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Efficacy of Conditional and Constitutive CRISPR/Cas9 in Rice Genome

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Efficacy of Conditional and Constitutive CRISPR/Cas9 in Rice Genome

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Abstract

Constitutive expression of Cas9 leads to a higher editing efficiency; however, it also increases the chances of off-target mutations. Thus, transient expression of Cas9 is a desirable approach to achieve higher targeting efficiency and to curb the off-target effects. It was previously shown that heat-inducible expression of Cas9 had an editing efficiency of 45% as compared to the strong constitutive expression, and the heat-shock induced mutations were inherited by the next generation. In this study, cold-inducible promoter, *AtRD29a*, was used for driving Cas9 expression, and evaluated for its editing efficiency upon cold-treatment on the *GUS* transgene loci. The expression analysis of Cas9 before and after cold-shock showed ~ 2.5x increase in the expression, which is ~1000x lower as that of constitutive expression of Cas9 by rice ubiquitin-1 promoter (OsUbi1). Further, the targeting efficiency of Cas9 expressed under constitutive (OsUbi1:Cas9), cold-shock (AtRD29a:Cas9) or heat-shock (HSP:Cas9) promoters was tested on rice Target of Rapamycin (*OsTOR*) gene. The *OsTOR* gene is known to regulate various anabolic processes such as cell cycle, ribosome biogenesis, and photosynthesis. The HEAT repeat regions and kinase domain region of *OsTOR* were targeted. Among 21 primary transgenic (T0) plants of OsUbi1:Cas9 representing 9 independent transgenic lines, HEAT site was found to be wild type (WT), while monoallelic or biallelic mutations were observed in the Kinase site in 4 plants representing 3 independent lines. The analysis of 33 T1 progeny studied from three T0 plants showed that 22 (66%) inherited the mutations at Kinase domain; but no *de-novo* mutations in HEAT site were observed. In HSP:Cas9 and AtRD29a:Cas9 lines, none of the 40 T0 plants, which represented 17 independent lines exhibited mutations in either site. The expression analysis of a subset of these lines, showed 2-11x and 2-43x induced transcript levels of Cas9 in HSP:Cas9 and AtRD29a:Cas9 lines, respectively, indicating proper regulation of the Cas9. Beside *OsTOR* gene,

the targeting efficiency of AtRD29a-, HSP- and OsUbi1-CRISPR/Cas9 was tested on the *OsPDS* gene, the key enzyme in carotenoid biosynthesis pathway. The analysis of Cas9 positive samples in these lines showed only WT sequences in all plants except for one OsUbi1:Cas9, which harbored biallelic mutations, indicating overall lower targeting efficiency in this experiment. In summary, the expression of Cas9 under heat and cold inducible promoters was under proper regulation as it did not exhibit any mutations at room-temperature. However, more analysis is needed to determine whether cold-induced expression of Cas9 by *AtRD29a* promoter is sufficient to create targeted mutations in the rice genome.

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Chapter 1

Introduction and literature review

1.1 CRISPR/Cas9 system as a gene editing tool

The CRISPR (clustered, regularly interspaced, short palindromic repeats)/Cas9 is a site-specific nuclease that has become a dominant genome editing tool, especially in creating targeted mutations in the genome. CRISPR/Cas9 is found in bacteria and archea, where it provides protection against invading plasmids and viruses by recognizing them through homology with CRISPR RNA and subsequent degradation by the Cas9 nuclease (Wiedenheft et al., 2012; Jinek et al., 2012; Mali et al., 2013). During this immunity process, some "spacers" fragments from the foreign DNA are incorporated into the CRISPR genomic locus, and act as a defender when other bacteria or viruses attack the host in the future. In this situation, the mentioned "spacers" are transcribed into the crRNAs. In the next step, crRNAs attach to the trans-activating crRNA (tracrRNAs) and bind Cas nuclease. This RNA-guided nuclease complex eventually creates the double stranded break (DSB) in the invading DNA, with a simple targeting rule: (a) the crRNA should have a short homology (e.g., 20 bp nucleotide) with target sequence, (b) on the target sequence, there should be a protospacer adjacent motif (PAM) at the 3' end of the target sequence (Gaj et al., 2013; Jinek et al., 2012; Mali et al., 2013; Voytas et al., 2013). In the breakthrough study by Jinek et al. (2012), the crRNA-tracrRNA complex was simplified into a shorter molecule called as single-guide (sg) RNA or guide (g) RNA that could bind Cas9 and target DNA. Cas9 is the most commonly used Cas type enzyme which is derived from *Streptococcus pyogenes* and recognizes the NGG PAM sequence (N can be any of A/T/C/G). In case of CRISPR/Cas9, the double stranded break (DSB) is created between the third and fourth nucleotide upstream of the PAM (Lee et al., 2019) **(Figure 1.1)**.

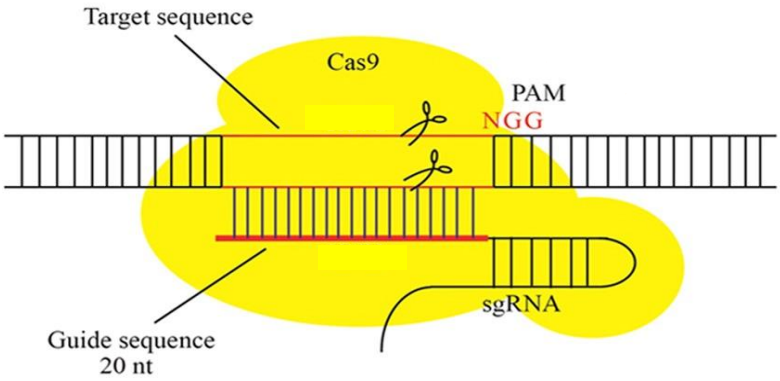


Figure 1.1 A schematic of CRISPR/Cas9 gene targeting. The sgRNA (black and red) being responsible for identifying the target gene, and then the two domains of Cas9 (yellow) cleave the target sequence in 3 nucleotides in upstream of NGG PAM (written in red). (Liu et al., 2017)

1.2 Off target effects of CRISPR/Cas9

In genome editing, there might be multiple DNA sequences in large genomes which bear similarity to the target sequence, so the CRISPR/Cas9 could also target them and create unwanted mutations or off-target effects. These mutations might lead to the genetic effects that can cause discrepancy between genotype and phenotype (Yee, 2016) or cause the cell death. Hence, it is important to remove or reduce them in the cell (Cong et al., 2013; Fu et al., 2013; Hsu et al., 2013; Xiao et al., 2014). To increase the specificity of Cas9 function, choosing the target site with the lowest off-target predictions or a lower rate of nuclease activity are most important. However, off-target effects to the regions of low homology could be created by high doses of gRNA:Cas9 complex (Hsu et al., 2013; Pattanayak et al., 2013).

Off-target effects have raised concerns for the practical applications of gene editing by CRISPR/Cas9. To avoid off-target effects in the genome that could lead to genetic destabilization and disruption of the normal cellular function, it is critical to enhance the precision and

effectiveness of genome editing (Corrigan-Curay et al., 2015; Mali et al., 2013). So far, scientists have not found any method to analyze the off target effects of CRISPR in essential genes because mutations in essential genes could cause lethality (Singh et al., 2016); however, various approaches have been employed to enhance on-target mutagenesis and reduce off-target activities on non-essential genes such as conditional expression of Cas9 under heat-shock promoter (Nandy et al., 2019), using *egg cell-specific* promoter and *Dmc1* maize promoter for germline expression of Cas9 (Feng et al., 2018; Wang et al., 2015), light-activated Cas9 (Hemphill et al., 2015; Chen et al., 2017), applying chemical changes in gRNA structure and temporarily inhibiting Cas9 expression (Wang et al., 2020; Ankenbruck et al., 2018; Luo et al., 2016; Kadina et al., 2018), and creating DDCas9 as a limited expressed Cas9 (Banaszynski, 2006; Senturk et al., 2017).

1.2.1 Strategies for enhancing the targeting efficiency and decreasing off target effects

There are various strategies devised to address the issues of enhancing on-target efficiencies and reducing off-target effects in eukaryotes. One of these strategies is the use of tissue-specific promoters. Wang et al. (2015) utilized a new *egg cell-specific* promoter with two different terminators for expressing Cas9 in *Arabidopsis*. It resulted in homozygous, biallelic, and triple mutations of *ETC2*, *TRY*, and *CPC* (an easily observable phenotype of clustered leaf trichomes) instead of only mosaic effects. According to the findings, *rbcSE9* terminator functioned more efficiently on Cas9 mRNA stability rather than *NOS* terminator in the egg cell. This outcome suggests that not only choosing the proper promoter is important but terminator codon is also important for gene regulation. Authors observed 8.3% bi-allelic mutations among T1 generation and 50% of the T2 plants harbored the same homozygous mutations. In another research, Feng et al. (2018) observed that *Dmc1* maize promoter that expresses in the meiocytes (Klimyuk & Jones, 1997), could also improve gene editing. In maize, *dmc1* and *U3* promoters were applied to drive

Cas9 and sgRNA, respectively, to target *zyp1, smc3,* and *zb7* genes. It showed that *dmc1* promoter was effective in generating 21.3%- 57.4% bi-allelic targeted mutations in all three genes. These mutations were also transmitted to progeny, and the genome resequencing data showed no off-target mutations in the sites bearing three or more mismatches with the target sites.

Light activated expression of CRISPR/Cas9 is another approach for controlling the expression of Cas9 and reducing the toxic effects of off-target mutations. In the light activated approach, a photo-chemically removable group known as "caged group" is attached to the amino acid of the protein, which renders the protein inactive. When the protein is exposed to specified light condition, the caged group dissociates and makes the protein functional. Using this approach, Hemphill et al. (2015) generated a 'caged lysine' at K866 position of Cas9. Authors used K866 site as this residue was observed to be the closest to the gRNA binding site. Using this approach, authors targeted the *TfR1* gene, a transmembrane transferrin receptor associated with leukemia and lymphoma in human cells. They compared the mutagenic frequency of the caged-Cas9 and non-caged Cas9 system and found that both were equally effective in targeting *TfR1* gene, where non-caged and caged Cas9 had repressed the transcript levels to 70% and 60% respectively. The caged-Cas9 became functional when it was exposed briefly for 120s at 365nm (UV light), and it was not active when it was not exposed to UV light. However, authors did not test the off-site mutations for caged-Cas9.

Another option for conditional expression of Cas9 and reducing the off-target effects is controlled activation of gRNA. Post synthetic masking of gRNA and its chemical activation can lead to a change in structure and the function of gRNA (Wang et al., 2020). It has been shown before that when a specific moiety is attached to RNA, it prevents the linkage of the RNA to other biomolecules (Ankenbruck et al., 2018). Wang et al. (2020) used this approach for controlling

gRNA expression by linking the azidomethylnicotinyl (AMN) groups to gRNA. This linkage would arrest gene editing by preventing the attachment of gRNA to Cas9. For removal of AMN, authors performed a treatment with phosphine (PH_3) - a colorless, flammable, and toxic gas, which acts on the azide group of AMN (Staudinger reduction), resulting in spontaneous loss of acyl group. This loss fully restores the RNA folding and activates the formation of Cas9-gRNA complex. Using this approach, authors obtained a controlled *in-vivo* gene editing of *HBEGF* and *ANTXR1* in the Cas9 expressing HeLa human cell lines.

Another study used the approach of conditional destabilization of Cas9. Sentruk et al. (2017) showed that attachment of the FKBP12- derived destabilizing domain protein to Cas9 creates DDCas9 that enables conditional expression of Cas9. By this way, the authors created a vector including *U6* and *EFS* promoter for driving sgRNA and DD-Cas9, respectively. The vector was transformed into *A549* cells and the level of Cas9 expression was measured in the presence and absence of shield-1 by western blot and RT-PCR. Here, Shield-1 is a synthetic ligand that while attaching to DD-Cas9 can protect it from degradation. Thus, it would be expected from RT-PCR results that the level of expression be high in the presence of shield-1 because there is no degradation of Cas9. In this concept, the cells treated by shield-1 for 2 hours showed a strong expression of Cas9 than the uninfected cells. Thus, this strategy provides a ligand mediated control on the function and stability of Cas9. Using this system, authors were able to edit multiple genes including *EGFR, TP53, CypD* and *RPA3* in a controlled manner, and with a similar efficiency when compared with Cas9 expressed under the constitutive promoter (Sentruk et al., 2017).

Employment of heat-shock inducible promoter for the expression of Cas9 enzyme is another successful approach in gene editing. Using this approach in our lab, Nandy et al. (2019) showed that rate of mutagenesis on *GUS* and *OsPDS* genes in rice could be controlled by simple heat

treatments. This study showed > 50% increase in the rate of targeted mutagenesis upon heat-treatment as compared to ~16% at the room-temperature. Moreover, the heat-treated lines showed no detectable mutations or a low rate of mutations on the off-target (OT) sites (no off-targeting in *GUS-OTs* and ~22% in *OsPDS-OTs*). In comparison, the rice lines expressing constitutive Cas9 showed 4-30% mutations in *GUS-OTs* and ~62% mutations in *OsPDS-OTs*. Most importantly, the on-target mutations in heat-treated lines were transmitted to the progeny at a high rate (Nandy et al., 2019). In general, the heat treatment has been shown to have a positive effect on genome editing efficiency in *Arabidopsis* and *Citrus* plants (Leblanc et al., 2018). In *Arabidopsis*, in targeting of *GFP* reporter gene, the plants were subjected to 37°C instead of 22°C. In comparison to the room temperature (22 °C), the treatment at 37 °C increased mutation rates by 5 folds in somatic tissues and >100 folds in germline when Cas9 was expressed by the *YAO* promoter. Also, a similar increase in the targeting efficiency of *CsPDS* in Citrus plants was observed, when these plants were exposed to the seven times repeated heat-stress at 37°C for 24 h, as compared to controls which were grown continuously at 24°C. Presumably, the positive effect of heat treatment on gRNA levels *in vivo* led to the increased targeting efficiency (Leblanc et al., 2018). According to these observations, heat-shock treatments can confer a synergistic effect in the overall efficiency of genome editing by CRISPR/Cas9. However, it remains to be tested, if heat-stress, in plants expressing high doses of Cas9 also increases the off-target activity.

