DOI: 10.31276/VJSTE.2024.0085

Design of the CRISPR/Cas9-based *OsNRAMP2* editing construct in TBR225 rice (*Oryza sativa* L.) variety

Duy Phuong Nguyen[1], Tran Tuyet Mai Le[2], Thi Thu Huong Phung[1], Anh Minh Nguyen[1], Phuong Ngoc Pham[1], Xuan Hoi Pham[1], Quynh Mai Le[2*]

[1]*Institute of Agricultural Genetics, Vietnam Academy of Agricultural Sciences, Km2 Pham Van Dong Road, Co Nhue Ward, Bac Tu Liem District, Hanoi, Vietnam*
[2]*University of Science, Vietnam National University - Hanoi, 334 Nguyen Trai Street, Thanh Xuan Trung Ward, Thanh Xuan District, Hanoi, Vietnam*

Received 20 August 2024; revised 13 January 2025; accepted 21 February 2025

Abstract:

Micronutrients are indispensable for plant growth and development. In rice, several protein-coding genes of the NRAMP (Natural resistance-associated macrophage protein) family have been identified as being involved in metal accumulation. In this study, a segment of the coding sequence of *OsNRAMP2* was isolated from the total DNA of the rice variety TBR225, one of the most popular varieties in Northern Vietnam. Based on the isolated DNA sequences, two crRNA sequences (CRISPR RNA), capable of binding two specific sites on exon II and exon III of *OsNRAMP2,* were designed using bioinformatics tools. Mutation sites in the early exons are more likely to cause sense changes in the target protein. The crRNAs were ligated to the *Btg*ZI and *Bsa*I sites on the pENTR4-V2 vector; the DNA fragment encoding both sgRNAs was inserted into the pCas9 vector to create a complete CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) editing construct, which may cause the *OsRAMP2* gene mutation in TBR225 rice. This will be the premise for studying the function of *OsNRAMP2* in TBR225.

Keywords: **CRISPR/Cas9, micronutrients, natural resistance-associated macrophage protein, *OsNRAMP2*, TBR225.**

Classification numbers: **3.1, 3.5**

1. Introduction

Iron (Fe) is a micronutrient essential for many cellular processes and various physiological functions in plants [1]. This metal plays an important role in the electron transport chain in both photosynthesis and respiration and in many enzymes. Although abundant in the Earth's crust, iron deficiency is frequent in humans due to the lack of iron absorption by food crops [2]. Slow plant growth and leaf yellowing (chlorosis) caused by iron deficiency are major agricultural problems worldwide [3]. Since excess iron is toxic to plants, the concentration of iron in cells is always very strictly controlled through the maintenance of homeostasis [4]. The natural resistance-associated macrophage protein (NRAMP) is a family of proteins that transport metals in living organisms and play a role in iron storage in plants, including rice. Seven OsNRAMP members have been identified and functionally described, including OsNRAMP1-7. OsNRAMP1 is involved in Fe transport and cadmium (Cd) accumulation. OsNRAMP4 (or NRAT1) is described as a trivalent aluminium transporter, unlike the other members that transport divalent ions. There have been many studies paying attention to the function of OsNRAMP5, which relates mainly to the absorption of Fe, Cd, and manganese (Mn) from the soil. Besides, OsNRAMP6 participates in the absorption of Mn, Fe, and disease resistance in rice. OsNRAMP7 is a major transporter in root cells involved in Fe, zinc (Zn), Mn, and Cd uptake [5].

OsNRAMP2 is a member of the NRAMP family; however, its function is not as well understood as other members. There have been studies showing that *OsNRAMP2* encodes proteins homologous to AtNRAMP3 and AtNRAMP4 [6]. Meanwhile, AtNRAMP3 and AtNRAMP4 are found on the vacuole membrane and have been shown to play a role in Fe and Cd transport. In iron-deficient environments, these two proteins participate in vacuole iron mobilisation, which is necessary for seed germination [7]. The role of OsNRAMP2 has been investigated and shown to be able to influence OsVIT1 expression to help maintain Fe homeostasis between the vacuole and cytoplasm of rice. Besides, OsNRAMP2 and OsIRT1 participate in the Fe transport process, affecting the germination of rice seeds [6]. Therefore, the mutation in the *OsNRAMP2* gene may likely reduce Fe accumulation in vegetative organs while

