Vietnam Journal of Biotechnology **22**(3): 437-449, 2024. DOI: 10.15625/vjbt-19559

CONSTRUCTION OF CRISPR/Cas9 VECTOR FOR EDITING *IaVQ9* GENE IN *Ipomoea Aquatica*

Huynh Thi Thu Hue[1,2]✉, Nguyen Thi Bich Ngoc[1], Le Tat Thanh[1,2], Nguyen Thi Hoa[3], Le Minh Tri[3], Le Thi Bich Thuy[4]

[1]*Institute of Genome Research, Vietnam Academy of Science and Technology, Hanoi, Vietnam.*
[2]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.*

[3]*Academy of Military Science and Technology, Hanoi, Vietnam.*

[4]*Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam.*

✉To whom correspondence should be addressed. E-mail: hthue@igr.ac.vn

Received: 04.12.2023
Accepted: 06.09.2024

ABSTRACT

The group of proteins containing the VQ motif (named VQ proteins) is a family of plant-specific proteins with a FxxhVQxhTG conservative VQ-motif region. VQ proteins regulate many developmental processes, including responses to biotic and abiotic stresses, and seed development. The VQ9 protein has an interaction with the WRKY8 factor, when this interaction occurs, it causes a decrease in the DNA binding ability of WRKY8 to DNA, which plays a role in the regulation function of the plant to stress. Some mutations in the *VQ9* gene increase salt tolerance in plants, suggesting that *VQ9* acts antagonistically to regulate responses to salt conditions. This antagonism is consistent with an increase or decrease in the Na+/K+ ratio. *Ipomoea aquatica* is commonly grown and used as a vegetable in Southeast Asia. The research involved RNA extraction from *I. aquatica* leaves, followed by PCR sequencing to confirm the presence of the *IaVQ9* gene. Subsequently, a specific guide RNA (gRNA) was designed using CRISPR-P ver.2.0 and inserted into the pRGEB31 vector, optimized for CRISPR/Cas9 applications. The gRNA-inserted vector was successfully transformed into *E. coli* DH10B and then into *Agrobacterium tumefaciens* EHA105, verified through colony PCR and restriction enzyme analysis. This process created a delivery system capable of editing the *VQ9* gene in *I. aquatica*. This research represents a significant step towards improving crop resilience to salinity, addressing a critical challenge for agriculture in salt-affected regions. Future studies will focus on transferring the construct back into *I. aquatica* plants to assess its impact on enhancing salt tolerance, potentially contributing to sustainable crop production in adverse environmental conditions.

Keywords: *Ipomoea aquatica*, gRNA, CRISPR/Cas9, VQ9, salt tolerance

INTRODUCTION

The group of proteins containing the VQ motif (named VQ proteins) is a family of plant-specific proteins with a FxxhVQxhTG conserved VQ-motif region. VQ proteins regulate many developmental processes, including responses to biotic and abiotic stresses, and seed development (Buscaill P, 2014). The VQ9 protein has an interaction with the WRKY8 factor, and this interaction causes a decrease in the ability of WRKY8 to bind DNA. The physical interaction between VQ9 and WRKY8, which overlaps with the DNA-binding domain of WRKY8, was found to diminish WRKY8's capacity to bind to W-box sequences (Hu *et al*., 2013; Tian *et al*., 2024). This was shown through electrophoretic mobility shift assays (EMSA), where the presence of VQ9 caused the complexes formed by WRKY8 and W-box DNA to exhibit reduced migration. As VQ9 increased, WRKY8's DNA-binding activity was further inhibited, demonstrating that the WRKY8-VQ9 interaction directly affects WRKY8's role in salt-stress response pathways by decreasing its DNA-binding activity. The VQ9 protein was determined to be present in the nucleus and expressed strongly when *Arabidopsis* plants were treated with saline conditions. Some mutations in the *VQ9* gene increase salt tolerance in plants, suggesting that *VQ9* acts antagonistically to regulate responses to salt conditions. This antagonism is consistent with an increase or decrease in the Na+/K+ ratio (Hu *et al*., 2013).

WRKY is the largest of the transcription factor family, which is found in many species, especially in higher plants (Ulber and Somssich, 2004). In plants, the WRKYs have long been known to participate in responses to biotic and abiotic stresses (Dong *et al*., 2003; Hu *et al*., 2012). In Arabidopsis, WRKY8 transcription factor is expressed mainly in roots and increases strongly under saline conditions. Under saline conditions, WRKY8 directly binds to the W-box on the RD29A promoter (a *RD29A* gene is related to abiotic stress resistance). Removing WRKY8 makes the plant susceptible to salt stress, slow germination, and leaf yellowing.

In recent years, by genome-wide analysis, the *VQ* family has been identified in many species, such as *Arabidopsis*, which includes 34 genes (Hu *et al*., 2013), *Eucalyptus grandis* includes 27 genes, in which *EgrVQ5* and *EgrVQ9* are homologous to *AtVQ9* and also tend to respond to salt stress (Yan *et al*., 2019). In each of the 4 species of the genus *Gossypium*, there are over 40 *VQ* genes per species (Chen *et al*., 2020); in maize, 61 *VQ* genes have been discovered and show that many genes respond to drought and salt stress, such as *ZmVQ1*, *ZmVQ11*, *ZmVQ25*, *ZmVQ37*, *ZmVQ51*, and *ZmVQ52*. These genes all respond to the factor *ZmWRKY* transcription (Song *et al*., 2016).

