DESIGN OF CRISPR/CAS9 SYSTEM FOR EDITING *OsNRAMP7* INVOLVED IN METAL TRANSPORT IN TBR225 RICE VARIETY

Phung Thi Thu Huong[1], Cao Le Quyen[1], Ngo Thi Van Anh[1], Pham Phuong Ngoc[1], Pham Thi Van[1], Le Quynh Mai[2], Pham Xuan Hoi[1], Nguyen Duy Phuong[1,*]

ABSTRACT

Rice (*Oryza sativa* L.) is one of the major food crops but is deficient in minerals such as Zn. In rice, there were eight genes belonging to *the Natural resistance-associated macrophage protein* (*NRAMP*) family, of which *OsNRAMP1*, *OsNRAMP4, OsNRAMP5, OsNRAMP6*, and *OsNRAMP8* were reported to transport metals such as iron (Fe), zinc, mangan (Mn). Recently, *AtNRAMP5*, closely related to *OsNRAMP7*, has been identified as a function in Zn uptake in *Arabidopsis*. To identify the function of *OsNRAMP7* in a local rice variety -TBR225, the expression of the interested gene was evaluated and a Clustered regular interspaced short palindromic repeats/CRISPR-associated Cas9 (CRISPR/Cas9) system for editing TBR225 *OsNRAMP7* was constructed. The accumulation of *OsNRAMP7* mRNA was increased in Zn-treated TBR225 rice plants. Two single guide RNAs for the *OsNRAMP7*-editing using CRISPR/Cas9 system was designed based on 0.52 kb DNA fragment containing the ExonII region of *OsNRAMP7* isolated from the genomic DNA of TBR225. The CRISPR/Cas9 system for directed editing TBR225 *OsNRAMP7* containing two CRISPR RNA (crRNA) sequences recognizing two different sites on the ExonII of *OsNRAMP57* was developed. The recombinant vector was validated by PCR and sequencing analysis. The results will be used for studying possible function of OsNRAMP7 via knocking out of the gene.

Keywords: *CRISPR/Cas9, OsNRAMP7, TBR225, Zinc.*

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1. INTRODUCTIONS

Rice (*Oryza sativa* L.) is one of the major food crops of the world in general and Vietnam in particular. Rice is a rich energy source, gluten-free, easy to digest, and low in fat. However, the most emerging issue is that rice is deficient in minerals, including zinc (Zn), which significantly affects the human immune system. This disadvantage results in micronutrient deficiencies in the diet, especially in countries where rice is the staple food [1]. One of the practical solutions to this problem is generating new rice varieties with high zinc content in the grain. TBR225 is one of the mega rice varieties for some northern provinces of our country with a significant proportion of rice production. Production of rice variety TBR225 with high Zn content will improve the value of this rice variety and bring many benefits to consumers.

The NRAMP (natural resistance-associated macrophage protein) transporter protein family has eight members in rice,

[1] Insitute of Agricultural Genetics
[2] VNU-University of Science
*Email: phuongnd.bio@gmail.com

including *OsNRAMP1*, *OsNRAMP2*, *OsNRAMP3*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsNRAMP7* and *OsNRAMP8* [2]. NRAMPs transport metal ions such as Zn2+, Mn2+, Fe2+, Cd2+, and others in plants. *OsNRAMP1*, *OsNRAMP2* and *OsNRAMP3* express primarily in roots, leaves and both tissues, respectively [3]. Furthermore, *OsNRAMP5* and *OsNRAMP2* are the major transporters responsible for Fe, Mn, and Cd, whereas *OsNRAMP4* functions as a transporter for Al [2, 4, 5]. However, relatively little information about these transporters is available, especially information regarding *OsNRAMP7*. The close genetic relationship between *OsNRAMP7* and the identified Zn transporter AtNRAMP4 in *Arabidopsis* [2] suggested the role of *OsNRAMP7* in Zn accumulation in rice.

