

EFFECT OF *OsgTL1* PROMOTER EDITING ON STOMATAL DISTRIBUTION PATTERN AND  
PHOTOSYNTHESIS IN RICE *Oryza sativa* L.



A Thesis Submitted in Partial Fulfillment of the Requirements  
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ผลของการปรับแต่งโปรโมเตอร์ของยีน *OsGTL1* ที่มีต่อรูปแบบการกระจายของปากใบและการ  
สังเคราะห์ด้วยแสงในข้าว *Oryza sativa* L.



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต  
สาขาวิชาพฤกษศาสตร์ ภาควิชาพฤกษศาสตร์  
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย  
ปีการศึกษา 2564  
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย



วิทยา ฟาจาโรนี : ผลของการปรับแต่งโปรโมเตอร์ของยีน *OsGTL1* ที่มีต่อรูปแบบการกระจายของปากใบและการสังเคราะห์ด้วยแสงในข้าว *Oryza sativa* L.. ( EFFECT OF *OsGTL1* PROMOTER EDITING ON STOMATAL DISTRIBUTION PATTERN AND PHOTOSYNTHESIS IN RICE *Oryza sativa* L. ) อ.ที่ปรึกษาหลัก : ศุภจิตรา ชัชวาลย์

ยีน *GT2-LIKE1 (GTL1)* เป็นตัวควบคุมเชิงลบในการพัฒนาปากใบ ซึ่งทำหน้าที่ควบคุมจำนวนปากใบในพืช งานวิจัยที่ผ่านมาได้มีการใช้ระบบ CRISPR/Cas9 เพื่อตัดแปรโปรโมเตอร์ของยีน *OsGTL1* งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อคัดกรองข้าวที่ได้รับการตัดแปรโปรโมเตอร์ *OsGTL1* และปราศจากยีน *Cas9* เพื่อตรวจสอบผลของการตัดแปรดังกล่าวต่อรูปแบบการกระจายของปากใบและกระบวนการสังเคราะห์ด้วยแสง ในการคัดกรองต้นข้าวที่ปราศจากยีน *Cas-9* ทำโดยการออกแบบไพรเมอร์ที่จำเพาะต่อ *Cas9* เพื่อใช้คัดกรองการคงอยู่ของยีน *Cas-9* ในทุกกอของข้าวในชั่วรุ่นที่ 3 จำนวน 8 สายพันธุ์ ซึ่งพบว่ามีเพียงสายพันธุ์เดียวเท่านั้นที่ปราศจาก *Cas9* ในทุกกอของต้น จากการหาลำดับนิวคลีโอไทด์ของโปรโมเตอร์ตัดแปรของยีน *OsGTL1* ในพืชที่ไม่มี *Cas9* พบการตัดแปร ได้แก่ การขาดหายเล็กน้อยของ DNA บางส่วน การแทรกเล็กน้อยของ DNA บางส่วน และการขาดหายของ DNA ชิ้นใหญ่ จากการสังเกตทางกายวิภาค พบว่าข้าวสายพันธุ์ตัดแปร 1A5A1-A, 20B9B1-A และ 20B13B2-B มีความหนาแน่นของปากใบต่ำกว่าและมีขนาดของปากใบเล็กกว่าพันธุ์ปกติซึ่งส่งผลต่อการสังเคราะห์ด้วยแสงที่แสดงด้วยอัตราการสังเคราะห์ด้วยแสงสุทธิ การนำของปากใบ อัตราการคายน้ำ ความเข้มข้นของแก๊สคาร์บอนไดออกไซด์ภายในปากใบ และประสิทธิภาพการใช้น้ำ การตัดแปรที่โปรโมเตอร์ของยีน *OsGTL1* พบว่าเกิดที่บริเวณ cis-element ที่เกี่ยวข้องกับการตอบสนองต่อความเครียด เช่น ABRE, GT1 Box, MYB, MYC, GTGA motif, E-box และ CGCG box ดังนั้น การตัดแปรในบริเวณโปรโมเตอร์ของ *OsGTL1* นี้จึงคาดว่าจะนำไปสู่การเปลี่ยนแปลงในการตอบสนองต่อความเครียด

สาขาวิชา พุทธศาสตร์

ปีการศึกษา 2564

ลายมือชื่อนิสิต .....

ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Widya Fajariani : EFFECT OF *OsGTL1* PROMOTER EDITING ON STOMATAL DISTRIBUTION PATTERN AND PHOTOSYNTHESIS IN RICE *Oryza sativa* L. .

Advisor: Prof. SUPACHITRA CHADCHAWAN, Ph.D.

*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 to investigate the modification effects on the stomatal distribution pattern and the photosynthesis process. *Cas9* specific primers were designed for *Cas9* screening in all tillers of 8 T<sub>3</sub> rice lines. Among them, only a single T<sub>3</sub> line was found to be *Cas9*-free in all tillers. The nucleotide sequences of *OsGTL1* promoter in some *Cas9*-free plants revealed the modification in *OsGTL1* promoter, which included small deletion, small insertion, and large deletion in the target region. Based on the anatomical observation, it was found that 1A5A1-A, 20B9B1-A, and 20B13B2-B edited lines had a lower stomatal density and smaller size than wild-type plants which also affected photosynthesis performance, including net photosynthesis rate, stomatal conductance, transpiration rate, internal CO<sub>2</sub> concentration, and water use efficiency. The *OsGTL1* promoter modification in the critical *cis*-elements involved in stress response, such as ABRE, GT1 Box, MYB, MYC, GTGA motif, E-box, and CGCG box was detected. Therefore, the edited *OsGTL1* promoters are expected to lead to changes in stress response.

Field of Study: Botany

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## CHAPTER I

### INTRODUCTION

Climate change has affected food security mainly through the increasing temperature (Islam & Kieu, 2020). This environmental change has a negative impact on the agricultural system by affecting growth, development, and productivity. Biotic and abiotic stresses can reduce average productivity by 65-87%, depending on crops (Shinozaki et al., 2015). In abiotic stress, primary food production is facing the experience of reduced water availability and increased intensity of drought in the future decades which will lead to the reduction of the critical food crop production including rice, wheat, and maize (Bertolino et al., 2019).

Drought is one of the most common stress-related to climate change. Plants have developed adaptation responses at the morphological, physiological, and biochemical levels to let them escape or adapt to the drought condition. Numerous independent studies have shown that the increase in temperature can reduce crop yields by about 50%. Nevertheless, even slightly drought stress can cause negative effects on crop yield involving several factors such as signalling, transcription factors, hormones, and secondary metabolites (Lamaoui et al., 2018).

In crop plants, especially rice, drought can be mitigated by the alteration of stomatal density, allowing the balance control between carbon acquisition and water loss. A series of molecular machinery plays a role in the regulation of stomatal development such as transcription factors, plasma membrane-associated proteins, and intercellular and extracellular signalling molecules (Buckley et al., 2019; Caine et al., 2019). The alteration of stomatal aperture in response to the environmental factors and internal signals regulate CO<sub>2</sub> uptake through the leaf for photosynthesis and water loss *via* transpiration. It can control gas exchange between the leaf interior and the external environment. Regulation of gas fluxes inside and outside the leaf is essential to maintain appropriate leaf temperature and conserve water status in the plant (Lawson et al., 2019).

According to Yoo et al. (2010), 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 the reduction of transpiration with similar biomass accumulation and net CO<sub>2</sub> assimilation, which improves integrated and instant WUE, leading to drought tolerance. They revealed that *GTL1* orthologs in monocots positively regulate stomatal density, transpiration and may play a role in regulating WUE. Research by Weng et al. (2012) and Yoo et al. (2011) reported that it was unclear how *GTL1* regulated the stomatal development in monocots and the consequences of altered stomatal density due to drought stress. Therefore, it is worth conducting further research to reveal these gene functions in monocots species such as rice.

One of the most advanced technology to generate the modification in the plant genome is the CRISPR/Cas9 system. CRISPR/Cas9 was firstly used in plants in 2013. This system has become popular in genome editing due to its simplicity, affordability, and efficiency (Stephens & Barakate, 2017). Instantaneous targeting of multiple genes can result in more than one improved trait in crops, and can also be used in basic research to infer the role of each gene in a complex network (Malzahn et al., 2017). According to Feng et al. (2013), they discover that the CRISPR/Cas9 system 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 breaks at specific sites in both dicot and monocot species. CRISPR/Cas9 rapidly moves as a viability study, promoting a reverse genetics revolution in plant research, and producing many desirable traits in major crops. Some of the experiments also showed that CRISPR/Cas9 had been successfully used for functional studies on rice genes (Malzahn et al., 2017).

The CRISPR/Cas9 system enables precise editing of the genome of the model plant, *Arabidopsis thaliana*, and likely of any other organisms (Hahn et al., 2017).

However, most of the reported analyses showed that the CRISPR/Cas9 did not directly segregate out from the plant genome. In this case, there are some concerns about the existence of the Cas9 of the mutants as it is difficult to determine whether the T<sub>2</sub> generation mutation comes from the mutation in the T<sub>1</sub> generation or newly produced in the T<sub>2</sub> generation. Moreover, the continued existence of the CRISPR/Cas9 construct in the mutants critically increases the risk of generating off-target mutations (Gao, Chen, Dai, Zhang, & Zhao, 2016). Therefore, the elimination of the transgene after genome editing is important to deliver the gene-edited plants with no recombinant gene-editing machinery (Aliaga-Franco et al., 2019).

The CRISPR/Cas9 system has been used in the previous study to modify the *OsGTL1* promoter (Hungsaprug, 2018). Screening the transgenic Cas9-free plants is very important to select the stable CRISPR-edited plants before the evaluation of *OsGTL1* promoter-edited effects. Characterization of the stable *OsGTL1* promoter modification will lead us to gain more understanding of the function of this gene. The results of this study will provide new information on the role of *OsGTL1* in the regulation of stomatal density and its effect on photosynthesis behavior, which will be beneficial for drought-tolerant rice cultivar development in the future.

The objectives of this study are:

1. To screen the presence of Cas9 in the transgenic rice genome and characterize the modification in *OsGTL1* promoter in the Cas9-free plants.
2. To investigate the stomatal distribution pattern and photosynthesis process of the Cas9-free rice plants with edited *OsGTL1* promoter.

## CHAPTER II

### LITERATURE REVIEW

#### 2.1 Climate change affects crop production and food security

Many crops have annual cycle and yield range according to weather conditions, notably rainfall and temperature. Maintaining food supply sustainability for seasonal production is challenging. Drought and flood create a special threat to food security, as they can result in both long-term and short-term crop losses. Changes in rainfall volume and timing throughout the season, as well as increased weather variability, are predicted to make local food systems more vulnerable (Al et al., 2008). Rice, wheat, and maize are the world's three major food crops, providing more than 42 percent of all calories consumed by the whole human population. Environmental changes, such as drought can have an impact on rice production (Bouman et al., 2013).

Climate change and the associated increase in atmospheric CO<sub>2</sub> concentration have a significant economic and social influence on rice production either now or in the future (Kumar et al., 2017). Reduced clean water supplies and increasing drought in the agriculture sector may threaten long-term global crop output. As a result, developing crops with higher water use efficiency (WUE), biomass production, or yield per unit of water consumed have become a global priority (Yoo et al., 2009).

#### 2.2 Drought stress

Plants are subjected to environmental stress regularly, which has a negative impact on their growth, development, and productivity. Stresses are caused by two conditions; abiotic stress is generated by environmental circumstances such as drought, salinity, heat or low temperatures, insufficient light, and phytotoxic chemicals that can harm plants, while biotic stress is triggered by other species (Shinozaki et al., 2015). When soil and atmospheric humidity are low and the ambient air temperature is high, drought stress occurs (Lamaoui et al., 2018). The cause of this condition is an imbalance between evapotranspiration flow and soil water intake (Lipiec et al., 2013).

Drought stress has a negative impact on photosynthetic process. The severity of the negative consequences is determined by the drought stress's intensity, duration, and rate of progression (Chaves et al., 2009). Many components of photosynthetic metabolism are temperature sensitive; therefore, starting with the photosynthetic machinery within a leaf is a sensible place to start when considering the impacts of temperature on crop photosynthesis. On a biochemical level, Rubisco efficiency and activation, as well as ribulose biphosphate (RuBP) regeneration, play a big role in net photosynthetic carbon assimilation ( $A$ ). The primary determinant is changed by  $\text{CO}_2$  in the chloroplasts. RuBP regeneration limits  $A$  at elevated  $\text{CO}_2$ , but Rubisco performance limits  $A$  at ambient and sub-ambient  $\text{CO}_2$ . PSII function can be inhibited by high-temperature enzyme breakdown, which lowers electron transport rates, inhibits Rubisco activase (Rca), and lowers chlorophyll content (Moore et al., 2021).

Abiotic stress, such as heat and drought, is frequently controlled by several genes, and the underlying mechanisms are more complex than biotic stress, which is typically characterized by monogenic resistance. Furthermore, various abiotic and/or biotic stimuli frequently have additive effects on heat and drought responses, making the study even more difficult (Lamaoui et al., 2018). Stress-related changes in metabolism and development are widely linked to altered gene expression patterns. When a plant detects stress at the cellular level, a stress response is triggered. Stress recognition activates signal transduction pathways, which transmit information from individual cells to the entire plant (Shinozaki et al., 2015).

Water use efficiency varies significantly between species and genotypes. Therefore, great efforts are now underway to identify what genetic factors influence WUE. Researchers are working hard to figure out what genetic factors influence WUE. Currently, genotypes in the primary gene pool show allelic variation for WUE *via* mechanisms that regulate transpiration, which is the flow of water *via* the stomata, cuticle, and boundary layer. Due to the differences in the characteristics of water and carbon dioxide ( $\text{CO}_2$ ) diffusion through this pathway, it is possible to raise WUE without affecting  $\text{CO}_2$  uptake by reducing transpiration. Since  $\text{CO}_2$  uptake and water loss primarily occur through the stomatal pores, it showed the genes involved in

stomatal development and stomatal opening/closure have an impact on WUE (Yoo et al., 2009).

### 2.3 Stomatal development and the role of *OsGTL1*

Plants acquire developmental adaptability to adjust their photosynthetic and water loss capacity as new leaves form. They can maximize fitness and survive in a variety of environments due to their developmental plasticity. In this adaptive process, stomata play a critical function. The gas exchange between the plant and its surroundings is controlled by small pores in the epidermal leaves. Stomata development is controlled through cell differentiation which results in appropriate stomatal density and spacing to enable efficient gas exchange. A complex signal cascade involving extracellular ligand-receptor interactions modulating the activity of three master transcription factors required for the development of stomata, which are SPEECHLESS (SPCH), MUTE, and FAMA (Zoulias et al., 2018). Changes in photosynthetic mesophyll tissues are caused by altered stomatal development caused by misexpression of *SPCH*, *EPF*, or *TMM* (Zhu et al., 2020).

Plants decrease transpirational water loss in response to water deficits by reducing stomatal aperture as a rapid plant response and repress stomatal development as a longer-term response, leading to reduced water loss and improved water-use efficiency (WUE) and drought tolerance (Franks et al., 2015; Yoo et al., 2009). The stomatal developmental process is subdivided into three cell division steps followed by four-cell differentiation: Meristemoids Mother Cells (MMCs) to meristemoids, meristemoids to Guard Mother Cells (GMCs), GMCs to Guard Cells (GCs), and Subsidiary Mother Cells (SMCs) to Subsidiary Cells (SCs), which are controlled by different transcription factors. Three cell division steps include entry division, SMC asymmetric division, and GMC symmetric division (Wu et al., 2019).

