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RESEARCH PAPER

# Application of ZFN for Site Directed Mutagenesis of Rice *SSIVa* Gene

**Yu-Jin Jung, Franz Marielle Nogoy, Sang-Kyu Lee, Yong-Gu Cho, and Kwon-Kyoo Kang**

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**Abstract** Many successful studies on genome editing in plants have been reported and one of the popular genome editing technology used in plants is Zinc Finger Nucleases (ZFN), which are chimeric proteins composed of synthetic zinc finger-based DNA binding domain and a DNA cleavage domain. The objective of this research was to utilize ZFNs to induce a double-stranded break in *SSIVa*, a soluble starch synthase involved in starch biosynthesis pathway, leading to the regulation of the *SSIVa* expression. The isoform *SSIVa* is not yet well studied, thus, by modifying the endogenous loci in *SSIVa*, we can explore on the specific roles of this gene in starch biosynthesis and other possible functions it might play. In this study, we used ZFN-mediated targeted gene disruption in the coding sequence of the *SSIVa* rice gene in an effort to elucidate the functional role of the gene. Generation of transgenic plants carrying premature stop codons and substitution events, revealed no *SSIVa* mRNA expression, low starch contents and dwarf phenotypes. Remarkably, based on our analysis *SSIVa* gene disruption had no effect on other starch synthesis related genes as their expression remained at wild type levels. Therefore, the engineered ZFNs can efficiently cleave and stimulate mutations at *SSIVa* locus in rice to affect plant height, grain filling and starch content.

**Keywords:** zinc finger nucleases (ZFN), *SSIVa* gene, starch biosynthesis, rice, starch content

Yu-Jin Jung, Kwon-Kyoo Kang*
Department of Horticulture Life Science, Hankyong National University, Ansung 17579, Korea
Tel: +82-31-670-5104; Fax: +82-31-670-5109
E-mail: kykang@hknu.ac.kr

Yu-Jin Jung, Kwon-Kyoo Kang
Institute of Genetic Engineering, Hankyong National University, Ansung 17579, Korea

Franz Marielle Nogoy, Yong-Gu Cho
Department of Crop Science, Chungbuk National University, Cheongju 28644, Korea

Sang-Kyu Lee
Department of Genetic Engineering, Kyunghee University, Yongin 17104, Korea

## 1. Introduction

About 90% of the world's rice is produced and consumed in Asia [1] and rice is an important source of commercial starch production. Starch constitutes largely of carbohydrates found in milled rice. Starch consists of two major glucans: amylose and amylopectin. Amylose is an essentially linear polysaccharide with α-1, 4 linked D-glucopyranose units and a few branches of α-1, 6 linkages, whereas amylopectin is a highly-branched polysaccharide consisting of short linear-chains connected by about 5% α-1, 6 branch linkages [2]. Starch biosynthesis pathway is complex and is not yet completely understood. Although there were many studies on characterization of enzymes involved in starch biosynthesis, the role of these enzymes on starch structures remains largely unknown [1]. It is known that there are seven starch synthesis enzyme classes, namely, ADP-glucose pyrophosphorylase (AGPase), granule bound starch synthase (GBSS), starch synthase (SS), branching enzyme (BE), debranching enzyme (DBE), starch phosphorylase (PHO) and glucose 6-phosphate translocator (GPT). These genes or enzymes contribute directly or indirectly to the production of starch granules [3]. *SSIVa* gene is one of the least characterized starch genes encoding one SS isoform. Previous study [3] had associated *SSIVa* to pasting temperature at a relatively medium to high level among their rice breeding lines.

In this study, *SSIVa* gene is targeted for gene editing by using zinc finger nucleases (ZFNs), one of the highly recognizable site-specific genomic modification techniques. ZFNs are chimeric proteins composed of a synthetic zinc

finger–based DNA binding domain and a DNA cleavage domain [4,5]. Engineered ZFNs are composed of two zinc finger arrays (ZFAs), each array is fused to a single subunit of a non-specific endonuclease, such as the nuclease domain from the *FokI* enzyme, which becomes active upon dimerization [4,6]. In general, a single array consists of 3 or 4 zinc finger domains, each one is designed to recognize a specific nucleotide triplet (GGC, GAT, *etc*.) [7]. Therefore, ZFNs made up of two "3-finger" ZFAs are capable of recognizing 18 base pair target site. In large genomes like in humans and plants, 18 base pair recognition sequence is generally unique. By leading the co-localization and dimerization of two *FokI* nuclease monomers, ZFNs generate a functional site-specific endonuclease that creates a double-stranded break (DSB) in DNA at the targeted locus [8]. DSBs are repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR).

