Robust CRISPR/Cas9 mediated genome editing tool for banana and plantain (Musa spp.)

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Robust CRISPR/Cas9 mediated genome editing tool for banana and plantain (Musa spp.)

Running title: Genome editing of banana and plantain

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Highlights

- CRISPR)/Cas9 system enables targeted and precise editing of banana and plantain genome
- Efficient CRISPR/Cas9 based genome editing protocol developed for banana and plantain using multiple gRNAs targeting phytoene desaturase (PDS) gene
- Edited plants showed albino and variegated phenotypes indicating mutations disrupting function of PDS
- Robust gene editing tools pave the way for the genetic improvement of banana and plantain, which are major staple food crops cultivated in Africa

Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system enables precision editing of the genome of many plant species. Developing robust gene editing tools in banana and plantain would pave the way for the improvement of these crops. Here, we developed efficient CRISPR/Cas9 genome editing protocol for banana and plantain using multiple gRNA targeting phytoene desaturase (PDS) gene. CRISPR/Cas9 construct containing two gRNAs was delivered into embryogenic cell suspension cultures of banana cultivar 'Sukali Ndiizi' and plantain cultivar 'Gonja Manjaya' by Agrobacterium-mediated transformation. The regenerated genome-edited events of 'Sukali Ndiizi' and 'Gonja Manjaya' showed albino and variegated phenotypes indicating mutations at the targeted sites disrupting the function of PDS gene. The majority of events (52/77 for 'Sukali Ndiizi' and 16/17 for 'Gonja Manjaya') were albino. Sequencing of the target sites confirmed presence of indels in all the 18 events sequenced demonstrating mutation efficiency of 100 % in both cultivars. The majority of events (6/8) of 'Gonja Manjaya' showed indels at both the target sites of PDS gene, however only 2/10 events of 'Sukali Ndiizi' showed indels at both sites, with one event (S24) having a knockout of large fragment (723 bp) indicating that both gRNAs were effective. Several of the albino events of both 'Sukali Ndiizi' and 'Gonja Manjaya' showed homozygous mutations. Further sequencing of four potential off-target sites in five events showed no mutations indicating CRISPR/Cas9 based editing in banana and plantain is targeted and precise with a very low probability of off-target sites. This study could provide a methodological framework for single or multiple knockouts in banana and plantain.

Keywords: Banana, plantain, genome editing, CRISPR/Cas9, multiplexing, phytoene desaturase

1. Introduction

Genome editing technologies using specific nucleases have been developed as effective genetic engineering methods for targeted mutations at specific sites in the genome of an organism [1]. CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated protein 9) based genome editing is the emerging most powerful tool with ability to create targeted mutations in the genome of crops and to understand the functional biology. These nucleases respond in a target-specific manner and induce a double-strand break (DSB) in the gene sequence [2]. The DSB is repaired either by homologous recombination (HR) or error-prone non-homologous end-joining (NHEJ) mechanisms, which may lead to disruption of gene function [3].

CRISPR/Cas9 system has emerged as a potent genome editing tool due to its simplicity, design flexibility, and high efficiency, and has been applied successfully in several plant species [4]. It is based on the type II CRISPR/Cas immune system of bacteria that protects against invading DNA viruses and/or plasmids. The CRISPR/Cas9 molecular immunity system is made up of the Cas9 endonuclease from Streptococcus pyogenes and a single guide RNA (sgRNA). The sgRNA directs the Cas9 endonuclease to a target sequence complementary to the 20 nucleotides preceding the protospacer-adjacent motif (PAM) NGG or NAG, which is required for Cas9 activity [5-6]. Multiplexing of CRISPR/Cas9 system by integrating two or more gRNAs, which are distance apart, is useful in generating large deletions in plants [7].

Banana including plantain (Musa spp.) is one of the world's staple food crops feeding millions of people and cultivated particularly by small holder farmers for home consumption and local or regional markets. Only about 15% of its total production goes to international markets. They are grown in more than 140 countries and ranked among the world's most valuable

agricultural commodities with a total production of 148 million ton (Mt) [8]. They are cultivated in different types of environments and produce fruits throughout the year making them important food security and cash crop [9]. One-third of its global production is from Africa with East Africa being the largest banana-producing and -consuming region.

Banana and plantain are polyploidy and originated from two wild diploid species, Musa acuminata (AA) and Musa balbisiana (BB) [10]. There are different types of bananas such as dessert (AAA, AAB group), cooking (AAA, AAB group), roasting (AAB group) and brewing (ABB group) types [11]. There are many cultivars of banana and plantain grown and consumed worldwide, but the 'Cavendish' (AAA group) type of dessert bananas are commonly grown by large-scale farmers for local, regional and international markets. However, other dessert banana varieties such as 'Sukali Ndiizi' (AAB group) and 'Gros Michel' (AAA group) are grown by small-holder farmers in Africa. 'Sukali Ndiizi', referred as "Apple banana", is commonly grown in East Africa. Plantain (AAB group) and East African Highland banana (EAHB, AAA group) are grown by small-holder farmers largely in Central and West Africa and East Africa respectively. **'**Gonja Manjaya', a roasting variety, is a false horn plantain commonly grown in Central and East Africa.

