Targeted mutation of *GA20ox-2* gene using CRISPR/Cas9 system generated semi-dwarf phenotype in rice

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Abstract. Recently, the engineered CRISPR/Cas9 system has been applied to rapidly and efficiently modify the targeted gene(s) in a wide variety of plants. Recent studies of successful targeted mutagenesis using the CRISPR/Cas9 system with a single gRNA expression in rice plants have been reported. *GA20ox-2* is a gene encoding an oxidase enzyme involved in the biosynthesis of gibberellin and linked to *sd1* locus. A previous study revealed that mutation of this gene resulted in shorter stature of rice plant due to defects in the gibberellin's signalling pathway. Here, we studied targeted mutation of *OsGA20ox-2* gene in rice using the CRISPR/Cas9 system with the expression of two gRNAs. In this study, we introduced a single plasmid vector of CRISPR/Cas9 system harboring dual gRNAs to modify *OsGA20ox-2* gene in a rice model cv. Kitaake via *Agrobacterium*-mediated transformation. Targeted mutagenesis of *OsGA20ox-2* gene using CRISPR/Cas9 generated nine mutated rice lines with a mutation frequency of 90%. Most mutated lines (50%) had mutations in both *OsGA20ox-2* gRNA. They resulted in homo-diallelic mutation type with 44 bp deletion, while three lines were heterozygous, one line was homo-diallelic with 2 bp insertion, and one line had no mutation. The K15 mutated rice line was identified as a homozygous two-nucleotide insertion and had the semi-dwarf phenotype, demonstrating that *OsGA20ox-2* gene had been disrupted.

Keywords: rice (*Oryza sativa* L.), CRISPR/Cas9 system, targeted mutagenesis, semi-dwarf.

1. Introduction

Rice improvement is essential to meet the increasing food demand due to rapid growth in population. Extreme weather events and the availability of natural genetic resources are other issues that have endangered the global food security [1]. Therefore, improving yield, biotic and abiotic tolerance is crucial to increase and stabilize the productivity of rice crop. Conventional breeding, such as hybridization and mutation techniques, have significantly contributed to increasing rice productivity. Molecular approaches, including marker-assisted backcrossing (MABC) and genetic engineering (GE) technology, have also played an important role in overcoming the limitations of conventional breeding and enhancing crop productivity. However, these technologies still have some disadvantages, such as

being time-consuming and labor-intensive. In the case of GE approach, the complex regulatory process followed by time-consuming and costly safety analysis, as well as the public acceptance of GE crops, are a great challenge [2]. Hence, a directed, rapid, and low-cost method is essential for developing high-yielding, biotic or abiotic stress resistant rice varieties. Targeted editing technology of key functional genes promises to be a powerful tool in accelerating varietal improvement [3].

Genome editing system can be applied to modify gene(s) rapidly in a precise and predictable manner. The technology has a great potential to accelerate crop basic research and improvement program. A more recently developed genome editing system is clustered regularly interspaced short palindromic repeat (CRISPR)/Cas (CRISPR-associated) based on RNA-guided engineered nucleases and a guide RNA complex. The most widely used system is the type II CRISPR/Cas9 employing a Cas9 endonuclease from *Streptococcus pyogenes* [4]. The system allows the creation of double-stranded breaks (DSBs), which can lead to gene mutations due to non-homologous end-joining (NHEJ) repair or gene replacement or correction as a result of homologous recombination-based repair (HR). In most cases, NHEJ causes random insertions or deletion (Indels), which can result in frameshift mutations if they occur in the coding region of the gene, effectively creating a gene knockout. The CRISPR/Cas9 system has been successfully used for efficient genome editing and applied to gene modification in plants, such as rice [5–8], wheat [6,9], maize [10], potato [11], tomato [12] and sweet orange [13].

GA20ox-2 is a gene encoding an oxidase enzyme involved in the biosynthesis of gibberellin, a plant growth hormone, and it is tightly linked to the *sd1* locus [14]. This gene was isolated from rice using degenerate primers based on the conserved domain of the *GA20ox* gene in rice (*OsGA20ox-1*) [15] and *Arabidopsis* (*GA5*) [16] and located on the chromosome 1 [14]. *OsGA20ox-2* is strongly expressed in leaf blades, stems, and unopened flowers. Increased expression of the gene in those tissues, especially in the leaf blade and stem, resulted in a semi-dwarf phenotype in the enzyme-defective *sd1* mutants, as indicated by shorter leaves and stems [17]. Further information explained that *sd1* mutants still allow the flowers to develop and be fertilized normally although the mutants experienced a loss of function of *GA20ox-2* gene. Because of the normal flower formation and fertilization, the yield of *sd1* mutant plants would be stable and not affected by the reduced plant height.

