

Binary Vector Construction for Site-Directed Mutagenesis of *Kafirin* Genes in Sorghum

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Abstract

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the world's leading cereal crops in agricultural production, which has a special importance in the arid regions. However, unlike other cereals, sorghum grain has a lower nutritional value, which is caused, inter alia, by the resistance of its seed storage proteins (kafirins) to protease digestion. One of the effective approaches to improve the nutritional value of sorghum grain is to obtain mutants with partially or completely suppressed synthesis or altered amino acid composition of kafirins. The employment of genome editing may allow to solve this problem by introducing mutations into the nucleotide sequences of the α - and γ -kafirin genes. In this study, genomic target motifs (23 bp sequences) were selected for the introduction of mutations into the α - and γ -*KAFIRIN* genes of sorghum. The design of the gRNAs was conducted using the online tools CRISPROR and CHOPCHOP. Two most suitable targets were chosen for α -*KAFIRIN* (*k1C5*) and two for γ -*KAFIRIN* (*gKAF1*) genes. The insertion of respective sequences in the generic vector pSH121 was performed at the *BsaI* (*Eco31I*) sites. Validation of the cloning procedure was performed by DNA sequencing. Subcloning of the resulting constructs was performed using the *SfiI* restriction sites into the compatible binary vector B479p7oUZm-LH. The correct assembly of binary vectors was confirmed by restriction analysis using the *MluI* and *SfiI* cleavage sites. The four vectors created (1C - 4C) were transferred by electroporation into the *Agrobacterium tumefaciens* strain AGL0. Currently, this vector series is used for stable transformation of sorghum using immature embryo explants.

Keywords

Sorghum bicolor (L.) Moench, CRISPR/Cas, Genome Editing, α -Kafirin, γ -Kafirin, Genetic Engineering, Grain Quality

1. Introduction

Among the many biotechnological approaches for improving the properties of agricultural plants, genome editing has the potential to play a key role. Unlike traditional strategies and breeding methods, Cas endonuclease technology provides a fast path to the creation of modified genotypes through site-directed mutagenesis or precise editing of the nucleotide sequences of respective genes [1] [2]. To date, this technology allowed to modify many agronomically important traits in major cultivated crops, such as corn, rice, wheat, potatoes, soybeans, sugarcane, etc. [3] [4].

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the most important drought-tolerant cereal crops in the arid regions of the Earth. Due to global warming of climate, the importance of this crop is expected to grow steadily. Sorghum grains do not contain gluten and can serve as a source of protein for people with gluten intolerances, which must follow a gluten-free diet. However, compared to other cereals, sorghum grain has a lower nutritional value, the main reason for which is the resistance of its grain storage proteins (kafirins) to protease digestion [5] [6] [7]. The poor digestibility of kafirins, in turn, reduces the access of amylolytic enzymes to starch granules and reduces the digestibility of starch and the nutritional value of sorghum grain [8].

Cas endonuclease technology offers to solve this problem. The targeted induction of mutations in genes encoding different classes of kafirins, including gene knockouts, using genome editing bears the potential to significantly improving the digestibility of proteins in sorghum grain and increase its nutritional value. The reduction of kafirin synthesis induces the changes in the ultrastructure of endosperm protein bodies and increases their digestibility by proteases [9] [10] [11]. As a further consequence, the proteome of caryopses may be rebalanced via enhanced synthesis of other proteins [10], including those with a higher content of essential amino acids such as lysine [11] [12]. Recently published work on the induction of mutations in the α -kafirin nucleotide sequence has shown the potential of Cas endonuclease technology to improve the nutritional value of sorghum grain [13].

Previous studies have revealed a multitude of aspects that have to be considered when generating transformation vectors for plant genome editing using Cas endonucleases [14] [15] [16]. The aim of this work was to create highly efficient vectors and agrobacterial clones containing these vectors to mutate the α - and γ -*KAFIRIN* genes of sorghum. Accordingly, major features of the constructs generated in the present study include the rice *U3* promoter and the maize

POLYUBIQUITIN 1 (UBI1) promoter to drive gRNA (guide RNA) and *cas9* expression, respectively. Further, the *Phosphinothricin phosphotransferase (Bar)* gene of *Streptomyces hygrosopicus* equipped with an intron to prevent agrobacterial expression and driven by the maize *UBI1* promoter was used as plant selectable marker.

