농 학 석 사 학 위 논 문

CRISPR/Cas9 매개 돌연변이 유발을 통한 자스모네이트-ZIM-도메인 단백질 9의 특성 규명

Characterization of Rice Jasmonate-ZIM-Domain Protein 9 through CRISPR/Cas9-mediated Mutagenesis

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Abstract

Characterization of Rice Jasmonate-ZIM-Domain Protein 9 through CRISPR/Cas9-mediated Mutagenesis

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CRISPR/Cas9 system is a powerful technique for genome editing. It is more effective and convenient than any other genome editing methods. In this study, I generate *JAZ9* knock-out mutant rice by using CRISPR/Cas9 system. The guide RNA was recombined with the rice U3 promoter in the *CRISPR/Cas9* binary vector pRGEB31 carrying the gRNA scaffold III. The guide RNA sequence was designed from the CRISPR-PLANT website (http://www.genome.arizona.edu/crispr) to avoid off-target effects. Total 26 individual T_0 transgenic rice were produced through *Agrobacterium*-mediated callus transformation. *Cas9*-mediated mutation was occurred upstream of 3-nucleotide position from protospacer adjacent motif site in the target region. Nucleotide sequence analysis showed that most T_0 transgenic plants were genetically chimeric mutants. Just two individual mutants

(approximately 8%) were homozygotic bialle chimera at the target site, and 16 individual mutants (approximately 62%) were heterozygotic chimeras. I classified these 26 individual lines into 4-groups according to the mutation pattern type. First, if no mutation occurs, 'only wild-type sequence'. Second, the cases of biallelic homozygotic chimeric mutations are grouped 'only mutated sequences'. Third, when a wild-type sequence and a mutated sequence coexist (codominant), the 'WT and mutated sequence together'. Finally, if the wild-type sequence and the mutated sequence coexist, but the ratio of the mutated sequence is much lower, the 'WT and the weak mutated sequence together'. Some chimeric mutant did not remain in the same group and mutations were observed or disappeared with the developmental stage. The mutation rate tended to increase with higher *Cas9* expression level. However, the copy number of *Cas9* transgene did not determine the level of *Cas9*. To obtain additional homozygotic mutant plants, I carried out further analysis with the T_1 and T_2 siblings. All T_1 siblings from T_0 mutant plants with homozygotic mutation still maintained homozygotic mutation. I also found that 60% of heterozygotic mutant plants in T_0 produce the homozygotic mutant in T_1. These suggest that homozygotic mutant can be obtained in T_1 generation from T_0 mutant of heterozygotic chimeras. The number of individual homozygotic mutant plants, therefore, can be increased through genetic segregation of T_0 heterozygotic mutants.

Key words: biallele, chimera, CRISPR/Cas9, guide RNA, JAZ9, mutant, transgenic rice.

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Introduction

For gene function research, the technologies have been developed to genome editing such as zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) (Bogdanove and Voytas, 2011; Carroll, 2011). Both ZFNs and TALENs are processes that cause site specific integration on the chromosome (Cristea et al., 2013). However, sites that can be specified have many limitations and are time-consuming and costly (Nemudryi et al., 2014). And the RNA interference method specifically suppresses gene expression by post-transcriptional gene silencing (PTGS), but this method does not knock-out genes completely (Elbashir et al., 2000; Elbashir et al., 2001). Transfer DNA (T-DNA) insertion has long been used to reverse genetics, but it may not be able to obtain the desired mutant because specific targeting of the desired gene is not possible (Krysan et al., 1999).

The clustered regularly interspaced short palindromic repeats (CRISPR) /CRISPR-associated protein 9 (Cas9) is a genome editing system that overcomes the limitations of other genome editing technologies. The Cas9 protein is complementarily targeted to DNA with a single guide RNA (gRNA) which locate in upstream side of the protospacer adjacent motif (PAM) (Jinek et al., 2012; Anders et al., 2014; Chavez et al., 2015). In the mutation process by CRISPR/Cas9 system, Cas9 nuclease generates DNA double strand breaks (DSBs) and induces random insertions or deletions (indels) by non-homologous end joining (NHEJ) repairing (Takata et al., 1998; Lieber, 2010). Because of these advantages, CRISPR/Cas9-mediated mutagenesis is widely used in bacteria, mammalian cells, human cells and plants. (Dicarlo et al., 2013; Mali et al., 2013; Ran et al., 2013).

Jasmonic acid (JA) and its volatile form, MeJA, is a phytohormone synthesized from fatty acids and play a role in the defense mechanisms of plants. (Seo et al., 2001;

Cheong and Do Choi, 2003; Farmer et al., 2003). Jasmonate ZIM-domain proteins (JAZs) repress the expression of JA-induced genes and act as substrates for the SCF^{COI1} complex (Devoto et al., 2002; Sheard et al., 2010; Singh et al., 2015). MYC2 act as a transcription factor of *bHLH* and interacts with JAZs to facilitate JA responses (Fernández-Calvo et al., 2011; Niu et al., 2011; Zhang et al., 2015). Therefore, in order to understand the JA signaling pathway, functional analysis of JAZs is essential, and this requires plants with impaired function of JAZs.

In this study, to generate knock-out mutants, I targeted the *JAZ9* gene in rice using CRISPR/Cas9 system. As a result, 18 T_0 transgenic rice showed four different patterns and chimerism, which were caused by the heterogeneity or biallelism of the cells. Furthermore, the editing pattern was found to be influenced by edited nucleotide (A, T, G or C) species, *Cas9* expression level and plant growth.

This patterns were also inherited to the next generation, confirming that the JAZ9 function of the mutants was actually inhibited. Therefore, the genotype of rice produced using the CRISPR/Cas9 system becomes more diverse as the growth and generation progresses, I suggest that the early analysis of genetic traits of transgenic rice is advantageous for obtaining homozygotic mutants.

Materials and Methods

1. Plant material, transformation and growth

Wild-type rice (Oryza sativa L ssp. japonica cv. Dongjin) were used for *Agrobacterium tumefaciens*-mediated callus transformation. Mature seeds induced embryonic calli after dehusk and sterilization, as described previously (Hiei et al., 1994). Total 26 T_0 transgenic plants were obtained through hygromycin selection and cultivated in green house with long day regime (16 h/8 h = light/dark) at 28℃.

2. Construction of DNA plasmid for *Cas*-mediated mutagenesis

For *JAZ9* mutagenesis, pRGEB31, a vector containing Cas9 coding region, was obtained from Addgene. *JAZ9*-specific guide RNA sequence was designed through the CRISPR-PLANT website (http://www.genome.arizona.edu/crispr). The gRNA was recombined with *Bsa*I (New England Biolabs) and T4 ligase (New England Biolabs) in the *Bsa*I site of the pRGEB31 vector. The recombinant plasmid pRGEB31-OsJAZ9 was verified by enzymatic digestion and nucleotide sequencing, which was transformed into *Agrobacterium tumefaciens* LBA4404.

3. Extraction of rice genomic DNA, PCR and nucleotide sequencing

Fresh rice leaves were sampled from 0.02 to 0.03 g, cut into small pieces using scissors and grinded in a 1.5 ml micro tube. Add 500 μl of extraction buffer (pH 8.0, 0.1 M Tris-HCl, 50 mM EDTA, 0.5 M NaCl and 0.001% 2-mercaptoethanol dissolve in dH_2O) to a 1.5 ml micro tube containing the sample and add 35 μl of 20% SDS solution after inverting and placed in a 65℃ heat block for 10 minutes. The supernatant was transferred to a new micro tube after centrifugation at 13000 rpm

for 10 min. 450ul of 2-propanol and 45ul of 3M NaOAC, place at -20℃ for 10 minutes, and centrifuge at 13000rpm for 10min. The supernatant is discarded and washed with 70% ethanol and diluted with dH_2O. From the extracted rice gDNA, *HPTII* and *Cas9* regions were amplified by PCR to confirm that the recombinant DNA was transformed. The *JAZ9* region was amplified and analyzed by nucleotide sequencing. Premixed-taq DNA polymerase (Bioneer) was used for PCR; Oligonucleotides and nucleotide sequencing were performed by Macrogen.

4. Quantitative RT-PCR and TaqMan PCR analysis

For analyses of expression level of transcripts, cDNAs of the plants were amplified by reverse transcriptase (Invitrogen) from total RNAs of 4-week-old and 6-week-old rice. *Tublin2* was used as an endogenous control to measure relative expression levels, SYBR green was used as a fluorescent material, and qPCR was performed using a 2X qPCR master mix (Bioneer). To analyze transgene copy number in T_0 transgenic rice, fluorescent probe was used to perform quantitative PCR. The hetero-single copy transgenic rice used as control. *NOS terminator* and *tublin2* were used TaqMan PCR as an endogenous control. Specific primers and probes were designed in the *Tublin2* and *NOS terminator* regions. Fluorophore HEX (for *Tublin2*) and FAM (for *NOS terminator*) were labeled at the 5'end of each probe. TaqMan PCR was performed with final 20ul reaction, rice gDNA was used 100ng per reaction, and 20X TaqMan PCR master mix (with probe, Macrogen) was used. The experiment was performed by the qPCR instrument of Roche Life Science.

5. Yeast Two-Hybrid assay

Matchmaker Gold Yeast Two-Hybrid System (Clontech) was used to perform yeast two-hybrid assay. pGBKT7 used as a bait plasmid contains Trp biosynthesis gene *TRP1* and pGADT7 used as a prey plasmid contains Leu biosynthesis gene *LEU2*.

OsJAZ9 and *OsCOI1* were amplified from wild-type Oryza sativa (Dongjin) cDNA and *AtCOI1* was amplified from wild-type Arabidopsis Thaliana (Col-0) cDNA. These cDNAs were recombined into the *Sma*1 enzyme site of pGBKT7 and pGADT7 using the gibson assembly system (New England Biolabs). The *OsJAZ9* bait and *COIs* prey plasmids were cotransformed in yeast strain (Y2H Gold, Saccharomyces cerevisiae). Cotransformed yeast were selected in synthetic double-dropout (DDO) minimal growth medium (without Trp and Leu). For the protein-protein interaction assay, Aureobasidin A, an antibiotic, was used at a concentration of 250 ng/mL, and yeast (OD = 0.5) selected from DDO medium was dropped into the Abs A-containing medium. Incubated for 4 days at 30℃ in dark condition and captured with a digital camera (Coolpix p300; Nikon).

