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DMR6 Gene Editing in Bananas: Protocol Development using CRISPR/Cas9 and Multiple Bud Clump Transformation

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ABSTRACT

This study established a CRISPR/Cas9-based genome editing protocol for the banana cultivars 'Hin' (ABB) and 'Hom Thong' (AAA), with the aim of enhancing resistance to diseases such as banana blood disease caused by *Ralstonia syzygii* subsp. *celebesensis* (Rsc). The editing target was the *downy mildew resistance 6* (*DMR6*) gene. A total of 416 potential single guide RNAs (sgRNAs) were identified, from which 26 were selected based on high On-scores, minimal predicted off-target effects, and favorable GC content. Five sgRNAs were successfully inserted into the CRISPR/Cas9 vector pRGEB32 and introduced into *Agrobacterium tumefaciens* strain EHA105. The transformation process involved co-cultivating banana shoot clumps with the CRISPR/Cas9 system under *in vitro* conditions. Transformation efficiency, determined by the presence of *Cas9* and *hptII* genes, ranged from 30–38% in 'Hin' and 13–25% in 'Hom Thong'. Gene editing was confirmed through deep sequencing, which detected base pair substitutions in the *DMR6* gene, including distinct mutations in both cultivars. The highest mutation frequencies were observed with sgDMR6-7, reaching 70.15% in 'Hin' and 33.24% in 'Hom Thong'. Overall, these findings demonstrated successful CRISPR/Cas9-mediated genome editing in bananas and provided a potential strategy for improving disease resistance in these economically important cultivars.

Abbreviation: Clustered regularly interspaced palindromic repeats (CRISPR), CRISPR-associated protein 9 (Cas9)

Introduction

Bananas (*Musa* spp.) are a vital crop in tropical and subtropical regions, serving both as a staple food and as a major economic commodity for numerous countries (Wang et al., 2022). Despite their global importance, banana production is threatened by banana blood disease (BBD), caused by *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*). This pathogen invades the vascular tissue of banana plants, producing symptoms such as leaf wilting, chlorosis, discoloration in the fruit flesh, peduncle, and pseudostem, as well as bacterial ooze from the flower bud. These symptoms progress to necrosis and ultimately result in plant death (Ray et al., 2021; Ray et al., 2022). *Rsc* spreads rapidly through multiple

pathways, including insect, bird, and bat vectors, as well as contaminated soil, water, and human activities. Long-distance dissemination is often facilitated by the movement of infected planting material, posing a persistent threat to banana-growing regions and causing severe economic losses (Buddenhagen, 2007; Ray et al., 2022).

Traditionally, breeding for stress resistance in plants has relied on phenotypic selection and genetic variation analysis, incorporating methods such as QTL mapping, genome-wide association studies (GWAS), marker–trait associations (MTAs), and meta-analysis to identify loci associated with desirable traits (Heidari et al., 2011; Shariatipur et al., 2021; Shahriari et al., 2024; Salami et al., 2024). While effective, these approaches are time-intensive and constrained by factors such as polygenic trait control, environmental interactions, and the requirement for large mapping populations (Ravi et al., 2021). Conventional gene transformation techniques enable more direct trait manipulation but often lack precision and may produce transgenic plants with unpredictable integration sites (Moazami-Goodarzi et al., 2020).

In contrast, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing provides a rapid, precise, and cost-effective method for targeted genetic modification without the need to introduce foreign DNA. This technology enables direct editing of susceptibility (S) genes to generate disease-resistant plants and facilitates functional validation of candidate genes identified through genomic analyses (Tripathi et al., 2021; Mahmood et al., 2022). Originating from the adaptive immune system of *Streptococcus pyogenes*, the CRISPR/Cas9 system achieves precision by creating site-specific double-strand DNA breaks. Guided by a synthetic single guide RNA (sgRNA) complementary to the target sequence, Cas9 endonuclease cleaves DNA at the designated locus. This targeted cleavage activates cellular repair mechanisms, enabling the deletion, insertion, or substitution of nucleotides for precise genetic modification (Gan and Ling, 2022; Tripathi et al., 2024).

The versatility of CRISPR/Cas9 extends well beyond plant research. For example, Heidari et al. (2024) demonstrated its high efficiency in biomedical science by knocking out the *Tissue Factor* gene in umbilical cord-derived mesenchymal stromal/stem cells, altering coagulation profiles and revealing therapeutic potential for regenerative medicine. Such cross-disciplinary applications underscore the robustness, precision, and adaptability of CRISPR/Cas9 as a genome editing platform.

The *Downy Mildew Resistance 6* (*DMR6*) gene represents one notable S gene target. It encodes a 2-oxoglutarate Fe(II)-dependent oxygenase that acts as a negative regulator of plant immunity, conferring recessive resistance to downy mildew in *Arabidopsis* (Van Damme et al., 2008). Upon pathogen attack, *DMR6* is upregulated, weakening plant defense responses. Together with its paralog *DMR6-Like Oxygenase 1* (*DLO1*), *DMR6* functions as a salicylic acid (SA) hydroxylase, converting SA into 2,5- and 2,3-dihydroxybenzoic acid (DHBA). This conversion maintains SA homeostasis, which is essential for balancing plant growth with immune defense against (hemi-)biotrophic pathogens (Li et al., 2012; Zhang et al., 2013; Zhang et al., 2017).

At the genomic level, *AtDMR6* belongs to the 2-oxoglutarate (2OG)-Fe(II)-dependent oxygenase superfamily, which includes over 150 genes in *Arabidopsis thaliana*. Phylogenetic analyses across 19 plant species place *AtDMR6* within a conserved clade of orthologous proteins, suggesting functional conservation across taxa (Zeilmaker et al., 2015). In several crops, including citrus and grapevine, mutations in *DMR6* have conferred resistance to bacterial, fungal, and oomycete pathogens (Hasley et al., 2021; Kieu et al., 2021; Shan et al., 2021; Thomazella et al., 2021; Tripathi et al., 2021; Parajuli et al., 2022).

For example, in grapevine, Djennane et al. (2024) edited *VvDMR6-1* in embryogenic calli and regenerated biallelic and chimeric edited plants. These plants exhibited reduced growth but lower susceptibility to *Plasmopara viticola*. Compared with mock-inoculated controls, edited lines displayed higher SA and cis-resveratrol levels. In potato, CRISPR/Cas9-mediated mutation of *StDMR6-1* via dual guide RNAs generated tetra-allelic knockouts, enhancing resistance to late blight (Kieu et al., 2021). In banana, editing *MusaDMR6* improved resistance to *Xanthomonas campestris* pv. *musacearum*, the causal agent of Banana Xanthomonas Wilt (BXW) (Tripathi et al., 2021).

The role of *DMR6* in plant immunity has also been demonstrated in tomato, where disruption of *SlDMR6-1* enhanced broad-spectrum resistance to bacterial, fungal, and oomycete pathogens (Thomazella et al., 2021). This increased resistance was associated with elevated SA levels and heightened expression of immune-related genes, indicating a stronger plant defense response. Similarly, frameshift mutations in *CsDMR6* using HypaCas9 conferred strong resistance to citrus canker, a major bacterial disease. Functional disruption of *CsDMR6* also increased expression of *NPR1*, a key regulator of systemic acquired resistance, suggesting that *CsDMR6* editing may enhance resistance to a broad range of citrus pathogens (Parajuli et al., 2022).

In the context of CRISPR/Cas9 technology, *in vitro* propagation plays a key role in bananas by facilitating germplasm distribution, conservation, and the maintenance of transgenic plants. Among the various *in vitro* methods, shoot tip and meristem

cultures are widely employed for large-scale clonal multiplication (Lee et al., 1997; Navarro et al., 1997; Madhulatha et al., 2004). Compared with field-grown materials, *in vitro* multiplication offers multiple advantages, making it the preferred approach for banana propagation (Vuylsteke and Ortiz, 1996).