*Corresponding author: Email: lequynhmai80@vnu.edu.vn

CC BY NC ND
Crossref Similarity Check Powered by iThenticate

increasing its translocation to grains. CRISPR/Cas9 was applied in gene editing to create mutations in target genes by the organisms themselves. The CRISPR/Cas9 complex consists of two components: single-guide RNA (sgRNA) and Cas9 nuclease, forming an endonuclease-RNA complex capable of cutting DNA precisely at the location specified by the sequence in sgRNA. This study was carried out on the rice variety TBR225, one of the commonly grown rice varieties in the North of Vietnam, to design structures that express the *OsNRAMP2* gene-edited RNA-protein Cas9 complex, thereby generating transgenic rice lines to characterise the function of OsNRAMP2 on micronutrient accumulation. The research results are the premise for creating rice plants with high levels of micronutrients, increased iron content, etc., to meet the needs for rice quality improvement in some key rice varieties in Vietnam.

2. Materials and methods

2.1. Materials

Rice variety TBR225 (*Oryza sativa* L. ssp. *indica*) was obtained from Thai Binh Seed Group Joint Stock Company and *Escherichia coli* DH5α and DB3.1 were purchased from Thermo Scientific (USA). Vectors pENTR4-V2 and pCas9 were provided by the research team of Dr. Sebastien Cunnac (Research Institute for Development, Montpellier, France), designed based on the framework of pCAMBIA1300 (Marker Gene Technologies, USA) and pENTR4 (Invitrogen, USA). The oligonucleotides used for polymerase chain reaction (PCR) were ordered from Sigma (USA).

2.2. Isolation of the OsNRAMP2 gene

Total plant DNA was extracted using the method described by J.J. Doyle, et al. (1987) [8] with a 2% CTAB (Cetyltrimethylammonium Bromide) buffer. All DNA samples were stored at -20°C for use in experiments.

The *OsNRAMP2* fragment (AP014959.1) was amplified by polymerase chain reaction (PCR) using the forward primer 5'-TTTCCTGTCTGTTTCTGCTGT-3' and reverse primer 5'-ACTGTCCAGCATTCTCAAGACC-3' from genomic DNA of TBR225 rice plants. The PCR mixture consisted of 0.5 µl template DNA, 0.6 µl each of 10 pmol/ml primer, 1.5 µl 2 mM dNTP and 1.5 µl Taq DNA polymerase 10X buffer, 0.5 µl Taq DNA polymerase, and 9.8 µl distilled deionised water. PCR was performed in 35 cycles: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s. PCR products were purified using the GenJET™ Gel Extraction kit (Thermo Fisher Scientific) and nucleotides were sequenced by Macrogen (Korea). BioEdit 4.0 software was used to process sequencing results. After processing, DNA sequences were compared with GenBank database and analysed using BLAST software (https://blast.ncbi. nlm.nih.gov/Blast.cgi).

2.3. sgRNA design

The *OsNRAMP2-TBR* edit-specific sgRNA sequence was determined using CRISPR-P v2.0 software (http://crispr.hzau.edu.cn/CRISPR2/). The GC content of the sgRNAs was also analysed using CRISPR-P v2.0 software. The secondary structure of the sgRNAs was analysed using Mfold 2.3 software (http://www.unafold.org/) and CRISPR-P v2.0 software. The specificity of the crRNA sequences (on/off-target) of the Cas9/sgRNA complex was analysed using the CRISPR tool in CRISPR-P v2.0 software. The DNA sequences in the rice genome homologous to the designed crRNA were determined using CCTop software (https://cctop.cos.uni-heidelberg.de:8043/). The rice genome sequences were referenced from the Ensembl Plants database. The specific sgRNA fragment was selected based on the GC content of the crRNA sequence, the ability to form and maintain the secondary structure, and the number, location, and nucleotide sequence characteristics of the crRNA homologous DNA fragment that occur in the reference genome [9].