6. Site-directed plasmid mutagenesis

EZchange™ Site-directed Mutagenesis Kit (Enzynomics) was used for the mutagenesis of prey plasmid. Primers for inverse PCR were designed from the enzynomics website (http://www.enzynomics.com/sub0405). Amplified PCR products were used to 300ng per reaction; 2X EZ-MIX Buffer, and 10X EZ-MIX enzyme. After reacting for 1 hour at 37℃, it was transformed into *Escherichia Coli* (DH5α).

Results

1. Generation of *osjaz9* mutant by CRISPR/Cas9 system

Using the CRISPR/Cas9 system to obtain rice mutants that are mutated in *JAZ9* gene, gRNA was designed from CRISPR-PLANT website (http://www.genome.arizona.edu/crispr). This process has helped to avoid the off-target effect. The JAZ9 specific gRNA was recombined into pRGEB31, a CRISPR/Cas9 binary vector. *JAZ9* gRNA is expressed by the U3 promoter, a rice internal promoter, and *Cas9* and *HPTII*, a hygromycin-resistant gene, are expressed by the 35S promoter (Fig. 1A).

Agrobacterium-mediated callus transformation was used to obtain transgenic rices, and the callus was derived from wild-type rice Dongjin. As a result, total 26 lines of T_0 rice were obtained. 26 individual lines of transgenic rice that had undergone the hygromycin selection process were verified again by PCR of the *HPTII* gene. Dongjin was used as NT control, and *HPTII* gene was amplified in all 26 lines except NT (Fig. 1B). This result mean that the recombinant DNA plasmid has been transformed into all T_0 rices. To confirm that mutation occurred in *JAZ9* of these T_0 plants, the gRNA target region of *JAZ9* was PCR amplified as much as 596 bp length and nucleotide sequencing analysis was performed using 3' antisense primer (Fig. 1C).

2. Genetic chimerism in T_0 generation

The chimerism of the *JAZ9* was shown from nucleotide sequencing analysis in the most of the T_0 transgenic plants. I amplified *JAZ9* of 26 transgenic plants by PCR and classified the *Cas9*-mediated genome editing into 4 types according to the

mutation pattern.

Group A, ‘Only WT sequence’ pattern (Fig. 2A). In this group, nucleotide sequencing results are in the same form as the result of NT nucleotide sequencing, and lines 10-13, 18, 19, 22 and 28 correspond to this group. In this case, the mutation did not occur at all or very little mutation was expected. Of the 26 lines, there were 8 lines in this group and account for about 31%.

Group B, ‘Only mutated sequence’ pattern (Fig. 2B). WT sequence is not observed at all, but only mutated sequence is observed, and line 2 and 20 correspond to this group. 2 plants account for about 8%. The mutation occurred in the 3-nucleotide upstream of the Protospacer Adjacent Motif (PAM) site. This sequence peak data suggests that biallelic homozygotic mutations were made in line 2 and 20 of *JAZ9*.

Group C, ‘WT and mutated sequence together’ pattern (Fig. 2C). WT sequence and mutated sequence are shown together and have the equal peak height. This suggests that the plant genome is showing chimerism. There were 6 lines in this group and these lines 1, 6, 17, 23, 24 and 27 account for about 23%.

Group D, ‘WT and weak mutated sequence together’ pattern (Fig. 2D). Although the WT sequence and the mutated sequence are all present like group C, the peak height of the mutated sequence is much lower than that of the WT sequence, and lines 3-5, 7, 9, 14-16, 21 and 26 are included in this group. And these account for about 38%. This indicating that the ratio at which the mutation occurred in *JAZ9* is much lower than the group C 'WT and mutated sequence together'. A comparison between the above 4 groups reveals that the assay results differ depending on the mutated portion of the *JAZ9* in cells of plant.

I further analyzed the line 2 homozygotic mutant of Group B to determine the correlation between the nucleotide sequencing peak height and the actual genome editing frequency. Sequencing of 11 independent colonies cloned in the TA-plasmid

resulted in 5 C additions (Fig. 2E, top) and 6 T additions. This result indicates that there is a correlation between the sequencing peak height and the genome editing frequency, and verifying that line 2 is a biallelic homozygotic mutant.

The results suggest that Cas9-mediated mutant plants consist of a mixture of genome edited cells and unedited wild-type cells; chimera of different genotypes of genome edited and unedited cells accounts for about 61%. Only 2 lines (lines 2 and 20) of the transgenic rices contained only genome edited cells without unedited cells. However, these were homozygotic biallelic mutants with different alleles on the genome; Line 2 and line 20 each have a C/T or A/T biallelic addition.

Overall, these results showed a chimerism of *JAZ9* in Cas9-mediated T_0 transgenic plants owing to heterogeneity of cells or biallelism in the same cell.
The proportions of these types are shown in Fig. 2F. Mutations occurred in 69% (18 lines out of 26 lines) of Cas9-mediated T_0 transgenic plants and no mutation occurred in the remaining 31% (8 lines). And T_0 homozygotic mutant was 8% (2 lines).

3. Correlation between edited nucleotide and mutation pattern

The frequency of the mutation was different depending on the type of added nucleotide. Deoxyadenylation (A addition) occurred on lines 17, 23 and 24, accounting for 17% of all mutants (Fig. 3A). And all 3 lines with A addition was group C, ‘WT and mutated sequence together’.

T addition occupies the largest portion with 54%, and lines 3-5, 7, 9, 14-16, 21 and 26 are included in this (Fig. 3B). In addition, all T additions showed group D, 'WT and weak mutated sequence' pattern, and there was no group D other than T addition.

G addition, as with A addition, occurred with a probability of 17% and lines 1, 6 and 27 are included (Fig. 3C). All the G added mutants are also included in group C. Conversely, group C only included G addition and A addition, and no other

nucleotide addition was observed.

The A/T addition and the C/T addition were line 20 and line 2, respectively, which together accounted for 12% (Fig. 3D and 3E). In the case of A/T addition, the frequency of A addition and T addition can be further increased, since A addition and T addition are independently generated on the same genome. Also, C addition could not be found, but C/T addition means C addition can occur with low frequency.

As a result, T addition was the most frequent, followed by A addition and G addition. And the A/T addition and C/T addition was the least. The frequency of each nucleotide addition is shown in Fig. 3F. The addition of one kind of nucleotide is 88% (A, T and G, 16 lines) and the addition of two kinds of nucleotides is 12% (A/T and C/T, 2 lines).

4. The genome editing pattern can vary depending on the developmental stage

Genomic patterns of the 6-week-old transformants were analyzed to compare whether the genome editing patterns could change with developmental stages and compared with the previous 4-week-old data (Figure 4). In most of T_0 transgenic plants showed no change in the genome editing pattern. However, I have shown changes in editing patterns along with development of lines 6, 15 and 20.

The line 6 transgenic plant at 4-week-old stage showed the group C 'WT and mutated sequence together' pattern; the added G sequence peak was clearly detected with the wt sequence peak. However, at 6-week-old stage, the peak is certainly disappeared (Fig. 4A).

Line 6 was the result of the opposite of line 15. In 4-week-old stage, line 15 was group A, ‘only WT sequence’ pattern; no edited sequence peak was found at all, but a peak appeared when G addition occurred in 6-week-old stage (Fig. 4B).

At 4-week-old stage, line 20 was group B, 'only mutated sequence' pattern and T

addition ratio was higher than A addition. But, as it grew up in the 6-week-old, the ratio of T was higher than A, and the genome editing pattern has changed to the combination of group B and group C (Fig. 4C).

These results suggest that the chimeric genotype of Cas9-mediated T_0 plant is due to the heterogeneity of the cell type. And the genotype can be changed by tissue and development by the continuous expression of Cas9.

5. Further analysis of genome editing pattern shows complexity of groups

To more precisely identify the genome editing status, 6-week-old line 20 mutant showing the combination form of group B and group C was cloned into TA-plasmid and analyzed.

In the analysis of 15 independent colonies cloned in TA-plasmid, there were 3 no mutation colonies (Fig. 5A), 2 T addition (Fig. 5B), and 8 A addition (Fig. 5C). This result shows that line 20, which is 6-week-old mutant, represents genomic chimerism and has both T addition and A addition in different cells. Especially, as mentioned in the previous results, it can be seen that T addition is group D pattern and A addition is group C pattern; the ratio means that T addition vs. A addition is 1: 4 (A addition is 4 times more.).

An unexpected substitution was also found (Fig. 5D). This indicates that substitution may be involved in the PCR product of the non-isolated genome. A substitution pattern cannot be noticed when sequencing with A addition. However, this result mean that Cas9-mediated genome editing may cause substitution as well as addition

Therefore, these results suggest that there is a correlation between sequencing peak height and genome editing frequency, and that point mutated mutants in Cas9-transformant can also be obtained.

6. *Cas9* expression level is correlated with the genome editing frequency

TaqMan PCR experiment were performed to determine the correlation between *Cas9* transgene copy number and *Cas9* expression. The transgene copy number of 4-week-old T_0 transgenic rice 26 lines was summarized (Fig. 6). The TaqMan PCR value ranged from 0 to 2.3. Among them, the single copy was 8 lines (30%).

There was a correlation between *Cas9* expression level and genome editing frequency. Line 2 of the 'Group B, Mutated sequence only' pattern showed the highest *Cas9* expression, and line 20 of the same group also higher than line 10 and line 11 of 'Group A, only WT sequence' and 'group D, WT and mutated sequence together' pattern line 3 and line 4. Similarly, line 6 and line 17 of the ‘Group C, WT and mutated sequence together’ patterns showed higher expression of *Cas9* than that of Group A and Group B lines. Group D also had higher Cas9 expression than Group A on average. These trends suggest that the Cas9 expression level is related to the genome editing frequency. As a result, the copy number of Cas9 transgene does not affect Cas9 expression level, but high Cas9 expression increases genome editing frequency.