Over the years, the CRISPR/Cas9 system has been used for gene editing in many organisms (Jiang *et al*., 2013). Bacteria have a restriction enzyme known as Cas that destroys the DNA of viruses that infect them. This enzyme is guided by a short RNA molecule, called a guide gRNA, that recognizes a specific sequence for cutting (Char *et al*., 2016). The Cas9 enzyme, an endonuclease isolated from *Streptococcus pyogenes*, combines with a guide RNA (gRNA) molecule and destroys foreign DNA (Cong *et al*., 2013). To manipulate plants, the genes encoding Cas9 and gRNA have been modified for expression in plant cells (Pan *et al*., 2016). The CRISPR/Cas9 system allows scientists to design gRNAs that

match the target site, thereby directing the Cas9 enzyme to create a cutting point at a specific location on the gene. Gene editing technology using the CRISPR/Cas9 system has been applied by many research groups to create new crop varieties such as corn, tomato and rice (Shi *et al*., 2017, Wang *et al*., 2017; Zhang *et al*., 2019).

In genome editing on plants, the Cas9 expression structure has undergone many changes to suit expression. Specifically, sgRNA sequences of about 98 nucleotides are often expressed under the control of U3 or U6 RNA promoters (Jiang *et al*., 2013). Both sgRNA expression constructs are usually small in size (300-600 bp) (Kadkhodaei *et al*., 2016). These cassettes can be directly attached to the gene transfer vector T-DNA region via Golden Gate or Gibson PCR techniques (Shan *et al*., 2013; Fan *et al*., 2015). In order to increase expression efficiency in plants, the codons on the Cas9 gene originating from *Streptococcus pyogenes* have been modified to optimize in plants (Jiang *et al*., 2013; Ma *et al*., 2015). The *Cas9* gene coding sequence is often expressed under strong promoters such as CaMV35S or Ubiquitin from maize, which can enhance the editing efficiency of the Cas9 protein. Then, both the sgRNA and Cas9 expression constructs were recombined on T-DNA and introduced into plant cells via *A. tumefaciens* (Shan *et al*., 2013; Fan *et al*., 2015; Ma *et al*., 2015). Salt tolerance is defined as the ability of a plant to grow under salinity. In plants, salt stress leads to changes in physiological processes and metabolism. The impact can be reduced in resistant cultivars because of a network of regulatory mechanisms within the plant. It involves the controlled expression of various genes and the coordination of metabolic, signaling and regulatory pathways (Wani et al., 2020). Enhancing salt tolerance in crops has been done by gene transfer in some plants, such as soybeans (Nguyen et al., 2019), and rice (Redillas et al., 2012). However, enhancing salt tolerance through gene editing technology is not popular.

The *Ipomoea aquatica* is commonly grown and used as a vegetable in Southeast Asia. This plant contains many nutrients, such as amino acids, vitamins A, C and iron micronutrients (Guo *et al*., 2020). In particular, *I. aquatica* plants are highly resistant to heat and humidity. However, the plant's salt tolerance level is quite weak. In this study, we designed gRNA aiming to edit the *VQ9* gene on water spinach plants - *I. aquatic* (*IaVQ9*) in the CRISPR/Cas9 system and generate *A. tumefaciens* containing the system.

MATERIAL AND METHOD

Material

The *Ipomoea aquatica* seeds were provided by the Fruit and Vegetable Research Institute; seeds were germinated, and young leaves were collected for RNA isolation. Plasmid pRGEB31 was provided by Addgene (MA, USA) (Figure 1). Oligonucleotide primers were provided by IDT (Singapore) (Table 1). Bacterial strains *E. coli* DH10B and *A. tumefaciens* EHA are stored at -80^{o}C.

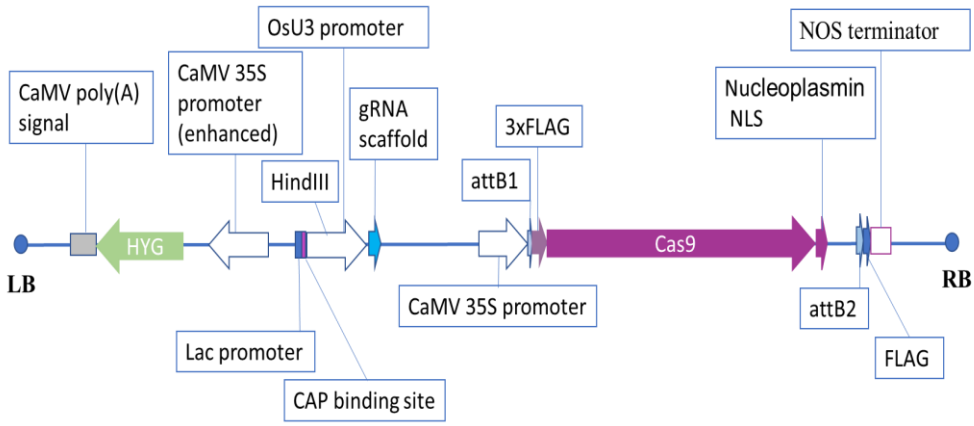

Figure 1. Schematic diagram of the left border (LB) to right border (RB) of pRGEB31 vector containing the CRISPR/Cas9 system.