*GT2-LIKE 1 (GTL1)* is one of the genes involved in stomatal development. *STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1)*, a negative regulator of stomatal density, is transcriptionally repressed by *GTL1*. *GTL1* regulates stomatal density, transpiration, and water use efficiency (WUE) by direct interaction with the

*SDD1* promoter (Yoo et al., 2011). Loss-of-function of *GTL1* in Arabidopsis mutation results in enhanced water deficit tolerance and greater integrated WUE by reducing daytime transpiration without a clear decrease in biomass accumulation (Yoo et al., 2010). They also identified *GTL1* orthologs that are similar to Arabidopsis in poplar (Pt01s45870), cucumber (Cs193210), grape (Vv01019722001), and peach (Pp001704). Interestingly, monocot plants such as rice, maize, and sorghum also have *GTL1* orthologs that are more similar to *AtGTL1* than *AtGT2* or *AtGTL2*.

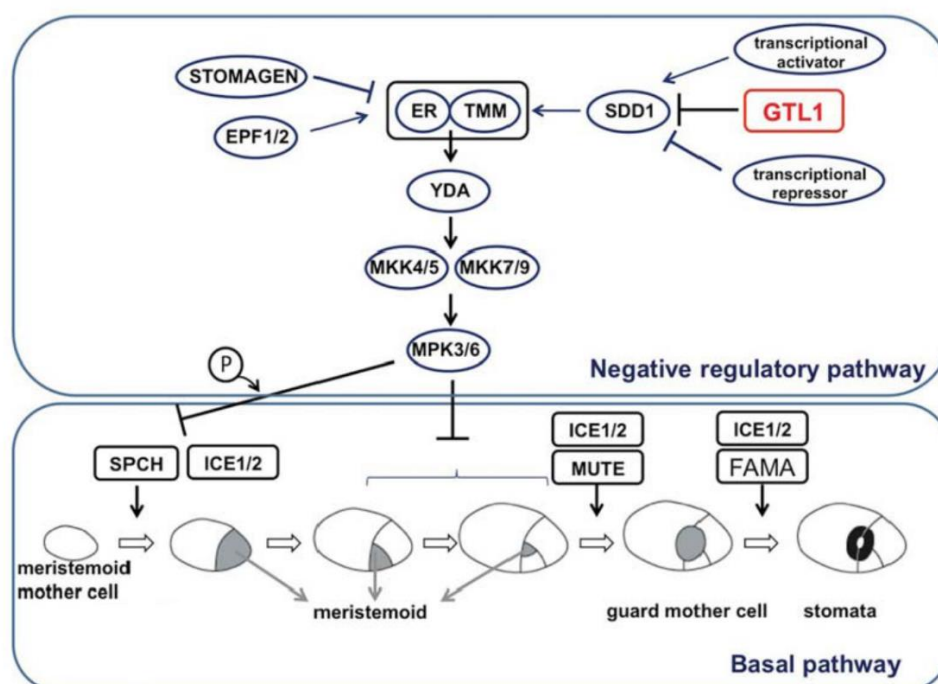


Figure 1. Diagram of the role of *GTL1* for the stomatal development pathway in Arabidopsis (Yoo et al., 2010).

Calmodulin (CaM), a calcium ( $\text{Ca}^{2+}$ ) sensor protein, decodes calcium  $\text{Ca}^{2+}$  signals and transmits them to  $\text{Ca}^{2+}$ /CaM-binding transcription factors, which directly regulate gene expression required for plant adaptation responses. The molecular mechanisms underlying  $\text{Ca}^{2+}$ /CaM signal transduction processes, as well as their functional importance, are still unknown. According to Yoo et al. (2019), they found out novel  $\text{Ca}^{2+}$ /CaM signal transduction mechanism for repressing stomatal growth in response to water stress by allosterically regulating the DNA-binding activity of *GTL1*. The interaction of  $\text{Ca}^{2+}$ /CaM with the second helix of the *GTL1* N-terminal trihelix DNA-binding domain (GTL1N) destabilizes a hydrophobic core of GTL1N and allosterically

inhibits third helix docking to the *SDD1* promoter, resulting in osmotic stress-induced  $\text{Ca}^{2+}/\text{CaM}$ -dependent activation (de-repression) of *SDD1* expression.

In *Arabidopsis*, hyperosmotic stress causes a reduction in stomatal development. In line with previous observations, they found that water deficit inhibited stomatal development, as demonstrated by a decrease in the stomatal index (Figure 2). *GTL1* is essential for transducing the signal from osmotic stress to stomatal development modulation. They demonstrated that the  $\text{Ca}^{2+}/\text{CaM}$ -*GTL1*-*SDD1* modular relay system functioned in water-deficit sensing (such as turgor decrease or low water potential). To promote stomatal development under well-watered conditions, *GTL1* is required. The  $\text{Ca}^{2+}/\text{CaM}$ -*GTL1*-*SDD1* module appears to be a transcriptional switch that allows plants to adapt to low water supply by suppressing stomatal development (Figure 2) (Yoo et al., 2019).

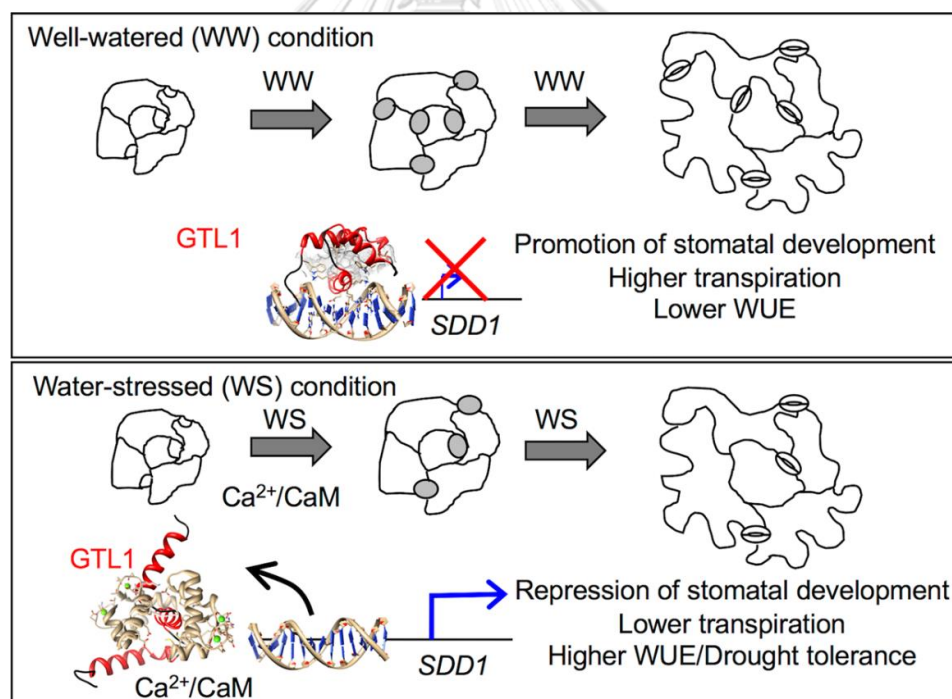


Figure 2. A proposed model for a  $\text{Ca}^{2+}/\text{CaM}$ -regulated transcriptional switch to repress stomatal development and improve water use efficiency and drought tolerance in *GTL1* (Yoo et al., 2019).

## 2.4 CRISPR/Cas9 Technologies

Using site-specific custom nucleases, it is now possible to change plant genomes by introducing double-strand breaks (DSBs) at a locus of interest using the advanced CRISPR/Cas system. DSBs are lethal to cells and must be fixed by plant internal DNA break repair processes such as homologous recombination (HR) or non-homologous end-joining (NHEJ) (Hahn et al., 2017). The CRISPR/Cas9 system, which uses the bacterial Cas9 nuclease from *Streptococcus pyogenes* and a single guide RNA (sgRNA) molecule to direct the nuclease to its specific target has greatly reduced the effort required to create DSBs at specific loci in living cells' genomes (Doudna & Charpentier, 2014; Jinek et al., 2012). The Cas9 system can make DSBs in almost any gene, and even multiple genes at the same time. It has already proven to be effective in a variety of model and agricultural plant species (Bortesi & Fischer, 2015).

CRISPR-Cas9 has been utilized to improve yield, quality, and nutritional value in monocot and dicot plant species, as well as to introduce or enhance resistance to biotic and abiotic challenges. Despite issues about biosafety, genome editing is a promising technology that has the potential to contribute to food production for the benefit of the world's rising population (El-Mounadi et al., 2020). Breeding resistant cultivars is a long-term strategy for mitigating abiotic stress. The development of resistant plant species and varieties requires a thorough understanding of the mechanisms of the abiotic stress response (Lamaoui et al., 2018). Though environmentally beneficial, the time-consuming screening and crossing protocol limits its use in dealing with stress and achieving the objective of global food security. The use of genomic engineering approaches to perform a site-specific change in the plant genome for strengthening adaptability, increasing yield, and conferring resilience against different stresses has been facilitated by technology advancements.

CRISPR/Cas has transformed biological research and has wider applicability to crop plants than other genome editing techniques. CRISPR/Cas has been demonstrated to be an effective technique for precisely editing genomes to achieve desired features (Bhat et al., 2021). Furthermore, various studies have used genes and

TFs involved with abiotic stress tolerance as target genes in the development of drought-tolerant plants using CRISPR/Cas strategies (Lamaoui et al., 2018).

CRISPR/Cas technology has also been employed to enable the stacking of numerous transgenic characteristics by creating huge numbers of site-specific integration (SSI) landing sites utilizing homologous recombination. Gene deletion using CRISPR/Cas has the potential to create the modification in a specific sequence (Chilcoat., 2017). Based on their research, CRISPR/Cas was employed to remove the complete *WX1* gene using 2 gRNAs. One Cas9–gRNA cut a nucleotide upstream of the transcriptional start point, while the other cut downstream of the stop codon (Fig. 3). The region between the two targets was removed, and the remaining distal chromosomal ends were connected for DNA damage repair using the nonhomologous end-joining (NHEJ) pathway, resulting in a *Wx1* null allele with the Waxy phenotype.

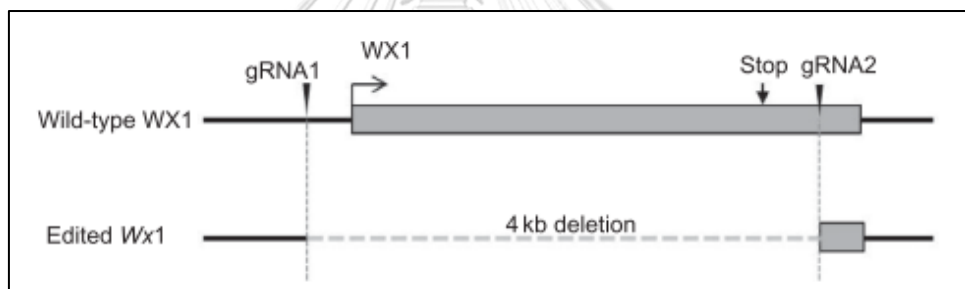


Figure 3. *Wx1* gene knockout design. A thick gray bar represents the *WX1* transcript, with a right-facing arrow indicating the transcript start site. Cas9–gRNA target cut sites are indicated with black arrows. A dotted gray line denotes the 4 kb region that is deleted in the desired *Wx1* modification (Chilcoat et al., 2017).

The application of genome editing in plants still becomes a major concern regarding methodological, biosafety, and social problems. It is mostly related to target gene site selection, guide RNA design, off-target effects, and delivery technique. The primary issue is the possibility of off-target mutations causing undesired genetic changes in plants (Liang et al., 2018). However, genome-modified plants, on the other hand, may or may not be transgenic. As previously stated, gene segregation may be used to eliminate the transgene carrying the CRISPR-Cas9 cassette (Figure 4). A genome-edited plant could be classified as non-transgenic if this is completed (El-Mounadi et al., 2020).

As a result, screening and elimination of the transgene-free after genome editing is critical for delivering stable gene-edited plants with no recombinant gene-editing machinery and providing more information to the community about the principles of genome editing has the potential to correct and prevent the spread of misconceptions of genome editing in the future (Aliaga-Franco et al., 2019; El-Mounadi et al., 2020).

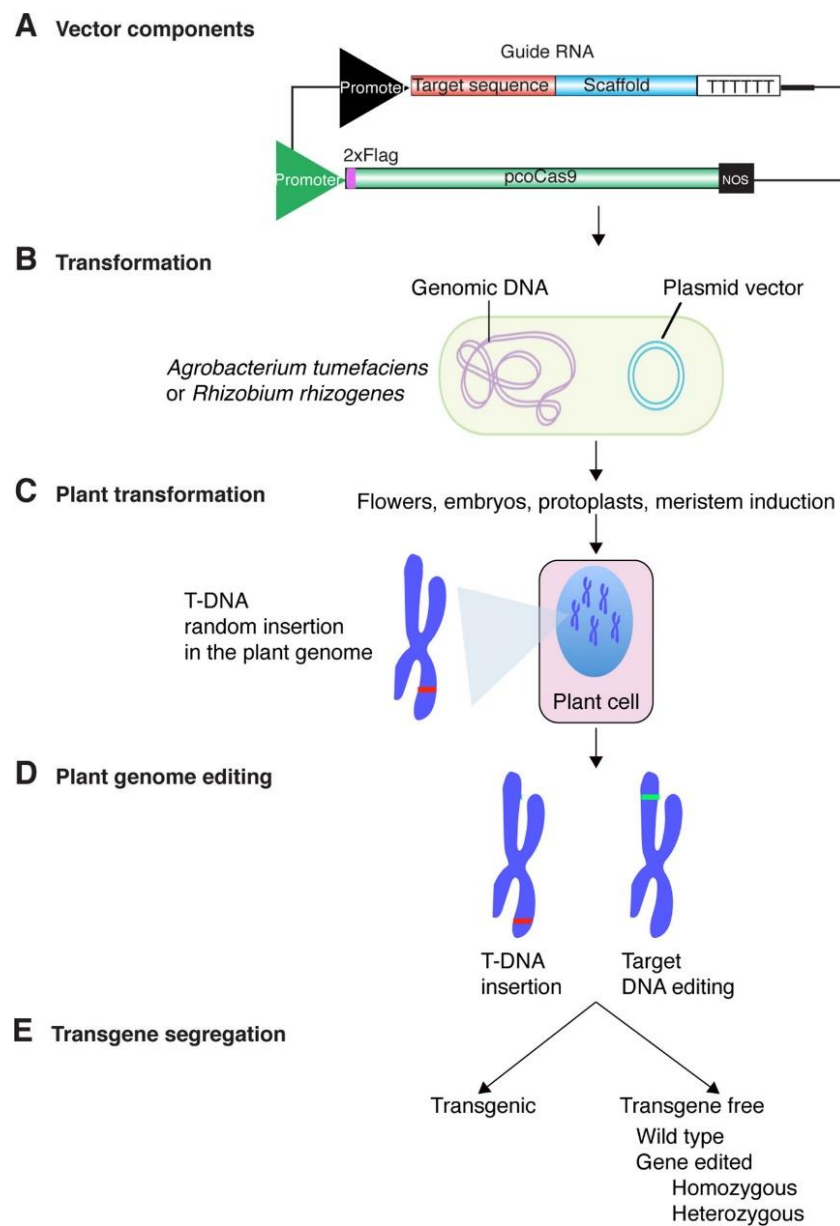


Figure 4. CRISPR-Cas9 to modify the genome. (A) The Cas9 protein and guide RNAs are cloned into a plasmid vector with T-DNA signals. (B) The plasmid vector

containing the Cas9 protein and guide RNAs expression cassette is transformed into *A. tumefaciens* or *R. rhizogens*. Bacteria are employed to alter embryos, protoplasts, roots, and leaf cells. The integration site of the T-DNA is random. (D) The Cas9 protein and guide RNAs cause the target DNA to be edited. The DNA target and the T-DNA insertion location are most likely non-linked. (E) Mendelian segregation can separate the T-DNA insertion and the altered region of the genome (El-Mounadi et al., 2020).