In this study, we designed ZFNs having a 3-finger on left array and 4-finger on right array based on the recognition site found in exon 4 of *SSIVa* gene to study the effect on starch content and to elaborate the role of *SSIVa* gene in starch biosynthesis pathway. We generated plants with targeted mutagenesis in *SSIVa* displaying deletion and substitution events. The transgenic plants exhibited lower starch content, dwarf phenotype and no grain filling. At molecular level, ZFN-treated plants showed no *SSIVa* gene expression while the expression of other starch related genes was unaffected.

## 2. Materials and Methods

### 2.1. ZFN design

The ZFNs were designed based on the binding site located in exon 4 of *SSIVa* gene from chromosome 1 of *Oryza sativa* Japonica group (see Figs. S1 and S2 for details). ZFNs were constructed by TOOLGEN company, Korea. The left and right arm of the ZFN construct is 9-bp and 12-bp long, respectively. The activity of the assembled ZFNs was tested using cell-based green fluorescent protein (GFP) reporter assay (Fig. S3) and the activity of 2A-linked *SSIVa* ZFN was also validated (Fig. S2). The overview of the ZFN construct in *SSIVa* gene is shown in Fig. 1A.

### 2.2. Vector construction

The coding sequences of ZF proteins were cloned between

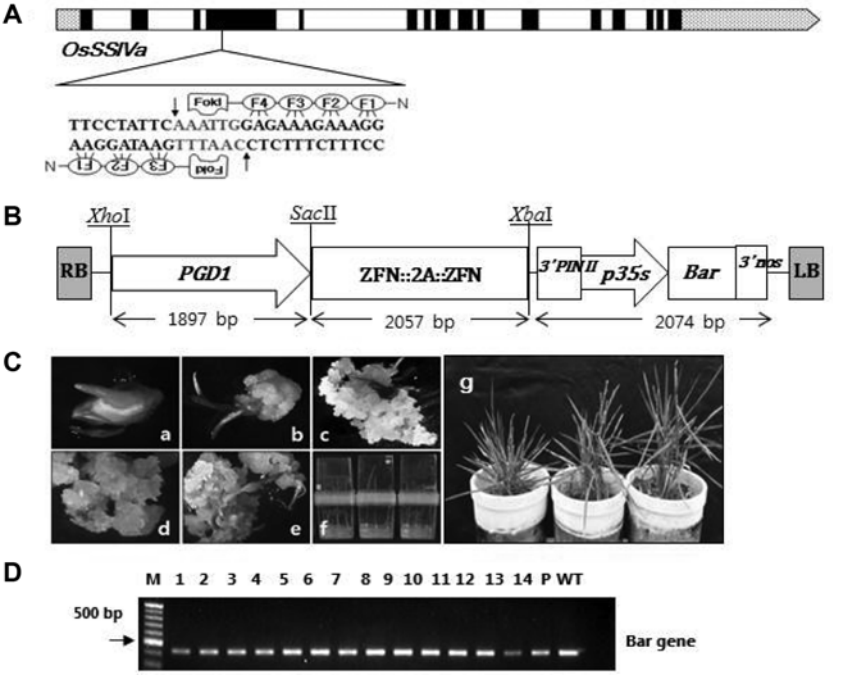


**Fig. 1.** Ti-plasmid of ZFN-*SSIVa* vector construct to cleave *SSIVa* gene in rice. (A) The gene models of *OsSSIVa* loci in chromosome 1. Black rectangles represent exons; white rectangles represent introns. The position of OPEN ZFN target site is indicated by triangles. The left and right arms consists of three and four zinc fingers (F1, F2, F3, F4), respectively, which together recognize a 21-bp target site. The site of cleavage in the target sequences is indicated by black arrowhead. (B) PGD1 promoter and CaMV 35s promoter gene, a 3'PINII: protease inhibitor II terminator gene, a herbicide-resistant gene Bar (phosphinotricine acetyltransferase gene) and a nopaline synthase terminator (3'nos). (C) *Agrobacterium*-mediated transformation procedures. (D) PCR amplification of transferred *bar* gene in transgenic rice lines. Lane M; DNA ladder, Lane 1-14; independent transgenic lines, Lane P; PCR products generated from the DNA template of vector plasmid that contains the pPZP::ZFN. Lane W; wild type plant.

the *Sac*II and *Xba*I sites of the PIN II Ti-plasmid vector. Promoters and terminators are as follow, PGD1, CaMV 35S, protease inhibitor II and nopaline synthase. The selectable marker used was *bar* (bialaphos resistance) gene (Fig. 1B).