1.3 Overall Goal and Specific Objectives:

In continuation to our previous study on heat-shock inducible CRISPR/Cas9 system (Nandy et al., 2019), this study investigated the targeting efficiency of another stress inducible *AtRD29a* promoter from *Arabidopsis*, which is responsive to cold, salt, and drought in rice. This promoter can induce the transcript levels up to 30-100-fold in rice, when exposed to cold-stress

(Pathak & Srivastava, 2020). This efficiency was also compared with the targeting efficiencies of Cas9 expressed under constitutive and heat-shock promoters using the same genomic target sites. Thus, specific objectives of this research were:

1- Efficacy of the cold-inducible CRISPR/Cas9 system consisting of the *AtRD29a:Cas9* gene in rice
2- Comparison of the cold-inducible Cas9 and heat-inducible Cas9 on targeting rice loci
3- Inheritance of CRISPR-induced mutations in *OsTOR*, an essential gene in rice

Chapter 2.

Efficacy of the cold-inducible CRISPR/Cas9 system consisting of the *AtRD29a:Cas9* gene in rice

2.1 Introduction

GUS is a gene from *Escherichia coli (E. coli)* that encodes β-Glucuronidase, a sugar consuming enzyme. *GUS* gene has been known as a screenable marker in plants since there is no expression of this gene in plants and the bacterial *GUS* gene would not disrupt the plant intrinsic gene expression. The only required step to make *GUS* gene as a reporter gene in plants is to drive it by the favorite promoter and then track its expression pattern to see the optimal function of the promoter in different experiments. As Jefferson et al. (1987) stated, GUS staining technique plays an important role in plant molecular biology and is usable for diverse applications.

In the current study, to determine the effect of cold-inducible expression of Cas9 on targeting *GUS* transgene, the cold-shock promoter, *AtRD29a* derived from *Arabidopsis*, was used to control the expression of Cas9. In our previous study, exposure of plants to cold conditions was shown to induce the expression of the *Arabidopsis AtRD29a* (Pathak & Srivastava, 2020).

Arabidopsis *RD29a* and *RD29b* are homologous genes, which respond to abiotic stress (Yamaguchi-Shinozaki & Shinozaki, 1993). The function of proteins encoded by these genes has not been clearly understood. Hence, Msanne et al. (2011) expressed RD29a and RD29b cDNA under the control of their native promoters in the rd29a and rd29b T-DNA knockout lines of *Arabidopsis*. To achieve this, four binary vectors with *RD29a* and *RD29b* promoters, responsible for driving the *RD29a* and *RD29b* cDNAs, were used for transformation. After cold, drought, and salt induction, *RD29a* was found to be more reactive to drought and cold stress and *RD29b* showed more response to salt stress (Msanne et al., 2011).

Further, it was shown in another study by Yamaguchi-Shinozaki & Shinozaki (1993) that *AtRD29a* induced the expression of *GUS* transgene not only in response to cold stress but also to

drought and salt conditions in both *Arabidopsis* and tobacco. Through histochemical analysis, they found that *AtRD29a* promoter functions in all tissues of the plants when exposed to drought stress.

2.2 Materials and Methods

2.2.1 Plasmid constructs

The pNS70 (AtRD29a:Cas9) and pNS62 (U3:GUS sgRNA) (**Figure 2.1a,b**) were co-transformed in the rice B1 line (Nandy & Srivastava, 2012) that contains a single copy of the *GUS* transgene driven by maize *ubiquitin* promoter (**Figure 2.1c**). The pNS70 contained *Arabidopsis RD29a* gene promoter driving the expression of *Streptococcus pyogenes* Cas9 with nos *3'* transcription terminator (T) (**Figure 2.1a**). The sgRNA construct, pNS62, contained rice *snoU3* promoter expressing two sgRNAs of *GUS* via tRNA processing mechanism (Xie et al., 2015). The Pol III terminator, TTTT, serves as a transcriptional terminator for sgRNA genes. For the plant selection, *35S* promoter driving the expression of *hygromycin phosphotransferase (HPT)* gene with nos *3'* transcription terminator was included in the pNS62 construct (**Figure 2.1b**) (Nandy et al., 2019).

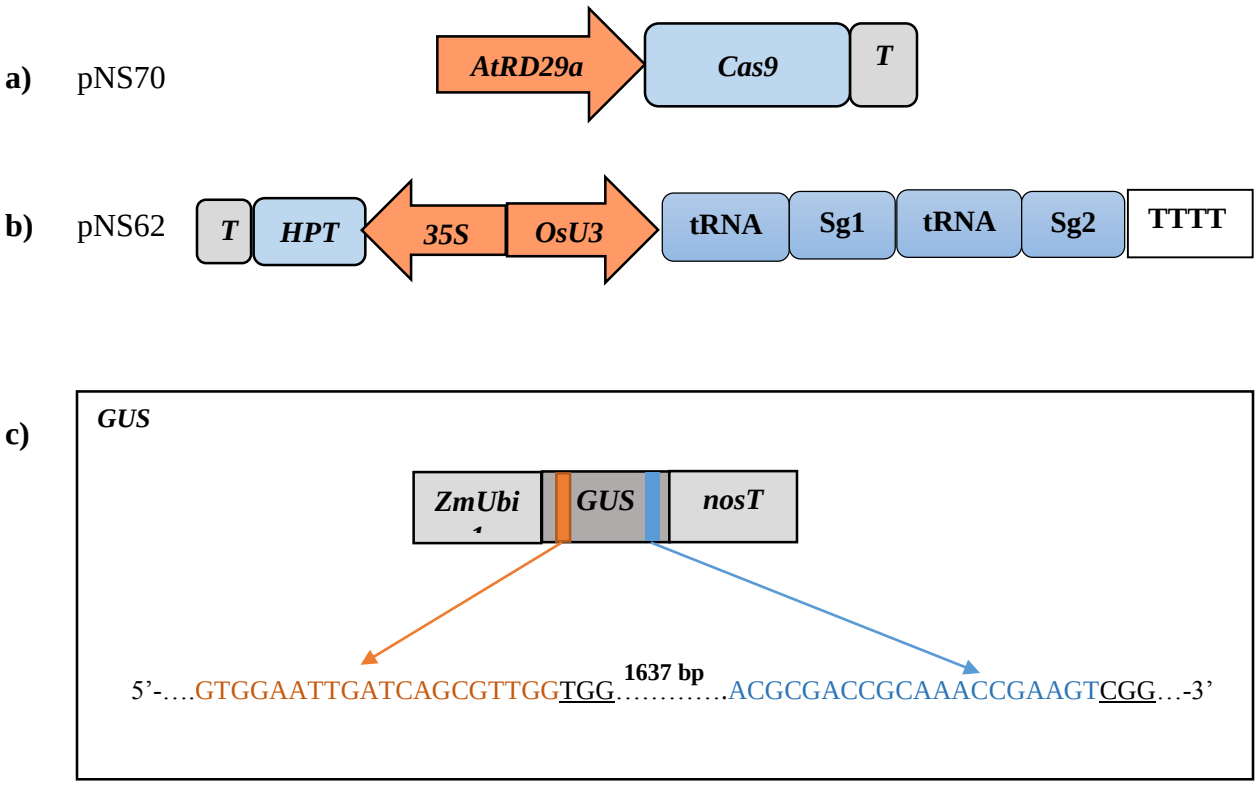


Figure 2.1 Vector constructs and target sites depiction. **[a]** pNS70 contains cold-inducible *Arabidopsis RD29a* gene promoter and *Streptococcus pyogenes* Cas9 coding sequence followed by nopaline synthase 3' sequence as the transcription terminator (T); **[b]** pNS62 GUS-sgRNA construct based on tRNA processing mechanism (Xie et al., 2015) consists of rice sno U3 promoter expressing a pair of *GUS* sgRNAs. *Hygromycin phosphotransferase* (HPT) consisting of *35S* promoter and nos 3' terminator (*T*) serves as the plant selection marker. Image is adapted from Nandy et al., (2019); **[c]** Position of target sites of *GUS* in this study**.** Target sites 1 and 2 are represented with orange and blue bars, respectively. The PAM site is underlined. The predicted double-stranded break site is indicated with hyphen. The distance between two target sites is shown in the bp. *ZmUbi1*: Maize Ubiquitin promoter; *T*: nopaline synthase 3' terminator; polyT: RNA Pol III terminator.

2.2.2 Rice transformation, selection, and regeneration

For *GUS* targeting, the calli was initiated from B1 seeds in Nipponbare background (Nandy & Srivastava, 2012) on 2N6D media. *B1* locus contains a homozygous *GUS* transgene driven by maize *ubiquitin* promoter and a *nos* terminator. The 4-week-old embryogenic calli was co-bombarded using biolistic gene gun. In this, a mixture of 25 µl gold suspension (60 mg/ml) was mixed with 25 µl of autoclave water and 10 µl of the plasmid DNA (1 mg/ml) in one 1.5 ml tube. Then it was properly mixed, and any bubble was removed. A drop of 50 µl CaCl2.2H2O (2.5M) and 20 µl Spermidine (0.1M) were added to the tube, and it was vortexed for 3 minutes. The solution was centrifuged, supernatant was removed, and it was washed twice with 200 proof ethanol. At last, 120 µl 200-proof Ethanol (for around every 10 plates) was added and dissolved by rubbing over pipette box (dissolving by pipetting leads to Ethanol evaporation) and it was used for bombardment using PDS1000/He gene gun.

After doing the bombardment, calli were transferred to 2N6D media for one week for the recovery. Then the putative transgenic calli were selected and regenerated on 2N6D and regeneration media, respectively containing hygromycin (50mg/L). The regenerated primary transgenic lines (T0) were subjected to rooting media for roots initiation. The T0 lines were later transferred to the greenhouse and were subjected to the downstream analysis.

2.2.3 GUS staining

The GUS staining was performed as described by Jefferson (1987). A small leaf tissue from regenerated primary transgenic lines were incubated overnight at 37 °C in the GUS staining solution consisting of the 1 mM X-Gluc (Gold Biotechnologies, St. Louis, MO, USA) and the results were visualized next day under Leica Stereoscope.

2.2.4 DNA extraction

For molecular analysis, DNA was extracted from the leaf tissue of transgenic plants by rapid gDNA extraction protocol (Edwards et al., 1991). In this method, 1 small leaf tissue was placed in 1.5 ml tube with some glass beads (50-100 µl) and it was ground for a few seconds (based on the tissue size) in a tissue lyzer. Later, 200 µl of SDS-Lysis buffer was added immediately to every sample and vortexed. Then samples were centrifuged in 15000 rpm for 10 min. In the next step, 100 µl of supernatant was transferred to the new tube and 100 µl of iso-propanol was added. The tube was incubated at room-temperature for 5 min and centrifuged for 10 min at 15000 rpm. Then supernatant was removed completely without disturbing the pellet. 150 µl of 70% ethanol was used for washing the pallet and centrifuged for 3 min in at 15000 rpm. Once again supernatant was removed, and the tubes were kept at RT for 5 min to be air dried. At the end, each sample was resuspended with 40 µl of water and used as genomic DNA for the rest of the experiment.

2.2.5 Polymerase chain reaction (PCR)

PCR was performed using Takara Max Amp RT kit in a 15µl volume consisting of 7.5µl Takara Premix, 5.5µl H_2O, 1µl each of forward and reverse primers (2-5 µM) and 1 µl of DNA. The PCR conditions used were 96°C for 1 min for denaturation, 56°C for 1 min for annealing, 72° C for 1 min for elongation. Cycle was repeated for 39x and then it was set for 72°C for 15 min followed by 4°C hold to make the samples cold.

Quantitative real time PCR (qRT-PCR) was performed for expression analysis of Cas9. Total RNA was isolated using Trizol reagent (Thermo-Fisher Scientific). Total RNA was treated with the DNase for the removal of any genomic DNA. One microgram of DNase treated RNA was used for the conversion of cDNA using PrimeScript RT reagent kit (Takara Bio, CA, USA). The *Cas9* gene expression analysis (RT-qPCR) using TB green Premix Ex Taq II (Takara Bio, CA,

USA) was performed on Bio-Rad CFX96 using the following program: 95°C for 30 sec and 40 cycles of 95°C for 5 sec + 60°C for 30 sec. The components for each qPCR reaction were: 4.25µl water, 6.25µl 2X Takara premix, 0.5 µl each of forward and reverse primers (10µM) and 1µl cDNA.

2.2.6 DNA sequencing

The target sites were amplified by the PCR primers flanking them as mentioned in table 2.1. The bands were eluted from 0.8%-1% gel depending on the product size. The DNA was purified from the gel using GeneJet Gel Extraction Kit (Thermo-Fisher Scientific) and eluted in 20µl water. Each of the 1µl eluted samples were visualized on the gel. Each of the 10µl extracted samples and 10µl of 2µM primers were sent to Macrogen USA for sanger sequencing.

