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Tolerance of T2 Generation 'Kitaake' Rice (*Oryza sativa* L.) CRISPR/Cas9-OsGA20ox-2 Mutant Strains to Drought Condition

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ABSTRACT

Rice is the most important staple crop in Indonesia. Food demand that continues to rise despite the limited land availability could be managed by developing drought tolerant superior cultivar using CRISPR-Cas9 system method. This experiment aimed to obtain non-transgenic mutant plants, to gain information on GA20ox-2 gene expression levels, and to study the tolerance levels of the CRISPR/Cas9-OsGA20ox-2 mutant strains 'Kitaake' T2 generation against drought conditions. Planting material used were a mutant gene GA20ox-2 'Kitaake' (K 23.1, K15, 29.1 K, K 19.1) and wild-type comparison. Among 20 plants tested for the presence of the Cas9 and hpt genes, strains K23.1, K15, K29.1, and K19.1 have 50%, 50%, 0%, and 45% of non-transgenic plants, respectively. DNA mutations were carried out in the form of deletion of 44 bases (K23.1, K29.1, K19.1) and insertion of two bases (K15) transcribed into RNA. The transcription resulted in a lower number of amino acids compared to its wild type (389 amino acids). Differences in the number of amino acids resulted in the different phenotypic expressions. K15 mutant strain had lower plant height and leaf length than the other mutant strains and wild type. However, the decrease in the plant height did not decrease the potential of the crop. Mutations in the K15 strain did not indicate better tolerant response to drought stress than other mutant strains (K23.1, K29.1, K19.1) and wild type in both vegetative and generative phase.

Keywords: genome editing, non-transgenic, insertion, deletion, expression, frameshift

INTRODUCTION

Oryza sativa is a cultivated species that feeds over half of the world's population (Zhao *et al*., 2011). A rising fertility rate has caused increasing food demand. The need for rice continues to increase along with the population growth rate that is faster than the growth of available food production (Badan Pusat Statistik, 2011).

In Indonesia, the population is increasing year by year and expected to increase (Worldbank, 2016). This leads to very high demand for rice in Indonesia (Sari, 2014). Rice imports are undertaken to cover the shortfall. From 2010–2015, the Indonesia's rice imports fluctuated with an upward trend (Statistic Indonesia, 2017). Therefore, it is necessary to increase national rice production through intensification and extensification (Abdurachman *et al*., 2008).

Agricultural intensification can be done by providing superior cultivar. Superior cultivars can be obtained from plant breeding program. Plant breeding is defined as a series of plant genetic research and development activities to produce superior cultivars. Through breeding activities, it is expected to produce a new variety of superior cultivars that have high productivity and have several other characters that support the efforts to improve quality and competitiveness (Carsono, 2008).

Development of a cultivar can be done through biotechnology. One of the biotechnology techniques that can be used in plant breeding is the editing of the genome through the CRISPR-Cas9 system. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeat) is associated to the protein 9

(Cas9) system, i.e. genome editing technology at specific points (Xu *et al*., 2014) specifically and predictably directly (Santoso *et al*., 2015). The editing of targeted genomes can be applied to help speed up plant breeding (Carsono, 2008). The CRISPR-Cas9 system has been applied to plants, for example in the rice plant (Xu *et al*., 2014).

Increased productivity through seed quality should be supported by land as the main factor of production. In Indonesia, drought-affected land continues to increase from year to year. In 1997 and 2015, rice plants that experienced drought were 513 thousand hectares (ha) and 597 thousand hectares (ha), respectively. The impact of the drought is that crop yields decrease by an average of around 40% (Surmaini *et al*., 2015). Therefore, it is necessary to conduct plant breeding program to obtain drought tolerance cultivars.

The GA20ox-2 gene has a function to produce giberelin (Qin *et al*., 2012). Genetic manipulation of gibberellin metabolism by mutating the GA20ox-2 gene may increase plant yield (Sakamoto *et al*., 2004) and make the plants shorter (Zhao *et al*., 2016). Smaller plants allow better water efficiency, thus they are able to tolerate and survive in drought conditions better (Serrano and Sarah, 2016).

