX2-HD2.

Co-Transformation	Number of Immature Embryo	Number of Calluses on Selection Medium	Number of Calluses on Pre-regeneration Medium	Number of Calluses on Regeneration Medium	Number of Shoots on rooting Medium	Number of Plantlets Acclimatized	Number of Putative Mutant Rice Lines T0
GA20ox2, OsSWEET	1181	484	133	65	59	59	53
OsCKX2, HD2	1105	447	70	40	26	26	24
GA20ox2, OsSWEET, OsCKX2 dan HD2	2043	1307	439	350	350	347	157

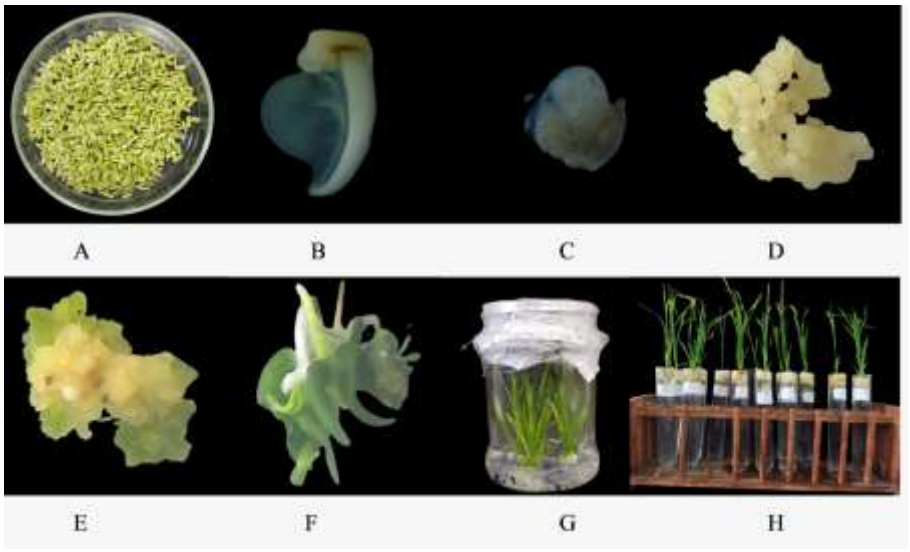

Figure 7. Transformation stages of immature embryos of Mentik Susu rice using *A. tumefaciens*. Description: A= Young rice embryo, B= Callus seven days after the co-cultivation period (infection), C= Callus on resting medium, D= Callus on selection medium, E= Callus with green spot on pre-regeneration medium, F= Shoots on regeneration medium, G= Plantlet on rooting medium, and H= Plantlet acclimatization in water medium.

DNA sequencing analysis of putative rice mutant generation T0

Sequencing analysis also materialized on the T0 generation putative mutant Mentik Susu rice plants that are PCR-positive for *hptII* and *Cas9* genes to identify the occurrence of mutagenesis of the target genes. The analysis results in T0 generation HD2-transformants lines showed no mutations in the target *HD2* gene, although the PCR analysis of the *hptII* and *Cas9* genes was positive. This occurrence might be due to resemblances in wild-type DNA sequences that the gRNA does not distinguish the target sequence previously designed. Wu *et al.* (2014) stated that the original characterization of the Cas9/sgRNA system showed that not every position in the guide RNA needs to match the target DNA, suggesting the existence of off-target sites. Amplification of the gRNA OsCKX2 (500 bp), HD2 (500 bp), OsGA20ox-2(800 bp), and SWEET11 (500 bp) flanking region arose for sequencing using the flanking primer pair