2.2.7 Sequence analysis

The sanger sequencing chromatograms (.abi) were analyzed by CRISP-ID program (Dehairs et al., 2016). Based on the sequence quality, the start and end position of the sequences were determined; and the background cut off % for noise removal was kept at 20-40% in order not to miss the mutation peaks. The parsed-out sequences were then aligned with the reference using CLUSTAL-OMEGA.

2.3 Results and discussion

The co-transformation of pNS62 and pNS70 on B1 callus resulted in 6 transgenic events that regenerated 13 primary transgenic plants (T0). A small leaf sample from regenerated primary transgenic plants was stained for the GUS activity and PCR was performed to verify the presence of Cas9 using Cas9F1- R1 primers **(Appendix).** The GUS activity was determined by staining leaf tissue in GUS staining solution. Plants derived from lines 3, 4 (4-1 to 4-4), and 6 (6-1, 6-2) lacked GUS activity, indicating targeting during the tissue culture and/or regeneration phases, while the plants derived from lines 1 and 5 (5-1 to 5-4) were GUS positive. All plants tested positive for

Cas9 except line #2, which was excluded from the analysis. Out of 13 plants, plant # 2, 3, 4-1, 4-2, 4-3, and 4-4 were albino; therefore, these plants could not be transferred to the greenhouse (**Table 2.1**).

To determine inducible expression of Cas9, the excised leaf sections from T0 plants were analyzed before and after cold-shock treatment by qRT-PCR. Cold-shock treatment was done on ice for 20 hours. Rice *7Ubiquitin* was used as a reference gene and the relative expression was calculated against B1 negative control using two delta delta C_t method (Livak & Schmittgen, 2001). The cold-shock induced Cas9 expression analysis showed 3.2- and 34-fold induction of Cas9 in lines 3 and 6 (plant #6-1 and #6-2), respectively, relative to the room temperature control of B1 plant, while other lines did not show any induced expression **(Figure 2.2; Table 2.1**).

Following the cold-stress, the plants were allowed to recover for one week, and subsequently leaf samples were collected for DNA isolation, GUS activity, and PCR across sg2 site using GusF82- NosR2 primers **(Appendix).** The PCR was performed on *GUS* sg2 target only, as our previous studies showed a higher targeting by *GUS* sg2 site than sg1 site (Nandy et al., 2019; Pathak et al., 2019). The PCR and sequencing at sgRNA2 target of these plants showed the presence of mutations in two lines (#4 and #6) that were GUS-negative even before cold-shock was imposed. This indicates establishment of mutation early in the plant development in these lines. All line #4 plants tested in this study were found to contain a homozygous 28 bp deletion at *GUS* sg2 target site **(Figure 2.3)**. However, since line #4 plants (4-1, 4-2, 4-3, 4-4) lacked detectable activity of Cas9 gene, the mutation is presumably induced by the transient expression activity of AtRD29a:Cas9 gene during plant cell transformation. Further since all line #4 plants exhibited the same mutation, they originated from the same cell that underwent gene targeting. Similarly, GUS-negative plants of line #6, 6-1 and 6-2, were found to contain mutations at sg2 site

(Table 2.1). Sequencing chromatogram showed the presence of two overlapping traces, which were analyzed by CRISP-ID program to reveal biallelic mutations consisting of a 5 bp deletion and a large indel **(Figure 2.3)**. Line #6 contained strong cold-inducible Cas9 activity. However, these plants could also express Cas9 in ambient conditions. Therefore, the observed mutations could be created by the cold-induced activity of Cas9; however, since clear biallelic mutations were identified instead of chimeric mutations, the mutations in line #6 plants were also likely established early in plant development by the ambient Cas9 activity. Plants of line #1, #3, #5 showed WT sequences at sg2 target site. Plants of line #1 and #5 did not show detectable Cas9 expression; therefore, these plants did not undergo gene targeting. Line #3 plant, on the other hand, was GUS-negative even before cold-shock treatment and showed inducible Cas9 expression **(Table 2.1)**. However, DNA sequencing revealed a WT sequence at sg2 target. Thus, absence of GUS activity in plant #3 was apparently not due to targeting at sg2 site. While the basis of GUS mutation in plant #3 is not clear, sg1 site may have been targeted or tissue culture may have induced mutations in the GUS gene.

The plants derived from line # 5 and #6 were transferred to greenhouse for maturity. However, all plants were sterile and did not produce any seeds. Therefore, inheritance studies at either/both target sites of *GUS* could not be pursued.

Overall, this study showed that AtRD29a:Cas9 expressed properly by showing cold-induction in rice lines and its transient/phase-specific expression generated targeted mutations in the rice genome. Therefore, this inducible Cas9 system could be useful for gene targeting in rice and curbing off-target effects by the constitutive expression of CRISPR/Cas9.

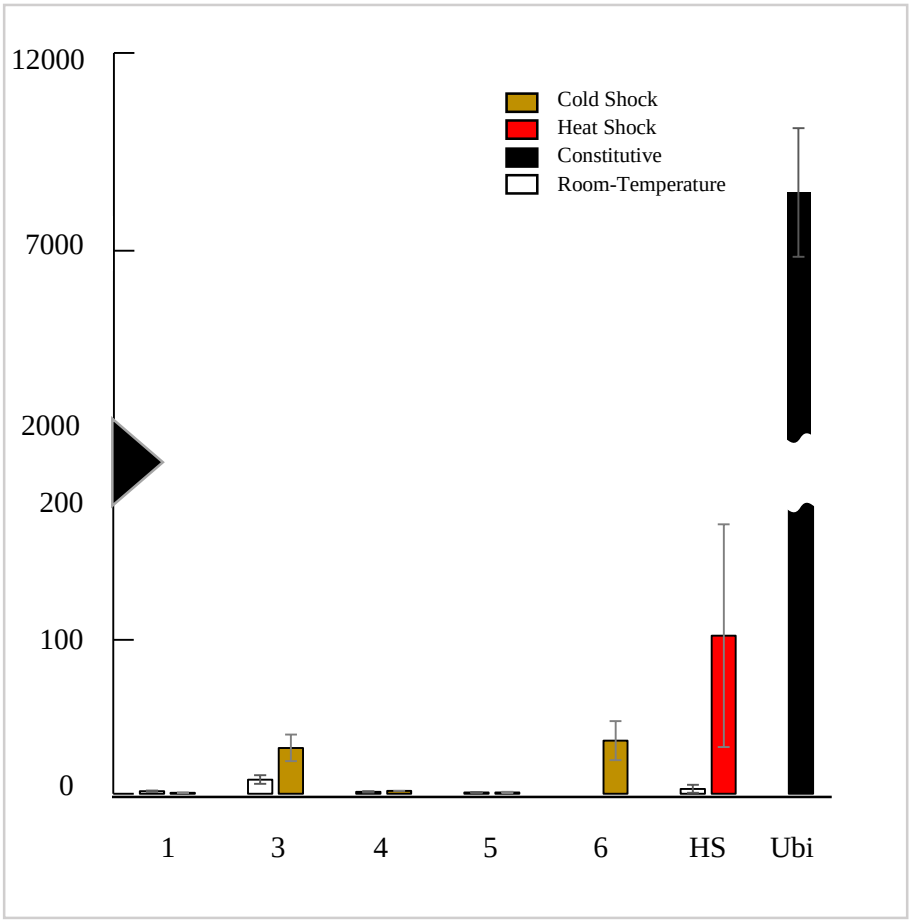


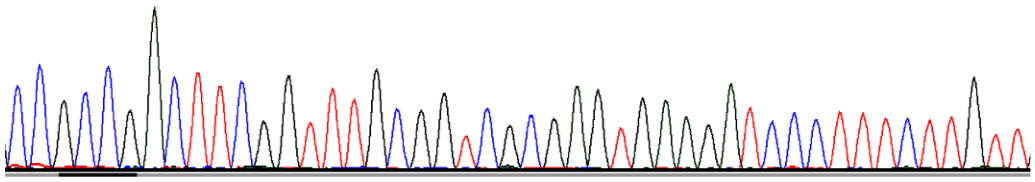
Figure 2.2 Expression of AtRD29a:Cas9 gene in rice lines (1 – 6) at room temperature (white) and upon cold-shock treatment relative to non-transgenic control. The data is compared to the reported Cas9 expression by heat-shock (HS) promoter (HSP:Cas9) and constitutive expression of OsUbi:Cas9 (Ubi) (Nandy et al., 2019). Error bars represent standard error.

Table 2.1 Analysis of AtRD29a:Cas9 targeted *GUS* transgene primary transgenic lines

Plant #	**GUS activity**				**Cas9 PCR**	**Cas9 expression relative to WT control**		**Sequence analysis at sg2 target site**	**Transfer to greenhouse**
	RT	CS	CS	CS		RT	CS		
1	+	+	+	+	+	1.745	0.639	WT	√
2	+	nd	nd	nd	-	nd	nd	nd	Albino
3	-	-	-	-	+	9.327	29.794	WT	Albino
4-1	-	-	-	-	+	nd	1.949	nd	Albino
4-2	-	-	-	-	+	nd	0.96	Homozygous 28 bp deletion	Albino
4-3	-	-	-	-	+	nd	1.911	homozygous 28 bp deletion	Albino
4-4	-	-	-	-	+	1.298	2.083	Homozygous 28 bp deletion	Albino
5-1	+	+	+	+	+	nd	0.581	WT	√
5-2	+	+	+	+	+	0.84	1.328	nd	√
5-3	+	+	+	+	+	nd	nd	WT	√
5-4	+	+	+	+	+	nd		WT	√
6-1	-	-	-	-	+	nd	34.582	Biallelic mutations	√
6-2	-	-	-	-	+	nd		Biallelic mutations	√

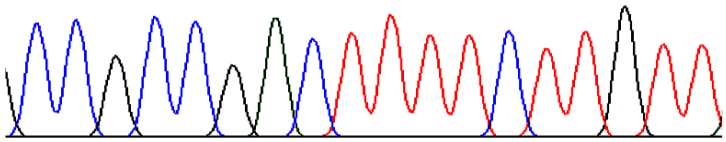
nd: not determined

(a): Ref



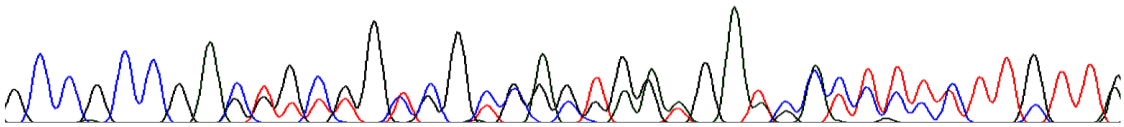
CCGCCGACTTCGGTTTGCGGTCGCGAGTGAAGATCCCTTTCTTGTT

(b): #4-2



CCGCCGACTTCGGTTTGCGGTCGCGAGTGAAGATCCCTTTCTTGTT ref
CCGCCGACT----------TTTCTTGTT (-28bp)

(c): #6-1



CCGCCGACTTCGGTTTGCGGTCGCGAGTGAAGATCCCTTTCTTGTT ref
CCGCCGA----------GTTTGGCGGCGGGGGGTGAAGCCCCTTTTGTT seq 1 (-5bp)
CCGCCGA------CGgctgtcgtcactaaagatcatttccttgt seq 2 (large indel)

Figure 2.3: CRISPR/Cas9 targeting using AtRD29a:Cas9 gene in *GUS* transgene located in rice genome. **[a]** Reference sequence (ref) and chromatogram of the target site; **[b]** Chromatogram and sequence analysis showing 28 bp homozygous deletion in plant 4-2; **[c]** Chromatogram and sequence analysis showing biallelic mutations in plant 6-1. Target site is shown in red, surrounding sequences in blue, PAM is underlined. Deletions are shown as dashed lines and insertions as small fonts.

Chapter 3.

Comparison of the cold-inducible Cas9 and heat-inducible Cas9 on targeting rice loci

3.1 Introduction

Several inducible systems for expression of transgene in plants have been defined especially for tobacco, rice, Arabidopsis, tomato, and maize. Inducible systems allow control on transgene expression. By applying an accurate control induction system, evaluation of the function of critical genes and modifications in gene expression level could be obtained (Borghi, 2010). Inducible systems could also allow conditional targeting of essential genes when constitutive systems would lead to non-viable cell or infertile plants. Regarding the temperature induction system, when organisms are exposed to the higher temperature rather, their cells react by fluctuation in transcription and translation (Schlesinger et al., 1982; Ashburner & Bonner, 1979). This heat shock phenomena was first observed in *Drosophila* (Ritossa, 1962) that led to the concept of temperature-induced regulation of gene expression. Heat-shock proteins (HSP) genes, found in most multicellular organisms including plants, are rapidly induced upon exposure to high temperature. Thus, HSP gene promoters are excellent heat-inducible systems that have been widely employed in plant biotechnology. One such system is the soybean HSP17.5E protein gene promoter (GmHSP17.5E) that shows excellent control and strong inducibility in rice (Nandy & Srivastava, 2012).

Cold stress is another form of abiotic stress, which can be implemented for testing the efficiency of Cas9 targeting. In Arabidopsis, AtRD29a gene promoter containing DRE elements has been effectively shown to respond to cold, drought and salt stress. In a study by Pathak and Srivastava (2020), AtRD29a:AtDREB1a had shown induction of AtDREB1a with a transcript levels up to 30-40 fold when exposed to cold stress for 20 hours on ice. Therefore, our rationale here is to evaluate the efficiency of AtRD29a:Cas9 and GmHSP17.5E:Cas9 for targeting of an essential Target of Rapamycin (TOR) and PDS gene, in comparison with OsUbi1:Cas9.