2. Materials and Methods

pSH121 (NCBI: txid2338066) (Figure 1(a)) [17] was used as the basic vector for the introduction of target-specific sequences of kafirin-encoding genes upon

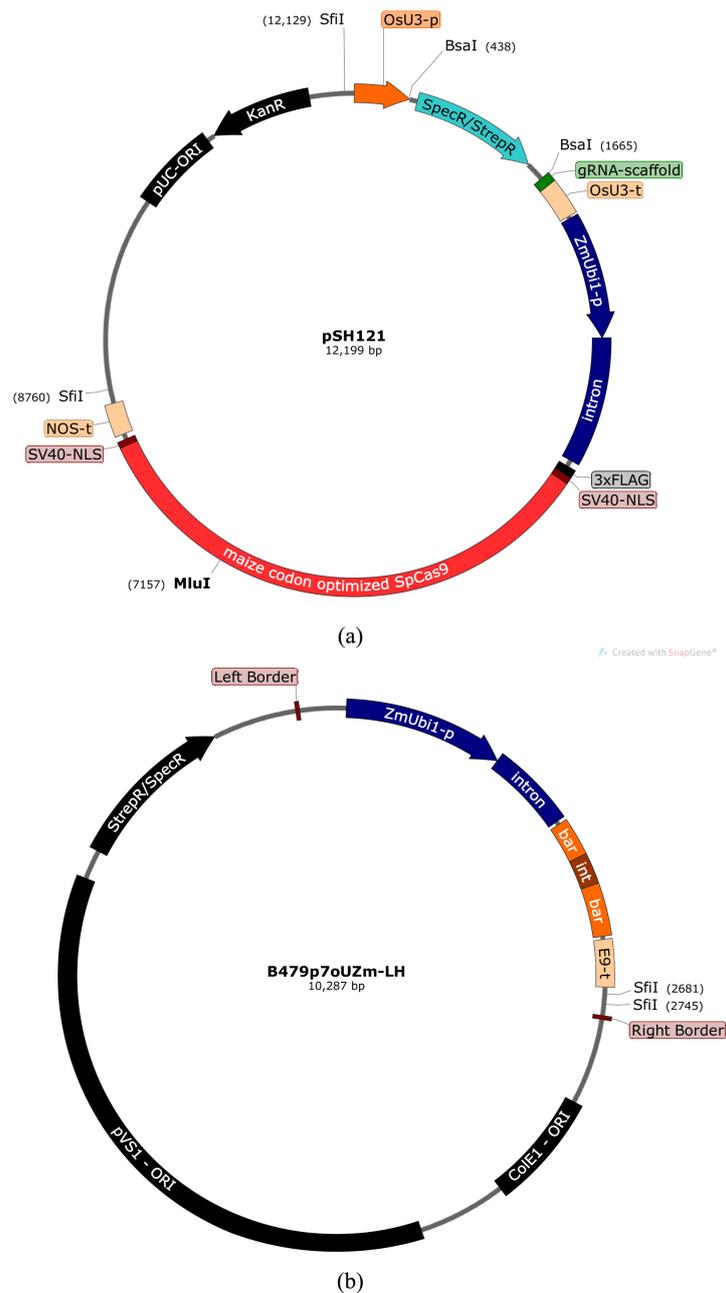


Figure 1. Vectors pSH121 (a) and B479p7oUZm-LH (b) used in this work.

cleavage with *BsaI* to complement the gRNA expression units. This vector contains the nucleotide sequence of a maize codon-optimized *cas9* gene under control of the maize *UB1* promoter and sites for the *SfiI* restriction enzyme for the directed transfer of a fragment containing the *cas9* and gRNA expression units into a binary vector of the p7i series. As a binary vector from this series, we chose B479p7oUZm-LH (**Figure 1(b)**) which contains the *bar* gene and also carries the *SfiA* and *SfiB* sites compatible with pSH121. This vector was purchased from DNA Cloning Service (<https://www.dna-cloning.com/>). Bioinformatics analysis of the nucleotide sequences of the pSH121 and B479p7oUZm-LH vectors was performed using the SnapGene Viewer software.