## CHAPTER III

### MATERIALS AND METHODS

#### I. Materials

##### 1. Plant Materials

1.1 Eight transgenic rice lines with background *Oryza sativa* L. c.v. 'Kitaake' was used as plant materials in this study. The seeds of wild type 'Kitaake' and transgenic lines including 1A5, 1A14, 2B3, 2B7, 7B3, 7B4, 20B9, 20B13 were provided from Miss Kansinee Hungsaprug.

##### 2. Equipment

###### 2.1 General use (planting, phenotyping, and sample collection)

- SPAD 502 Plus, Konica Minolta, Japan
- Plastic pot
- Scissor
- Waterproof label
- Envelope
- Ruler
- Marker

###### 2.2 DNA extraction and purification

- Grinding machine Homogenizer (Mixer Mill MM 400, Retsch, Germany)
- Freezing block
- -80°C deep freezer (Forma 700 series, Thermo Scientific, USA)
- -20°C freezer (Mitsubishi, Japan)
- Magnetic beads
- Micropipette (Pipet-Lite XLS, Rainin, USA)
- Micropipette tips
- Autoclave (Taichung, Taiwan)
- Liquid nitrogen container
- Centrifuge (Sorvall Legend Micro 21, Thermo Scientific, USA)
- Vortex (Labnet, USA)
- Spin down machine

- Nanodrop (Nanodrop 2000, Thermo Scientific, USA)
- Dry bath (MD-01N model, Major Science, Taiwan)
- Microtube 1,5 ml
- Ice box
- Forceps
- Spatula
- Plastic tray
- Aluminium foil
- Scissor

### 2.3 Polymerase Chain Reaction (PCR) and Gel electrophoresis for Cas9-free screening and *OsGTL1* characterization

- Thermocycler (Bio-Rad T100, USA)
- Micropipette (Pipet-Lite XLS, Rainin, USA)
- Micropipette tips
- Microtube 1,5 ml
- Microtube for PCR
- Spin down
- Plastic tray
- Paper box
- Parafilm
- Balance (Mettler Toledo AG285, Mettler Toledo, Switzerland)
- Gel documentation system (Geldoc) (Nippon Genetics FAS-Digi PRO, Japan)
- Horizontal gel electrophoresis unit (Mini Run GE-100, Hangzhou BIOER Technology, China)
- Microwave (Toshiba, Thailand)
- Erlene Meyer 100ml
- Cylinder
- Ice box

## 2.4 Stomatal observation

- Microscope (Olympus BX-51, Japan)
- Aluminium foil
- Forceps
- Scissor
- Object glass
- Cover glass

## 2.5 Photosynthesis observation

- LICOR LI-6400/XT Portable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA)
- Ruler

## 3. Chemicals and reagents

### 3.1 Rice planting, phenotyping and sample collection

- Fertilizer (Osmocote 13-13-13, Osmocote 12-25-6+1)
- Fipronil
- Liquid nitrogen

### 3.2 DNA extraction and purification

- DNA extraction kit (GENEAID, Taiwan)
- DNA purification kit (TIANGEN Biotech, Beijing, China)
- Distilled water

### 3.3 Polymerase Chain Reaction (PCR) and Gel electrophoresis for Cas9-free screening and *OsGTL1* characterization

- Taq DNA polymerase (Thermo Fisher Scientific, USA)
- 100 mM dNTP mix (Thermo Fisher Scientific, USA)
- Taq buffer (Thermo Fisher Scientific, USA)
- $MgCl_2$  (Thermo Fisher Scientific, USA)
- EDTA 0.5M pH 8 (Appendix table 2)
- Tris Base (Gold Biotechnology Inc, USA)
- Boric acid (Merck, Germany)
- 5X TBE buffer (Appendix table 2)
- 6X Loading dye (Appendix table 2)

- Gene ruler 1KB (Thermo Fisher Scientific, USA)
- Midori green staining gel (NIPPON Genetics, Japan)
- Distilled water
- Agarose (LE Agarose, SBio by Smart Science, Thailand)

### 3.4 Stomatal observation

- Dental resin (Vinylight, BMS Dental, Italy)
- Nail polish (Ten Ten, Thailand)
- Ethanol 70%

## II. Methods

### 1. Identification of Cas9-free plants

#### 1.1 Rice grown condition

The identification of the Cas9-free plants started by screening from eight T<sub>3</sub> transgenic rice lines created by Hungsapruk (2018). These transgenic lines were created by using pRGEB32 vector. The wild type (WT) rice (*Oryza sativa* L. cv. 'Kitaake') was used as the negative controls. At least 5 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 (Zhang et al., 2019) and osmocote fertilizer.

#### 1.2 Phenotypic Observation

Phenotypic parameters in vegetative and reproductive stages, which are plant height, the number of leaves, the number of tillers, panicle initiation date, the number of seeds per plant, and filled grain number per plant were determined. Leaf greenness of the youngest fully expanded leaf and the oldest leaf were determined by SPAD chlorophyll meter (SPAD 502 Plus, Konica Minolta, Japan) in the main tiller of four-week-old plants.

### 1.3 Primer Design for Cas9-free screening

The Cas9 primers were designed from the sequence of pRGEB32 plasmid sequences from the Addgene website by using Primer3plus tools.

### 1.4 DNA Extraction

The young leaves from each tiller of T<sub>3</sub> plants were collected for genomic DNA extraction. The genomic DNA was extracted by using a homogenizer machine and Genomic DNA GENE AID Kit (Geneaid Biotech Ltd, Taiwan). DNA concentration and quality were measured by using Nanodrop.

### 1.5 Screening Cas9-free plants

For Cas9-free transgenic plant screening, Cas9 specific primers were used to amplify Cas9 fragments by using the plant genomic DNA as a template. WT genomic DNA and pRGEB32 vector were used as negative and positive controls for the amplification, respectively. The existence of the Cas9 fragment was monitored by using gel electrophoresis.

The existence of *Cas9* gene in each tiller was detected by polymerase chain reaction (PCR) using the primers specific to *Cas9* gene, which was designed according to the *Cas9* sequence in pRGEB32 plasmid. Two rounds of *Cas9* amplification were performed. The first round was performed by using primer set 1 (Cas9-1 and Cas9-2). With these primers, the *Cas9* fragment with the size of 532 bp could be amplified. The sample that cannot be amplified by Cas9—1 and Cas9—2 primers was confirmed for the Cas9-free by amplification of the second pair of Cas9 primers, Cas9-3 and Cas9-4. This pair of primers will result with 4445 bp *Cas9* gene. The PCR conditions and primer sequences were shown in Appendix table 1.

## 2. Determination of *OsGTL1* promoter modification in Cas9-free transgenic lines

### 2.1 Design of *OsGTL1* promoter-specific primers

The specific primers for *OsGTL1* promoter were designed based on the sequence obtained from the Rice Genome Annotation Project (MSU) database (LOC\_Os03g02240) by using Snap gene software Figure 5.

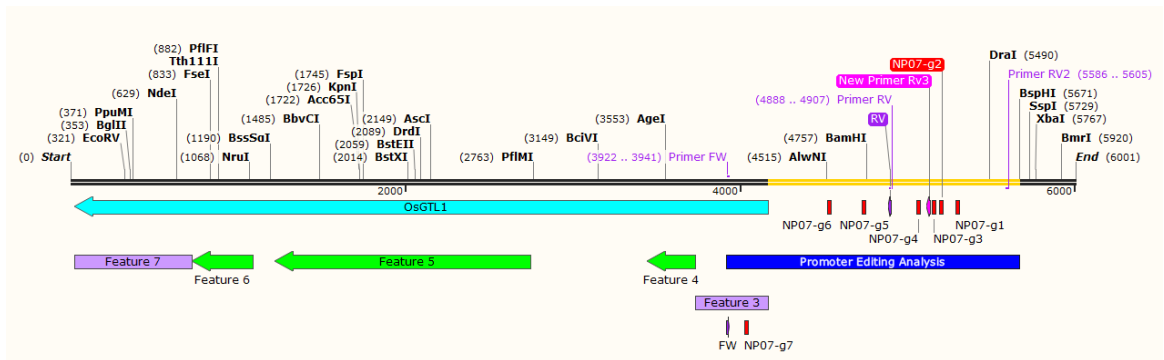


Figure 5. Map of the gRNAs position in the *OsGTL1* promoter (red square represents gRNA no.1-7 from right to the left-hand side).

## 2.2 Determination of *OsGTL1* promoter modification

*OsGTL1* promoter-specific primers (Appendix table 1) were used to amplify the *OsGTL1* promoter fragment from the Cas-9 free genomic DNA. The size of the amplified fragments was determined by gel electrophoresis and the DNA fragment were purified by using TIANGEN Purification Kit (Beijing, China). At least 2 fragments with different sizes from each selected Cas9-free transgenic line 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. Assessment of *OsGTL1* promoter modification effects on the stomatal distribution pattern

### 3.1 Plant materials

Four *Cas9*-free lines with different modifications on the *OsGTL1* promoter including 1A5, 2B3, 20B9, and 20B13 were used to determine stomatal distribution patterns on the flag leaves and the second leaves at the reproductive stage.

### 3.2 Stomatal characterization

The plants were grown with randomized complete block design (RCBD) with 4 replications under natural environment. At the reproductive stage, stomatal imprints of the abaxial epidermis were collected with dental resin from 3 positions, near leaf tip, middle, and at the base of the leaf blade. The clear nail polish was used to

imprint the epidermal pattern from the dental resin. The stomatal density (SD: number of stomata per area) was the number of stomata per leaf area. A leaf area of 0.156 mm<sup>2</sup> used for SD calculation was obtained from the field of view observation (length x width). The stomatal distribution pattern was determined by indicating the number of stomata per micrometer square (mm<sup>2</sup>) from left to right and from top to bottom from each imprinted image. Stomata size was observed by measuring the width and length of the stomatal complex. The stomatal distance was determined by measuring the distance between each stoma vertically and horizontally. The pictures were captured three times from each imprint from three positions in flag leaf and second leaf. Then, the pictures were observed and captured under a 20X objective lens with a high-resolution microscope Olympus BX-51. The photograph pictures of the epidermal pattern were analyzed by the cell counter plugin of ImageJ (FIJI v.1.51u) (Mohammed et al., 2019).

### 3.3 Statistical analysis

The stomatal size, density, and stomatal distance of these plant lines were tested by analysis of variance (ANOVA) at  $p < 0.05$  with SPSS Statistics 20.0 software, and the means were compared by Duncan's multiple range test (DMRT). The significant difference was accepted at  $p \leq 0.05$ .

## 4. Assessment of *OsGTL1* promoter modification effects on photosynthesis

### 4.1 Light response curve measurement

The same plant materials, experimental design, and growing condition as indicated in 3 were used in this experiment. The light response curve of each plant line was determined before the photosynthesis measurement. We set a loop for changing through a series of light intensities, 2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400, 200, 100, 50, 25 and 0  $\mu\text{mol.m}^{-2}.\text{s}^{-1}$  for 2 minutes before logging at each iteration. For each logging, we recorded data every other second 3 times. The mean value was used as the measured data to represent the measurement variation (Tiangen et al., 2017).

#### 4.2 Photosynthesis assessment

Photosynthesis rate, stomatal conductance, internal CO<sub>2</sub> concentration, transpiration rate,  $A/C_i$ , and water use efficiency (A/E) of these plants were determined using a portable photosynthesis system, LI-6400 (Li-Cor, USA) with saturating light according to the light response curve. The air CO<sub>2</sub> concentration ( $C_a$ ) of 400  $\mu\text{mol mol}^{-1}$ , chamber block temperature of 30°C, and relative humidity 70–75% were used for photosynthesis measurement (Chintakovid et al., 2017). At the reproductive stage, the photosynthetic parameters were determined in the flag leaves and second leaves at the booting stage, flowering stage, and grain filling stage.

#### 4.3 Statistical analysis

Photosynthesis rate ( $A$ ), stomatal conductance ( $g_s$ ), internal CO<sub>2</sub> concentration ( $C_i$ ), and transpiration rate ( $E$ ) data were analyzed with analysis of variance (ANOVA) and the means were compared by Duncan's multiple range test (DMRT). The significant difference was accepted at  $p \leq 0.05$ .

## CHAPTER IV

### RESULTS

#### 1. Identification of *Cas9*-free plants

##### 1.1 Determination of *Cas9*-free plants

Eight transgenic lines inserted with sgRNA targeted to *OsGTL1* and *Cas9* gene, namely 1A5, 1A14, 2B3, 2B7, 7B3, 7B4, 20B9, and 20B13 (Hungsaprug, 2018), were used in this study. A total of 63 plants with 215 tillers was evaluated for the existence of *Cas9* gene in the tillers. The tiller was named by addition of an alphabet A to Z to the name of the line; for example, 1A5A1-A, 1A5A1-B and 1A5A1-C were from different tillers in 1A5A-1 transgenic lines. 1A5A-1 and 1A5A-2 are siblings from the original transgenic line, 1A5.

The first screening of *Cas9*-free tillers, performed with *Cas9* – 1 and *Cas9* – 2 primers, are shown in Figure 1. In 1A5A lines, only 1A5A-1 and 1A5A-2 showed the *Cas9*-free tillers (Figure 6A). In the 7B4B lines, all progenies, 7B4B-1 to 7B4B-7 contained *Cas9* gene in every tiller tested (Figure 6B). Based on the amplification with *Cas9* – 1 and *Cas9* – 2 primers, 20B9B line was the *Cas9*-free plants as every tiller contained no *Cas9* fragment (Figure 6C). The *Cas9*-free lines were also detected in another 3 lines, 2B3, 2B7, and 20B13 (data shown in Appendix figure 1). In 20B13B line, some plants such as 20B13B-1 and 20B13B-2 were *Cas9*-free, but others 20B13Bs still had *Cas9* gene in the genome (Figure 6D). All of the *Cas9*-free tillers were validated again with *Cas9* – 3 and *Cas9* – 4 primers. The results were shown in Appendix Figure 1. It was confirmed that *Cas9* gene could not amplified in all the *Cas9*-free tillers from the first screening which means that no *Cas9* gene was left in these genomes. The summary of the total number of *Cas9*-free plants and tillers were shown in Table 1.