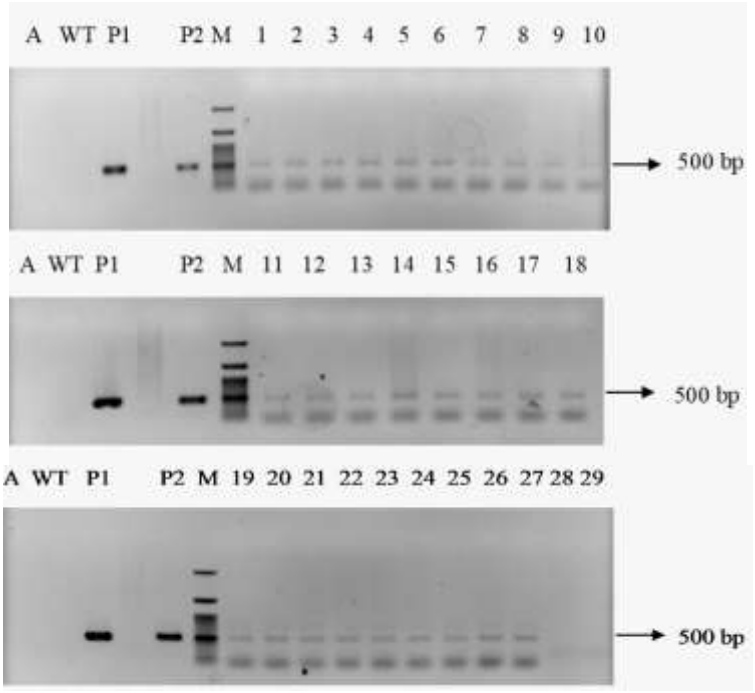

Figure 8. The results of DNA amplification of plants with the *hptII* gene primer pair for gene target four genes *GA20ox2*, *OsSWEET*, *OsCKX2*, and *HD2*. Notes: A= water, WT= wild type, P1= vector pCAMBIA1300, P2= vector pDIRECT_25H, M= Marker 100 bp, 1-29= Sample number.

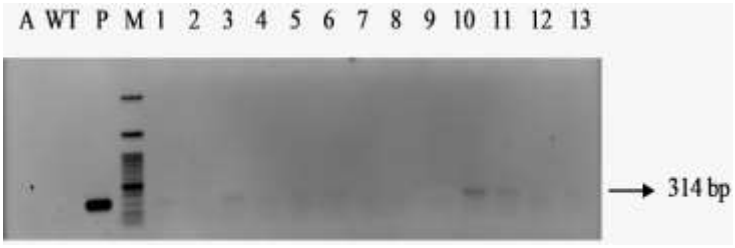

Figure 9. The results of DNA amplification of plants with the *Cas9* gene primer pair to detect plasmid pDIRECT21A target four genes *GA20ox2*, *OsSWEET*, *OsCKX2*, and *HD2*. Notes: A= water, WT= wild type, P1= vector pDIRECT21A, M= Marker 100 bp, 1-13= Sample number.

Figure 10. The results of DNA amplification of plants with the *Cas9* gene primer pair to detect plasmid pDIRECT25H target four genes *GA20ox2*, *OsSWEET, OsCKX2,* and *HD2*. Notes: A= water, WT= wild type, P1= vector pDIRECT25H, M= Marker 100 bp, 1-13= Sample number.

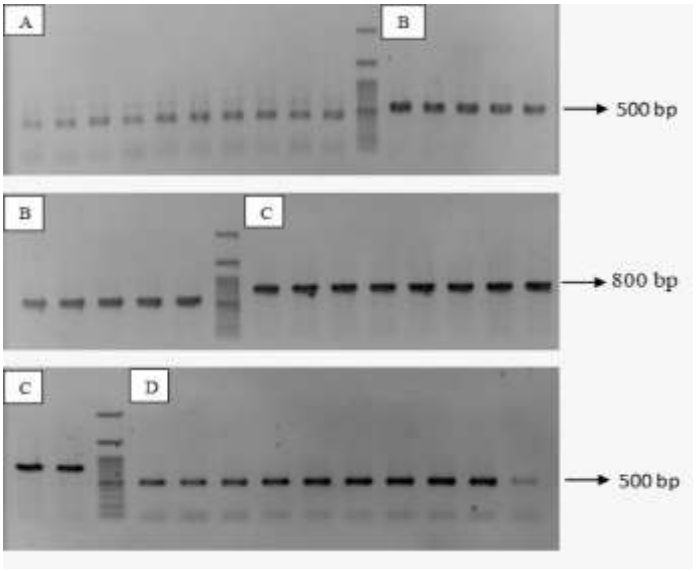

Figure 11. DNA amplification results of the Mentik Susu rice putative mutant generation T0 in the target gene region. A. Amplicon of gRNA OsCKX2 flanking region, B. Amplicon of HD2 gRNA flanking region, C. Amplicon of OsGA20ox-2 gRNA flanking region, and D. Amplicon of SWEET11 gRNA flanking region.

