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RESEARCH PAPER

Design and construction of single guide RNA for CRISPR/Cas9 system based on the *xa13* resistance gene in some varieties of rice (*Oryza sativa*)

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ABSTRACT

The *xa13* gene is a recessive resistance gene against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) found in several rice varieties. Activation of this gene will trigger the formation of sucrose as a nutrient supply to *Xoo* for their growth in the plant. The disruption of this recessive gene expression in the plant can affect the negative impact of the gene, and recently can be created using clustered regularly interspaced short palindromic repeats (CRISPR) system using CRISPR-associated protein-9 (CRISPR/Cas9) technology that requires gRNA to recognize the targeted-sequence. This study aimed to design and construct the gRNA-targeting *xa13* gene in rice using bioinformatics tools. CHOPCHOP was used for generated the gRNA candidates according to the target gene sequence. Two candidates of gRNA-targeted xa13 have been selected based on the analysis of bioinformatics data. Each candidate of gRNA consisted of 20 nucleotides (nt) of the target sequence upstream 3 nt of the protospacer adjacent motif (PAM) sequence (5'-NGG) targeting two exons in the *xa13* gene. The gRNA1 will target exon 1 and the gRNA2 will target exon 2, with an efficiency of 52.51% and 44.63% respectively. Data showed that the GC content of all gRNA candidates ranged from 55–70% with no target-off location in the whole genome of rice. The transformation and confirmation test based on the physiological and genomic characteristics of transformants confirmed that the design has been successfully constructed.

Key words: CRISPR/Cas9, gRNA, *Oryzae sativa*, *xa13* gene, *Xanthomonas oryzae*

INTRODUCTION

Bacterial leaf blight (BLB) is caused by the bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the phytopathogenic bacteria that infect plants at various stages of growth, both vegetative and generative. On average, disease severity due to BLB can reach 68.5 to 90.3% in the vegetative phase, especially in susceptible varieties (Khaeruni et al., 2014). Moreover, disease severity tends to increase from 56 to 84 days after planting and depends on resistance genes presented in each variety (Chen et al., 2020; Pinem & Syarif, 2018). Recently, about 46 genes have been identified in rice that play role in rice to its resistance against *Xoo* including gene *xa13* (Chen et al., 2020; Kumar et al., 2020; Nadhira et al., 2022).

One of the genes that affect the resistance of rice against *Xoo* is the gene, which belongs to the sugar will eventually be exported to the transporters (*SWEETs*) family (also known as *OsSWEET11*, *Os8N3* or *xa13*) (Breia et al., 2021). The gene is activated through transcription activator-like effectors (TALEs) presented in *Xoo*. TALEs are known to bind to effector binding elements (EBEs) in the promoter region of the *OsSWEET11,* consequently, nutrients formed from plants are diverted to supply the nutritional needs of *Xoo* (Zafar et al., 2020). The activation of the gene causes the rice to become more susceptible to the *Xoo* infection (Kim et al., 2019). Chu et al. (2006) reported that the lower the expression level of *xa13*, the higher the resistance level of rice against *Xoo.* Moreover, the disruption or deactivation of *xa13* in rice exhibits a broad-spectrum resistance against *Xoo* (Xu et al., 2019). Therefore, it is interesting to inhibit or deactivate this gene to create a resistant plant. The

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most recent technology to realize this idea is genome editing (Kim et al., 2019).

Genome editing technology is an effective and efficient way of changing the composition of deoxyribonucleic acid (DNA) in cell organisms including plants through several techniques such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system in addition to Zinc Finger (ZF) or Transcription activator-like effectors nuclease (TALEN) system (Gaj et al., 2013) Utilization of the CRISPR/Cas system, particularly CRISPR/Cas9, has been widely reported to alter the nucleotide arrangement in the plant genome such as the rubisco gene (*rbs*C) in tobacco for more efficient assimilation (Donovan et al., 2020). In addition, gene *Os8N3* knockout succeeded to enhance the resistance of Japonica rice against *Xoo* (Kim et al., 2019). In practice, CRISPR/Cas9 system will target genes in an organism guided by noncoding RNA that is easily adapted and commonly called guide RNA (gRNA) which is also one of the keys to successful genome editing through the CRISPR/Cas9 approach (Liu et al., 2020). The function of gRNA is very important in protecting the template and recognizing specific pairs of target sequences which are then tasked with sending signals to Cas9 to break the double bond chain through the double-strand break (DSB) mechanism for further gene editing processes in the CRISPR/Cas9 system (Zafar et al., 2020).