Table 1: List of primers used in the study.

Primer name	Sequence (5'-3')	Target gene
VQ9-G1-F	GTAACAGCAAGAAAAGAGCG	Spacer of gRNA
VQ9-G1-R	CGCTCTTTTCTTGCTGTTAC	
VQ9 F2	ACAATATCCAAACTGAAGCCCC	*VQ9* gene
VQ9 R1	TTGTCGGCCGGATTGTAGAC	
OsU3-F1	GAACACTGGGTACGTTGGAAAC	OsU3 promoter
OsU3-R1	CTGGAGATTATTGCTCGGGTAGA	

Methods

The gRNA generation experiments in CRISPR/Cas9 targeting the *VQ9* gene (*IaVQ9*) were performed according to Xie et al., with minor modifications (Xie *et al*., 2014).

RNA extraction

Total RNA was extracted using TRIzol reagent which has been innovated appropriately with the seedling *of I. aquatica* and the concentration of RNAs was later determined using NanoDrop™ One (US).

Sequencing the IaVQ9 gene

The *IaVQ9* gene was amplified by the primer pairs VQ9G1 (F2-R1) listed in Table 1 at an annealing temperature of 61°C. The volume of the 20 µl reaction contained 8.1 µl H_2O, 10 µl DreamTaq Master Mix 2x (Thermo Fisher Scientific, MA, USA), 0.4 µl of 10

µM primer each and 0.5 µl cDNA template. The 30 amplification cycles included an annealing temperature of 61^{o}C for 25 seconds and a chain extension of 30 seconds. The purified PCR product was sequenced using an ABI 3500 sequencer (USA). The sequence reading results were compared with some VQ9 gene sequences of species in the genus *Ipomoea*.

Designing specific gRNA for the IaVQ9 gene

The gRNA–spacer sequence was designed according to the web tool CRISPR-P ver.2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR) with input information being the *IaVQ9* sequence. The gRNA sequence was selected with a high on-target index, a low off-target index and no mispairing with the CDS position of another gene. After selecting the target DNA fragments, an appropriate adapter was added to the 5' ends of the corresponding primers. Specifically, the forward primer of gRNA is appended with the sequence 5'-GGCA-3' or 5'-GGC-3' while the reverse primer is appended with the sequence 5'-AAAC-3' depending on the nucleotide of the primer.

Introduce gRNA oligonucleotides into an expression vector

Each 1 µl of VQ9-G1-F and VQ9-G1-R primer were mixed together in 48 µl H_2O. The reaction was carried out for 5 minutes at 95^{o}C and immediately cooled on ice for 20 minutes. Then, 1 µl of the product was mixed with 100 ng of circularized pRGEB31 vector. The mixture was supplemented with 3 µl 10x T4 ligase buffer, 3 µl BSA 1 mg/ml, 0.5 µl B*sa*I, 1 µl T4 ligase (Thermo Scientific) and adjusted by water to 30 µl. The ligation reaction was conducted starting at 37^{o}C for 5 minutes, followed by 40 cycles: 20^{o}C 5 minutes, 37^{o}C 5 minutes and finished with 50^{o}C 10 minutes - then 80^{o}C for 10 minutes and stored at 16^{o}C. The ligation product was transformed into *E. coli* DH10B then *A. tumefaciens* competent cells by electroporation. Colony PCR was used to check the presence of the gRNA in the pRGEB31 vector using two primer pairs OsU3-R1 and VQ9G1F; OsU3-F1 and VQ9G1R. Then, recombinant vectors were cut by restriction enzymes *Hind*III and *Bsa*I.

RESULT AND DISCUSSION

Amplifying and sequencing the *IaVQ9* gene

RNA extraction is the first important experiment for the next steps in the research. The total RNA from the leaves was shown (Figure 2A) with two clear 18S and 28S bands. Simultaneously, the quality of the total RNAs was also demonstrated by analysis with the A260/280 UV ratio, the result shown is 1.93. The concentration of total RNAs was 500 ng. Thus, total RNAs are qualified to perform cDNA synthesis for further experiments.

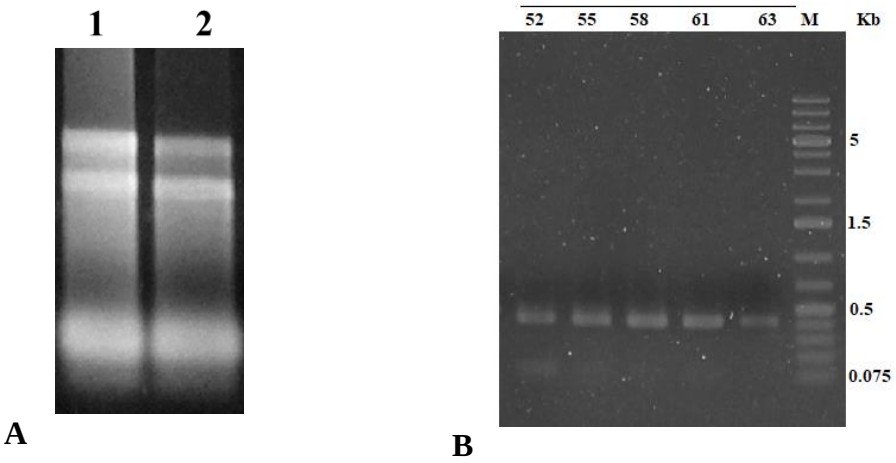

Figure 2. Total RNAs and the PCR of *IaVQ9* gene. (A) Total RNA of two *I. aquatica samples*; (B) Optimizing conditions for amplifying *IaVQ9* gene. 52, 55, 58, 61, 63: Tested temperature from 52-63oC, respectively, M: 1kb Marker.