Despite so much importance, production of banana and plantain is seriously affected by several factors particularly biotic and abiotic stresses. Diseases and pests are one of the major factors affecting yields worldwide. There is a big yield gap in banana production in the areas where several of the pathogens and pests are present together [12]. In addition, climate change is also having a significant effect on banana yields, particularly in African countries where the crop is grown with minimal or no irrigation [13]. The improvement of banana and plantain will be of great importance for improving food security as these crops feed more people per unit area of

production than cereal crops [14]. Developing improved varieties of banana and plantain using traditional breeding is challenging due to low genetic variability in Musa germplasm, sterility of majority of the cultivars commonly grown by farmers, polyploidy, and lengthy production cycle [15]. The application of innovative biotechnological approaches such as genome editing can complement traditional breeding for developing improved varieties of banana and plantain with resistance to diseases and pests, tolerance to abiotic stresses and higher yields. However, this requires robust genome editing tool which can be applied for both banana and plantain.

The phytoene desaturase (PDS) gene has been used as a marker to establish CRISPR/Cas9-mediated genome editing in several plant species [16-21]. PDS is a key enzyme in the carotenoid biosynthesis pathway, catalyzing the desaturation of colorless phytoene into ζ-carotene, which is further converted into lycopene, a colorful compound in the pathway [22]. The disruption of PDS affects photosynthesis, gibberellin and carotenoid biosynthesis causing albinism and dwarfing.

Recently, genome editing has been demonstrated in banana cultivar 'Rasthali' (AAB group) using a single gRNA targeting the PDS gene [23]. However, the reported mutation efficiency (59%) was quite low. Further, Naim et al. [24] reported genome editing of 'Cavendish' banana (AAA group) using CRIPSR construct with two gRNAs targeting the PDS gene. Although the authors obtained a mutation efficiency of 100%, only one gRNA was effective. Currently, genome editing capacity is mostly restricted to advanced laboratories in the USA, Australia, Asia and Europe; and its execution in African laboratories has remained limited. Considering that banana and plantain are a major source of food security and income in Africa, it is imperative to develop highly efficient genome editing protocol which could be applied for improvement of various cultivars of these crops. Here, we report successful establishment of a robust genome editing platform for banana and plantain at International Institute of Tropical Agriculture (IITA)

hosted by Biosciences eastern and central Africa (BecA) hub in Nairobi, Kenya using the multiplexed CRISPR/Cas9 system. The robust and improved CRISPR/Cas9 based genome editing system was developed targeting the PDS gene with the same multiplexed gRNAs in both banana and plantain cultivars commonly grown in Africa. Multiplexing of gRNAs targeting the conserved region of the PDS gene resulted in high mutation efficiency of 100% multiple knockouts or deletion of big fragment (about 723 bp). Analysis of the edited events for potential off-target sites using web tool Breaking-Cas and BLASTN program in the Banana Genome Hub indicated a very low probability of off-target mutations. Further sequencing of four off-target sites in five edited events confirmed the mutations in the PDS gene to be targeted and precise. This study provides a methodological framework for genome editing of banana and plantain for knocking out of single or multiple targets.

2. Methodology

2.1. Plant materials

Embryogenic cell suspension of banana cultivar ‘Sukali Ndiizi’ (AAB) and plantain cultivar ‘Gonja Manjaya’ (AAB) were used in this study.

2.2. Target gene and gRNA design

PDS gene sequences were downloaded from banana genome A (Musa accuminata, gene Ma08_g16510) and genome B (Musa balbisiana, gene ITC1587_BchrUn_random_T36676) from the Banana Genome Hub [25]. The PDS gene sequences from A and B genome were aligned using Multalin [26] to identify conserved region. Two gRNAs were designed from the conserved region of exon 5 and 6 of A genome and exon 2 and 3 of B genome using ATUM CRISPR DNA

Design Tool (https://www.atum.bio). These gRNAs (gRNA1: GTATCAATGATCGCTTGCAA and gRNA2: TTTTGCCAGCCATGCTTGGA) targeting the PDS gene in both A and B genomes were 723 bp apart. The gRNAs were chosen based on their specificity to their targets with minimum possibility of off target effect. The oligos with gRNAs and the corresponding reverse sequences were synthesized after adding appropriate adaptors at the 5' end (supplementary Table S1) to enable cloning in the gRNA expression plasmids.

The gRNAs were also blasted against the PDS gene sequences from 'Rasthali' and 'Cavendish' bananas to check if they are conserved across different banana cultivars. The PDS gene sequences for 'Rasthali' (AAB) (Genbank accession No. MF574096.1 and Genbank Accession No. MF574097.1) [23] and 'Cavendish Williams' (AAA) (Genbank accession No. JQ762260.1) were downloaded from National Center for Biotechnology Information (NCBI).

