BIODIVERSITAS
Volume 25, Number 8, August 2024
Pages: 2783-2790

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250852

Evaluating the phenotypic responses of CRISPR/Cas9-edited Mentik Wangi rice mutants T1 focusing on *GA20ox2* and *OsCKX2* gene knockout

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Manuscript received: 13 June 2024. Revision accepted: 28 August 2024.

Abstract. *Fathin TS, Sisharmini A, Apriana A, Santoso TJ, Yunus A. 2024. Evaluating the phenotypic responses of CRISPR/Cas9-edited Mentik Wangi rice mutants T1 focusing on* GA20ox2 *and* OsCKX2 *gene knockout. Biodiversitas 25: 2783-2790.* Mentik Wangi is a local Indonesian rice variety with tall plant stature, making it susceptible to lodging and yield loss, necessitating trait improvements to overcome these weaknesses. Gene editing of *GA20ox2* and *OsCKX2* using CRISPR/Cas9 was conducted to improve the characteristics of Mentik Wangi. This study aimed to evaluate the phenotypic responses of T1 Mentik Wangi mutant lines generated through gene editing using the CRISPR/Cas9 system. Eighty-five mutant lines and wild-type plants were grown and observed in a greenhouse. Phenotypic traits were observed as quantitative traits, such as plant height, panicle length, total number of tillers, number of productive tillers, total number of grains, 1000-grain weight, heading date, and harvesting age; and qualitative traits, such as panicle type and panicle exsertion type. The results showed a decrease in plant height and panicle length in Mentik Wangi rice mutant lines. Several lines exhibited improvements in total tiller number, productive tiller number, total grain number, and 1000-grain weight. Mutant lines required longer to reach the generative phase and displayed differences in panicle type and panicle exsertion type. This study reported that the T1 mutant lines demonstrated distinct phenotypic responses indicating improvements in the desired traits.

Keywords: Gene editing, Mentik Wangi, phenotypic traits, plant height, rice mutants

INTRODUCTION

Rice is a primary carbohydrate source that dominates food consumption globally. The increase in population every year as well as the global climate crisis greatly impacts the demand and productivity of rice. Efforts to maintain food security include boosting local aromatic rice productivity. Developing biotechnology in agriculture can be an innovation to overcome these challenges. Variety development can be carried out by utilizing advances in the field of biotechnology, such as gene editing, short hairpin RNAs (Yeh et al. 2015), and *Agrobacterium tumefaciens* mediated transformation (Sisharmini et al. 2019).

Mentik Wangi is a local Indonesian aromatic rice variety that produces sticky rice, making it highly sought after by consumers (Sudrajat 2020). This rice variety has low productivity with around 3.78 tons/ha (Yunus et al. 2021) compared to some other rice varieties, such as Inpari 13 (4.59 tons/ha), Ciherang (5.03 tons/ha), and Mekongga (5.62 tons/ha) (Hambali and Lubis 2017). One key element contributing to the low productivity of Mentik wangi is yield loss due to lodging. Mentik wangi is known for its tall plant phenotype. This tall stature makes it susceptible to lodging. The unpredictable weather conditions, further exacerbate the risk of lodging in Mentik Wangi. Cereal crops are particularly vulnerable to lodging due to their root systems and stems. Lodging significantly disrupts plant physiology, impacting flowering, reducing photosynthesis, and consequently limiting carbohydrate assimilation (Singh et al. 2020). Moreover, lodging hinders nutrient transport, ultimately affecting grain formation. To improve the low productivity of Mentik Wangi, trait enhancement can be done by controlling the levels of phytohormones, such as gibberellin and cytokinin.

Cytokinins and gibberellins are phytohormones that play an important role in the growth and development processes of plants. Gibberellins have a role in the elongation process of stem meristem cells, stimulate flowering and fruit formation, and increase resistance to abiotic stress (Gao and Chu 2020). Cytokinins play a role in the division of apical meristem cells, the formation and maintenance of chloroplasts, the mobilization of nutrients within the plant, and increased resistance to stress (Jameson 2023). The levels of endogenous cytokinins and gibberellins will affect physiological activities that impact the phenotypic response of the plant. The sd-1 gene is a

gene that regulates the formation of gibberellins which play a role in plant growth processes such as increased plant height. The *gn-1a* gene encodes *OsCKX2*, an enzyme that degrades bioactive cytokinins (Ashikari et al. 2005).

