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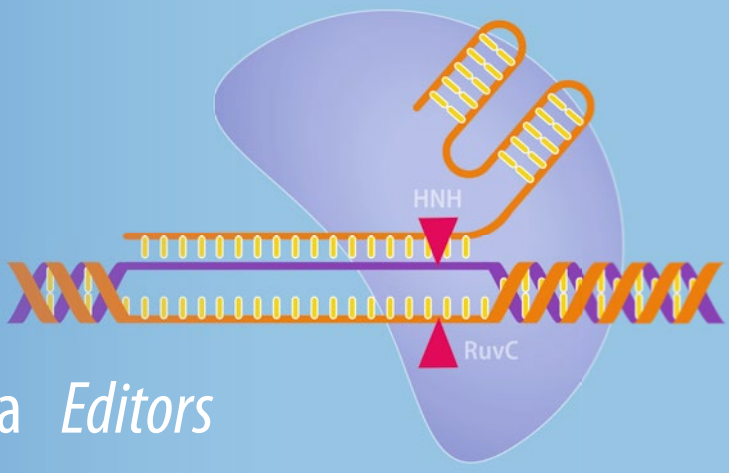

M. Tofazzal Islam
Pankaj K. Bhowmik
Kutubuddin A. Molla *Editors*

CRISPR-Cas Methods

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CRISPR-Cas Methods

Edited by

M. Tofazzal Islam

Institute of Biotechnology and Genetic Engineering (IBGE), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, Bangladesh

Pankaj K. Bhowmik

Department of Aquatic and Crop Resource Development, National Research Council of Canada, SASKATOON, SK, Canada

Kutubuddin A. Molla

Crop Improvement Division, ICAR-National Rice Research Institute, Cuttack, Odisha, India

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Editors
M. Tofazzal Islam
Institute of Biotechnology and Genetic Engineering (IBGE)
Bangabandhu Sheikh Mujibur Rahman Agricultural University
Gazipur, Bangladesh

Kutubuddin A. Molla
Crop Improvement Division
ICAR-National Rice Research Institute
Cuttack, Odisha, India

Pankaj K. Bhowmik
Department of Aquatic and Crop Resource Development
National Research Council of Canada
SASKATOON, SK, Canada

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Chapter 14

CRISPR-Cas9-Based Genome Editing of Banana

Leena Tripathi, Valentine Otang Ntui, and Jaindra Nath Tripathi

Abstract

Genome editing is an emerging powerful new breeding tool, which can be applied for genetic improvement of banana for important agronomic traits such as resistance to biotic stresses, adaptation to climate change, and high yielding. Banana is an important staple food and cash crop, feeding millions of people in tropical and subtropical countries. Recently, CRISPR-Cas9-based genome editing system has been established for banana in a few laboratories. Here, we describe the procedures for generation of genome-edited events of banana, detection of targeted and potential off-target mutations, and phenotyping for important traits such as disease resistance. This chapter will provide readers strategy for applying CRISPR-Cas9-based genome editing for improvement of banana.

Key words Banana, Genome editing, CRISPR-Cas9, gRNA

1 Introduction

Banana (*Musa* spp.) is one of the world's most important staple food crops cultivated in tropics and subtropics with an annual production of 145 million tons [1]. One-third of its global production is from Africa, with East Africa being the largest banana-growing and banana-consuming region. They are of different types such as dessert, cooking, roasting, and brewing types, providing food to millions of people. Banana production is seriously affected by several factors, particularly biotic and abiotic stresses. Improved varieties of banana with resistance to diseases and pests, tolerance to abiotic stresses, and higher yields can be developed more precisely using new breeding tools like genome editing.

Genome editing technologies using specific nucleases have been developed as effective genetic engineering methods to target and digest DNA at specific locations in the genome of an organism [2]. The most commonly used tools for targeted genome editing in plants are zinc finger nucleases (ZFNs) [3, 4], TAL effector proteins (TALENs) [5–7], and RNA-guided endonucleases (RGENs)

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or CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) [8–10]. These nucleases respond in a target-specific manner and induce a double-strand break (DSB) in the gene sequence [11]. The DSB is repaired either by homologous recombination (HR) or error-prone nonhomologous end-joining (NHEJ) mechanisms, which may lead to mutations disrupting gene function [12].