A segment in the CDS region of the *IaVQ9* gene was amplified by PCR with the VQ9F2 and VQ9R1 primers, which were designed based on the *VQ9* sequences of some species in the *Ipomoea* genus. To optimize the conditions for *VQ9* gene PCR, as shown in Figure 2B, the results show that all temperature conditions produced DNA bands with the right size, but the most specific and clear products with the theoretical size (415 bp) were at 61^{o}C.

The *IaVQ9* PCR from the cDNA of *Ipomoea aquatica* was purified using the PCR purification kit and then sequenced. The sequencing of the *IaVQ9F2* gene segment was compared with the reference sequence of the *Ipomoea* genus published on the NCBI database, with a similarity rate of about 90.2% (Figure 3). The VQ9F2 nucleotide sequence was converted to the amino acid sequence, and the VQ motif region (FxxhVQxhTG) (in the yellow frame in Figure 4) consisted of the deduced protein sequence. This motif functions to mediate transcriptional regulation and protein-protein interactions in the VQ9 protein signaling pathway (only in plants) for responses to biotic and abiotic stresses, grain growth and photochemical processes. After determining the *VQ9* nucleotide sequence, the gRNA was designed as shown in Figure 5 (frame in yellow).

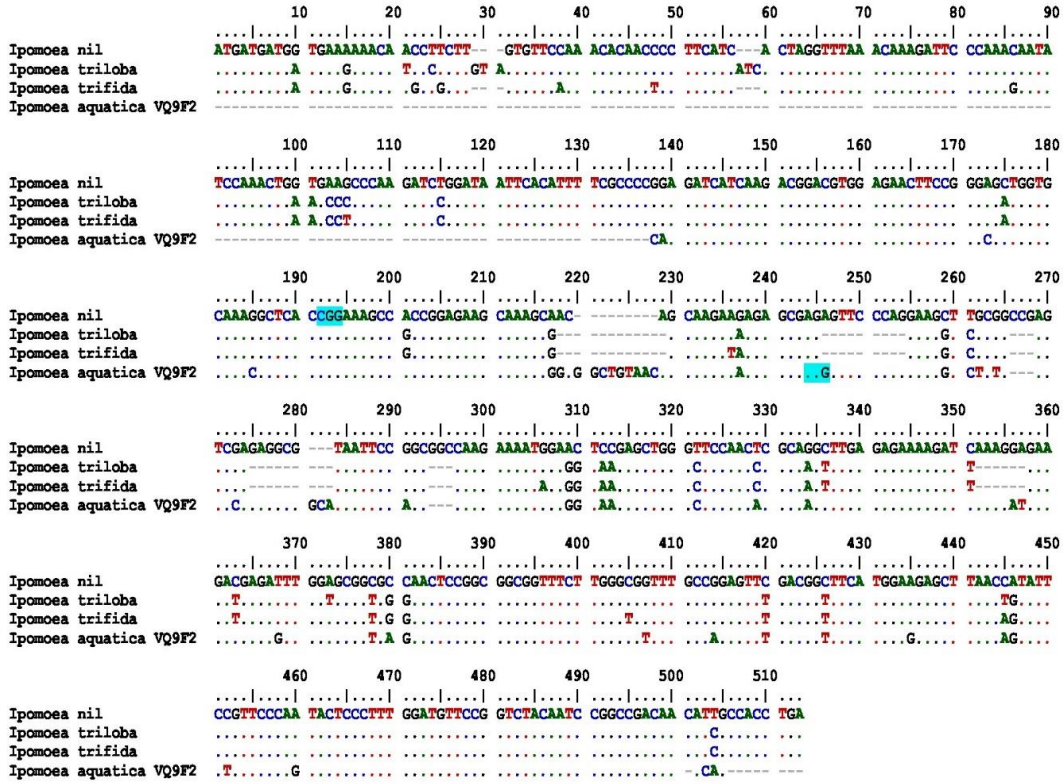

Figure 3. Comparison sequenced VQ9F2 segment of the *Ipomoea aquatica* with reference Ipomoea sequence published on the NCBI database.