On the other hand, rice (*Oryza sativa*) cultivars are grouped into subspecies (subsp.) *japonica* and *indica* which are genetically different in 11 out of 12 chromosomes representing the variation of sequences in the chromosomes including gene sequences (Zhang et al., 1992). More specifically, Yu et al. (2016) determined about 51 polymorphisms occur in the BLB resistance locus *xa13* sequence among 217 rice cultivars. However, genome editing through CRISPR/Cas9 approach requires gRNA that is designed specifically for the target gene according to the target gene sequence (Kim et al., 2019; Zafar et al., 2020). Therefore, designing and constructing an appropriate gRNA based on *the xa13* gene sequence from both cultivars of *japonica* and *indica* are necessary to obtain a CRISPR/Cas9 system for editing the rice genome for various varieties.

MATERIALS AND METHODS

Research Site. All experiments were carried out at the Center for Development of Advanced Science and Technology (CDAST) and Pusat Unggulan IPTEKS (PUI) Bio-Tin University of Jember.

Sequence Selection for gRNA Candidates Targeting *xa13* Gene Sequence in Rice. Sequences of the *xa13* gene in the chromosomes of rice *japonica* such as Nipponbare (Accession No. NC_029263.1), Nipponbare (PAC) (Accession No. AP005439.3), Nipponbare (BAC) (Accession No. AP005528.2) and *indica* such as IR64 (Accession No. DQ421395.1), IR24 (Accession No. DQ421396.1), IRBB13 (Accession No. DQ421394.1) were collected from the GenBank and aligned for determining the conservative areas using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the sequence consensus among sources of *xa13* genes was visualized using a web-based tool, WebLogo version 2.8.2 (Crooks et al., 2004). The intron and exon presented in the *xa13* sequence in the chromosomal DNA sequences were determined by aligning the chromosomal sequence with mRNA_ref sequences obtained from the GenBank using the Clustal Omega. The gap presented between two sequences was determined as intron sequences.

Ideally, the gRNA should consist of 20 nucleotides (nt) based on the target sequence and three nucleotides based on the Protospacer Adjacent Motif (PAM) sequence (Xie & Yang, 2013). The oligonucleotides of gRNA were designed using the most cited CRISPR-based technology supporting tool (Bhagwat et al., 2020). CHOPCHOP version 3 (Labun et al., 2021) generated information related to PAM, the efficiency of gRNA, GC content, genome location, number of mismatches, and their location in the coding sequence (CDS) (Montague et al., 2014). The selected gRNA sequences (designed by adding GGCA at the 5' end of one strand and CAAA at the 5' end of the complementary strand resulting in 23 nt) were synthesized as single-stranded oligonucleotides at PT. Genetika Science (Indonesia), namely gRNA-Exon1 and gRNA-Exon2.

Plasmid pRGEB32 Isolation. The plasmid pRGEB32 carrying a designated gRNA site was a gift from Yinong Yang (Addgene plasmid #63142; http://n2t.net/addgene:63142; RRID: Addgene_63142) (Xie et al., 2015). This gRNA site will be inserted with the designed duplex of gRNA-Exon1 and gRNA-Exon2 fragments as described below. The plasmid was isolated from pRGEB32-carrying *Escherichia coli* DH5α using the High-Speed Miniprep Kit (Geneaid, Taiwan) according to the manufacturer's protocols. The purified plasmid was then eluted with 30 µL elution buffer/TE buffer and stored at -20 ºC for further steps.

Construction of gRNA-targeted *xa13* Gene into pRGEB32 Harboring CRISPR/Cas9. Purified plasmid pRGEB32 was cleaved using the *Eco*311 (*Bsa*I) enzyme (Thermo Fisher Scientific, U.S) for vector linearization. The *Eco*311 (*Bsa*I) enzyme recognizes the sequence of 5'-GGTCTC-3' and cleaves it producing overhangs 5'-GTTT-3' and 3'-CCGT-5' for the complementary. Briefly, the 2 µL of *Eco*311 (*Bsa*I) was added into a 38 µL mixture of 20 µL of pRGEB32, 4 µL of Buffer G (10×), and 14 µL of dH_2O in an Eppendorf tube and incubated at 37 ºC overnight followed by visualizing the product in a 0.8% agarose electrophoresis. Linearized plasmid pRGEB32 was then extracted and purified from agarose gel using Gel Extraction Miniprep Kit (Biobasic, Canada) according to the manufacturer's protocols.