CRISPR-Cas9 system has emerged as a potent genome editing tool due to its simplicity, design flexibility, and high efficiency and has been successfully applied in many organisms, including several plant species [13]. It is based on the type II CRISPR-Cas immune system in bacteria that protects against invading DNA viruses and/or plasmids. The CRISPR-Cas9 molecular immunity system comprises the Cas9 endonuclease of *Streptococcus pyogenes* and a synthetic single guide RNA (gRNA), which directs the Cas9 endonuclease to a target sequence complementary to the 20 nucleotides preceding the protospacer adjacent motif (PAM), which is required for Cas9 activity [8, 14]. Multiplexing of CRISPR-Cas9 system, by integrating two or more gRNAs, which are a distance apart, is useful in generating large deletions and knocking out multiple genes in plants [15].

Genome editing using CRISPR-Cas9 technology has been recently developed for banana [16–18]. The steps involved in the genome editing of banana [18] are illustrated in Fig. 1. This chapter presents protocols routinely used in the authors' laboratory for genome editing of banana for knocking out the genes and characterization of edited events.

2 Materials

2.1 Reference Banana Genome

All the banana cultivars including plantains are polyploid clones derived from *Musa accuminata* (A genome) or/and *Musa balbisiana* (B genome). The cultivars can be grouped as diploids (AA, BB, AB), triploids (AAA, AAB, ABB), and tetraploids (AAAA, AAAB, AABB, ABBB) based on genomes. The wholegenome sequence of *Musa accuminata* (DH Pahang) and *Musa balbisiana* (Pisang Klutuk Wulung) are publicly available at the Banana Genome Hub database (http://bananagenome-hub.southgreen.fr/) [19]. These genome sequences can be used as reference genome for editing of the potential targets in banana.

2.2 Tools for Designing gRNA

Many tools are available to design gRNA and most of them provide information on the location and efficiency of the gRNA as well as off-target effects. Researchers have their preference for using the tool. Some of the commonly used tools for gRNA design are listed below:

http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx

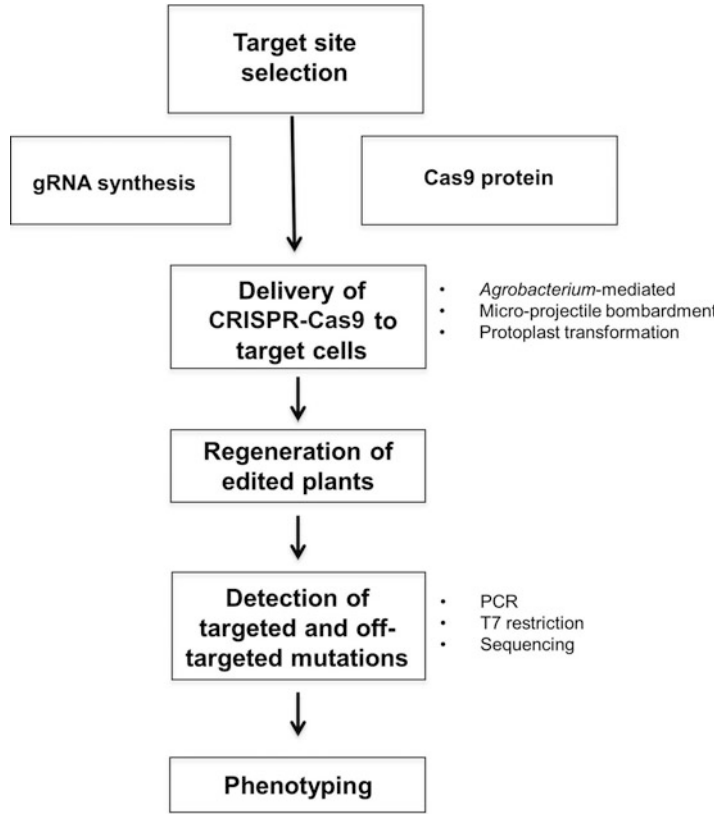

Fig. 1 Illustration of various steps involved in genome editing of banana

https://chopchop.rc.fas.harvard.edu/

http://www.genome-engineering.org/crispr/

http://crispor.tefor.net/

https://benchling.com/crispr

https://www.deskgen.com

Alt-R Custom Cas9 crRNA Design Tool (https://eu.idtdna.com/site/order/designtool/index/CRISPR_CUSTOM).