### 2.3. Rice transformation

The ZFN-*SSIVa* carried by a binary vector was transformed in rice (*Oryza sativa L*. ssp. Japonica cv. Dongjin) by *Agrobacterium*-mediated transformation adopted from a different study [9] (Fig. 1C).

### 2.4. DNA isolation

DNA was extracted from transformed callus using CTAB method. Calluses were grinded until juice goes out from tissue. The juice was mixed with 1 mL CTAB extraction buffer (2% CTAB (hexadecyl trimethyl-ammonium bromide), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0) and incubated for 30 min at 65°C water bath. Afterwards, the samples were centrifuged for 15 min at 12,000 rpm at room temperature. The collected aqueous phase was transferred in a 1.5 mL centrifuge tube and equal volume of chloroform:isoamyl (24:1) was added and then mixed by inverting. Samples were centrifuged again and the aqueous phase was collected in a new tube and isopropanol was added. The samples were incubated in 4°C for an hour. DNA was pelleted after centrifugation for 15 min. The pellet was washed with 70% ethanol and dried. Then the pellet was suspended in autoclaved water supplemented with 10 mg/mL RNase.

### 2.5. PCR analysis and T7E1 assay

For amplification of the ZFN target site, primers were designed based on PGD1 promoter and pinII terminator sequences. The purified product was then digested with *Tsp*RI enzyme (Figs. 2A and 2B). The generated $T_0$ transgenic plants were again collected for DNA extraction and were confirmed by amplifying the *bar* gene. Using the same $T_0$ DNAs, T7 endonuclease 1 (T7E1) assay was used with primers 5'-ctgctcctaaatcaagctcgtc-3' and 5'-tgtttcttgatggcctgcttggt-3'.

### 2.6. Sequence analysis

To check if there are sequence alterations in *SSIVa,* we used primers (PGD1 promoter forward and pinII terminator reverse) flanking the ZFN target site for amplification and used the product for direct sequencing. Samples were sequenced by GGbio (Green Gene Bio) company. DNA sequencing results were aligned with the original target site of *SSIVa* gene.

### 2.7. Flanking sequence tag (FST)

FST analysis was done based on another study [10]. After DNA extraction, restriction digest of genomic DNA was done followed by ligation of an adapter to the genomic DNA. PCR amplification of the regions flanking the T-DNA insert(s) using primers specific to the adapter and the T-DNA followed afterwards. PCR products were sequenced and lastly, identification of the flanking sequence tags (FSTs) characterizing the T-DNA inserts.

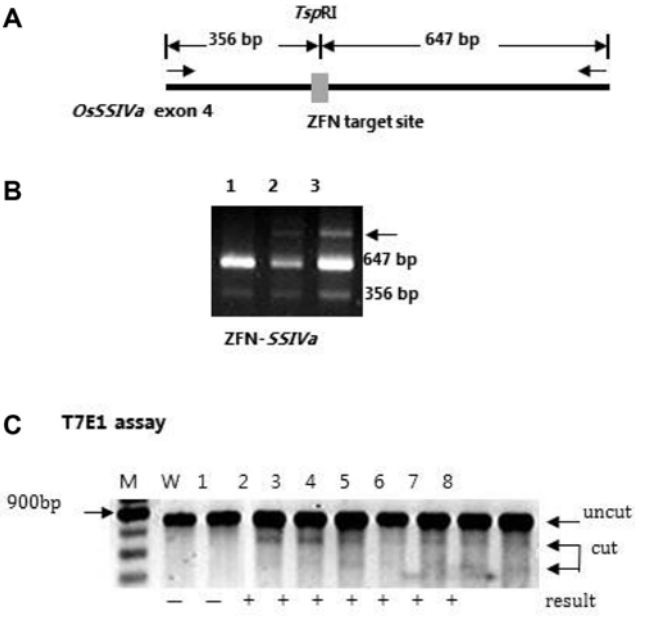


**Fig. 2.** Detection of ZFN-induced mutations in transgenic rice. (A) Schematic strategy on how to amplify and identify mutations in the ZFN target sites (gray rectangle). Restriction enzyme site present in the ZFN cleavage site was *Tsp*RI for *SSIVa*. Short black arrows indicate the positions of PCR primers relative to the target sites. (B) Restriction endonuclease assay to detect ZFN-induced mutations in rice callus. Mutations introduced by NHEJ are resistant to restriction enzyme digestion due to loss of restriction sites and result in uncleaved PCR products (indicated by arrows). Genomic DNA was cleaved with restriction enzymes before PCR amplification to enrich ZFN-induced mutations. Lanes: 1, control sample without transformation; 2, callus sample transformed with pPZP::ZFN after 24 h of incubation; 3, callus sample transformed with pPZP::ZFN after 48 h incubation (C) T7E1 assay with PCR products. The amplification products were separated using a 2% agarose gel. Lane M; DNA ladder, Lane W; wild type plant, Lane 1~7; independent lines.