The CRISPR/Cas9 genome editing technology is directed to convert GA20ox-2 genes into rice. Gene-generated T1 generation of GA20ox-2 gene produces a premature stop codon, causing alteration of the GA20ox-2 gene metabolism pathway. This leads to a decrease in GA20 product, which further causes the change in the posture of rice plants to be shorter (dwarf). This short posture of plants has a pleiotropic effect on the number of more saplings. First, the leaf architecture is upright so as to capture more light energy. Secondly, the plants are more responsive to nitrogen fertilization, and especially to the higher harvest index. Some T1-generation plants do not contain the CRISPR/Cas9 constructs to obtain a non-transgenic T1 generation plant (Santoso, 2015). Plants with the GA30ox-2 mutated gene have an effect on the drought tolerance properties (Vikram *et al*., 2016). Therefore, further research is needed to obtain non-transgenic mutant plants, to gain GA20ox-2 expression level information, and to study tolerance levels of T2 generation of 'Kitaake' CRISPR/Cas9-OsGA20ox-2 mutant strains against drought conditions. This study is expected to obtain mutant strains that have a higher tolerance to drought than the wild type based on genotypic and phenotypic criteria.

MATERIALS AND METHODS

The materials used were T2 generation of 'Kitaake' rice seeds of GA20ox-2 (GA1, K15, K29.1, and K19.1) genes and wild type of 'Kitaake' paddy seeds as comparison. Study on the tolerance of T2 generation of 'Kitaake' CRISPR/Cas9-OsGA20ox-2 mutant strains to the drought conditions was conducted in several steps, consisting of rice cultivation, drought stress application (vegetative and generative phase in different experimental unit), soil moisture test, morphological observation, DNA isolation, RNA isolation, RNA quantification, RT-PCR, PCR, gel electrophoresis, gel electrophoresis, and harvesting. The data obtained were analyzed using analysis of variance with several mutant strains as treatments for each observed variable. If there was any significant difference, the analysis was then continued with Tukey's test. T test was also used in this study.

The T2 generation of 'Kitaake' rice CRISPR/Cas9-OsGA20ox-2 mutant strains (40 seeds/each strain) and control (40 seeds) were planted in media containing soil and manure with a ratio of 1: 1. Each hole of tray was planted with 5 seeds. After the 21st day, planting was performed on a bucket containing 1 kg of soil with 600 mL water added. Each bucket was planted with 1 plant. Each bucket was labeled

Table 1. Scoring Scrolling Leaves and Drought.

Scale	Level of tolerance	Scoring of leaf curl
0	Very tolerant	leaves were healthy
1	Tolerant	leaves begin to fold (shaped somewhat V)
3	Somewhat tolerant	leaves folded (very-shaped V)
5	little sensitive	leavestruly bud (U-shaped)
7	Sensitive	ends of the leaf contact (form O)
9	Very delicate	leaves of thetight curl

Source: IRRI (1996) *cit*. Silitonga and Risliawati (2011).

with strains and plant code. Watering was carried out every other day (110 mL) until the plant was 30 HSS. When the plant was 30 HSS (vegetative phase), 20 plants of each strain were treated with drought stress by stopping the watering for 15 days (80% of wild-type plants had leaf rolling scale of 9 and leaf drying scale of 9 (Table 1)), while 20 other plants were still given watering and treated with drought stress at age of 64 HSS-70 HSS (generative phase).