The successful development of transgenic bananas depends on efficient regeneration systems and transformation protocols, with meristematic tissues and somatic embryogenic cell cultures serving as primary sources for plant regeneration. Somatic embryogenic cell suspensions are particularly advantageous for genetic transformation due to their totipotency. In contrast, meristem-based transformation faces limitations, including the low abundance of totipotent cells and the difficulty of selecting non-chimeric transgenic tissues.

Recent advances, such as the use of multiple bud clumps (*Mbcs*), have enabled the production of transgenic banana cultivars through both *Agrobacterium*-mediated transformation and particle bombardment, as demonstrated in the cultivar 'Rastali' (AAB) (Sreeramanan et al., 2006a; Sreeramanan et al., 2006b).

In the present study, CRISPR/Cas9 technology was applied to edit the *DMR6* gene in bananas, targeting the susceptible cultivars 'Hom Thong' (AAA) and 'Hin' (ABB) to *Ralstonia syzygii* subsp. *celebesensis*. Multiple bud clumps were used in the *Agrobacterium*-mediated transformation process, enabling efficient gene editing and subsequent plant regeneration. This strategy allowed the introduction of targeted mutations in *DMR6*, with the potential to reduce susceptibility to bacterial wilt and other diseases. By harnessing the precision of CRISPR/Cas9 to modify key susceptibility genes, this work aims to accelerate the development of banana varieties resistant to bacterial pathogens, providing a promising avenue to safeguard global banana production against persistent threats.

Materials and Methods

The CRISPR/Cas9-based genome editing of the *DMR6* gene was carried out in *Rsc*-susceptible banana cultivars 'Hin' and 'Hom Thong'. To provide a clear overview of the experimental workflow, a graphical summary outlining the major steps in the CRISPR/Cas9-mediated transformation process is presented in Figure 1. This includes explant preparation, vector construction, *Agrobacterium tumefaciens* transformation via vacuum infiltration, and post-transformation analysis.

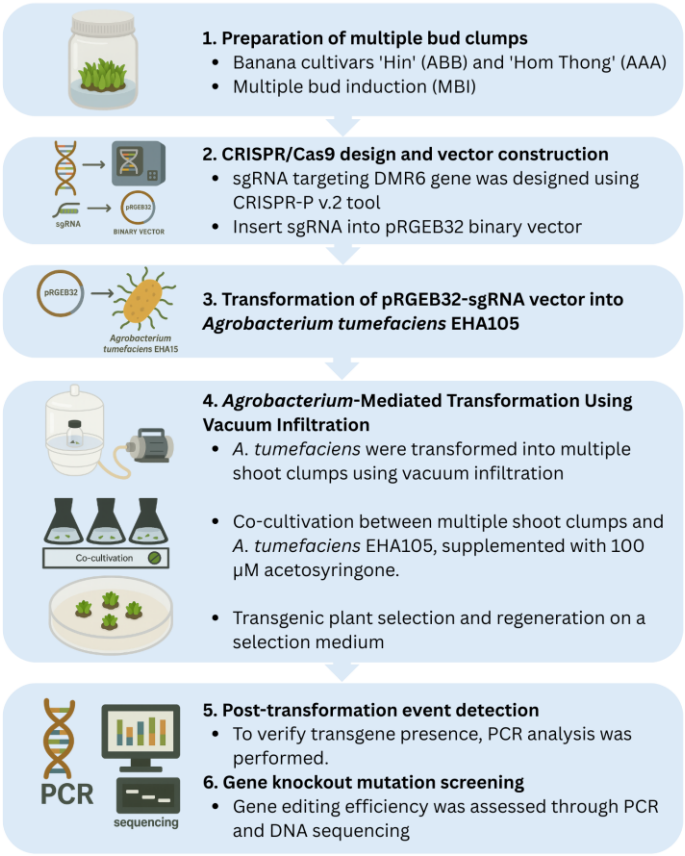

Fig. 1. Graphical flowchart of *DMR6* editing based CRISPR/Cas9 in banana.

Preparation of plant materials and explants

The banana cultivars 'Hin' (ABB) and 'Hom Thong' (AAA) were used in this study. Their resistance to *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) has been previously assessed, with both cultivars classified as highly susceptible (HS) (Nitayaros et al., 2023; Kawicha et al., 2025). Banana suckers were trimmed and thoroughly washed under running tap water. The pseudostems were cut to a final length of approximately 10 cm and surface-sterilized by immersion in 70% ethanol for 1 min, followed by treatment with 30% hydrogen peroxide containing 0.1% Tween 80 under shaking conditions for 30 min. This sterilization step was repeated once under identical conditions. The explants were rinsed three times with autoclaved distilled water, and any remaining leaf sheaths and necrotic tissues were carefully removed. Explants were then trimmed to a diameter of approximately 1–2 cm.

Prepared explants were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 5 mg L^{-1} benzyladenine (BA) and incubated for one month. For multiple bud induction, explants previously cultured on MS medium with 5 mg L^{-1} BA for three months were transferred to Multiple Bud Induction (MBI) medium containing MS salts, MS vitamins, 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 100 mg L^{-1} ascorbic acid, 22 mg L^{-1} 6-benzylaminopurine (BAP), and 3 g L^{-1} gelrite, adjusted to pH 5.8 (Tripathi et al., 2012). Cultures were incubated in the dark at 26 ± 2 °C for 3–4 months, with monthly subculturing. Multiple bud clumps obtained from this process were subsequently used for CRISPR/Cas9 transformation.

CRISPR/Cas9 design and vector construction

Potential 20-nucleotide (nt) target sites with the 5′-NGG-3′ protospacer adjacent motif (PAM) were identified for CRISPR single guide RNA (sgRNA) targeting the *DMR6* gene using the CRISPR-P v.2 tool (http://crispr.hzau.edu.cn/CRISPR2/). Candidate sgRNAs were selected based on high (Sguide score > 50) and moderate (20 < Sguide score < 50) scoring criteria, while minimizing predicted off-target effects (Lei et al., 2014). Forward and reverse oligonucleotides encoding the sgRNAs were modified by adding ATT and AAAC sequences at the 5′ ends to generate *Bsa*I recognition sites. Five sgRNA oligonucleotides—sgDMR6-2, sgDMR6-4, sgDMR6-5, sgDMR6-6, and sgDMR6-7—were designed and synthesized (Table 1).

Table 1. Forward and reverse oligonucleotides encoding the sgRNAs, and primer sequences used to detect *Cas9* and hygromycin genes.

sgRNA/Primer	Sequence (5'-3')	Aim
sgDMR6-2F	**GGCA**GCCGGATGTAGTTTTCCGGG	Cloning
sgDMR6-2R	**AAAC**CCCGGAAAACTACATCCGGC	
sgDMR6-4F	**GGCA**GTACAGGAGCTACACCTACG	
sgDMR6-4R	**AAAC**CGTAGGTGTAGCTCCTGTAC	
sgDMR6-5F	**GGCA**GAGGCCTTTGAGATTCCGGC	
sgDMR6-5R	**AAAC**GCCGGAATCTCAAAGGCCTC	
sgDMR6-6F	**GGCA**GTGAGCTCAGATCGACGGCG	
sgDMR6-6R	**AAAC**CGCCGTCGATCTGAGCTCAC	
sgDMR6-7F	**GGCA**GACCACAAGCCAGTAACACG	
sgDMR6-7R	**AAAC**CGTGTTACTGGCTTGTGGTC	
Cas9-F	AGCATCGGCCTGGACATCGGC	Cas9 detection
Cas9-R	GGAAGTGGCCCCGGAACTTG	
M13 Rev	CAGGAAACAGCTATGAC	sgRNA detection
HygR-F2	GATGTTGGCGACCTCGTATT	Hygromycin detection
HygR-R2	GTGCTTGACATTGGGGAGTT	
Sq_D1_F	TCCTCTCCACTGTCCCTCAC	Sequencing
Sq_D1_R	ACTGGCACTCCATGGTTCA	
Sq-D5-F	CGAGCTGTGGTGAACTCAGA	
Sq-D5-R	CATGCCTTCTTTCCCAACAT	

