OsGTL1 promoter editing using CRISPR/Cas9 in rice *Oryza sativa* L.

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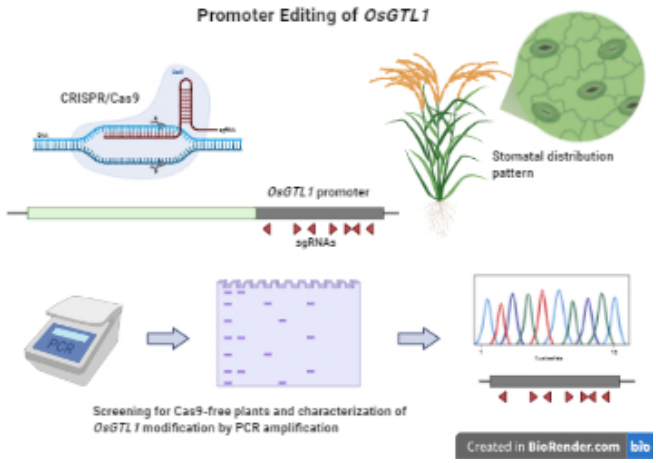
Abstract: *GT2-LIKE1* (*GTL1*) gene is a negative regulator of stomatal development. It regulates stomatal number in plants. The CRISPR/Cas9 system has been used to modify the *OsGTL1* promoter. This research aims to screen *Cas9*-free rice with *OsGTL1* promoter modification. *Cas9* specific primers were designed for *Cas9* screening in all tillers of 8 T_3 rice lines. Only a single T_3 line is the Cas9-free in every tiller, while 3 out of 8 lines had Cas9 in all tillers. Seeds from Cas9-free tillers could be obtained from 5 independent lines. No significant difference in leaf greenness, tiller number per plant and leaf number per plant among the modified plants and wild type (WT). However, 7 out of 8 modified lines were the significantly smaller than WT. The nucleotide sequences of *OsGTL1* promoter in some Cas9-free plants revealed the modification in *OsGTL1* promoter, which included small deletion, insertion, and big deletion in the target region.

Graphical abstract:



Keywords: CRISPR/Cas9; *OsGTL1*; *Oryza sativa* L.

1. Introduction

Drought is one of the most common events associated with climate change. Increasing water use efficiency is supposed to be advantageous under very dry climates and severe drought conditions [1]. Plants have created adaptation mechanism at the morphological, physiological, and biochemical levels to let them escape or adapt to the drought condition. Numerous independent studies have revealed that drought stress can reduce crop yields by involving several factors such as signaling, transcription factors, hormones and secondary metabolites [2].

In crop plants, especially rice, drought can be mitigated by altering stomatal density, allowing the balance control between carbon acquisition and water loss. Several molecular machines play a role in the regulation of stomatal development, such as transcription factors, plasma membrane-related proteins, and intercellular and extracellular signaling molecules [3, 4]. Stomata aperture changes in response to environmental factors and internal signals regulating CO_2 uptake through leaves and water loss through transpiration. It can control the gas exchange between the interior of the leaf and the external environment. The regulation of the gas flux inside and outside the leaf is very important to maintain a suitable leaf temperature and maintain the water status in the plant [5].

The identification of *GT2-LIKE 1* (*GTL1*) in Arabidopsis, which is a transcriptional repressor of *Stomatal Density and Distribution 1* (*SDD1*) indicating that *GTL1* directly interacts with the *SDD1* promoter regulating stomatal density, transpiration, and water use efficiency (WUE). Loss of *GTL1* function results in reduced transpiration with similar biomass accumulation and net CO_2 assimilation, which improves integrated and instant WUE, leading to drought tolerance [6]. Some studies reported that it was unclear how *GTL1* regulated the stomatal development in monocots and the consequences of altered stomatal density due to drought stress [7, 8]. Therefore, it is worth conducting further research to reveal this gene functions in monocots species such as rice.

CRISPR/Cas9 system considered as one of the most advanced technology to generate the modification. This system has become popular in genome editing due to its simplicity, affordability, and efficiency [9]. The instantaneous targeting of multiple genes can yield more than one trait that is better in plants, and can also be used in basic research to infer the role of individual genes in complex networks [10]. This technology also can work efficiently to generate targeted gene mutation and correction in plants. They showed that the engineered CRISPR/Cas9 could create transient expression in Arabidopsis protoplasts and stable expression in transgenic Arabidopsis and rice plants. The results reveal the possibility of using engineered CRISPR/Cas9 as molecular scissors to modify the targeted genome by creating double strand break at specific sites in both dicot and monocot species [11].