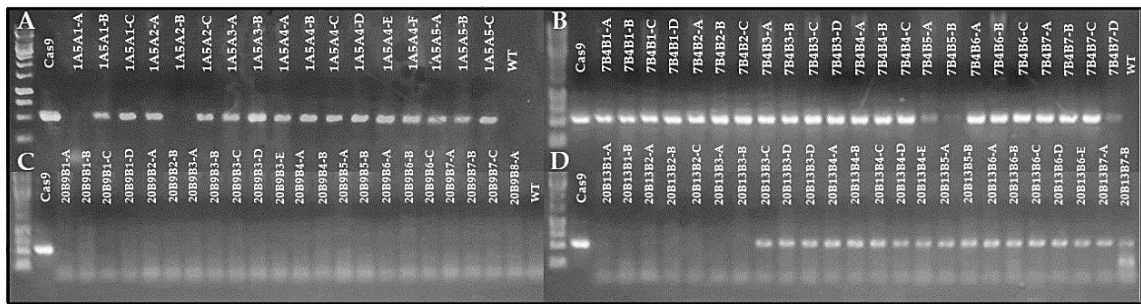


Figure 6. Gel image for screening of *Cas9*-free tillers from 1A5A (A), 7B3B (B), 20B9B (C), and 20B13B (D). pRGEB32 which contains *Cas9* was used as positive control and the wild-type sample (WT) was used as a negative control.

Table 1. The number of *Cas9*-free plants which were obtained from T<sub>3</sub> seeds.

Lines	Number of tested plants	<i>Cas9</i> -free Plants		Number of tested tillers	<i>Cas9</i> -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	

## 1.2 Phenotype Observations

The leaf greenness of the youngest fully expanded leaf and the oldest leaf were determined by SPAD chlorophyll meter (SPAD 502 Plus, Konica Minolta, Japan) in the main tiller of four-week-old of the putative *OsGTL1* edited lines and WT plants. Three spots at position upper, middle and bottom parts of each leaf blade were used to determine leaf greenness by SPAD value. The SPAD values of all rice plants at the age of 4 – 8 weeks after germination were ranged from 28.88 to 37.86 (Figure 7A). No significant difference of SPAD values among the putative *OsGTL1* edited lines and WT and the greenness remained during the period of study. It showed that the modification in *OsGTL1* did not affect the chlorophyll content in the modified lines.

The leaf number of all lines including WT was increased by time (Figure 2B). No significant difference in leaf number was detected during the first 4 weeks after germination. After 5 weeks of germination, the significant difference in leaf number was detected among the putative *OsGTL1* edited lines. Line 2B3 and 2B7 had the highest leaf number (10-11 leaves per plant) and lines 7B3 and 7B4 had the lowest leaf number (6-7 leaves per plant). However, after 6 weeks of germination, leaf number of all lines were similar (Figure 7B).

The maximum tiller number of all lines tested was similar, ranging from 11 to 18 tillers per plant. These plants ended the tillering stage by reaching the highest number of tillers 7 weeks after germination. Line 2B3, 2B7, and 7B3 had the highest tiller number, while lines 1A5 and 1A14 had the lowest tiller number (Figure 7C). The statistical analysis showed no significant difference between the putative *OsGTL1* edited lines and WT every time point of observation.

Plant height was increased by time and reached the maximum in 5 weeks after germination. WT was taller than the putative *OsGTL1* edited lines. Some lines, 2B3 and 2B7 had the similar height to WT, but some lines, 1A5 and 7B3 were significantly smaller than other lines. Their average height was 58.2 and 56.3 cm, respectively in the last week of observation. It is worth mentioning that almost all edited lines became smaller compared to WT plant (Figure 7D and Appendix Figure 2).

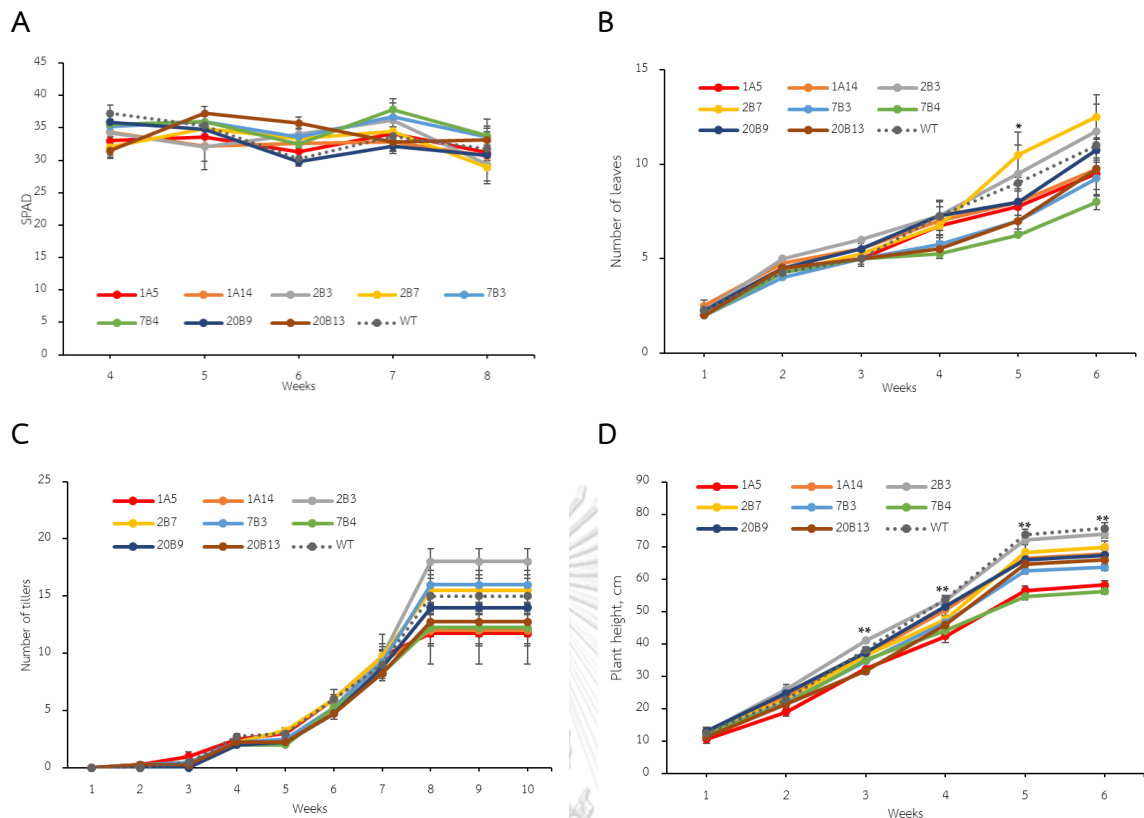


Figure 7. Phenotypes of the putative *OsGTL1* edited lines in comparison with wild type (WT) including leaf greenness by SPAD (A), number of leaves (B), number of tillers (C), and plant height (D). Statistical analysis was done by the analysis of variance (ANOVA) at  $p < 0.05$  with SPSS Statistics 20.0 software and the means were compared by Duncan's multiple range test (DMRT). The data were presented as the mean  $\pm$  SE with significantly different data ( $p < 0.05$ ). \* indicates the significant difference among means was detected.

## 2. Determination of *OsGTL1* promoter modification in Cas9-free transgenic lines

Seeds from *Cas9*-free tillers were planted to investigate *OsGTL1* promoter modification. *OsGTL1-F* and *OsGTL1-R* primers were used to amplify *OsGTL1* promoter from genomic DNA extracted from the *Cas9*-free plants. Based on the gel electrophoresis, the amplified fragments showed the size difference approximately ranging from 1 to 1.5 kb (Figure 8). The amplified fragment from 20B9B line's progeny showed approximately the same sizes, which was smaller than the fragment amplified from WT's genomic DNA (Figure 8A), while the fragments with

approximately the same size as the fragment from WT were detected in 2B7B's progeny (Figure 8B). The PCR fragments from 20B13B's progeny were smaller than the fragment amplified from WT's DNA (Figure 8C). Interestingly, amplified fragments from 1A5A's progeny were in various sizes (Figure 8D). The PCR product with different sizes from each line was isolated and sent out 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.

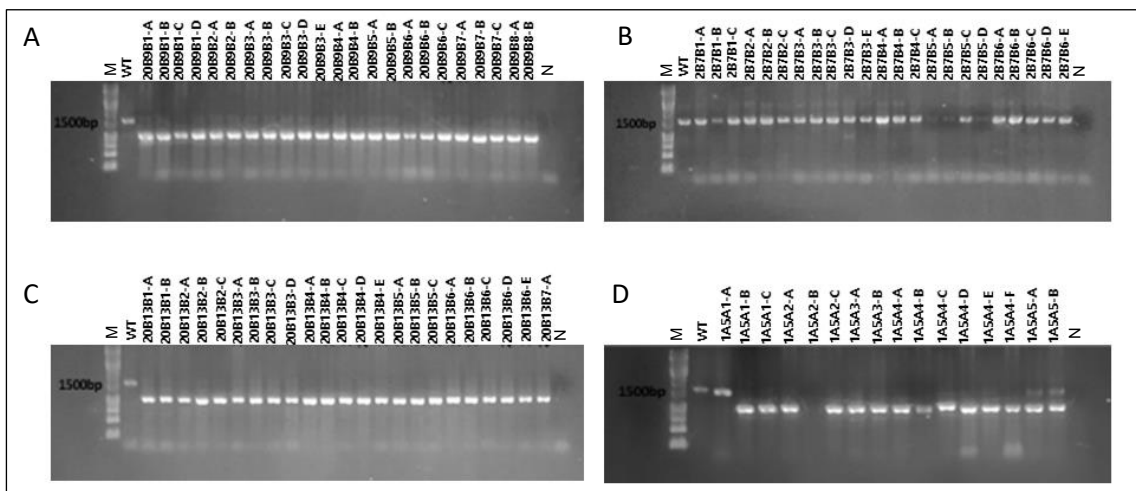


Figure 8. Amplification of *OsGTL1* promoter from Cas9-free plants line 20B9B-1 (A), 2B7B-1 (B), 20B13B-1 (C), and 1A5A1-A (D). *OsGTL1* promoter from WT is 1684 bp, shown in the far-left lane next to molecular marker (M). N is the negative control.

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 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. The same deletion 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 was not close to the PAM sites (Figure 9). It can be assumed the efficiency of the gRNAs can be different in each line resulting in different modifications. The results also showed sgRNA can work independently or worked together to modify the plant genome.

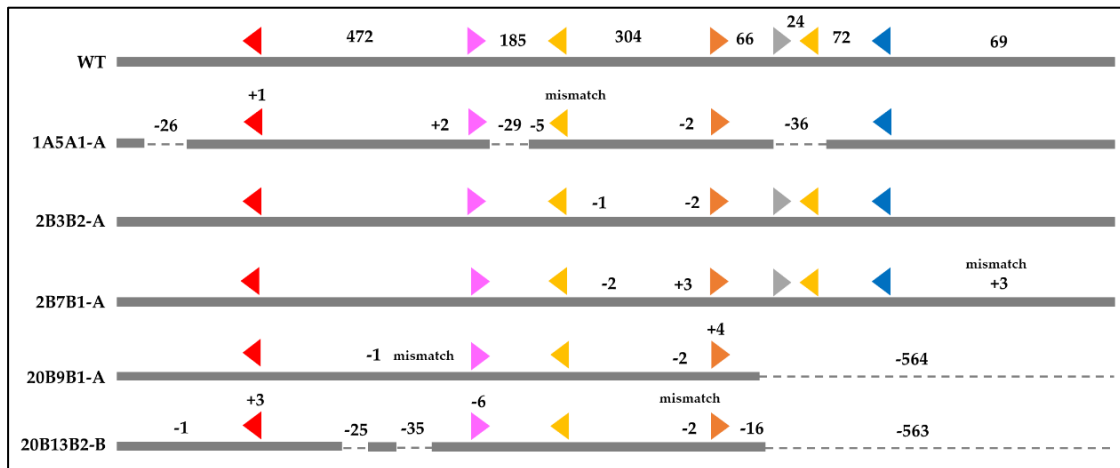


Figure 9. Diagram of the modification in *GTL1* alleles ( $T_3$  generation). There are different types of modification on the *GTL1* promoter including insertion (+), deletion (-), big deletion (dash line), and mismatch of the base. The triangles with different colors represent gRNAs no.1-7 from the right to the left-hand side. The number between the triangle showed the distance between gRNAs in base pairs.

Cis-regulatory elements have an important role in gene transcription process. Therefore, it is important to analyse the presence of cis-acting elements in the promoter of the gene. The promoter sequences were analysed by using the PLACE database (<https://www.dna.affrc.go.jp/PLACE/>) to identify the presence of cis-acting elements in the *OsGTL1* promoter. Furthermore, the resulting data were retrieved to TB tools software to visualize the position of cis-acting elements in the *OsGTL1* promoter Figure 10.

For line 1A5A1-A, the big deletion was found on gRNA 2-3 and 5-6. However, there were also some small deletions and insertions nearby the other gRNAs. Line 2B3B2-A and 2B7B1-A showed only small deletion and insertion nearby gRNA 4-5 while line 20B9B1-A and 20B13B2-A showed big deletion between gRNA 1-4. Several cis-elements were detected in that modified position such as ABRE, GT1 Box, MYB,

MYC, GTGA motif, E-box, and CGCG box (Figure 10). Therefore, the modification in these positions might affect the ability of the plant to survive under stress conditions.

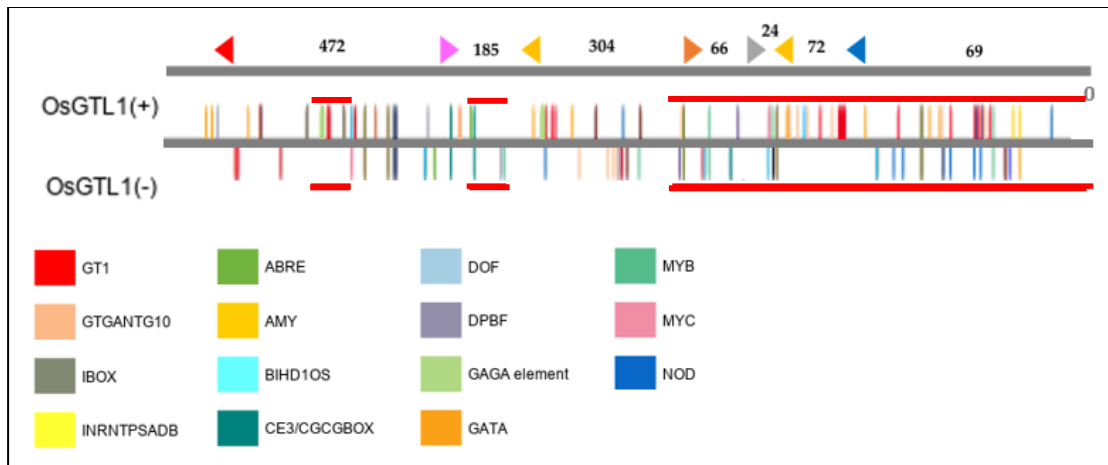


Figure 10. Promoter analysis from PLACE database. Red lines represent the modification that occurs in edited lines. Colour bars represent the Cis-acting elements in the *OsGTL1* promoter. This picture was visualized by TB tools software (<https://bio.tools/tbtools>).

### 3. Assessment of *OsGTL1* promoter modification effects on the stomatal distribution pattern

Four lines with *OsGTL1* promoter modification, 1A5A1-A, 2B3B2-A, 20B9B1-A, and 20B13B2-B, were selected to study the modification effects on stomatal distribution patterns in comparison with WT. All of these four lines contain different deleted region in the promoter (Figure 9). The investigation was performed at booting stage on flag leaf and second leaf. Imprinting on the adaxial surface was difficult to do because of the presence of dirt such as dust and insects on the upper surface of the leaf so that the resulting stomata image is not clear. Therefore, we only present the abaxial epidermal imprints in this research which were shown in Figure 11.