ATUM CRISPR DNA Design Tool (https://www.atum.bio).

In our laboratory, we routinely use ATUM CRISPR DNA Design Tool or Alt-R Custom Cas9 crRNA Design Tool from IDT for editing of banana. These tools provide us with highly efficient gRNAs with minimal off-target effects.

2.3 Plasmid Constructs

For preparing CRISPR-Cas9 constructs for editing of banana, four different plasmid vectors are used for cloning [20]. Each plasmid contains a complete expression cassette for gRNA and is regulated by the OsU6 promoter. The first plasmid is the Golden Gate entry vector. This plasmid also contains tetracycline efflux protein, which

confers resistance to tetracycline and enables the selection of positive clones. The second plasmid is the Golden Gate recipient vector. In this plasmid, the LacZ gene is readily replaced by gRNA expression cassettes via Golden Gate reactions. It contains the Gateway recombination sites to facilitate the integration of the gRNAs and *Cas9* gene cassette into the binary plasmid. It also contains amino glycoside adenylyltransferase, which confers resistance to spectinomycin and streptomycin. Only two gRNAs at a time can be integrated into this plasmid. The third plasmid is the vector containing the *Cas9* gene cassette. This is a Gateway-compatible plasmid. This plasmid contains a plant codon-optimized *Cas9* gene with high GC content and aminoglycoside adenylyltransferase that confers resistance to spectinomycin and streptomycin. The fourth plasmid is the binary vector such as pMDC32, which is also a Gateway-compatible plasmid. It contains *nptII* gene, which confers resistance to kanamycin and enables selection of positive clones. This plasmid also contains a *hpt* selection marker for *in planta* selection.

2.4 Banana Cell Suspension

An ideal explant for delivering CRISPR-Cas9 plasmid constructs to the banana genome is embryogenic cell suspensions. These are single cells with the potential to regenerate into complete plantlets in 7–9 months. However, the generation of embryogenic cell suspensions is a lengthy process, laborious, and cultivar-dependent [21, 22]. Therefore, the availability of embryogenic cell suspensions of different cultivars is very critical. The generation of embryogenic cell suspension of banana has been reported from various explants, especially male flowers and multiple buds [21, 23–27].

Embryogenic cell suspensions of several cultivars such as Gonja Manjaya, Dwarf Cavendish, Sukali Ndiizi, Zebrina, Gross Michel, Agbagba, Orishele, and Obino l'Ewai available at the International Institute of Tropical Agriculture (IITA), Nairobi, Kenya, can be used as starting material for genome editing.

2.5 Banana Protoplast

Protoplast is required for delivering CRISPR-Cas9 ribonucleoprotein (RNPs) for DNA-free editing of banana. The protoplast of banana can be isolated using the protocol developed earlier [28]. The protoplasts are isolated from embryogenic cell suspensions of banana. The complete plantlets can be regenerated from viable protoplast cultured on regeneration medium [29]. The accomplishments with protoplast culture are still limited, and plant regeneration from protoplast is an inefficient technique with a majority of banana cultivars due to lack of fine embryogenic cells.

3 Methods

3.1 Design of gRNA

Once a target gene for editing identified, the gene sequences are downloaded from genome A or/and B, depending upon the genome composition of the cultivar. The sequences are then aligned to identify conserved regions as target sites for editing. Primers specific to the gene, based on the reference genome, are designed, and the gene is re-sequenced from the interested cultivar to get the exact sequences without any changes. Single or multiple gRNAs can be designed for the target site in the gene using the tools listed above.

Multiplexing of gRNA can enhance mutation efficiency and create large mutations if Cas9 cleaves at two target sites simultaneously and also can knock out multiple genes at once [18]. The gRNA tool design several potential gRNAs from which the gRNAs with higher efficiency and having minimal or no potential off-target effect should be selected.

3.2 Plasmid Construct Preparation

In order to clone the gRNA into the expression vector, adaptors are added to the 5′ end of the gRNAs and the corresponding reverse sequences. The gRNA sequences are then synthesized as oligos and used for cloning through Golden Gate and Gateway techniques [18, 20]. The gRNA expression vectors are usually linearized to produce overhang; this facilitates the cloning of gRNAs. Forward and reverse oligos of each gRNA are phosphorylated and annealed and ligated to the gRNA expression vectors using T4 ligase. The ligated product is then sequenced to confirm the insert.