CRISPR/Cas9 (Clustered Regular Interspaced Short Palindromic Repeats/ CRISPR-Associated Cas9) is a commonly used technique in reverse-genetics research to knock out a gene of interest [4]. Because of its simplicity, efficiency, and versatility, the CRISPR/Cas9 system has revolutionized gene editing and is widely used. The generation of target gene-inactivating mutants based on the specific double-stranded DNA cleavage of the sgRNA-Cas9 complex and the strand break repair machinery is one of the most widely used applications of the CRISPR/Cas9 system. The cell's non-homologous end joining (NHEJ) mechanism causes a double-strand break (DSB) [6]. Many valuable agro-biological traits of plants, such as tolerance to abiotic and biotic stresses, yield, and quality, have been improved by this method [7].

In order to understand the possible involvement of *OsNRAMP7* as Zn transporter in rice plants, the expression of *OsNRAMP7* under Zn treatment was investigated and a CRISPR/Cas9 system-mediated *OsNRAMP7* editing was developed for lightening up *OsNRAMP7* function in TBR225 rice in coming studies

2. MATERIALS AND METHODS

2.1. Materials

The rice variety TBR225 was provided by Thaibinh Seed Corporation. The *Escherichia coli* strains DH5α and DB3.1 were purchased from Thermo Scientifics (USA).

Vectors pENTR4-V1 and pCas9 were provided by the research team of Dr. Sebastien Cunnac (Research Center for Development, Montpellier, France).

The primer pairs used in the study were designed based on the reference sequences (Os12g0581600 and Os03t0718100-01) and ordered from PHUSA Biochem Cor. (Vietnam).

2.2. Methods

2.2.1. Expression analysis

Zn treatment experiments and gene expression analysis were carried out according to the description of Sasaki *et al.* (2012) [8]. Four-week-old rice plants were grown in a nutrient solution containing 10 µM $ZnSO_4$ for 0.5 h, 1 h, 3 h, and 6 h. Total RNA was extracted from the treated plant and used for first-strand cDNA with primers oligo dT. Expression of *OsNRAMP7* was evaluated by amplifying 177 bp of the gene using primers NRAMP7-RT-F: 5'-TTGAAAACTACGGGGTG AGG-3' and NRAMP7-RT-R: 5'- AACAACCCCA ACTGCTTGTC-3'. PCR mixture contains 0.5 µL of cDNA template (100 ng), 0.3 µL of each 10 pmol/ml primer (1.5 µL of 2 mM dNTP and 1.5 µL of 10 X *Taq* DNA polymerase buffer, 0.5 µL of 5 U/µL *Taq* DNA polymerase, 10.3 µL of deionized sterilized water. Meanwhile, *OsActin* was used as a internal control using *Actin1* specific primers (Actin-F: 5'-

TGATGGTGTCAGCCACACT-3', and Actin-R: 5'-TGGTCTTGGCAGTCTCCATT-3'; 221 bp). The RT-PCR was performed in 3 independent experiments.

2.2.2. OsNRAMP5 isolation

Total DNA was extracted from TBR225 plants as described by Doyle and Doyle (1990) [9] using a 2% CTAB solution. The rice leaf sample (100 mg) was ground in liquid nitrogen to a fine powder, then 500 µL of 2% CTAB solution (containing 40 mg/mL RNase) was added and centrifuged at 13,000 rpm to collect the solution. Five hundred µL of a mixture of phenol:chloroform:isoamyl (25: 24: 1) was added to the solution to precipitate the protein. The mixture was then centrifuged at 13,000 rpm to remove the protein. DNA was precipitated by adding 500 µL of isopropanol to the solution and finally redissolved in TE buffer.

A part of *OsNRAMP7* (referred to as *OsNRAMP7-TBR*) was isolated from TBR225 genomic DNA using specific primers (NRAMP7-F: TCTCAACGCTTCACGGACTT-3' and NRAMP7-R: 5'-TGAGGATTTCT GCCTTGTGCT-3'; Os12g0581600; 517 bp). The PCR conditions were as follows: denaturation at 95˚C for 30 s and 35 cycles of 95˚C for 30 s, 55˚C for 30 s, and 72˚C for 60 s. The PCR products were directly sequenced by Marorgen Cor. (Korea). Sequencing results were analyzed using BioEdit 4.0 software; a homology search was performed using the NCBI BLAST program on GenBank.