CRISPR/Cas9 constructs targeting the coding region of the *DMR6* gene were developed using the pRGEB32 binary vector (Addgene plasmid #63142), following the protocol of Xing et al. (2015). To generate double-stranded sgRNAs, 25 µL of 10 µM forward and reverse sgRNA oligonucleotides (Table 1) were annealed in a thermal cycler (PCRmax Alpha Cycler, UK) by heating at 95 °C for 3 min, followed by stepwise cooling at 1 min intervals from 90 °C to 20 °C, as described by Binyameen et al. (2021). The

annealed sgRNAs were cloned into the *Bsa*I restriction site of the pRGEB32 vector—containing the *OsU3* promoter and sgRNA scaffold—using Golden Gate cloning (Xing et al., 2014).

The 15 µL Golden Gate assembly reaction contained 6 ng of double-stranded sgRNA, 200 ng of pRGEB32 vector, 1X T4 DNA ligase buffer (NEB, USA), 1X rCutSmart™ buffer (NEB, USA), 10 U of *Bsa*I-HF®v2 (NEB, USA), and 300 U of Hi-T4™ DNA ligase (NEB, USA). The reaction was incubated at 37 °C for 15 min, followed by 50 °C for 10 min, and enzyme inactivation at 80 °C for 10 min. The resulting recombinant plasmids were designated as pRGEB32-sgDMR6-2, pRGEB32-sgDMR6-4, pRGEB32-sgDMR6-5, pRGEB32-sgDMR6-6, and pRGEB32-sgDMR6-7.

Transformation of pRGEB32-sgDMR6 vector into Agrobacterium

The pRGEB32-sgDMR6-2, -4, -5, -6, and -7 plasmids were introduced into competent *A.tumefaciens* EHA105 cells (Gold Biotechnology, USA) using the heat-shock method. Briefly, *A. tumefaciens* cells were thawed on ice for 10–15 min. One microliter of pRGEB32-sgDMR6 plasmid was gently mixed with 50 µL of competent cells and placed on ice for 5 min. The mixtures were flash-frozen in liquid nitrogen, then incubated in a 37 °C water bath for 5 min. Subsequently, 950 µL of Luria–Bertani (LB) broth was added, and the cultures were incubated at 30 °C for 4 h with shaking at 200 rpm.

Following incubation, 200 µL of each bacterial culture was spread onto LB agar selective medium containing 15 µg mL^{-1} rifampicin (for *A. tumefaciens* selection) and 50 µg mL^{-1} kanamycin (for plasmid selection). Plates were incubated at 30 °C for 2 d. Positive clones were identified by colony PCR in a 25 µL reaction containing one bacterial colony, 1X PCR buffer S (Vivantis, Malaysia), 0.2 mM dNTPs (Vivantis, Malaysia), 0.2 µM each of forward and reverse primers (M13 Rev and sgDMR6-reverse primers) (Table 1), and 1 U of Taq DNA polymerase (Vivantis, Malaysia). PCR amplification was performed in a thermal cycler (PCRmax Alpha Cycler, UK) with the following conditions: 95 °C for 2 min; 30 cycles of 95 °C for 45 s, 60 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR products were resolved on a 1% agarose gel in 1X TBE buffer at 100 V for 90 min and visualized by staining with ViSafe Green Gel Stain (Vivantis, Malaysia).

Agrobacterium-mediated transformation via vacuum infiltration

Prior to transformation, the hygromycin concentration was optimized for shoot induction and selection of gene-edited banana plants. Multiple shoot clumps from tissue culture were maintained on MS medium containing 5.0 mg L^{-1} BA and varying hygromycin concentrations (0, 10, 20, and 30 mg L^{-1}) for 30 d, after which growth and development were evaluated.

Transformation was performed using vacuum infiltration-assisted *Agrobacterium*-mediated gene transfer. A single colony of *A. tumefaciens* EHA105 harboring the pRGEB32-sgDMR6 plasmid was cultured overnight on LB agar supplemented with 15 µg mL^{-1} rifampicin and 50 µg mL^{-1} kanamycin. Colonies were scraped and suspended in MS medium, and the bacterial density was adjusted to an OD_{600} of 0.2. Multiple shoot clumps from one-month-old tissue cultures were sectioned and immersed in the bacterial suspension, which was supplemented with 100 µM acetosyringone to enhance gene transfer efficiency.

The tissues were subjected to vacuum infiltration in a desiccator connected to a vacuum pump (Rocker 300, Taiwan) at 400 mmHg for 10 min. After releasing the vacuum, they were incubated for an additional 35 min in the desiccator (Rustagi et al., 2015). For co-cultivation, the tissues were transferred to liquid MS medium containing 5.0 mg L^{-1} BA and 100 µM acetosyringone and incubated in the dark with shaking for 24 h.

To eliminate residual bacterial cells, the tissues were washed twice in liquid MS medium supplemented with 5.0 mg L^{-1} BA and 300 mg L^{-1} Timentin, with each wash lasting 1 h under continuous shaking. The washed tissues were then transferred to selection medium consisting of MS medium with 5.0 mg L^{-1} BA, 30 mg L^{-1} hygromycin, and 200 mg L^{-1} Timentin, and cultured for approximately 30 d, with subculturing every two weeks. Surviving shoots were screened via PCR analysis, and confirmed transgenic shoots were further cultured on hygromycin-containing MS medium to promote development into mature plants.

Post-transformation event detection

To verify the presence of the transgene, PCR analysis was conducted on genomic DNA extracted from plants maintained on selection medium for 30 d. DNA extraction followed a modified cetyltrimethylammonium bromide (CTAB) protocol (Porebski et al., 1997). Briefly, young leaves were ground in liquid nitrogen and mixed with CTAB buffer containing 1% SDS. Proteins were removed using chloroform:isoamyl alcohol (24:1, v/v), and the upper aqueous phase was collected for DNA precipitation. The resulting DNA pellet was dissolved in TE buffer. DNA quality and concentration were determined using a NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific, USA) and verified by agarose gel electrophoresis.

PCR was performed to confirm the presence of *Cas9* and hygromycin phosphotransferase (*hptII*) genes. Each 20 µL PCR reaction contained 50 ng of DNA

template, 1X PCR buffer S (Vivantis, Malaysia), 0.2 mM dNTPs (Vivantis, Malaysia), 0.2 µM of each primer (CAS9-F and CAS9-R for *Cas9*; HygR-F2 and HygR-R2 for *hptII*) (Table 1), and 1 U of Taq DNA polymerase (Vivantis, Malaysia). Amplification was performed in a thermal cycler (PCRmax Alpha Cycler, UK) under the following program: pre-denaturation at 95 °C for 2 min; 30 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s; followed by a final extension at 72 °C for 5 min. PCR products were resolved by electrophoresis on a 1.2% agarose gel in 1X TBE buffer at 100 V for 90 min and visualized using ViSafe Green Gel Stain (Vivantis, Malaysia).