2.4. Design of T-DNA carrying a polygenic edited sgRNA expression construct

Two oligonucleotide pairs BtgZI-crRNA1 (forward: 5'-TGTT[GTTTGGTGAAACAAAGCCAAG]-3', reverse: 5'- AAAC[CTTGGCTTTGTTTCACCAAAC]-3') and BsaI-crRNA2 (forward: 5'-GTGT[GTATGGATCTGAAC AAGCTGA]-3', reverse: 5'-AAAC[TCAGCTTGTTCAGA TCCATAC]-3') were linked together by DNA denaturation/renaturation (95°C - 10 min, 4°C - ∞) to form two complete double-stranded crRNAs (*crRNA1-OsNRAMP2* and *crRNA2-OsNRAMP2*). *crRNA1-OsNRAMP2* and *crRNA2-OsNRAMP2* were inserted at the *Btg*ZI and *Bsa*I sites located after the promoter regions, *U6.1* and *U6.2,* on the vector pENTR4-V2, respectively (Fig. 1). The restriction reaction mixture included: 17 µl plasmid (50-100 ng/µl), 2.0 µl FastDigest Green 10X buffer, 1.0 µl enzyme, and was left at 37°C for 1 hour for the reaction to occur. The digested product was checked by electrophoresis on a 1% agarose gel. The GenJET™ Gel Extraction kit (Thermo Fisher Scientific) was used to purify PCR products from agarose gels. The ligation reaction mixture (10 µl) contained: 0.5 ng of DNA product, 25 ng of purified linear vector, 1.0 µl of 10X T4 DNA ligase buffer, 0.5 µl of T4 DNA ligase enzyme (5 U/µl), 0.5 µl of 10 mM ATP, and the remainder deionised distilled water. The DNA ligation reaction mixture was incubated at 22°C for 30 minutes, then at 4°C overnight. The recombinant vector was checked by PCR using the primer pair BtgZI-crRNA1-F/BsaI-crRNA2-R (0.45 kb). Next, the sequence containing two constructs *[U6.1::crRNA1-OsNRAMP2]* and *[U6.2::crRNA2-OsNRAMP2]* (0.45 kb) was inserted into the binary vector pCas9 using the Gateway kit (Invitrogen). The recombinant pCas9/sgRNA-*OsNRAMP2* vector was examined by PCR

with primer pairs BtgZI-crRNA1-F/BsaI-crRNA2-R (0.45 kb), Ubi-F (5’-CCCTGCCTTCATACGCTATT-3’) /Cas9-t-R (5’-GCCTCGGCTGTCTCGCCA-3’) (0.36 kb) and by DNA sequencing.

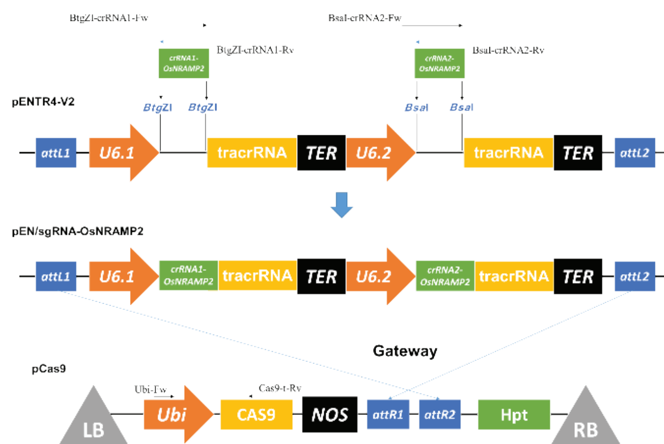

Fig. 1. Design diagram of *pCas9/sgRNA-OsNRAMP2* expression vector. *U6.1; U6.2*: OsU6 promoter; TER: Terminator; Ubi: ZmUbiquitin promoter; NOS: nopaline synthase terminator; LB: Left border; RB: Right border; Hpt: Hygromycin phosphotransferase gene; attL1/attL2; attR1/attR2: Recognition sites of Gateway LR clonase.

3. Results and discussion

3.1. Isolation of the OsNRAMP2 gene of TBR225

The total DNA from TBR225 was used as a template for polymerase chain reaction cloning the regions containing exon II and exon III of *OsNRAMP2* with the specific primer pair NRAMP2-F/NRAMP2-R. The PCR results obtained showed a single DNA band with a size of about 0.68 kb (Fig. 2), corresponding to the theoretical size of the sequence to be isolated.

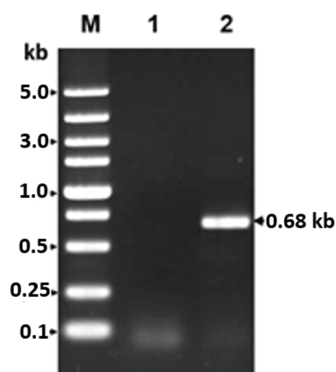

Fig. 2. Results of isolation of *OsNRAMP2* from TBR225 by polymerase chain reaction technique. M: 1 kb DNA standard scale (iNtRON); 1: Negative control (no template DNA); 2: Template is the total DNA of TBR225.