The two gRNAs are then assembled into the Golden Gate recipient and Gateway vector by digestion with the appropriate enzyme(s) depending on the vector and ligation with T_4 DNA ligase. The Golden Gate assembly reaction together with a *Cas9* entry plasmid are inserted into the Gateway binary plasmid. The plasmid is then mobilized to *Agrobacterium tumefaciens* through electroporation [30]. The various steps involved in the cloning of gRNA(s) and *Cas9* gene into the binary plasmid are summarized in Fig. 2.

3.3 Preparation of CRISPR-Cas9 Ribonucleoproteins (RNPs)

Preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) can also be delivered directly into the plant cells [31–34]. These RNA-guided endonucleases (RGENs)-RNPs direct genome editing of the target sites immediately after transfection and are degraded rapidly by endogenous proteases in cells, thus reducing off-target effects and leaving no traces of foreign DNA elements [31, 35].

RNPs can be assembled following the protocol from IDT (www.idtdna.com). Each RNA oligo (crRNA and tracrRNA) is suspended in nuclease-free TE buffer to a final concentration of

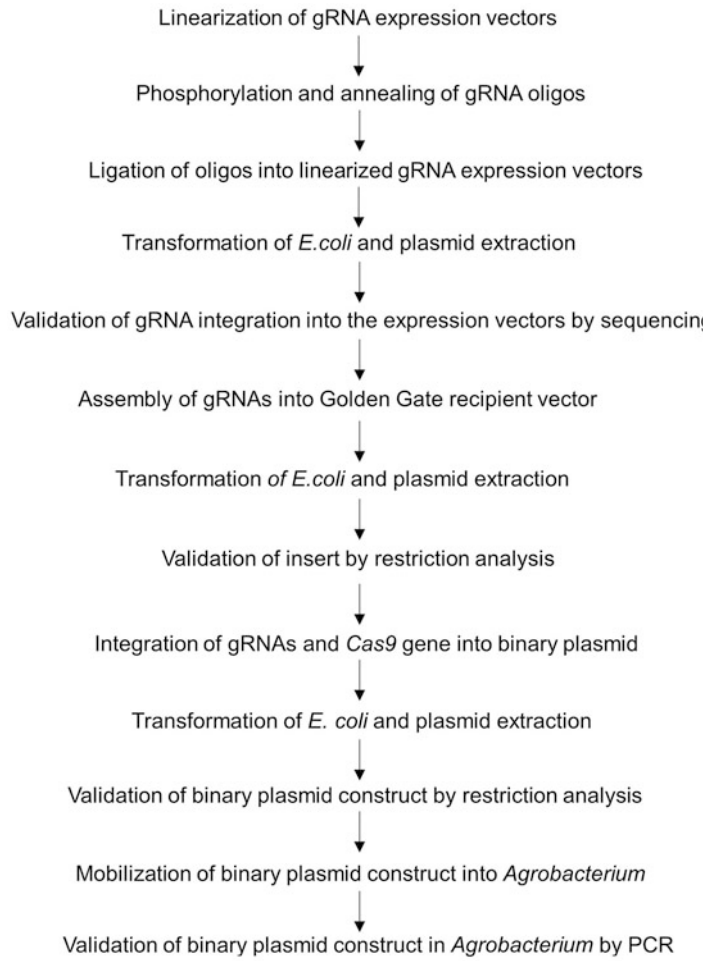

Fig. 2 Flow diagram showing steps for producing the CRIPSR-Cas9 plasmid construct

100 μM. The two RNA oligos are then mixed in a PCR tube to a final duplex concentration of 3 μM (3 μL of each oligo and 94 μL of nuclease-free duplex buffer). Using a thermal cycler, the mixture is heated at 95 ° C for 5 min and cooled to 25 ° C. The Cas9 protein is also diluted to a working concentration of 5 μM in the Cas9 working buffer (20 mM HEPES, 150 mM KCI, 5% glycerol and 1 mM DTT, pH 7.5). To assemble the RNP complex, 1.5 pmol of duplexed RNA oligos are mixed with 1.5 pmol of Cas9 protein in Opti-MEM media to a final volume of 12.5 μL. The mixture is incubated at room temperature for 5 min. The RNP complex can then be delivered to plant cells.