7. Homozygotic mutant can be obtained in T_0 generation

T_0 line 2 transgenic rice was found to be a biallelic homozygotic mutant. I harvested the seeds from the line 2 mutant and analyzed the genotypes of the T_1 siblings of 8 plants (Fig. 8). PCR was used to amplify the *Cas9* region of the transgene to obtain a T_1 sibling mutant in which *Cas9* transgene was segregated. Of the 8 T_1 siblings, only one sibling (line 2-3) did not amplify *Cas9* region. To analyze the editing pattern in T_1 siblings, I amplified the endogenous *JAZ9* region and proceeded with nucleotide sequencing (Fig. 8A). As a result, all T_1 siblings of line 2 mutants were still mutated in *JAZ9*, and mutated nucleotides of line 2, which were C/T additions, were isolated into three types. Lines 2-1, 2-2 and 2-4 were homozygotic C addition

patterns and lines 2-6 and 2-7 were homozygotic T addition patterns. And, line 2-3, 2-5 and 2-8 were observed in the C/T addition pattern as T_0 line 2 mutant (Fig. 8B). These results indicate that the genotype of the T_0 mutant was inherited stably as T_1 siblings. Plus, cas9 segregation out mutant line 2-3 harvested the seeds and the harvested 4 individual T_2 siblings analyzed the genotypes (Fig. 8C). T_2 siblings were all cas9 segregation out mutants and mutated nucleotides were also divided into three types. Similar to the type in T_1 siblings, the C/T addition pattern was also separated by C addition, T addition and C/T addition patterns in T_2 siblings. C addition was 1 line 2-3-4, T addition was 1 line 2-3-2, and C/T addition was 2 lines 2-3-1 and 2-3-3. Taking all the results so far into account, I suggest that each allele of the T_0 biallelic homozygotic mutant using Cas9-mediated transformation is inherited to the next generation based on Mendel's law of genetics. In addition, since mutants with fixed genome are no longer targeted to guide RNA, additional mutations do not occur or mutations disappear. Finally, the *Cas9* transgene can obtain the segregation out mutant from the T_1 generation.

8. Homozygotic mutants were obtained even from T_0 heterozygotic mutant

The nucleotide sequencing results of Group C-line 6 showed that additional homozygotic mutants could be obtained. Of the 20 T_1 siblings in Line 6, 4 individuals showed homozygotic G addition (Fig. 9a, left), 13 individuals were mixed with the WT sequence and the mutated sequence like the T_0 genomic pattern, and the remaining 3 individuals showed only the WT sequence. The homozygotic G addition of Line 6-27 was inherited in all T_2 siblings (Fig. 9a, right).

In the case of Group D-line 21, T_1 homozygotic mutants were also obtained. In line 21, 30 siblings were analyzed, 7 of them were 'Group B, homozygotic A addition' patterns (Fig. 9b, left), 16 individuals were 'Group C, WT and mutated

sequence together' patterns, 3 individuals were 'Group D, WT and weak mutated sequence together' patterns, and the remaining 4 individuals were 'Group A, only WT sequence' patterns. Similarly, line 21-29 homozygotic mutation pattern was inherited in all T_2 siblings (Fig. 9b, right). These results indicate that even though homozygotic mutants cannot be obtained from T_0 Cas9-mediated mutants, they can be obtained from T_1 siblings. Also, it can be seen that the added nucleotide types of the T_0 mutant are not changed but are inherited to the next generation.

The analysis of Group D-line 3 and line 4 indicated that there were lines that could not obtain additional mutants in T_1 siblings. 1 weak mutated plant was obtained from 8 individual T_1 siblings, each of line 3 and line 4, and all the rest were WT sequence plants. This result suggests that homozygotic mutants can be obtained in the next generation even if they are in the same T_0 group D line. In addition, a line with no additional mutations in the T_1 generation means that mutation does not occur in the next generation.

Group A-line 15 also failed to acquire homozygotic mutants in T_1 and T_2 generations. Line 15 and line 6 were lines with different genotypes on the 4-week-old plant and the 6-week-old plant, both T_1 lines having the same genetic tendency as the results of the 4-week-old T_0 plant. This suggests that mutations that occur early in the growth are important rather than additional mutations that occur according to the developmental stage. In other words, mutations in somatic cells are not inherited to the next generation. In conclusion, the analysis of T_0 transgenic rice early in the growth is useful for obtaining homozygotic mutants.

9. Mutated OsJAZ9 protein did not interact with JA receptors

Yeast two-hybrid assay was performed to see what changes occurred in the function of OsJAZ9 mutant rice from Cas9-mediated genome editing. Endogenous *OsJAZ9* and 2 mutant forms of *OsJAZ9* were inserted into the prey plasmid, respectively (Fig.

11a). And, AtCOI1 and OsCOI1, which are known to interact with JAZ9 protein, were inserted into the bait plasmid, respectively. I analyzed whether each prey plasmids interacted with bait plasmids. As a result, mutated OsJAZ9 did not interact with COI1s (Fig. 11b). The result means that the early generation of the stop codon due to 1 nucleotide addition may interfere with the interaction between OsJAZ9 and COI1s. This suggests that genome editing using the CRISPR/Cas9 system can interfere with the specific function of the gene; and Jasmonate signal pathway of the Cas9-mediated *OsJAZ9* mutant may have been interrupted.

10. *OsJAZ9* negatively regulate JA response in rice

To determine what changes occurred in the JA signal of the mutant with the loss of OsJAZ9 function, I analyzed the expression levels of JA-induced genes in line 2-3 mutants by qRT-PCR (Fig. 11).

The expression levels of *OsbHLH148, OsDIP1* and *OsMYC2* were analyzed when JA was applied to Dongjin wild-type rice (Fig. 11a). As a result, the expression level of *OsbHLH148* was 82 fold, *OsDIP1* increased 45 fold and *OsMYC2* increased 4 fold. These JA-induced genes were used to compare expression levels in wild-type and line 2-3 mutants (Fig. 11b). The gene expressions were all increased in line 2-3 mutants. Among them, increased degree of the expression level of *OsMYC2* was lower in JA-treated wild-type plants than in other genes; in line 2-3 mutants, increased degree of the expression levels were similar or slightly high.

Thus, the expression of *OsMYC2* appears to be most influenced by OsJAZ9 knock-out of line 2-3 mutants. These results suggest that OsJAZ9, which acts as a repressor in the Jasmonate signaling pathway, has lost the function of the repressor protein as a result of mutation, and this lost of function has led to increased JA-induced gene.

Discussion

Recently, US Department of Agriculture (USDA) announced that products using the CRISPR/Cas9 system will not be considered in the category of GMOs (Waltz 2016a; Waltz 2016b). This case is suitable for producing and practicing crops with excellent traits of CRISPR/Cas9-mediated transformation, and it is important to find Cas segregant out mutant plants. I constructed 26 lines of T_0 transgenic rice and obtained 18 lines of chimeric mutants. These lines showed 3 patterns in chimeric genotype which generated by heterogeneity or biallelism, among which biallelic homozygotic mutants were able to obtain 2 lines. Nucleotide sequencing peak height correlated with mutation frequency and there was a difference in the rate of mutant according to genome editing pattern. For example, the genomes of the T_0 mutant line 2 were C/T biallelic homozygotic mutations, and the nucleotide sequencing analysis showed that the peak heights of C and T were equal. As a result of randomly separating the PCR product, 5 C addition and 6 T addition were obtained. Also, the analysis results of the T_0 mutant line 20 showed the same tendency as above.

Previous studies have shown that Cas9 expressed in rice protoplasts resulted in several to hundreds of indels at the targeted site (Xie and Yang, 2013). However, in our results of Agrobacterium-mediated callus regeneration, the genome editing type of all rice mutants was 1 nucleotide addition; mutant ratios were different depending on the type of nucleotide. These results suggest that the mechanisms of Cas9 acting on the chromosome of the protoplast and the chromosome of the callus-regenerated cell could be different. These Cas9 mechanism differences in different chromosomes have not yet been clarified, but if you want to obtain a large number of nucleotide deletion mutants, it may be advantageous to produce protoplast-regenerated mutant

plants.

A comparison of 4-week-old and 6-week-old plants showed that the genome editing pattern was dependent on the developmental stage of the plant. Mutations that did not exist or mutations disappeared, and even portions of mutated nucleotides were reversed. These results suggest that Cas9 endogenous mutants may be mutated and genotype may be different depending on tissue. The frequency of mutation mentioned above was higher at higher Cas9 expression level, and the specificity of the editing pattern was inherited in the next generation. In particular, the 4-week-old stage and the 6-week-old stage showed that the lines with different genotypes tend to inherit the 4-week-old stage genotype in the next generation.

JAZ9 expression level of Cas9-mediated *JAZ9* mutant was not different from WT plant. This means that the mutation generation by 1 nucleotide addition did not affect the degradation of the *JAZ9* transcript. However, the results of the protein-protein interaction assay of JAZ9 and COI1s showed that the early generation of stop codon caused by frame shift destroys the inherent functions of JAZ9. Functional defects in JAZ9 acted on the JA signaling pathway to promote expression of JA-induced genes.

There are many researches applying CRISPR/Cas9 system. For example, studies on endonuclease-deficient Cas9 (dCAS9), engineering combined with t-RNA processing system and CRIPR/Cas9 system, and frequency of occurrence of Cas9-mediated mutation (Farzadfard et al., 2013; Xie et al., 2015; Lu and Zhu, 2017). These techniques can be used to study the function of genes by creating mutants that mutate or out of function only the desired domains of a particular protein and to study crosstalk between phytohormones.

In this study, 26 T_0 transgenic rice plants were constructed using the CRISPR/Cas9 system, two lines of T_0 homozygotic mutants were obtained. Since the frequency of obtaining T_0 homozygotic mutant rice is 8%, it is suggested that 30 to 40 independent T_0 transgenic rice should be produced if more than 3 lines of T_0

homozygotic mutants are to be obtained.

If not, there is a way to obtain additional homozygotic mutants through analysis at the T_1 generation. The method of making rice mutant using callus-regeneration is time-consuming and labor-intensive. Also, growing and harvesting all of the rices produced is time and space constrained. Therefore, if you want to obtain a larger number of mutants, it will be an efficient way to quickly analyze transgenic plants within the 4-week-old stage.

Researches utilizing and applying CRISPR/Cas9 are being carried out all over the world, and its utilization is very valuable for biological research.