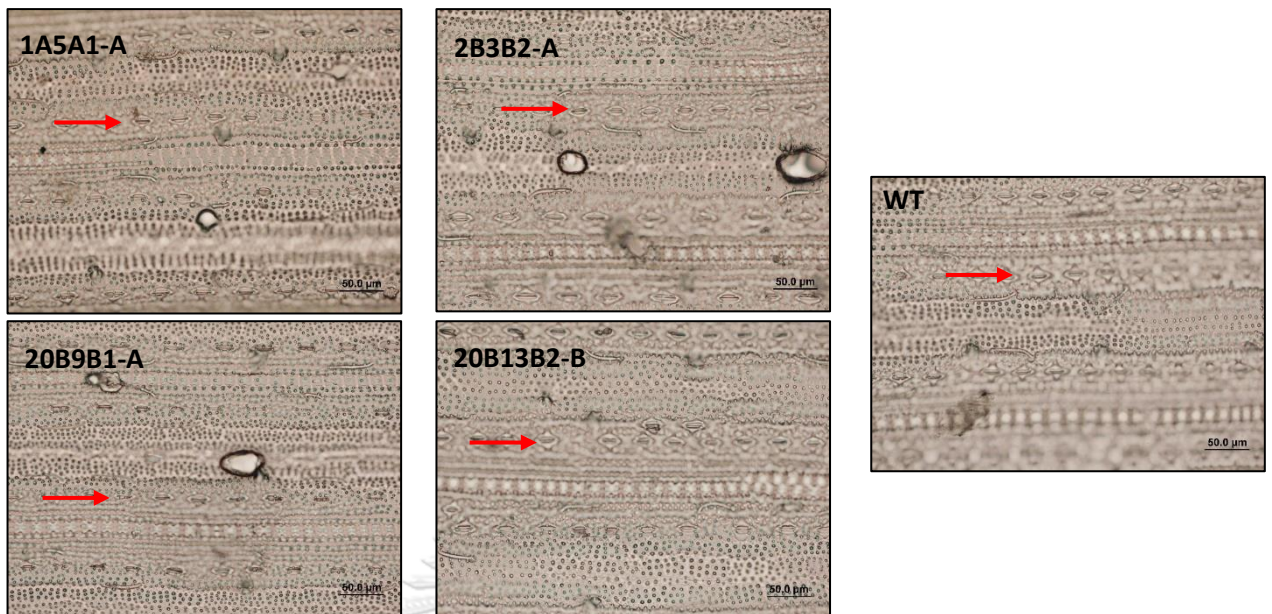


Figure 11. Images of the abaxial surface imprint of edited lines and wildtype. These pictures showed the middle part of the flag leaf. Stomata are shown in the red arrow.

The result showed some edited lines that have a big deletion in their sequences such as lines 1A5A1-A, 20B9B1-A, 20B13B2-B have a lower number of stomata and SD both in the flag and second leaf while line 2B3B2-A has a similar number with wild type plants. The stomatal density at the base, middle and tip regions on the leaf was similar in each line. The statistical analysis also showed edited lines 1A5A1-A, 20B9B1-A, 20B13B2-B were significant differences with line 2B3B2-A and wild-type plants in the bottom and middle position of the flag leaf while in the second leaf it showed significant differences in the bottom and upper position.

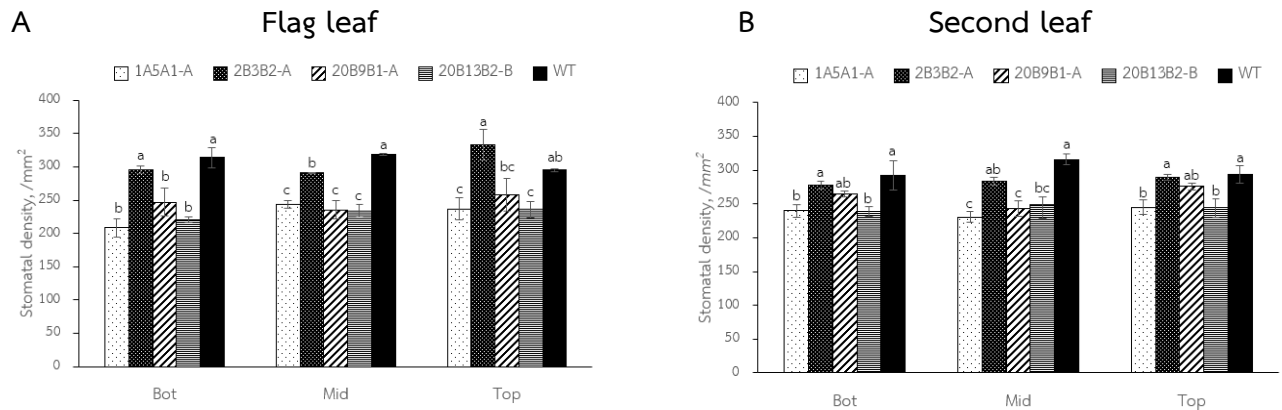


Figure 12. Stomatal observations were measured in the flag leaf and second leaf at the reproductive stage. Stomatal density in the flag leaf (A) and stomatal density in the second leaf (B). The imprints were collected from tip, middle and base of the leaf blade. The different letters above the bars represent the significant difference of the mean at  $p < 0.05$ , analyzed with DMRT. Error bars present SE.

The stomatal sizes were also investigated. Stomatal width and length were measured in at least 30 stomatal cells per imprints and the average number from each imprint was used for calculations. Stomatal morphology was modified due to the *OsGTL1* promoter modification. All edited lines showed the smaller size stomata, when compared to WT (Figure 13). All edited lines showed the smaller width. For the stomatal length, the stomata of 2B3B2-A at the base and the tip had the similar length to the stomata of WT in the same region, but the other three edited lines had the significantly smaller stomatal length than WT (Figure 13 A,B). However, these effects were clearly detected in the flag leaves. No significant difference in stomatal length was detected in the second leaves of all lines (Figure 13 C,D).

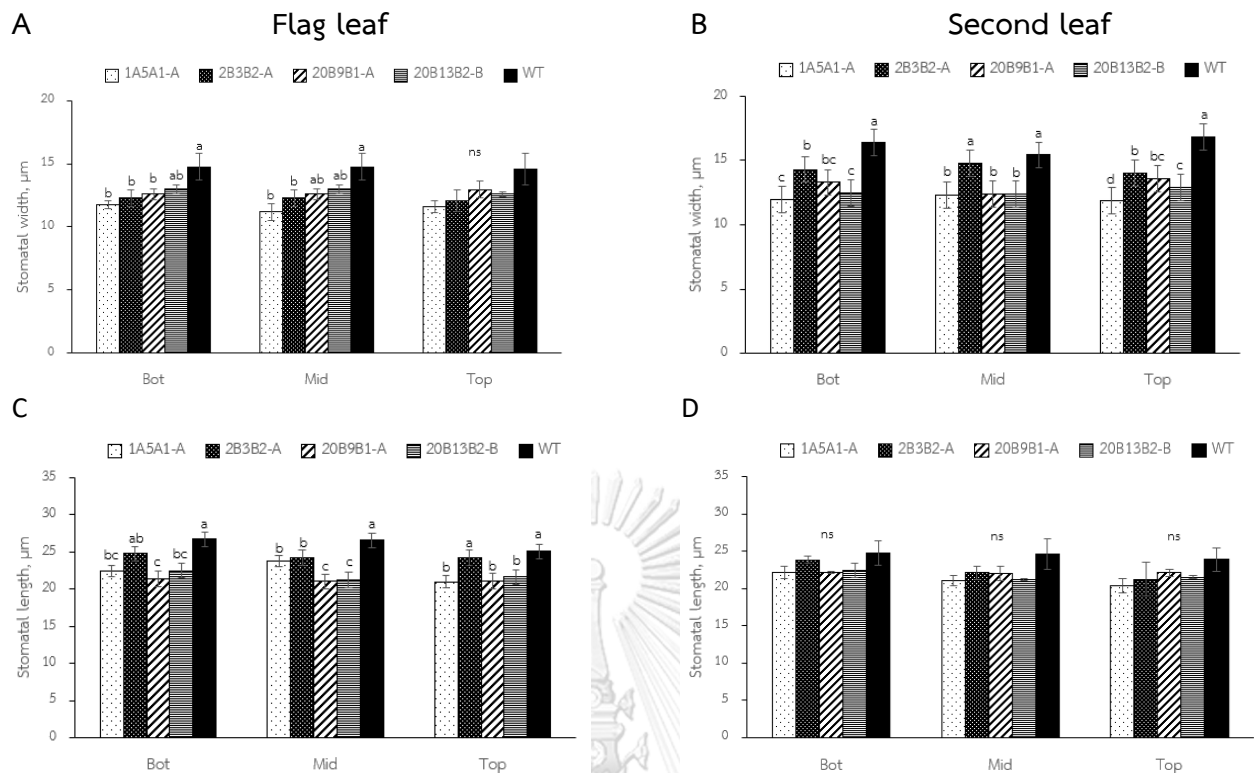


Figure 13. Stomatal observations were measured in the flag leaf and second leaf at the reproductive stage. Stomatal size includes stomatal width (A) and stomatal length (B). The different letters above the bars represent the significant difference of the mean at  $p < 0.05$ , analyzed with DMRT. Error bars present SE.

Stomatal distance in vertical and horizontal directions was measured in this experiment. The distances were measured in at least 10 spots per image and the average number from each imprint was used for calculations. The result showed some edited lines such as 1A5A1-A, 20B9B1-A and 20B13B2-B have larger distances vertically (Figure 14 A, B) but no significant difference was detected based on statistical analysis.

Stomatal horizontal distance showed line 2B3B2-A and wild type have smaller distance between the stomata compared to line 1A5A1-A, 20B9B1-A and 20B13B2-B. However, statistical analysis showed no significant difference between edited lines and wild-type plants (Figure 14 C, D).

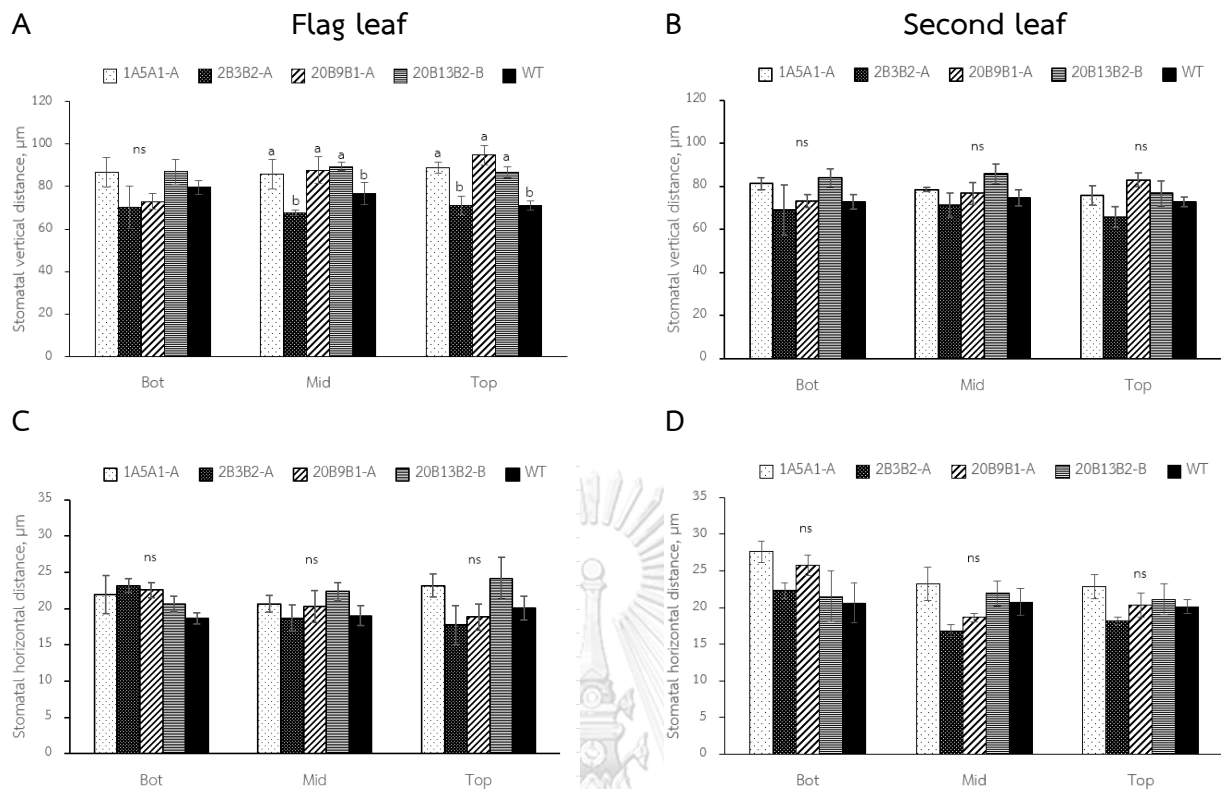


Figure 14. Stomatal observations were measured in the flag leaf and second leaf at the reproductive stage. Stomatal distance includes stomatal vertical distance (A) and (B), stomatal horizontal distance (C), and (D). The different letters above the bars represent the significant difference of the mean at  $p < 0.05$ , analyzed with DMRT. Error bars present SE.

In order to determine if the stomatal values detected in the flag leaf and second leaf are different in these lines, the student t-test was analysed and the results were shown in Table 2. Only the stomatal width and the stomatal vertical distance were significantly different between the flag and second leaves. The other parameters, such as stomatal density, stomatal length, and stomatal horizontal distance showed no significant difference between the flag leaf and second leaf. These results indicated the morphological and distribution changes by *OsGTL1* promoter modification.

Table 2. Result of T-Test Analysis to compare stomatal parameters between flag leaf and second leaf.

Parameters	Flag leaf	Second leaf	Significance level (P)
Stomatal density	264.43	265.77	0.773
Stomatal width	12.90	13.66	0.000
Stomatal length	22.96	23.16	0.489
Stomatal distance (Vertical)	75.92	81.22	0.000
Stomatal distance (Horizontal)	20.79	21.48	0.251

#### 4. Assessment of *OsGTL1* promoter modification effects on photosynthesis

The stomata are the open pores for gas exchange between plants and environment. The changes in morphology and distribution of stomata can lead to the changes in gas exchange activity. Therefore, the photosynthesis parameters were determined among these lines to reveal the effects of *OsGTL1* promoter modification. As mentioned in the material and method section, the leaf gas exchange parameters were measured in two types of leaf including flag leaf and second leaf in the main tiller of each plant. Photosynthesis activity of the flag leaf and second leaf at the reproductive stage including booting, flowering, and grain filling stage were measured repeatedly in each stage.

Before the data collection, we made a light response curve to select the suitable light intensity for the measurement. Based on the light response curve analysis, it showed that all edited lines reached the light saturation point at 1200  $\mu\text{mol.m}^{-2}\text{s}^{-1}$ , while the WT plant reached the saturation point at 1,800  $\mu\text{mol.m}^{-2}\text{s}^{-1}$  (Figure 15). Therefore, we selected light intensity at 1200  $\mu\text{mol.m}^{-2}\text{s}^{-1}$  for the photosynthesis parameter measurement. Based on the light response curve, it revealed the lower net photosynthesis rate (A) of all the edited lines, when compared to WT at light intensity higher than 800  $\mu\text{mol.m}^{-2}\text{s}^{-1}$ .