To rule out any allelic variations in the target sites of PDS gene in the cultivars used in our study, a fragment (994 bp) of the PDS gene containing both target sites was sequenced. Genomic DNA was extracted from leaves of 'Sukali Ndiizi' and 'Gonja Manjaya' wild-type plants using cetyltrimethylammonium bromide (CTAB) method [27]. The fragment (994 bp) of the PDS gene containing both target sites was amplified using the primers (PDS_F: CAGCTAACTGAGATCAGTTT, and PDS_R: AGATGGCTATATTTCGGTAC) flanking both gRNAs. The primer set was designed based on PDS gene (Ma08_g16510) from Musa acuminata reference genome using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/). This primer set was then blasted to PDS gene sequence (ITC1587_BchrUn_random_T36676) from Musa balbisiana, to confirm 100% homology. This primer was able to amplify a DNA fragment of approximately 994 bp covering both gRNAs in A and B genomes. The PCR reaction was performed in a 20 µl 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 were as follows: initial denaturation at 95°C for 15 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were purified and cloned to pCR8/GW/TOPO® (Invitrogen) according to the manufacturer's instruction, transformed to chemical competent cells of E. coli strain DH5α and selected on LB plates containing spectinomycin. Ten colonies were selected for each cultivar and used for Sanger sequencing using BigDye Terminator v3.1. The sequencing data were analyzed using SnapGene software (www.snapgene.com) for detecting any SNPs in the target sites for each cultivar. Further the sequences of PDS gene fragment from 'Sukali Ndiizi' and 'Gonja Manjaya' were aligned with those of Musa acuminata and Musa balbisiana using Multalin to detect any SNPs in the target sites.

2.3. Construction of CRISPR/Cas9 plasmid

The gRNA expression plasmid pYPQ131C (for gRNA1) and pYPQ132C (for gRNA2) were linearized by restricting with the enzyme BsmBI to produce 4 bp overhang. The linearized products were purified using PCR purification kit. Forward and reverse oligos of each gRNA were phosphorylated and annealed using T_4 polynucleotide kinase according to Lowder et al. [28]. The phosphorylated and annealed oligos were then ligated to the linearized plasmids using T4 ligase at room temperature for 1 h. The ligated products were transformed to E. coli strain DH5α and selected on LB medium containing tetracycline (10 mg/l). Colonies were selected, plasmid DNA extracted and verified by Sanger sequencing.

Clones with the correct insert were selected and the two gRNAs were assembled into the Golden Gate recipient and Gateway vector pYPQ142 by digestion with Bsa1 and then ligating with T_4 DNA ligase. The product was transformed to E. coli strain DH5α and selected on LB medium containing spectinomycin (100 mg/l). Plasmid DNA were extracted from these colonies and verified by restriction digestion with EcoRI and NcoI. One of the positive plasmids resulting from the Golden Gate assembly above and together with the Cas9 entry vector pYPQ167 were cloned into the Gateway binary vector pMDC32 [29] by LR clonase™ (Invitrogen, New Zealand) recombination reaction. The reaction mixture was incubated at room temperature overnight. After incubation, 1 µl of proteinase K was added and incubated at 37°C for 10 min. The reaction mixture was transformed into chemical competent cells of E. coli strain DH5α and selected on LB medium containing kanamycin (50 mg/l). Clones were verified by isolating plasmid DNA and digesting with KpnI. The final CRISPR/Cas9 plasmid pMDC32_Cas9_MaPDS has hpt gene as an in planta selection marker, Cas9 gene and two gRNAs, each driven by the rice Pol III promoter, OsU6. The Cas9 gene used in this plasmid is plant codon optimized and is regulated by double CaMV35S promoter.

The pMDC32_Cas9_MaPDS was mobilized to Agrobacterium tumefaciens strain EHA105 by electroporation and transformed colonies were selected on LB medium containing kanamycin (50 mg/l) and rifampicin (25 mg/l). Plasmid DNA was extracted from resulting transformed colonies and validated by PCR for the presence of Cas9 gene. Validated colony of Agrobacterium tumefaciens strain EHA105 containing pMDC32_Cas9_MaPDS was maintained on LB medium at 4°C and glycerol stocks at -80°C and used for transformation of banana and plantain.

2.4. Delivery of CRISPR/Cas9 plasmid into banana and plantain through Agrobacterium-mediated transformation

Transgenic events were generated by transforming the embryogenic cell suspensions of 'Sukali Ndiizi' and 'Gonja Manjaya' with CRISPR/Cas9 plasmid construct pMDC32_Cas9_MaPDS following the method described by Tripathi et al. [30]. The plantlets regenerated on selective medium were maintained and multiplied by sub- culturing every 8 weeks on proliferation medium [31]. The regenerated plantlets were visualized for the albino phenotype due the disrupted function of PDS.

2.5. Molecular analysis of gene edited plants

2.5.1. PCR analysis to confirm integration of Cas9 gene

Total genomic DNA was extracted from 100 mg of fresh leave samples collected from all the regenerated plants (albino, variegated and green) and wild-type plants using cetyltrimethylammonium bromide (CTAB) method [27]. The concentration and quality of the DNA was checked with Nanodrop and approximate 100 ng of DNA was used as template for PCR analysis of the Cas9 gene using the primers 35S_F: GCCTCTGCCGACAGTGGTCC, and Cas9_R: GCAGATGCGGTTCTTGCGGC. PCR was performed 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 were performed as follows: initial denaturation step at 95˚C for 5 min, followed by 34 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s, extension at 72˚C for 1 min, and final extension at 72˚C for 10 min. After amplification, 10 µl of PCR product was resolved on 1% agarose gel stained with gel red.

2.5.2. PCR analysis to detect band shift

The mutations in the PDS gene were confirmed by PCR band shift analysis using the gene specific primers (PDS_F: CAGCTAACTGAGATCAGTTT, and PDS_R: AGATGGCTATATTTCGGTAC) flanking both gRNAs. The primers were designed as described above. PCR amplification conditions were performed as follows: initial denaturation step at 95°C for 15 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. After amplification, PCR amplicons were resolved on 1% agarose gel, stained with gel red and observed for shift in movement of bands on agarose gel.