The genomic sequences of the α - and γ -*KAFIRIN* genes were taken from the site <https://phytozome.jgi.doe.gov> (α -*KAFIRIN* (*k1C5*): Sobic.005G193100, Chr05: 67654898 ... 67655764; γ -*KAFIRIN* (*gKAF1*): Sobic.002G211700, Chr02: 60423442 ... 60424313). The selection of target motifs was carried out using the online tools CRISPOR (<http://crispor.tefor.net/>) and CHOPCHOP (<https://chopchop.cbu.uib.no/>) [18] [19].

For molecular cloning, conventional techniques were used if not specified otherwise [20]. The restriction endonucleases *Eco31I*, *MluI* and *SfiI* were purchased from Thermo Scientific. Restriction endonuclease *SfiI* is unique in that it recognizes a 13-nucleotide site and forms sticky ends, which is particularly useful to transfer DNA fragments in directed fashion. Fractionation of linearized plasmid DNA was carried out in agarose gel in 1x TAE buffer. Subsequent purification of DNA was performed using the ISOLATE II PCR and Gel Kit (BIOLINE) along with Quantum Prep™ Freeze'n Squeeze DNA Gel Extraction Spin Columns (Bio-Rad Laboratories). Ligation of targets and plasmids with 5' and 3'-overhangs was performed using T4 DNA ligase (Thermo Scientific). The created constructs were introduced into *E. coli* XL-1 Blue bacterial cells. The presence of target-specific inserts was monitored by DNA sequencing on an ABI 3130 genetic analyzer using the OsU3p-F3 sequencing primer GACAGGCGTCTTCTACTGGTGCTAC. To validate the correct assembly of the cloned binary plasmids, restriction endonuclease analysis was performed using the enzymes *MluI* and *SfiI*. The created vectors were transferred by electroporation into the *A. tumefaciens* strain AGL0.

3. Results and Discussion

Transformation vectors for site-directed mutagenesis of kafirin genes were created by the following steps:

- 1) Retrieve kafirin gene sequences from databases and select target motifs within their coding sequences.
- 2) Clone the target-specific parts of the gRNAs into the generic vector pSH121.
- 3) Perform the verification of cloned DNA targets by sequencing.
- 4) Subclone a fragment containing the *cas9* and gRNA expression units into the generic binary vector B479p7oUZm-LH.

5) Perform restriction endonuclease analysis to confirm the correct generation of vectors.

The genetic maps of the pSH121 and B479p7oUZm-LH vectors used in this study are shown in **Figure 1**.

3.1. Bioinformatics Analysis and Oligonucleotide Design for the gRNA Expression Units

Signal sequences play an important role in the packaging of kafirins into protein bodies, and, consequently, in the accumulation of storage proteins in sorghum grain. For example, a single nucleotide substitution (G → A) at position 61 relative to the first nucleotide of the start codon of α -KAFIRIN gene distinguishes the *hdhl* mutant with a high digestibility of kafirins and high lysine content from other sorghum varieties [21]. This missense mutation results in the amino acid alanine (Ala) instead of a threonine (Thr) at the last position of the signal peptide. This mutation is thought to render the protein resistant to processing and to trigger the unfolded protein response (UPR) and the formation of irregular protein bodies [21]. Therefore, we chose nucleotide sequences of these parts of α - and γ -kafirins as target motifs for the RNA-guided Cas9 used in this study.

Using the CRISPOR and CHOPCHOP online tools to analyze the 63 bp signal sequence of α -kafirin made it possible to identify four target motifs, from which the two with the best features, such as specificity score, predicted efficiency, outcome of out-of-frame mutations and number of off-targets, were selected (**Table 1**). The same procedure was pursued for the 57 bp signal sequence of γ -kafirin, which revealed five target motifs, from which another two were selected (**Table 2**). The results provided by the two platforms were very similar and therefore, only the data delivered by the CRISPOR tool are shown here.

The nucleotides of the signal sequences of α -KAFIRIN (*k1C5*) and γ -KAFIRIN (*gKAF1*) genes with the location of target sites are shown in the scheme (**Figure 2**).

Table 1. Selection of target motifs within the signal peptide-encoding sequence of the α -KAFIRIN gene using the CRISPOR online tool.