The aim of this research was to study targeted gene mutation of *OsGA20ox-2* using CRISPR/Cas9 system to generate semi-dwarf phenotype in rice. Our recent work has demonstrated that we have successfully introduced the single plasmid vector of the CRISPR/Cas9 system harboring dual gRNAs to modify *OsGA20ox-2* gene in a rice model cv. Kitaake via *Agrobacterium*-mediated transformation. Here, we showed the CRISPR/Cas9 had induced mutations in the targeted endogenous plant gene *GA20ox-2* and they were detectable in T_0 rice plant. The K15 mutated rice line was identified as a homozygous two-nucleotide insertion and had the semi-dwarf phenotype, demonstrating that *OsGA20ox-2* gene had been disrupted.

2. Materials and methods

2.1. Plant material and plasmid

The rice variety Kitaake (*Oryza sativa* L. spp. *japonica*) was used as material for *Agrobacterium*-mediated transformation as previously described. The plant transformation vector was based on pBY02-Cas9 (kindly provided by Dr. Bing Yang), a binary vector for *Agrobacterium*-mediated rice transformation containing a maize *ubiquitin* 1 promoter driving the rice codon-optimized *Cas9* gene and a CaMV 35S promoter driving *hpt*II gene expression for hygromycin resistance.

2.2. Construction of guide RNA of OsGA20ox-2 gene

Construction of guide RNA gene was performed using an intermediate vector pENTR-gRNA that can express two different gRNAs [18]. The pENTR-gRNA vector containing two cloning sites, the first cloning site is facilitated with 2×*Btg*ZI downstream of one rice U6 promoter and another cloning site with 2×*Bsa*I downstream of the second rice U6 promoter. CRISPR/Cas9 construct was designed in a binary T-DNA vector for co-expression of *Cas9* gene and guide RNA. *OsGA20ox-2* gene-specific

gRNAs sequences (gRNA1-GA20ox-2 and gRNA2-GA20ox-2) were cloned into entry vector pENTR-gRNA. Two complementary oligonucleotides (24 nucleotides) from each gRNA that target a specific genomic locus of *OsGA20ox-2* gene were annealed to generate a double-stranded DNA oligonucleotide (dsOligo). Both guide RNAs of *OsGA20ox-2* gene were then inserted into pENTR-gRNA through the 2×*Btg*ZI and 2×*Bsa*I cloning sites, respectively. The gRNA cassette containing two gRNA of *OsGA20ox-2* was finally combined with pBY02-Cas9 by using the Gateway LR Clonase (Thermo Fisher Scientific, USA). The CRISPR/Cas9 construct containing the *Cas9* gene and guide RNA was transformed into *A. tumefaciens* strain LBA4404, and the transformation vector was then used for rice transformation.

2.3. Rice in vitro culture and transformation

Mature *japonica* cv. Kitaake rice seed was dehusked, surface sterilized and placed onto callus induction medium (4 g/l NG salt, 300 mg/l casamino acid, 2.8 g/l L-proline, 30 g/l sucrose and 4 g/l Gelrite, pH 5.8) containing 2 mg/l 2,4-D. The callus tissue derived from the mature embryo is then used as the starting material for transformation. *Agrobacterium*-mediated rice transformation steps followed the protocol as previously described [19]. The transgenic rice lines were grown in a greenhouse with standard maintenance as recommended. Mature seeds were collected from T_0 plants and used for further genotyping analysis.

2.4. Genotyping transgenic rice plant

Total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method from the transgenic rice plants [20]. The genomic DNA was used as a template to amplify the specific region surrounding the CRISPR/Cas9 target sites of *OsGA20ox-2* gene using a pair of specific primer, i.e. OsGA20ox-2-F (5'-TCATGTCTGTCCAGTGGCAAC-3') and OsGA20ox-2-R (5'-CACCATCGTTTTAATTACCCCATT-3'). The PCR fragments were directly sequenced to identify the pattern of mutations by sending the fragment to a Lab Service at PT Genetika Science. Sequence data were analyzed by alignment to the target sequence of transgenic line compared to one of the wild type plants using the BLAST program.