The soil moisture test was performed in 1 plant life cycle by gravimetric method. The tools and materials used were petridishes, analytical scales, ovens, soil samples, and labels. In the vegetative phase, Doyle and Doyle (1990) method to isolate DNA was used with modifiocation, followed by PCR for hpt and Cas9 gene checks. RNA isolation was also performed in the vegetative phase of the plant using the RNA isolation kit method (QIAGEN). Quantification of RNA was carried out using a nanodrop device. RT-PCR was performed to convert RNA to cDNA as a template in GA20ox-2 gene expression. The DNA amplification process was done by PCR (Polymerase Chain Reaction). Results from the PCR process were electrophoresed using 1% agarose gel. The agarose gel was stained in a bromide etidium solution for 10 minutes and washed with water for 5 minutes. The agarose gel was then visualized with Chemidoc Gel System (Biorad). The visualization results were saved with the image format jpg.

The observations were conducted on genotypic and phenotypic characteristic. Genotypic observations were performed on the presence of CRISPR/Cas9 (Cas9 and hpt) and the presence of cDNA bands of GA20ox-2 gene expression on T2 generation of 'Kitaake' rice. Phenotypic observation was performed by observing the response of plant leaves when treated with drought stress (Table 1). In addition, measurements of plant height, leaf length, number of filled grains per plant, number of empty grains per plant, total grains per plant, and number of tillers. During 1 plant life cycle, soil samples were taken once a day to measure the moisture content.

The results obtained from the data analysis will be related to the genotype observation (GA20ox-2 gene expression). The genotypic observations were performed on the presence of the GA20ox-2 gene band on the T2 generation of 'Kitaake' rice, so that the linkage between GA20ox-2 gene and the resulting phenotype will be known.

RESULTS AND DISCUSSION

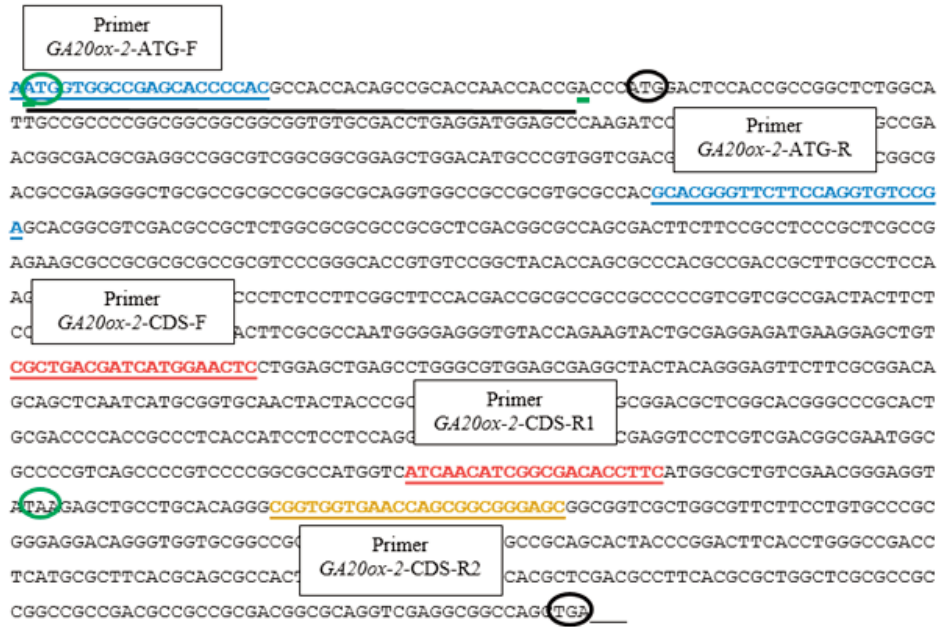
The mutant plants, T2 generation of K23.1 strain, K29 strain, K19 strain (mutation with 44 base reduction), K15 strain (mutation with addition of two bases), and wild type as comparison were used in this study. The mutant strains had passed through segregation in previous generations (T0 to T1, T1 to T2), thus affecting the presence of the Cas9 and hpt genes. The selected segregation type was free of the Cas9 and hpt genes so that the selected plant is a non-transgenic mutant plant.