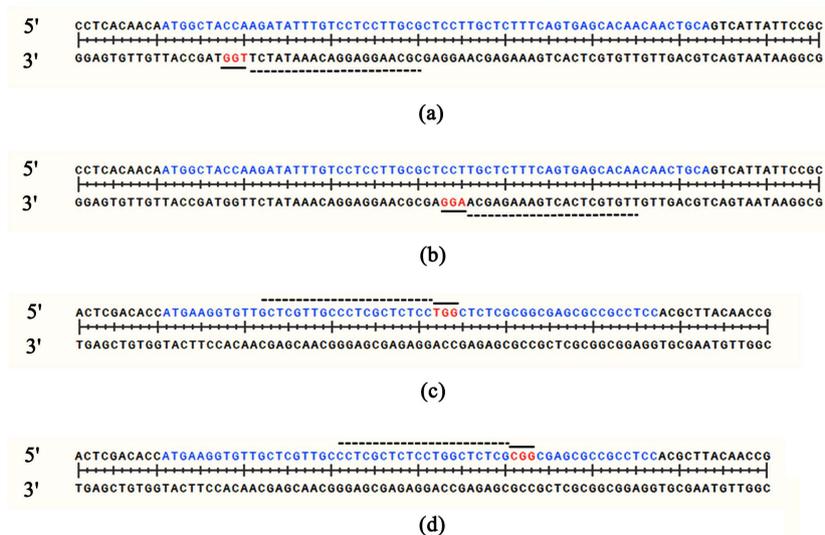
##	Position/Strand	Target motif	MIT Specificity Score	CFD Spec. score	Predicted Efficiency, Doench'16	Predicted Efficiency, Mor.-Mateos	Outcome Out-of-Frame	Off-targets for 0-1-2-3-4 mismatches
1	8/rev	CGCAAGGAGGACAAATATCT <u>TGG</u>	17	86	39	39	61	3-2-9-6-89 109 off-targets
2	33/rev	TTGTGCTCACTGAAAGAGCA <u>AGG</u>	9	86	61	51	64	8-2-5-4-27 46 off-targets
3	21/rev	AAAGAGCAAGGAGCGCAAGG <u>AGG</u>	8	72	67	57	46	11-4-3-16-131 165 off-targets
4	24/rev	CTGAAAGAGCAAGGAGCGCA <u>AGG</u>	7	86	51	64	43	10-4-3-3-27 47 off-targets

The 63 bp input sequence ATGGCTACCAAGATATTTGTCCTCCTTGCCTCCTTGCTCTTTTCAGTGAGCACAACAACCTGCA was used from *Sorghum bicolor* (pz9Sbicolor), chromosome_5:58133820-58133882, reverse genomic strand. It contains four possible target motifs. Expected cleavage positions are located -4 to -3 bp upstream of the Cas9-bound triplet (PAM).

Table 2. Selection of target motifs within the signal peptide-encoding sequence of the γ -*KAFIRIN* gene using the CRISPOR online tool.

#	Position/Strand	Target motif	MIT Specificity Score	CFD Spec. score	Predicted Efficiency, Doench'16	Predicted Efficiency, Mor.-Mateos	Outcome Out-of-Frame	Off-targets for 0-1-2-3-4 mismatches
1	32/fw	GCTCGTTGCCCTCGCTCTCC <u>IGG</u>	94	97	37	22	53	0-0-0-7-37 44 off-targets
2	41/fw	CCTCGCTCTCCTGGCTCTCG <u>CGG</u>	89	93	57	42	58	0-0-1-15-94 110 off-targets
3	30/rev	CGGCGCTCGCCGCGAGAGCC <u>AGG</u> (high GC content)	84	83	32	59	53	0-0-0-8-125 133 off-targets
4	20/rev	CGCGAGAGCCAGGAGAGCGA <u>GGG</u>	77	88	54	51	40	0-0-0-14-151 165 off-targets
5	21/rev	CCGCGAGAGCCAGGAGAGCG <u>AGG</u>	59	80	49	59	41	0-0-2-9-259 270 off-targets

The 57 bp input sequence ATGAAGGTGTTGCTCGTTGCCCTCGCTCTCCTGGCTCTCGCGGCGAGCGCCGCCTCC was used from *Sorghum bicolor* (pz9Sbicolor), chromosome_2:60425298-60425354, forward genomic strand. It contains five possible target motifs. Expected cleavage positions are located -4 to -3 bp upstream of the Cas9-bound triplet (PAM).