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Table 1. Identification of genes used in this study

Gene	Accession No	Oryzabase gene name synonym
OsJAZ9	Os08g0428400	Jasmonate ZIM-domain protein 9
OsCOI1	Os01g0853400	Coronatine insensitive 1a
AtCOI1	At2g39940	Coronatine insensitive 1
OsbHLH148	Os03g0741100	Basic helix-loop-helix protein 148
OsMYC2	Os10g0575000	Basic helix-loop-helix protein 009

Table 2. Oligonucleotides sequence of guide RNA duplex for vector construction

Name	Sequence (5′ – 3′)
gRNA sequence	GTCCTTCAGGTCGTCGGCGG
gRNA Duplex-F	GGCAGTCCTTCAGGTCGTCGGCGG
gRNA Duplex-R	AAACCCGCCGACGACCTGAAGGAC

Table 3. List of primer sets used in this study

Name	Sequence (5′ – 3′)	Purpose
JAZ9-F	GCAGAGATGAATGCGTGCAGCT	Sequencing
JAZ9-R	GCACACAGCTCAGACCGTTAGAATC	Sequencing
HPTII-F	ATTTGTGTACGCCCGACAGT	Genotype
HPTII-R	ACCGCAAGGAATCGGTCAAT	Genotype
Cas9-F	AGAACCTGCCCAACGAGAAG	Genotype
Cas9-R	AGGATTGTCTTGCCGGACTG	Genotype
TUB2-F	CCGAGTTCGACGATGGTGACG	qRT-PCR
TUB2-R	AGCCCACACGGACAGATCATAGGATA	qRT-PCR
Cas9 RT-F	GCACCAAAGAGGTGCTGGAC	qRT-PCR
Cas9 RT-R	CTAGATATCTCGAGTGCGGC	qRT-PCR
JAZ9 RT1-F	CGCGTGGGATGGAGAGGGA	qRT-PCR
JAZ9 RT1-R	TTGGTCGCCGGAAACTGCC	qRT-PCR
JAZ9 RT2-F	ACTAGAGAGCAGTGACACCATTGGCA	qRT-PCR
JAZ9 RT2-R	TCATATCTGTAACTTTGTGCTGGGGG	qRT-PCR
Bait-OsCOI-F	GCCATGGAGGCCGAATTCCCGATGGG TGGCGAGGTGCCG	Y2H
Bait-OsCOI-R	CTGCAGGTCGACGGATCCCCTCACGC AGGATGCAAGGGG	Y2H
Bait-AtCOI-R	GCCATGGAGGCCGAATTCCCGATGGA GGATCCTGATATCAAG	Y2H
Bait-AtCOI-R	CTGCAGGTCGACGGATCCCCTCATATT GGCTCCTTCAGGAC	Y2H
Prey-JAZ9-F	GAGGCCAGTGAATTCCACCCGATGGA GAGGGATTTTCTTGGC	Y2H
Prey-JAZ9-R	TCCCGTATCGATGCCCACCCTCATATC TGTAACTTTGTGCTG	Y2H
JAZ9m-F	TCGGCGGCGGCGAGGGA	Mutagenesis
JAZ9m-R	CCGACGACCTGAAGGACATG	Mutagenesis

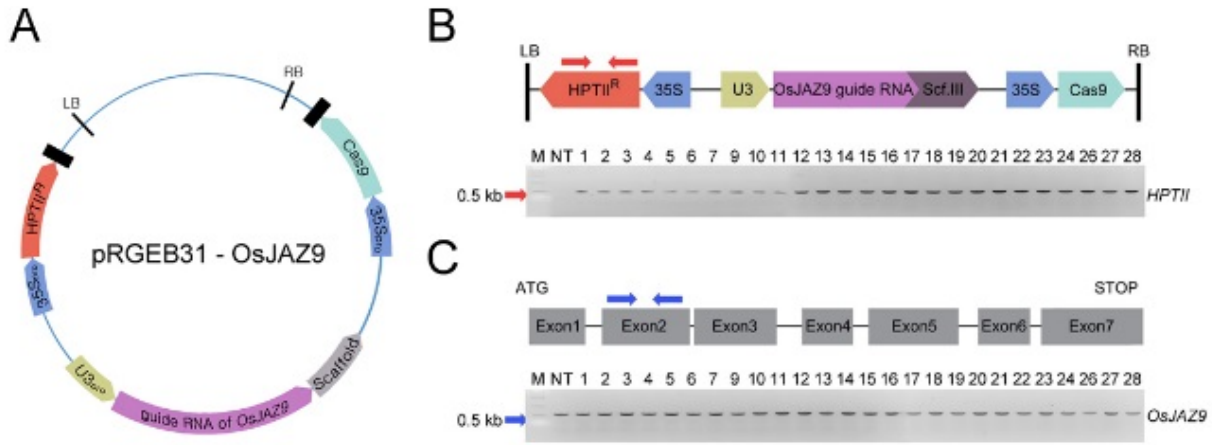

A
RB
LB
Cas9
35Spro
Scaffold
Guide RNA of OsJAZ9
U3pro
35Spro
HPTIIpro
pRGEB31 - OsJAZ9
B
LB
RB
HPTII^R
35S
U3
OsJAZ9 guide RNA
Scf.III
35S
Cas9
M NT 1 2 3 4 5 6 7 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 26 27 28
0.5 kb
HPTII
C
ATG
STOP
Exon1
Exon2
Exon3
Exon4
Exon5
Exon6
Exon7
M NT 1 2 3 4 5 6 7 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 26 27 28
0.5 kb
OsJAZ9

Figure 1. Production of JAZ9 rice mutant using CRISPR/Cas9 system

Plasmid was constructed to mutate the *JAZ9* of rice, and 26 individual plants obtained from this plasmid were verified by PCR. A. The plasmid diagram for mutating *JAZ9*. *OsJAZ9* guide RNA is expressed from the U3 promoter, *Cas9* and *HPTII* from 35S promoter. B. *HPTII* verification PCR for 26 individual plants obtained from *Agrobacterium tumefaciens*-mediated transformation. Arrows indicate the PCR primers of the *HPTII* showing hygromycin-B resistance. C. PCR results of the *JAZ9* on the genome of 26 lines tested in Figure 1b. Arrows indicate the PCR primers of the *JAZ9*. The wild-type rice for callus regeneration used Dongjin and the cell line of *Agrobacterium tumefaciens* used LBA4404.

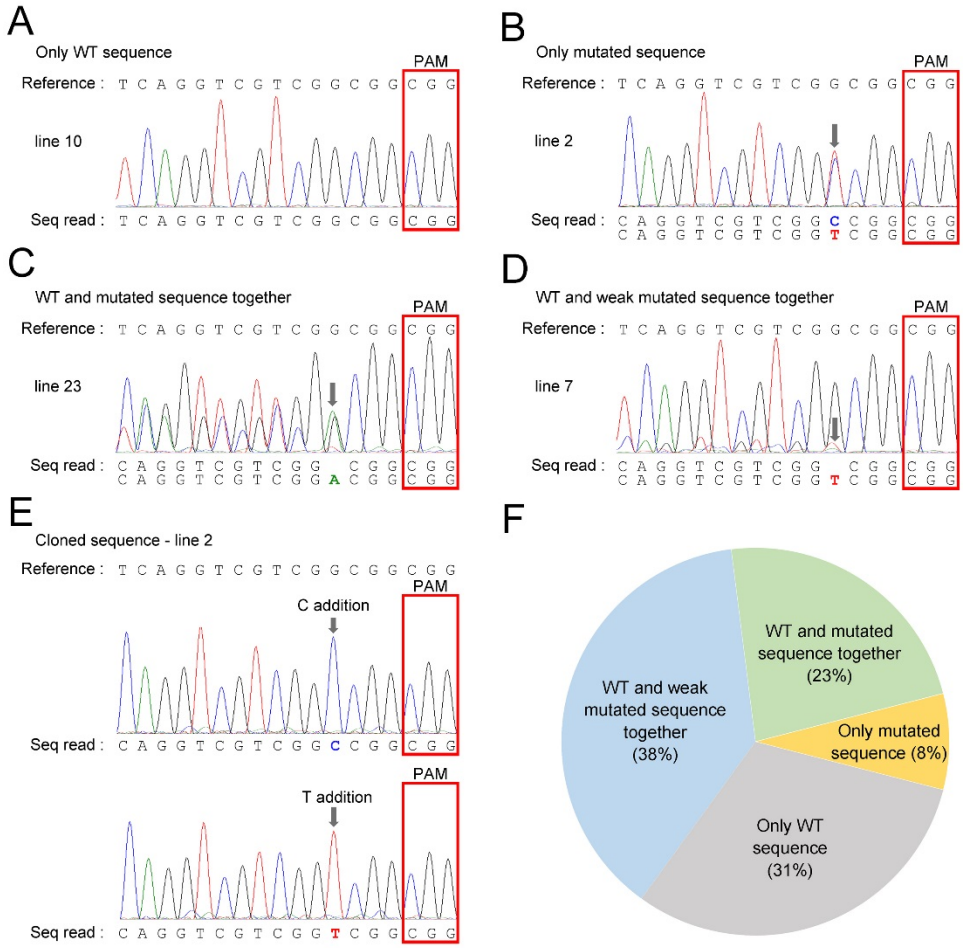

A
Only WT sequence
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 10
Seq read : T C A G G T C G T C G G C G G C G G
B
Only mutated sequence
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 2
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G T C G G C G G
C
WT and mutated sequence together
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 23
Seq read : C A G G T C G T C G G _ C G G C G G
C A G G T C G T C G G A C G G C G G
D
WT and weak mutated sequence together
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 7
Seq read : C A G G T C G T C G G _ C G G C G G
C A G G T C G T C G G T C G G C G G
E
Cloned sequence - line 2
Reference : T C A G G T C G T C G G C G G C G G
PAM
C addition
Seq read : C A G G T C G T C G G C C G G C G G
PAM
T addition
Seq read : C A G G T C G T C G G T C G G C G G
F
WT and mutated sequence together (23%)
Only mutated sequence (8%)
Only WT sequence (31%)
WT and weak mutated sequence together (38%)

Figure 2. Four chimeric mutation patterns of T_0 transgenic rice

JAZ9 of 26 T_0 transgenic rice genomes were analyzed by PCR and nucleotide sequencing. Arrows indicate the added nucleotide. A. Group A, only WT sequence pattern. Analysis of a total of 26 lines showed that 8 lines had the same sequencing peak as wild-type analysis results. B. Group B, only mutated sequence. Two lines showed a homozygotic chimeric mutation pattern. C. Group C, WT and mutated sequence together. Both the WT sequence and the mutated sequence appeared on the 6 lines and showed equal height of the sequencing peak. D. Group D, WT and weak mutated sequence together. In 10 lines, both WT sequence and mutated sequence were present as in Group C, but the mutated sequence showed a much lower peak height. E. Analysis of group B chimeric DNA. *JAZ9* PCR products of line 2 were cloned into TA-vector and analyzed by nucleotide sequencing. F. The pie diagram for four patterns. The frequency of each genome editing pattern for 26 T_0 transgenic rices was summarized.