3.1.1 Rice phytoene desaturase (PDS) gene

Carotenoid biosynthesis is critical for many physiological pathways in plants such as biosynthesis of chlorophylls, phytohormones, and vitamins. They also take part in photosynthesis by β-carotene pigments and xanthophyll production (Qin et al., 2007). Phytoene synthase (PSY) and phytoene desaturase (PDS) are the most critical enzymes which have an effect on carotenoids biosynthesis pathway in plant (Giuliano et al., 1993). Hence, depletion of PDS enzyme by knocking out the PDS gene leads to the suppression of the carotenoid biosynthesis in plant. PDS gene has been targeted by CRISPR/Cas9 in previous studies (Xie et al., 2015).

In the current study, we studied the targeting efficiency of cold- and heat-inducible CRISPR/Cas9 systems consisting of AtRD29a:Cas9 and GmHSP17.5E:Cas9, respectively, on generating targeted mutation in the PDS gene and compared it with the constitutive CRISPR/Cas9 consisting of OsUbi1:Cas9. The two *PDS* target sites 1 and 2 are located on exons 5 and 7, respectively, separated by 987 bp **(Figure 3.1).**

3.1.2 Target of Rapamycin (TOR) gene

TOR is an essential protein that exists in all eukaryotes including humans. TOR kinase positively regulates anabolic processes and promotes growth and development in nutrient sufficient condition (Fu et al., 2020). TOR phosphorylates transcription factors that regulate the anabolic processes. In plants, many advances have been done to understand TOR signaling but more research is needed to understand the underlying mechanisms. We developed CRISPR lines of rice to target the *TOR* gene *(OsTOR)* and attempted to compare the targeting efficiency of heat shock and cold shock induced CRISPR/Cas9 on *TOR* gene. *OsTOR* target sites 1 and 2 are located on exons 9 and 50 separated by 14 kb **(Figure 3.2)**.

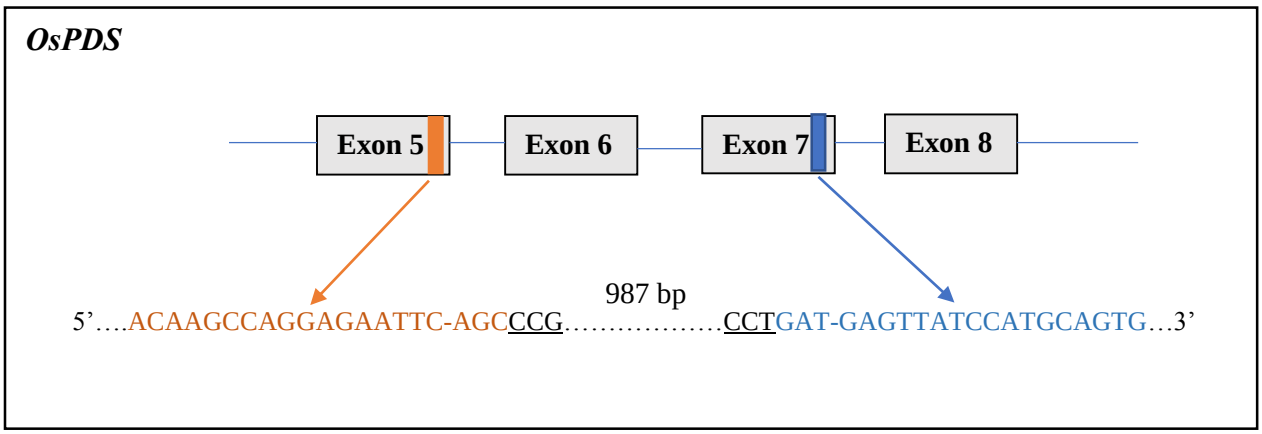


Figure 3.1 Position of target sites of *OsPDS* used in this study**.** Target sites 1 and 2 are represented with orange and blue bars, respectively. The PAM site is underlined. The predicted double stranded break site is indicated with hyphen. The distance between two target sites is shown in the bp.

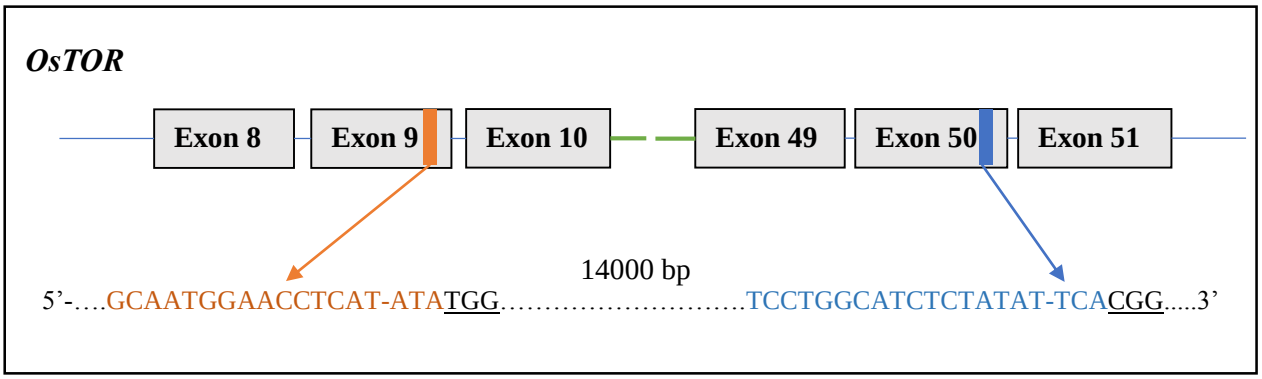


Figure 3.2 Position of target sites of *OsTOR* used in this study**.** Target sites 1 and 2 are represented with orange and blue bars, respectively. The PAM site is underlined. The predicted double-stranded break site is indicated with hyphen. The distance between two target sites is shown in the bp.

3.2 Materials and methods

3.2.1 Plasmid constructs

The constructs used in TOR and PDS experiments are listed as following:

(a) pJU34 + pHPT

(b) pNS66 + pNS65 — (*OsPDS* gene)

(c) pNS66 + pNS70

(d) pNS74 + pNS65 — (*OsTOR* gene)

(f) pNS74 + pNS70

pJU34: It contains constitutive Cas9 expression system under rice *Ubiquitin* promoter, and *PDS* sgRNA1 and 2 under rice *U3* promoter (**Figure 3.1, 3.3c**).

pNS65: It contains Cas9 under soybean heat-shock promoter (*GmHSP17.5e*) (**Figure 3.3a**)

pNS66: It contains *PDS* sgRNA1 and 2 under rice *U3* promoter along with *HPT* selection marker under *35S* promoter (**Figure 3.1, 3.3b**).

pNS70: It contains Cas9 under *Arabidopsis RD29a* promoter **(Figure 3.3a)**

pNS74: It contains *35S* promoter driving *HPT* selection marker gene and *TOR* sgRNA1 and 2 under rice *U3* promoter (**Figure 3.2, 3.3b)**.

pHPT: Contains HPT marker gene under 35S promoter.

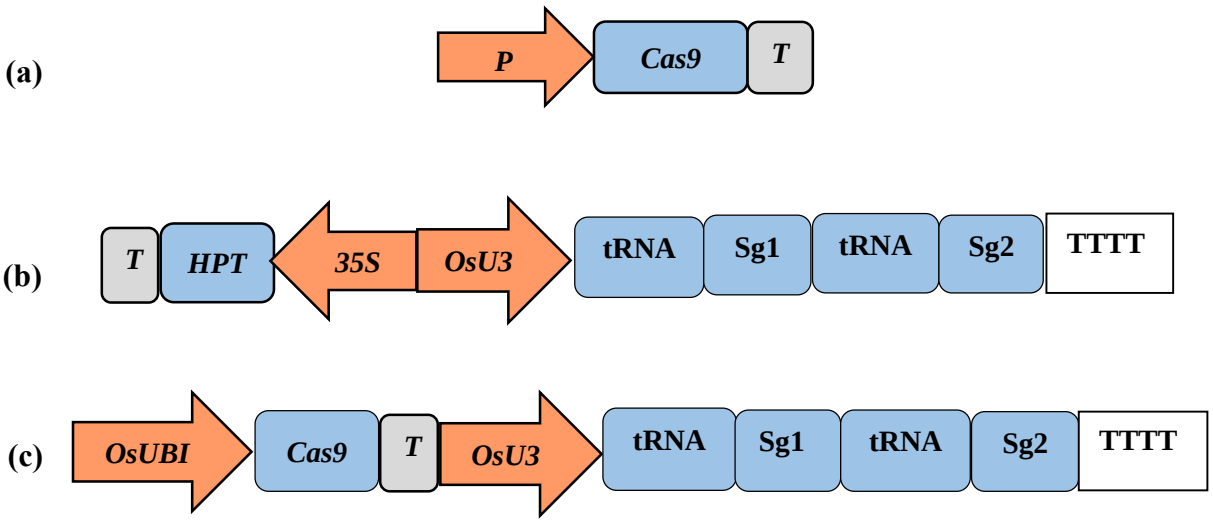


Figure 3.3 Vector constructs used in the study. **[a]** Promoters (P): Cold-inducible *Arabidopsis rd29a* gene promoter (pNS70)/ heat inducible Soybean heat-shock promoter (*GmHSP17.5e*, pNS65) driving *Streptococcus pyogenes* Cas9 expression; **[b]** sgRNA construct based on tRNA processing strategy (Xi et al., 2015) consists of rice sno *U3* promoter expressing a pair of sgRNAs of *OsPDS (*pNS66*).* The construct also contains hygromycin phosphotransferase (*HPT*) gene consisting of *35S* promoter for the plant selection. Poly T is the transcription terminator for *U3* promoter. **[c]** Constitutive vector design showing Cas9 expression by rice Ubiquitin-1 promoter (*OsUbi*) and multiplexed sgRNA by rice U3 promoter (*OsU3*). Constitutive CRISPR constructs targeting *OsPDS* and *OsTOR* are named pJU34 and pNS71, respectively. All sgRNA constructs were made in pRGE32 background (Xie et al., 2015). The nopaline synthase 3' terminator (T) is shown as light grey box in each construct. Image is adapted from Nandy et al. (2019)

3.2.2 Rice transformation, selection, and regeneration

For gene targeting, the calli was initiated from rice Kitaake seeds on 2N6D media. The 4-week cold embryogenic calli were co-bombarded using biolistic gene gun. In this, a mixture of 25 µl gold suspension was mixed with 25 µl of autoclave water and 10 µl of favorable plasmid in one 1.5 ml tube. Then it was properly mixed, and any bubble was removed. A drop of 50 µl CaCl2.2H2O (2.5M) and 20 µl Spermidine (0.1M) were added to the tube, and it was vortexed for 3 minutes. The solution was centrifuged, supernatant was removed, and it was washed twice with 200 proof ethanol. At last, 120 µl 200-proof Ethanol (for around every 10 plates) was added and dissolved by rubbing over pipette box (dissolving by pipetting leads to Ethanol evaporation) and it was used for bombardment.

After doing the bombardment, calli were transferred to 2N6D media for one week for the recovery. Then the putative transgenic calli were selected and regenerated on 2N6D and regeneration media, respectively containing hygromycin (50mg/L). The regenerated primary transgenic lines (T0) were subjected to rooting media for roots initiation. The T0 lines were later transferred to the greenhouse and were subjected to the downstream analysis.

3.2.3 DNA extraction

For molecular analysis, DNA was extracted from the leaf tissue of transgenic plants by rapid gDNA extraction protocol (Edwards et al., 1991). In this method, 1 small leaf tissue was placed in 1.5 ml tube with some glass beads (50-100 µl) and it was ground for a few seconds (based on the tissue size) in a tissue lyzer. Later, 200 µl of SDS-Lysis buffer was added immediately to every sample and vortexed. Then samples were centrifuged in 15000 rpm for 10 min. In the next step, 100 µl of supernatant was transferred to the new tube and 100 µl of iso-propanol was added. The tube was incubated at room-temperature for 5 min and centrifuged for 10 min at 15000 rpm.

Then supernatant was removed completely without disturbing the pellet. 150 µl of 70% ethanol was used for washing the pallet and centrifuged for 3 min in at 15000 rpm. Once again supernatant was removed, and the tubes were kept at RT for 5 min to be air dried. At the end, each sample was resuspended with 40 µl of water and used as genomic DNA for the rest of the experiment.

3.2.4 Polymerase chain reaction (PCR)

PCR was performed using Takara Max Amp RT kit in a 15µl volume including 7.5µl Takara Premix, 5.5µl H_2O**,** 1µl of forward and reverse primers (2-5 µM) and 1 µl of DNA. The Cas9 PCR conditions were 95°C for 1 min for denaturation, 60° C for 1 min for annealing, 72° C for 1 min for elongation. Cycle was repeated for 29x and then it was set for 72°C for 15 min followed by 4°C hold to make the samples cold. The PCR condition used for *OsPDS* and *OsTOR* target sites was 95°C for 1 min for denaturation, 58° C for 1 min for annealing, 72° C for 1 min for elongation. Cycle was repeated for 39x and then it was set for 72°C for 15 min followed by 4°C hold.

Quantitative real time PCR (qRT-PCR) was performed for expression analysis of Cas9. Total RNA was isolated using Trizol reagent (Thermo-fisher Scientific). Total RNA was treated with the DNase for the removal of any genomic DNA. One microgram of DNase treated RNA was used for the conversion of cDNA using PrimeScript RT reagent kit (Takara Bio, CA, USA). The Cas9 gene expression analysis (RT-qPCR) using TB green Premix Ex Taq II (Takara Bio, CA, USA) was performed on Bio-Rad CFX96 using the following program: 95°C for 30 sec and 40 cycles of 95°C for 5 sec + 60°C for 30 sec. The components for each qPCR reaction were: 4.25µl water, 6.25µl 2X Takara premix, 0.5 µl each of forward and reverse primers (10µM) and 1µl cDNA.