The CRISPR/Cas9 system enables precise editing of the genome of the model plant *Arabidopsis thaliana* and likely of any other organisms [12]. There are some concerns about the existence of the Cas9 in the modified plants as it is difficult to determine whether the T_2 generation mutation comes from the mutation in the T_1 generation or newly produced in the T_2 generation. Moreover, the continued existence of the CRISPR/Cas9 construct in the mutants critically increases the risk of generating off-target mutations [13]. Therefore, after genome editing, eliminating the transgene is essential to deliver the gene-edited plants with no recombinant gene-editing machinery [14].

The CRISPR/Cas9 system has been used in the previous study to modify the *OsGTL1* promoter [15]. Screening the transgenic Cas9-free plants is very important to select the stable CRISPR-edited plants before evaluating *OsGTL1* promoter-edited effects. Characterization of the *OsGTL1* promoter modification as stable edited

rice will lead us to understand the function of this gene. The objectives of this research are to screen the presence of *Cas9* in the transgenic rice and characterize the modification in *OsGTL1* promoter in *Cas9*-free plants.

2. Materials and Methods

2.1 Plant Materials

The Cas9-free plants were screened from 8 T_3 transgenic rice lines created by Hungsapruk (2018). The wild type (WT) rice (*Oryza sativa* L. cv. 'Kitaake') was used as the negative control. At least five seeds from each transgenic line were germinated for seven days and transferred for further growth in the plastic pots containing clay soil. All the transgenic plants were grown in the transgenic greenhouse at the Department of Botany, Faculty of Science, Chulalongkorn University under normal growth conditions (28-35°C) and 60% relative humidity with an appropriate supply of water [16] and fertilizer.

2.2 Phenotypic Observation

Phenotypic parameters of the modified plants, which were leaf greenness, number of leaves, number of tillers, and plant height were collected. Leaf greenness of the youngest fully expanded leaf and the oldest leaf were measured by SPAD chlorophyll meter (SPAD 502 Plus, Konica Minolta, Japan) in the main tiller of four-week-old plants. Leaf number and tiller number were determined in weekly basis. Plant height was measured from soil level to the highest leaf tip at tillering stage.

Table 2. Primer sequences and PCR condition for Cas9 screening and *OsGTL1* amplification

Primers	Sequences	Product size	PCR Condition
Cas9-Fw	TCCTGCAGACAGTGAAGGTG	532 bp	Pre-denaturation: 95˚C for 3 min
Cas9-Rv	GCCTTATCCAGTTCGCTCAG		Denaturation: 95˚C for 30 sec
			Annealing: 57˚C for 30 sec
			Extension: 72˚C for 40 sec
			Final extension: 72˚C for 5 min
			Hold: 12˚C
			Cycle: 35 Cycles
Cas9-Fw (Full)	CTTACCCTATCTGTTTGGTG	4445 bp	Pre-denaturation: 95˚C for 3 min
			Denaturation: 95˚C for 30 sec
Cas9-Rv (Full)	CGCTGTTATCAACCACTTTG		Annealing: 56,5˚C for 30 sec
			Extension: 72˚C for 4,45 min
			Final extension: 72˚C for 15 min
			Hold: 12˚C
			Cycle: 35 Cycles
GTL1-Fw	GCTTGAAGGAGATGGAGAGC	1684 bp	Pre-denaturation: 95˚C for 3 min
GTL1-Rv	GTATAAAGCGAAAGCGTGTG		Denaturation: 95˚C for 30 sec
			Annealing: 59˚C for 30 sec
			Extension: 72˚C for 2 min
			Final extension: 72˚C for 10 min
			Hold: 12˚C
			Cycle: 40 Cycles

2.3 Screening Cas9-free plants

For Cas9-free transgenic plant screening, the young leaves from each tiller of T_3 plants were collected for DNA extraction. The genomic DNA was isolated by using the genomic DNA GENEAID Kit (Geneaid Biotech Ltd, Taiwan). *Cas9* specific primers (Table 1) were designed from pRGEB32 plasmid sequences. The primers were used to amplify Cas9 fragment by using the plant genomic DNA as a template. Wild type (WT) genomic DNA and pRGEB32 vector were used as negative and positive controls for the amplification, respectively. The existence of the *Cas9* gene was monitored by using gel electrophoresis.