On the other hand, to produce an insert of oligo duplex gRNAs, a single-strand oligonucleotide of forward and reverse synthetic nucleotides gRNA-Exon1 and gRNA-Exon2 were mixed in a PCR tube and annealed at a temperature of 95 ºC for 3 min in a thermal block followed by placing at room temperature for 45 min (Lee et al., 2019). Each oligo-duplex gRNAs was ligated into linearized pRGEB32 (mentioned above) with a ratio of 2:1. The ligation reaction was a mixture of 1 µL KAPA T4 DNA ligase, 2 µL linearized plasmid, and 2 µL ligation buffer (10×) (KAPA Biosystems, U.S) in a total reaction volume of 20 µL. The sample was incubated at 4 ºC overnight before transformation into *E. coli* TOP10.

Cloning of pRGEB32::gRNAs and confirmation of transformants. Cloning of pRGEB32::gRNAs (pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2) was done in *E. coli* TOP10 competent cells as follows. The ligated pRGEB32::gRNAs were transformed into competent cells following a heat shock protocol. Briefly, the sample (mixture of ligated pRGEB32::gRNAs and competent cells) was heat shocked at 42 ºC for 90 s on a thermal block, chilled on ice for 15 min, and shaken in an incubator at 37 ºC for 60 min. Then, a 100 µL sample was spread on Luria Bertani (LB) agar containing 50 mg L^{-1} of kanamycin and incubated at 37 ºC overnight.

Confirmation of transformants was done by visualizing the DNA restriction pattern of the *Eco*311 (*Bsa*I) enzyme on an agarose gel electrophoresis in addition to the observation of colony grown on the selection LB medium containing selectable marker, kanamycin 50 mg L^{-1} (Marillonnet & Grützner, 2020). Briefly, a single colony grown on a selection medium was picked up, transferred into the 4 mL of LB broth containing 50 mg L^{-1} kanamycin, and incubated at 37 ºC overnight. The culture grown on the medium was then subjected to plasmid isolation using a High-speed Plasmid Mini-kit Kit (Geneaid, Taiwan). Pure plasmids were then digested and visualized as described above.

RESULTS AND DISCUSSION

Design and Construction of Oligonucleotides as gRNA Candidates Targeted Exon1 and Exon2 in the *xa13* Gene Sequence. Targeting the *xa13* gene or also known as the *OsSWEET11* gene, which is a recessive resistance (R) gene, aims to improve rice resistance to *Xoo* pathogens as the cause of bacterial leaf blight (BLB). SWEET belongs to the sugar efflux transporter family and has a very important role in biological processes, including the supply of nutrients for pollen and seed filling. Meanwhile, the sugar will be diverted to supply the nutritional needs of *Xoo* (Jiang et al., 2020; Zafar et al., 2020). Since this gene affects rice susceptibility against *Xoo*, the deactivation of this gene using the CRISPR/Cas9 system could provide an efficient and precise tool via gRNA and PAM recognition.

According to the *xa13* gene sequence organization, five exons have been determined with some variation in nucleotides. The variation of nucleotides was found in 3 out of 5 exons such as in IRBB13, the guanine (G) presented in exon 3 instead of cytosine (C), and adenine (A) instead of guanine (G) in exon 5. In addition, in most *indica* rice (IR64 and IR24), the thymine is presented in exon 4 instead of cytosine (C) of *japonica* rice (Figure 1A). The selection of specific sequences of gRNAs was based on the conserved sequences of gene *xa13* from group *japonica* and *indica*. These exons are selected since its clean from nucleotide variations in *xa13* sequences that are appropriate for gRNAs. Previous studies reported the selection of specific gRNAs from conservative sequences of various *Tomato yellow leaf curl virus* (TYLCV) coding and non-coding sequences was the best way to target various geminivirus genomes (Ali et al., 2015). In contrast, designing gRNA based on non-conserved sequences within the various sequences among the target DNA results in potential off-target activities (Zhang et al., 2016). Therefore, the sequence of exon 1 and exon 2 of *xa13* is proposed as the target of further constructed gRNA oligonucleotides.