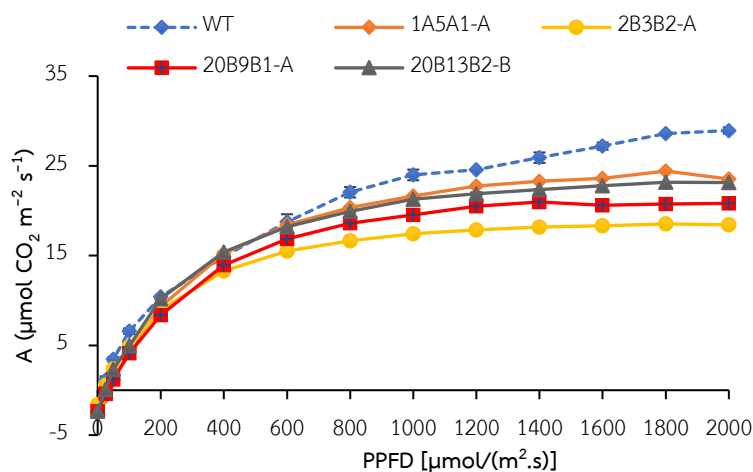


Figure 15. The light response curve of edited lines and wild type.

From the stomatal observation, some of the edited lines showed a lower stomatal density, and sizes. Therefore, the photosynthesis parameters were determined to investigate if the stomatal changes affected the photosynthesis activities in these lines. Therefore, in this study, four edited lines, 1A5A1-A, 2B3B2-A, 20B9B1-A and 20B13B2-B, together with WT were investigated.

The net photosynthesis rate ( $A$ ) at booting stage was higher than  $A$  at flowering and grain filling stage in all lines. However, no significant difference of  $A$  among these lines could be detected in flag leaf and second leaf at booting stage and flowering stages. At grain-filling stage, the significant difference in  $A$  was found in the flag leaf of the edited lines and WT. However,  $A$  of the edited line was similar to WT.  $A$  of 2B3B2-A flag leaf was significantly lower than  $A$  of 1A5A1-A and 20B9B1-A at this stage (Figure 16A). In the second leaf,  $A$  of all lines showed no significant difference in all stages (Figure 16A).

For the stomatal conductance ( $g_s$ ), no significant difference was detected among lines at each developing stage in both flag leaf and second leaf (Figure 16B). However, it is worth mentioning that  $g_s$  at booting stage and flower stage were higher than  $g_s$  at grain-filling stage.

Although the significant difference in  $A$  and  $g_s$  among lines could not be detected at booting stage, which tended to possess the higher level of  $A$  than flowering and grain-filling stage, the transpiration rate ( $E$ ) of these lines at booting stage was significantly different (Figure 16C).  $E$  of 1A5A1-A was the lowest among all

lines and it was significantly lower than WT. However,  $E$  in flag leaves of all lines, except 20B9B1-A and 1A5A1-A, became lower, when they were at flowering and grain-filling stages. The similar pattern of changes in  $E$  by developmental stages was also detected in second leaves of all lines, except that  $E$  in second leaves of 20B13B2-B was quite stable through the development at reproductive stage (Figure 16C).

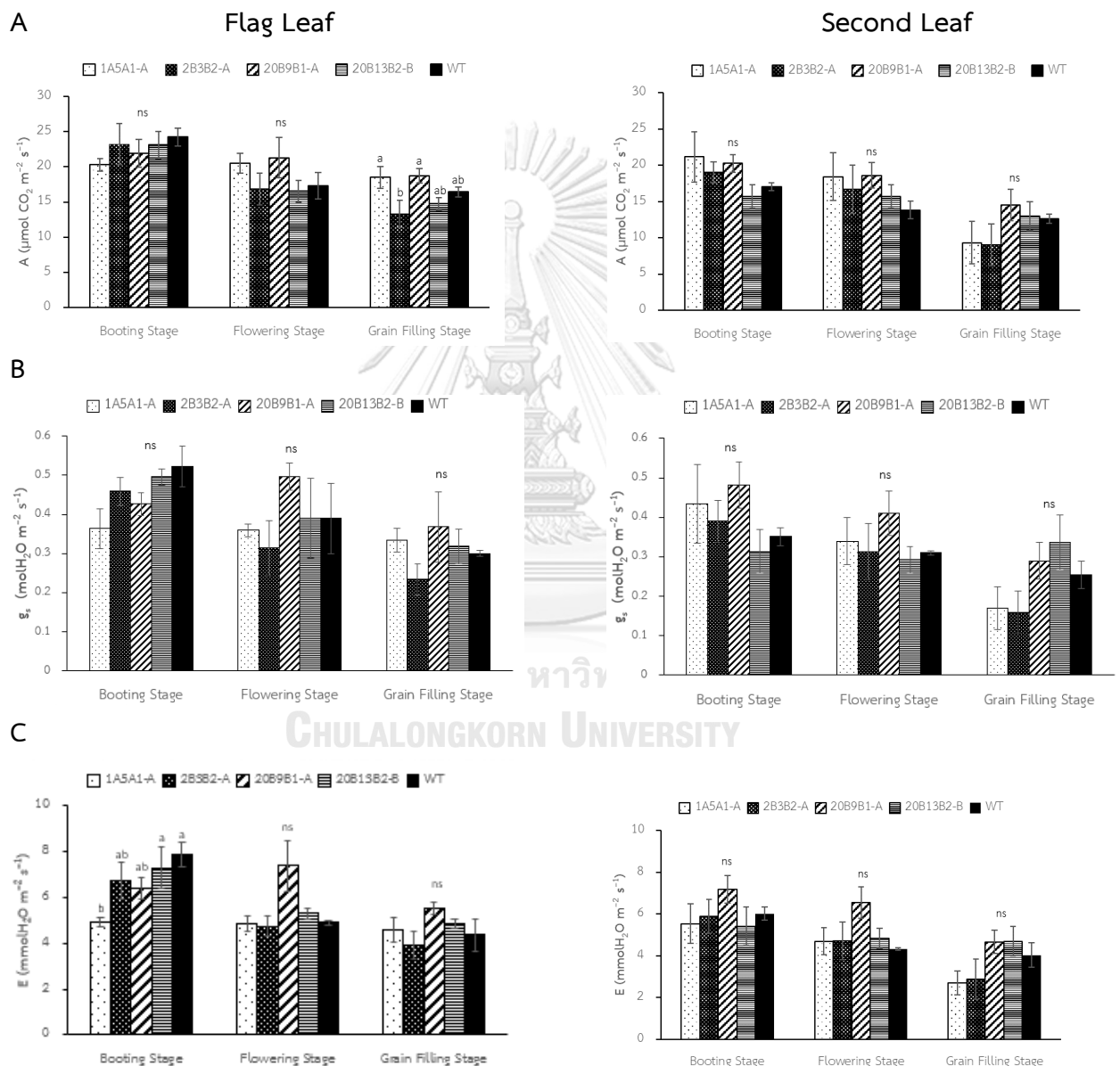


Figure 16. Photosynthesis parameters were measured in the flag leaf and second leaf. Net photosynthetic rate (A) (A), stomatal conductance ( $g_s$ ) (B), transpiration rate (E) (C), of modified plants and wildtype at reproductive stage. The different letters

above the bars represent the significant difference of the mean at  $p < 0.05$ , analyzed with DMRT. Error bars present SE.

The intercellular  $\text{CO}_2$  concentration ( $C_i$ ) was quite stable through the development at reproductive stage.  $C_i$  was kept at 280-290  $\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in all edited lines and wildtype both in the flag and second leaf during the booting stage. Then, the pattern was changed in the flowering stage where lines 20B9B1-A and 20B13B2-B have the highest  $C_i$  than other lines and wildtype plants about 299-306  $\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in the flag and second leaf. During the grain filling stage, line 20B13B2-B and wildtype have the highest  $C_i$  compared to others both in the flag and second leaf. Statistical analysis showed no significant difference in the flag leaf during the booting stage and no significant differences were detected in the second leaf at all stages. However, the data showed  $C_i$  was maintained at around 280-314  $\mu\text{molCO}_2 \text{ m}^{-2} \text{ s}^{-1}$  in all stages (Figure 17D).

$A/C_i$  ratio demonstrates the  $\text{CO}_2$  fixation capacity or ability. As  $C_i$  concentration increase, the rate at which carbon is incorporated into carbohydrate in the light-independent reaction also raise thus  $A$  will also increase until it is limited by another factor.  $A/C_i$  ratio in flag leaf and second leaf of all lines were similar at booting and flowering stage. This similarity was also maintained in the second leaf at grain-filling stage. The significant difference in  $A/C_i$  ratio was detected in the flag leaf at grain-filling stage.  $A/C_i$  ratio of 1A5A1-A and 20B9B1-A was higher than the ratio of other lines (Figure 17E).

Instantaneous water use efficiency (WUE) parameter was determined by the ratio of net photosynthesis rate ( $A$ ) and transpiration rate ( $E$ ), which implied how much  $\text{CO}_2$  could be fixed per a molecule of  $\text{H}_2\text{O}$  loss. It was clearly seen that WUE of 1A5A1-A was significantly higher than others at booting stage. Although the significant difference in WUE could be detected only at booting stage of flag leaf, WUE of 1A5A1-A tended to be higher than others' (Figure 17F).

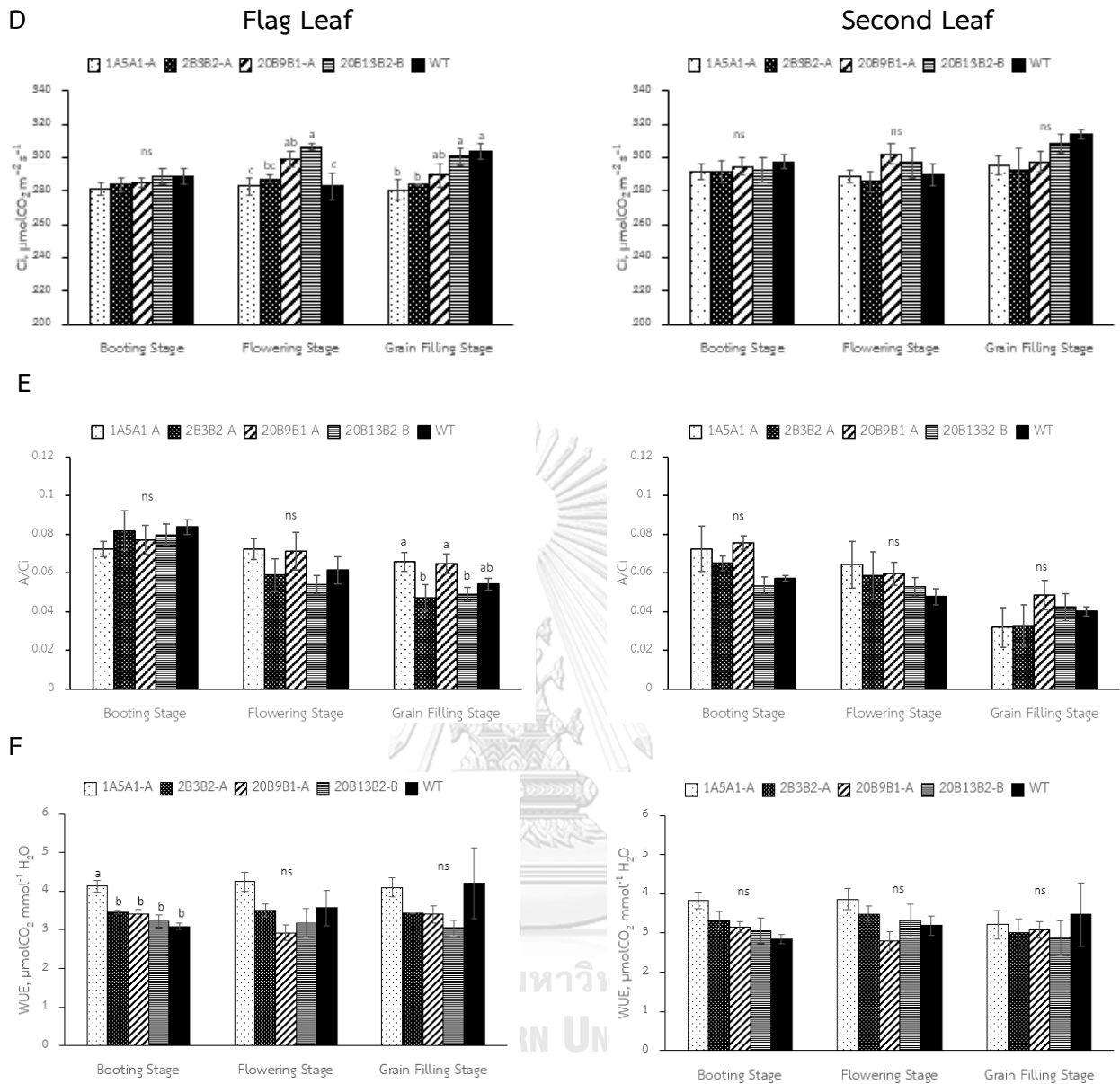


Figure 17. Intercellular  $\text{CO}_2$  concentration ( $C_i$ ) (D), ratio  $A/C_i$  (E), and water use efficiency (WUE) (F) of modified plants and wildtype at reproductive stage. The different letters above the bars represent the significant difference of the mean at  $p < 0.05$ , analyzed with DMRT. Error bars present SE.

## CHAPTER V

### DISCUSSIONS

#### 1. Identification of *Cas9*-free plants

This study shows that seven sgRNAs were successfully designed to alter the *OsGTL1* promoter. To ensure that the CRISPR/Cas9 construct has been segregated out from the genome, screening of the *Cas9*-free transgenic plants is required (Aliaga-Franco et al., 2019). Additionally, besides increasing the risk of off-target mutation, the presence of the CRISPR/Cas9 construct genome can cause difficulties to confirm whether mutations are inherited from previous generations or generated in new generations (Gao et al., 2016). Our data demonstrated that the tillers from the same plant could be free of *Cas9* or contained it in the genome (Figure 6). This raised the possibility that the chimera could form in the embryo. We, later, confirmed that the seedlings, which were the progeny from the *Cas9*-free tiller were *Cas9*-free (Appendix Figure 5).

#### 2. Determination of *OsGTL1* promoter modification in *Cas9*-free transgenic lines

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. It was previously demonstrated that two independent alleles could be obtained by using two sgRNAs (L. Liu et al., 2021). In addition, another research reported the 193 bp to 240 bp deletion could occur using two or more sgRNAs (Aliaga-Franco et al., 2019). We also identified several small deletions, insertions, and mismatches in the target site (Figure 9). Normally, Cas9 created a double-stranded break at a few base pairs upstream of the PAM site. The error-prone nonhomologous end-joining DNA repair can cause small deletion, insertion, or mismatch in the target site (Gao et al., 2016).

Editing the regulatory region of the gene has been considered as 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 (Huang et al., 2020). The editing of several critical nucleotides in regulatory elements might also have an advantage over significant phenotype variations (L. Liu et al., 2021). Another research has demonstrated the use of the CRISPR/Cas editing technique to replace the native *ARGOS* promoter sequence with the *GOS2* promoter sequence in maize to generate a constitutive expression of an auxin-regulated gene implicated in organ size *ARGOS8*. Furthermore, the *ARGOS8* modified varieties demonstrated a significant increase in grain yield during drought conditions while maintaining yield under optimal growth conditions (Shi et al., 2017).