2.4 Determination of OsGTL1 promoter modification in Cas9-free transgenic lines

The specific primers for the *OsGTL1* (*LOC_Os03g02240*) promoter (Table 1) were designed based on the sequence obtained from the Rice Genome Annotation Project (MSU) database by using Snap gene software. *OsGTL1* promoter-specific primers were used to amplify the *OsGTL1* promoter fragment from the Cas-9 free genomic DNA. The amplified fragments size was determined by using gel electrophoresis and the DNA fragment were purified by using TIANGEN Purification Kit (Beijing, China). The fragments with different sizes from each selected Cas9-free transgenic lines were selected for sequencing. The sequence information was compared to the DNA sequence in the Rice Genome Annotation Project (MSU) database to determine the modification occurring in the *OsGTL1* promoter.

3. Results

3.1 Screening Cas9-free plants

Based on 8 transgenic lines, 63 plants with 215 tillers were investigated (Table 2). Transgenic plant DNA was extracted from leaf tissue in each tiller. Five seeds from 1A5 plant were grown, indicated as 1A5A-1, 1A5A-2, 1A5A-3, 1A5A-4 and 1A5A-5. Only one tiller from 1A5A-1 and 1A5A-2 were Cas9-free (Figure 1A). In 7B4 line, all progenies, 7B4B-1 to 7B4B-7 contained Cas9 in every tiller (Figure 1B). The Cas9-free plants, meaning that every tiller in the plant was Cas9-free, were detected in 4 lines, 2B3, 2B7, 20B9 and 20B13. All of 20B9Bs were Cas9-free, while 20B13B line showed that some plants, 20B13B1 and 20B13B2 were Cas9-free plants, but others 20B13Bs were not (Figure 1D). The summary of the number of Cas9- free plants and tillers were shown in Table 2.

3.2 OsGTL1 promoter modification in Cas9-free transgenic lines

In this case, we targeted the promoter region using seven sgRNA as shown in Figure2. The DNA of all 80 Cas9-free plants were used to amplify the *GTL1* promoter region from each sample. Based on the gel electrophoresis, the amplified fragments showed difference in sizes, approximately ranging from 1 to 1.5 kb.

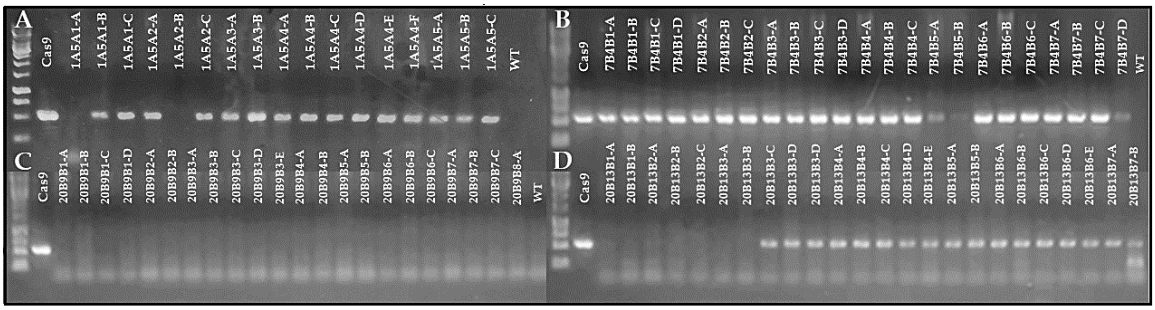


Figure 1. Gel image for screening of Cas9-free tillers by using polymerase chain reaction (PCR). Several samples showed no specific band which means those were Cas9-free. pRGEB32 which contains Cas9 was used as positive control and the wild-type sample was also used as a negative control.

Table 2. The number of Cas9-free plants which were obtained from T_3 seeds.