To obtain and confirm the suitability of sequence consensus *xa13* as the target gene of designated gRNA, the result of CHOPCHOP was analyzed (Table 1). Two candidate gRNAs (gRNA-Exon1 and gRNA-Exon2) were obtained consisting of 20 nt targeting *xa13* gene sequence located in Chromosome 8. Both gRNAs have different PAM sequences (TGG in gRNA-Exon1

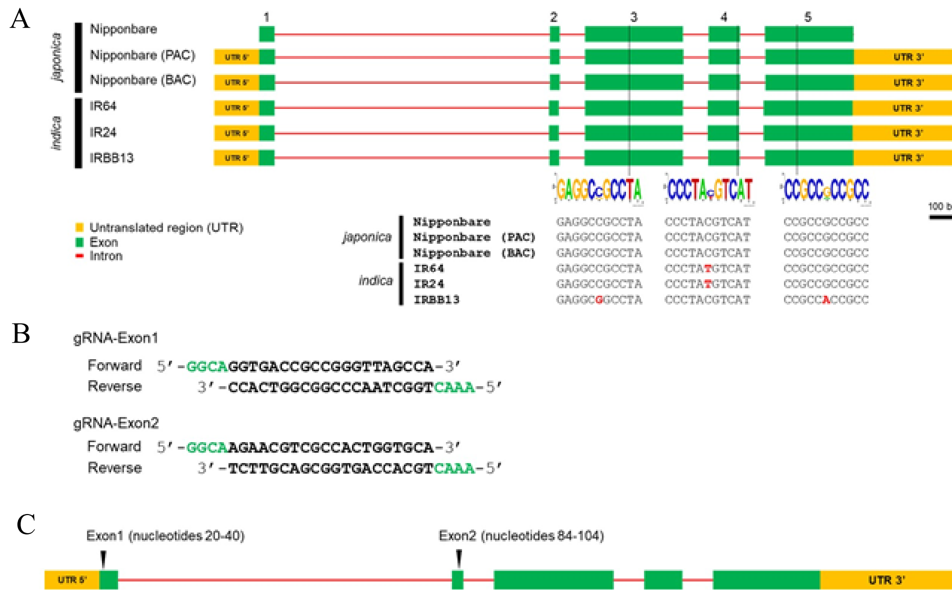

Figure 1. Designing gRNAs-targeted *xa13* gene in rice. A. Schematic diagram of *xa13* gene structure among indica and japonica rice. The consensus sequences were generated as a Pictogram to show the non-conserved nucleotide in exons (indicated with the red letters); B. Sequences of gRNAs, forward and reverse, added with 4-nucleotides (letters in red) overhanging at the 5'-end of the 20 bp of designed –gRNAs; C. Schematic diagram of *xa13* with gRNA-targeted sites labeled with black arrowheads.

Table 1. General information of gRNAs sequences for *xa13*-targeted gene

Code	gRNA-Sequence	PAM	Strand	Location in CDS	Genome location in Chromosome	% GC content	Mismatch	Efficiency of gRNA
gRNA-Exon1	GGTGACCGCC GGGTTAGCCA	TGG	-	20–40 (Exon1)	8	70%	1	52.51%
gRNA-Exon2	AGAACGTCGC CACTGGTGCA	AGG	-	84–104 (Exon2)	8	60%	0	44.63%