3.2.5 DNA sequencing

The target sites were amplified by the PCR primers flanking them as mentioned in table 3.1 and 3.2. The bands were eluted from 0.8%-1% gel depending on the product size. The DNA was purified from the gel using GeneJet Gel Extraction Kit (Thermo-Fisher Scientific) and eluted in 20µl water. Each of the 1µl eluted samples were visualized on the gel. Each of the 10µl extracted samples and 10µl of 2µM primers were sent to Macrogen USA for sanger sequencing.

3.2.6 Sequence analysis

The sanger sequencing chromatograms (.abi) were analyzed by CRISP-ID program (Dehairs et al., 2016). Based on the sequence quality, the start and end position of the sequences were determined; and the background cut off % for noise removal was kept at 20-40% in order not to miss the mutation peaks. The parsed-out sequences were then aligned with the reference using CLUSTAL-OMEGA.

3.3 Results and discussion

To understand the targeting efficiencies of Cas9 expressed under heat inducible (*GmHSP17.5e*), and cold-inducible promoters (*AtRD29a*), rice Kitaake calli was initiated on 2N6D media. The 3-4-weeks-old embryogenic calli were transformed with specific constructs using the PDS1000/He gene gun and selected on hygromycin containing media. The hygromycin resistant calli were grown further and subjected to plant regeneration. All tissue culture protocols were performed according to Nishimura et al. (2006). In the cold shock, heat shock, and constitutive *PDS* experiments, the transformed calli selected on hygromycin were subjected to Cas9 PCR and target site analysis by PCR/sequencing **(Table 3.2; Figure 3.5)**. Primer sequences are given in **(Appendix)**. Also, these clones were allowed to regenerate into plants.

In the cold shock and heat shock *TOR* experiments, first Cas9 PCR was done followed by target site analysis**.** Then one Cas9+ plant per line was randomly selected and exposed to heat-shock at 42^{o}C of incubator for 3 hours or cold-shock on ice for 20 hours. Then samples were used for RNA isolation for RT-qPCR using Cas9F1-R1 primers. The Cas9-negative lines obtained in the same experiment were used as the negative controls. Simultaneously, the respective RT samples from the same plants were also included in the experiment for comparison. The relative expression was done against the *7Ubiquitine* gene.

3.3.1 Targeting of rice *OsPDS* gene

Three sets of co-transformation experiments were done to generate constitutive, heat-inducible, and cold-inducible PDS targeting CRISPR lines. These included co-transformation with pJU34 + pHPT (constitutive), pNS65+66 (heat-inducible), and pNS66+70 (cold-inducible) that generated transformation efficiencies of 45%, 50% and 30% respectively **(Table 3.1)**. In absolute numbers, 4 calli events for OsUbi1:Cas9, 5 events for HSP::Cas9, and 3 events for AtRD29a::Cas9 were obtained after two rounds of selection on hygromycin **(Table 3.1)**. In the calli, the transformed and non-transformed events can co-exist; therefore, two separate events from each calli were analyzed. First, Cas9 PCR was done for the verification of transformation. In the OsUbi1:Cas9, events #1 and 3; in HSP: Cas9, events #2, 3, and 4; and AtRD29a: Cas9, events #1, 2, and 3 were Cas9 positive. In HSP:Cas9 and AtRD29a:Cas9, event #3 and event #2 and 3, respectively, contained both non-transformed and transformed calli type **(Table 3.2)**.

Next, only the Cas9 positive events from each experiment maintained at the room temperature were analyzed for the mutations at the targeted sites. First, we analyzed only target site 1, using the sequencing primer PDSF2240 **(Appendix).** Further, since both the target sites in *OsPDS* are targeted at an equal rate (Pathak et al., 2019), analysis of one presumably reports the

targeting of the other. Accordingly, we observed that OsUbi1:Cas9 event #3 contained biallelic 4bp and 3bp deletions **(Figure 3.4),** while events #1, 2, and 4 were WT and all the remaining Cas9 positive events tested in HSP/AtRD29a:Cas9 were WT. While a low targeting efficiency of *OsPDS* gene was observed in the calli as only one of the 4 OsUbi1:Cas9 events harbored mutations, it also showed that heat/cold-inducible Cas9 did not create mutations at the ambient temperature in the calli. We were unable to regenerate the plants from any of the constructs, hence this study was restricted only to the calli.

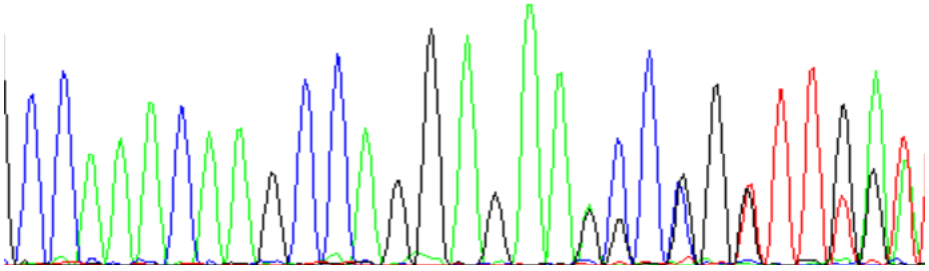
Table 3.1 PDS experiment

Construct	**type**	**# of shots**	**Calli events on selection**		**Transformation efficiency %**
			1st round	2nd round	
pJU34 + pHPT	OsUbi:Cas9	9	7	4	45
pNS66 + pNS65	HSP:Cas9	10	12	5	50
pNS66 + pNS70	AtRD29a:Cas9	10	7	3	30

Table 3.2 Cas9 PCR analysis of *OsPDS* CRISPR lines

Event #	**Sub-sections**	**Constitutive (pJU34+pHPT)**	**Heat-inducible (pNS66+pNS65)**	**Cold-inducible (pNS66+pNS70)**
1	1a	+	-	+
	1b	+	-	+
2	2a	-	+	+
	2b	-	+	-
3	3a	+	+	-
	3b	+	+	+
4	4a	-	-	na
	4b	-	+	na
5	5a	na	-	na
	5b	na	-	na

na: not applicable



ACAAGCCAGGAGAATTCAGCCGGTTT ref
ACAAGCCAGGAGA--------AGCCGGTTT -4bp
ACAAGCCAGGAGA-------**a**AGCCGGTTT -3bp

Figure 3.4: OsUbi1:Cas9 targeting *OsPDS* gene. Plant # 3 maintained at the room temperature shows biallelic 4 bp and 3 bp deletion at the target site. The PAM is underlined, red and light blue fonts represent the target site and surrounding sequences, respectively.

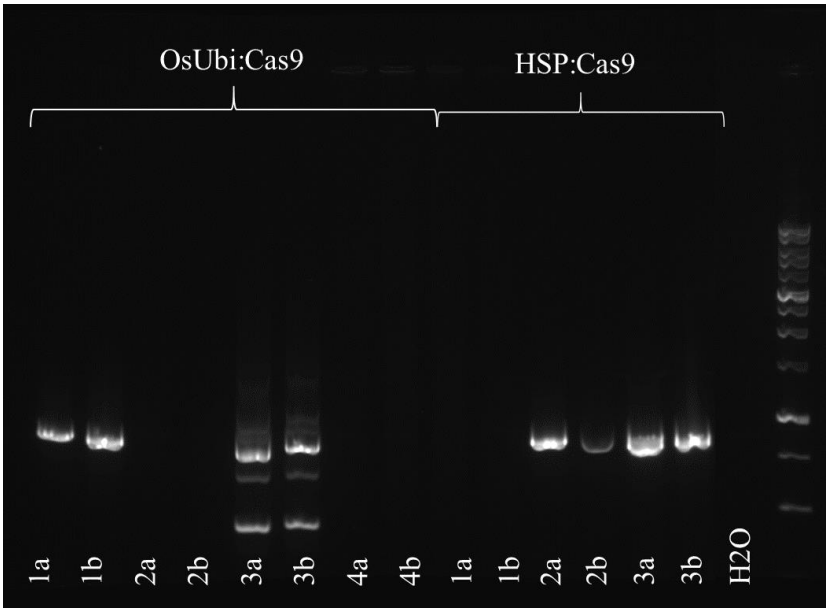


Figure 3.5: Cas9 PCR analysis of calli lines transformed with (pJU34+pHPT & pNS66+pNS65) targeting *OsPDS* gene. 1kb ladder is shown on the right

3.3.2 Targeting of rice *OsTOR* gene

In *OsTOR* gene experiments, 20 and 10 shots of plasmids pNS74+65 (HSP:Cas9) and pNS74+70 (AtRD29a:Cas9), respectively, were made on the calli of Kitaake that generated 16 plants (6 lines) and 24 plants (11 lines), respectively. All these plants were subjected for Cas9 verification by PCR **(Table 3.3)**. Nine plants from three independent transgenic lines of HSP:Cas9 (# 8, 13 and 16), and 10 plants from 8 independent transgenic lines of AtRD29a:Cas9 (#1, 4, 5, 6, 8, 13, 14, and 15) were found to be Cas9 positive **(Table 3.3).** These Cas9 positive plants were then subjected to target sites amplification. The sg2 target site was analyzed as no targeting of sg1 was found in the TOR-CRISPR lines using OsUbi1:Cas9 gene. However, none of the inducible CRISPR plants maintained at the ambient conditions harbored mutations in the *OsTOR* sg2 site. This analysis shows (a) inducible CRISPR systems are too weak at ambient conditions to induce targeting at the *OsTOR* site, (b) targeting at the *OsTOR* site occurs at a low efficiency as indicated by constitutive (OsUbi:Cas9) TOR-CRISPR lines, which is further suppressed using inducible systems.

Next, Cas9 expression analysis was performed by real-time PCR (qPCR) on 7 AtRD29a:Cas9 and 3 HSP:Cas9 plant lines after applying cold and heat shock **(Figure 3.6)**. The HSP:Cas9 lines showed induced expression of 2-11x with respect to the negative control obtained from the Cas9 negative plant, and AtRD29a:Cas9 showed induced expression of 2-43x with respect to the negative control line **(Figure 3.6)**. Importantly, these lines showed minimal expression at the room temperature and abundant induction upon heat or cold treatment, indicating proper regulation of the promoters in the rice genome.

For analysis of T1 progeny, 3 Cas9 positive lines of each heat-shock (13-1, 13-2, and 16-1) and cold-shock (1-1, 14-3, and 15-1) CRISPR plants, which had enough number of seeds, were

selected, and germinated in 1/2 MS media after sterilization with 70% ethanol and 30% bleach. Nine seeds of 13-1, nine seeds of 13-2, eight seeds of 16-1, ten seeds of 1-1, nine seeds of 14-3, and six seeds of 15-1 were used in the study. When plants germinated, a small leaf tissue was taken for DNA extraction, and subjected to Cas9 PCR with Cas9F8-R3 primers **(Appendix).** The result is shown in (**Table 3.4)**. To continue, the Cas9 positive samples were subjected to two rounds of heat-shock of 42°C for 3 hours in an incubator or one round of cold -hock for 20 hours in ice package depending on the type of CRISPR constructs they harbored. Plants were allowed to rest for 1 week before leaf samples were collected from the treated plants for DNA extraction. Next, PCR was done to amplify sg2 target site with TORF2-R2 **(Appendix)**. The PCR amplicon was submitted to Psomagen for Sanger sequencing to detect any possible induced mutations. This analysis showed that all cold-shocked CRISPR lines contained WT in sg2 target. The sequencing of heat-shock CRISPR lines was inconclusive, and therefore kept for reanalysis by PCR sequencing.