3.4 Delivery of Plasmid Construct into Banana Cells and Generation of Complete Plants

Highly efficient transformation and regeneration system is required for gene editing in banana [36]. The regeneration of plant cells into complete plants after delivering the CRISPR-Cas9 plasmid is the biggest challenge in the application of genome editing in several crops [37]. The CRISPR-Cas9 plasmid construct can be delivered to embryogenic cells of bananas through either *Agrobacterium*-mediated transformation or microprojectile bombardment. Protocols for microprojectile bombardment technique are available for modifying banana [38, 39]. *Agrobacterium*-mediated transformation protocols have also been established for many cultivars of banana and plantains and routinely used in several laboratories to deliver plasmid construct into banana cells [18, 21, 22, 40–43].

Embryogenic cells of banana can be transformed with *Agrobacterium tumefaciens* harboring CRISPR-Cas9 plasmid construct following the protocol described by [18] (Fig. 3). A single colony of *Agrobacterium* containing the CRISPR-Cas9 construct is grown with shaking for 48 h at 200 rpm in liquid LB medium containing 50 mg/L kanamycin and 50 mg/L rifampicin. Two hundred microliters of the liquid culture is refreshed in the same medium and grown for 24 h at the same conditions. The fresh culture is centrifuged at 3,200 × *g* for 10 min, and the pellet is resuspended in 25 mL bacterial suspension medium containing 200 μM of acetosyringone. The bacterial suspension is incubated at room temperature for 2 h at 60 rpm to an OD_{600} of 0.5–0.6. Embryogenic cell suspensions of banana are cocultivated with the *Agrobacterium tumefaciens* for 3 days in the dark at 22 °C in cocultivation media containing 200 μM of acetosyringone. After 3 days, *Agrobacterium*-infected cell suspensions are washed with liquid callus induction medium (MS containing 1 mg/l 2,4-D and 300 mg/L cefotaxime) and transferred to embryo development medium without selection antibiotic for 1 week. After 1 week, the cells are transferred to embryo regeneration medium supplemented with 300 mg/L cefotaxime and 25 mg/L hygromycin for 8 weeks

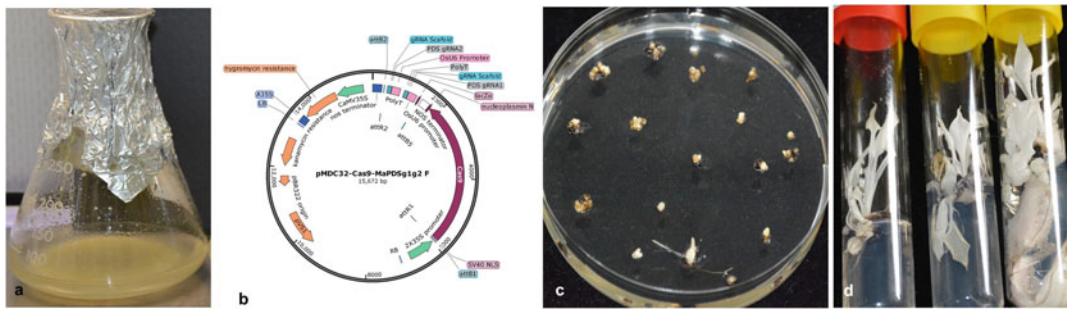

Fig. 3 Generation of genome-edited banana plants through *Agrobacterium*-mediated delivery of CRISPR-Cas9 plasmid. (**a**) Embryogenic cell suspension, (**b**) plasmid construct containing gRNAs targeting *PDS* gene and *Cas9* gene, (**c**) *Agro*-infected embryos regenerating on selective medium, and (**d**) plants with edits in *PDS* gene showing albino phenotype

in the dark. The medium is refreshed every 2 weeks. Developed embryos are then transferred to hormone-free medium for 4–6 weeks in the dark for embryo maturation. Mature embryos are transferred to embryo germination medium and exposed to light at 26–28 °C and a photoperiod of 16 h/8 h (day/night) for 4 weeks. Embryo development medium, maturation medium, and germination medium are supplemented with 300 mg/L cefotaxime and 25 mg/L hygromycin. Finally, regenerated shoots are transferred to the proliferation medium for maintenance and multiplication with subculturing every 4–6 weeks. All media apart from the liquid callus induction medium are solidified with 3 g/L gelrite. Figure 3 describes the generation of genome-edited banana plants targeting the knockout of *phytoene desaturase (PDS)* gene as a marker.