3. Results and discussion

Two guide RNAs have been designed from exon 1 of *OsGA20ox-2*. To enhance mutagenesis success in the targeted exon of *OsGA20ox-2* gene, two closely located target sites in *OsGA20ox-2* gene were selected for gRNA construction. The position of the gRNAs of *OsGA20ox-2* is shown in Figure 1. The gRNA1-GA20ox-2 was designed in sense orientation and contained the start codon ATG in order to enhance the chances of mutation of the target gene. Modification of ATG start codon will make the gene unable to start the transcription process, and therefore, the gene will malfunction.

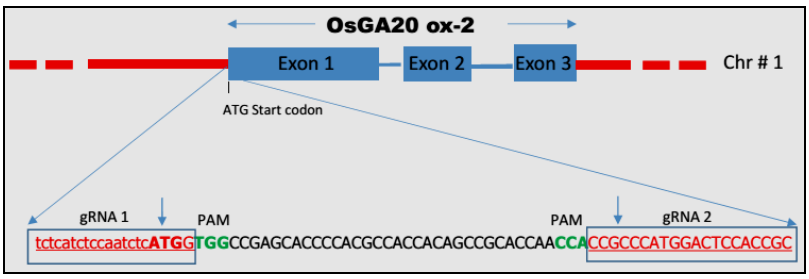

Figure 1. Position of dual gRNAs of *OsGA20ox-2* gene in chromosome 1. Both gRNAs are located in exon 1 of the gene. The gRNA1 was designed in sense orientation containing start codon ATG, while the gRNA2 was in antisense orientation with 44 bp distance from gRNA1.

The intermediate vector pENTR-gRNA, which has two different rice U6 small nuclear RNA gene promoter (PU6-1 and PU6-2), was used to express the dual gRNAs of *OsGA20ox-2*. The gRNA1-GA20ox-2 was cloned at 2×*Btg*ZI sites from the first gRNA scaffold in a tail-to-tail orientation downstream of PU6-1. Meanwhile, the gRNA2-GA20ox-2 was inserted at 2×*Bsa*I cloning sites that were part of the second gRNA scaffold in a tail-to-tail orientation downstream of PU6-2. Two sequential steps of cloning enabled the insertion of the custom two gRNAs into *Btg*ZI dan *Bsa*I restriction enzyme sites in the pENTR vector to produce the intermediate construct pENTR-gRNA-OsGA20ox-2 (Figure 2).

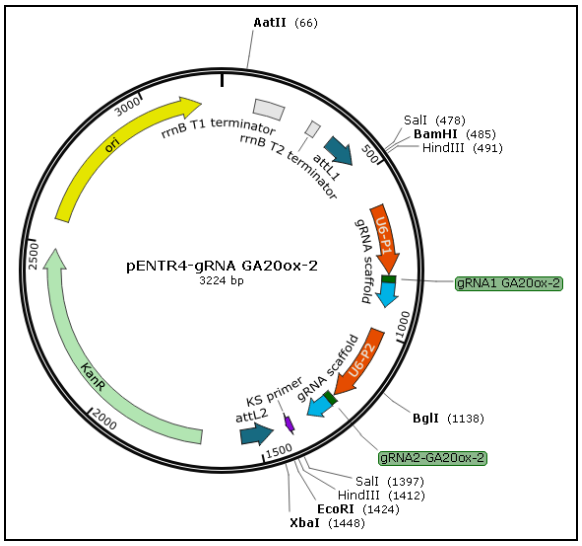

Figure 2. The intermediate vector pENTR-gRNA-OsGA20ox-2 carrying dual guide RNA of *OsGA20ox-2* gene, i.e. gRNA1-GA20ox-2 and gRNA2-GA20ox-2.

To evaluate the efficacy of the CRISPR/Cas9 system in inducing double-strand breaks (DSB) and initiating target gene mutation in rice, we selected *OsGA20ox-2* gene as the target. This gene encodes an oxidase enzyme involved in the biosynthesis of gibberellin. Rice has only one copy of the *OsGA20ox-2* gene and if this gene is knocked out, it would result in a semi-dwarf phenotype indicated by shorter leaves and stems. The standard protocol of *Agrobacterium*-based rice transformation [19] (Figure 3) was used to introduce the CRISPR/Cas9-gRNA-GA20ox-2 to generate 10 independent rice lines.