The selection used to detect the presence of the Cas9 and hpt genes was PCR selection using primers for both genes. Selection results showed that from 20 selected plants of each strain, K23.1 strain has 10 non-transgenic plants (50%), K15 strain has 10 non-transgenic plants (50%), K.29.1 strain has 0 non-transgenic plants (0%), and K19.1 strain has 9 non-transgenic plants (45%).

Wild-type of 'Kitaake' rice was used as a comparison of mutant plants. The GA20ox-2 gene on wild-type plants has a complete DNA base sequence. Therefore, the transcription process of 2844 bases of genomic DNA and translations of 1170 bases of RNA produce a 389 complete amino acid (Kawahara *et al*., 2013), whereas the GA20°x-2 in mutant plants has a number of bases of DNA, RNA, and different type of amino acid products.

GA20ox-2 gene expression testing used cDNA templates for amplification that used PCR process. The primer used 3 primer pairs designed from the wild-type cDNA sequence of the GA20ox-2 gene, based on the mutation point in the mutant strain (the point of mutation seen from the DNA sequencing of mutant strains) (Figure 1).

The strands of K23.1, K29.1, and K19.1 experienced a mutation in the form of 44-base deletion (black strain) in the GA20ox-2 gene (Figure 1). The cDNA base sequence showed that the GA20ox-2-ATG-F/R primer had no attachment region especially for GA20ox-2-ATG-F primers because the complementary base was deleted. This inhibits the amplification process. This was proven by the absence of amplified bands. The other primers were GA20ox-2-CDS-F/GA20ox-2-CDS-R and GA20ox-2- CDS-F/GA20ox-2-CDS-R2, having attachment areas resulting in bands (Figure 2). Mutations in K23.1, K29.1, and K19 strains cause the amino acid to decrease from 389 to 373 amino acids.



Remark: GA20ox-2-CDS-F/GA20ox-2-CDS-R1 = 278 bp; GA20ox-2-CDS-F/GA20ox-2-CDS-R2 = 345 bp; A20ox-2-ATG-F/R = 299 bp

Figure 1. The primary composition of GA20ox-2 gene expression testing on wild-type cDNA. The black strain is a mutation in the K23.1, K29.1, and K19.1 strains (deletion of 44 bases). The green strain is a mutation in the K15 strain (2 bases insertion). The black circle represents the start codon and stop codon of K23.1, K29.1, and K19.1. The green circle is the start codon and the K15 strain is stop codon.

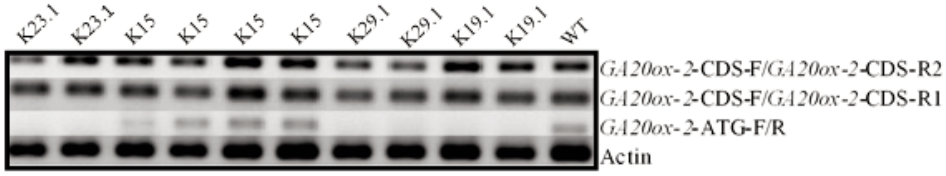


Figure 2. Gene expression GA20ox in rice mutants 'Kitaake' strain K23.1, K15, K29, K19.1 and the wild type (WT)

Table 2. DNA, cDNA, and protein produced by strains of mutant and wild-type.

Strain	Mutation Type	Total bases (bp)		Amino acid
		DNA	cDNA	
Wild type	No mutation	3140	1170	389
K23.1	-44 homo-dialelic	3096	1126	373
K15	+2, homo-dialelic	3142	1172	300
K29.1	-44, homo- dialelic	3096	1126	373
K19.1	-44 homo-dialelic	3096	1126	373

The K15 strain mutates on the GA20ox-2 gene in the form of insertion of a two-A base (green strain) (Figure 1). Based on the GA20ox-2 gene sequence cDNA in the K15 strain, it shows that the GA20ox-2-ATG-F/R, GA20ox-2-CDS-F/GA20ox-2-CDS-R1, and GA20 ox-2-CDS-F/GA20 ox-2-CDS-R2 have a complementary base sequence that enable the PCR amplification process work. This is proven by the emergence of cDNA amplified bands (Figure 2). GA20ox-2-ATG-F/R primary amplified bands had one additional base. The size of tape is not visible, because there was only one base added . K15 strain was also experiencing frameshift so that the type of amino acid produced is different from the wild type. Mutations in the K15 strain cause a decrease in amino acids from 389 to 300 amino acids.