Plant breeding aims to enhance plant productivity by modifying these inherited traits. Conventional plant breeding relies on random processes and limits the ability to target specific traits for improvement. However, advancements in gene editing technologies, such as CRISPR/Cas9, offer a more precise and efficient approach (Jung et al. 2019; Manghwar et al. 2019; Biswas et al. 2020; Zhang et al. 2020; Nurhayati et al. 2021). CRISPR/Cas9, inspired by bacterial defense mechanisms, is a highly accurate, efficient, and user-friendly method compared to other techniques (Miki et al. 2018; Manghwar et al. 2019). It is applicable across diverse organisms, including plants (Zhang et al. 2020).

Successful applications of CRISPR/Cas9 have been demonstrated in crops, like rice and corn. Ruzyati et al. (2022) conducted a study utilizing CRISPR/Cas9 technology to develop a semi-dwarf Mentik Wangi rice variety with enhanced productivity. The study focused on disrupting targeting genes, the sd-1 and gn-1a, for achieving a semi-dwarf rice plant with a higher grain yield, and yielded 15 transformed lines carrying the CRISPR/Cas9 gRNA-*OsCKX2* and CRISPR/Cas9 gRNA-*OsGA20ox-2* constructs. This study aimed to evaluate the phenotypic responses of T1 mutant rice focusing on *GA20ox2* and *OsCKX2* gene knockout.

MATERIAL AND METHODS

Plant materials

The seeds used were Mentik Wangi (wild-type) and 10 Mentik Wangi mutant lines resulting from gene editing, MW 6.2 and MW 10.3 (mutation in *OsGA20ox2* gene in T0 generation); MW 7.1 and MW 12.1 (mutation in *OsCKX2* gene in T0 generation); MW 5.1; MW 5.2; MW 6.1; MW 7.2; MW 9; and MW 12.2 (no mutation in T0 generation). The scheme of this research begins with assembling the CRISPR/Cas9 gRNA-*GA20ox2* and *CKX2* constructs. These constructs are then transformed into Mentik Wangi via *Agrobacterium* using immature embryo explants The immature embryos are selected on media containing antibiotics (hygromycin). The selected immature embryos will grow into callus and form shoots, then become plantlets. Acclimatization is carried out after the plantlets have sufficiently long roots (Ruzyati et al. 2022). The plants resulting from acclimatization are then planted in a screen house and produce T0 seeds. The T0 seeds are then planted, and their growth and yield are observed. Mentik Wangi mutant lines were selected based on the results of PCR analysis (positive *hptII* gene and *cas9* gene), sequencing, and T0 plant phenotypes.

Procedures

Cultivation

Wild-type and selected mutant lines seeds were germinated on petri dishes for two days, then transferred to trays with a growing medium mixture of sand and fertilizer. The sand used is first sun-dried and left to incubate for 7 days. After that, organic fertilizer was added with a ratio of 1:1. The plants were transferred to pots with soil growing medium after 21 days. Planting is done by planting 1 plant in 1 pot. The planting medium used after seedling is a soil planting medium mixed with organic fertilizer. The soil used is of the lithosol type and the ratio of organic fertilizer to soil is 1:10. The mixture of soil and organic fertilizer is put into each pot approximately 17 cm high and then flooded with water. Further fertilization is carried out 10 days after planting (DAP) with 0.5 g/pot of KCl fertilizer and 0.7 g/pot of urea fertilizer, 31 days after planting with 0.5 g/pot of KCl fertilizer and 0.7 g/pot of urea fertilizer, and 59 days after planting with 0.7 g/pot of urea fertilizer.

Phenotype analysis

Eighty-five plants were observed, and their phenotypic characters were analyzed. Eight quantitative characters observed and divided into 3 parts, growth performance (plant height and panicle length), agronomic traits (total number of tillers, number of productive tillers, total number of grains, and 1000-grain weight), and delayed senescence (heading date and harvesting time). The 2 qualitative characters observed were panicle type and panicle exsertion type. Observation of qualitative variables refers to the guidelines by Rost (1997).

Data analysis

Quantitative data were analyzed using analysis of variance (ANOVA) at α 5%, followed by Dunnett's test using R studio software. Qualitative data were analyzed descriptively and presented using boxplots.