3.5 Delivery of CRISPR-Cas9 Ribonucleoprotein (RNP) Complexes into Banana Protoplast and Regeneration of Complete Plants

Direct delivery of RNPs into plant cells could be achieved by electroporation, particle bombardment, and protoplast transfection by polyethylene glycol (PEG) cell penetrating peptides or mesoporous silica nanoparticle (MSN)-mediated direct protein delivery. Transient expression of a gene has been reported in banana after electroporation or PEG-mediated delivery of DNA into the protoplast [39, 44]. However, the transformation efficiency was quite low.

The PEG-mediated delivery of RNPs into cells is most commonly reported for several crop species. However, there is no protocol reported for the regeneration of complete plantlets after PEG-mediated transfection of banana protoplast. Therefore, the knowledge generated from other crops can be applied for the transfection of RNPs into banana protoplasts and then regeneration of complete plants. The protocol for transfection described here is according to [31, 45]. For the transfection experiment, 5×10^5 of plant protoplasts are resuspended in 200 μL of MMG solution (4 mM MES pH 5.7, 0.4 M mannitol, 15 mM $MgCl_2$). The protoplast suspension is mixed with the 5–20 μL of RNP complex prepared as above. An equal volume (5–20 μL) of freshly prepared PEG solution (40% [w/v] PEG 4000, Sigma no. 95904, 0.2 M mannitol, and 0.1 M $CaCl_2$) is added to the protoplast suspension and mixed gently. The mixture is incubated at room temperature in the dark for 15 min. After incubation, 950 μL of W5 solution (2 mM MES pH 5.7, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl) is added, mixed well by inverting, and centrifuged at 320 × *g* for 3 min. The supernatant is discarded, and the pellet is resuspended gently in 1 mL WI solution (0.5 M mannitol, 20 mM KCl, and 4 mM MES pH 5.7) and incubated in the dark at 25 °C for 24–48 h. After 48 h, protoplasts are collected and regenerated on the regeneration medium to complete plants.

3.6 Molecular Characterization

3.6.1 DNA Extraction

Total DNA is extracted from the leaves of wild-type and putative edited events using modified cetyltrimethylammonium bromide (CTAB). About 100 mg of fresh leaves sample is ground to a fine powder using mortar and pestle. The ground tissue is transferred to 2 mL Eppendorf tubes and 500 μL of extraction buffer (100 mM Tris–HCl, pH 8.0; 20 mM EDTA (pH 8.0); 1.4 M NaCl; 2% PVP-10; 0.8% cetyltrimethylammonium bromide and 0.001% mercaptoethanol) is added. The samples are vortexed vigorously, incubated at 65 °C for 30 min, and then centrifuged for 10 min at 30,000 × *g*. The supernatant is subsequently mixed with an equal volume of chloroform-isoamyl alcohol (24:1) and the mixture centrifuged at 30,000 x *g* for 10 min. After a second chloroform-isoamyl alcohol extraction, the supernatant is mixed with an equal volume of isopropanol and incubated at room temperature for 10 min. Nucleic acids are pelleted by centrifugation as described above. The pellets are washed with 70% ethanol, air-dried for 30 min, and resuspended in 50 μL of TE buffer containing RNase. The samples are stored at −20 °C. The concentration and quality of the DNA is checked with Nanodrop.

3.6.2 PCR Analysis to Confirm Integration of Cas9 Gene

The integration of the *Cas9* gene should be confirmed in the events generated through *Agrobacterium*-mediated transformation using plasmid constructs. PCR analysis is performed with genomic DNA using primers specific to the *Cas9* gene (Fig. 4a). The reaction is set up in a 20 μL reaction volume containing 1 μL genomic DNA (100 ng/μL), 10 μL of HotStarTaq master mix, 1 μL of 10 μM of each primer, and 7 μL nuclease-free water. PCR amplification conditions will depend upon the primers used. After amplification, 10 μL of PCR product is resolved on 1% agarose gel stained with gel red.