Different chemical compounds of DNA, cDNA, and amino acids between mutant strains and wild type (Table 2) were further observed on the phenotypic characteristic of each mutant strain and its relation to drought stress treatment. Treatment of drought stress was given in the vegetative and generative

Table 3. Further test results of plant height and leaf length at the end of the drought stress treatment (45 HSS) with Tukey test.

Strain	Plant high (cm)	Leaf length (cm)
Wild Type	50.3ab	28.4ab
K29.1	54.4a	31.2ab
K23.1	52.3ab	32.5a
K19.1	48.2b	27.3b
K15	37.8c	23.0c

Remark: Means followed by the same letters in the same column are not significantly different according to the Tukey test at a significance level of α= 5%.

Table 4. DNA, cDNA, and protein produced by strains of mutant and wild-type.

Strain	High average of plant (cm)		Significance	HSS
	Normal	Drought		
Wild Type	53.8	50.3	*	45
K23.1	57.4	52.3	*	41
K15	43.0	37.8	*	43
K29.1	58.5	54.4	*	42
K19.1	52.4	48.2	*	45

Remark: (*) indicates significant difference according to T test at a significance level of α= 5%.

phases (different experimental units). During the treatment of drought stress in vegetative phase (30–45 HSS), several agronomic traits were observed, including plant height, leaf length, leaf rolling, and leaves drying. During the treatment of drought stress generative phase (64–70 HSS), the variables observed were the number of filled grains, the number of empty grains, total grains, and number of tillers.

Further test results on the plant height and leaf length, showed that wild type did not differ significantly from mutant strains K23.1, K29.1, and K19.1 (Table 3). The product of amino acid of mutant strains was decreased compared to the wild type due to deletion (Table 2).

The K15 strain has the highest average of plant height and leaf length than any other strain. Mutation in the form of a two-base insertion makes the K15 strain experience frameshift. It also causes translational product to decrease, i.e. 89 amino acids so that the strain had a significant difference compared with other mutant strains and wild type. This is in accordance with the research of Colebrook *et al*. (2014) stating that mutating the GA20ox-2 gene can reduce plant height. According to Qiano and Chen (2013), GA20ox-2 mutant strains that had decreased plant height (dwarfs) were caused by a decrease in gene expression. The data of plant height and leaf length in this study showed a coefficient of variance of 13% and 17%, indicating that the research was done according to the standard rules.

Treatment of drought stress affects plant height in all strains (Table 4). This indicates that all strains tested either mutants or controls suffer from drought stress. Wild-type strains, K23.1, K15, K29.1, and K19.1 respectively showed a decrease in plant height due to drought stress at 45 HSS, 41 HSS, 43 HSS, 42 HSS, and 45 HSS. There was no significant difference in the plant height as well as in the tolerance to drought between mutant and wild-type strains. Shorter plant (dwarf) of K15 mutant strains did not show better tolerance to drought stress.

The analysis results on the plant height and leaf length showed that not all mutation types that occur in GA20ox-2 genes decreased the plant height and leaf length that differed from those of wild type. Mutations in strains K23.1, K29.1, and K19.1 (deletion of 44 bases) did not show differences in plant height and leaf length compared with wild type. The K15 strain has a lower value of plant height and leaf length compared to the wild type. Therefore, the K15 strain mutation corresponds best with the role of the sd1 allele in 'Dee-geo-woo-gen' and 'IR8', which decreases GA20 product and reduces plant height and leaf length (dwarf) compared to the wild type.