2.5.3. Sequence analysis to detect targeted mutations in PDS gene

The PCR products of 994 bp size, as described above, were purified with QIAquick PCR purification Kit (Qiagen) according to the instruction manual and used for Sanger sequencing. The sequencing reaction constituted of 4 µl PCR product, 4 µl of 5X sequencing buffer, 1 µl of Big Dye Terminator, 1 µl of 100 ng/µl of either PDS_F or PDS_R primer and 10 µl of nuclease free water. PCR amplification was performed in thermal cycler with; initial denaturation step at 95˚C for 2 min, followed by 40 cycles of denaturation at 94˚C for 10 s, annealing at 50 ˚C for 10 s, extension at 60˚C for 4 min, and final extension at 72˚C for 4 min. After PCR amplification, the sequencing product was purified by adding 50 µl of 100% ethanol, 2 µl of 3 M Sodium Acetate and 2 µl of 125 mM EDTA, and incubated at room temperature for 15 min. The mixture was centrifuged at 13000 rpm for 25 min. The pellets were washed with 70% ethanol and air dried for 15 min in the hood. The pellets were then resuspended in 10 µl HiDi formamide and

incubated at 65 ˚C for 5 min, 95 ˚C for 2 min and cooled in ice. The samples were then sequenced using ABI 3130 DNA sequencer (Applied Biosystems, California, USA). The target sequences of mutant events were aligned with the wild type sequence using SnapGene software (www.snapgene.com).

2.6. Evaluation of Off-target mutation

The CRISPR/Cas9 analysis web tool Breaking-Cas [32] was used to check the potential off target mutations in the edited events. Each gRNA, a 23 bp-long sequence which included the PAM motif was blasted against banana genome A (Musa acuminata) and B (Musa balbisiana) using the BLASTN program in the Banana Genome Hub [25] to identify the genes with potential off-target sites. Primers were designed specific to four potential off-targets sites (ITC1587_Bchr3_T08224, ITC1587_Bchr5_T14510, ITC1587_Bchr7_T19647, and ITC1587_Bchr10_T29488), which showed high sequence similarity with either the gRNAs alone or gRNAs with the PAM motif (supplementary Table S2). PCR was performed with five edited events (S1, S17, S24, G3 and G11) and wild-type non-edited control using these primers (Table S1). PCR products were purified and sequenced. The sequence chromatograms were analyzed with SnapGene software (www.snapgene.com).

3. Results

3.1. Target sites in the PDS gene

The banana and plantain cultivars, ‘Sukali Ndiizi’ and ‘Gonja Manjaya’, used for genome editing are triploid (AAB). The PDS is supposed to be single copy gene [23] having two alleles on A genome and one allele on B genome.

Complete genome sequences of PDS gene were downloaded from Musa accuminata (A genome) and Musa balbisiana (B genome) from the Banana Genome Hub [25]. The PDS gene (Ma08_g16510) of Musa accuminata is 27,944 bp with 14 exons, while PDS gene (ITC1587_BchrUn_random_T36676) in Musa balbisiana is 1849 bp containing 4 exons. The alignment of these sequences shared 98% nucleotide homology starting from the start to the stop codon of the PDS gene. A conserved block of sequence was identified within this region, which was used to design the gRNAs (Fig. 1, supplementary Fig. S1). The gRNAs were then blasted against PDS gene sequences of other banana cultivars such as 'Rasthali' and 'Cavendish', and both the target sites were found to be conserved with no SNPs (supplementary Fig. S2).

In addition, allelic variations in the target sites of cultivars 'Sukali Ndiizi' and 'Gonja Manjaya' was ruled out by sequencing the cloned 994 bp fragment of the PDS gene. The sequences of all the ten clones of the PDS fragment containing both target sites for each cultivar were same confirming no allelic variations in the target sites (supplementary Fig. S3). Further, aligning the sequences for 994 bp fragment of PDS gene of 'Sukali Ndiizi' and 'Gonja Manjaya' with Musa accuminata and Musa balbisiana showed 97% nucleotide identity with no SNPs in the target sites.

3.2. CRISPR/Cas9 plasmid construct

Two gRNAs were designed targeting the PDS gene based on the most conserved regions of Musa acuminata and Musa balbisiana reference genome using ATUM CRISPR DNA Design Tool (https://www.atum.bio). gRNA1 (GTATCAATGATCGCTTGCAA) and gRNA2 (TTTTGCCAGCCATGCTTGGA) were designed to target exon 5 and 6 of the PDS gene in A genome (Ma08_g16510) (Fig. 2A) and exon 2 and 3 in B genome

(ITC1587_BchrUn_random_T36676) (Fig. 2B). Both the gRNAs were integrated into the T-DNA region of the binary vector pMDC32 producing the CRISPR/Cas9 construct pMDC32_Cas9_MaPDS (Fig. 2C). The detailed method of preparing the CRISPR/Cas9 construct is summarized in Fig 3.

3.3. Generation and validation of transgenic banana and plantain with edited PDS gene

The transgenic events were generated by delivering construct pMDC32_Cas9_MaPDS into embryogenic cell suspensions of banana cultivar 'Sukali Ndiizi' (AAB) and plantain cultivar 'Gonja Manjaya' (AAB) through Agrobacterium-mediated transformation. In total, 81 independent putative transgenic events of 'Sukali Ndiizi' and 18 events of 'Gonja Manjaya' were regenerated on selective media containing hygromycin (25 mg/l).