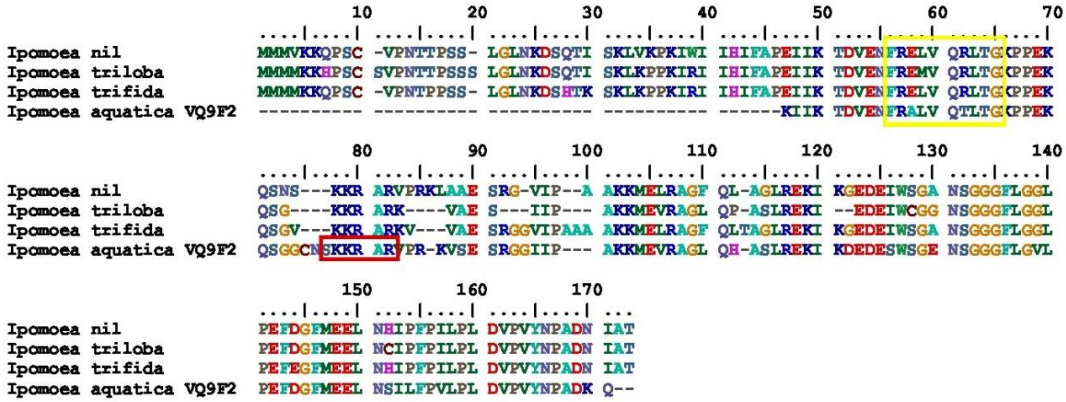

Figure 4. Comparison of the deduced VQ9F2 protein with reference sequences. The yellow frame region is the conserved VQ9 motif, the red frame region is the gRNA region

Designing gRNA targeted to the *IaVQ9* gene

The purified PCR product was sequenced, showing similarities with the reference sequences of the *VQ9* gene of *Ipomoea* on the database. Based on the sequence, we designed a gRNA, targeting the CDS of the *IaVQ9* gene. The spacer sequence of gRNA was designed according to the web tool CRISPR-P ver.2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR). The designed gRNA located on the sense segment of the *IaVQ9* gene is 5'-GTAACAGCAAGAAAAGAGCG -3' with a high on-target index and no off-target in the *I. aquatica* genome. Because this sequence begins with nucleotide G, it is connected to adapters 5'-GGCA-3' on the forward primer (VQ9G1-F) and 5'-AAAC-3' on the reverse primer (VQ9G1- R).

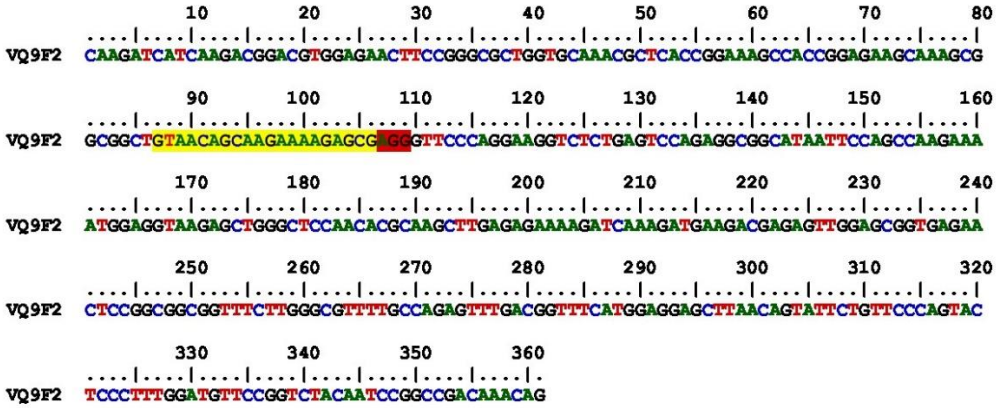

Figure 5. The sequenced segment of VQ9F2 gene of *Ipomoea aquatica.* The yellow area is the designed gRNA sequence; The red area is the PAM location

Creating *E. coli* and *A. tumefaciens* strains carrying the pRGEB31 vector containing gRNA

The ligated pRGEB31 with the gRNA fragment was transformed into *E. coli* DH10B and cultured with 50 mg/µl kanamycin on LB medium. Plasmids were isolated from five colonies and used as templates for PCR using VQ9G1-F and OsU3-R1 primers. A recombinant pRGEB31 vector had positive results, the product was about 230 bp (Figure 6A). To repeat the check of the gRNA inserted into the vector, the OsU3-F1 and VQ9G1-R primers were used for the PCR with the same DNA plasmid and a 270 bp PCR band was correctly calculated for size (Figure 6B). Thus, the recombinant pRGEB31-VQ9G1 plasmid was successfully transferred into DH10B. The plasmids are sequenced and have no difference in the gRNA sequence compared to the designed gRNA. The sequence of the inserted fragment on this T-DNA was sequenced using the Sanger method on an ABI 3500 machine. The results showed that the spacers were correctly inserted between the two *Bsa*I restriction enzyme cut sites (adjacent to the OsU3 promoter and just before the gRNA scaffold) and no nucleotide variations were detected, as shown clearly in Figure 6C when comparing the gRNA region inserted into the vector with the pRGEB31 vector and

the gRNA sequence. Thus, the RNA spacer-scaffold structure will be expressed under the control of the OsU3 promoter, which is specifically used for RNA expression (Figure 6C).