Lines	Number of tested plants	Cas9-free Plants		Number of tested tillers	Cas9-free Tillers		
		Number	%		Number	% of all	% per plant
1A5	5	0	0	16	2	12.5	13.3
1A14	7	0	0	16	0	0	0
2B3	8	2	25	36	15	41.7	32.5
2B7	9	7	77.8	32	29	90.6	90.7
7B3	9	0	0	32	0	0	0
7B4	7	0	0	22	0	0	0
20B9	9	9	100	27	27	100	100
20B13	9	2	22.2	34	7	20.6	27.8
Total	**63**	**20**	**31.75**	**215**	**80**	**37.21**	

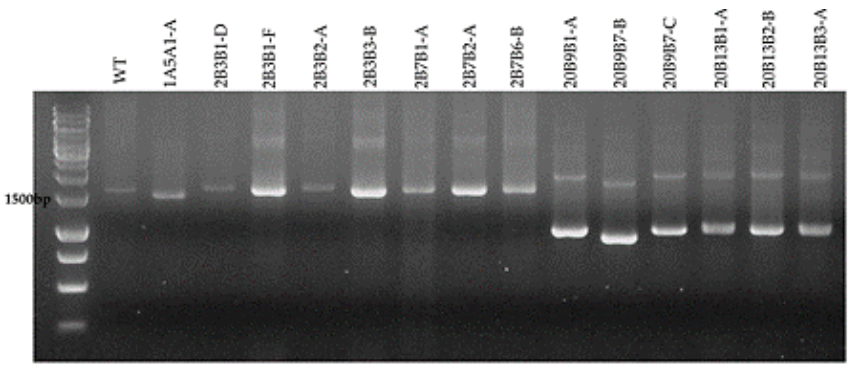


Figure 2. Gel image of GTL1 promoter amplification by polymerase chain reaction (PCR) from DNA samples. The expected band size from wild-type (WT) is 1684 bp.

The PCR product with different sizes from each line in Figure 2 was selected for sequencing. The sequencing result was analyzed and aligned with the wild-type sequence from the Rice Genome Annotation Project (MSU) database as a reference to verify the modification occurring in each selected line. Based on sequence analysis, the modification on *OsGTL1* promoter included small deletion, insertion, big deletion, and combination between insertion and deletion at the same time. Line no. 1A5A1-A (from plant no.1A5, seeds no.1, tiller no.1) showed 36 bp deletion between gRNA no. 2 and 3 which worked together and cut the DNA perfectly at 3-4 base upstream of PAM sites. It also occurred in gRNA no. 5 and 6 which caused 29 bp deletion. We also found small deletion and insertion nearby the other gRNAs. Line no. 20B9B1-A and 20B13B2-B showed big deletion between gRNAs no. 1 to 4 which resulted in 564 bp and 563 bp deletion in both lines, respectively. Line no. 20B13B2-B also showed an additional 25 bp and 35 bp deletion between gRNAs no. 6 and 7. In contrast, Line no. 2B3B2-A and 2B7B1-A showed only small deletion and insertion in several regions which not close to the PAM sites (Figure 3). It can be assumed that the efficiency of the gRNAs was different. The sgRNA can work independently or worked together to modify the plant genome.

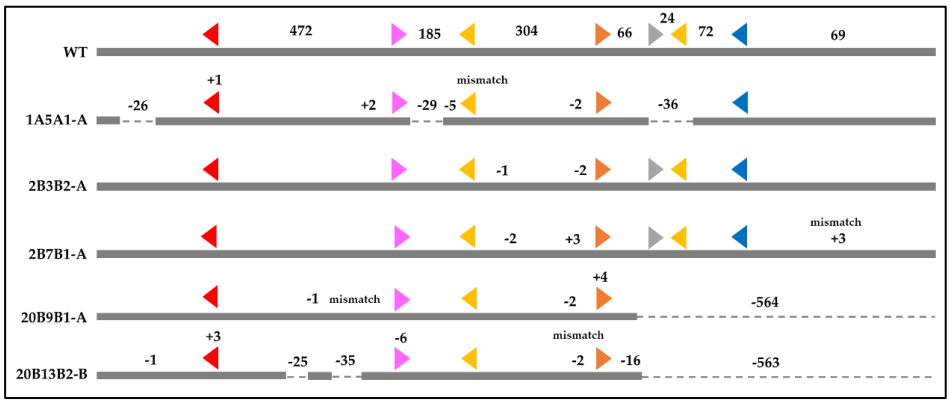


Figure 3. Diagram of the modification in *GTL1* alleles (T_3 generation). There are different types of modification on the GTL1 promoter including insertion (+), deletion (-), big deletion (dash line), and mismatch of the base. The triangles with different colors represent gRNAs no.1-7 from right to the left-hand side. The number between the triangle showed the distance between gRNAs in base pairs.