Gene knockout mutation screening

Gene editing efficiency was evaluated via PCR amplification of the target *DMR6* locus followed by DNA sequencing. PCR reactions (20 µL) contained 50 ng of genomic DNA, 1X Phusion HF buffer (NEB Inc., USA), 0.2 mM dNTPs (Vivantis, Malaysia), 0.4 µM of each primer (Sq_D1_F and Sq_D1_R for sgDMR6-2, sgDMR6-5, sgDMR6-6, and sgDMR6-7; Sq_D5_F and Sq_D5_R for sgDMR6-4) (Table 1), and 1 U of Phusion High-Fidelity DNA Polymerase (NEB Inc., USA). Amplification was carried out in a thermal cycler (PCRmax Alpha Cycler, UK) with the following program: pre-denaturation at 95 °C for 2 min; 30 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s; and a final extension at 72 °C for 5 min.

PCR products were examined by 1% agarose gel electrophoresis and purified using the BioFact Gel & PCR Purification System (BIOFACT, South Korea). Product quality and concentration were determined using a NanoDrop™ Lite spectrophotometer. The purified amplicons were sequenced using Barcode-Tagged sequencing (BTSeq™) (Celemics, Inc., Korea). Resulting FASTQ data were analyzed using the Galaxy web-based platform (https://usegalaxy.org/). Target sequences from transgenic lines were aligned and compared with wild-type sequences using AliView version 1.28 (Larsson, 2014).

Statistical analysis

The Chi-square test was applied to compare the mutation frequencies and gene transformation efficiency between 'Hin' and 'Hom Thong'. A *P*-value < 0.05 was considered statistically significant and calculated using Chi-square test or Fisher's Exact Test. The statistical analyses were performed using IBM SPSS Statistics 25.0.

Results

Target site design and CRISPR/Cas9 vector constructs

The nucleotide sequence of the *DMR6* gene (locus GSMUA_Achr1G03620_001) was used to design single-guide RNA (sgRNA) target sites for CRISPR/Cas9-mediated gene editing. Using the CRISPR-P v.2 tool (http://crispr.hzau.edu.cn/CRISPR2/), 416 potential sgRNA target sites were identified, each comprising a 20-nucleotide guide sequence followed by the NGG protospacer adjacent motif (PAM). The On-Score values for these sgRNAs ranged from 0.0047 to 0.9151. From this set, 26 sgRNAs were selected based on high On-Score values (0.2272–0.8803) and fewer than 30 predicted off-target sites. The sgRNA with the lowest number of predicted off-targets had only two potential sites. The GC content of the selected sgRNAs ranged from 50% to 70%.

Five sgRNAs were chosen for further analysis, all targeting exons 1 and 4 of the *DMR6* gene. These had guide scores ranging from 0.6653 to 0.8803, GC contents between 55% and 65%, and predicted off-target sites ranging from 5 to 13. Of these, sgDMR6-2, sgDMR6-5, sgDMR6-6, and sgDMR6-7 were located on the sense strand, whereas sgDMR6-4 was located on the antisense strand (Fig. 2A).

To enable *Agrobacterium*-mediated transformation, five CRISPR/Cas9 plasmid constructs (pRGEB32-sgRNAs) were developed to express the selected sgRNAs: sgDMR6-2, sgDMR6-4, sgDMR6-5, sgDMR6-6, and sgDMR6-7. Each sgRNA was inserted into the vector's *Bsa*I site under the control of the *OsU3* (rice snoRNA U3) promoter. The expression vectors also carried the *Cas9* gene driven by the ubiquitin (UBI) promoter and the *hptII* gene, controlled by the cauliflower mosaic virus 35S (CaMV35S) promoter, for transgenic plant selection (Fig. 2B).

Confirmation of pRGEB32-sgRNA vector cloning in E. coli DH5α and A. tumefaciens (EHA105)

Successful cloning of double-stranded sgRNAs into the pRGEB32 vector was verified by colony PCR. Primer pairs M13-Rev and sgDMR6-2R, sgDMR6-4R, or sgDMR6-5R were used for amplification. Positive recombinant *E. coli* DH5α colonies produced a 439 bp PCR product, confirming the insertion of the respective sgRNAs (Fig. 3). Similarly, transformation of the pRGEB32-sgDMR6-2, -4, -5, -6, and -7 expression vectors into *A. tumefaciens* strain EHA105 was confirmed by colony PCR, which also yielded an amplicon of approximately 439 bp (Fig. 3).

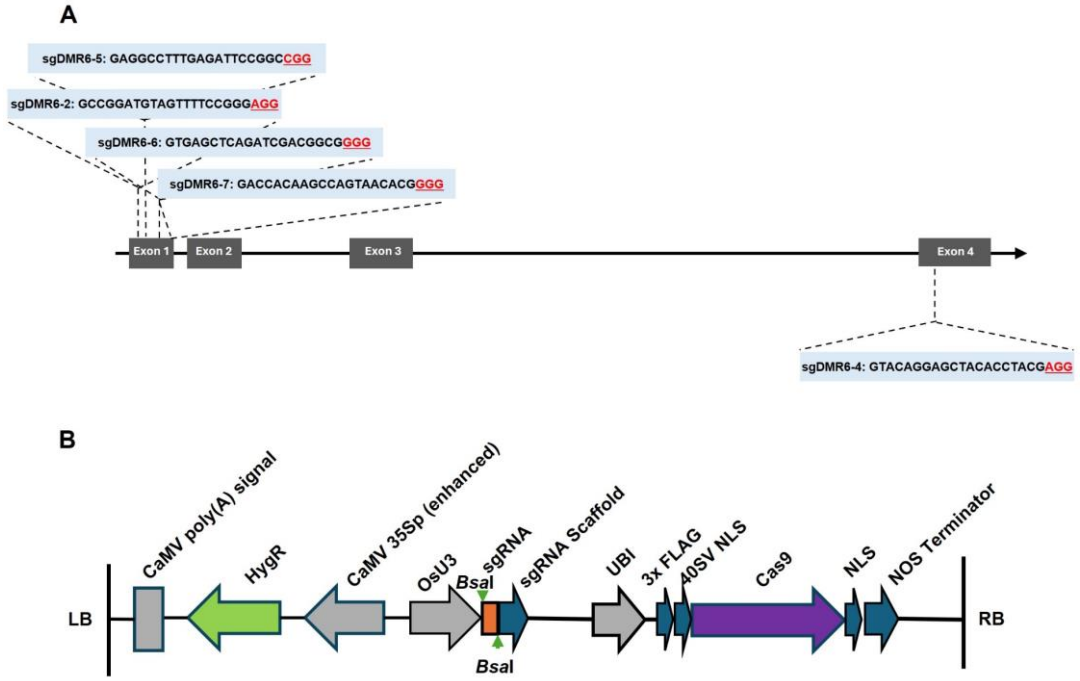

Fig. 2. sgRNA target sites and T-DNA constructs used for *DMR6* gene editing. (**A**) Schematic diagram of five selected sgRNA targeting sites located on exons. Shaded boxes indicate the exons and the black line represents the intron. The PAM sequences are marked in underlined red letters; (**B**) Schematic representations of T-DNA in pRGEB32-sgRNA expression cassette carrying the sgRNAs: sgDMR6-2, -4, -5, -6, and -7. The elements include *Cas9* controlled by the double UBI promoter and the *hptII* (HygR) gene expressed by the CaMV35S promoter.