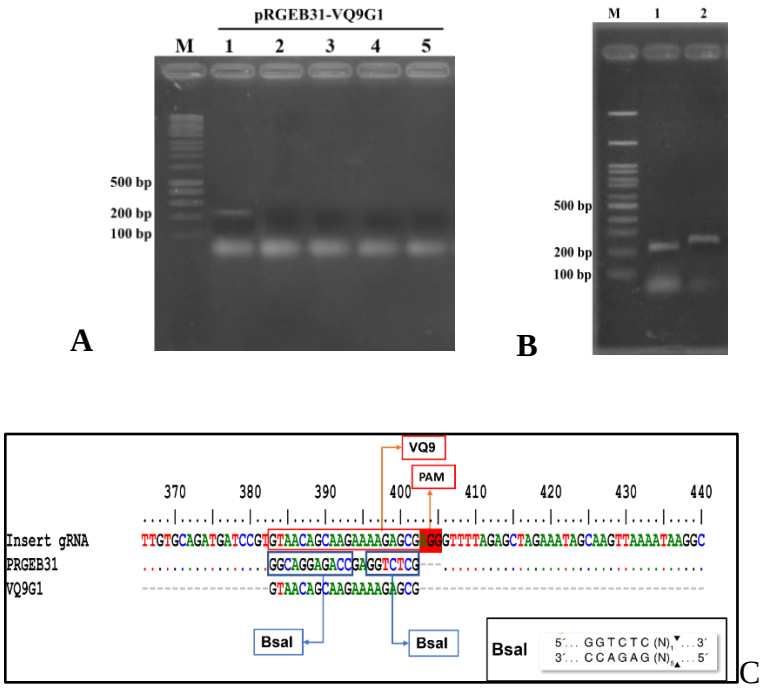

Figure 6. Colony PCR-gel electrophoresis of pRGEB31- VQ9G1 in *E. coli*. (A) Test plasmids with VQ9G1-F2 and OsU3-R1 primers; (B) Test number 1 with two primer pairs: VQ9G1-F and OsU3-R1 (well 1); OsU3-F1 and VQ9G1-R (well 2); (C) The insertion site of the spacer sequence and the sequencing results of a T-DNA fragment carrying the spacer sequence on the plasmid pRGEB31-VQ9G1.

The electroporation method was used to transform plasmid pRGEB31-VQ9G1 into *A. tumefaciens* strain EHA105. The appearance of relatively many colonies in antibiotic culture is shown in Figure 7A. Then, six colonies were selected by plasmid isolation and restriction enzyme cutting. The results showed that the plasmid DNAs were clear with a length higher than the 10 kb band in Figure 7B.

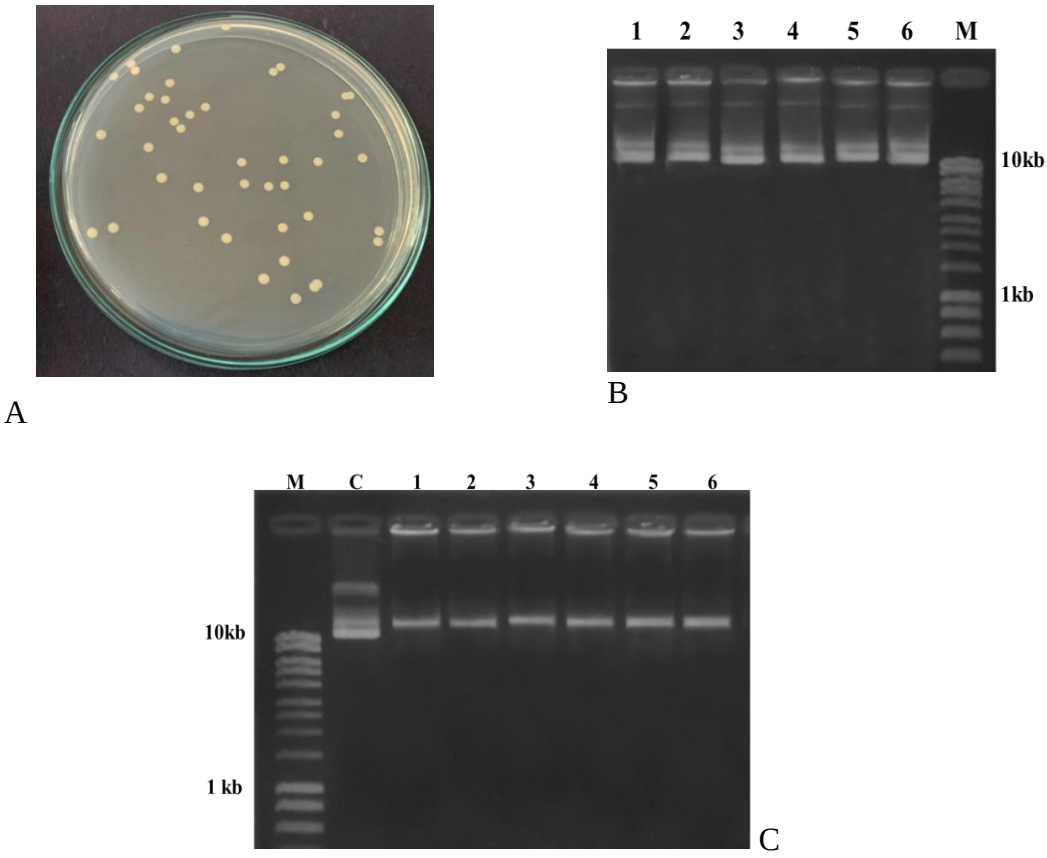

Figure 7. Checking recombinant plasmid pRGEB31-VQ9 in *A. tumefaciens.* (A) Colonies after transformation; (B) Plasmid pRGEB31-VQ9G1 from *A. tumefaciens;* (C) Cutting the pRGEB31-VQ9G1 plasmid with *Hind*III and *BsaI.* 1,2,3,4,5,6: Plasmids pRGEB31-VQ9G1 were cut with *Hind*III and *Bsa*I; c: uncut pRGEB31 plasmid.