### 2.8. Starch content determination

The starch content of leaves was assayed according to the enzymatic method described in another study [11]. Briefly, rice leaves (200 mg) were grinded with liquid nitrogen until fine powder is achieved and then extracted with 10% perchloric acid. Insoluble solids in neutralized perchloric acid extracts were then separated by centrifugation (20,000 *g* for 5 min). The resultant sediment was used for the determination of starch content levels. Here we measured in three replications, one replication consists of six samples

comprised of the wildtype, mock plant and four transgenic samples.

### 2.9. Statistical analysis

Analysis of variance was performed using SAS version 9.1(SAS Institute Inc., Cary, NC, USA). Significant differences were determined using *t*-test (LSD) for comparison of means.

## 3. Results

### 3.1. ZFN-induced mutagenesis in *SSIVa* gene

To locate the possible ZFN binding sites in *SSIVa* gene, the whole target sequence was surveyed using TOOLGEN Genomics Toolmaker. From the analysis made, one candidate binding site located in exon 4 of *SSIVa* gene from chromosome 1 was selected (Supplemental Fig. S1). A "3-finger" array was designed on the left side of 6 bp spacer and "4-finger" array on its right side creating a 9 bp and 12 bp recognition sites (Fig. 1A). Binding of the arrays to the target sequences enables the *FokI* nuclease monomers to dimerize and cleave within the spacer. After proper validation of the ZFN construct (Supplemental Fig. S2), it was digested from its plasmid using *SacII* and *XbaI* enzymes and subcloned into a binary vector with PGD1 (phosphogluconate dehydrogenase) as promoter of ZFN expression. After studying the gene promoter activities in rice, the spatial activities of the PGD1 promoter were found to be strikingly similar to those of the *ZmUbi1* (Maize ubiquitin1), a widely used constitutive promoter [12]. The final vector was transformed by *Agrobacterium* into rice (*Oryza sativa L.* ssp. Japonica cv. Dongjin) (Figs. 1B and 1C) (Supplemental Fig. S3).

After growing the transformed calli into mature plants, DNA was extracted from the leaf samples and PCR genotyping was done using *bar* gene primers to confirm the transformation events (Fig. 1C). Fig. 2A shows a schematic illustration of the ZFN target site and the expected sizes of amplicon after digesting with *TspRI*. In Fig. 2B, the restriction