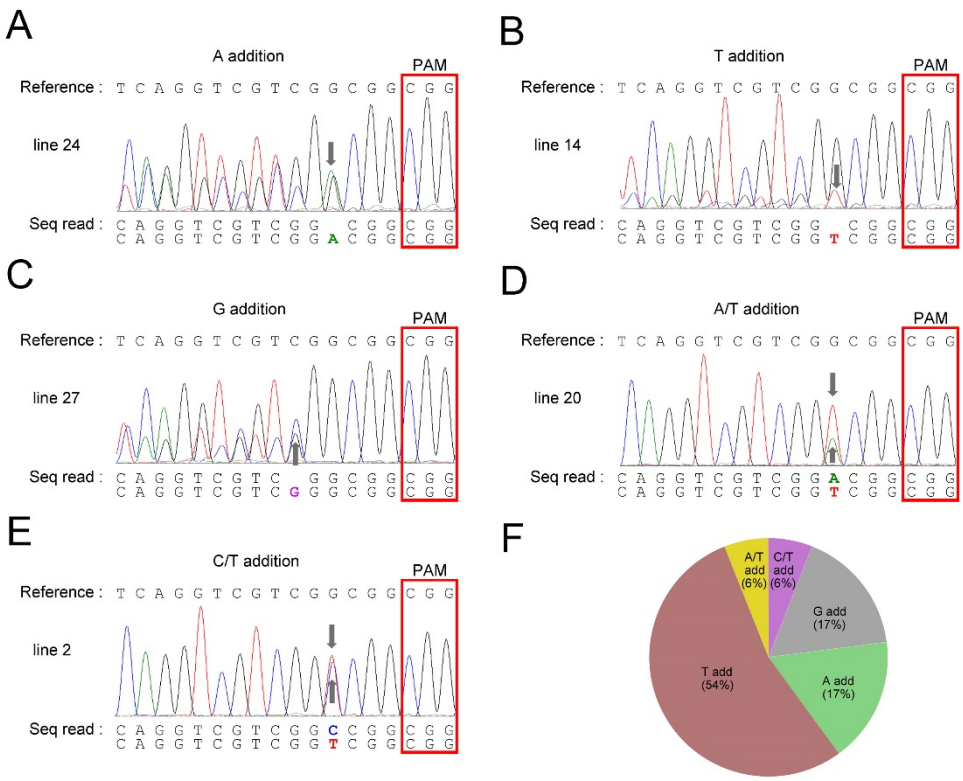

A
A addition
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 24
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G A C G G C G G
B
T addition
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 14
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G T C G G C G G
C
G addition
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 27
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G G C G G C G G
D
A/T addition
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 20
Seq read : C A G G T C G T C G G A C G G C G G
C A G G T C G T C G G T C G G C G G
E
C/T addition
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 2
Seq read : C A G G T C G T C G G C C G G C G G
C A G G T C G T C G G T C G G C G G
F
A/T add (6%)
C/T add (6%)
G add (17%)
A add (17%)
T add (54%)

Figure 3. Analysis of the types of mutated nucleotides in T_0 transgenic rice

Classification of mutants according to the type of nucleotide edited in *JAZ9*. A. Deoxyadenylated mutant sequence. The nucleotide sequencing result in which adenine is added upstream of 3-nucleotide in the PAM site. B. Deoxythymidylated mutant sequence. The sequencing result in which thymine is added upstream of 3-nucleotide in the PAM site. C. Deoxyguanylated mutant sequence. The sequencing result in which guanine is added upstream of 5-nucleotide in the PAM site. D. A mutant sequence that is both deoxyadenylated and deoxytrimidylated. The sequencing result in which adenine and thymine are added upstream of 3-nucleotide in the PAM site. E. A mutant sequence that is both deoxycytidylated and deoxythymidylated. The sequencing result in which cytosine and thymine are added upstream of 3-nucleotide in the PAM site. F. A pie diagram for five cases of all 26 T_0 transgenic rices. Each nucleotide addition ratio corresponds to the sequencing peak color.

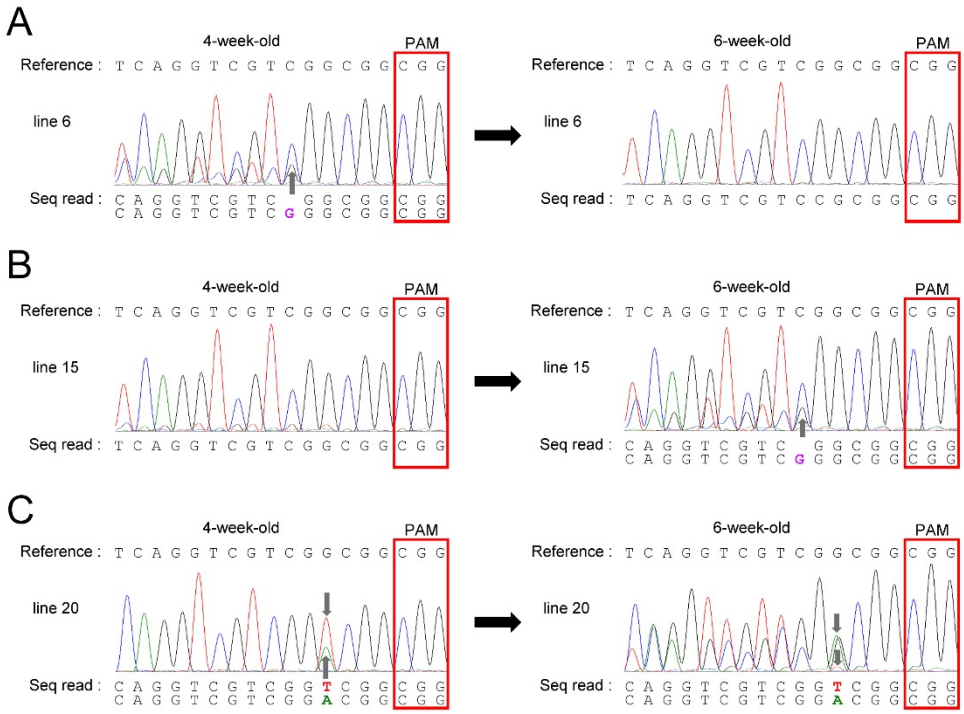

A
4-week-old
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 6
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G C G G C G G
6-week-old
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 6
Seq read : T C A G G T C G T C C G C G G C G G
B
4-week-old
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 15
Seq read : T C A G G T C G T C G G C G G C G G
6-week-old
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 15
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G C G G C G G
C
4-week-old
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 20
Seq read : C A G G T C G T C G G T C G G C G G
C A G G T C G T C G G A C G G C G G
6-week-old
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 20
Seq read : C A G G T C G T C G G T C G G C G G
C A G G T C G T C G G A C G G C G G

Figure 4. Mutation pattern changes of T_0 transgenic rice according to developmental stage

Four-week-old leaf and 6-week-old leaf of same plant were subjected to PCR and nucleotide sequence analyzed, respectively. a. The case of mutation disappearing during plant development. Line 6, which showed group C pattern at the 4-week-old stage, shows group A pattern at the 6-week-old stage. b. The case showing additional mutation during plant development. In contrast to line 6, line 15 showed group A pattern at the 4-week-old stage, but G addition detected at the 6-week-old stage. c. When mutated nucleotide ratio change as plant growth. The peak height of deoxythymidylated mutation was higher than that of deoxyadenylated mutation in line 20 at the 4-week-old stage. And it showed A/T addition of the group B pattern. However, the peak height of A was higher than T in the 6-week-old stage. Moreover, it has changed to the combined form of group B and group C patterns.

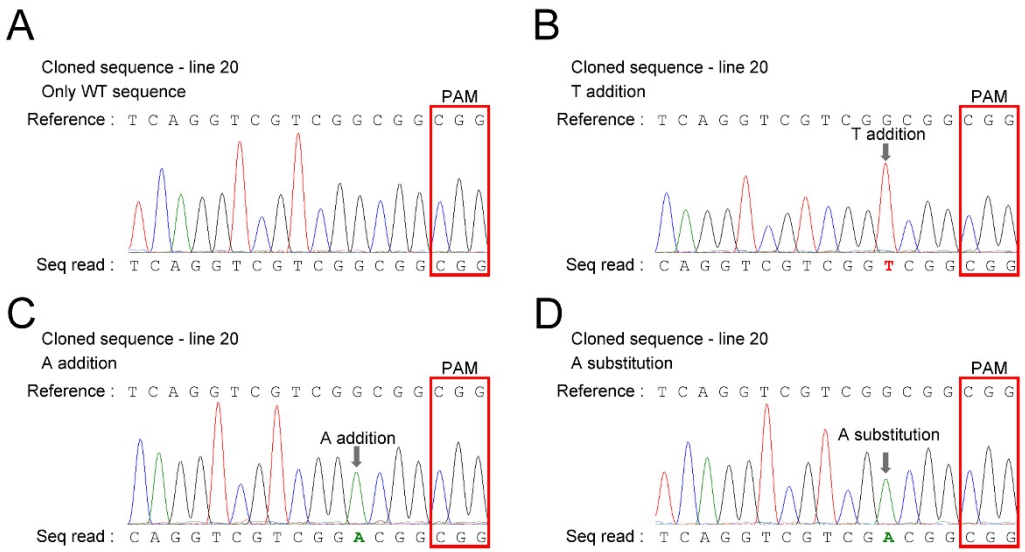
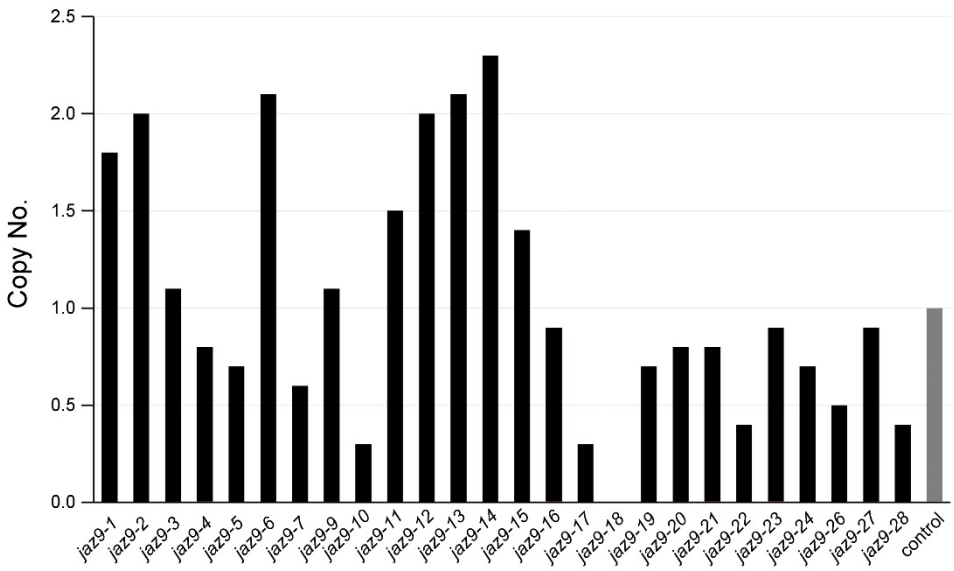