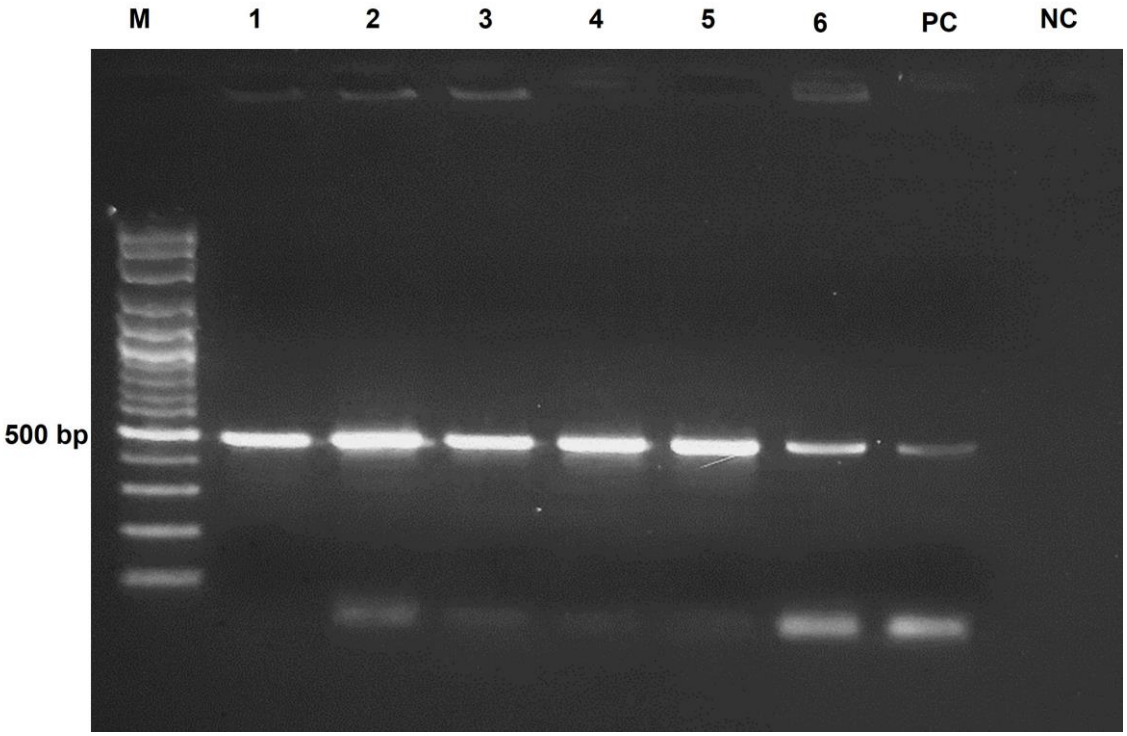

Fig. 3. Colony PCR verification of pRGEB32 plasmids containing sgRNA inserts transformed into *Escherichia coli* DH5α and *Agrobacterium tumefaciens* EHA105. M: ONE MARK 100 DNA ladder (BIO-HELIX, Taiwan). Lane 1: *E. coli*-sgDMR6-2, lane 2: *E. coli*-sgDMR6-4, lane 3: *E. coli*-sgDMR6-5, lane 4: *A. tumefaciens*-sgDMR6-2, lane 5: *A. tumefaciens*-sgDMR6-4, lane 6: *A. tumefaciens*-sgDMR6-5. PC: positive control (pRGEB32 plasmid), and NC: negative control.

Agrobacterium-mediated CRISPR vector transformation

CRISPR vector transfer was performed via vacuum infiltration-assisted *Agrobacterium*-mediated transformation. Multiple shoot clumps obtained from tissue culture were sectioned and immersed in an *A. tumefaciens* EHA105 cell suspension supplemented with 100 µM acetosyringone to enhance gene transfer efficiency. The tissues were then subjected to vacuum infiltration at 400 mmHg for 10 min, followed by a 45 min incubation in a desiccator.

To determine the optimal hygromycin concentration for the selection of gene-edited banana tissues, a preliminary experiment was conducted using MS medium supplemented with 5.0 mg L^{-1} BA and varying hygromycin concentrations (10, 20, and 30 mg L^{-1}). Multiple shoot clumps were cultured under these conditions, and their responses were evaluated after four weeks. At 10 mg L^{-1} hygromycin (Fig. 4A), partial shoot regeneration was observed, indicating insufficient selection pressure. Increasing the concentration to 20 mg L^{-1} (Fig. 4B) reduced regeneration efficiency, although some tissue growth persisted. In contrast, explants cultured on medium containing 30 mg L^{-1} hygromycin (Fig. 4C and D) exhibited complete inhibition of shoot regeneration, accompanied by visible tissue browning and necrosis, indicating effective suppression of non-transformed cells.

Based on these results, 30 mg L^{-1} hygromycin was selected as the optimal concentration for subsequent selection experiments. This concentration was incorporated into the regeneration medium (MS medium supplemented with 5.0 mg L^{-1} BA, 30 mg L^{-1} hygromycin, and 200 mg L^{-1} timentin) to ensure effective selection of transformed tissues while preventing regeneration of non-edited cells.

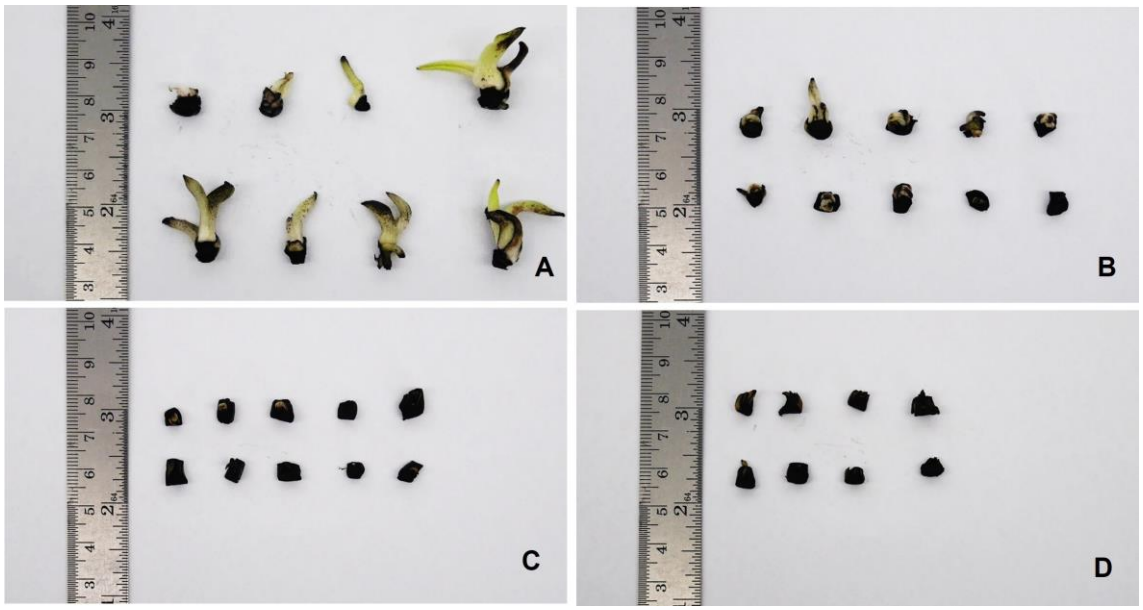

Fig. 4. Shoot clumps of 'Hom Thong' grown on MS media supplemented with varying concentrations of hygromycin: (**A**) 0 mg L^{-1}; (**B**) 10 mg L^{-1}; (**C**) 20 mg L^{-1}; (**D**) 30 mg L^{-1} for 30 d.

The efficiency of shoot regeneration following *A.tumefaciens* strain EHA105-mediated transformation was evaluated in two banana cultivars, 'Hin' and 'Hom Thong', using multiple bud clumps (Mbcs) as explants. Five CRISPR/Cas9 constructs—pRGEB32-sgDMR6-2, -4, -5, -6, and -7—were independently introduced into the tissues. Explants were cultured on MS medium supplemented with 5.0 mg L^{-1} BA, 30 mg L^{-1} hygromycin, and 200 mg L^{-1} timentin for 30 d. Both cultivars demonstrated shoot regeneration on the selection medium across all constructs, confirming the successful use of Mbcs as transformation-competent tissues. Shoot regeneration frequencies varied by construct and cultivar, ranging from 30 to 60% (Table 2). Although 'Hin' generally exhibited slightly higher regeneration frequencies than 'Hom Thong', statistical analysis using the Pearson Chi-square test revealed no significant differences between the two cultivars ($P > 0.05$ for all comparisons). These findings support the applicability of Mbcs as effective explants for *Agrobacterium*-mediated CRISPR/Cas9 gene transfer in bananas, offering a reproducible tissue system for future gene-editing efforts.