These events showed different phenotypes; 52 events of 'Sukali Ndiizi' were albino, 20 events were variegated with mosaic pattern and 5 events showed a mixture of albino and variegated leaves (Fig. 4A-C; Table 1). Similarly, 16 events of 'Gonja Manjaya' were albino and 1 event was variegated (Fig. 4D-E; Table 1). There were 5 green events (4 'Sukali Ndiizi' and 1 'Gonja Manjaya') similar to wild-type (Fig. 4F).

All the regenerated events were validated for the presence of Cas9 gene by PCR analysis using gene specific primers. As expected, all the events having the albino and variegated phenotypes showed amplified fragment of 587 bp, confirming the presence of the Cas9 gene in transgenic events of both 'Sukali Ndiizi' and 'Gonja Manjaya' (Fig. 5A and B). All the five green events did not show amplification of Cas9 gene similar to control wild-type plantlet indicating that these events were probably escapes. The PCR analysis confirmed transgenic nature of 77 events of 'Sukali Ndiizi' and 17 events of 'Gonja Manjaya'.

All the PCR positive transgenic events were maintained by sub-culturing on proliferation medium every 8 weeks. The albino plants grew slowly and after several sub-cultures started turning brown. However, the variegated events produced multiple shoots and in several of the variegated events developed mixed shoots with some albino and others a mixture of albino and variegated indicating high level of chimerism with mutations happening at different stages of plant regeneration.

3.4. Molecular analysis of gene edited events

3.4.1. PCR to detect band shift

Two gRNAs targeting PDS gene were introduced into the banana and plantain cultivars. The gRNAs targets were 723 bp apart in the PDS gene of both cultivars, therefore if Cas9 cleaved both the gRNAs simultaneously, a band shift of about 723 bp was expected. All the regenerated events confirmed for the presence of the Cas9 gene, were analyzed for shift in size of amplicons using primers flanking the two gRNAs. Only one event (S24 event of 'Sukali Ndiizi') showed the predicted band shift of 723 bp (Fig. 5C) suggesting simultaneous cleavage at both target sites. This event also showed a second amplicon similar to that of the wild-type control suggesting heterozygous mutations. All the other events of both the cultivars showed amplicons similar to that of the wild-type indicating small or no indels (Fig. 5C and D).

3.4.2. Detection of mutations by sequencing

Ten PCR positive events of 'Sukali Ndiizi' (S1, S2, S6, S12, S17, S21, S24, S36, S46, and S50) and 8 events of 'Gonja Manjaya' (G3, G4, G5, G6, G7, G8, G9 and G11), which were tested by PCR for band shift, were selected for further analysis by sequencing to detect

mutations. Two escape plants (ES19 and EG13) along with wild type controls were also included as control along with wild type plant. Sequencing of the purified PCR products confirmed targeted mutations in all the events with albino and variegated phenotypes. As expected, the green escape events (ES19 and EG13), which also did not show Cas9 amplification, were similar to wild type control with no mutations confirming that all the indels in the transgenic events were due to the editing of the target sites (Fig. 6 and 7). All three types of mutations, deletions, insertions and substitutions were observed in the events sequenced. Seven events of 'Sukali Ndiizi' (S2, S12, S17, S21, S36, S46 and S50) showed only insertion (+1-3 bp), while both insertion (+3-178 bp) and deletion (Δ2-723 bp) were observed in three events (S1, S6 and S24) (Fig. 6). The mutations were 3 bp upstream of the PAM sequence in the majority of events. Out of all the events analyzed, only event S24 showed deletion of large fragment of 723 bp confirming the result of band shift analysis. Some of the amplicons of S24 showed addition of 178 bp and deletion of 182 bp indicating the allelic variations in this event.

All events of 'Gonja Manjaya' showed indels with small deletions, insertions and/or substitutions. Six events (G3, G4, G5, G6, G7 and G8) of 'Gonja Manjaya' showed indels with insertion (+1 bp) and substitution (s1 bp) (Fig. 7). Event G9 showed all types of mutations, insertion (+3 bp), deletion (Δ2 bp) and substitution (s1 bp), whereas event G11 had insertion (+8) and deletion (Δ11 bp). Several albino events for both 'Sukali Ndiizi' (S2, S6, S12, and S36) and 'Gonja Manjaya' (G4, G5, G6, G7 and G8) showed same mutations for all the replicates sequenced confirming the homozygous mutations in all the functional alleles of PDS gene.

The majority of events (6/8, G3, G4, G6, G7, G8, and G9) of 'Gonja Manjaya' and 2/10 events (S21, and S24) of 'Sukali Ndiizi' showed indels at both target sites of the PDS gene confirming that both gRNAs were effective. These results confirmed that the albino and

variegated phenotype of the events were due to mutations disrupting the function of PDS gene through the action of gRNAs/Cas9.

3.5. Off-target analysis

Even though the gRNAs were selected based on minimal potential for off-target effect based on the ATUM CRISPR DNA Design Tool. The gRNAs used for generating edited events were further confirmed for low potential off-target hits using the CRISPR web-tool Breaking-Cas [32]. This tool identified 12 and 14 off-target sites hitting 7 and 10 Musa genes for gRNA1 and gRNA2, respectively. To further get the details on the off-targets hit in the A or B genome, the gRNA+PAM (23 nt) sequences were blasted against the respective banana genomes. The blast search resulted in 3 and 7 putative off-targets for gRNA1 and gRNA2 respectively, for the A-genome (M. accuminata) and 5 and 12 potential off-targets in B genome (M. balbisiana), for gRNA1 and gRNA2, respectively (Table 2, supplementary Table S2). All the potential off-target sites had matches of 60-82% to the gRNA+PAM sequences. Sequencing of four potential off-target sites in five events showed no mutation in any of the events. Our results showed no or very low chances of off target effect for the gRNAs used for editing of banana and plantain.