(Figure 11). Overall, sequencing analysis of Mentik Susu rice putative mutant lines produced eight mutations: two mutations in the *OsGA20ox2* gene sequence, five mutations in the *SWEET11* gene promoter sequence, and one in the *OsCKX2* gene. All these mutations appeared in the form of base substitutions.

The *OsGA20ox2* gene sequence had a mutation in Region I of the MS-GA_C100.1 line, where the adenine base incurred replacement with cytosine (Table 3). In contrast, adenine substitution occurred for guanine in Region II of the MS-GA_C173.1 line (Table 4). Further observations need attention on the T1 generation mutant line to confirm the mutation effects on the desired agronomic traits. Beyene *et al.* (2022) stated that the tef plants that experienced a mutation in the *SD1* gene showed a shorter plant height by 28%–42% compared with the wild type. Mutants of the Mentik Susu rice line, which had mutations in the target gene *OsGA20ox2*, continued further analysis to determine the effect of the mutations on the amino acid structure (Table 5). The mutation in rice line MS-GA_C100.1 showed a base substitution from adenine (A) to cytosine (C). However, this substitution did not change the amino acid sequence of alanine. Likewise, the mutant rice line MS-GA_C173.1 experienced a substitution from the base adenine to guanine, and this substitution caused a change in the amino acid composition from lysine (K) to glutamate (E). The base substitution in the MS-GA-C100 rice line occurs in the third codon; hence, the substitution does not cause amino acid changes. Relatedly, a base substitution surfaced in the rice line MS-GA_C173.1 in the first codon, resulting in a change in the amino acid sequence. This study's results align with Schmitz *et al.* (2002), who stated that the substitution in the third codon does not cause a change in the amino acid because the substitution is silent. Thus, the amino acid lysine, an uncharged/polar neutral R group amino acid, incurred conversion to glutamate, a positively charged R group amino acid. Furthermore, mutagenesis of the *GS2E* gene with CRISPR/Cas9 technology produced a 6-bp deletion mutation and 1-bp substitution, causing an increase in rice grain size compared with the wild type (Wang *et al.,* 2022).

Table 3. Sequence of the *OsGA20ox2* gene in the Mentik Susu rice showing mutations in Region I.

Lines	gRNA flanking nucleotides sequence of gene *OsGA20Ox2*	Mutation Type
WT_MS_GA	CCCGTCGTCGCAGACTACTTCTCCAGCACCCTCGGCCCC	Wild type sequence
MS_GA_C100.1	CCCGTCGTCGCCGACTACTTCTCCAGCACCCTCGGCCCC	Single base substitution

Table 4. Sequence of the *OsGA20ox2* gene in the Mentik Susu rice showing mutation in Region II.

Lines	gRNA flanking nucleotides sequence of gene *OsGA20Ox2*	Mutation Type
WT_MS_GA	TTCGCGCCAATGGGGTAATTAAAACGATGGTGGACGACATT	Wild type sequence
MS_GA_C173.1	TTCGCGCCAATGGGGTAATTAGAACGATGGTGGACGACATT	Single base substitution

Table 5. Amino acid sequence of the *OsGA20ox2* gene in the Mentik Susu rice with mutation.