Following the genetic transformation, T_0 independent lines that showed resistance to hygromycin were identified. To detect mutations in the targeted sequence regions in the T_0 plants, the DNA of ten independent T_0 rice lines were amplified using specific primers for the specific region surrounding the CRISPR/Cas9 target site of *OsGA20ox-2*. PCR amplification showed that all ten plants produced DNA fragment or amplicon with a size of 768 bp (Figure 4). The amplicon from each plant was then subjected to DNA sequencing analysis to identify mutagenesis of the target mutation sites.

Based on DNA sequencing analysis, it was found that there were various combinations of mutations in the representative rice lines as illustrated in Figure 5. Nine mutated rice lines were identified from the ten independent transgenic lines, which means that the mutagenesis frequency was 90%.

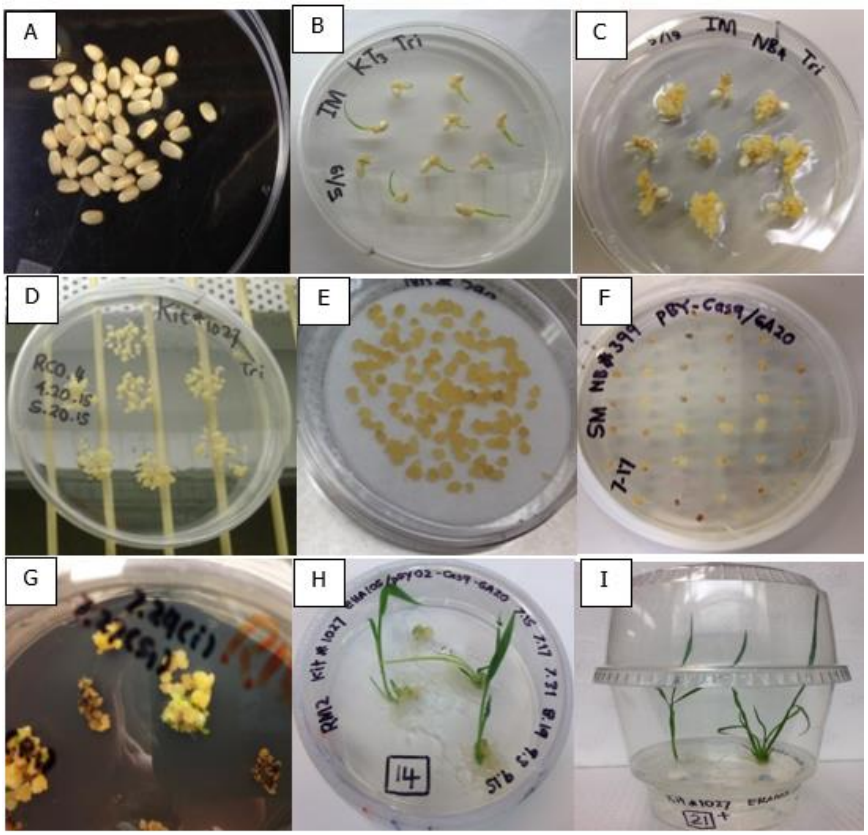

Figure 3. *Agrobacterium*-based rice transformation using mature embryos of rice cv. Kitaake. (A–D) Callus induction steps to produce embryogenic calli. (E) Co-cultivation the calli with *A. tumefaciens* suspension. (F) Selection of resistant callus in hygromycin-containing medium (50 mg/l). (G) Selected callus formed green spots. (H) Regenerated calli. (I) Plantlet in rooting medium.