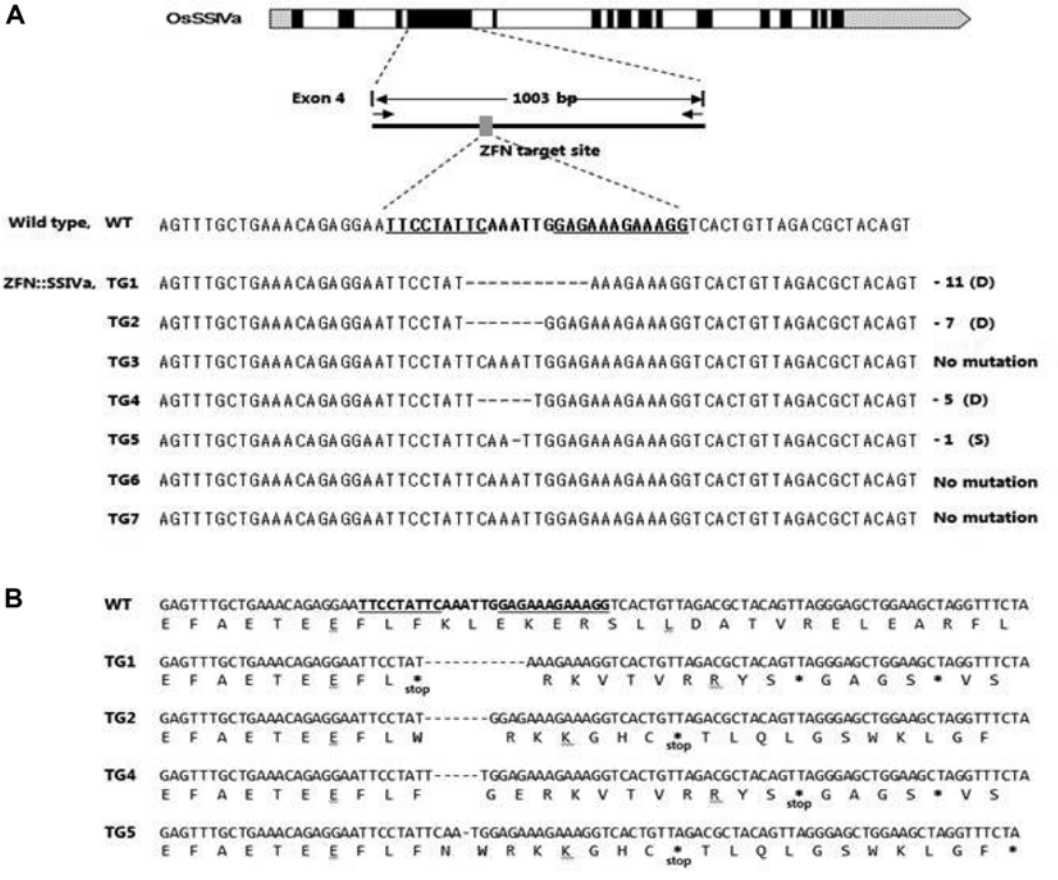


**Fig. 3.** (A) Sequences of germinally transmitted mutations induced by ZFNs. For each target gene, the wild-type sequence is shown at the top with the ZFN recognition sites underlined. Deletions are shown as hyphens. (B) Alteration of amino acid sequences after ZFN cleavage in *SSIVa* gene. Each lines were recovered from initially detected mutated $T_0$ plants for the *SSIVa* gene. Target sites of ZFN monomers are underlined. Predicted amino acid sequences of this region of the *SSIVa* gene in the four mutant lines are shown in letter codes.

sites are destroyed by most NHEJ-induced mutations, the mutated sequences are resistant to restriction digestion and are amplified preferentially in a subsequent round of PCR with primers flanking the target site. When PCR products are further digested by the same restriction enzymes and subjected to gel electrophoresis, uncleaved sequences with mutations can be distinguished from cleaved wild-type sequences. Using this assay, mutated alleles were observed in DNA samples derived from the transformed $T_0$ plants.

To confirm ZFN-mediated cleavage in the target site of *SSIVa*, we used T7E1 assay to identify mutations coupled with a mismatch-specific endonuclease. Taken together, the data in Fig. 2 confirms that the designed ZFNs can be used to drive targeted mutagenesis into exon 4 of *SSIVa* gene. To check if there are sequence alterations in *SSIVa,* we used primers flanking the ZFN target site for amplification and used the product for direct sequencing. From seven $T_0$ PCR products sent for sequencing, four of them contained 1 to 11 bases deletion, these events reveal the site directed mutagenesis. The other three $T_0$ plants revealed no mutations (Fig. 3A). The detected mutations at *SS1Va* locus were deletions and substitutions in the target site resulting in premature stop codons and presumably truncated variants of the encoded protein (Fig. 3B).

### 3.2. Phenotype of mutated plants and location of ZFN-SSIVa

When the mutated plants were grown in greenhouse, abnormal morphological features were observed like dwarf phenotype and unfilled grains (Fig. 4A). To determine if there were T-DNA insertions in genic regions, flanking sequence tag (FST) analysis was done. In Fig. 4B, the T-DNAs of ZFN-*SSIVa* vector were found in different chromosomes in each ZFN-induced mutated plant and they were located in the intergenic regions. Thus, we found that there were no T-DNA insertions in other genic regions during ZFN cleavage in *SSIVa* target site.

Fig. 5A, depicts the starch synthesis pathway in rice as it occurs in the plastid. From UDP-glucose and ADP-glucose, there are various starch synthesis genes differentiating the formation of amylose and amylopectin. We measured the gene expression of nine enzymes (*AGPL1, AGPL3, SSI, SSIIc, SSIIIa, SSIIIb, GBSSI, GBSSII, BEIIb and PUL)* involved in starch synthesis among the wildtype, mock plant and four transgenic plants having deletion (TG1, TG2, TG4) and substitution (TG5) events (Fig. 5). The mock plant used in this study was transformed with an empty vector only. The expression patterns of all genes in transgenic plants are relatively similar to wildtype and