A
Cloned sequence - line 20
Only WT sequence
PAM
Reference : T C A G G T C G T C G G C G G C G G
Seq read : T C A G G T C G T C G G C G G C G G
B
Cloned sequence - line 20
T addition
PAM
T addition
Reference : T C A G G T C G T C G G C G G C G G
Seq read : C A G G T C G T C G G T C G G C G G
C
Cloned sequence - line 20
A addition
PAM
A addition
Reference : T C A G G T C G T C G G C G G C G G
Seq read : C A G G T C G T C G G A C G G C G G
D
Cloned sequence - line 20
A substitution
PAM
A substitution
Reference : T C A G G T C G T C G G C G G C G G
Seq read : T C A G G T C G T C G A C G G C G G

Figure 5. Analysis of 6-week-old line 20 mutant

Line 20, The analysis of 6-week-old leaf showed that the WT and mutated sequence together pattern is distinct. but, the WT and weak mutated sequence together pattern is also shown. a, b, c, d. Analysis of chimeric DNA of line 20 mutant plant. *JAZ9* PCR products of line 20 were cloned into TA-vector and analyzed by nucleotide sequencing. Of the 15 clones, A addition and T addition were 8 and 2, respectively. And there were 2 A substitutions and 3 WT sequence. Arrows refers to the mutated nucleotide.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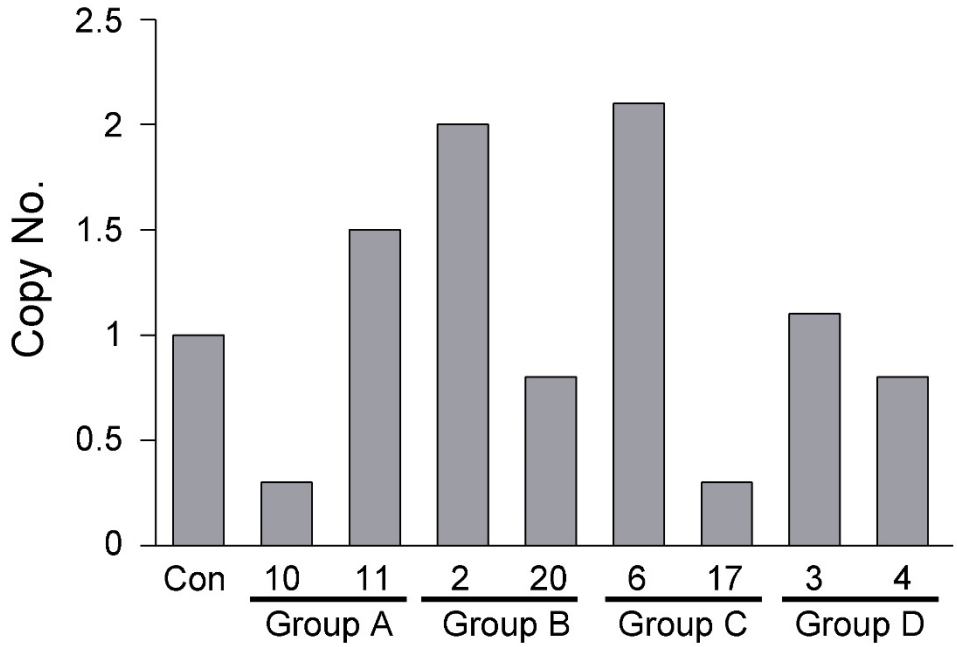
jaz9-19
jaz9-20
jaz9-21
jaz9-22
jaz9-23
jaz9-24
jaz9-26
jaz9-27
jaz9-28
control

Figure 6. Analysis of transgene copy number in T_0 transgenic rice

The transgene copy numbers of 26 individual transgenic rice were determined using TaqMan PCR. *NOS terminator* and *tublin2* were used for TaqMan PCR analysis. Control marked in gray used transgenic rice as a hetero-single copy.


Copy No.
2.5
2
1.5
1
0.5
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Con
10
11
2
20
6
17
3
4
Group A
Group B
Group C
Group D

Figure 7. Correlation of mutation patterns according to *Cas9* expression level

Measurement of *Cas9* expression level from total RNAs using quantitative RT-PCR. *Tublin2* was used for reference for qRT-PCR analysis. *Cas9* expression levels were measured relative to *Tublin2*. When the *Cas9* expression level of *Line 10* is set to 1, it represents the relative *Cas9* expression level of the other *lines*.

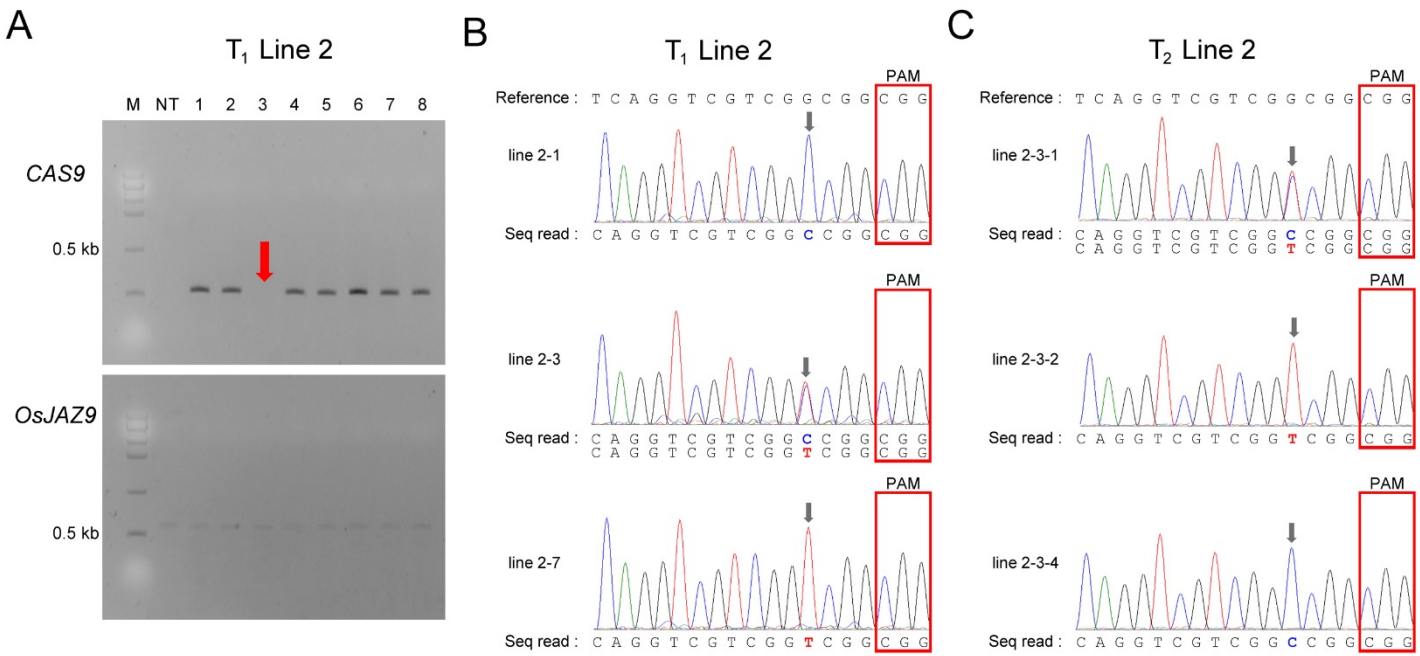
A
T1 Line 2
M NT 1 2 3 4 5 6 7 8
CAS9
0.5 kb
OsJAZ9
0.5 kb
B
T1 Line 2
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 2-1
Seq read : C A G G T C G T C G G C C G G C G G
line 2-3
Seq read : C A G G T C G T C G G C C G G C G G
C A G G T C G T C G G T C G G C G G
line 2-7
Seq read : C A G G T C G T C G G T C G G C G G
C
Reference : T C A G G T C G T C G G C G G C G G
line 2-3-1
Seq read : C A G G T C G T C G G C C G G C G G
C A G G T C G T C G G T C G G C G G
line 2-3-2
Seq read : C A G G T C G T C G G T C G G C G G
line 2-3-4
Seq read : C A G G T C G T C G G C C G G C G G

Figure 8. Analysis of homozygotic mutants in T_1 and T_2 transgenic rice

Cas9 transgene was analyzed in T_1 and T_2 sibling plants. A. Analysis of *Cas9* transgene segregation by PCR amplification. The PCR data of the T_1 siblings of *Line 2* showed that *Cas9* transgene was segregated out in T_1 *line 2-3* (up). *JAZ9* also amplified to identify mutation patters in T_1 and T_2 siblings (down). B. Nucleotide sequences of *JAZ9* in T_1 siblings. The C/T addition from *line 2* isolated into three types. C. Nucleotide sequences of *JAZ9* in T_2 siblings. The C/T addition from *line2-3* isolated into three types.