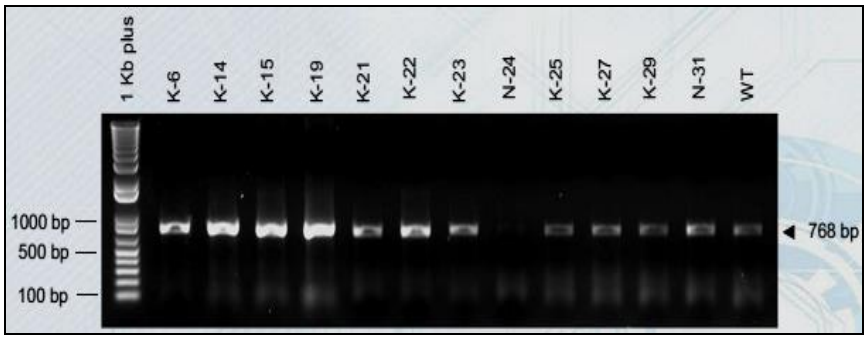

Figure 4. PCR amplification of the specific region in *OsGA20ox-2* gene using a pair of specific primers flanking the mutation target site.

Lines	Nucleotide	Mutation type
WT	AATCTCATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGCCCATGGAC	
K-6	AATCT---GGCGGCCGAACACCCCCCGCCGCCACGGCCGCGCCAACCCCCGAGCCAGGGAC	nd, heterozygous
	AATCTCA----------CCCATGGAC	
K-14	AATCTCA----------CCCATGGAC	- 44, homo-diallelic
K-15	AATCTCAaTGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGaCCCATGG	+ 2, homo-diallelic
K-19	AATCTCA----------CCCATGGAC	-44, homo-diallelic
K-21	AATCTCATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGCCCATGGAC	No mutation
K-22	AATCTCA----------CCCATGGAC	-44, homo-diallelic
K-23	TCTCTCG----------CCCGTGTC----------TGGCC	nd, heterozygous
K-25	AATCTCA----------CCCATGGAC	-44, homo-diallelic
K-27	AATCTCAATGGTGGACGAGCACCCCACGCCAGCACAGGCGCACCAACCACCGCGCCCGCG	nd, heterozygous
	AATCTCA----------CCCATGGAC	
K-29	AATCTCA----------CCCATGGAC	-44, homo-diallelic

Figure 5. CRISPR/Cas9-gRNA-induced mutations at the target sites of *OsGA20ox-2* from 10 transgenic lines. Various mutations combinations were found in selected T_0 rice plants. Nucleotide in red represent the gRNA1 and gRNA2 and green underlined nucleotides indicate PAM. Dashes for deletion and blue lowercase letter for insertion.

Half of the mutated lines had alterations in both OsGA20ox-2 gRNA and resulted in homo-diallelic mutation type with 44 nt deletion, while 3 lines were heterozygous, one line was homo-diallelic with 2 nt insertion, and one line had no mutation (Figure 5). These results indicated that CRISPR/Cas9 technology could generate transgenic rice lines with mutated gene of interest easily with high efficiency in the T_0 generation. The high efficiency of targeted mutagenesis in this study is possibly due to the unique characteristics of the CRISPR/Cas9 system. The specificity of CRISPR/Cas9 is not affected by DNA methylation of target gene sequences [21]. The CRISPR/Cas9 technology is advantageous for gene modification in plants with high GC content in their genome such as rice. Hsu et al. (2014) proposed that the high mutation rate was also affected by the GC content of the target gene. We observed that *OsGA20ox-2* gene has 56.9% GC content, which might lead to the high mutagenesis frequency of *OsGA20ox-2* gene.

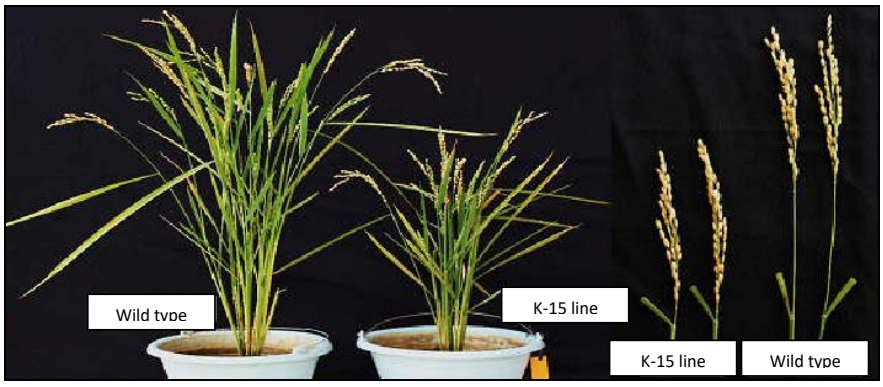

Figure 6. Comparison of CRISPR/Cas9 mutant line K-15 and wild type cv. Kitaake in plant height and panicle length characters.