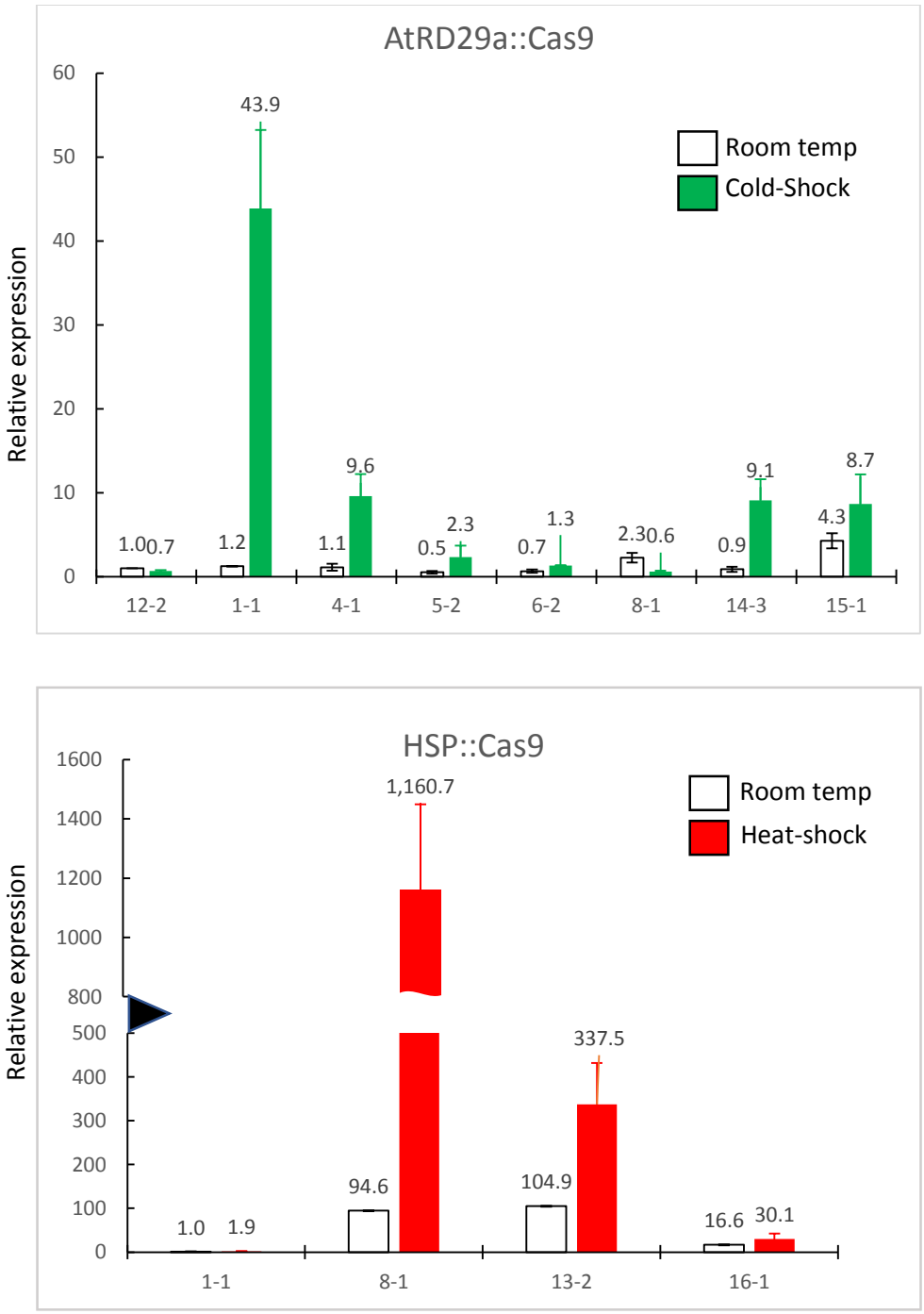
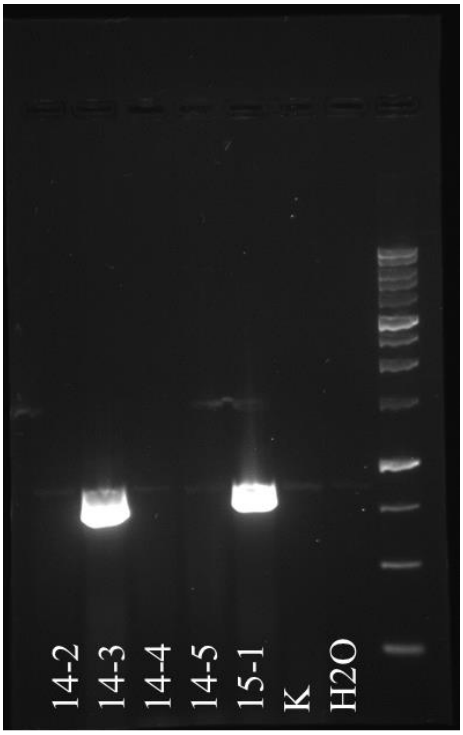


Figure 3.6. Inducible expression of Cas9 driven by *AtRD29a* or *HSP* promoter in rice. Primary transgenic lines (T0) were treated with appropriate condition of cold or heat to determine fold-induction (numbers above each bar) compared to the non-transgenic controls. Error bars indicate standard errors.

(a)



(b)

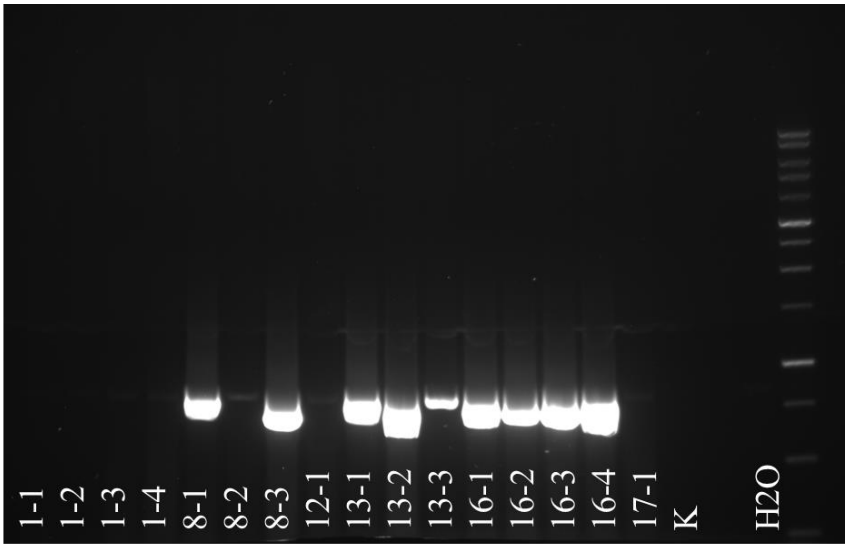
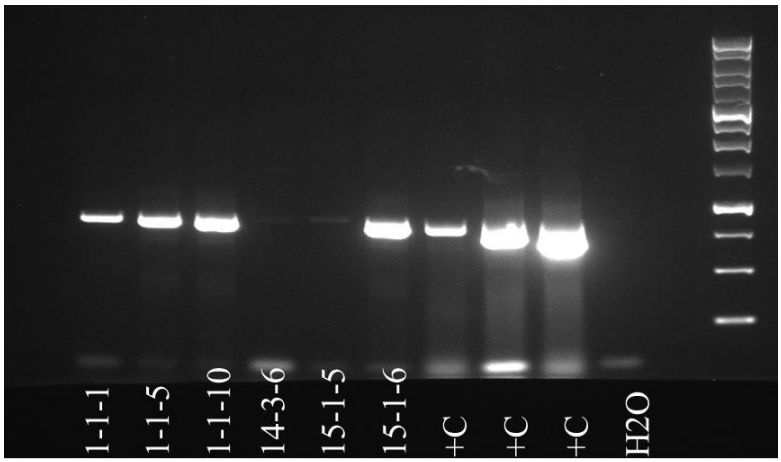


Figure 3.7. Representative image of Cas9 PCR analysis of *OsTOR* primary transgenic lines transformed with **(a)** AtRD29a:Cas9 (pNS74+pNS70) **(b)** HSP:Cas9 (pNS74+pNS65). Kitaake (K) and water are negative controls

(a)



(b)

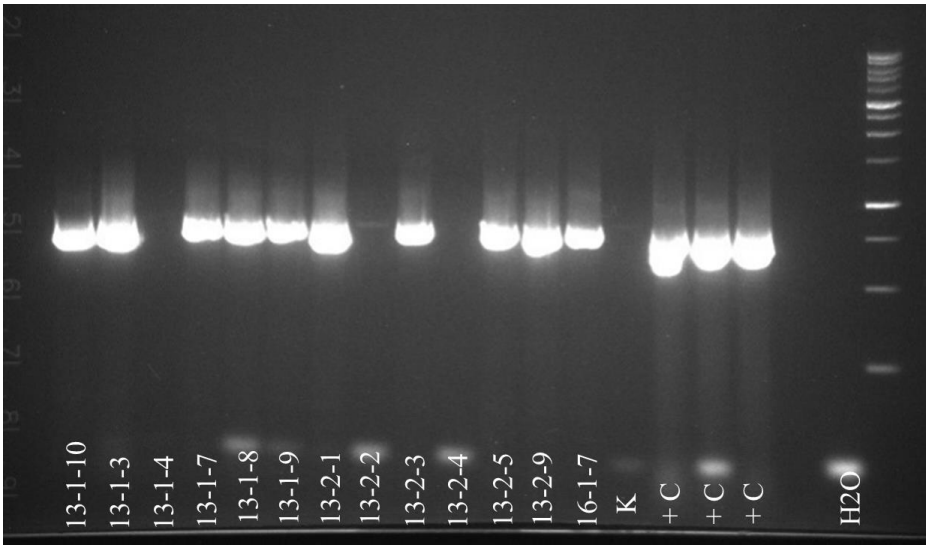


Figure 3.8. Representative image of Cas9 PCR analysis of *OsTOR* T1 transgenic lines transformed with **(a)** AtRD29a:Cas9 (pNS74+pNS70) **(b)** HSP:Cas9 (pNS74+pNS65). Kitaake (K) and water are negative controls

Table 3.3 *OsTOR* T0 Cas9 analysis

Construct	Event #	Cas9 analysis
pNS74+pNS65	1-1	-
	1-2	-
	1-3	-
	1-4	-
	8-1	+
	8-2	-
	8-3	+
	12-1	-
	13-1	+
	13-2	+
	13-3	+
	16-1	+
	16-2	+
	16-3	+
	16-4	+
	17-1	-
pNS74+pNS70	1-1	+
	3-1	-
	4-1	+
	4-2	-
	5-1	-
	5-2	+
	6-1	+
	6-2	+
	6-3	+
	8-1	+
	9-1	-
	9-2	-
	9-3	-
	12-1	-
	12-2	-
	13-1	-
	13-2	-
	13-3	+
	14-1	-
	14-2	-
	14-3	+
	14-4	-
	14-5	-
	15-1	+

Table 3.4 *OsTOR* T1 Cas9 analysis

Construct	Event #	Cas9 analysis
pNS74+pNS65	13-1-1	+
	13-1-2	+
	13-1-3	+
	13-1-4	-
	13-1-6	-
	13-1-7	+
	13-1-8	+
	13-1-9	+
	13-1-10	+
	13-2-1	+
	13-2-2	-
	13-2-3	+
	13-2-4	-
	13-2-5	+
	13-2-6	+
	13-2-7	+
	13-2-8	-
	13-2-9	+
	16-1-1	-
	16-1-2	+
	16-1-3	+
	16-1-4	-
	16-1-6	+
	16-1-7	+
	16-1-8	+
	16-1-10	+
pNS74+pNS70	1-1-1	+
	1-1-2	-
	1-1-3	-
	1-1-4	+
	1-1-5	+
	1-1-6	-
	1-1-7	+
	1-1-8	+
	1-1-9	+
	1-1-10	+
	14-3-1	+
	14-3-2	+
	14-3-3	-
	14-3-4	+
	14-3-5	+
	14-3-6	-

Table 3.4 (Cont.)

Construct	Event #	Cas9 analysis
pNS74+pNS70	14-3-7	+
	14-3-8	-
	14-3-9	+
	15-1-1	+
	15-1-2	+
	15-1-3	+
	15-1-4	+
	15-1-5	-
	15-1-6	+

Chapter 4.

Inheritance of CRISPR-induced mutations in *OsTOR*, an essential gene in rice

4.1 Introduction

In genome editing, not only a high targeting efficiency for making a double-stranded break is important but also inheritance of pointed mutations to the progeny has been a critical issue.

In a study, Durr et al. (2018) used *pUbiCAS9-Red*, a seed specific red fluorescence reporter gene along with Cas9 to study inheritance rate of somatic deletions. In this study, authors first targeted >10 genes and studied the targeting efficiency in somatic cells, which ranged from 4-69%. Later, they selected the seeds, which did not contain Cas9 and lacked red fluorescence from high rate of somatic deletion lines to study the inheritance rate of mutations. It was observed that the inheritance rate ranged from 0.5-5%. Hence, it was concluded that though CRISPR/Cas9 creates heritable deletions in *Arabidopsis,* the inheritance rate is very low. Some recent reports have shown that usage of promoters, which have a high activity during the early stages of sexual reproduction of plants, can enrich the efficiency of mutations created by CRISPR/Cas9 (Wang et al., 2015, Mao et al., 2016). Therefore, Durr et al. (2018) modified their construct towards having synthetic EC1 promoter, which has expression in egg cells. Using this promoter, the efficiency of heritable deletions increased to 6-100% in some of the target sites, and in several cases the progeny ended up with having homozygous genomic deletions.

In another study, it was shown that heat-shock (HS) inducible Cas9 can create heritable targeted mutations and limiting off-target effects. They noticed transfer of mutations to the T1 progeny as monoallelic and biallelic at a high rate independent of the Cas9 gene. Moreover, off-target activity was either minimized or undetectable in HS CRISPR/Cas9 transgenic lines (Nandy et al., 2019).

However, none of the studies have so far investigated CRISPR/Cas9 targeting rate of the essential gene and its heritability into the progeny. Here, we investigated the inheritance rate of

the mutation created in the *OsTOR* gene in rice, and the occurrence of any *de novo* mutations in the progeny. Rice genome contains a single copy of *OsTOR* gene that is essential for regulating growth and development through the anabolic processes (Maegwa et al., 2015).