2.2.3. Design of sgRNA

The *OsNRAMP7-TBR* editing sgRNA was determined using CRISPR-P v2.0 software (http://crispr. hzau. edu. cn/ CRISPR2). The secondary structure of sgRNA was predicted by Mfold 2.3 software (http://unafold.rna. albany.edu/). The DNA sequences homologous to the crRNA region of designed sgRNA in the rice genome were identified using CCTop software (http://crispr.cos.uni-heidelberg.de). The crRNA sequences were then synthesized by PHUSA Biochem Cor. (Vietnam).

2.2.4. Construction of T-DNA vector

Two oligonucleotide pairs BtgZI-crRNA1-F/BtgZI-crRNA1-R (5'-tgttGTATGGCTGTCT CCTTTGCA-3'/5'-aaacTGCAAAGGAGACAG CCATAC-3') and BsaI-crRNA2-F/BsaI-crRNA 2-R (5'-gtgtGTGTCTCTTGAAAA CTACG-3'/5'-aaa cCGTAGTTTTCAAGAGAC AC-3') were annealed to each other by heat treatment at 95°C for 10 min to form two double-stranded crRNAs (called as *crRNA1-OsNRAMP7* and *crRNA2-OsNRAMP7*). These DNA fragments were then inserted at the *Btg*ZI and *Bsa*I sites on the pENTR4-V2/sgRNA vector, respectively (Figure 1). After PCR validation of recombinant vector with BsaI-crRNA2-F/pENTR4-R (5'- ATGGC TCATAACACCCC TTG-3') (443 bp in length) and pENTR4-F (5'- CTACAAACTC TTCCTGTTAGTTAG-3')/BtgZI -crRNA1-R (527 bp in length), the DNA region containing two sgRNA expression constructs driven by *U6* promoter (0.45 kb) was ligated into the pCas9 binary vector using the Gateway kit (Invitrogen). The pCas9/sgRNA-OsNRAMP7 recombinant vector was tested by PCR with primer pairs BtgZI-crRNA1-F/BsaI-crRNA2-R (0.45 kb in length), Ubi-F (5'-CCCTGC CTTCATAC GCTATT-3')/Cas9-t-R (5'-GCCTCGGCTGTC TCGCCA-3') (0.35 kb in length) and Hpt -F (5'-AAGGAGGTGATC CAGCC-3')/Hpt-R (5'-GAGTTTGATCCTGGCT CAG-3') (0.8 kb in length) and DNA sequencing.

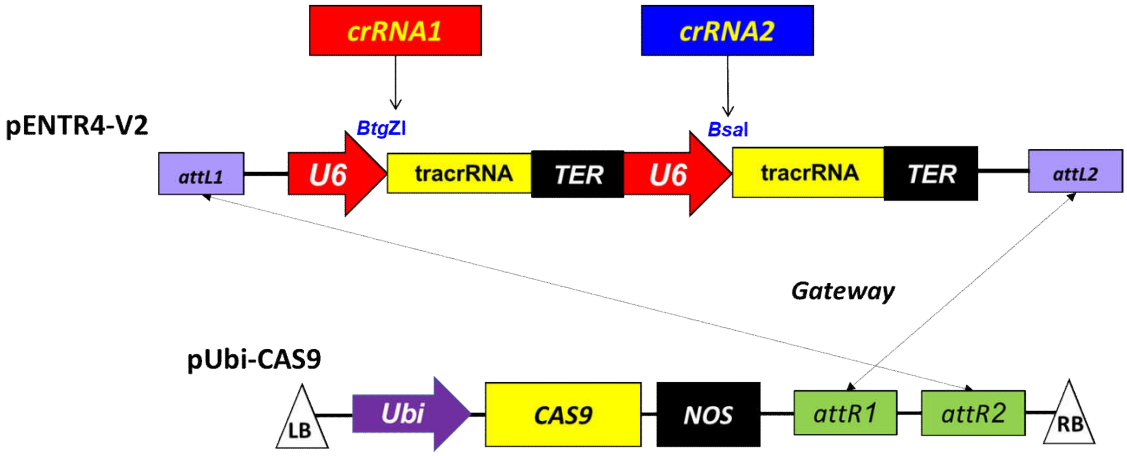

Figure 1. Schematic illustration of pCas9/sgRNA-*OsNRAMP7* vector construction

Note: (U6) OsU6 promoter; (TER) Terminator; (Ubi) ZmUbiquitin promoter; (NOS) nopaline synthase terminator; (LB) Left border; (RB) right border; (attL1/attL2; attR1/attR2) recognition sites of Gateway LR clonase