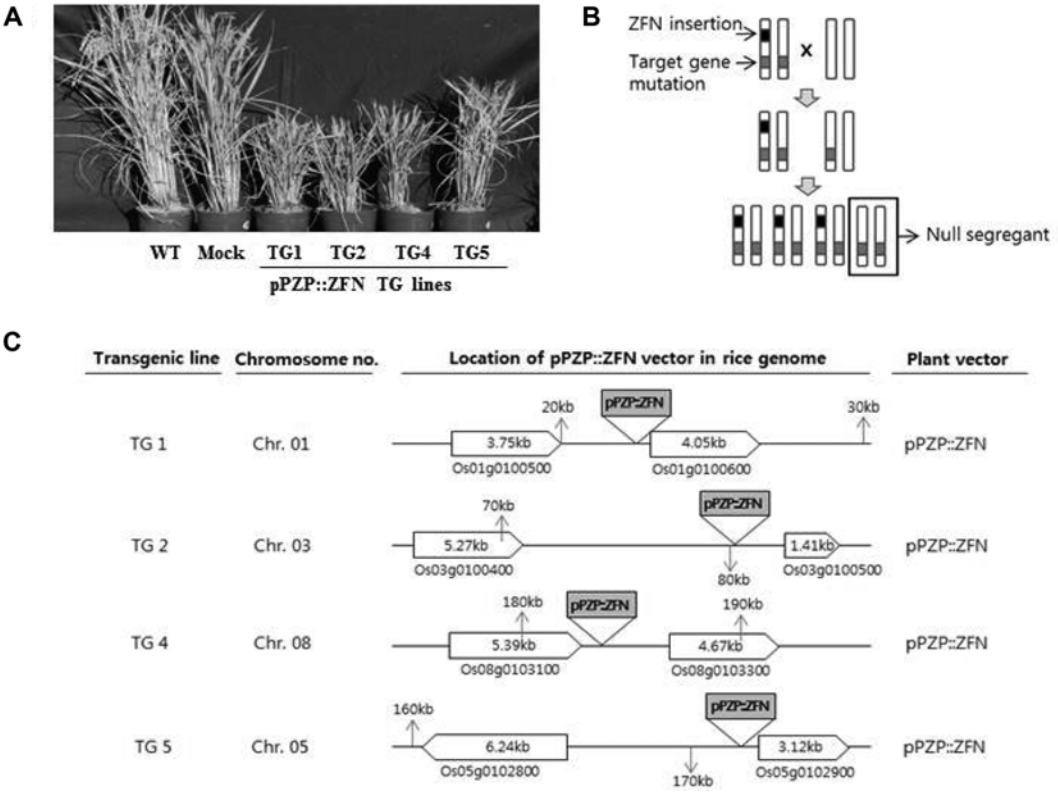


**Fig. 4.** (A) Phenotype of wild type, mock control and ZFN-*SSIVa* transgenic rice plants. The mock plant used in this experiment was transformed with an empty vector only. (B) Schematic of the null segregant selection from target gene mutation. (C) Analysis of flanking regions adjacent to the left or right border of the T-DNA.