4.2 Materials and Methods

4.2.1 Plasmid constructs

In this objective, the construct pNS71, which contain constitutive Cas9 expression system under rice *Ubiquitin* promoter and *OsTOR* sgRNA1 and 2 under rice *U3* promoter, was co-bombarded with the selection marker vector, pHPT **(Figure 4.1)**. pHPT contains *hygromycin phosphotransferase* gene under *35S* promoter. The resulting primary transgenic lines (T0) are given in **Table 4.1.**

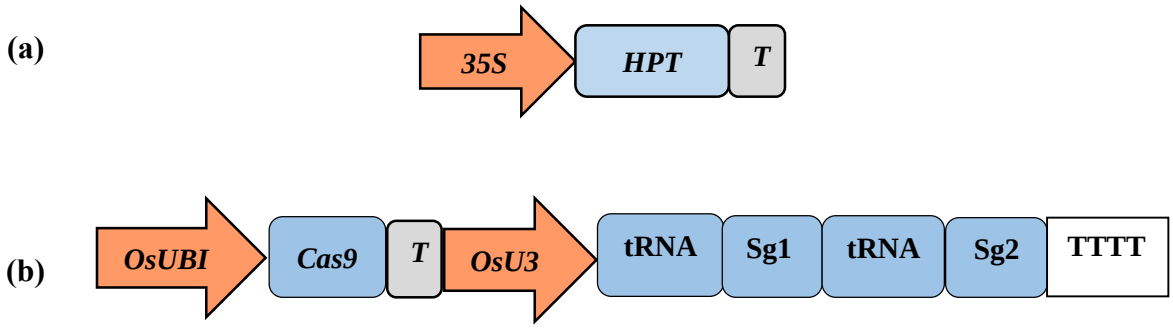


Figure 4.1. Vector constructs used in the study. **[a]** Selection marker gene vector, pHPT, contains *35S* promoter driving hygromycin phosphotransferase (*HPT*) gene; **[b]** CRISPR/Cas9 vector pNS71 in pRGE32 background (Xie et al., 2015) contains Cas9 expressed by rice Ubiquitin promoter (*OsUbi*) and two sgRNA by rice *U3* promoter. T, nos 3' terminator; TTT, Pol III terminator.

4.2.2 Rice transformation, selection, and regeneration

For gene targeting, the calli was initiated from Kitakee seeds on 2N6D media. The 4-week cold embryogenic calli was co-bombarded using biolistic gene gun. In this, a mixture of 25 µl gold suspension was mixed with 25 µl of autoclave water and 10 µl of favorable plasmid in one 0.5 ml tube. Then it was properly mixed, and any bubble was removed. A drop of 50 µl CaCl (2.5M) and 20 µl Spermidine (0.1M) were added to the tube, and it was vortexed for 3 minutes. The solution was centrifuged, supernatant was removed, and it was washed twice with 200 proof ethanol. At last, 120 µl 200-proof Ethanol (for around every 10 plates) was added and dissolved by rubbing over pipette box (dissolving by pipetting leads to Ethanol evaporation) and it was used for bombardment.

After doing the bombardment, calli were transferred to 2N6D media for one week for the recovery. Then the putative transgenic calli were selected and regenerated on 2N6D and regeneration media, respectively containing hygromycin (50mg/L). The regenerated primary transgenic lines (T0) were subjected to rooting media for roots initiation. The T0 lines were later transferred to the greenhouse and were subjected to the downstream analysis.

4.2.3 DNA extraction

For molecular analysis, DNA was extracted from a small leaf tissue of transgenic plants by Rapid Arabidopsis gDNA extraction protocol. In this method, 1 small leaf tissue was placed in 1.5 ml tube with glass beads (50-100 µl) and it was ground for a few seconds (based on the tissue size) with single tube grinder machine. 200 µl of SDS-Lysis buffer was added immediately to every sample and vortexed. Then samples were centrifuged in 15000 rpm for 10 min. In the next step, 100 µl of supernatant was transferred to the new tube and 100 µl of iso-propanol was added to the same tube, incubated at room-temperature for 5 min and centrifuged them again for 10 min at

15000 rpm. Then supernatant was removed completely without disturbing the pallet. 150 µl of 70% ethanol was used for washing the pallet per tube and centrifuged for 3 min in at 15000 rpm. Once again supernatant was removed, and the tubes were kept at RT for 5 min to be air dried. At the end, each sample was resuspended with 40 µl of water and used as genomic DNA for the rest of the experiment.

4.2.4 Polymerase chain reaction (PCR)

PCR was performed using Takara Max Amp RT kit in a 15µl volume including 7.5µl Takara Premix, 5.5µl H_2O**,** 1µl of forward and reverse primers (2-5 µM) and 1 µl of DNA. The Cas9 PCR conditions were 95°C for 1 min for denaturation, 60° C for 1 min for annealing, 72° C for 1 min for elongation. Cycle was repeated for 29x and then it was set for 72°C for 15 min followed by 4°C hold to make the samples cold. The PCR condition used for *TOR* target site was 95°C for 1 min for denaturation, 58° C for 1 min for annealing, 72° C for 1 min for elongation. Cycle was repeated for 39x and then it was set for 72°C for 15 min followed by 4°C hold.

4.2.5 DNA sequencing

The target sites were amplified by the PCR primers flanking them as mentioned in Appendix. The bands were eluted from 0.8%-1% gel depending on the product size. The DNA was purified from the gel using GeneJet Gel Extraction Kit (Thermo-Fisher Scientific) and eluted in 20µl water. Each of the 1µl eluted samples were visualized on the gel. Each of the 10µl extracted samples and 10µl of 2µM primers were sent to Macrogen USA for sanger sequencing.

4.2.6 Sequence analysis

The sanger sequencing chromatograms (.abi) were analyzed by CRISP-ID program (Dehairs et al., 2016). Based on the sequence quality, the start and end position of the sequences were determined; and the background cut off % for noise removal was kept at 20-40% in order not

to miss the mutation peaks. The parsed-out sequences were then aligned with the reference using CLUSTAL-OMEGA.

4.3 Results and discussion

After the co-bombardment of pNS71 and pHPT, the calli were selected on hygromycin and the selected calli were subjected to regeneration of plants. The regenerated plants were transferred to the greenhouse for further analysis.

A total of 21 plants derived from 9 independent lines were generated and transferred to the greenhouse. These plants were subjected to target site analysis at both sg1 and sg2 targets **(Table 4.1).** None of the plants showed mutations at the sg1 site but 4 plants (10-1, 23, 34-1 and 34-2) showed mutations at sg2 site **(Table 4.1; Srivastava et al., unpublished data)**. This data suggests that sg1 target, one of the HEAT domains, in the *OsTOR* gene, is refractory to mutations. Plant *TOR* contains up to 20 tandem HEAT repeats. HEAT stands for **H**untingtin, **E**longation factor 3, Protein phosphatase 2**A**, yeast PI3-kinase **T**OR1, and participates in protein-protein interactions to initiate TOR signaling (John et al., 2011). However, sg2 target, the Kinase domain at the C-terminal of TOR could be targeted at a low rate of 4 plants out of 21 representing 3 independent lines from a total of 9. This low rate of targeting is not surprising given TOR plays essential functions in plant development by integrating environmental and nutrient signals (McCready et al., 2020).

The analysis of mutations at sg2 target site in the 4 T0 plants showed that plant #10-1 contained monoallelic in-frame -6 bp deletion at the predicted DSB site, plant #23 contained monoallelic +1 bp insertion leading to early stop codon, plants #34-1 and #34-2 harboring mutations that could not be parsed out. (**Figure 4.4**; **Table 4.1**).

Next, progeny plants of four TOR-CRISPR plants were analyzed: T0 plants #6-1, 23, 34-1, and 34-2, whereas T0 plant #10-1 was completely sterile as it did not develop any seeds. It is likely that -6 bp in-frame deletion in 10-1 led to major defects in embryo development (McCready et al., 2020). However, tissue-culture induced somaclonal variations causing plant sterility cannot be ruled out. Overall, progeny analysis of 10-1 plant could not be done.

T0 plant #6-1 did not show mutations but was Cas9 + in the PCR (**Figure 4.2**; **Table 4.1)**. To determine whether *de novo* mutations could occur in the germline that are subsequently inherited by the T1 plants, 31 T1 plants of #6-1 were sown in the greenhouse, 11 of which were subjected to sg2 target site analysis. None of the 11 plants showed any mutations in the sg2 target. While Cas9 activity was determined in 6-1 plants **(Figure 4.3**), this observation suggests that very low rate, if any, *de novo* targeting occurred on *OsTOR* kinase domain. This observation aligns with the T0 analysis in which low rate of targeting was observed on the sg2 target.

T0 plant #23 set only a few seeds indicating possible effects of the +1 mutation, leading to early stop codon, on embryo survival. Nevertheless, 13 seeds were sown, and 11 T1 plants were analyzed by Cas9 PCR **(Figure 4.3)** and PCR-sequencing. Since plant #23 contained monoallelic +1 mutation, ¾ of these plants are expected to inherit this mutation. However, none of the 11 T1 plants showed mutations at sg2 site. This indicates that the early stop codon is a strong mutation that possibly causes defects in embryo development. Not much is known about loss of function *TOR* mutants, but embryo development in Arabidopsis *TOR* null mutants is arrested at the globular stage (Menand et al., 2002). Thus, *TOR* null mutations cause major defects in embryogenesis in plants.

T0 plants 34-1 and 34-2 originated from a single line (#34). However, since gene targeting could occur at variable stages of plant regeneration, the two plants could harbor variable mutations.

In both plants, the sequence chromatogram showed overlapping traces near sg2 DSB site **(Figure 4.4c)** but they could not be parsed out to clearly identify mutations. Therefore, 20 T1 seeds each of 34-1 and 34-2 were sown in the greenhouse for target site analysis in the progeny. Eleven plants of each (a total of 22) were subjected to the target site analysis **(Table 4.2).** All 22 plants harbored biallelic-heterozygous or biallelic-homozygous mutations, indicating the presence of biallelic-heterozygous mutations in the parent T0 plants. Further, both sets of T1 plants contained the same type of mutations (-3 or -9 bp deletions) suggesting the clonal origin of the two plants, T0 #34-1 and T0 #34-2. Nine of the 22 T1 plants contained biallelic homozygous mutations showing either -3 bp or -9 bp in-frame deletions **(Figure 4.5a,b).** The remaining possibly contained biallelic-heterozygous -3/-9 bp deletions **(Figure 4.5c)**, although sequences could not be clearly parsed out by the CRISP-ID program. Eight of the homozygous mutant T1 plants were also subjected to sg1 target site analysis as one of the 9 homozygous plants was too dwarf and sterile, and therefore removed from the analysis. None of the 8 T1 plants showed mutations in sg1 target **(Table 4.2).** This corroborates with our earlier observation that sg1 targeting was undetectable in T0 plants. Most T1 plants derived from 34-1 and 34-2, including homozygous mutant plants, generated abundant T2 seeds, suggesting a minimal effect, if any, of the observed mutations on embryogenesis in these plants. To understand the effect of mutation on TOR protein sequence, sequence alignments were done using CLUSTAL-OMEGA program, which indicated in-frame deletion of Y (Tyr) with a base substitution [Glu (E) to Asp (D)] in -3 bp allele, and in-frame and EYR (Glu-Tyr-Arg) deletion in the Kinase domain of -9 bp allele **(Figure 4.6).** These amino acids, especially Y, appear to be semi-conserved between plants and non-plant species and conserved among plants (K. Jamsheer, pers. Comm.). However, since no obvious morphological defects were observed in these plants, these mutations could cause growth suppression or conditional

defects as TOR responds to environmental and nutrient signals. Accordingly, *Arabidopsis* TOR mutants show arrested growth and embryogenesis (McCready et al., 2020; Menand et al., 2002), while TOR suppression leads to upregulation autophagy and reduced growth (Pu et al., 2017).

Table 4.1 Point-mutations in primary transgenic (T0) lines_ *OsTOR* OsUbi: Cas9

line	**Cas9**	**Targeting**	
		Sg1 site	**Sg2 site**
4-1	-	WT	WT
6-1	+	WT	WT
6-2	+	WT	WT
6-3	NA	WT	WT
6-4	+	WT	WT
6-5	+	WT	WT
7-1	-	WT	WT
7-2	-	WT	WT
7-3	NA	WT	WT
7-4	NA	WT	WT
7-5	-	WT	WT
7-6	NA	WT	WT
9	NA	WT	WT
10-1	+	WT	Monoallelic -6
10-2	NA	WT	WT
10-3	NA	WT	WT
23	+	WT	Monoallelic +1
32-1	NA	WT	WT
34-1	+	WT	[1]Biallelic -9; -3
34-2	+	WT	[1]Biallelic -9; -3
61	+	WT	WT

NA (not available): Cas9 PCR was not done for these lines since there was no seeds for them.
[1]based on T1 analysis

Table 4.2 Point-mutations in the T1 progeny_ *OsTOR* Ubi: Cas9

T0 plant	No. of T1 plants tested	Sg1 mutations[1]		Sg2 mutations		Eff. (%)	
		Non-targeted	Targeted	Non-targeted	Targeted	Sg1	Sg2
6-1	11	ND		11	0	-	0
23	11	ND		11	0	-	0
34-1	11	5	0	0	11	-	100
34-2	11	3	0	0	11	-	100

ND: not determined;
[1]Only lines with homozygous mutation at sg2 were tested for sg1 mutations