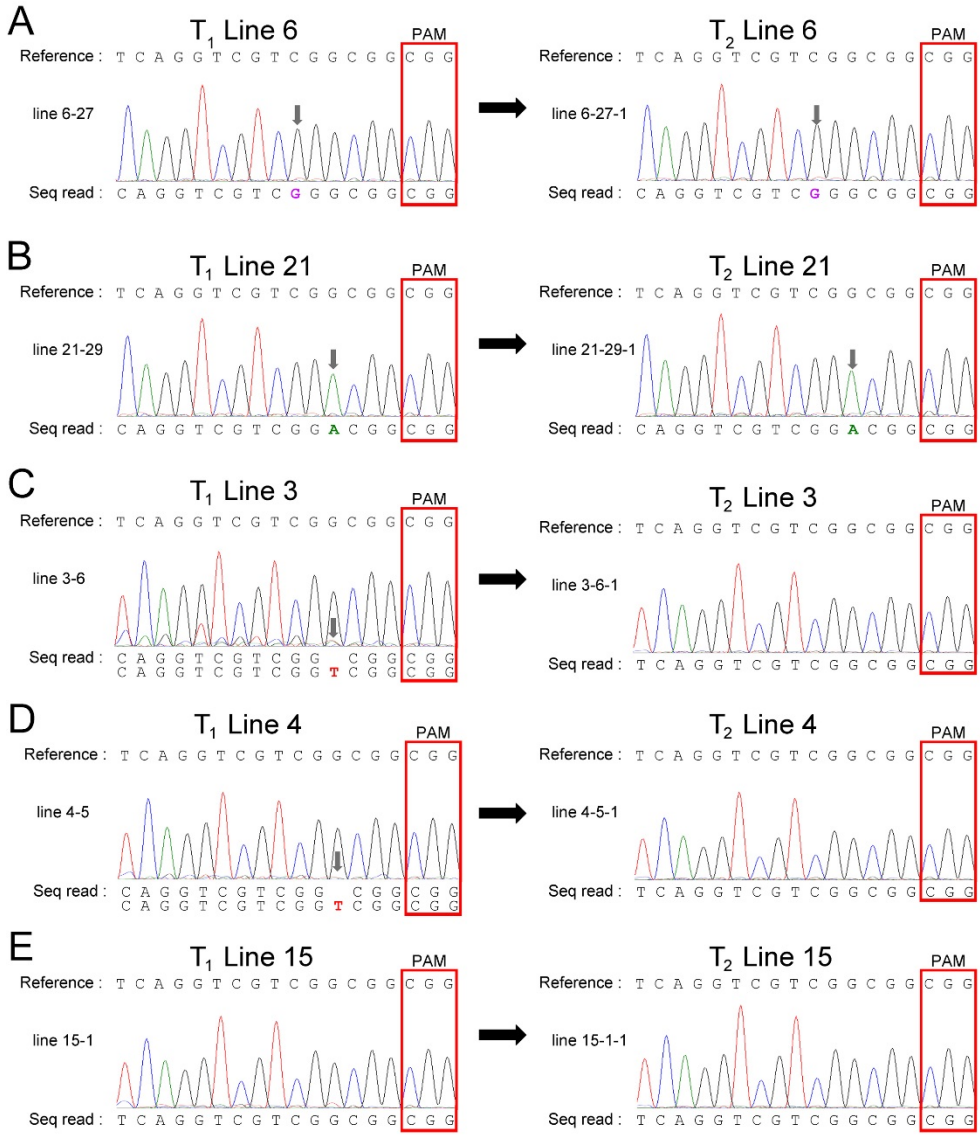
A
T1 Line 6
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 6-27
Seq read : C A G G T C G T C G G C G G C G G
T2 Line 6
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 6-27-1
Seq read : C A G G T C G T C G G C G G C G G
B
T1 Line 21
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 21-29
Seq read : C A G G T C G T C G G A C G G C G G
T2 Line 21
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 21-29-1
Seq read : C A G G T C G T C G G A C G G C G G
C
T1 Line 3
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 3-6
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G T C G G C G G
T2 Line 3
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 3-6-1
Seq read : T C A G G T C G T C G G C G G C G G
D
T1 Line 4
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 4-5
Seq read : C A G G T C G T C G G C G G C G G
C A G G T C G T C G G T C G G C G G
T2 Line 4
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 4-5-1
Seq read : T C A G G T C G T C G G C G G C G G
E
T1 Line 15
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 15-1
Seq read : T C A G G T C G T C G G C G G C G G
T2 Line 15
PAM
Reference : T C A G G T C G T C G G C G G C G G
line 15-1-1
Seq read : T C A G G T C G T C G G C G G C G G

Figure 9. Analysis of heterozygotic mutants in T_1 and T_2 transgenic rice

A. Group C-*line 6* nucleotide sequences of *JAZ9* in T_1 and T_2 siblings. 4 of the 20 siblings were homozygotic G addition mutants (left), all T_2 siblings from *line 6-27* were all homozygotic G addition mutants (right). B. Group D-*line 21* in T_1 and T_2 siblings. 7 of the 30 siblings were homozygotic A addition mutants (left), all T_2 siblings from *line 21-29* were all homozygotic A addition mutants. c, d. Group D-*line 3* and *line 4* in T_1 and T_2 siblings. Each 1 of the 8 siblings from *line 3* and *line 4* were weak T addition mutants (C,D, left). The other siblings from *line 3* and *line 4* were all only WT sequences in T_1 siblings. Four T_1 siblings from *line 3-6* were all only WT sequence (C, right). And T_2 siblings from *line 4-5* were same as *line 3-6* (D, right). E. Group A-l*ine 15* in T_1 and T_2 siblings. All 8 siblings were only WT sequence in T_1 (left), and the same was true in T_2 (right).

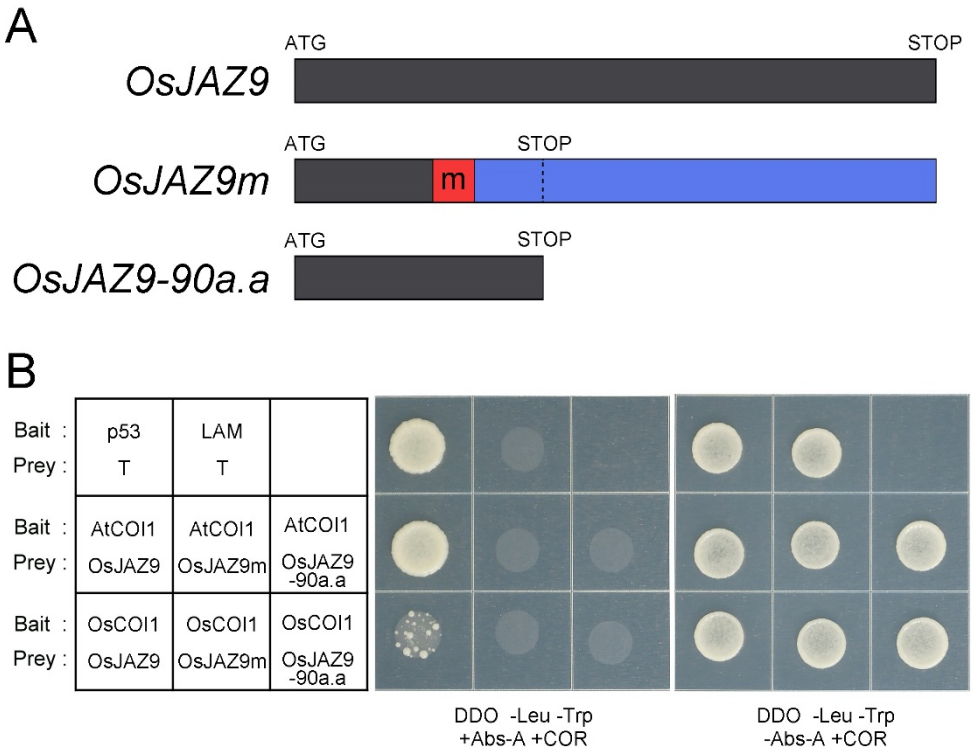
A
ATG
STOP
OsJAZ9
ATG
STOP
OsJAZ9m
m
ATG
STOP
OsJAZ9-90a.a
B
Bait :
Prey :
p53
T
LAM
T
Bait :
Prey :
AtCOI1
OsJAZ9
AtCOI1
OsJAZ9m
AtCOI1
OsJAZ9
-90a.a
Bait :
Prey :
OsCOI1
OsJAZ9
OsCOI1
OsJAZ9m
OsCOI1
OsJAZ9
-90a.a
DDO -Leu -Trp
+Abs-A +COR
DDO -Leu -Trp
-Abs-A +COR

Fig 10. Protein-protein interaction between OsJAZ9 or OsJAZ9 mutant proteins and COI1

Yeast two-hybrid assay was used to analyze whether OsJAZ9 or OsJAZ9 mutants, OsJAZ9 and OsJAZ9-90a.a, interact with COI1 protein. A. Schematic of OsJAZ9, OsJAZ9m and OsJAZ9-90a.a protein structure. *OsJAZ9m* is frame-shifted *OsJAZ9* (colored with blue) by 1 nucleotide addition (M box) and stop codon is generated early. *OsJAZ9-90a.a* is a construction that generates stop codon early without frame shift unlike *OsJAZ9m*. B. Yeast two-hybrid assay of COI1 with JAZ9 modified proteins. *AtCOI1* or *OsCOI1* was used as a Bait plasmid, and *OsJAZ9* or *OsJAZ9m* or *OsJAZ9-90a.a* was used as a prey plasmid. In the presence of interactions between proteins in transformed yeast lines, they survive in the Abs-containing DDO medium. The DDO medium without Abs A shows that the prey and bait plasmids are properly transformed. Both types of media contain 120uM coronatine. The Bait-p53 plasmid with the prey-T plasmid was used as positive control and the Bait-LAM plasmid with the prey-T plasmid was used as negative control.