RESULTS AND DISCUSSION

Growth performance

Eighty-five plants were cultivated and observed in a greenhouse to investigate the phenotypic responses of the T1 mutant lines compared to the wild-type. Based on the ANOVA test, there is a significant value in plant height (Table 1). This indicates that gene editing had a significant impact on plant height. Dunnett's test revealed significant effects of gene editing on the target genes *GA20ox2* and *OsCKX2*, impacting the plant height of the T1 mutant lines (Figure 1.A). Compared to the wild-type (WT), the T1 mutant lines exhibited reduced plant height (Figure 2). This difference was particularly evident in lines MW 10.3, MW 12.1, MW 12.2, MW 5.2, MW 7.2, and MW 9, with height reductions of 12.5%, 10.5%, 8.7%, 9.4%, 12%, and 14.2%, respectively. Individual MW 10.3.8 displayed the shortest plant height at 77.3 cm. ANOVA test showed a significant value in panicle length (Table 1). This indicates that gene editing had a significant impact on panicle length. The Dunnett's test revealed significant differences in panicle length between the T1 mutant lines and the WT (Figure 1.B). Panicles of lines MW 10.3, MW 12.1, MW 12.2, MW 5.1, MW 7.1, and MW 7.2 were significantly shorter than the WT, exhibiting reductions of 11.6%, 7.9%, 7%, 5.4%, 5.4%, and 7.7%, respectively. The shortest panicle was detected in individual MW 10.3.8 at 18.21 cm, while the longest panicle was found in individual MW 6.1.2 at 24.93 cm.

Table 1. ANOVA test of quantitative variables

Variable	df1	df2	F Value	α = 5%		α = 1%	
				F Table	Significance	F Table	Significance
PH	10	74	5.003	2.00	*	2.58	*
PL	10	74	8.854	2.00	*	2.58	*
TTN	10	74	2.771	2.00	*	2.58	*
PTN	10	74	2.273	2.00	*	2.58	ns
OGW	10	74	2.229	2.00	*	2.58	ns
TNGPP	10	74	7.852	2.00	*	2.58	*
HD	10	74	6.191	2.00	*	2.58	*
HT	10	74	2.042	2.00	*	2.58	ns

Note: PH: plant height, PL: panicle length, TTN: total tiller number, PTN: productive tiller number, OGW: 1000 grain weight, TNGPP: total number of grains per panicle, HD: heading date, HA: harvesting age. *: significance, ns: not significance