and AGG in gRNA-Exon2) with an efficiency and GC content of more than 40% and 60%, respectively. However, only gRNA-Exon1 has one possible mismatch. The gRNA sequences were chosen based on the CHOPCHOP analysis (Table 1, Figure 1B) since the design meets the criteria for appropriate gRNA design. As previously described that the efficiency of gRNA consisting of 20 nt is affected by their percentage of GC content (ranging from 40–80%) with more than 50% (G) adjacent to PAM will increase the high rate of mutagenesis and on-target efficiency (Gagnon et al., 2014; Labun et al., 2016; Montague et al., 2014; Zhu & Liang, 2019). In addition, gRNA-Exon1 and gRNA-Exon2 have different targets of PAM sequences such as TGG and AGG, respectively. The targeted PAM sequence of CRISPR/Cas9 recognizes 5'-NGG-3' as the NGG motif which follows the base pair region on the target complement DNA strand (Xie & Yang, 2013). On the other hand, due to the *Cas*9 potentially having off-target activities, it is necessary to find the number of mismatches tolerated. Single and double mismatches tolerance is dependent on their position along gRNA. Increasing the number of mismatches can reduce the *Cas*9 affinity to the matching sites of gRNA (Xie et al., 2014). In addition, the gRNAs targeting Exon 1 and Exon 2 in the *xa13* gene sequence were designed by adding 4 nt overhanging at the 5'-end of each strand to target nucleotides 20–40 in Exon 1 and nucleotides 84–104 in Exon 2 (Table 1, Figures 1B and C). The overhang nucleotides at the DNA insert improve and increase ligation fidelity in their ligation strands partner (Potapov et al., 2018).

Moreover, the construction of pRGEB32 carrying gRNAs indicated that both designed gRNAs were proposed to be inserted and ligated into the *Bsa*I restriction site at pRGEB32 (Figure 2A). Since the *Bsa*I recognition sequence is presented upstream of the restriction site, the resulting strands remained an

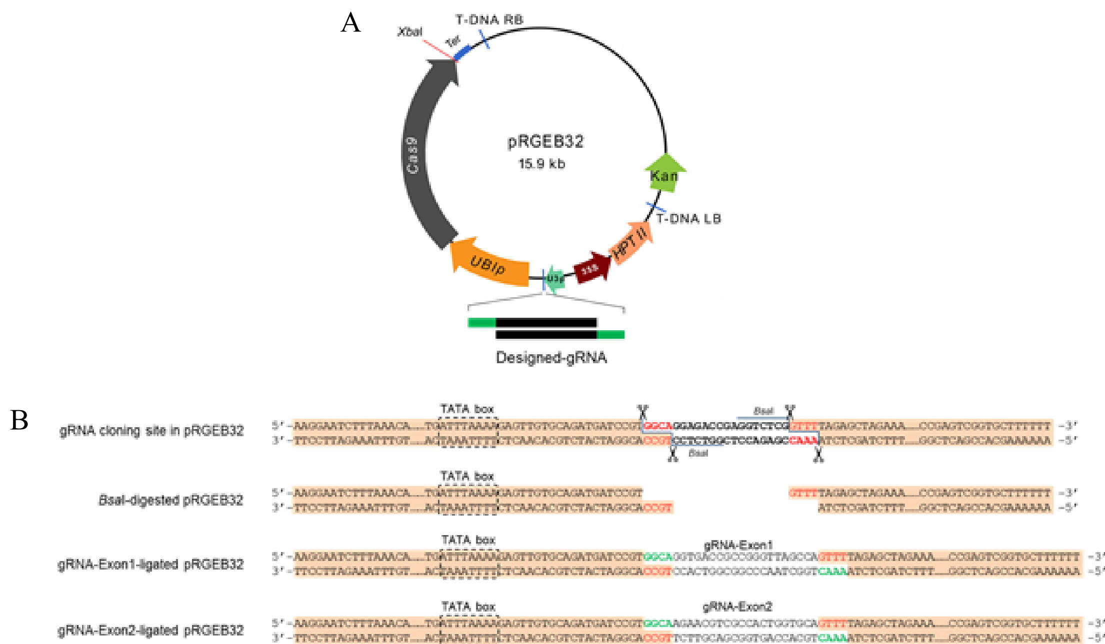

Figure 2. Construction of pRGEB32 carrying gRNA sequence. A. Schematic figure of the pRGEB32 vector carrying CRISPR/Cas9 with designed-gRNA cloning site and an *Xba*I recognition and restriction site at downstream of *Cas*9. The gRNA cloning site is located upstream of the U3 promoter with *Bsa*I recognition and restriction sequences; B. Schematic illustration of introducing designed-gRNAs into *Bsa*I-digested site in pRGEB32 vector. The *Bsa*I-digested site providing overhang 4 nt GTTT and CCGT at each 5′-end (letters in red) were ligated with the oligo duplex gRNA-Exon1 and gRNA-Exon2 containing 5′ compatible CAAA and GGCA (letters in green) overhang 4 nt.

overhanging pattern at the 5'-end at the *Bsa*I-recognized strand against its complementary strand (Xie et al., 2015). Consequently, the designed gRNAs were exactly a match for insertion and ligation with no *Bsa*I recognition sequence and restriction site (Figure 2B).