The isolated sequences, when compared using the BLAST tool on NCBI, showed query coverage from 74 to 97% with the top five significant alignments in the database, and the percent identity was over 99%. The results of nucleotide sequencing indicated that the isolated gene fragment was 100 and 99% similar to the corresponding sequences of the rice varieties *Indica* Shuhui498 (CP018159.1) and *Japonica* Nipponbare (AP014959.1), which were published in GenBank. The isolated *OsNRAMP2-TBR* gene fragment contained the full exon II coding region and part of the *OsNRAMP2* exon III sequence (Fig. 3). This sequence region was selected for use in the *OsNRAMP2*-editing sgRNA design experiment. Consequently, the vector constructs will be capable of editing the gene not only in TBR225 but also in other varieties.

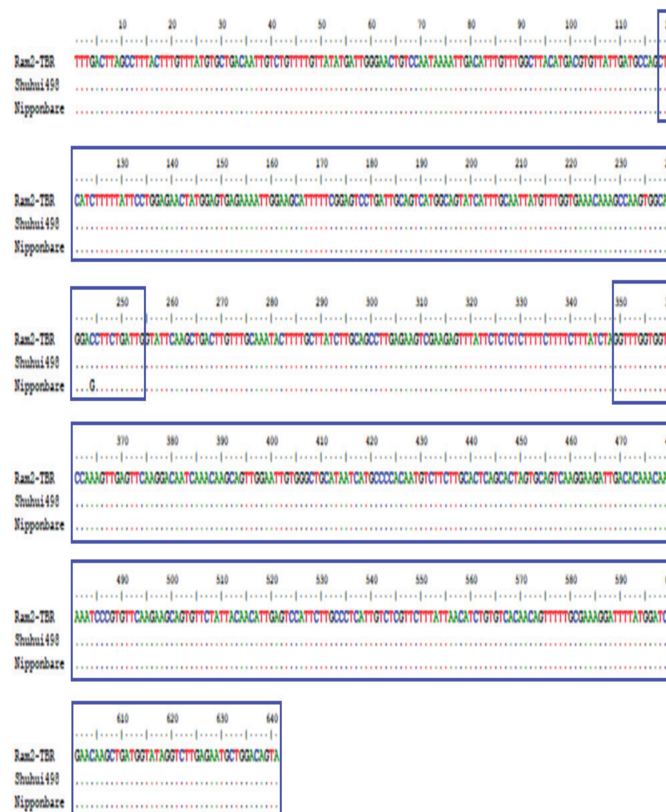

Fig. 3. *OsNRAMP2* sequence comparison. The *OsNRAMP2* region sequences of the rice varieties TBR225, Shuhui498 (CP018159.1), and Nipponbare (AP014959.1) were compared. Exon II and exon III sequences are framed in blue.

3.2. Design of sgRNA sequences editing OsNRAMP2-TBR

The ability of the CRISPR/Cas9 system to precisely cut double-stranded DNA at the desired location is based on the activity of the CRISPR/Cas9 protein/RNA complex, which includes two main components: (1) the Cas9 protein, which has endonuclease activity, and (2) sgRNA molecules

that guide the complex to the correct DNA site to be cut. In particular, the specificity of the CRISPR/Cas9 complex is determined by an approximately 20-nucleotide sequence (crRNA) on the sgRNA molecule [9]. To optimise the expression of the crRNA structure controlled by the U6 promoter and ensure the DNA-cutting ability of the Cas9-sgRNA complex, the crRNA sequences must comply with the following conditions: (1) the 5'-end starts with G (or either complements or replaces the G), (2) the 3'-end must be serial with the conserved sequence PAM (NGG), (3) has a length of 19-22 nucleotides, and (4) the DNA cleavage site on the target sequence. CRISPR-P v2.0 software was used in this study to design *OsNRAMP2* gene-editing crRNA sequences of TBR225 that satisfy the above requirements and have an on-score (predictability of target location recognition) >0.1. Next, to ensure the ability to maintain the activity and specificity of the CAS9-sgRNA complex in the cell nucleus, the sgRNA must have a GC content (%GC) in the sequence of 30-80%, be capable of forming stable secondary structures (retains loop structure RAR, SL2, and SL3), total base pairs (TBP) <13, number of consecutive base pairs (CBP) <8, number of base pairs inside the crRNA structure (IBP) <7. From the above characteristics, the two crRNAs with the highest specificity, crRNA-1 and crRNA-11, were selected for the next step (Table 1).