3. RESULTS AND DISCUSSION

3.1. Analysis of *OsNRAMP7* expression

Many members of the *NRAMP* gene family have been shown to increase their expression under metal ions treatment [8, 00]. In this study, the responses of *OsNRAMP7* expression in the plants exposed to the addition of micronutrient Zn in the hydroponic solution were investigated. The RT-PCR results revealed the accumulation of *OsNRAMP7* transcripts in the different Zn treatment time points (0.5 h, 1 h, 3 h, and 6 h) (Figure 2). The *OsNRAMP7* expression increased immediately in response to the addition of Zn and peaked at 3 h after treatment. Despite the slight decrease, the high expression level of *OsNRAMP7* still remained at 6 h point. These observations initially suggested that *OsNRAMP7* involves in Zn transportation in rice variety TBR225.

Among the *OsNRAMPs* involved in metal transportation in rice, some are strongly expressed under normal conditions, such as *OsNRAMP4* and *OsNRAMP6*, while others are weakly expressed (*OsNRAMP1*, *OsNRAMP2*, and *OsNRAMP5*) or no expression (*OsNRAMP6* and *OsNRAMP7*) [11]. In the current study, *OsNRAMP7* expression was only observed under the Zn treatment condition but not the normal growth condition. The expression of *OsNRAMPs* in response to different metal treatments also varies widely. *OsNRAMP1* strongly expressed in addition of Fe [3]; in contrast, *OsNRAMP2* and *OsNRAMP5* were induced in deficiency of Fe, Cu, Mn, and Zn [4]. Here, the accumulation of *OsNRAMP7* mRNA increased significantly by Zn addition treatment, suggesting that *OsNRAMP7* may have a certain role in the Zn transportation of TBR225 rice.

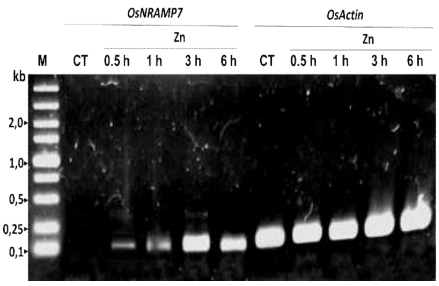

Figure 2. Expression of *OsNRAMP7* in Zn-treated TBR225 rice plants

Note: RT-PCR analysis of OsNRAMP7 and OsActin (internal control) expression in Zn-treated TBR225 rice plants at 0.5 h, 1 h, 3 h, and 6 h post-treatment. (Zn) Zn treated rice samples; (CT): rice samples without Zn treatment; (M) 1.0 kb DNA ladder (Cleaver Scientific).

3.2. Isolation of TBR225 *OsNRAMP7*

To design an *OsNRAMP7*-editing CRISPR/Cas9 system for functional identification of TBR225 *OsNRAMP7* through gene knocking-out, the ExonII-containing region of *OsNRAMP7* was isolated from the total DNA of TBR225. The PCR amplification produced a single DNA band corresponding to approximately 0.52 kb (Figure 3) as the theoretically calculated size of the expected *OsNRAMP7* fragment.

The sequencing analysis showed that the isolated DNA fragment was 99% homology to both Indica rice variety Shuhui498 (CP018168.1) and Japonica rice variety Nipponbare (AP014968.1) published on GenBank (Figure 4). These indicated that *OsNRAMP7* was successfully isolated from TBR225. The complete ExonII region of *OsNRAMP7* with the size of 137 bp was selected from the amplified *OsNRAMP7-TBR* fragment for designing *OsNRAMP7*-edited sgRNA.

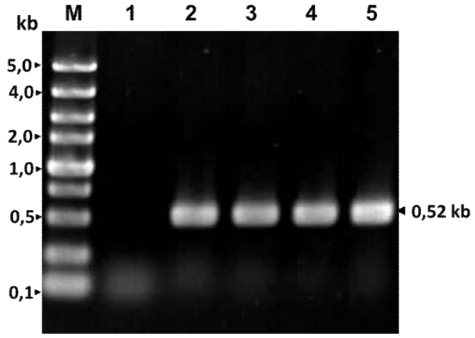

Figure 3. Isolation of *OsNRAMP7-TBR* by PCR

Note: PCR products were electrophoresed on 1% agarose gel. Lane M: 1.0 kb DNA ladder (Cleaver Scientific); lane 1: blank control (no DNA template); lane 2-5: templates were TBR225 genomic DNAs with dilutions of 1: 1, 1: 5, 1: 20 and 1: 50.