4. Discussion

Banana and plantain are among the most important staple food crops in Africa. Application of genome editing has huge potential for developing improved varieties of banana and plantains addressing the challenges with biotic and abiotic stresses and closing the yield gap particularly in Africa. In this study, we established CRISPR/Cas9 based gene editing system for both banana and plantain targeting the PDS gene as marker. PDS regulates key enzymes in the

biosynthesis of carotenoids and its disruption leads to albino phenotype [33]. Therefore, PDS gene has been used as a marker to establish genome editing platform in many plant species [20, 23-24, 34-36]. In order to maximize the chances of mutations and also deletion of large fragment, we developed a CRISPR/Cas9 construct expressing two gRNAs targeting two different exons of PDS gene in both cultivars. This construct was delivered into embryogenic cells of banana and plantain using existing transformation system at IITA [37]. Albino phenotype was observed in several of edited events generated for both the cultivars 'Sukali Ndiizi' and 'Gonja Manjaya' (Fig. 4). Our results are in line with previous reports from other plant species [18-19]. The frequency of generation of uniform albino mutants with no variegation was very high, 67-94% of the regenerated transgenic events of 'Sukali Ndiizi' and 'Gonja Manjaya', respectively (Table 1). We also obtained edited plants with mixture of variegated and albino leaves, which might be due to chimeric mutations happening at different stages of plant regeneration. As the delivery of CRISPR/Cas9 reagent in this study was plasmid based through Agrobacterium-mediated transformation, it remained integrated into the plant genome and editing should have been continued from within a given cell until the target site was mutated. This could have resulted in chimeric plants consisting of cells with no mutations or carrying different mutations. Similar results are reported in other crops such as rice [38]. In order to avoid this type of pattern in future, we are developing DNA-free genome editing system for banana and plantain by delivering preassembled Cas9 protein-gRNA ribonucleoproteins (RNPs) directly into plant cells. These RNPs directly edit the target sites immediately after delivery and then are rapidly degraded by endogenous proteases in cells, thus reducing the chances of generation of plants with chimeric mutations [39]. The major advantage of this system is that the RNP based editing leads to generation of transgene-free mutants, which are not regulated in several countries.

All the regenerated events were validated by PCR analysis amplifying Cas9 gene. Sequencing of 18 PCR positive transgenic events showed a mutation frequency of 100% in both the cultivars 'Sukali Ndiizi' and 'Gonja Manjaya'. All the mutant plants showed frameshift mutations preventing proper translational of PDS and subsequently resulting in photo-bleaching leading to albino phenotype. Similar result with high mutation rate was obtained in our previous study of knocking out of endogenous Banana Streak Virus (eBSV) integrated in 'Gonja Manjaya' [40]. The high mutation frequency recorded in this study is also similar to that reported for 'Cavendish' banana by Naim et al. [24]. However, Kaur et al. [23], reported a significantly low (59%) mutation frequency using CRISPR/Cas9 to edit PDS in the banana cultivar 'Rasthali', which is in the same genomic group (AAB) as 'Sukali Ndiizi' and 'Gonja Manjaya'. Differences in mutation frequencies in various plants have been shown to reflect a variety of factors that influence NHEJ-mediated mutagenesis. Prominent among them is the promoter that direct the spatiotemporal expression of Cas9 gene and gRNAs, methods of Cas9/gRNA delivery that result in different transgene copy numbers, the specific target gene sequence and chromosomal location, which affects the accessibility of the target gene to Cas9/gRNA to cause double strand breaks [41]. Our result indicates that the protocol developed here is very efficient for genome editing of banana cultivars containing both A and B genome.

In this study, indels mostly occurred at 3 bp upstream of the PAM sequence in the majority of events irrespective of the cultivar. All types of mutations (deletion, insertion and substitution) were observed in the edited events. About 50% events (9/18) sequenced for both cultivars 'Sukali Ndiizi' (S2, S6, S12, and S36) and 'Gonja Manjaya' (G4, G5, G6, G7 and G8) showed homozygous mutations fully disrupting the function of PDS leading to albino phenotype. Simultaneous expression of two or more gRNAs (multiplexing) has been reported to enhance

mutation efficiency as well as creating large mutations. Here, we expressed two gRNAs and observed high mutation efficiency of 100% and also a large deletion of 723 bp in one of the events indicating that both gRNAs were effective (Fig. 6 & 7). Even though both gRNAs showed efficacy, their mutation potency differed with cultivars. All events of 'Sukali Ndiizi' showed mutations in gRNA1 target, however only 2 events (S21 and S24) had mutations at both targets (Fig. 6). On the contrary, majority of events (6/8) of 'Gonja Manjaya' had mutations in both target sites. Differences in mutations at both target sites between the two cultivars could be due to inherent differences in the genome of these cultivars. Sequencing result with 18 events suggested that gRNA1 was probably a more effective guide than gRNA2. A similar study by Naim et al. [24] using two gRNAs targeting PDS gene in 'Cavendish' banana (AAA group) reported that none of the events showed the predicted band shift as only one gRNA was effective. In our study, both gRNAs were found to be effective, but the frequency of deletion of a large fragment is low. This system, however, demonstrates that deletion of large gene fragment of 700 bp or larger is possible in banana using CRISPR/Cas9.