3.6.3 PCR Analysis to Detect Band Shift

If the edited events are generated using two gRNAs and Cas9 cuts simultaneously at both target sites, a large fragment gets deleted, which can be detected by resolution of amplicons on agarose gel as band shift (Fig. 4b). Primers are designed flanking the gRNAs using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The targets are amplified using these primers, resolved on 1% agarose gel, stained with gel red, and checked for shorter band in comparison to wild-type non-edited control.

3.6.4 T7 Analysis

T7 endonuclease I (T7E1) assay should be performed to detect the smaller indels (Fig. 4c). The DNA fragments containing the targeted sites are amplified by PCR using a pair of primers flanking the gRNAs and Phusion High-Fidelity DNA Polymerase (NEB). The PCR product is then denatured-annealed at 95 °C for 5 min, ramped down to 25 °C at 0.1 C s^{-1}, and incubated at 25 °C for another 30 min. The annealed PCR products are then digested with

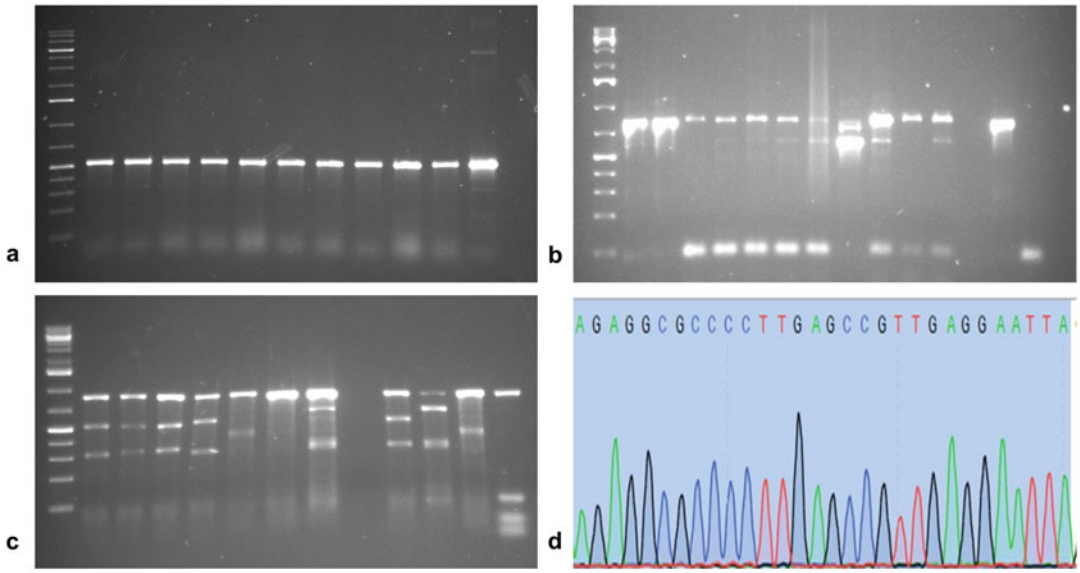

Fig. 4 Molecular characterization of genome-edited events of banana. (**a**) PCR analysis to confirm the presence of *Cas9* gene. (**b**) Detection of band shift in edited events by PCR. This is only possible if Cas9 cleaves the two gRNAs simultaneously in case of multiplexing. (**c**) T7 endonuclease I assay to detect mutations in edited events. (**d**) Sanger sequencing of edited events to detect the mutations

5 U of T7E1 for 2 h at 37 °C. The T7E1-digested products are separated on1% agarose gel stained with gel red.

3.6.5 Sequencing to Detect Targeted Mutations

The targeted mutations in the edited events can be further confirmed by sequencing [18] (Fig. 4d). The PCR-amplified fragment of the target site is purified with QIAquick PCR Purification Kit (Qiagen) according to the instruction manual. The purified products are cloned into *pCR™8/GW/TOPO®* according to the manufacturer's instruction, transformed into DH5α chemical competent *E. coli* cells, and selected on LB plates containing spectinomycin. About ten clones for each event are prepared for Sanger sequencing. The purified PCR products can be directly (without cloning) sequenced by next-generation sequencing. The sequencing reactions are prepared in a 96-well plate and sent for sequencing. The sequencing data are analyzed using software such as SnapGene (WWW.snapgene.com) or vector NTI [46]. The sequences of edited events and wild-type non-edited control are aligned to detect indels and type of mutations (deletions, additions, or/and replacements) at the target site(s).