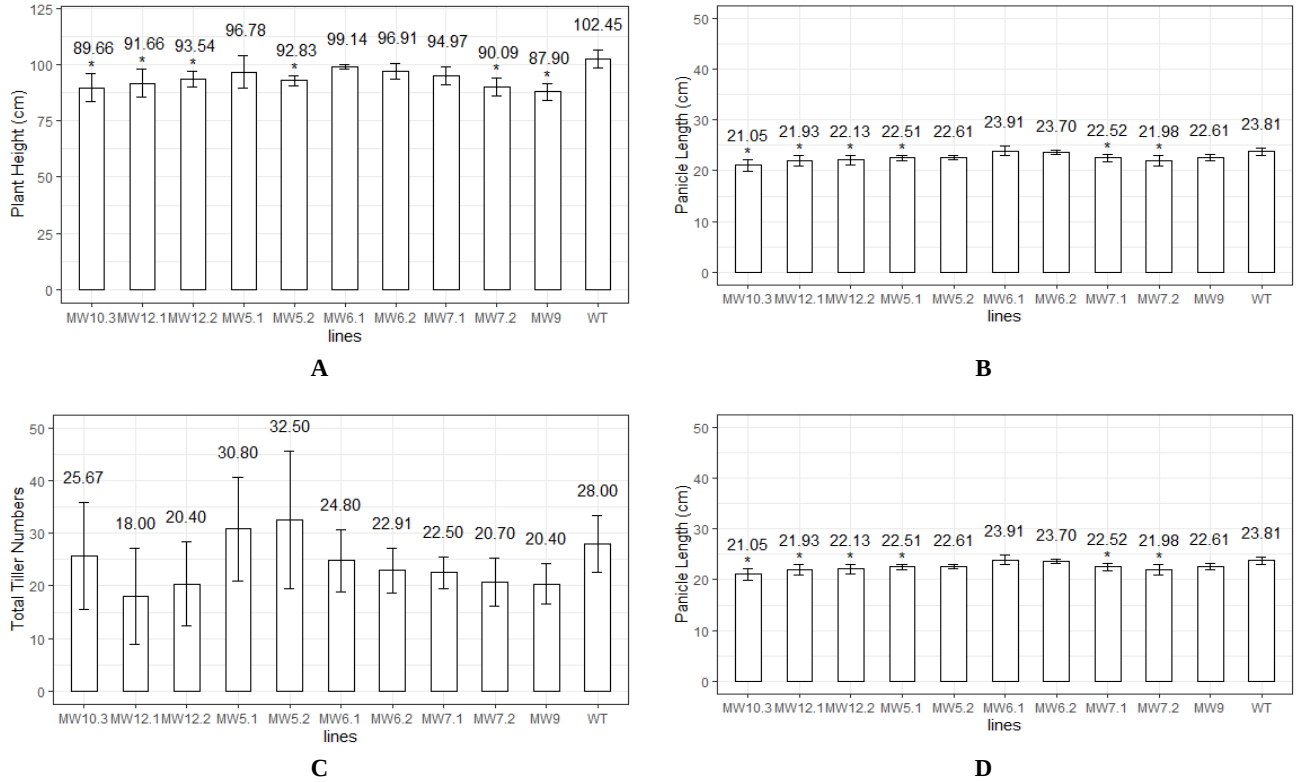
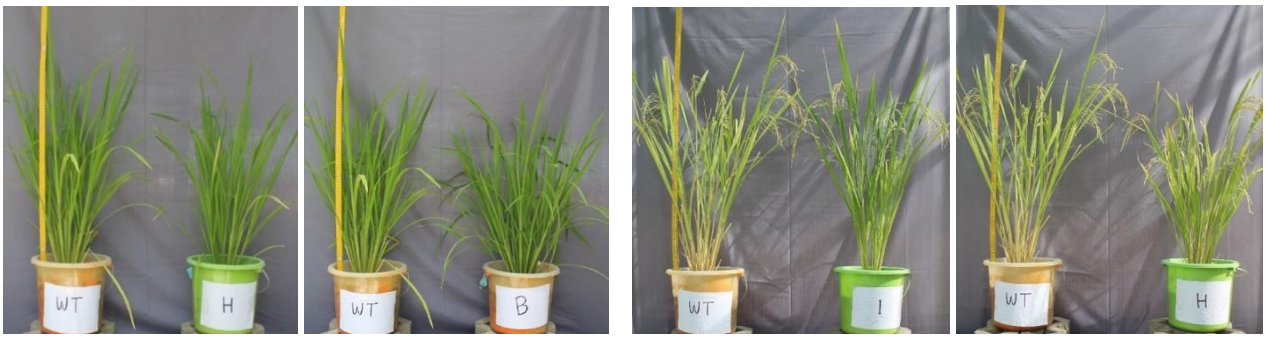


Figure 1. Growth of Mentik Wangi wild-type and T1 mutant lines: A. Plant height; B. Panicle length; C. Total number of tillers; D. Number of productive tillers. *: significantly different from wild-type



WT – MW 10.3 WT – MW 5.2 WT – MW 12.1 WT – MW 5.2

A **B**

Figure 2. The representative phenotype of growth in T1 mutant lines and wild-type line: A. Vegetative phase; B. Generative phase

The phenotypic responses observed in the T1 mutant Mentik Wangi lines highlight the impact of gene editing. The reduction in plant height and panicle length is a direct consequence of editing the target *GA20ox2* gene. Studies by Wang et al. (2024) also demonstrated a significant decrease in panicle length in gene-edited rice lines compared to the wild-type (WT). Previous research on improving rice plant stature to semi-dwarf using gene editing has produced mutants with semi-dwarf phenotypes, such as Murai et al. (2004), Biswas et al. (2020), Nawaz et al. (2020), Santoso et al. (2020), and Nurhayati et al. (2021). The CRISPR/Cas9 constructs successfully edited the target genes in previous transformations. Mutations at the sd-1 locus can lead to shorter panicles and reduced plant height (Murai et al. 2004). These mutations disrupt gibberellin metabolism in the plant. Genetics plays a crucial role in determining the phenotype of individual plants. Regulated genes influence plant hormone metabolism. These hormones are essential for characterizing the phenotype of an individual (Lan et al. 2023). Several genes regulate plant hormones involved in rice growth (Spielmeyer et al. 2002; Zegeye et al. 2022; Sitoe et al. 2022). Gibberellin is a plant hormone that plays a role in plant growth, particularly stem elongation. Gibberellin is synthesized from geranyl geranyl diphosphate (GGDP). Gibberellin biosynthesis requires various enzymes, including 2-oxoglutarate-dependent dioxygenases (2ODDs). The transformation of GA12 to GA4 requires the enzymes GA 20-oxidase (*GA20ox*) and GA 3-oxidase (*GA3ox*), which are soluble 2ODDs (Yamaguchi 2008). The *GA20ox* enzyme is expressed by the *GA20ox-1* and *GA20ox-2* genes (Sasaki et al. 2002). Knocking out the genes that regulate the *GA20ox* enzyme can reduce the enzyme's levels, inhibiting endogenous gibberellin production. Lower endogenous gibberellin levels can disrupt cell division in rice meristems, leading to semi-dwarf plants (Nawaz et al. 2020).