The effectiveness of both gRNAs in this study suggested the designing of gRNA and selection of guide with high efficiency is critical step in genome editing. First we designed the gRNA using the ATUM CRISPR Design Tool, which is a powerful tool for selecting gRNAs with predicted high efficiency score. gRNA1 had 100% efficiency and gRNA2 had 64% efficiency based on ATUM CRISPR Design Tool. This might explain the reason for gRNA1 to be more effective in comparison to gRNA2.

In Cas9-mediated genome editing the possibility of off-target mutations in the regions similar to the target sites is a major limitation. To minimize off-target effects, the gRNAs were selected based on their specificity to the target site and minimal potential off targets in the Musa genome.

Sequencing of selected off-target sites did not show any mutations in the events. Our results showed no or very low potential off-target effect indicating that CRISPR/Cas9 induced mutagenesis in banana is precise and efficient.

5. Conclusion

We have succeeded in establishing CRISPR/Cas9 based genome editing platform in banana and plantain targeting the PDS gene as marker. This is the first study reporting disruption of PDS in two different banana groups with same CRISPR-Cas9 cassette, and generation of mutants with the deletion of larger fragment (Δ723 bp). The genome editing tool established in our laboratory at IITA-Kenya paves the way for functional genomics and developing improved varieties by knockout single or multiple genes of banana and plantain, which are important staple food crops. This platform can also provide facility for capacity building of researchers in Africa in new breeding tools.

Authors' contribution

L.T. conceived the original concept and led the study. V.O.N. designed the gRNAs and prepared the plasmid construct. J.N.T. generated the genome-edited events. V.O.N. and J.N.T. performed molecular characterization of edited events. All authors wrote the manuscript.

Conflict of Interest

The authors declare no competing interest.

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Figure legends

Fig. 1. Alignment of partial sequences of PDS gene from reference genome of Musa accuminata (AA) and Musa balbisiana (BB). Only the segment containing the gRNAs and primers used for sequencing is shown here. Brown nucleotides indicate primers flanking the gRNAs which were used for sequencing, red nucleotides indicate gRNAs, green nucleotides indicate PAM (protospacer adjacent motif) segment and blue nucleotides indicate mismatches between M. accuminata (Ma) and M. balbisiana (Mb).

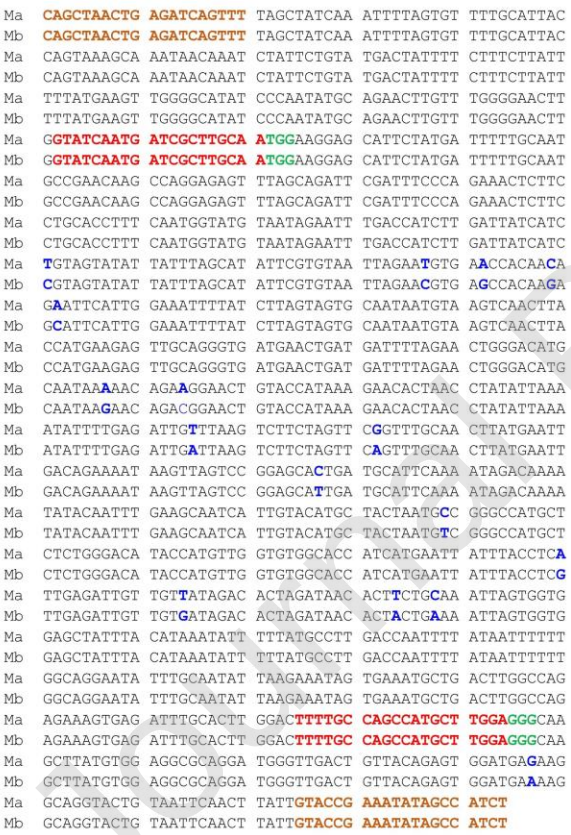


Fig. 2. Schematic representation of the PDS gene and CRISPR/Cas9 construct used for editing of banana. A-B) Position of gRNAs for targeting the PDS gene in both A and B genome of banana. Blue boxes represent exons; digits indicate exon number while introns are indicated by line. gRNAs and PAM (Protospacer Adjacent Motif) sequences are indicated in red and green respectively. C) Schematic map of T-DNA region of binary vector pMDC32_Cas9_MaPDS. LB, Left border; RB, Right border, hpt, hygromycin phosphotransferase gene; 35S P, CaMV35S promoter, OsU6 p, Oryza sativa U6 promoter; 2X35S P, double CaMV35S promoter. Primers used for amplification of fragment of Cas9 gene for validation of transgenic events are indicated.