Table 1. *OsNRAMP2*-edited crRNA sequence.

crRNA	crRNA-PAM sequence (5'-3')	Restriction site	TBP	CBP	IBP	%GC	On score
crRNA-1	GTTTGGTGAAACAAAGCCAAGTGG	Exon II	10	6	0	40%	0.4609
crRNA-2	GATACTGCCATGACTGCAATCAGG	Exon II	13	6	0	45%	0.3452
crRNA-3	GTGAAACAAAGCCAAGTGGCAAGG	Exon II	9	6	3	50%	0.1130
crRNA-4	AGAACTATGGAGTGAGAAAATTGG	Exon II	10	5	0	35%	0.1116
crRNA-5	AATCAGAAGGTCCTTGCCACTTGG	Exon II	7	3	0	50%	0.0927
crRNA-6	TCGGAGTCCTGATTGCAGTCATGG	Exon II	9	4	0	55%	0.0594
crRNA-7	AGTATCATTTGCAATTATGTTTGG	Exon II	4	3	3	25%	0.0516
crRNA-8	TTTCTCACTCCATAGTTCTCCAGG	Exon II	11	7	0	40%	0.0364
crRNA-9	CTTTTTATTCCTGGAGAACTATGG	Exon II	6	4	0	35%	0.0238
crRNA-10	GAGAAAATTGGAAGCATTTTTCGG	Exon II	14	6	0	30%	0.0155
crRNA-11	TTATGGATCTGAACAAGCTGATGG	Exon III	11	5	0	40%	0.6840
crRNA-12	AGAACACTGCTTCTTGAACACGGG	Exon III	14	10	0	40%	0.3713
crRNA-13	TAGAACACTGCTTCTTGAACACGG	Exon III	7	4	0	40%	0.2793
crRNA-14	TGAGTGCAAGAAGACATTGTGGGG	Exon III	8	3	0	40%	0.2756
crRNA-15	GATTGTCCTTGAACTCAACTTTGG	Exon III	16	9	0	40%	0.2534
crRNA-16	AAGGACAATCAAACAAGCAGTTGG	Exon III	7	4	0	40%	0.2533
crRNA-17	ACGAGACAATGAGGGCAAGAATGG	Exon III	13	8	0	50%	0.1436
crRNA-18	CTGAGTGCAAGAAGACATTGTGGG	Exon III	9	7	0	45%	0.1407
crRNA-19	TAATAAAGAACGAGACAATGAGGG	Exon III	9	4	0	30%	0.1375
crRNA-20	TGTCACAACAGTTTTTGCGAAAGG	Exon III	10	5	0	40%	0.1313

Besides the advantages of simplicity, convenience, and broad applicability, a disadvantage of CRISPR/Cas9 gene editing compared with other gene editing systems (TALENs, ZFNs, etc.) is specificity, due to crRNA being capable of recognising only about 20 nucleotides and Cas9 still functioning even if the target DNA sequence has several nucleotides different from the crRNA [9, 10]. Mispairing and miscutting that cause mutations in genes other than the target gene can lead to unwanted traits in the gene-edited line. To minimise the possibility of off-targeting of the Cas9/sgRNA complex, the sequences of the crRNAs were further analysed using CCTop software (Table 2). The analysis results showed that both crRNA-1 and crRNA-11 sequences have only one homologous sequence and are not located in the coding region (exon) of the functional gene (Table 2). These mutation sites have little effect on physiological and biochemical processes in gene-edited plants. However, at the mutation screening step in the next generation of gene-edited plants, these crRNA homologous sites need to be checked to ensure that no unwanted mutations occur [11].

Table 2. DNA sequences homologous to crRNA in the rice genome.

crRNA	Homologous DNA sequences	Location	Distance*	Targeted gene ID
crRNA-1	**T**TTTGGT**A**A[AACAAAG**A**CAAG]	-	1390	Os03g0680300
crRNA-11	**G**ATGGAT**A**[T**T**AACAAGCTGA]	-	235	Os10g0578050
	T**T**TGGA**A**C[TGAA**G**AAGCTGA]	-	NA	NA
	TAT**TT**ATC[TGAAC**C**AGCTGA]	-	490	Os06g0472300

*: Distance to the nearest exon in the rice genome; letters in [ ] represent sequence homology to the core region (12 Nu) of crRNA; bold letters represent positions that are different from the corresponding Nu sites on crRNA; (-): Neither exon nor intron.