Agronomic traits

ANOVA test showed a significant in total tiller number (Table 1). This indicates that gene editing had a significant impact on the total tiller number. The total tiller number in several mutant lines was higher compared to the WT. Two T1 mutant lines, MW 5.1 and MW 5.2, exhibited a significantly higher total tiller number than the WT, with 30.8% and 16.07% increases, respectively. The highest total tiller number was observed in individual MW 5.1.8 with 44 tillers, while the lowest was found in individual MW 12.1.9 with only seven tillers (Figure 1.C). ANOVA test showed a significant in productive tiller number (Table 1). This indicates that gene editing had a significant impact on productive tiller numbers. The number of productive tillers in several T1 mutant lines was also higher than the WT, specifically in lines MW 10.3, MW 12.1, MW 12.2, MW 5.1, MW 5.2, MW 6.1, MW 6.2, MW 7.1, and MW 9. These lines showed increases of 54.6%, 4.4%, 9.6%, 45.2%, 24.6%, 14.8%, 21%, 20.3%, and 2.6%, respectively. However, one mutant line, MW 7.2, displayed fewer productive tillers than the WT. The highest number of productive tillers was observed in individual MW 10.3.5, with 28 tillers, while the lowest was found in individual MW 12.1.9, with only four tillers (Figure 1D).

ANOVA test showed a significant in 1000-grain weight (Table 1). This indicates that gene editing had a significant impact on 1000-grain weight. Data analysis revealed no significant difference in 1000-grain weight between the WT and T1 mutant lines (Figure 3.B). The highest weight was observed in individual MW 6.2.4 at 28 g, while the lowest was found in individual MW 6.1.5 at 17.4 g. ANOVA test showed a highly significant difference in the total number of grains per panicle (Table 1). This indicates that gene editing had a significant impact on the total number of grains per panicle. Two mutant lines, MW 6.1 and MW 6.2, exhibited a significantly higher total number of grains per panicle than the WT. The highest total grain number was observed in individual MW 6.1.2, with 174 grains, while the lowest was found in individual MW 10.3.8, with 65.43 grains (Figure 3.A).

Rice productivity is evaluated based on parameters such as tiller number, grain number, and 1000-grain weight (Lan et al. 2023). The observed increases in total tiller number, productive tiller number, total grain number per panicle, and 1000-grain weight in the T1 mutant lines are phenotypic responses to gene editing targeting the *OsCKX2* gene. Knocking out the *OsCKX2* gene has also been shown to enhance tiller number (Tsai et al. 2012; Yeh et al. 2015; Rong et al. 2022), total grain number per panicle (Huang et al. 2018), and 1000-grain weight (Zheng et al. 2023). Cytokinin is a phytohormone involved in various physiological processes in plants, including cell division, root and shoot formation, and leaf and flower development (Vankova 2014). Endogenous cytokinin levels in plants are influenced by cytokinin oxidase/dehydrogenase enzymes. These enzymes catalyze the degradation of cytokinins by breaking the side chains of active cytokinins, leading to a decrease in active endogenous cytokinin levels (Cortleven et al. 2019). Reducing the levels of cytokinin oxidase/ dehydrogenase enzymes can increase endogenous cytokinin levels in plants (Ashikari et al. 2005; Li et al. 2022). Some T1 mutant lines exhibited a decrease in tiller number, total grain number per panicle, and 1000-grain weight. The lower 1000-grain weight in the mutant lines could be attributed to reduced seed-setting rates (Rong et al. 2022). Plant phenotypes are influenced not only by genetics but also by environmental factors. High temperatures can suppress endogenous cytokinin levels in plants. There is a decrease in endogenous cytokinin content in maize kernels exposed to 35°C (Cheikh and Jones 1994). Increased temperature hinders grain filling, resulting in reduced grain weight (Dou et al. 2017).