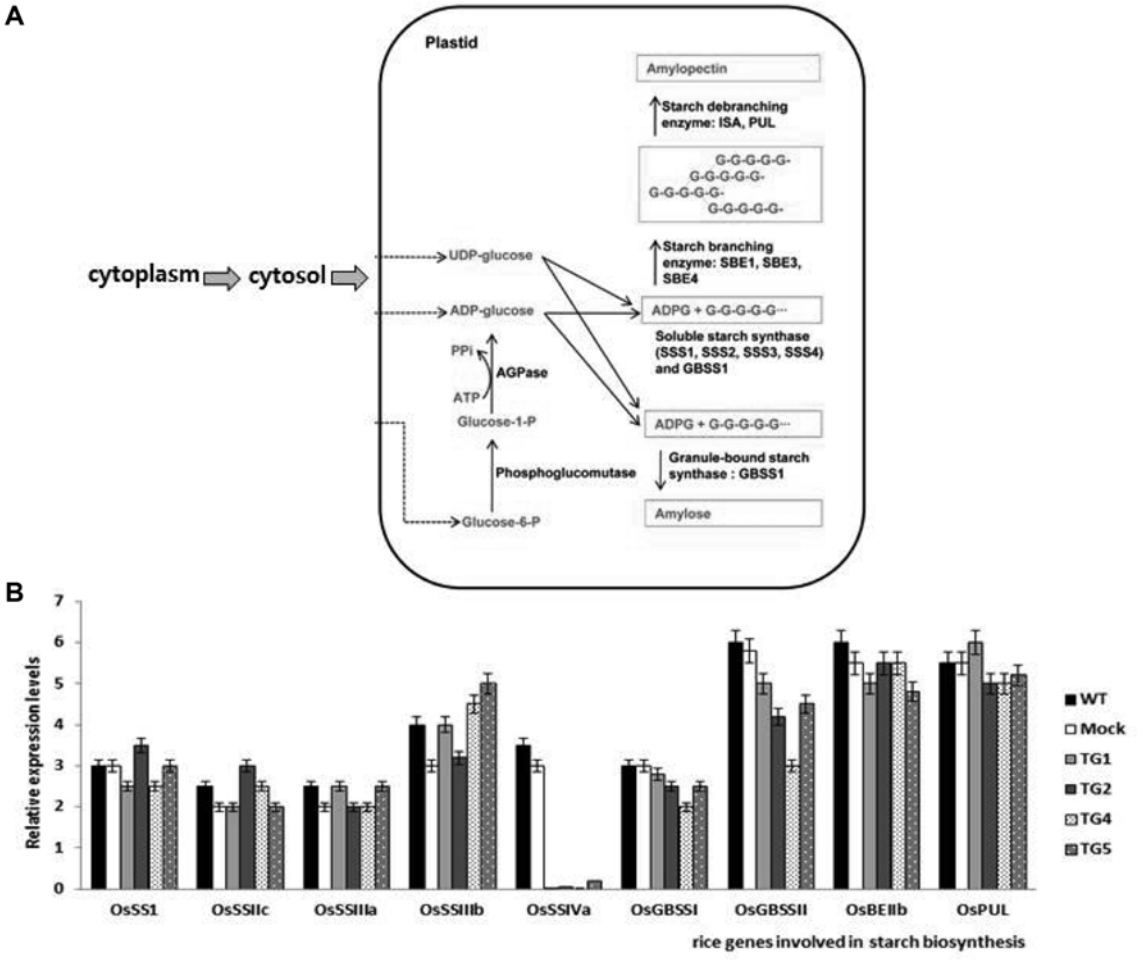


**Fig. 5.** (A) Main pathway of starch biosynthesis including sucrose synthesis, sucrose degradation and starch synthesis in rice. FBP1, fructose-1,6-bisphophatase1; PGM, phosphoglucomutase; AGPase, ADP-glucose pryophosphorylase; PPi, pyrophosphate [26]. (B) Quantitative RT-PCR analysis of nine genes associated with starch synthesis pathway. As a loading control, the samples were also amplified with specific primers for rice *actin* gene for qRT-PCR values. CT values were calculated using actin expression level as a control. Error bars show the standard error of the mean for three replications. The amplified products were separated using a 1.5% agarose gel. Lane M- DNA ladder, Lane WT- wild type plant, Lane Mock- transformed plant with an empty vector, Lane TG1, TG2, TG3, TG4-plants.

mock plant, but it is noticeable that TG1, TG2, TG4 and TG5 have no *SSIVa* gene expression.

### 3.3. Starch synthesis and its consumption in mutated plants

One striking phenotypic change in the transgenic plants was the unfilled grains or no grain filling. To measure the starch content, we collected leaves instead of rice grains to measure the starch production during day time and starch consumption at night time. The starch contents in wild type (Dongjin) and mock plant at the end of day were 5.81 and 5.52 mg per 1 g of fresh weight. While lower (2.14 ~ 2.75 mg) starch production in transgenic rice plants was observed. Meanwhile, at the end of the night the starch

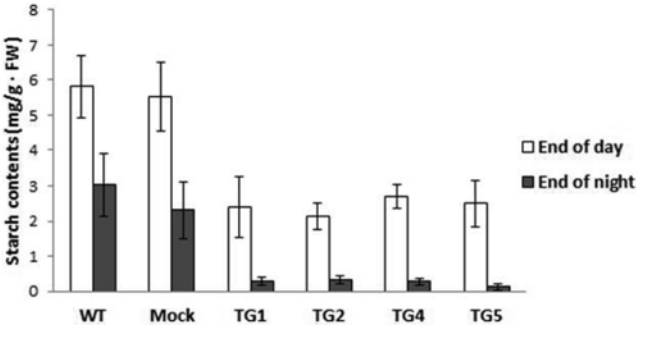


**Fig. 6.** Change of total starch contents of the wild type, mock and ZFN-*SSIVa* transgenic plants, TG1, TG2, TG4, and TG5. The difference is significant at 0.05 level by LDS test with $t_{0.05(10)} = 2.228$.

contents in wild type was 3.03 mg per 1g of fresh weight and mock plant had 2.32 mg, and 0.07 ~ 0.34 mg in transgenic rice plants (Fig. 6). The *t-test* analysis was done between wild type and transgenic plants. Computed *ts* values of wild type and transgenic plants were 11.16 and 9.84 at the end of day, while 7.50 and 8.38 at the end of night, respectively, with significance ($t_{0.05(10)} = 2.228$). So the expression of *SSIVa* by ZFN was effectively knocked-out.