Lines	Amino acid sequence of gene *OsGA20ox2* of Mentik Susu rice	Amino Acid Sequence Change
WT OsGA20ox2	FHDRAAAPVVADYFSSTLGPDFAPMG-LKRWWTTLHFKFKTNSKHTDRDY	Wild type
MS-C100.1	FHDRAAAPVVADYFSSTLGPDFAPMG-LKRWWTTLHFKFKTNSKHTDRDY	Does not cause amino acid change
WT OsGA20ox2	FHDRAAAPVVADYFSSTLGPDFAPMG-LKRWWTTLHFKFKTNSKHTDRDY	Wild type
MS-C173.1	FHDRAAAPVVADYFSSTLGPDFAPMG-LERWWTTLHFKFKTNSKHTDRDY	K (lysine) mutate to E (glutamic acid)

Table 6. Sequence of the *SWEET11* gene promoter in the Mentik Susu rice that show mutations in Regions -18 to -64.

Lines	Flanking nucleotides sequence of gRNA from gene promoter of OsSWEET11	Mutation type
WT_MS_OsSW	TCTTCCTAACCTTCTCACTGATTAACACCTTAAAGTTAGTTAATAAC	Wild type sequence
MS_SW-C_3_1	TCTTCCTAACCTTCTCACTGATTAACACCTTAGAGTTAGTTAATAAC	Single base substitution
MS_SW_21.2	TCTTCCTAACCTTCTCACTGATTAACACCTTAGAGTTAGTTAATAAC	Single base substitution
MS_SW_4.1	TCTTCCTAACCTTCTCACTGATTAACACCTTAGAGTTAGTTAATAAC	Single base substitution

Mutations in the three rice lines MS_SW-C3.1, MS_SW-A21.2, and MS_SW-A4.1 occurred in the SWEET11 gene promoter in the -18 to -64 regions, and the mutation that happened was a substitution from adenine to guanine (Table 6). In the MS-SW-C48.1 rice line, the mutation appeared at sequence positions -125 to -185, and the mutation was also in the form of substitution and conversion of guanine to cytosine (Table 7). Meanwhile, the rice line MS_SW_C137.1 had a mutation in the form of substituting the base adenine for guanine (Table 8). Yang *et al*. (2018) reported that rice mutants with mutations in the SWEET promoter function as sucrose transporters when working with the sucrose sensor in HEK293T cells. Rice lines, cv. Mentik Susu, which had mutations in the promoter sequence of the *SWEET11* gene, requires further scrutiny, especially concerning their resistance to the bacteria *Xanthomonas oryzae* (*Xoo*). Suppose the mutant rice line shows resistance to *Xoo*. In that case, the mutation due to the genome editing has successfully changed the plant's phenotype from susceptible to BLB resistant.

Table 7. Sequence of the *SWEET11* gene promoter in the Mentik Susu rice that show mutations in Regions -125 to -185.

Lines	Flanking nucleotides sequence of gRNA from gene promoter of OsSWEET11	Mutation Type
WT_MS_OsSW	GCCAAGGCCAAACCACACATGCAGTTGTAGTAGCACTTAAGCCTTCCTCTCTAGCTAGCA	Wild type sequence
MS_SW_C48.1	GCCAAGGCCAAACCACACATGCAGTTGTAGTACCACTTAAGCCTTCCTCTCTAGCTAGCA	Single base substitution

Table 8. Sequence of the *SWEET11* gene promoter in the Mentik Susu rice showing mutations in Regions -52 to -88.

Lines	Flanking nucleotides sequence of gRNA from gene promoter of OsSWEET11	Mutation type
WT_MS_OsSW	TCCTCTTCCTAACCTTCTCACTGATTAACACCTTAAAGTTAGTTAATAACCTTCATCACC	Wild type sequence
MS_SW_C137.1	TCCTCTTCCTAACCTTCTCACTGATTAACACCTTAGAGTTAGTTAATAACCTTCATCACC	Single base substitution

Table 9. Sequence of the *OsCKX2* gene in the Mentik Susu rice showing the presence of mutations.

Lines	gRNA flanking nucleotides sequence of gene *OsCKX2*	Mutation type
WT_MS_ OsCKX2	GGTATAGGTGTTTTGACGTATACATGTATCTGCAGGTGTCGGGGAGATGG TG------AC	Wild type sequence
MS_ OsCKX2_128.1	GGTATAGGTGTTCTGACGTATACATGTATCTGCACGTGTCCGGGAGATGG TG------AC	Substitution of 3 base pairs

Table 10. Amino acid sequence of *OsCKX2 gene* in the Mentik Susu rice with mutation.