Delayed senescence

The T1 mutant lines displayed a delay in senescence. Based on the ANOVA test, there is a significant in heading date (Table 1). This indicates that gene editing had a significant impact on heading date. Dunnett's test revealed significant effects of gene editing on the target genes *GA20ox2* and *OsCKX2*, impacting the heading date of the T1 mutant lines. Data analysis (Figure 4.A) indicated significant differences in heading date between the WT and T1 mutant lines. Line MW 6.2 required six days longer to

flower compared to the WT. Individual MW 9.6 had the fastest heading date, seven days earlier than the WT. MW 10.3.8 and MW 12.1.9 exhibited the longest heading date, 14 days later than the WT. ANOVA test showed a significant in harvesting age (Table 1). This indicates that gene editing had a significant impact on harvesting age. Significant differences in maturity time were also observed between the WT and T1 mutant lines. Line MW 6.1 had a 10-day longer maturity time compared to the WT. One T1 mutant line, MW 9, displayed an earlier maturity time, eight days faster than the WT. The earliest maturity time was detected in individual MW 5.1.6 at 128 days after transplanting (DAT), while the latest maturity time was observed in individuals MW 5.2.4 and MW 7.2.11 at 191 DAT (Figure 4.B).

The transition from the vegetative to the generative phase is initiated by chlorophyll degradation. This process is triggered by phytohormones, including abscisic acid (ABA). ABA has an antagonistic effect on cytokinin (Zhang et al. 2021). Knocking out the *OsCKX2* gene increases endogenous cytokinin levels, which are involved in chlorophyll biosynthesis and chloroplast development (Cortleven and Schmülling 2015). Higher chlorophyll levels in plants can delay senescence. Lower endogenous ABA levels in plants can also slow down flowering initiation (Martignago et al. 2020). The T1 mutant lines exhibited delayed flowering and maturity times compared to the WT. Previous research has also shown delayed senescence in mutant lines (Yeh et al. 2015; Rong et al. 2022; Zheng et al. 2023).

Panicle morphology

The distribution of panicle type and panicle exsertion type was observed to assess the impact of gene editing. Data analysis revealed that two T1 mutant individuals, MW 6.1.2 and MW 9.1, exhibited 100% compact panicle type, while MW 12.1.9 displayed 100% intermediate panicle type (Figure 5). Regarding panicle exsertion type, one individual, MW 7.1.11, showed 71.4% well-exserted type, MW 9.5 displayed 90.9% just-exserted type, and no enclosed type was found (Figure 6).

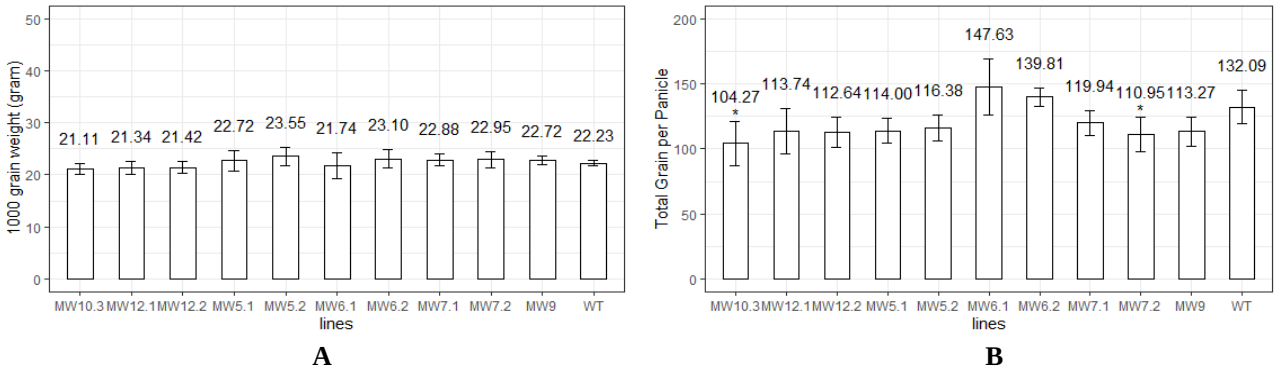


Figure 3. Agronomic characters of Mentik Wangi wild-type and T1 mutant lines: A. Total number of grains/panicle; B. 1000-grain weight. *significantly different from wild-type