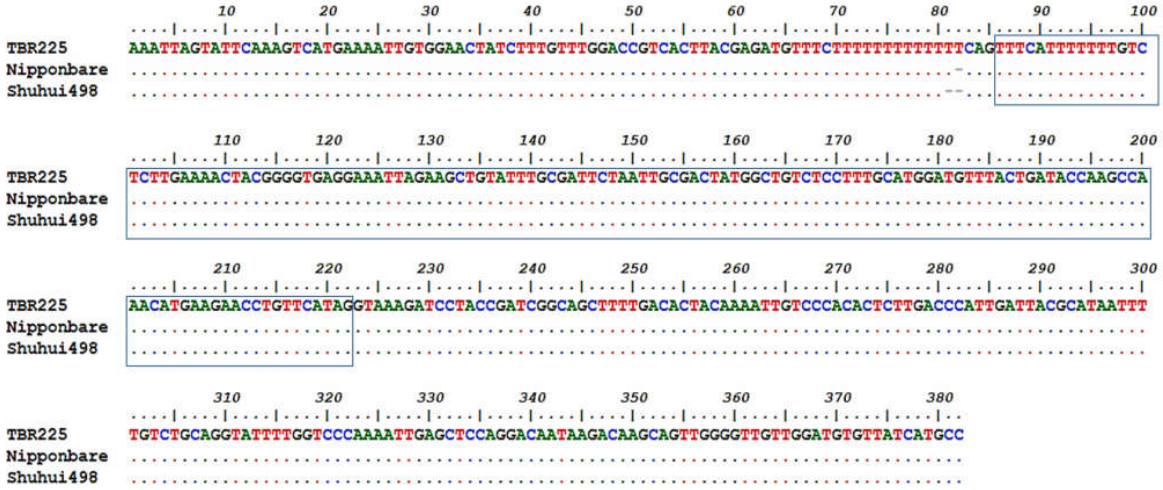

Figure 4. Alignment of *OsNRAMP7* fragments

Note: Dots indicate the similar nucleotides. ExonII regions are showed in the box. (TBR225, Nipponbare, Shuhui498) OsNRAMP7 sequences of TBR225, Nipponbare (AP014968.1) Shuhui498 (CP018168.1) rice varieties.

3.3. Design of *OsNRAMP7*-editing sgRNA sequences

The gene editing CRISPR/Cas9 system works on its ability to precisely cut double-stranded DNA at the desired location of the RNA-protein complex. This complex consists of two main components: (i) the Cas9 protein with endonuclease activity and (ii) the single guide RNA (sgRNA) that guides the complex to the targeted DNA site. The specificity of the CRISPR/Cas9 complex depends on the 17 - 22 nucleotide CRISPR RNA (crRNA) sequence on the sgRNA molecule [12].

In order to ensure the best performance of the sgRNA expression construct and the DNA cutting ability of the CRISPR/Cas9 system, the crRNA sequences must comply with the following conditions: (i) target DNA sequence complementary to crRNA located upstream the protospacer adjacent motif (PAM) sequence (NGG), (ii) 19-22 Nu length, (iii) the DNA cut site is on the target sequence (ExonII of *OsNRAMP7*) [12]. Using CRISPR-P v2.0 software, 11 sgRNAs satisfying the above requirements were determined (Table 1). Furthermore, to maintain the activity and specificity of the Cas9-sgRNA complex in the cell nucleus, in the secondary structure of the sgRNA molecule, three structural loops, RAR, SL2, and SL3, must be remained [13]. The total base pairs (TBP - total base pairs), the number of consecutive base pairs (CBP - consecutive base pairs) and the number of base pairs inside the crRNA structure (IBP - internal base pairs) should be less than 13, 8 and 7, respectively [14]. The content of GC in crRNA sequences ranges from 30-80% [12]. Two sgRNAs (sgRNA-1 and sgRNA-6) (Table 2) satisfying all these requirements and having the highest on-score were selected for the design of a T-DNA vector expressing the TBR *OsNRAMP7*-editing CRISPR/Cas9 system.