PCR analysis was conducted on plants maintained on the selection medium for 30 d post-transformation to confirm the presence of the *Cas9* and *hptII* genes. Amplification products corresponding to the *Cas9* gene (~490 bp) and the *hptII* gene (~468 bp) were detected in both banana cultivars (Fig. 5). Samples

from ‘Hin’ and ‘Hom Thong’ cultivars transformed with the five different pRGEB32-sgDMR6 constructs exhibited variable frequencies of transgene integration. The highest detection rate was recorded for sgDMR6-6, with 10 out of 12 samples from ‘Hom Thong’ and all 10 samples from ‘Hin’ testing positive for both *Cas9* and *hptII*. In contrast, lower integration rates were observed for sgDMR6-5 and sgDMR6-2 in ‘Hom Thong’.

Statistical analysis using the Chi-square test revealed significant differences in transgene detection rates between cultivars for several constructs (sgDMR6-2, sgDMR6-5, sgDMR6-6, and sgDMR6-7), with *p* values ranging from 0.008 to 0.035. These findings confirm successful transformation and highlight genotype-dependent variability in transgene integration efficiency (Table 3).

Table 2. Shoot regeneration frequency in ‘Hom Thong’ and ‘Hin’ banana cultivars following transformation with five pRGEB32-sgDMR6 vectors via *Agrobacterium tumefaciens* EHA105.

pRGEB32-sgDMR6 vector	Cultivar	Shoot regeneration frequency [a]				Pearson Chi-Square	p-value [c]
		-	+	Total	% [b]		
sgDMR6-2	‘Hom Thong’	14	6	20	30	1.667	0.197
	‘Hin’	10	10	20	50		
sgDMR6-4	‘Hom Thong’	10	10	20	50	0.404	0.525
	‘Hin’	12	8	20	35		
sgDMR6-5	‘Hom Thong’	10	10	20	50	0.921	0.337
	‘Hin’	13	7	20	40		
sgDMR6-6	‘Hom Thong’	8	12	20	60	0.404	0.525
	‘Hin’	10	10	20	50		
sgDMR6-7	‘Hom Thong’	8	12	20	60	2.506	0.113
	‘Hin’	13	7	20	40		

[a] Shoot regeneration was recorded after 30 d on selection medium. (+) indicates the number of explants that regenerated shoots; (−) indicates no shoot regeneration.
[b] Percentages represent the proportion of shoot-regenerating explants.
[c] p-values were calculated using the Pearson Chi-square test to compare regeneration between cultivars.

Targeted mutagenesis of the DMR6 gene in transgenic bananas

To confirm successful gene editing, DNA from plants testing positive for the *Cas9* and *hptII* genes was subjected to deep sequencing using Barcode-Tagged sequencing. The analysis compared the base sequences of the edited banana cultivars, ‘Hom Thong’ (HT-T_0) and ‘Hin’ (Hin-T_0), with their respective wild-type sequences, focusing on the sgRNA-targeted regions adjacent to the PAM sequence. Sequencing revealed base substitutions at specific target sites. For sgDMR6-2, C/G substitutions were detected at frequencies of 28.81% in ‘Hom Thong’ and 34.13% in ‘Hin’. For sgDMR6-7, G/T substitutions were observed at frequencies of 33.24% in ‘Hom Thong’ and 70.15% in ‘Hin’. No base alterations were detected at the target sites for sgDMR6-4, sgDMR6-5, or sgDMR6-6, and no insertion/deletion (Indel) mutations were identified (Table 4; Fig. 6).

Discussion

Genome editing using the CRISPR/Cas system has revolutionized plant genetics. Over the past decade, this technology has been widely applied to modify plant genomes, facilitating detailed investigations of biosynthetic pathways, functional characterization of specific genes, and advancement of breeding programs in both model and non-model crop species (Cardi et al., 2023). In the present study, a CRISPR/Cas9-based genome editing protocol was successfully developed and applied to target the susceptibility gene *downy mildew resistance 6* (*DMR6*) in banana varieties ‘Hin’ (ABB) and ‘Hom Thong’ (AAA), both of which are highly susceptible to *Ralstonia syzygii* subsp. *celebesensis*. The identification and selection of single guide RNAs (sgRNAs) targeting the *DMR6* gene provided a robust foundation for the editing strategy (Wang et al., 2023). The sgRNAs were selected for their high on-target scores and low off-target potential, ensuring precision in genome editing. Five sgRNAs were ultimately selected, each with 5 to 13 predicted off-target sites, reflecting a strategic approach to balance efficiency and specificity.

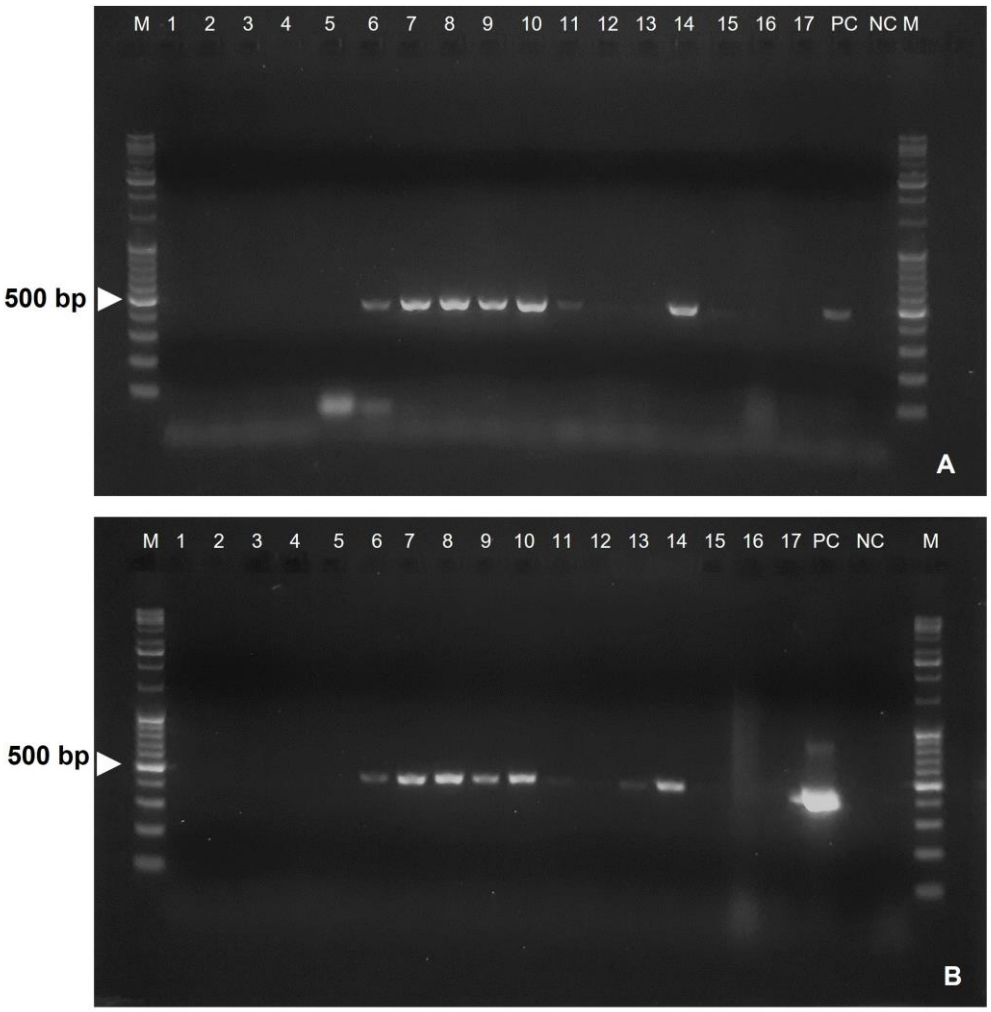

Fig. 5. Verification of *Cas9* and *hptII* genes in 'Hom Thong' following pRGEB32-sgDMR6 vectors transfer via *A. tumefaciens* EHA105. (A) DNA amplification using primers CAS9-F and CAS9-R. (B) DNA amplification using primers HygR-F2 and HygR-R2. M: VC DNA Ladder Mix (Vivantis, Malaysia); 1-5: non-transgenic plants; 6-10: plants transformed with pRGEB32-sgDMR6-2; 11-17: plants transformed with pRGEB32-sgDMR6-4; PC: positive control (pRGEB32 plasmid); NC: negative control.