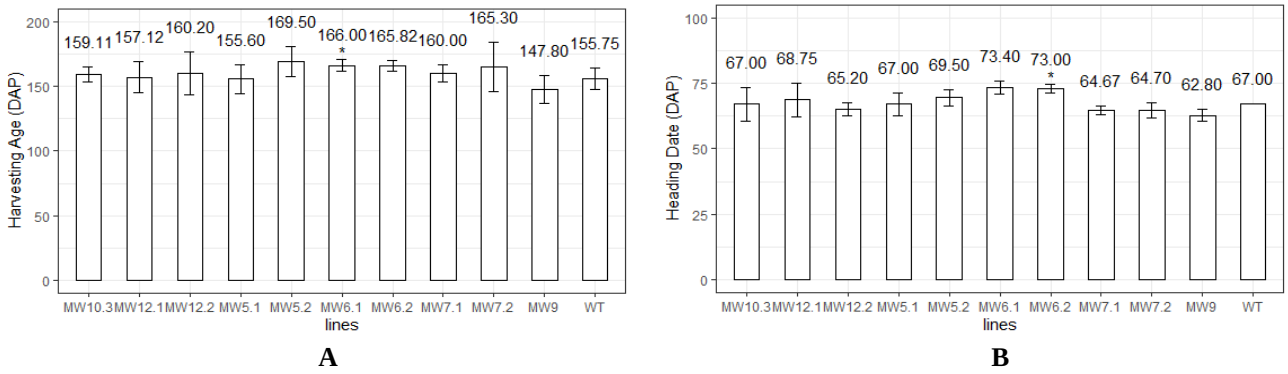


Figure 4. Delayed senescence of Mentik Wangi wild-type and T1 mutant lines: A. Days to flowering; B. Days to harvest. *significantly different from wild-type

Panicle formation marks the transition from the vegetative to the generative phase. The apical bud meristem transforms into a flowering meristem, triggered by the florigen signal (Tsuji 2017). The panicle, the compound flower of rice, consists of a main axis, primary branches, secondary branches, and spikelets (Tanaka et al. 2023). Panicle morphology influences productivity, particularly the angle formed between the main axis and the primary branches. Panicle types are categorized into three types: compact, intermediate, and open, based on the angle between the main axis and the primary branches. This angle formation is influenced by the interaction of phytohormones, such as auxin, cytokinin, and gibberellin (Kurakawa et al. 2007; Liao et al. 2019). Changes in cytokinin and gibberellin levels in the mutant lines affect the resulting panicle type. High cytokinin levels in the flowering meristem stimulate higher cell division activity, leading to a larger panicle angle. Reduced gibberellin levels in the plant meristem reduce cell elongation activity, impacting optimal panicle angle formation (resulting in compact panicles). Compact panicles tend to yield higher harvests as they reduce lodging, but they are more susceptible to pathogen attacks. Open panicles exhibit greater pathogen resistance due to their looser spacing, reducing humidity levels.

Panicle exsertion can also influence rice productivity. Panicle exsertion is determined by the position of the panicle base relative to the flag leaf (Figure 7). Based on this position, panicle exsertion can be classified into several types: well-exserted, moderately well-exserted, just-exserted, partly-exserted, and enclosed. Panicles that cannot fully emerge (enclosed by the flag leaf) tend to produce fewer grains (Sitaresmi et al. 2019). Enclosed panicles can occur due to mutations in the target *GA20ox2* gene, leading to reduced endogenous gibberellin levels and disrupted flowering meristem elongation. However, this does not always occur in every panicle. Within a single plant, variations in panicle exsertion type can occur due to differing hormone levels (Zhan et al. 2019).