Transformation of pRGEB32::gRNAs into *E. coli* TOP10 and Confirmatory Test of Transformant Carrying CRISPR/Cas9 System. Transformation of ligated oligo-duplex gRNAs into pRGEB32 (pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2) into competent cells of *E. coli* TOP10 showed that all cells transformed with non-digested pRGEB32::gRNA grown on LB agar plates containing 50 mg L^{-1} of kanamycin as well as transformed with the original non-digested pRGEB32 (Figure 3A). This indicates that the ligated plasmid has been successfully transformed into competent cells. As a proof of this concept, transformed the ligated oligo-duplex gRNAs with pRGEB32 (pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2) into *E. coli* TOP10 resulting in the growth of the colony, hence the transformation using of empty TOP10 cells resulting no colonies in LB plate containing kanamycin 50 mg L^{-1}. It is indicated only transformants colonies containing ligated oligo-duplex gRNAs harboring pRGEB32 can grow in a medium containing kanamycin 50 mg L^{-1}. In contrast, *E. coli* TOP10 has only antibiotic-free plasmid which caused the cell is unable to survive in media containing antibiotics (Cranenburgh et al., 2001; Liakopoulos et al., 2018). The pRGEB32 plasmid is designed to have the *Kan* gene that supports the growth of colonies when it is grown on a media containing kanamycin (Zhang et al., 2021).

Confirmation of clones was conducted using enzyme digestion pattern analysis as well as Kostylev et al. (2015). The restriction pattern of all extracted plasmids with *Eco*311 (*Bsa*I) showed that only the original pRGEB32 exhibited a single band with a size of 15,888 bp (Figure 3B). This occurs since the original pRGEB32 is successfully digested with *Bsa*I generating a linearized plasmid due to the existence of *Bsa*I recognization and restriction site at the gRNA site in the pRGEB32 (Fischer et al., 2018; Xie et al., 2015). Meanwhile, no digestion pattern was observed on both pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2 while added with the *Bsa*I enzyme. As predicted, Since the inserts (oligo-duplex gRNAs) have no *Bsa*I recognition and restriction site, both pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2 remain undigested.