Table 3. Detection of *Cas9* and *hptII* transgenes in 'Hom Thong' and 'Hin' banana cultivars following transformation with pRGEB32-sgDMR6 vectors via *Agrobacterium tumefaciens* EHA105.

pRGEB32-sgDMR6 vector	Cultivar	No. of samples exhibiting or lacking the target genes [a]							
		Cas9			*p* [b]	*hpt*II			*p* [b]
		-	+	Total		-	+	Total	
sgDMR6-2	'Hom Thong'	1	5	6	0.035	1	5	6	0.035
	'Hin'	8	2	10		8	2	10	
sgDMR6-4	'Hom Thong'	7	3	10	0.603	7	3	10	0.603
	'Hin'	6	1	7		6	1	7	
sgDMR6-5	'Hom Thong'	2	8	10	0.015	2	8	10	0.015
	'Hin'	7	1	8		7	1	8	
sgDMR6-6	'Hom Thong'	2	10	12	0.008	2	10	12	0.008
	'Hin'	8	2	10		8	2	10	
sgDMR6-7	'Hom Thong'	2	10	12	0.019	2	10	12	0.019
	'Hin'	6	2	8		6	2	8	

[a] Number of samples exhibiting (+) or lacking (−) the target genes.
[b] p-values were calculated using Fisher's Exact Test.

Table 4. The percentage of base substitutions at the target sites sgDMR6-2 and sgDMR6-7 in 'Hom Thong' and 'Hin' bananas.

Banana cultivars	Base substitution (%)	
	sgDMR6-2 C/G substitution	sgDMR6-7 G/T substitution
'Hom Thong' (HT-T0)	28.81	33.24
'Hin' (Hin-T0)	34.13	70.15

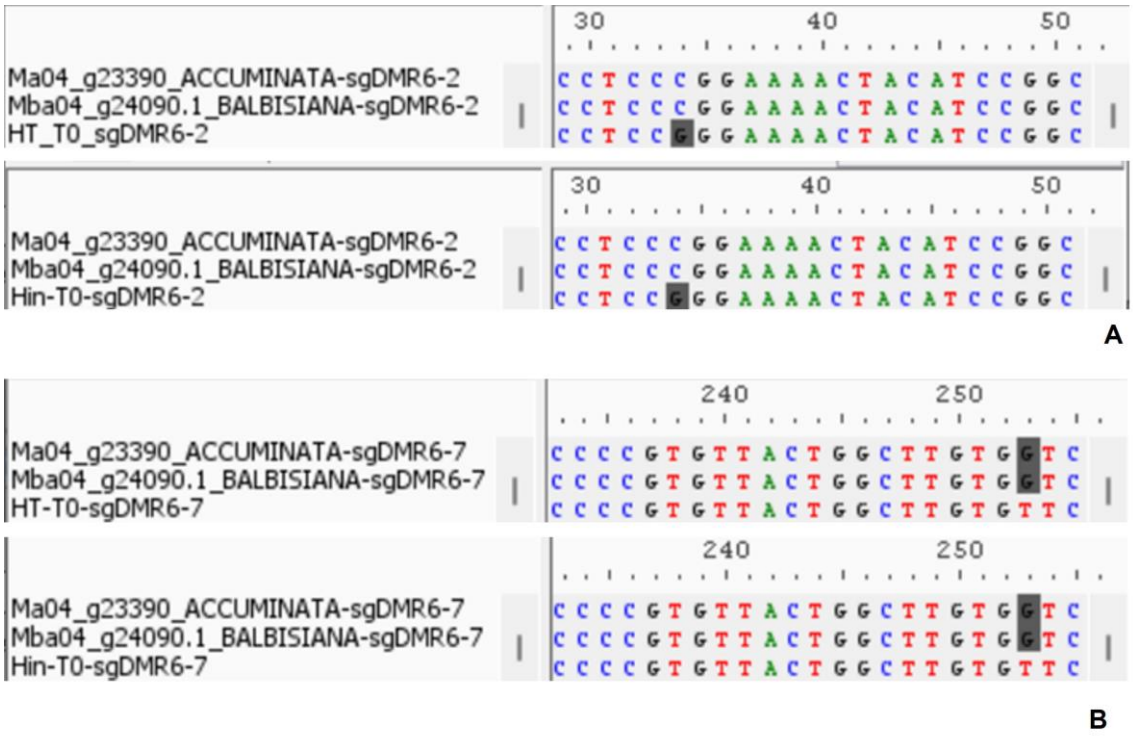

Fig. 6. (**A**) Sequence analysis of the *DMR6* gene targeting sgDMR6-2 and; (**B**) sgDMR6-7 in gene-edited H0 'Hom Thong' and 'Hin'. Shaded boxes highlight base substitutions in the mutants compared to the wild-type sequence.

The specificity of CRISPR/Cas9-mediated genome editing is highly influenced by the off-target potential of sgRNAs. High off-target activity can result in unintended mutations at genomic loci with sequence similarity to the target site, potentially altering gene function or regulatory elements and compromising phenotypic stability. Conversely, sgRNAs with low off-target potential are designed to minimize mismatches—particularly near the PAM-proximal region—thereby enhancing the accuracy and reproducibility of edits (Guo et al., 2023). Advances in computational design tools and the development of high-fidelity Cas9 variants have further improved sgRNA design, reducing off-target activity in both plant and animal systems (Asmamaw Mengstie et al., 2024). In this study, the selected sgRNAs enabled efficient targeting of the *DMR6* gene, producing precise point mutations without detectable insertion/deletion (Indel) events. These results underscore the importance of rational sgRNA design for achieving clean, efficient, and reproducible genome edits in crop improvement. Factors such as sgRNA sequence context and secondary structure may have contributed to differences in guide efficiency (Ma et al., 2015; Liang et al., 2016). Therefore, pre-validating Cas9/sgRNA constructs prior to plant transformation can markedly improve the success rate and overall efficiency of CRISPR/Cas9-mediated genome editing in plants (Wang et al., 2023).

The use of multiple bud clumps (Mbcs) as explants for transformation represented a key methodological component of this study. Mbcs offer several advantages over traditional single-bud or meristematic tissue approaches (Yang et al., 2004; Sreeramanan et al., 2006a; Sreeramanan et al., 2006b). They provide a greater number of initial cells, thereby increasing the likelihood of successful transformation events and improving overall regeneration efficiency. Furthermore, Mbcs facilitate the generation of multiple independent transformants from a single explant, which can be advantageous for achieving uniformity and consistency in transgenic lines.

Despite these advantages, the application of Mbcs presents certain challenges. The selection of optimal bud clumps and their preparation for transformation require meticulous attention to detail to maximize transformation efficiency and minimize issues such as chimerism. Chimeric plants, containing both transformed and non-transformed tissues, can complicate the identification of true transgenic lines and reduce reproducibility.