Thus, the synthesised structural and specificity prediction analyses determined that crRNA-1 and crRNA-11 (replacing the T-terminus with the G-terminus) could be used for *OsNRAMP2-TBR* mutagenesis by non-homologous end joining (NHEJ) [10-13] using the CRISPR/Cas9 gene editing system [14, 15]. Ultimately, crRNA-1 and crRNA-11 were chosen due to their higher specificity and lower off-target risk.

3.3. Design of T-DNA carrying the OsNRAM2-TBR-editing sgRNA expression construct

To design the *OsNRAMP2-TBR*-recognised sgRNA expression construct, the pENTR4-V2 system carrying the sgRNA expression construct was treated with BtgZI and BsaI (Figs. 4A, 4B) before insertion of *crRNA1-OsNRAMP2* and *crRNA2-OsNRAMP2*, carrying the crRNA-1 and crRNA-11 sequences, respectively. The

recombinant plasmid was examined by PCR with the specific primers BtgZI-crRNA1-F/BsaI-crRNA2-R. The results of electrophoresis showed the appearance of a single band approximately 0.45 kb in size (Fig. 4C), which is consistent with the theoretically calculated size of the DNA fragments. This proves that the obtained recombinant vector (referred to as pEN/sgRNA-OsNRAMP2) contains the two sequences *crRNA1-OsNRAMP2* and *crRNA2-OsNRAMP2* at the desired position.

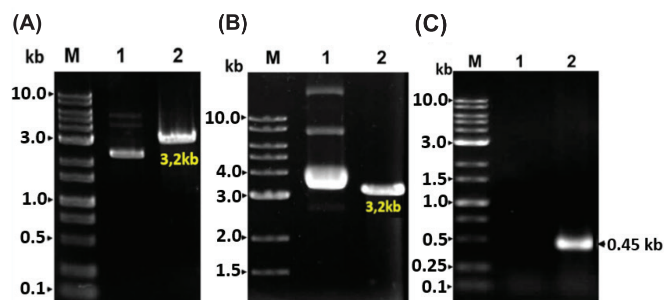

Fig. 4. Coupling *crRNA1-OsNRAMP2* and *crRNA2-OsNRAMP2* into the expression construct on pENTR4-V2. Restriction of pENTR4/gRNA by *BtgZ*I **(A)** and *Bsa*I **(B)**; 1: Original vector, 2: Restriction product. **(C)** pEN/sgRNA-*OsNRAMP2* by polymerase chain reaction with primer BtgZI-crRNA1-F/BsaI-crRNA2-R. 1: Negative control (no template DNA); 2: Template is pEN/sgRNA-*OsNRAMP2*. M: 1 kb DNA ladder (iNtRON).

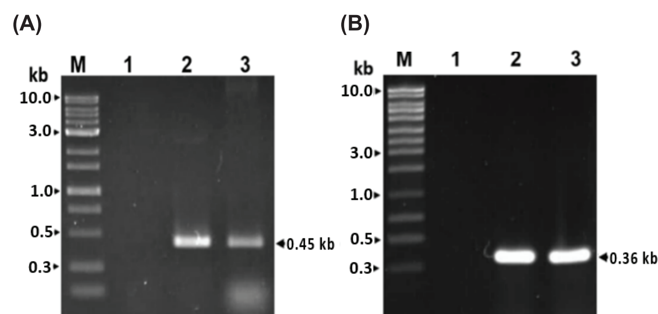

Fig. 5. Testing of pCas9/sgRNA-*OsNRAMP2* by polymerase chain reaction. (A) PCR with primers BtgZI-crRNA1-F/BsaI-crRNA2-R; **(B)** PCR with primers Ubi-F/Cas9-t-R. 1: Negative control (no template DNA), 2: Template is pCas9/sgRNA-*OsNRAMP2* vector, 3A: Template is pEN/sgRNA-*OsNRAMP2* vector, 3B: Template is pCAS9 vector.