## 4. Discussion

In genome editing with engineered nucleases specific target recognition is very important. Any off-target effects generated by custom nucleases are generally toxic, and unexpected mutations throughout the genome cannot be monitored easily [13]. Paired nickases have been utilized in ZFN-mediated genome editing, in which the ZFN nickases reduced off-target mutagenesis in the same way as Cas9n [14-16]. Detecting the optimum target site in *SSIVa* involved designing the ZFN targeting vector for *SSIVa* gene. Analysis of *SSIVa* gene structure revealed ZFN binding sites which were found in exon 4. The synthesis of ZFN vector was confirmed by using a cell-based green fluorescent protein (GFP) reporter assay to determine if they are active in cell-culture-based system (data not shown). There was a report that some of the starch synthesis enzymes and their isoforms assembled into functional complexes [17], thus, disruption of the coding sequence of *SSIVa* may lead to drastic change in phenotype. Also, individual starch synthase isoforms can have unique roles [18] or overlapping functions in starch biosynthesis [17].

The transgenic plants with deletions and substitution in *SSIVa* showed no gene expression. Also, the plants have lower starch contents compare to the wild type and mock plants. *SSIV* gene is considered to be important for the initiation of starch granules [19] and is also essential for the coordinated formation of starch granules that occurs during leaf expansion [20]. However, in our results, the expressions of each gene involved in starch synthesis are relatively similar among wildtype, mock plant and the transgenic plants. Thus, *SSIVa* disruption has no direct effect on the expression of upstream and downstream genes in starch synthesis pathway (Fig. 5).

No production of filled grains in transgenic rice plants was observed. So we measured starch contents in leaves to compare the starch synthesis and its consumption during day time and night time (Fig. 6). The reduction of total starch content in transgenic plants was attributed to the ZFN-based disruption of *SSIVa* gene. However, the observed reduced plant height and no grain filling might also be the results of modified expression of the isoforms of starch synthase (SS) genes. Among the SS isoforms, *SSIV* is important in starch formation but it may also play an essential role in plant height and grain filling in rice.

The phenotype of *SSIVa* knock out plants displayed dwarfism compared to the average height of wildtype and mock plants as shown in Fig. 4A. In one study [21] bigger growth of *Arabidopsis* plants overexpressing *SSIV* gene was observed. The transgenic plants that they produce grow faster and had more biomass. In an earlier study [22], the rate of plant growth was correlated with starch degradation, so that the plants with reduced level of degradation during the night also displayed low rates of growth. Also, in a separate work [23], the elimination of *SSIV* decreases the rate of starch degradation during night time and leads to the reduced plant growth. Thus, the overexpression of *SSIV* increments the levels of starch accumulated by the end of the day, which were completely mobilized during the night, leading to an increment in the rate of starch degradation. Thus, the increase in the carbon skeletons and energy available during the night would determine a higher rate of growth in overexpressed *SSIV Arabidopsis* lines [21]. Fig. 6 showed a low starch content in night time among ZFN-induced mutation plants which reduces the starch degradation and in effect, causes a lower growth rate.

Pairwise comparison of starch content between the wildtype and the transgenic plants showed significantly ($P < 0.05$, Student's *t*-test) lower content at the end of the day and at the end of the night (Fig. 6). The *t-test* analysis revealed that the starch levels were higher at the end of the day compared to those at the end of the night for both wild type and transgenic plants. In our previous study, RNAi-*SSSI* transgenic plants showed different enzyme activities in rice grains [24]. This could be due to the RNAi vector effecting low expression of SSSI which subsequently affected other various enzymes (SSS, SBE and SDBE) in amylopectin synthesis. So the results indicated that we could get similar result as in RNAi experiments in this study. *SSIVa* is an enzyme that is involved in elongating the long chains with a DP (degree of polymerization) of glucosyl units therefore, it plays a crucial role in the biosynthesis of amylopectin [25].

This study showed that regulation of endogenous *SSIVa* through gene editing is an invaluable technique that could aid in developing rice variety with improved eating quality.

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