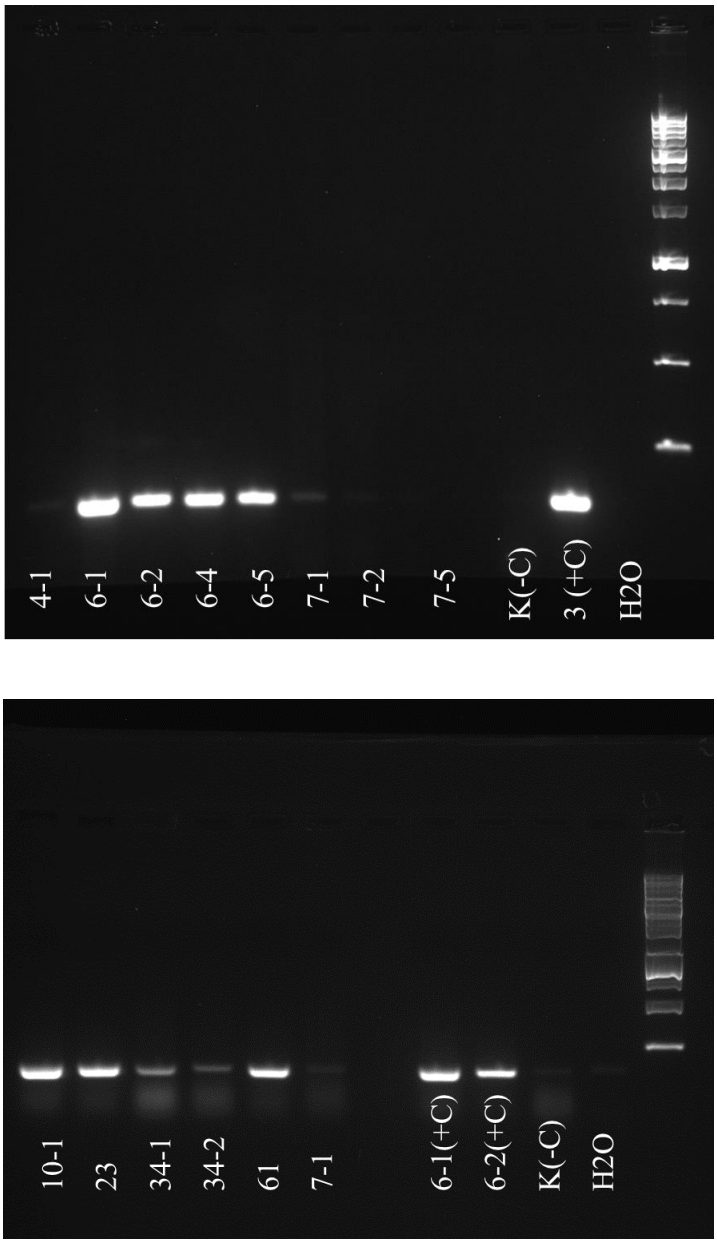
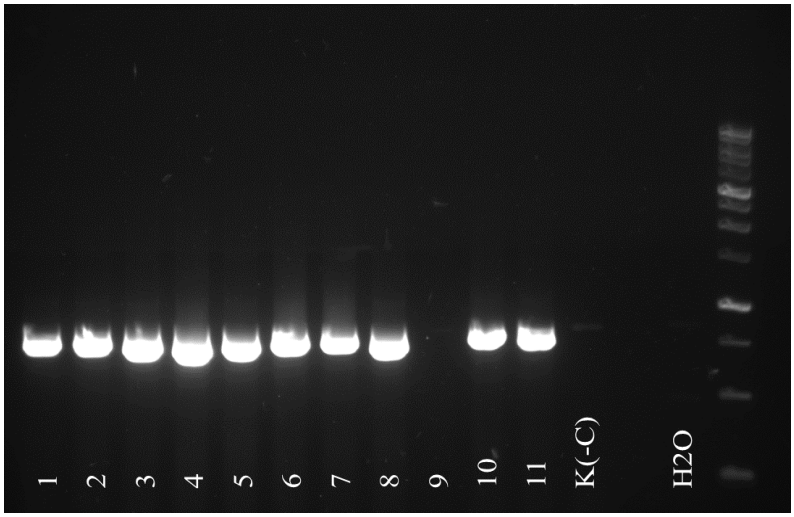


Figure 4.2. Representative image of Cas9 PCR analysis of OsUbi:Cas9 *OsTOR* primary transgenic lines transformed with pNS71+pHPT, Kitaake (K) and water are negative controls

(a)



(b)

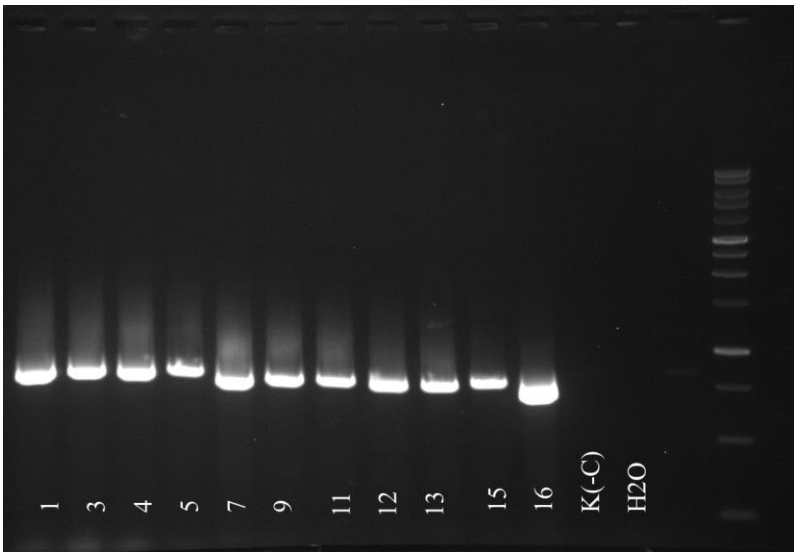
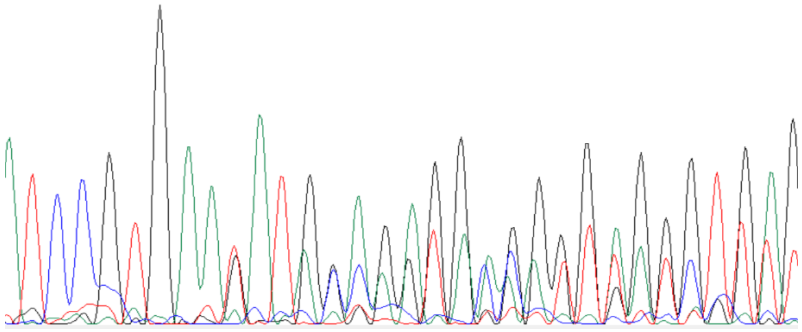


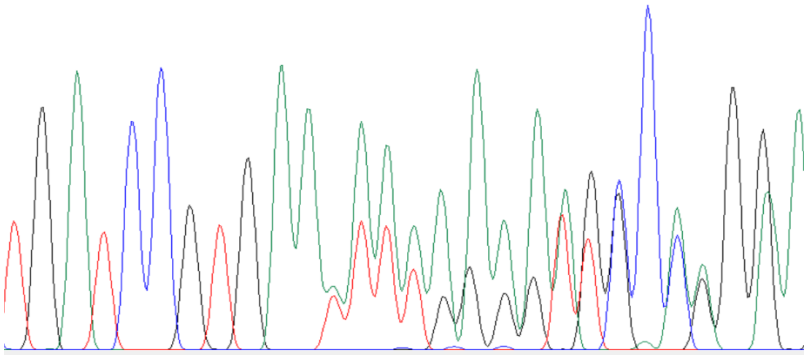
Figure 4.3. Representative image of Cas9 PCR analysis of OsUbi:Cas9 *OsTOR* T1 transgenic lines transformed with pNS71+pHPT **(a)** Line #23 **(b)** Line #6-1. Kitaake (K) and water are negative controls

(a)



```
ref             CACACTTCATGCCCTGATCCGTGAATATAGAGATGCCAGGAAGgtttgtctgatgcaac
Sequence_1      CACACTTCATGCCCTGATCCGTGAA------GATGCCAGGAAGGTTTGTCTGATGCAAC
Sequence_2      CACACTTCATGCCCTGATCCGTGAATATAGAGATGCCAGGAAGGTTTGTCTGATGCAAC
                ************************      ****************************
```

(b)



```
Sequence_2      CACACTTCATGCCCTGATCCGTGAAATATAGAGATGCCAGGAAGGTTTGTCTGATGCAAC
Sequence_1      CACACTTCATGCCCTGATCCGTGA-ATATAGAGATGCCAGGAAGGTTTGTCTGATGCAAC
ref             CACACTTCATGCCCTGATCCGTGA-ATATAGAGATGCCAGGAAGgtttgtctgatgcaac
                ********************** ***********************************
```

(c)

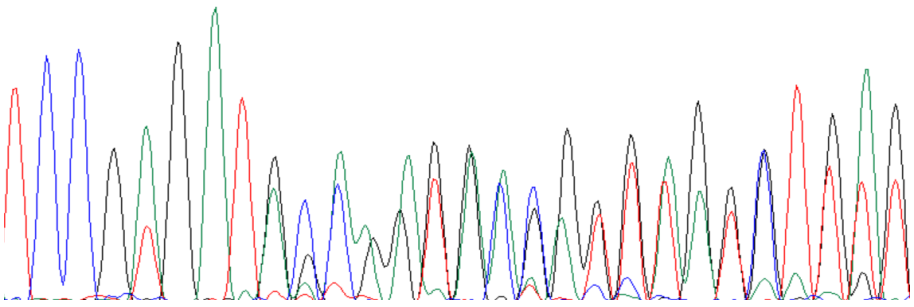
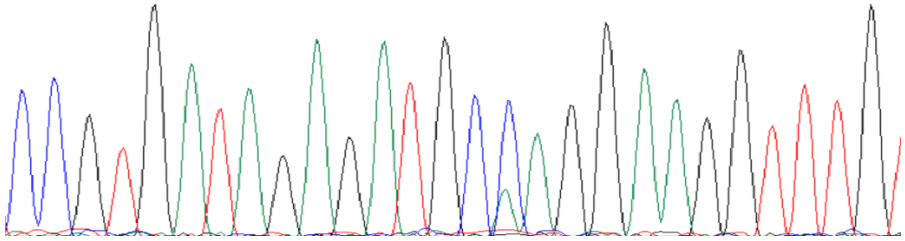


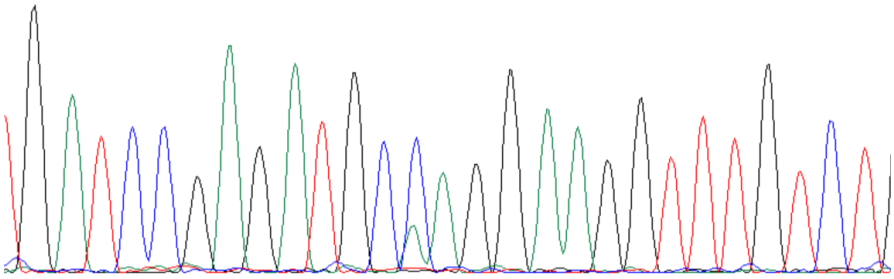
Figure 4.4. *OsTOR* T0 OsUbi:Cas9 sg2 analysis **(a)** Plant #10-1: Monoallelic -6 bp deletion (in-frame) **(b)** Plant #23: Monoallelic +1 bp insertion (early stop codon) **(c)** Chromatogram and sequence alignments of 34-1 harboring mutations that could not be parsed out

(a)



```
ref             ACACACTTCATGCCCTGATCCGTGAATATAGAGATGCCAGGAAGgtttgtctgatgcaac
sequence_1      ACACACTTCATGCCCTGATCCGTGA---TAGAGATGCCAGGAAGGTTTGTCTGATGCAAC
                ************************   ********************************
```

(b)



```
ref             ACACACTTCATGCCCTGATCCGTGAATATAGAGATGCCAGGAAGgtttgtctgatgcaac
Sequence_1      ACACACTTCATGCCCTGATCCG---------AGATGCCAGGAAGGTTTGTCTGATGCAAC
                **********************         ****************************
```

(c)

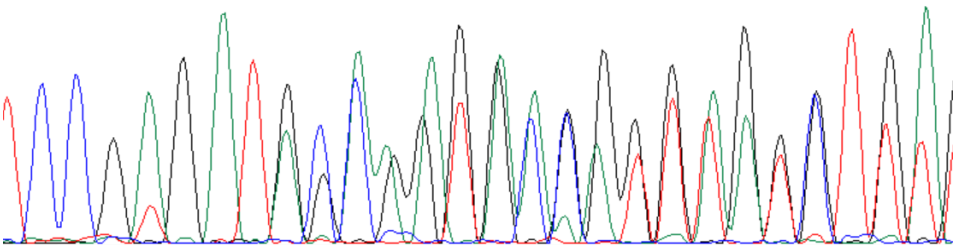


Figure 4.5. Chromatogram and sequence alignments of *OsTOR* T1 OsUbi:Cas9 sg2 site of line 34-1 **(a)** Plant #34-1-5: Homozygous 3 bp deletion **(b)** Plant #34-1-7: Homozygous 9 bp deletion **(c)** Plant #34-1-10 harboring mutations that could not be parsed out.

Figure 4.6. Amino acid sequence alignment of sg2 target site of *OsTOR* for T1 progeny. The mutation at DNA level is mentioned on the right. The box highlights the 3 and 1 (+1 substitutions) amino acid deletions

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Appendix

Primer sequences

Primer	**Sequence (5' – 3')**	**Application**
GusF82	ACCTCGCATTACCCTTACGC	Genotyping sg2 target site in *GUS* gene
NosR2	GCGGGACTCTAATCATAAAAACCC	
Cas9F1	AAAGACCGAGGTGCAGACAG	Cas9 gene expression analysis
Cas9R1	ACCAGCACAGAATAGGCCAC	
PDS-F1982	GGTAGAAATGCCATGCGGGA	Genotyping *OsPDS* locus
PDS-R100	TGGAACTCAAATAACTGGAGATGC	
PDS-F2240	TCCTTCCATGCTCACACTGT	
Cas9-F8	CAGTCCGGCAAGACAATCCT	Cas9 genotyping
Cas9-R3	CACTTCCCGGATCAGCTTGT	
TOR-F2	GGTGAATACTCTGCTGGA	Genotyping of target site 2 in *OsTOR* gene
TOR-R2	CTG ACA CAA GTT TGT TCA GG	