**Figure 2.** The nucleotides of signal sequences (highlighted in blue) of α -*KAFIRIN* (*k1 C5*) ((a), (b)) and γ -*KAFIRIN* (*gKAF1*) ((c), (d)) genes with the location of PAM sites (underlined with a solid line) and selected target motifs (underlined with a dotted line).

According to the chosen target motifs, oligonucleotides were designed for subsequent cloning of gRNA/*cas9* vectors. The sequences of the oligonucleotides are shown in **Table 3**.

3.2. Design and Cloning of gRNA/Cas9 Vectors

Canonical target motifs for *U3* promoter-driven guide RNAs and Cas9 have the generic sequence AN₁₉NGG (encompassing the target motif-specific part of gRNA and the PAM (protospacer adjacent motif)). For efficient transcription of gRNA under the control of the RNA polymerase III-processed *OsU3* promoter,

Table 3. Targets of kafirin genes used in the work.

Target motif no.	Target-specific oligo name	Nucleotide sequence of target-specific oligo	Name and target gene of the resultant vector
1	<i>α8r_F</i>	TGGCAcgcgaaggaggacaaatatct	1C (<i>α-KAFIRIN</i> editing)
	<i>α8r_R</i>	AAACagatattgtcctccttgcgT	
2	<i>α33r_F</i>	TGGCAttgtgctcactgaaagagca	2C (<i>α-KAFIRIN</i> editing)
	<i>α33r_R</i>	AAACtgctctttcagtgagacaaT	
3	<i>γ32f_F</i>	TGGCAgctcgttgccctcgtctcc	3C (<i>γ-KAFIRIN</i> editing)
	<i>γ32f_R</i>	AAACggagagcgagggcaacgagcT	
4	<i>γ41f_F</i>	TGGCAcctcgtctcctggctctcg	4C (<i>γ-KAFIRIN</i> editing)
	<i>γ41f_R</i>	AAACcgagagccaggagagcaggT	

an A was used as an additional 5'-terminal nucleotide in all gRNAs, because useful target motifs starting themselves with an A are not available in the targeted gene regions.

The principles of cloning target-specific derivatives of vector pSH121 are shown in **Figure 3**. The sequences of the generic and derived vectors pSH121 differ in size (12,396 bp and 12,199 bp, respectively). The design of forward (single strand) oligonucleotides was as follows: 5'-TGGCA (or G) N2-20-3'. The design of reverse single strand oligonucleotides was accordingly as follows: 5'-AAAC (complementary to N20-2) T (or C)-3'. A double-stranded nucleotide fragment for integration in pSH121 can be easily created by annealing these two complementary single-stranded oligonucleotides (see **Table 3**). The double-stranded insert fragment has sticky ends compatible with the *BsaI*-created DNA-ends of the linearized vector pSH121.

The cloning protocol included the following steps.

1) Plasmid pSH121 was digested with *BsaI* (*Eco31I*) restriction enzyme to allow for the insertion of the target-specific insert. Restriction products of *BsaI* fragments 1227 bp (SpecR) and 10,972 bp were separated on a 1% agarose gel. The latter fragment was isolated and purified from the gel.

2) The assembly of the target-specific double-stranded (ds) oligonucleotide was performed by heating a mixture of an equimolar amount of each of the single-stranded F and R oligonucleotides followed by their annealing via slow cooling.

3) The assembled ds oligonucleotide was ligated using compatible overhangs with the 10,972 bp *BsaI* (*Eco31I*)-fragment of plasmid pSH121.

4) The ligation products were transformed into competent *E. coli* cells, which were then grown and selected on LB medium with kanamycin. The plasmids isolated from the selected colonies were cleaved using endonuclease *MluI* and then sequenced to confirm the presence of the insert.

5) Upon digestion using *SfiI*, the fragment containing expression units for gRNA and *cas9* was ligated with the *SfiI*-linearized vector B479p7oUzm, thereby combining all functional elements and both borders of the T-DNA. The resultant binary vector also carries a bacterial selectable marker gene conferring re-

3.3. Restriction Analysis

To control the successful assembly of binary vectors, restriction endonuclease analysis was performed using the enzymes *MluI* and *SfiI*. The *MluI* recognition site is unique in pSH121 and absent in B479p7oUZm-LH, while both of the generic vectors pSH121 and B479p7oUZm-LH have two *SfiI* restriction sites each. In **Figure 8**, digestion of each of the newly created vectors (1C, 2C, 3C, 4C) is displayed. The vectors have a size of 17,846 bp. Whereas *MluI* produced one fragment, cleavage with the *SfiI* yielded two fragments, the sizes of which correspond to the expected values (10,223 bp and 7623 bp).