3.3 OsGTL1 promoter modification resulted in changes in plant height.

The phenotypes of the modified plants, which were leaf greenness, leaf number per plant, tiller number per plant and plant height at tillering stage were investigated. No significant changes in leaf greenness, leaf number per plant and tiller number per plant were detected. However, 7 out of 8 lines of the transgenic plants became smaller than WT (Figure 4). Only 2B3 line had the similar plant height to WT. Other lines became smaller than WT after 3 weeks of growth.

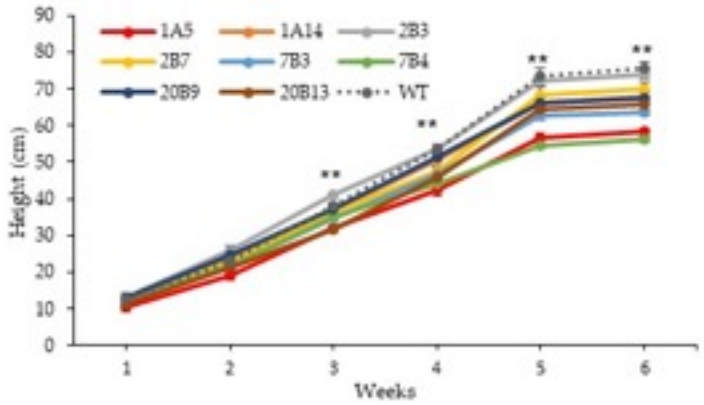


Figure 4. Plant height of transgenic lines and wild type (WT) at tillering stage. The data were presented as the mean ± SE with significantly different data ($p < 0.01$) shown by **.

4. Discussion

This research reveals the successful design of 7 sgRNAs to modify *OsGTL1* promoter. Screening of the Cas9-free transgenic plants is necessary to ensure that the CRISPR/Cas9 construct has been segregated out from the genome [14]. In addition, besides increasing the risk of off-target mutation, the existence of the CRISPR/Cas9 construct genome can make it difficult to distinguish whether the mutations that occur are inherited from previous generations or are produced in new generations [13]. It was clearly shown by our data that the tillers from the same plant could be free of Cas9 or contain it in the genome (Figure 1). This suggested the chimera could

occur in the embryo. We, later, confirmed that the seedlings, which were the progeny from the Cas9-free tiller were Cas9-free.

Variability of CRISPR/Cas9 efficiency must be concerned especially when we use more than one sgRNA to modify the gene. In this case, we can observe that several sgRNAs worked together and generated the big deletion or worked independently in the target site. Following several studies that have been conducted before, they obtained two independent alleles by using two sgRNAs [17]. In addition, another research also found out the 193 bp to 240 bp large deletion using two or more sgRNAs [14]. We also identified several small deletions, insertions, and mismatches in the target site (Figure 3). Normally, Cas9 created a double-strand break a few base pairs upstream of the PAM site. The error-prone of nonhomologous end-joining DNA repair can cause small deletion, insertion, or mismatch in the target site [13].

Editing the regulatory region of the gene is considered to be the best strategy to modify the gene function. Recent research showed that editing conserved *cis*-acting elements on a promoter region can create novel *Wx* alleles with fine-tuned amylose levels and improve the grain quality [18].

The editing of several critical nucleotides in regulatory elements might also have an advantage over significant phenotype variations [17]. In this case, we have observed that most of the *OsGTL1* edited plants became smaller. This may be due to the decrease in carbon fixation ability. *GTL1* in the Arabidopsis and Poplar affects the efficiency of the plants that have a lower number of stomata increasing water deficit tolerance by reducing the transpiration rate under drought stress [6, 7]. As *OsGTL1* regulates stomatal formation; therefore, it is important to observe the stomatal number and photosynthesis behavior in the next step.

5. Conclusions

These results demonstrate the variation in the number of Cas9-free plants in T3 plant lines. The nucleotide sequences of the amplified fragments revealed the modification in the *OsGTL1* promoter, which included small deletion, insertion, and big deletion in the target region. The phenotype parameters showed that most of the transgenic lines became smaller that WT at tillering stage. The further investigation on the stomatal number and photosynthesis capacity should be performed in the future.

Author Contributions: Conceptualization, S.C, L.C; methodology, S.C., L.C; validation, W.F.; formal analysis, W.F.; investigation, W.F.; resources, S.C., K.H., P.G.L.; data curation, W.F.; writing—original draft preparation, W.F.; writing—review and editing, S.C.; visualization, W.F., S.C.; supervision, S.C., L.C.; project administration, S.C.; funding acquisition, S.C., L.C. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest in this research.

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