The transformation efficiencies achieved in this study using Mbcs and *A.tumefaciens* strain EHA105 were promising when compared to previous reports, although direct comparisons are limited by differences in cultivar genomes and transformation protocols. In our work, successful shoot regeneration was obtained from transformed explants, with shoot formation rates ranging from 30% to 60%. These results demonstrate the feasibility of using *in vitro* methods for large-scale clonal multiplication of genetically modified banana plants. The transformation rate observed was comparable to that reported for embryogenic cell suspension (ECS) transformation in the banana cultivar 'Rasthali' (AAB genome; 59%) (Kaur et al., 2018), but lower than the ECS transformation rate (100%) reported for the banana cultivar 'Sukali Ndiizi' (AAB genome) and plantain cultivar 'Gonja Manjaya' (AAB genome) (Ntui et al., 2020).

Differences in transformation efficiency between 'Hin' (ABB) and 'Hom Thong' (AAA) in this study may be attributed to genetic variation affecting responsiveness to transformation and regeneration protocols. Similar genotype-dependent variation has been reported previously (Kaur et al., 2018; Ntui et al., 2020). Additionally, the use of vacuum infiltration to enhance *Agrobacterium* penetration likely contributed to the relatively high transformation efficiencies observed here. These findings suggest that Mbcs, when combined with vacuum infiltration, represent a reliable and efficient alternative to ECS-based transformation methods for CRISPR/Cas9-mediated gene editing in banana. Optimizing protocols for specific varieties remains essential for improving efficiency further.

Deep sequencing confirmed successful gene editing, revealing C/G and G/T base pair substitutions at specific target sites. Editing frequencies were 28.81% and 34.13% for C/G substitutions in 'Hom Thong' and 'Hin', respectively, and 33.24% and 70.15% for G/T substitutions in the same varieties. These mutations are expected to disrupt *DMR6* gene function, potentially conferring increased resistance to blood disease. Van D. et al. (2008) demonstrated, through expression analysis of *DMR6* mutants using quantitative PCR and DNA microarrays, that loss of *DMR6* function resulted in the upregulation of several defense-related genes.

In this study, no insertion/deletion (Indel) mutations were detected, indicating that the CRISPR/Cas9 system primarily generated point mutations. Such precision is advantageous for targeted modifications, as it minimizes unintended disruptions to gene function while still impairing susceptibility. The predominance of point mutations over Indels suggests that *DMR6*-mediated resistance may result from the activation of defense responses rather than large-scale genomic alterations. Furthermore, deep sequencing enabled the identification and quantification of specific editing events across different transgenic lines, providing valuable insights into editing efficiency and the potential phenotypic consequences of these genetic modifications.

Differences in gene editing efficiency between the banana cultivars 'Hin' (ABB) and 'Hom Thong' (AAA) are likely attributable to multiple factors. A major contributor is genotype-dependent variation in responsiveness to *Agrobacterium*-mediated transformation and tissue regeneration, which has been well documented in banana (Tripathi et al., 2019). Variations in explant sensitivity, hormonal responsiveness, and regeneration potential can influence both shoot regeneration and editing frequency. Additionally, differences in genomic background may affect CRISPR/Cas9 access to target sites and the efficiency of double-strand break repair pathways, such as non-homologous end joining (Sandhya et al., 2020). Collectively, these biological and technical factors likely account for the cultivar-specific variation observed in editing outcomes.

Although targeted mutations—specifically C/G and G/T substitutions in the *DMR6* gene—were confirmed by sequencing, phenotypic validation of the gene-edited plants could not be conducted due to unsuccessful regeneration of mutant lines during later developmental stages. The failure to regenerate mutant lines in this study is likely attributable to a combination of factors previously reported in banana transformation research. These include genotype-specific differences in regeneration potential (Tripathi et al., 2015), tissue browning and necrosis resulting from stress induced by selection agents such as hygromycin (Permadi et al., 2024), and physiological damage associated with *Agrobacterium*-mediated transformation procedures (Levengood et al., 2024). Furthermore, targeted disruption of *DMR6*, a gene involved in both plant defense and developmental processes, may have further reduced regeneration capacity.

Despite these challenges, gene editing of susceptibility (*S*) genes in banana and plantain has shown promise elsewhere. Researchers at the International Institute of Tropical Agriculture (IITA) in Kenya have developed lines with enhanced resistance to bacterial diseases through *S* gene targeting. These gene-edited plants have been validated under controlled conditions and are now

progressing to field trials for potential deployment (Tripathi et al., 2024).

The release of such gene-edited plants raises important ethical and regulatory considerations. Although DNA-free edits may be exempt from GMO regulations in certain countries, public perception, ecological safety, and compliance with biosafety guidelines remain crucial, particularly for widely consumed, vegetatively propagated crops such as banana (Tripathi et al., 2024).

Conclusion

This study presented an efficient CRISPR/Cas9-based protocol for editing the *DMR6* gene in banana, employing multiple bud clumps (Mbcs) in combination with vacuum infiltration. This methodological approach proved effective in facilitating transformation and regeneration in the cultivars 'Hin' (ABB) and 'Hom Thong' (AAA), both of which are highly susceptible to *Ralstonia syzygii* subsp. *celebesensis*. The use of Mbcs increased the availability of target cells for transformation, reduced the likelihood of chimerism, and enabled the production of multiple independent transformants from a single explant. Vacuum infiltration likely enhanced *Agrobacterium* penetration, contributing to the observed transformation efficiency. Together, these features made the protocol suitable for generating gene-edited lines while maintaining scalability for potential breeding applications. Although validated in two contrasting genomic backgrounds, the protocol demonstrated strong potential for application across other banana genotypes. Nonetheless, the genotype-dependent nature of both transformation and regeneration suggested that further optimization of key factors—such as hormonal composition, culture media formulation, and transformation parameters—was likely necessary to maximize efficiency in other varieties. This was particularly relevant given the observed differences in transformation and editing outcomes between 'Hin' and 'Hom Thong'.

Beyond its utility for *DMR6* editing, the protocol offered a valuable platform for broader functional genomics research and precision breeding in banana. The ability to produce targeted point mutations illustrated the precision achievable with well-designed sgRNAs and highlighted the importance of rational guide selection for minimizing off-target effects. Furthermore, the methodological framework established in this study could be adapted for editing other susceptibility or resistance genes, accelerating the development of cultivars with improved disease resistance and agronomic performance. Overall, the combination of Mbcs and vacuum infiltration represented a practical, reproducible, and scalable approach to CRISPR/Cas9-mediated genome editing in banana. By integrating rigorous sgRNA selection with an efficient transformation system, this study provided both a technical advance and a foundation for future efforts in crop improvement. The findings not only advanced the toolkit available for banana biotechnology but also underscored the importance of tailoring transformation strategies to the genetic and physiological characteristics of each target cultivar.

Future work should prioritize multi-season field trials to evaluate the durability of *DMR6*-mediated resistance under natural disease pressure. Such trials would be key in assessing agronomic performance, potential fitness trade-offs, and trait stability across diverse environmental conditions. Equally important are the ethical and regulatory considerations surrounding the deployment of gene-edited bananas, including biosafety compliance, public acceptance, and equitable access. Advancing these cultivars toward responsible and sustainable agricultural use requires transparent communication, active stakeholder engagement, and strict adherence to national and international biosafety guidelines.

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Author contributions

PK, AS, and TT designed the experiments, analyzed the data, and wrote the manuscript. LR conducted the experiments and analyzed the data. RT, AAT, PT, and PD provided experimental samples and contributed to data analysis. All authors read and approved the final manuscript.

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Conflict of Interest

The authors indicate no conflict of interest in this work.

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