Several functions of *GTL1* have been investigated in some species such as *Arabidopsis*, wheat, and poplar. Based on Yoo et al. (2010) findings, *GTL1* loss-of-function mutants (*gtl1-4*) in *Arabidopsis thaliana* had a greater integrated WUE, resulting in a better survival rate in the mutant after a water shortage. It shows that *AtGTL1* repressed *SDD1* which regulates stomatal density.

Several elements related to various stress responses such as oxidation, light, cold, drought, dehydration, heat, and low temperature have been discovered in *A. thaliana* and *O. sativa* promoter sequences (Kaur et al., 2017). In this case, we have observed that most of the *OsGTL1* edited lines that have big deletion such as line 1A5A1-A, 20B9B1-A, and 20B13B2-B shows decreased stomatal density and smaller size. This modification is assumed to affect the important cis-acting elements in the promoter sequence which also affects the development of stomata. Several cis-elements are deleted such as ABRE, GT1 Box, MYB, MYC, GTGA motif, E-box, and CGCG box.

Many genes expressing various functions have been revealed to have GT-elements in their promoter regions. In the promoter of light-responsive function genes, the GT-element is typically present. However, light does not control many of the GT functions demonstrating that the GT-element is not only a light-specific regulatory module (Zhou, 1999). The highly conserved motif "CGCG box" plays important role in dehydration and calmodulin-binding for signal transduction under dehydration and the other is also related to abiotic stress (Meaux, 2018). ABRE and G-box were highly conserved motifs in dehydration-inducible *Arabidopsis* promoters.

According to Liu et al (2014), in the dehydration-inducible promoters, the ABRE and G-box motifs appeared more frequently. According to their findings, DRE is a conserved motif in dehydration-inducible soybean and rice promoters, with single or multiple DRE core sequences found in 22% (soybean) and 49% (rice) of dehydration-inducible promoters. Therefore, not only morphological change but the modification of these important regions can lead to the adaptation mechanism of the plant response to dehydration stress.

### **3. Assessment of *OsGTL1* promoter modification effects on the stomatal distribution pattern**

Stomatal development in dicotyledonous such as Arabidopsis starts with a subset of protodermal cells called meristemoid mother cells (MMCs), which have a stem-cell fate. Several rounds of amplifying division are performed on these MMCs, with asymmetric division occurring in an inward spiral pattern. Each round of asymmetric division produces a meristemoid, a small daughter cell, a large daughter cell, and a stomatal lineage ground cell (SLGC). Meristemoids differentiate into guard mother cells (GMCs), which go through a single symmetric division and differentiation cycle. Two mirror-symmetric guard cells with a pore in the center develop from this process. SLGCs can either continue spacing division, dividing asymmetrically to form satellite stomata or develop into pavement cells (Han et al., 2021).

Monocotyledonous grass stomatal complexes differ from eudicot stomatal complexes. Rice stomatal complex (SC) consists of two dumbbell-shaped guard cells (GCs) parallel with two subsidiary cells (SBs) (Chatterjee et al., 2020). It is accompanied by subsidiary cells that develop without stem cell-like meristemoid cells and arranged parallel to the leaf vein. GMCs are produced directly when the stomatal lineage is initiated by an asymmetric division of the precursor cells. The nearby cells of newly GMCs take on the fate of subsidiary mother cells (SMCs) and develop polarity toward the adjacent GMC. The GMCs divide symmetrically to create a pair of guard cells after the SMCs asymmetrically divide and develop into subsidiary cells (Han et al., 2021; Hepworth et al., 2018).

The disruptions of core region of the promotor in the gene upstream can have big effect to the stomatal development in the downstream pathway. As the function of *GTL1* as molecular transrepressor of *SDD1* under water deficit condition. According to (Yoo et al., 2010) *GTL1* transcript was abundant when plants were growing with adequate water supply but expression was downregulated by water stress or dehydration. Therefore, we assumed that disruption of important region of *OsGTL1* can reduce its expression and induce the *SDD1* expression. Abundance transcript of *SDD1* initiates more subtilisin-like protease signal to activate ERR and TMM receptors that are known as receptors that negatively regulates the basal stomatal development pathway affecting the lower SD. However, *SDD1* repression by *GTL1* is still required for the formation of stomata during development and to optimize transpiration and CO<sub>2</sub> assimilation (Yoo et al., 2010). Thus, eliminating the *GTL1* function completely is not the best option to increase the plant's ability to survive under water stress condition. The gene expression analysis should be conducted in the next experiment to proof this hypothesis and observe how these modifications affect the *OsGTL1* expression under drought stress conditions.

According to the results, not only change the stomatal density but the modification of *OsGTL1* promoter also affect the morphological change of the stomata which reduced the stomatal size (SS). Decreased SD can lead to smaller GCs showing the positive relationship between SD and SS in some cereal crops such as barley and rice (Hughes et al., 2017; Caine et al., 2019). We assumed that the reduction of stomatal size occurs due to heat exposure during plant growth. It has reported that plants cultivated under water limitation in *Arabidopsis* show no changes in SD, but they do show reductions in SS (guard cell area, based on guard cell pair length and width) (Doheny-Adams et al., 2012). These plastic changes in stomatal number and size allow plants to adapt their stomatal pore area in response to their surroundings, affecting their maximum and minimum gas exchange (Bertolino et al., 2019). Several research also reported that temperature stress including heat stress and chilling stress can reduce the SS in *Brassica oleracea* (Rodríguez et al., 2015). In respond to the high-temperature environment, the size of the stomata has

been reduced in two *Rhododendron* cultivars. Not only reduced the size and they also have lower percentage of open stomata to slow water loss as an adaptive response (Shen et al., 2017).

#### 4. Assessment of *OsGTL1* promoter modification effects on photosynthesis

*GTL1* in the Arabidopsis and poplar affects the efficiency of the plants to have a lower stomatal density (SD) and increase water deficit tolerance by reducing the transpiration rate under drought stress (Weng et al., 2012; Yoo et al., 2010). Therefore, the investigation to determine how the stomatal density affected the photosynthesis capacity was performed.

According to Yoo et al. (2009), lower stomatal density caused by *gtl1* did not affect CO<sub>2</sub> assimilation and biomass accumulation, but it resulted in higher integrated and instantaneous plant WUE. Net CO<sub>2</sub> assimilation rate saturates as stomatal conductance increases due to nonstomatal limitations, such as the regeneration of ribulose 1,5-bisphosphate in most C<sub>3</sub> plants. Transpiration, on the other hand, continued to increase linearly with stomatal conductance across the same stomatal conductance range. As a result, a moderate reduction in stomatal density could significantly reduce transpiration without the CO<sub>2</sub> assimilation effect resulting in increased WUE. It is possible that a more significant reduction in stomatal density would significantly affect CO<sub>2</sub> uptake, as well as WUE and biomass accumulation (Yoo et al., 2010).

In this study, it demonstrated from three edited lines that have lower SD such as 1A5A1-A, 20B9B1-A, and 20B13B2-B, but only 1A5A1-A line showed the decreased stomatal conductance and transpiration rate. Moreover, net photosynthetic rate (*A*) was similar in all lines. This led to the increased WUE as hypothesized. Another research reported earlier on the increase of WUE and soil water conservation, owing to the reduced *E* and *g<sub>s</sub>* rates (Honda et al., 2021). Importantly, *OsEPF1* overexpression reduces stomatal density, which has a proportionally bigger effect on *g<sub>s</sub>* rather than on *A*, or any significant reduction in carbon gain (Mohammed et al., 2019). The similarity in *A* in the flag leaf suggested the importance of photosynthesis at reproduction stage, especially at booting stage. The result also suggested that flag

leaf is the most essential leaf in the rice canopy during reproductive stage. According to Adachi et al. (2017), due to its position at the top provides maximum light availability and it has a higher photosynthetic capacity than lower leaves in the canopy. Although the edited plants contained lower stomatal density than WT, they tried to maintain the photosynthesis capacity during the reproduction stage. Thus, it helped to increase WUE significantly in some line.

We would like to mention about the high temperature condition during the experimental period. This factor might affect the photosynthesis performance. From the results, most of the edited lines such as 1A5A1-A, 20B9B1-A, and 20B13B2-B showed different phenotypes as hypothesized under normal conditions. Some edited lines have lower SD even without drought treatment. It could be due to high temperature during the growing time as shown in the appendix which can cause dehydration and induce stomatal changes. The highest daylight temperature reached 42.9°C during the observation time which was not an optimum temperature for japonica rice. Several studies have been investigated that heat stress mostly affected the reproductive stage rather than the vegetative stage. In crops, the vegetative stage is relatively more tolerant to high temperatures, while heat stress effects are largely limited to reduced reproductive capacity and seed set during flowering, and decreasing grain filling rate and the ripening stage period (Chaturvedi et al., 2017). Therefore, we assumed that even these edited plants had the lower stomata density, but they need to adjust the photosynthesis capacity to fulfill energy requirements for the seed development during the reproductive stage.

## CHAPTER VI CONCLUSION

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. Based on the anatomical observation, it was found that 1A5A1-A, 20B9B1-A, and 20B13B2-B edited lines had a lower stomatal density and smaller size than wild-type plants which also affected photosynthesis performance, including photosynthesis rate, stomatal conductance, transpiration rate, internal CO<sub>2</sub> concentration, and water use efficiency (WUE). The *OsGTL1* promoter modification in the critical *cis*-elements involved in stress response, such as ABRE, GT1 Box, MYB, MYC, GTGA motif, E-box, and CGCG box was detected. Therefore, the edited *OsGTL1* promoters are expected to lead to changes in stress response, which should be studied in the future.

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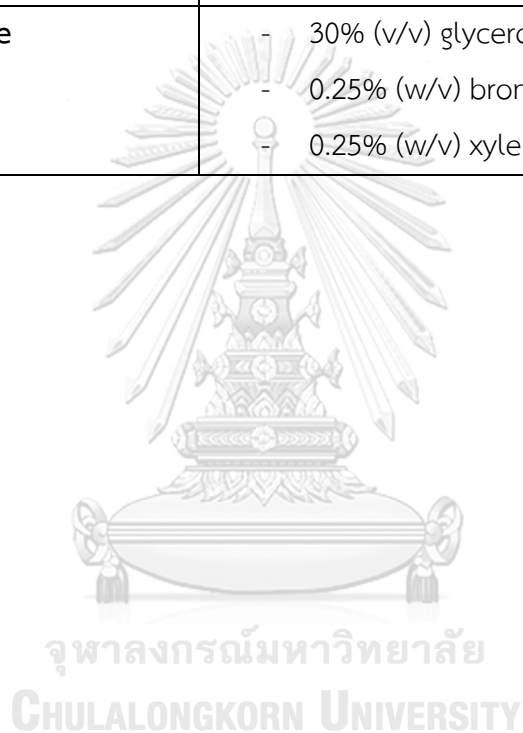
## APPENDIX

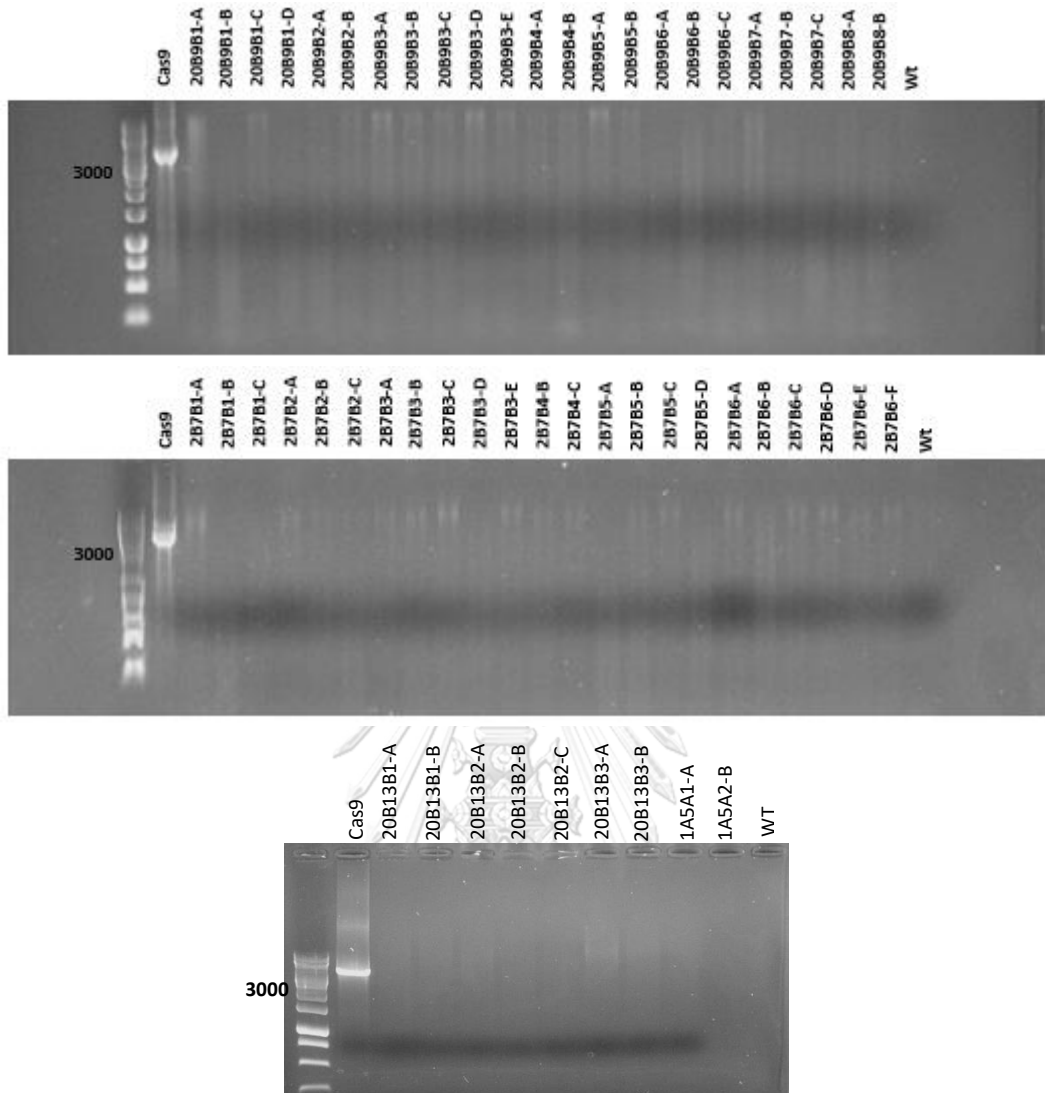
**Appendix Table 1.** Primer sequences and PCR condition for Cas9 screening and *OsGTL1* amplification

Primers	Sequences	Product size	PCR Condition
Cas9-Fw	TCCTGCAGACAGTGAAGGTG	532 bp	Pre-denaturation: 95°C for 3 min 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-Rv	GCCTTATCCAGTTCGCTCAG		
Cas9-Fw (Full)	CTTACCCTATCTGTTTGGTG	4445 bp	Pre-denaturation: 95°C for 3 min Denaturation: 95°C for 30 sec 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
Cas9-Rv (Full)	CGCTGTTATCAACCACTTTG		
<i>OsGTL1</i> -Fw	GCTTGAAGGAGATGGAGAGC	1684 bp	Pre-denaturation: 95°C for 3 min 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
<i>OsGTL1</i> -Rv	GTATAAAGCGAAAGCGTGTG		