The DNA fragment carrying two gRNA expression constructs (*[U6.1::crRNA1-OsNRAMP2]* and *[U6.2::crRNA2-OsNRAMP2]*) was coupled to the vector and pCas9 via the Gateway reaction. The recombinant plasmid was extracted from the positive transformation and checked for the presence of constructs expressing two gRNA by PCR. The results show that the PCR product bands with primers BtgZI-crRNA1-F/BsaI-crRNA2-R (specific for the two *OsNRAMP2-TBR*-editing crRNA sequences) and Ubi-F/Cas9-t-R (specific for the structure *[Ubiquitin::Cas9]*) have sizes of 0.45 and 0.36 kb, respectively (Figs. 5A, 5B), which match the theoretically calculated sizes. This result provides initial confirmation that the pCas9/sgRNA-*OsNRAMP2* vector carries the full Cas9 expression structure (controlled by the Ubiquitin promoter) and the successfully designed *OsNRAMP2-TBR* editing complex expression structure.

The pCas9/sgRNA-*OsNRAMP2* recombinant vector was sequenced and analysed using BioEdit 2.0 software (Fig. 6) to confirm that the DNA fragments were correctly inserted into the target vector and that no mutation occurred during the pairing process. The results of nucleotide sequence analysis showed that the obtained vector carried the two sequences *crRNA1-OsNRAMP2* and *crRNA2-OsNRAMP2* editing *OsNRAMP2-TBR*, located precisely between the promoters (*U6.1* and *U6.2*) and *tracrRNA* in the sgRNA expression construct. The complete *OsNRAMP2-TBR* edited sgRNA expression construct was recombined into the pCas9 vector framework. A recent study used CRISPR/Cas9 technology to inactivate the *OsNRAMP2* gene, designing two sgRNAs and successfully evaluating the reduction of Cd accumulation in grains of mutant rice lines [16]. The study showed that *OsNRAMP2* affects the content of Zn, Mn, and Fe minerals in different tissues, and also influences some yield traits [16]. The designed editing construct in our study can serve as material for further research to evaluate those effects in Vietnamese TBR225 rice.

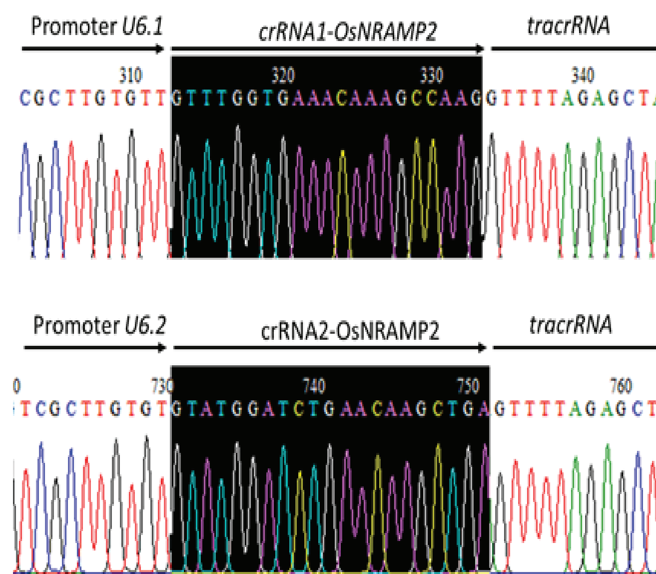

Fig. 6. Sequencing pCas9/sgRNA-*OsNRAMP2*. Part of the pCas9/sgRNA-*OsNRAMP2* sequencing results. The crRNA sequences are blacked out. The crRNA, part of the promoter *U6.1*, *U6.2*, and *tracrRNA* are shown by arrows (→).

4. Conclusions

The 680 bp *OsNRAMP2* gene fragment isolated from TBR225 rice shares 99 and 100% identity with the published sequences of Nipponbare (AP014959.1) and Shuhui498 (CP018159.1), respectively. Two *OsNRAMP2-TBR* editing sgRNAs were designed using a bioinformatics tool. The plant transgenic vector pCas9/sgRNA-*OsNRAMP2*, expressing the *OsNRAMP2-TBR* editing CRISPR/Cas9 complex, was successfully created in this study for further research on the *OsNRAMP2* function and improving the quality of the TBR225 rice variety.

CRediT author statement

Duy Phuong Nguyen: Data curation, Formal analysis, Visualisation, Validation, Writing original draft; Tran Tuyet Mai Le: Methodology, Formal analysis, English editing; Thi Thu Huong Phung: Methodology, Data curation; Anh Minh Nguyen: Methodology, Software; Phuong Ngoc Pham: Formal analysis; Xuan Hoi Pham: Supervision, Conceptualisation; Quynh Mai Le: Investigation, Project administration, Funding acquisition, Supervision, Conceptualisation, Data curation, Writing - Reviewing and Editing.