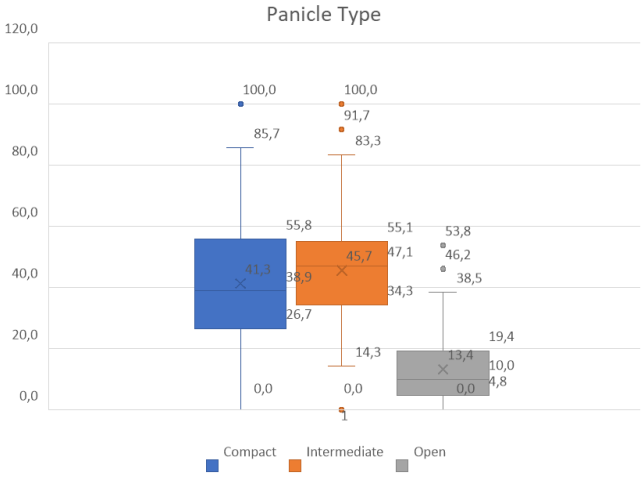


Figure 5. Distribution of panicle type in T1 mutant lines and wild-type

Figure 6. Distribution of panicle exsertion type in T1 mutant lines and wild-type

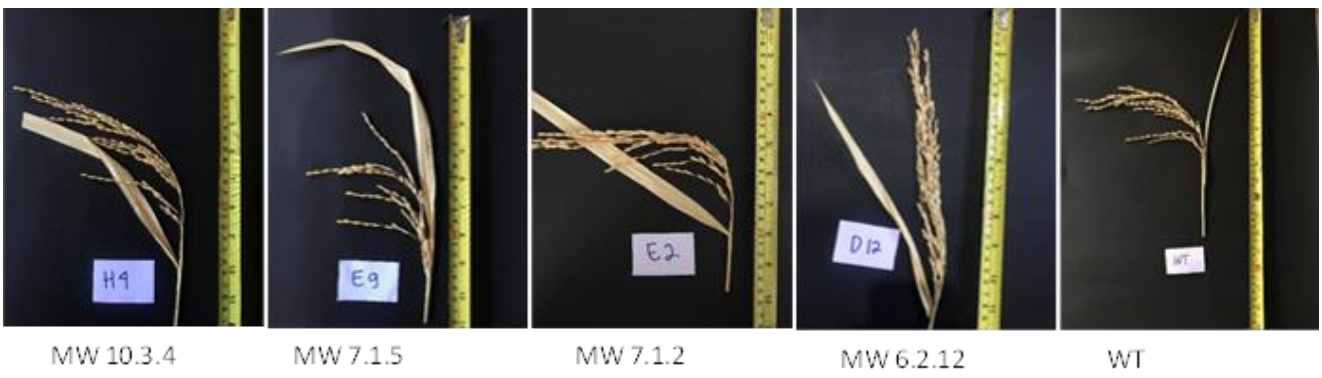


Figure 7. Representative panicle phenotype of T1 mutant lines and wild type line

Gene editing presents a viable solution for improving these traits. CRISPR/Cas9, a precise gene editing method, has proven effective in modifying plant phenotypes (Jung et al. 2019; Nawaz et al. 2020; Das et al. 2022). Previous research successfully introduced the CRISPR/Cas9 gRNA-*GA20ox2* and CRISPR/Cas9 gRNA-*OsCKX2* constructs into Mentik Wangi rice, resulting in 15 lines that tested positive for these constructs (Santoso et al. 2020; Ruzyati et al. 2022). Two Mentik Wangi mutant lines, MW 6.2 and MW 10.3, exhibited mutations in the downstream region of the gRNA targeting the *GA20ox2* gene, involving a single base substitution. Two other lines showed mutations in the upstream region of the first exon of the *OsCKX2* gene, with one line displaying an insertion of one base and the other line showing a substitution of two bases (Ruzyati 2022). Genes encode specific functional proteins within plant cells. These proteins determine specific phenotypic characteristics, from the molecular and cellular levels to organ morphology.

In conclusion, phenotypic character analysis of T1 mutant lines of Mentik Wangi rice revealed differences compared to the wild-type. This indicates that the knockout of *GA20ox2* and *OsCKX2* genes influences plant phenotypic responses, including decreasing plant height and panicle length, increasing and also decreasing in several lines the total tiller number, productive tiller number, total grain number per panicle, and 1000-grain weight; and creating variations in panicle type and panicle exertion type. Additionally, the knockout of *GA20ox2* and *OsCKX2* genes affects leaf senescence, delaying flowering and harvesting age. The knockout of the *GA20ox2* gene can transform the characteristics of Mentik Wangi into a semi-dwarf variety.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from RISPRO Invitation Batch 2 (Registration No. RISPRO/ B2/1/20/2020) LPDP (Indonesia Endowment Fund for Education Agency) in collaboration between Universitas Sebelas Maret and National Research and Innovation Agency (BRIN).

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