Table 1. Characteristics of TBR *OsNRAMP7*-editing sgRNAs

Name	Sequences of crRNA (5'-3')	Size	TSL	GSL	CBP	TBP	IBP	% GC	On-score
sgRNA-1	CTATGGCTGTCTCCTTTGCA	20	3	0	7	11	0	50	0.5766
sgRNA-2	ATGAACAGGTTCTTCATGTT	20	3	0	3	9	0	35	0.1029
sgRNA-3	ATCAGTAAACATCCATGCAA	20	3	0	3	5	0	35	0.0736
sgRNA-4	TGCGATTCTAATTGCGACTA	20	4	1	5	10	5	40	0.0305
sgRNA-5	CTCTTGAAAACTACGGGGTG	20	3	0	5	10	0	50	0.0266
sgRNA-6	TTGTCTCTTGAAAACTACG	19	3	0	3	9	0	40	0.6158
sgRNA-7	GCGATTCTAATTGCGACTA	19	4	1	6	11	5	40	0.3845
sgRNA-8	TCTTGAAAACTACGGGGTG	19	3	0	5	10	0	45	0.1228
sgRNA-9	ACATGAAGAACCTGTTCAT	19	3	0	5	7	0	35	0.1065

sgRNA-10	TGAACAGGTTCTTCATGTT	19	3	0	3	10	0	35	0.1029
sgRNA-11	TCAGTAAACATCCATGCAA	19	3	0	3	5	0	35	0.0736

(TBP) total number of base pairs, (CBP) number of continuous base pairs, (IBP) number of base pairs of the crRNA sequence in the secondary structure of sgRNA, (TSL) total number of loops, (GSL) number of loops containing crRNA; (% GC) content of GC in the crRNA sequence; (On-score): the score predicts the ability to correctly recognize the target location.

The specificity of CRISPR/Cas9 is dependent on the crRNA sequence (~20 Nu); however, Cas9 can still function even if there are a few mismatches between the target DNA sequence and crRNA [7, 12, 15]. Therefore, the specificity of the two engineered sgRNAs was further analyzed using CCTop software. The analysis showed that one and six sequences in the rice genome were homologous to the crRNA sequence of sgRNA-1 and sgRNA-6, respectively. All these homologs locate in the non-coding region of different gene (Table 2); therefore, they will be less likely to affect the biochemical and physiological processes if CRISPR/Cas9-induced off-target mutations occur. Nevertheless, these potential off-target sites still need to be validated (by sequencing analysis) at a later step in screening the gene-edited rice plants to ensure that there will be no unexpected mutations in the genome [6, 12, 13, 14].

Table 2. DNA sequences homologous to *OsNRAMP7*-TBR-editing crRNAs

Name	crRNA homologous sequences	Coordinates	Distance (bp)*	Gene ID
sgRNA-1	C**C**AT**CT**CT[GTCTCCTT**G**GCA]	-	1868	Os04g0207501
sgRNA-6	TT**TCA**TC[TTGAAAA**T**TACG]	-	31040	Os09g0311600
	T**CC**TCTT[TTGAAAA**A**TACG]	-	232	Os03g0111200
	TT**A**TT**A**C[TTGAAAA**T**TACG]	-	1022	Os09g0516300
	T**GT**TC**C**C[TTGAAAAC**A**ACG]	I	261	Os04g0495400
	TTGT**GT**T[TTGAAAACTA**T**G]	-	645	Os04g0565200
	TTGT**GT**T[TTGAAAACTA**T**G]	-	6624	Os11g0472500

The letters in brackets [] represent sequences homologous to the core region (12 nu) of crRNA; bold letters represent positions that are different from the corresponding nucleotide sites on crRNA; (I) intron; (-): neither exon nor intron.

**Distance to nearest Exon in rice genome.*

Additionally, in this study, the *U6* promoter was used in the sgRNA expression construct (Figure 1), from which transcription necessarily starts from the +1 (G) position

[12]. Thus, two pairs of oligonucleotide were synthesized with a 4-nu sequence complementary to the end of the *U6* promoter and tracrRNA sequences, followed by a G nucleotide at the 5' end and designed crRNA sequence for the next vector construction experiment.