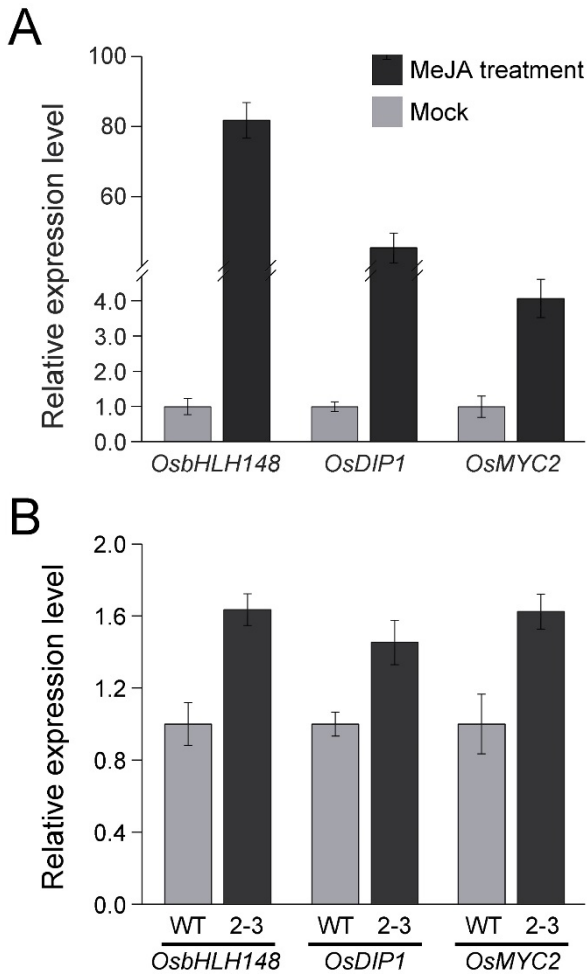
A
100
80
60
4.0
3.0
2.0
1.0
0.0
Relative expression level
MeJA treatment
Mock
OsbHLH148
OsDIP1
OsMYC2
B
2.0
1.6
1.2
0.8
0.4
0.0
Relative expression level
WT
2-3
OsbHLH148
OsDIP1
OsMYC2

Figure 11. qRT-PCR analysis of expression levels of JA-induced genes

Expression levels of JA-induced genes were measured in OsJAZ9 mutant (line 2-3) and JA treated wt, respectively. A. The expression levels of *OsbHLH148*, *OsDIP1* and *OsMYC2* were measured using qRT-PCR after 2-week-old wt rice was treated with 100uM MeJA for 1 hour and total RNAs were extracted. Gray indicates mock-infected rice (Mock) and black indicates rice treated with 100uM MeJA. B. Relative expression level of JA-induced genes in wt and *JAZ9* mutant. 2-week-old rice plants were used in this experiment, gray indicates wt, and black indicates line 2-3 mutant. Both panel A and B used *Tublin2* as a reference.

국문초록

크리스퍼/카스9 시스템은 유전자 편집에 매우 유용한 기술이다. 다른 유전자 편집 기술보다 효과적이고 편리하다. 본 연구에서는 크리스퍼/카스9 시스템을 이용하여 *JAZ9* 유전자의 넉-아웃 돌연변이 벼를 제작하였다. 가이드 RNA는 가이드 RNA 스캐폴드Ⅲ를 갖는 크리스퍼/카스9 바이너리 벡터 pRGEB31에 재조합 하였으며, 이는 벼의 U3 프로모터에 의해 전사된다. 가이드 RNA 서열은 비의도적 오프타겟 효과를 방지하기 위해 CRISPR-PLANT 웹사이트 (http://www.genome.arizona.edu/crispr)를 통해 고안되었다. 아그로박테리움을 매개로 캘러스 형질전환법을 이용해 총 26 개의 개별적인 T_0 형질 전환 벼를 생산하였다. 카스9 매개 돌연변이는 표적부위의 인접한 모티프 지역으로부터 3-뉴클레오타이드 상류에서 일어났다. 염기서열 분석 결과 대부분의 T_0 형질전환 식물은 유전적으로 키메라 돌연변이체임을 보였다. 오직 두 개의 개별 돌연변이체 (약 8%)만 표적부위의 이중 대립형질 동형접합 키메라 였고, 16 개의 독립 돌연변이체 (약 62%)는 이형접합 키메라였다. 우리는 이 26 개의 독립 계통을 돌연변이화 유형에 따라 4개의 집단으로 분류했다. 첫째, 돌연변이가 발생하지 않은 경우는 '야생형 서열만 존재'로. 둘째, 이중 대립형질 동형접합 키메라의 경우는 '돌연변이형 서열만 존재'로

분류했다. 셋째, '야생형과 돌연변이형 서열이 함께 존재'는 야생형 서열과 돌연변이형 서열이 함께 분석되었다. 마지막으로, 야생형 서열과 돌연변이형 서열이 공존하지만, 돌연변이형 서열의 비율이 훨씬 낮은 경우를 '야생형과 일부 돌연변이형 서열이 함께 존재'로 분류했다. 일부 키메라 돌연변이체는 식물의 발달에 따라 돌연변이가 새로이 관찰되거나 사라지기도 했다. 돌연변이 비율은 카스9의 발현 수준이 높을수록 증가하는 경향을 보였다. 그러나, 카스9 도입 유전자의 카피 수가 카스9의 발현 수준을 결정하지는 않았다. 추가적인 동형접합 돌연변이 식물을 얻기 위해, 우리는 T_1과 T_2 세대의 자매 식물체를 추가로 분석했다. 동형접합 돌연변이가 있는 T_0 돌연변이 식물의 모든 T_1 자매 식물체는 여전히 동형접합 돌연변이를 유지했다. 또한, T_0에서 이형접합 돌연변이 식물의 60%가 T_1에서 동형접합 돌연변이체를 생산한다는 사실을 발견했다. 이는 이형접합체를 전개하여 수확하면, 다음 세대에서 동형접합체를 얻을 수 있음을 시사한다. 따라서 독립적인 동형접합 돌연변이 식물체의 수는 T_0 이형접합 돌연변이체의 유전적 분리를 통해 증가 될 수 있다.

주요어 : 가이드 RNA, 돌연변이체, 이중 대립형질, 크리스퍼/카스9, 키메라, 형질 전환 벼, JAZ9

학 번 : 2016-22322

감사의 글

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분자생물학에 대해 무지했던 저를 인내와 사랑으로 가르쳐 주신 최양도 교수님께 먼저 감사의 말씀을 올립니다. 선생님을 처음 뵈었을 때가 생각납니다. 무작정 이사부터 오고 나서 선생님을 뵈러 갈 만큼 어렸던 저를 웃으며 맞아 주셨던 것 정말 감사드립니다. 많이 긴장한 모습이 보이셨는지 넥타이 매는 법은 더 연습해야 하겠다고 친근하게 말씀해주신 것이 아직도 기억에 남습니다. 입학 후에 실수한 일도 있었고 자책할 일도 있었는데 그 때마다 독려해 주셔서 버텨낼 수 있었습니다. 그리고 항상 저에게 관심과 조언의 말을 아끼지 않으신 장규필 박사님께 감사드립니다. 지쳐서 나태 해 지려 할 때 해주신 따끔한 조언들 덕분에 저는 한 꺼풀 어린 모습을 벗어낼 수 있었습니다. 저의 앞날에 대해서도 함께 고민해주시고 도와주신 것들을 잊지 않겠습니다. 그리고 제가 연구를 함에 있어 물심양면 큰 도움을 주신 김주곤 교수님과 연구실의 구성원 분들께도 감사드립니다. 저의 방문을 충분히 귀찮게 여길 수 있었지만 항상 반갑게 맞아 주시고 같은

식구처럼 대해주셔서 진심으로 고마웠고 행복했습니다. 응용생명화학부 모든 교수님들께도 감사드립니다. 실험 재료를 빌리러 다닐 때도 많았고 실험 방법에 대해서 조언을 구하러 다니기도 했는데 응생화 교수님들과 학위과정중인 동료 분들 덕분에 모두 수월히 해결 할 수 있었습니다. 가장 많은 시간을 같이 보냈던 태영이형 에게도 감사합니다. 같이 출장도 많이 다니고 술도 한잔 하면서 서로 진솔한 얘기 많이 할 수 있었고 들을 수 있었습니다. 실험적인 것뿐만 아니라 다방면으로 조언 해주셔서 감사합니다. 실험실 막내로 같이 있어준 똑순이 순현. 사실 나보다 먼저 입학해서 선배인데 친구로 동료로 지낼 수 있어서 좋았고 많은 일들을 나눠서 할 수 있어서 든든했어. 실험실 선배님이자 큰형님이신 서주석 박사님께 감사드립니다. 형님이 해주신 조언의 말들 다 기억하고 있습니다. 감사합니다. 그리고 철호형, 아름누나 정말 힘든 시기에 함께 해주고 다독여줘서 감사합니다. 큰 힘이 되었고 옆방에 두 분이 있다는 게 정말 축복이었어요. 동기로 친구로 동생으로 함께한 진이. 같이 실험 얘기 고민 얘기 할 수 있는 동기가 너라서 다행이야. 앞으로도 친하게 지내자. 재민이형, 편하게 대해주고 가끔씩 해준 따뜻한 말 감사했습니다. 가장 중요한 우리 가족 아버지, 어머니, 형. 마음에 들지 않으면 벽에 머리를 박고, 포도는 껍질만 빨아먹던 막내아들이 애교 없고 틱틱대기만 하던 청소년기를 지나 번듯한 성인이 되었습니다. 제 스스로 번듯한 성인이라 말하는 것도 민망하지만 아버지, 어머니 두 분께는 어디에 내놓아도 아깝지 않은 아들이겠지요. 저는 참 유복한 가정에서 자랐습니다. 저에게 역시 아버지 어머니가 최고의

부모님 이십니다. 2009년 1월이 저에게는 잊혀지지 않는 시간 중 하나입니다. 대학 합격 후 아버지께서 제게 하신 한마디 말씀 때문입니다. '올 겨울은 추워도 춥지가 않고 늦게까지 일해도 피곤하지가 않다. 고맙다.' 살가운 말이나 애교가 너무도 어색해져 버린 저에게 아버지께서 민망함을 붙잡고 말씀하셨을 줄 알고 있습니다. 앞으로도 제가 살아가는 동안 그 순간이 잊혀지지 않을 것 같습니다. 감사합니다. 어머니, 제 작년에 아프시다는 소식을 듣고 하늘이 무너진다는 참 뜻을 알았습니다. 엄마는 내 하늘 이었나봐요. 무사히 완쾌하신 것 너무도 축하드리고 고마워요. 딸은 못되더라도 전화 자주하는 아들이 될게요. 그리고 형, 빨리 취직해서 아직까지 공부하는 동생 용돈 챙겨주느라 고생했어. 엄마가 하늘이었다면 형은 가뭄에 단비 같은 존재였어...ㅎㅎㅎ 조카 낳으면 내가 용돈 많이 챙겨 줄게. 그리고 주희. 5년째 옆에 있어줘서 고마워. 앞으로도 서로 힘들 때 손 잡아주자. 한 70년 정도만.

오늘의 제가 있는 것은 이 모든 분들이 계셨기에 가능했다고 믿습니다. 미처 적지 못한 분들도 많지만 마음속으로나마 감사함을 전하겠습니다. 감사합니다.