In addition, the vector in *A. tumefaciens* was also checked with restriction enzymes. Vector pRGEB31 originally hadtwo *Bsa*I cutting sites at positions 382 and 402, corresponding to the gRNA insertion site. When the gRNA sequence is inserted into the vector, there is no longer a recognition site for the *Bsa*I enzyme. On the vector, the *Hind*III recognition site is unique, so the enzyme is used to cut pRGEB31-VQ9G1. Thus, six colonies of *A. tumefaciens* were examined for pRGEB31-VQ9G1 structure by cutting with two enzymes *Hind*III and *Bsa*I. After cutting, a linear plasmid DNA larger than 15 kb was obtained in six tested plasmids (Figure 8C). It shows that the colonies carry the pRGEB31-VQ9G1 construct. In summary, the pRGEB31-VQ9G1 vector was transferred into *A. tumefaciens* strain EHA105.

The *VQ* gene family has also been shown by many studies to have characteristics related to resistance to abiotic stresses such as heat and salinity in cotton, corn, and Eucalyptus (Chen et al., 2020; Song et al., 2016; Yan et al., 2019). The mutated *VQ9* gene has been shown in *Arabidopsis* to have a positive effect on salt tolerance in plants (Hu *et al.* 2013). Therefore, we hope that by mutating the *VQ9* gene we can target water spinach

selection. It can increase salt tolerance through CRISPR/Cas9 technology applied to the plant. Thus, by creating *A. tumefaciens* containing the pRGEB31-VQ9G1, the current study has created a source of vector material for such research purposes. The gene segment encoding the *VQ9* gene of *I. aquatica* was sequenced and contains the entire conservative VQ motif, which is typical for the *VQ* gene family. All nucleotides of the motif were found identical in the compared species, confirming that the isolated fragment accurately represents the *VQ9* gene of water spinach. However, the designed gRNA region is only specific to the decoded VQ9 gene of water spinach in this study. Because the *VQ9* gene in some plants is one exon, it may be the same in water spinach. The pRGEB31-VQ9G1 was strictly constructed when introduced into the water spinach plant, will cause specific mutations in the target region. Off-target sites identified did not show any clear bias towards conversion or transposition compared to the intended target sequence from sequencing results. Additionally, off-target effects have been minimized through the use of advanced gRNA design and analysis tools during the gRNA design process (Anthon *et al*., 2022). Moreover, in the off-target assessment process for gRNAs using the CRISPRs tool that do not match the reference, it is possible to mask the input and exclude from the search genomic regions that could potentially interfere with the on/off-target lists and scores.

CONCLUSION

In summary, the *VQ9* gene segment from *I. aquatica* was isolated and sequenced. The sequence contains a conservative sequence region that is typical of plant *VQ* genes. A specific gRNA was created based on this sequence in order to mutation the *VQ9* of *I. aquatica* by genome editing. A vector pRGEB31-VQ9G1 carrying the gRNA fragment was created and *A. tumefaciens* containing the system was generated. Future studies will focus on transferring the construct back into *I. aquatica* plants to assess its impact on enhancing salt tolerance.

ACKNOWLEDGEMENTS

This work was funded by Vietnam Ministry of Science and Technology under Grant No. ĐTĐL.CN-77/21-C.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

Anthon C, Corsi GI, and Gorodkin J (2022) CRISPR on/off: CRISPR/Cas9 on- and off-target gRNA design. *Bioinformatics* 38: 5437-5439. http://doi.org/10.1093/bioinformatics/btac697

Buscaill P, Rivas S (2014) Transcriptional control of plant defence responses. *Curr Opin Plant Biol* 20*: 35-46.* http://doi.org/10.1016/j.pbi.2014.04.004.

Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini L A, Zhang F (2013) Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339: e819. http://doi.org/10.1126/science.1231143.

Char SN, Neelakandan AK, Nahampun H, Frame B, Main M, Spalding MH, Becraft PW, Meyers BC, Walbot V, Wang K and Yang B. (2016) An *Agrobacterium*-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. *Plant Biotechnol J* 15:257-268. http://doi.org/ 10.1111/pbi.12611.

Chen P, Wei F, Cheng S, Ma L, Wang H, Zhang M, Mao G, Lu J, Hao P, Ahmad A, Gu L, Ma Q,

Wu A, We H, Yu S (2020) A comprehensive analysis of cotton VQ gene superfamily reveals their potential and extensive roles in regulating cotton abiotic stress. *BMC Genomics* 21: e795. http://doi.org/10.1186/s12864-020-07171-z.

Dong JX, Chen CH, Chen Z X (2003) Expression profiles of the *Arabidopsis WRKY* gene superfamily during plant defense response. *Plant Mol Biol* 51: 21-37. http://doi.org/10.1023/a:1020780022549.

Doudna JA, Charpentier E (2014) Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 346: e1258096. http://doi.org/10.1126/science.1258096

Fan D, Liu T, Li C, Jiao B, Li S, Hou Y, Luo K (2015) Efficient CRISPR/Cas9-mediated Targeted Mutagenesis in Populus in the First Generation. *Scientific reports* 5: 12217-12217. http://doi.org/10.1038/srep12217.