Although the mutations that occured in the 'Kitaake' strain K15 differ from mutations in 'Dee-geo-woo-gen' (deletion of 383 bases) and 'IR8' (deletion of

Table 5. Results of T-test between wild-type and mutant strains of GA20ox-2 against the leaves dry.

Strain	Strain	Mean	P value
Wild type (3.7)	K15	5.0	0.11
	K23.1	5.6 *	0.04
	K19.1	4.1	0.63
	K29.1	3.1	0.43

Remark: (*) indicate significant difference according to T test at a significance level of 5%.

Table 6. Number of grain content, empty grain, and total grain

Strain	Number of filled grain	Total of empty grain	Grain total number
wild type	39.0ab	50.1bc	89.0a
K23.1	39.5ab	48.4bc	87.9a
K15	46.3a	39.4c	85. 7a
K19.1	26.7b	62.1ab	88.7a
K29.1	25.8b	77.5a	103.3a

Remark: Means followed by the same letters in the same column are not significantly different according to the Tukey test at a significance level of α= 5%.

Table 5. T test results between wild-type and mutant strains of GA20ox-2 to the number of tillers.

Strain	Strain	Mean	P value
Wild type (4.5)	k23.1	4.2	0.294
	K15	4.1	0.330
	k19.1	5.1	0.125
	k29.1	5.0 *	0.049

Remark: (*) indicate significant difference according to T test at a significance level of α= 5%.

383 bases), they all have the same effect on the plant height and leaf length.

The effect of further mutations was proven by analyzing leaf rolling scales under drought stress. According to Sujinah and Jamil (2016), the leaf rolling shows a reduction in the transpiration rate of water savings by closing stomata and minimizing leaf surface area. The results showed that all strains showed the same scale of leaf rolling (not significantly different) under drought stress. This is shown through Kruskall Wallis test with p value of 0.1179.

The T-test result on the leaf drying scales showed that the survival ability under drought stress was not significantly different amongst each other (Table 5). According to Serrano and Sarah, (2016), dwarf plants are more able to tolerate and survive in drought conditions than normal plants. This has not been demonstrated in this study, probably because the 'Kitaake' rice genotypically has short phenotype so that there was no significant difference observed when compared with 'Kitaake' mutant rice.

Drought stress in generative phase was set up during the pollination period. According to Akram *et al*. (2013), the number of filled and empty grains is most affected when the drought stress occurs during generative phase (pollination period). In that phase, lack of water causes pollen to become barren so that it is not able to fertilize. Failure of fertilization will lead to empty grain.

The result of Tukey test showed that K15 strain had the highest number of filled grains as well as the lowest number of empty grains, however, the values were not significantly different compared to those of the wild type (Table 6). There was no significant difference on the total number of grains either. Therefore, it has not been demonstrated that mutant strains have better self-defense abilities under drought stress during generative phase (pollination) stress compared to the wild type.

The influence of mutation was then seen from the

number of tillers. The result of T-test on the number of tillers indicated that the majority of mutant strains were not significantly different from the wild type (Table 7). This is also in accordance with the research of Santoso (2015), stating that the pleiotropic effect on the number of tillers has not been seen in short posture of K15 mutant rice. It is probably based on the characteristic of the wild type of 'Kitaake', which is short plant, thus, the mutations in the GA20ox-2 gene are not enough to produce pleiotropic effects.

Overall, genotypic and phenotypic observation shows that the K15 strain has the most distinct character compared to the other mutant strains (K23.1, K29.1, and K19.1) and the wild type. Types of mutations in the K15 strain (reduced amino acids and frameshift) result in lower plant height and leaf length compared to the wild type, as well as decrease the number of leaves, thus do not increase the yield potential and show no better tolerance to drought.

CONCLUSIONS

Differences in the number of amino acids result in different phenotypic expressions. K15 mutant line has lower plant height and leaf length. However, the decrease is not followed by the decrease in the yield potential of the crop. Mutations in the K15 line do not indicate better tolerant response to drought stress than other mutant lines and wild type in both vegetative and generative phase.

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