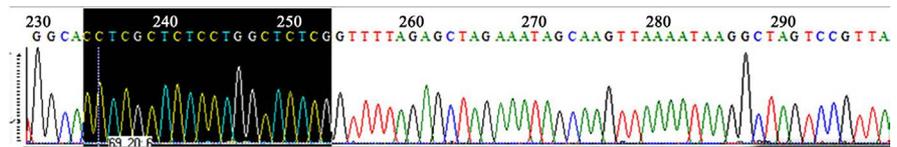


Figure 7. Confirmation by Sanger sequencing of the correct insertion of the target motif #2 (γ 41)-specific part (indicated by black background) into the gRNA expression unit of pSH121 for the targeted mutagenesis of the γ -*KAFIRIN* gene.

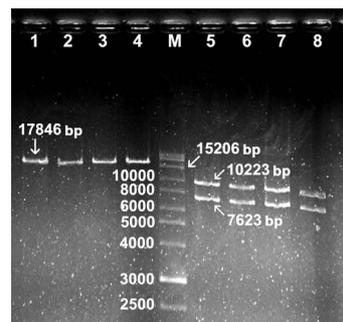


Figure 8. Restriction analysis of newly constructed binary vectors. Lanes 1 - 4: binary vectors 1C, 2C, 3C and 4C after digestion with *MluI* restriction enzyme; M—DNA fragment length marker (“SibEnzyme” Russia, cat. No. M30 <http://russia.sibenzyme.com/info815.php>); 5 - 8: binary vectors 1C, 2C, 3C and 4C after digestion with restriction endonuclease *SfiI*.

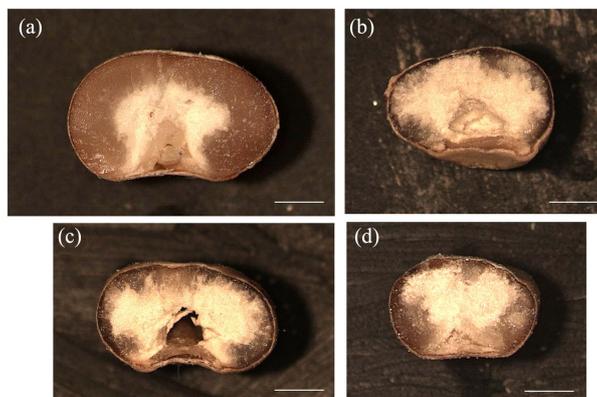


Figure 9. Cross sections of kernels set on the panicle of the sorghum plant #2C-1.2.5 carrying a genetic construct for α -*KAFIRIN* gene editing (target #2C) ((b), (c), (d)) ((a)—kernel of original cv. Avans). Bar 1 mm.

4. Conclusion

It is expected that the population of the Earth will reach 9.6 billion people by the middle of this century. The demand for staple crops thus will increase by up to 60% [22]. To cope with this challenge, a significant improvement of plant breeding and plant production methods is required. In this regard, genome editing belongs to the most promising approaches [23], with Cas endonucleases being the currently most powerful platform. Using this technology, the improvement of grain quality via targeted mutagenesis of the *KAFIRIN* genes of sorghum may be achieved in a comparatively short time [24]. The vectors we have created represent an important step towards this goal. One of these vectors, 2C for α -*KAFIRIN* gene editing, was used to transform sorghum via *Agrobacterium* (strain AGL-0)-mediated DNA transfer to immature embryos of cv. Avans. In these experiments, we have obtained four plants (T_0 generation) with modified endosperm texture (Figure 9) that should be expected in the case of disturbed synthesis of α -kafirins, and improved *in vitro* digestibility of endosperm proteins [11] [12] [13] [21]. The incorporation of vectors during transformation was confirmed by PCR analysis. Amplification and sequencing of the target regions from the transgenic plants are in progress.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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