Guo R, Wang X, Han X, Chen X, Wang-Prusk G (2020) Physiological and transcriptomic responses of water spinach (*Ipomoea aquatica*) to prolonged heat stress. *BMC Genomics* 21:533 https://doi.org/10.1186/s12864-020-06953-9.

Hu Y, Chen L, Wang H, Zhang L, Wang F, Yu D (2013) Arabidopsis transcription factor WRKY8 functions antagonistically with its interacting partner VQ9 to modulate salinity stress tolerance. *The Plant Journal* 74: 730-745. http://doi.org/10.1111/tpj.12159.

Hu YR, Dong QY, Yu DQ (2012) *Arabidopsis* WRKY46 coordinates with WRKY70 and WRKY53 in basal resistance against pathogen *Pseudomonas syringae*. *Plant Sci* 185: 288-297. http://doiorg/10.1016/j.plantsci.2011.12.003.

Jiang W, Zhou H, Bi H, Fromm M, Yang B, Weeks DP (2013) Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic acids research* 41: e188. http://doi.org/10.1093/nar/gkt780.

Kadkhodaei S, Memari HR, Abbasiliasi S, Rezaei MA, Movahedi A, Shun TJ, Ariff AB (2016) Multiple overlap extension PCR (MOE-PCR): an effective technical shortcut to high throughput synthetic biology. *RSC Advances* 6: 66682-66694. http://doi.org/10.1039/C6RA13172G.

Ma X, Zhang Q, Zhu Q, Liu W, Chen Y, Qiu R, Wang B, Yang Z, Li H, Lin Y, Xie Y, Shen R, Chen S, Wang Z, Chen Y, Guo J, Chen L, Zhao X, Dong Z, Liu YG (2015) A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. *Molecular Plant* 8: 1274-1284. http://doi.org/10.1016/j.molp.2015.04.007.

Nguyen NT, Vu HT, Nguyen TT, Nguyen L-AT, Nguyen M-CD, Hoang KL, Nguyen KT, Quach TN (2019) Co-expression of Arabidopsis AtAVP1 and AtNHX1 to Improve Salt Tolerance in Soybean. *Crop Sci* 59:1133-1143. http://doi.org/10.2135/cropsci2018.10.0640.

Pan C, Ye L, Qin L, Liu X, He Y, Wang J, Chen L, Lu G (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Sci Rep* 6: e24765. http://doi.org/10.1111/pbi.12832.

Redillas MCFR, Park SH, Lee JW, Kim YS, Jeong JS, Jung H, Bang SW, Hahn TR, Kim JK (2012) Accumulation of trehalose increases soluble sugar contents in rice plants conferring tolerance to drought and salt stress. *Plant Biotechnol Rep* 6:89-96. http://doi.org/10.1007/s11816-011-0210-3.

Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology* 31: e686. http://doi.org/10.1038/nbt.2650.

Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M, Hakimi SM, Mo H, Habben JE (2017) ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield under field drought stress conditions. *Plant Biotechnology Journal* 15: 207-216. http://doi.org/10.1111/pbi.12603.

Song W, Zhao H, Zhang X, Lei L and Lai J (2016) Genome-Wide Identification of VQ Motif-Containing Proteins and their Expression Profile Under Abiotic Stresses in Maize. *Front. Plant Sci* 5: e1177. http://doi.org/10.3389/fpls.2015.01177.

Tian J, Zhang J, Francis F (2024) The role and pathway of VQ family in plant growth, immunity, and stress response. *Planta* 256: e16. http://doi.org/10.3389/fimmu.2020.612452.

Ülker B, Somssich IE (2004) WRKY transcription factors: from DNA binding towards biological function. *Curr opin plant biol 7*: 491-498. http://doi.org/10.1016/j.pbi.2004.07.012.

Wani SH, Kumar V, Khare T, Guddimalli R, Parveda M, Solymosi K, Suprasanna P, Kishor PK (2020) Engineering salinity tolerance in plants: progress and prospects. *Planta* 251:1-29. http://doi.org/10.1007/s00425-020-03366-6.

Wang L, Chen L, Li R, Zhao R, Yang M, Sheng J, Shen L (2017) Reduced Drought Tolerance by CRISPR/Cas9-Mediated SlMAPK3 Mutagenesis in Tomato Plant. *J Agric Food Chem.* 65: 8674-8682. http://doi.org/10.1021/acs.jafc.7b02745.

Xie K, Yang Y (2013) RNA-Guided Genome Editing in Plants Using a CRISPR-Cas System. *Molecular Plant* 6: 1975-1983. http://doi.org/10.1093/mp/sst119.

Yan H, Wang Y, Hu B, Qiu Z, Zeng B, Fan C (2019) Genome-Wide characterization, Evolution, and Expression Profiling of VQ Gene Family in Response to Phytohormone Treatments and Abiotic Stress in *Eucalyptus grandis*. *International Journal of molecular sciences* 20: e1765. http://doi.org/10.3390/ijms20071765.

Zhang A, Liu Y, Wang F, Li T, Chen Z, Kong D, Bi J, Zhang F, Luo X, Wang J, Tang J, Yu X, Liu G, Luo L (2019) Enhanced rice salinity tolerance via CRISPR/ Cas9-targeted mutagenesis of the OsRR22 gene. *Mol Breeding* 39: e47. http://doi.org/ 10.1007/s11032-019-0954-y.