3.4. Design of T-DNA construct and CRISPR-mediated TBR *OsNRAMP7* editing

To design an *OsNRAMP7-TBR*-recognized sgRNA expression construct, the *crRNA1-OsNRAMP7* (containing crRNA of sgRNA-1) and *crRNA2-OsNRAMP7* (containing crRNA of sgRNA-6) DNA fragments were inserted into the pENTR4-V2 vector (Figure 1), which was previously cut by *Btg*ZI and *Bsa*I, respectively (Figure 5 A&B, lane 2). The recombinant plasmid was tested by PCR with primer pairs BsaI-crRNA2-F/pENTR4-R, pENTR4-F/BtgZI-crRNA1-R, respectively; the obtained PCR products were approximately 0.44 kb (Figure 5 C, lane 3) and 0.53 kb (Figure 5 C, lane 6) in sizes, consistent the theoretical calculation of the DNA fragments. This result proves that the recombinant vector (pEN/sgRNA-OsNRAMP7) contains two sequences, *crRNA1-OsNRAMP7* and *crRNA2-OsNRAMP7*, at the desired position.

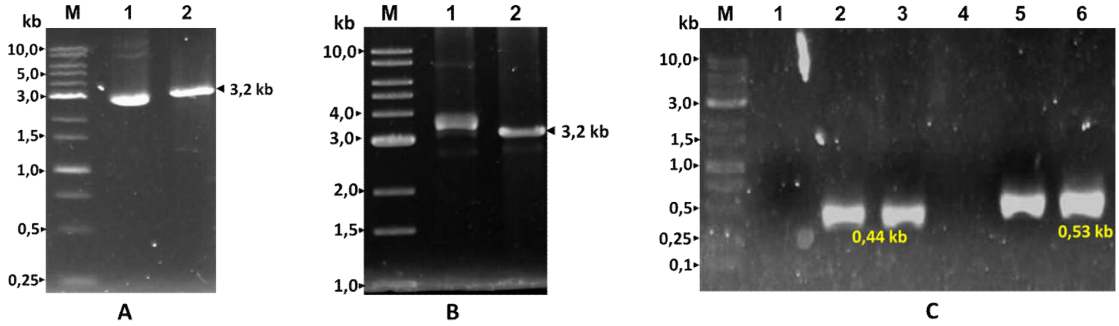

Figure 5. Insertion of *crRNA1-OsNRAMP7* and *crRNA2-OsNRAMP7* into pENTR4-V2

Notes: (A) Digestion of pENTR4-V2 with BtgZI; lane 1: original vector, lane 2: digested vector. (B) Digestion of pENTR4-V2/crRNA1 with BsaI; lane 1: original vector, lane 2: digested vector. (C) Validation of pEN/sgRNA-OsNRAMP7 by PCR; lanes 1-3: primers were BsaI-crRNA2-F/pENTR4-R; lanes 4-6: primers were pENTR4-F/BtgZI-crRNA1-R; lanes 1 & 4: negative control (template was pENTR4-V2); lanes 2 & 5: template was cells transformed ligation products; lanes 3 & 6: template was pEN/sgRNA-OsNRAMP7. Lane M: 1.0 kb DNA ladder (Cleaver Scientific and Thermo Scientific).

Next, to design the plant transformation vector containing the sgRNA expression cassette and the Cas9 gene, sgRNA-OsNRAMP7 from donor vector pEN/sgRNA-OsNRAMP7 was ligated to the destination pCas9 vector using Gateway LR reaction. The recombinant transformation vector was validated by PCR for the presence of sgRNA and *Cas9* expression cassettes with the specific primer pairs (BtgZI-crRNA1-F/BsaI-crRNA2-R, Ubi-F/Cas9-t-R, respectively). The PCR product analysis in Figure 6 (lane 4) shows bands with approximately 0.45 kb (Figure 6, lane 4) and 0.36 kb (Figure 6, lane 8) in size, corresponding with the theoretical size of the amplicons for sgRNA and Cas9. These results revealed that the final vector pCas9/sgRNA-OsNRAMP7 was constructed habouring the complete Cas9 gene (driven by *Ubiquitin* promoter) and TBR *OsNRAMP7*-editing sgRNA expression construct.