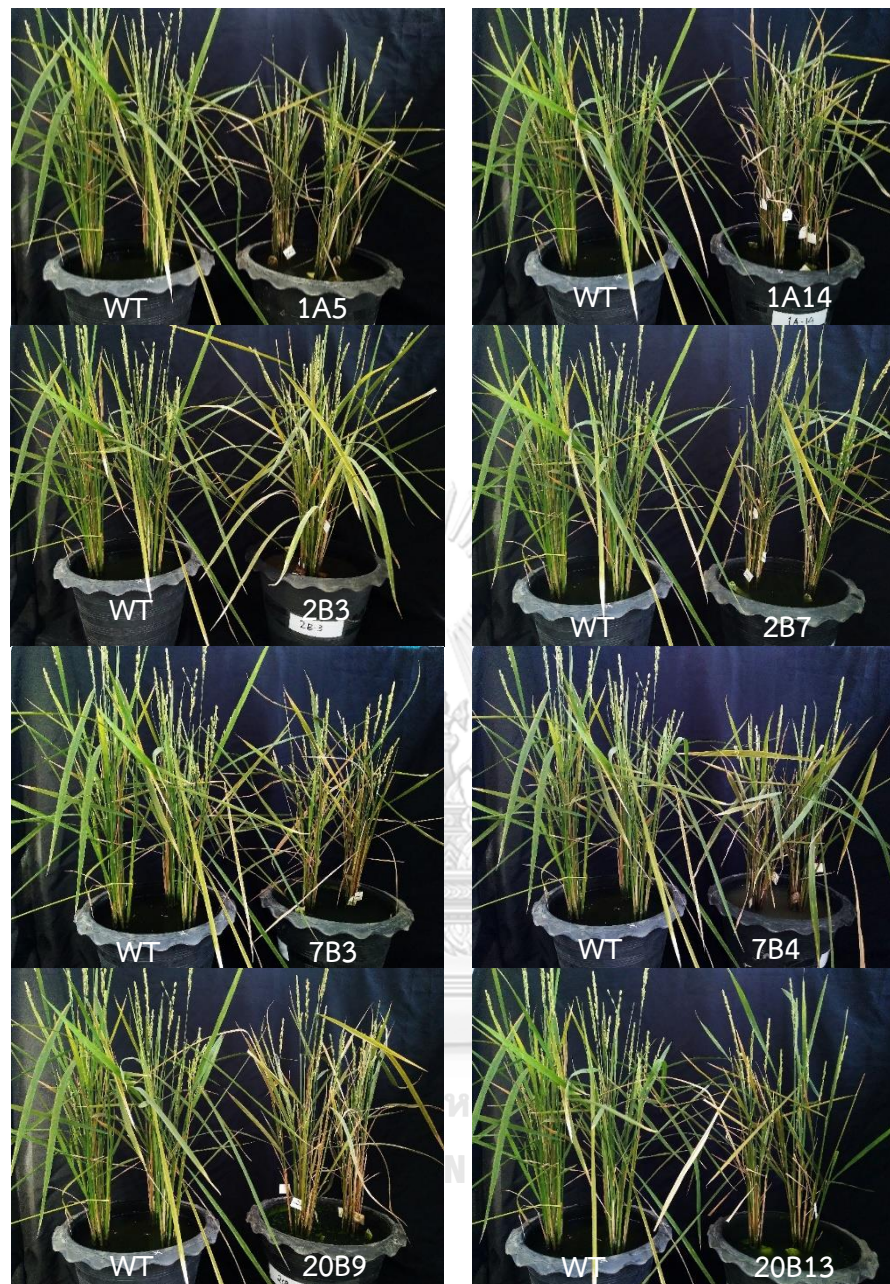
Appendix Table 2. Chemicals, reagent and buffer preparation

1. EDTA 0.5M pH 8	<ul style="list-style-type: none"> <li>- Disodium EDTA•2H<sub>2</sub>O 186.1 g</li> </ul> Adjust the volume to 1000ml with distilled H <sub>2</sub> O Adjust pH with NaOH or HCl
2. 5X TBE buffer	<ul style="list-style-type: none"> <li>- Tris base 54 g</li> <li>- Boric acid 27.5 g</li> <li>- 0.5 M EDTA pH 8.0 20 ml</li> </ul> Adjust the volume to 1000ml with distilled H <sub>2</sub> O
3. 6X Loading dye	<ul style="list-style-type: none"> <li>- 30% (v/v) glycerol in water</li> <li>- 0.25% (w/v) bromophenol blue</li> <li>- 0.25% (w/v) xylene cyanol FF</li> </ul>





**Appendix Figure 1.** Gel image for screening of Cas9-free tillers by using polymerase chain reaction (PCR). The product size of this primer was approximately 4445bp.



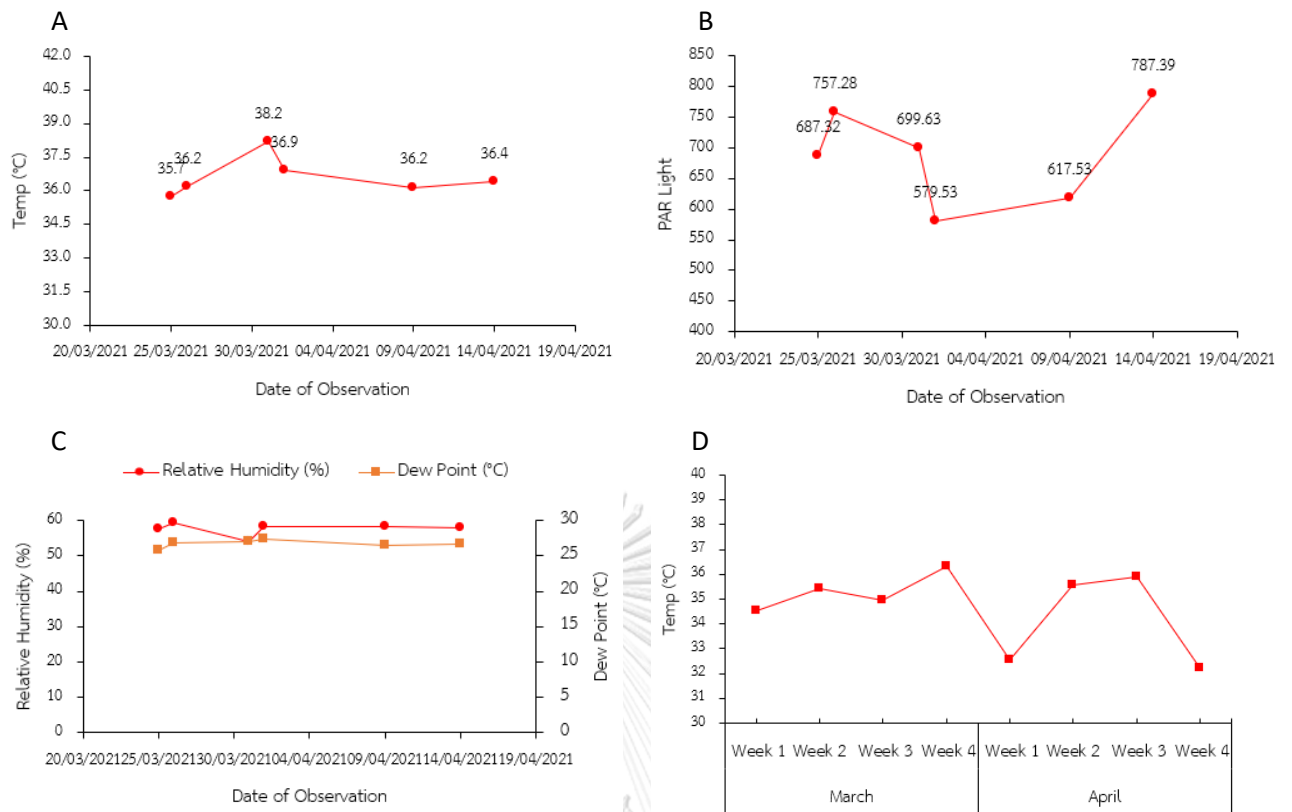
**Appendix Figure 2.** Phenotype parameters observations in edited lines and wild-type plants.

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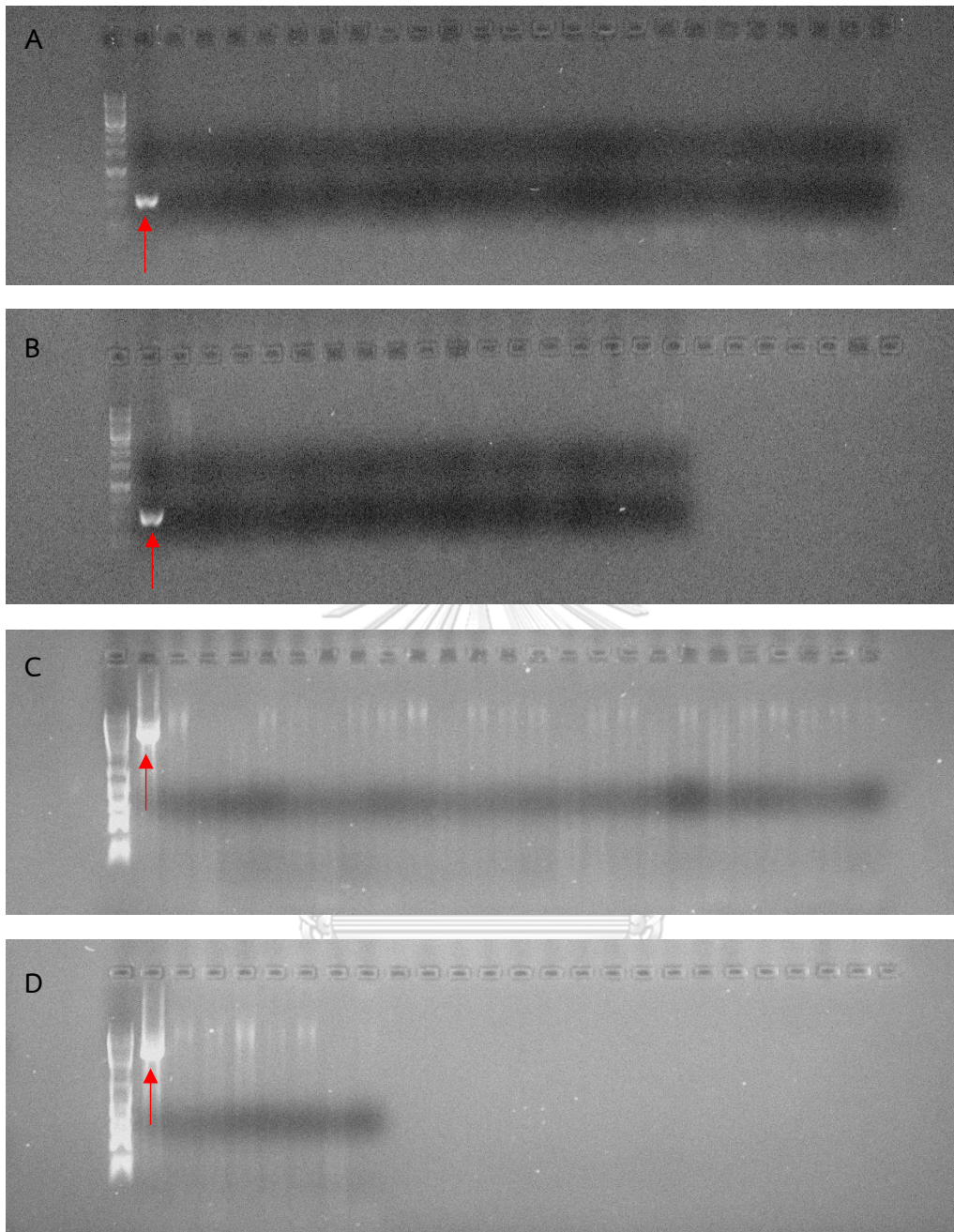
WT          GATAGAGGAGAATTAAGCGGTAGGAGGGTGGACGAGAGTGGCCAGACAAGGCCCACTGT
1137
1A5A1-A    --TAGAGGAGAATTAAGCGGTAGGAGGGTGGACGAGAGTGGCCAGACAAGGCCCACTGT
1080
          *****
          gRNA 4
WT          AACAGCCTGTGTGGTGGGCCCGGCAGGGCAGGGGTAGAGAGAGAGAGATGGTGGTGGTGG
1197
1A5A1-A    AACAGCCTGTGTGGTGGGCCCGGCAGGGCAGGGGTAGAGAGAGAGAGATGGTGGTGGTGG
1140
          *****
          gRNA 3
WT          CGCGCAACGTGCATCCAGAGAGGGATGTGAGTGAGTATAAACGCGAACGGACCCCTC
1257
1A5A1-A    CGCGCAACGTGCATCCAGAGAGGGATGTGAGTGAGTATAAACGCGA-----
1186
          *****
          gRNA 2
WT          TCGCTAACCCACACGCCACGTTGCGTACCACACAGGTCACTCCCATTCTAGCTCAGC
1317
1A5A1-A    -----GTTGCGTACCACACAGGTCACTCCCATTCTAGCTCAGC
1224
          *****
          gRNA 1
WT          TAAGCTTGATTAGGTACTAGTGCTAGTTGCTAGTAACATGCATGCATGCATCCACATGTT
1377
1A5A1-A    TAAGCTTGATTAGGTACTAGTGCTAGTTGCTAGTAACATGCATGCATGCATCCACATGTT
1284
          *****
WT          TTTGATTCTTGAACCTTATTCATTTTACTGCTAATTAATCAAATGGTATAGTAGAGAA
1437
1A5A1-A    TTTGATTCTTGAACCTTATTCATTTTACTGCTAATTAATCAAATGGTATAGTAGAGAA
1344
          *****

```

Appendix Figure 3. Alignment of *OsGTL1* promoter in Line no. 1A5A1-A compared to the wild-type sequence.



**Appendix Figure 4.** The trend graph of environmental conditions during daylight time of photosynthesis observation. Temperature (A), Light intensity (B), and Relative humidity (C), and weekly temperature (D). The data shown above is an average of daylight data from 8.00 a.m. to 5.00 p.m. the highest temperature is 42.9°C. These data were retrieved from Watchdog weather forecast.



**Appendix Figure 5.** Gel image for screening of Cas9-free plants by using polymerase chain reaction (PCR) in  $T_4$  generations. Cas9 short primer (A, B) product size 532bp, full Cas9 primer (C, D) product size 4445bp. All the tested plants are Cas9-free. Red arrow shows the positive control (pRGEB32).

## VITA

**NAME** Widya Fajariani

**DATE OF BIRTH** 08 June 1996

**PLACE OF BIRTH** Samarinda

**INSTITUTIONS ATTENDED** Mulawarman University

**HOME ADDRESS** Phetchaburi 7, Ratchatewi, Bangkok 10400

**PUBLICATION** Fajariani, W., Hungsaprug, K., Lynagh, P. G., Comai, L., & Chadchawan, S. 2021. OsGTL1 promoter editing using CRISPR/Cas9 in rice *Oryza sativa* L. Proceedings of the 7th International Conference on Biochemistry and Molecular Biology (6-7 July 2021), Bangkok, Thailand

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