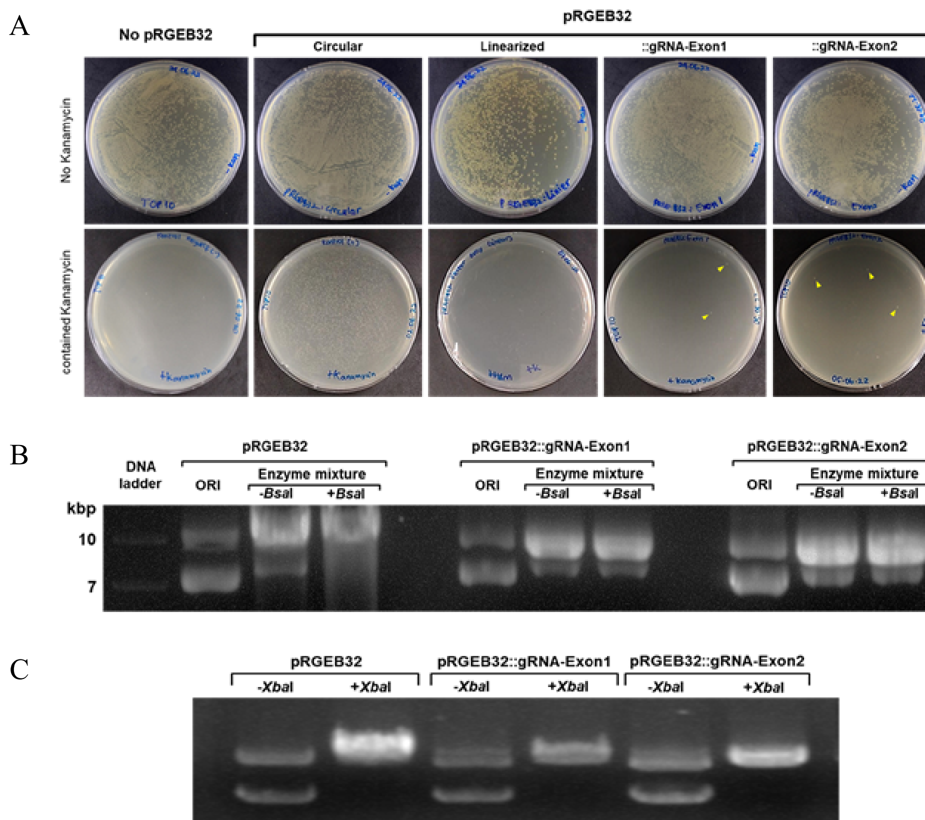

Figure 3. Confirmatory test of transformants. A. Growth of *E. coli* TOP10 transformed with no pRGEB32 and pRGEB32 on LB agar media with no kanamycin and contained kanamycin. Colonies carrying expected ligated gRNAs-pRGEB32 are shown in yellow arrowheads; B. The *Bsa*I restriction pattern of the plasmid pRGEB32, and ligated gRNAs-pRGEB32 plasmid (pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2) isolated from the transformants on agarose gel electrophoresis. The enzyme reaction mixture without *Bsa*I (-*Bsa*I) and the original plasmid (ORI) of pRGEB32 or its ligated gRNAs in eluent were compared to Its *Bsa*I (+*Bsa*I) restriction pattern; C. The *Xba*I restriction pattern of the plasmid pRGEB32 compared with the ligated gRNAs-pRGEB32 plasmid (pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2).

However, all plasmids in the enzyme reaction mixture without restriction enzyme (*Bsa*I) exhibited slightly distinct size and mobility on agarose gel electrophoresis compared with its original plasmid (ORI) (Figure 3B). The distinction indicates that the mobility of DNA fragments in agarose gels is influenced by their topological shapes such as open circular (OC), supercoiled (SC) or covalently closed circular (CCC), and linear (Hintermann et al., 1981). Plasmids with a supercoiled topology move faster than linear plasmids, since the molecules are more knotted and compacted, the migration moves faster through agarose gels with low concentrations (Cebrián et al., 2015). Slower movement of DNA fragments in all plasmids in the enzyme mixture without restriction enzyme (-*Bsa*I) compared to original pRGEB32 indicates that the supercoiled plasmid was affected by digestion with *Bsa*I enzyme which resulted in the compact supercoiled form being uncoiled or the relaxed state becoming open circular (OC) (Nitiss et al., 2012). Moreover, transformant confirmation with *Xba*I digestion pattern showed that all plasmids such as native pRGEB32, pRGEB32::gRNA-Exon1, and pRGEB32::gRNA-Exon2 digested with *Xba*I showed single bands (Figure 3C). It is known that the original pRGEB32 plasmid contains an *Xba*I restriction site downstream of the Cas9 gene (Figure 3A) (Xie et al., 2015). Thus, all enzyme restriction experiments strongly confirmed that the designed gRNA was successfully ligated to pRGEB32 resulting in pRGEB::gRNA-Exon1 or pRGEB::gRNA-Exon2.

CONCLUSION

In conclusion, two plasmids carrying the CRISPR/Cas9 system and gRNA to edit the *xa13/OsSWEET11/Os8N3* gene in rice have been successfully

designed and constructed. The design was constructed with the gRNAs effectively proposing exon 1 and exon 2 of *xa13* presented in either *O. sativa* subsp. *japonica* or subsp. *indica*. Both constructed-gRNAs have different PAM sites targeting TGG and AGG on exon 1 and exon 2 in the *xa13* sequence, respectively. The plasmids were named pRGEB32::gRNA-Exon1 and pRGEB32::gRNA-Exon2.

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AUTHORS' CONTRIBUTIONS

HSA, AW, and WIDF designed the research and provided the fund for the experiments. WAH and HSA executed the experiments and analyzed the data. All authors contributed to the interpretation of data, manuscript preparation, and writing. All authors have read and approved the final manuscript.

COMPETING INTEREST

The authors declare that no competing interest regarding this publication.

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