The results also revealed that targeted mutagenesis of *OsGA20ox-2* with dual gRNA can be generated with high efficiency. Dual gRNA construct could efficiently generate targeted mutagenesis since all mutated lines experienced mutation in targeted sites for the dual of gRNA-GA20ox-2. In this experiment, we adopted a strategy to enhance the efficiency of mutagenesis for genome editing. We designed and constructed two gRNAs targeting *OsGA20ox-2* gene to increase the success rate or

improve the possibility that at least one gRNA will be active for mutagenesis [10]. Another important utility of 2-gRNAs-for-1-gene approach is to enable large deletion mutations in the targeted gene.

In our experiment, homozygous mutations were found in the T_0 plants, accounting for 60% of all T_0 plants (Table 1). It was also observed that half of the homozygous mutations were deletion and all deletions were 44 bp in length (Figure 5). The deletion was caused by the cleavage of double-stranded DNA by Cas9 at a position three base pairs upstream of the PAM sequence from each of gRNA. Homozygous insertion mutation occurred in one plant (K-15) with 2 bp insertion. This result revealed the tendency that the targets showing higher mutation rates were more likely to have homozygous mutations at T_0 [22].

Table 1. Agronomic characters observed on CRISPR/Cas9-T_1 mutant rice lines.

Line	Indel (T_0)	Plant height (cm)	Number of tiller	Exsertion	Leaf flag length (cm)	Length panicle (cm)	Number of fertile seed	Number of sterile seed
WT	Control	82.48	10.50	19.93	36.61	12.74	39.96	4.14
K6.1	Hetero-diallelic	67.94	5.00	20.39	27.04	13.14	46.78	4.22
K14.1 +	44 bp deletion	81.45	9.40	21.55	28.59	12.59	46.10	4.23
K15 +	2 bp insertion	54.31	8.27	14.60	21.79	11.48	32.88	3.75
K19.1 +	44 bp deletion	72.89	8.44	18.36	32.85	12.13	39.04	5.53
K21 +	No mutation	76.40	8.00	21.94	30.5	13.35	48.13	4.27
K23	Hetero-diallelic	73.77	7.58	20.26	29.77	13.26	45.90	3.62
K25.1	44 bp deletion	66.41	5.88	19.77	25.90	12.13	36.24	4.57
K27.1 +	Hetero-diallelic	65.03	6.50	17.02	23.99	11.31	28.00	5.67
K29	44 bp deletion	82.07	8.79	16.09	30.33	15.50	28.66	19.78
N24.2	No mutation	75.72	14.08	15.29	28.57	15.23	37.23	13.8
N31.1	44 bp deletion	69.86	8.85	16.09	30.33	15.50	28.66	19.78

WT = wild type.

To evaluate their phenotypic characters, nine T_1 mutant lines were planted in a greenhouse. Observations indicated that the mutant lines had agronomic characters that were almost similar with the wild type. However, there was one line that showed significant differences from the wild type, i.e. mutant line K-15, especially for plant height and flag leaf length characters (Table 1, Figure 6). It proved that knocking out *OsGA20ox-2* resulted in a semi-dwarf phenotype that was indicated by shorter leaves and stems, which is similar to results found by another group [17]. Based on this result, we summarized that CRISPR/Cas9 system was highly efficient for genome editing in rice. Homozygous mutant alleles were readily found in T_0 plants. Similar result was also obtained by Zhang et al. [22]. This finding confirms CRISPR/Cas9 system as a technology for inducing rapid and accurate modifications in plant genome, thus reducing the breeding time when compared with conventional breeding or genetic engineering. This study also proves that the CRISPR/Cas9 system is a powerful tool in rice improvement through targeted gene editing.

4. Conclusions

Targeted mutagenesis of *OsGA20ox-2* gene using CRISPR/Cas9 generated nine mutated rice lines with a mutation frequency of 90%. Half of the mutated lines had alterations in both OsGA20ox-2 gRNAs and resulted in homo-diallelic mutation type with 44 bp deletion, while 3 lines were heterozygous, one line was homo-diallelic with 2 bp insertion, and one line had no mutation. The K15

mutated rice line was identified as a homozygous two-nucleotide insertion and had the semi-dwarf phenotype.

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