Lines	Amino acid sequence of gene *OsCKX2* Mentik Susu rice	Mutation type
WT_MS_ OsCKX2	GIGVLTYTCICRCRGDGDV-VF-RIHVSA	Wild type sequence
MS_ OsCKX2_128.1	GIGVLTYTCICTCPGDGDV-VF-RIHVSA	1 mutation does not cause change of amino acid, R (arginine) changed to T (threonine) and R (arginine) changed to P (proline)

Rice line MS_OsCKX2_128.1 showed a three-base substitution mutation of the *OsCKX2* gene: one thymine base to cytosine and two guanine bases to cytosine (Table 9). However, the T-to-C base substitution in the *OsCKX2* gene does not cause amino acid changes. Substitution of the base G to C causes changes in the amino acids R (arginine) to T (threonine) and R (arginine) to P (proline) (Table 10). The effect of base substitutions that caused changes in these amino acids on phenotypic changes must also show proof in T1 generation mutant plants. The study hoped that changes in the amino acid sequence would produce rice mutants with high productivity in rice cv. Mentik Susu. According to Han *et al.* (2019), sd1 mutant rice decreased cell length, width, and size, while the number of cells increased compared with wild-type cells. SD1 mutant rice plants appear shorter than wild rice plants (Tomita and Ishii, 2018). Arginine is a positively charged R-group amino acid. At the same time, threonine is a polar R group amino acid with no charge, and proline is a

nonpolar aliphatic R group amino acid. Over-expression of the gene may stimulate the allocation of neutral amino acids and, ultimately, increase grain growth and yield (Fang *et al*., 2021). Based on the results of amino acid changes in the mutant rice lines, expected positive findings in the T1 generation mutant lines will show higher productivity.

In this study, the mutagenesis process in rice mutant lines occurred expectedly. This mutation succeeded in modifying the sequence at the location of the target gene. Shelake *et al.* (2019) stated that CRISPR/Cas-mediated DSBs can cause frameshift mutations, which, in turn, cause a premature stop on codons or changes in the mRNA, causing changes in the protein's amino acid sequence. Mutations occurring in the *OsGA20ox2*, *OsCKX2*, and *SWEET11* genes (promoter) may likely lead to good mutations using CRISPR/Cas9 genome editing. CRISPR/Cas9 is an unconventional technology that can be an alternative to editing plant genomes according to scientists' wishes. According to Wang *et al.* (2020), the CRISPR/Cas9 mutant obtained can improve the desired quality of Japonica rice and can better serve as a new strategy in plant breeding. Initially, the CRISPR/Cas9 system is an essential component of adaptive immunity in bacteria and archaea involved in removing viral and plasmid DNA from invading parasites (Sardesai and Subramanyam, 2018).

In the next generation, mutant rice lines that are products of genome editing need more analysis to ensure that the mutant lines are transgene-free, ascertaining these mutant lines will not receive categorization as GMO (genetically modified organism) products. Ishizaki T (2016) stated that removing Cas9 through segregation is a delicate process to ensure stable mutant inheritance.

CONCLUSIONS

The CRISPR/Cas9 plasmid vector carrying gRNA to edit the *HD2* gene for early maturity has succeeded in constructing and transforming into the *A. tumefaciens* vector. The CRISPR/Cas9-HD2 cassette construct has successfully transformed into the rice genome cv. Mentik Susu. Eight rice lines resulting from genome editing have experienced mutations in the target genes: two lines in the *OsGA20ox2* gene, five in *SWEET11*, and one in the *OsCKX2* gene. This mutation results in a change in the amino acid sequence: lysine (K) becomes glutamate (E) in the *OsGA20ox2* gene, R (arginine) becomes T (threonine), and R (arginine) becomes P (proline) in the *OsCKX2* gene. Mutant rice lines resulting from CRISPR/Cas9 genome editing need further analysis to identify expected phenotypic changes.

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