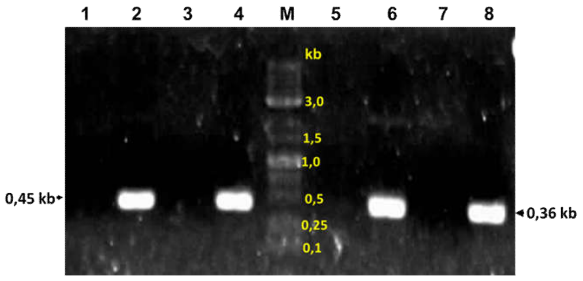

Figure 6. Validation of recombinant vector pCas9/sgRNA-OsNRAMP7

Note: PCR products were electrophoresed on 1% agarose gel. Lanes 1-4: primers was BtgZI-crRNA1-F/BsaI-crRNA2-R; lanes 5-8: primers was Ubi-F/Cas9-t-R; lanes 1 & 5: blank control (no template DNA); lanes 2 & 7: template was pEN/sgRNA-OsNRAMP7; lanes 3 & 6: template was pCas9; lanes 4 and 8: template was pCas9/sgRNA-OsNRAMP7. Lane M: 1.0 kb DNA ladder (Clever Scientifics).

To confirm the expected DNA fragments were correctly inserted into the targeted vector without changes, the recombinant pCas9/sgRNA-OsNRAMP7 vector was sequenced and analyzed using BioEdit 2.0 software (Figure 7). The sequencing analysis showed that two sequences crRNA1-OsNRAMP7 and crRNA2-OsNRAMP7 were accurately inserted between the promoter (*U6.1* and *U6.2*) and the tracrRNA encoding sequence in the sgRNA expressing structure and the complete OsNRAMP7-TBR-editing sgRNA expression construct were ligated into the pCas9 vector.

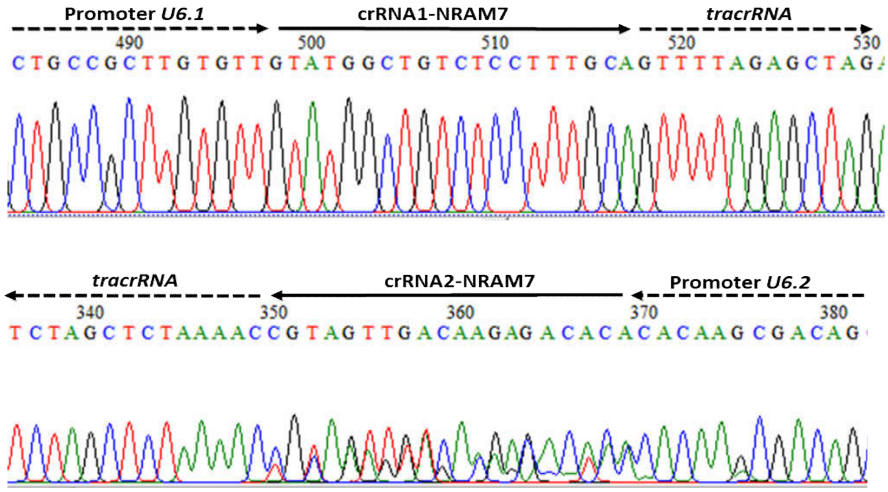

Figure 7. Sequencing pCas9/sgRNA-OsNRAMP7

Note: Part of pCas9/sgRNA-OsNRAMP7 sequencing results. The crRNA sequences are shown by arrows (→), the sequences of promoter U6 and tracrRNA are shown by the broken arrows (⇢).

4. CONCLUSIONS

In this study, the expression of *OsNRAMP7* of rice TBR225 was found to be increased under Zn treatment. Two single guide RNA (sgRNAs) was selected from potential target sites based on exon II sequence of *OsNRAMP7*isolated from rice TBR225 using the bioinformatics tool. The plant transformation vector expressing the OsNRAMP7-TBR editing CRISPR/Cas9 complex was developed for further evaluating *OsNRAMP7* function as Zn or other metal transporters in TBR225 rice variety.

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