3.6.6 Evaluation of Off-Target Mutations

There are several tools available for the detection of potential off-target mutations. We use the Cas9/gRNA analysis software Breaking-Cas (http://bioinfogp.cnb.csic.es/tools/breakingcas/) [47] to check for potential off-target sequences [18]. Further,

each gRNA including the PAM sequence is blasted against banana genomes A and B using the BLASTN program in the Banana Genome Hub (http://banana-genome-hub.southgreen.fr/organism/Musa/acuminata-ssp.-burmannica) to identify the genes with potential off-target sites. Once potential off-target sites are identified, if any, the fragment having these sites are amplified using the primers flanking the potential off-target sites. The PCR products are purified and sequenced to confirm the off-target mutations. The sequencing data is then analyzed using SnapGene software, Vector NTI, or any sequence analysis and design tool. From our experience, although the software usually picks potential off-targets, in most cases, such off-targets are not detected by sequencing. This is because we usually select gRNAs with minimal or no potential off-target effects.

3.7 Phenotyping of Genome-Edited Events

Phenotyping of edited events depends upon the target traits. This section describes the methods for phenotyping of genome-edited events developed for resistance to banana streak virus (BSV) and banana Xanthomonas wilt (BXW).

3.7.1 Phenotyping for BSV

BSV is a badnavirus which is integrated into the banana genome and known as endogenous BSV (eBSV). The plants with integrated eBSV do not show any disease symptoms. These proviruses can be activated into the infectious episomal BSV by various stress conditions. When the banana plants are stressed, the eBSV produces a functional episomal viral genome and infectious viral particles, and as a result, the plant develops disease symptoms. Environmental conditions such as water stress triggers its activation resulting to infection. The genome-edited banana plants were developed by knocking out the viral genome of eBSV integrated into the host genome [48]. To phenotype for this disease, edited plants confirmed by sequencing are acclimatized and grown in pots in the greenhouse. About 4-month-old potted plants are subjected to water stress for 2 weeks to induce BSV symptoms [48]. At the end of the stress period, the plants are assessed by visual observation for the presence or absence of BSV symptoms, which appeared in the form of broken or continuous streaks on the leaf lamina and vary from yellow to chlorotic, black, or brown color. The wild-type non-edited control plants are included in all the experiments. The BSV symptoms are further confirmed by molecular diagnostics to confirm the presence of a virus [48].

3.7.2 Phenotyping for BXW

The edited events developed with the aim to develop resistance to BXW caused by *Xanthomonas campestris* pv. *musacearum* (Xcm) can be phenotyped using in vitro plantlets under laboratory conditions or potted plants in glasshouse [49, 50]. Bacterial culture is grown for 48 h in YTS media composition (1% yeast extract, 1% tryptone, 1% sucrose). The culture is then centrifuged for 5 min

at 3,560 × *g*. The supernatant is discarded, and the pellets are dissolved in sterile water to $OD_{600} = 1.0$. For rapid assay using in vitro plantlets, 100 μL of the fresh bacterial culture is injected in the pseudo stem of 4-week-old well-rooted plantlets using an insulin syringe. Inoculated plantlets are incubated at 26 ± 2 °C in the growth room and checked for the presence of symptoms as yellowing, necrosis, and complete wilting of plants up to 6 weeks [49].

For glasshouse screening, regenerated plants are weaned in a disposable cup containing sterile soil and incubated in the humid chamber for 6 weeks and transferred to pots. About 100 μL of the fresh bacterial culture is injected in the first fully opened leaf of 3-month-old plants. The plants are assessed for 8 weeks for the development of disease symptoms of chlorosis or necrosis of the leaves and final symptoms of complete wilting of the plants [50] with preliminary symptoms. The relative resistance of transgenic plants to BXW is evaluated 8 weeks after inoculation based on the reduction in wilting in comparison with control non-edited plants.

The wild-type control plants are included in all the experiments.

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