ACKNOWLEDGEMENTS

We thank all material donours for contributing to this research. This research is funded by the Vietnam Ministry of Science and Technology under grant number NDT/CN/21/24 for the project "Research on micronutrient biofortification in rice using biotechnology".

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] Shumayla, S.K. Upadhyay (2022), "Mechanism of iron uptake and homeostasis in plants", *Cation Transporters in Plants*, **9**, pp.147-165, DOI: 10.1016/B978-0-323-85790-1.00015-4.

[2] M. Takahashi, Y. Terada, I. Nakai, et al. (2003), "Role of nicotinamine in the intracellular delivery of metals and plant reproductive development", *Plant Cell*, **15(6)**, pp.1263-1280, DOI: 10.1105/tpc.010256.

[3] L. Gao, J.D. Chang, R.J. Chen, et al. (2016), "Comparison on cellular mechanisms of iron and cadmium accumulation in rice: Prospects for cultivating Fe-rich but Cd-free rice", *Rice*, **9(39)**, DOI: 10.1186/s12284-016-0112-7.

[4] G.Z. Akmakjian, N. Riaz, M.L. Guerinot (2021), "Photoprotection during iron deficiency is mediated by the bHLH transcription factors PYE and ILR3", *Proceedings of The National Academy of Sciences*, **118(40)**, 11pp, DOI: 10.1073/pnas.2024918118.

[5] M. Naz, Z. Dai, M. Tariq (2022), "Role of NRAMP transporters for Fe, mineral uptake, and accumulation in rice and other plants", *Metals Metalloids Soil Plant Water Systems*, pp.331-348, DOI: 10.1016/B978-0-323-91675-2.00004-4.

[6] Y. Li, J. Li, Y. Yu, et al. (2021), "The tonoplast-localized transporter OsNRAMP2 is involved in iron homeostasis and affects seed germination in rice", *Journal of Experimental Botany*, **72(3)**, pp.4839-4852, DOI: 10.1093/jxb/erab159.

[7] V. Lanquar, F. Lelievre, S. Bolte (2005), "Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron", *The EMBO Journal*, **24(23)**, pp.4041-4051, DOI: 10.1038/sj.emboj.7600864.

[8] J.J. Doyle, J.L. Doyle (1987), "A rapid DNA isolation procedure for small quantities of fresh leaf tissue", *Phytochemical Bulletin*, **19**, pp.11-15.

[9] G. Liang, H. Zhang, D. Lou, et al. (2016), "Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing", *Scientific Reports*, **6**, 8pp, DOI: 10.1038/srep21451.

[10] X. Liu, S. Wu, J. Xu, et al. (2017), "Application of CRISPR/Cas9 in plant biology", *Acta Pharmaceutica Sinica B*, **7(3)**, pp.292-302, DOI: 10.1016/j.apsb.2017.01.002.

[11] J.G. Doench, N. Fusi, M. Sullender, et al. (2016), "Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9", *Nature Biotechnology*, **34(2)**, pp.184-191, DOI: 10.1038/nbt.3437.

[12] B. Shen, W. Zhang, J. Zhang, et al. (2014), "Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects", *Nature Methods*, **11(4)**, pp.399-402, DOI: 10.1038/nmeth.2857.

[13] L.M. Smith, J.Z. Sanders, R.J. Kaiser, et al. (1986), "Fluorescence detection in automated DNA sequence analysis", *Nature*, **321(6071)**, pp.674-679, DOI: 10.1038/321674a0.

[14] L. Bortesi, R. Fischer (2015), "The CRISPR/Cas9 system for plant genome editing and beyond", *Biotechnology Advances*, **33(1)**, pp.41-52, DOI: 10.1016/j.biotechadv.2014.12.006.

[15] X. Ma, Q. Zhang, Q. Zhu, et al. (2015), "A robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants", *Molecular Plant*, **8(8)**, pp.1274-1284, DOI: 10.1016/j.molp.2015.04.007.

[16] W. Yang, L. Chen, Y. Ma, et al. (2023), "*OsNRAMP2* facilitates Cd efflux from vacuoles and contributes to the difference in grain Cd accumulation between *japonica* and *indica* rice", *The Crop Journal*, **11(2)**, pp.417-426, DOI: 10.1016/j.cj.2022.09.013.