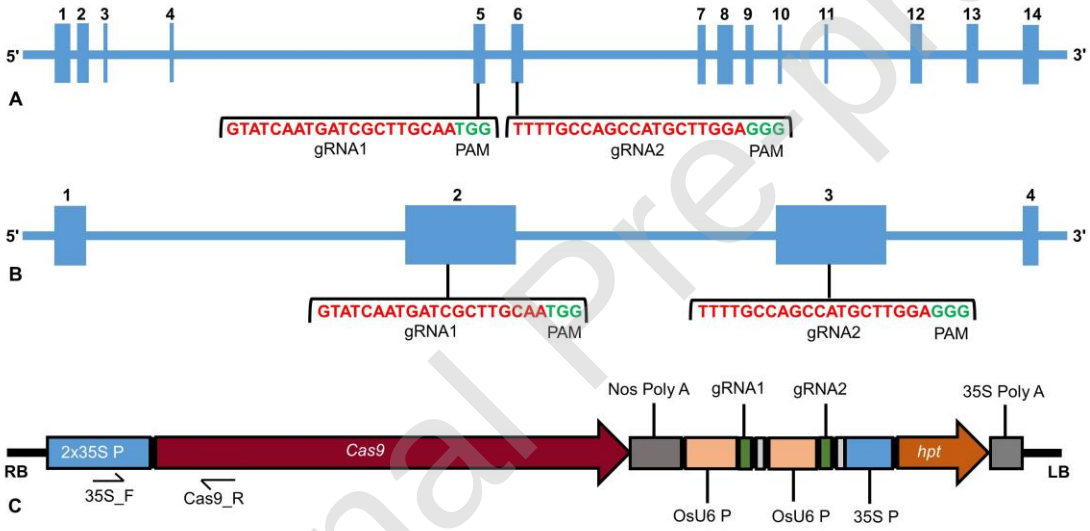


Fig. 3. Schematic flow chart showing steps for developing the CRIPSR/Cas9 construct.

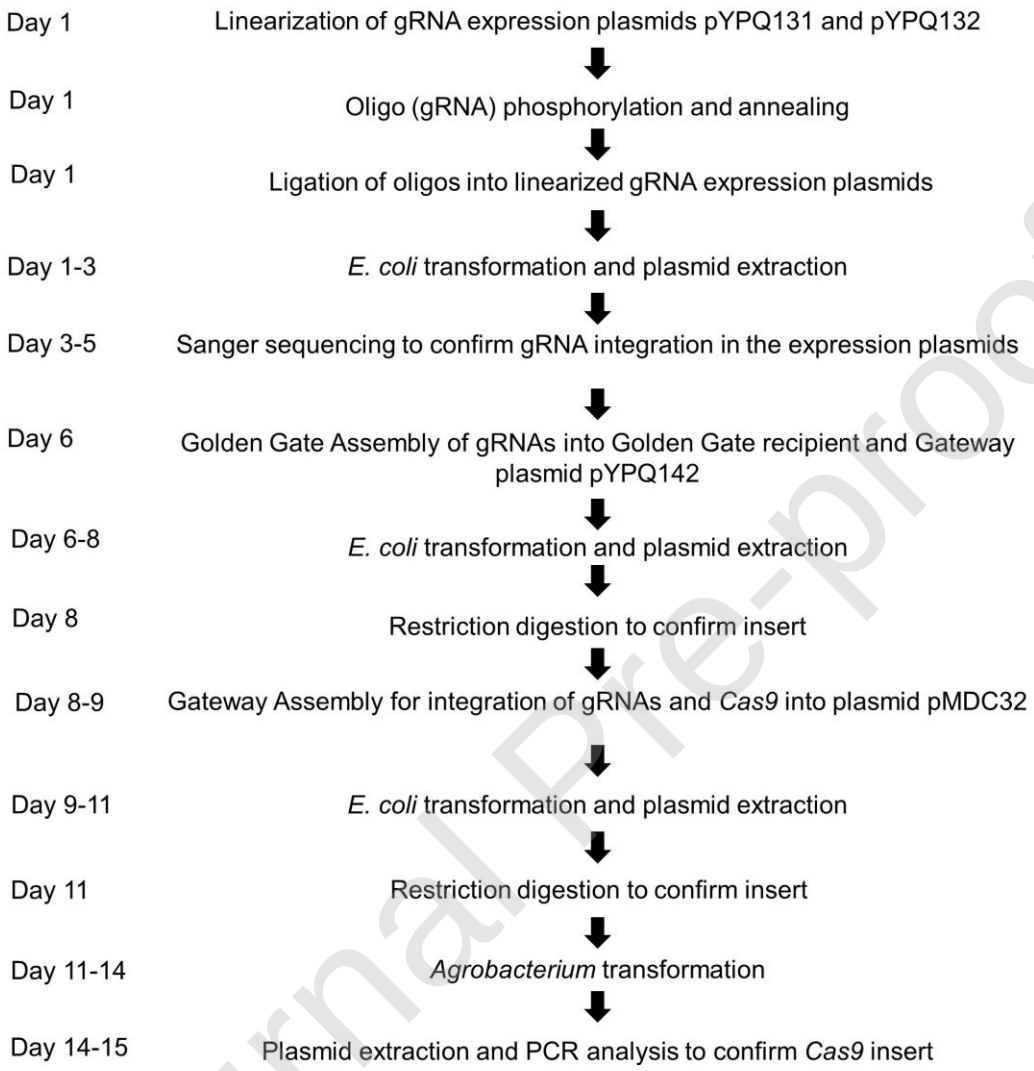


Fig. 4. Genome edited events of ‘Sukali Ndiizi’ (upper panel) and ‘Gonja Manjaya’ (Lower panel) showing albino phenotype. A & D) Albino plants, B & E) Variegated plants, C) Mixture of albino and variegated phenotype, F) Wild-type control.

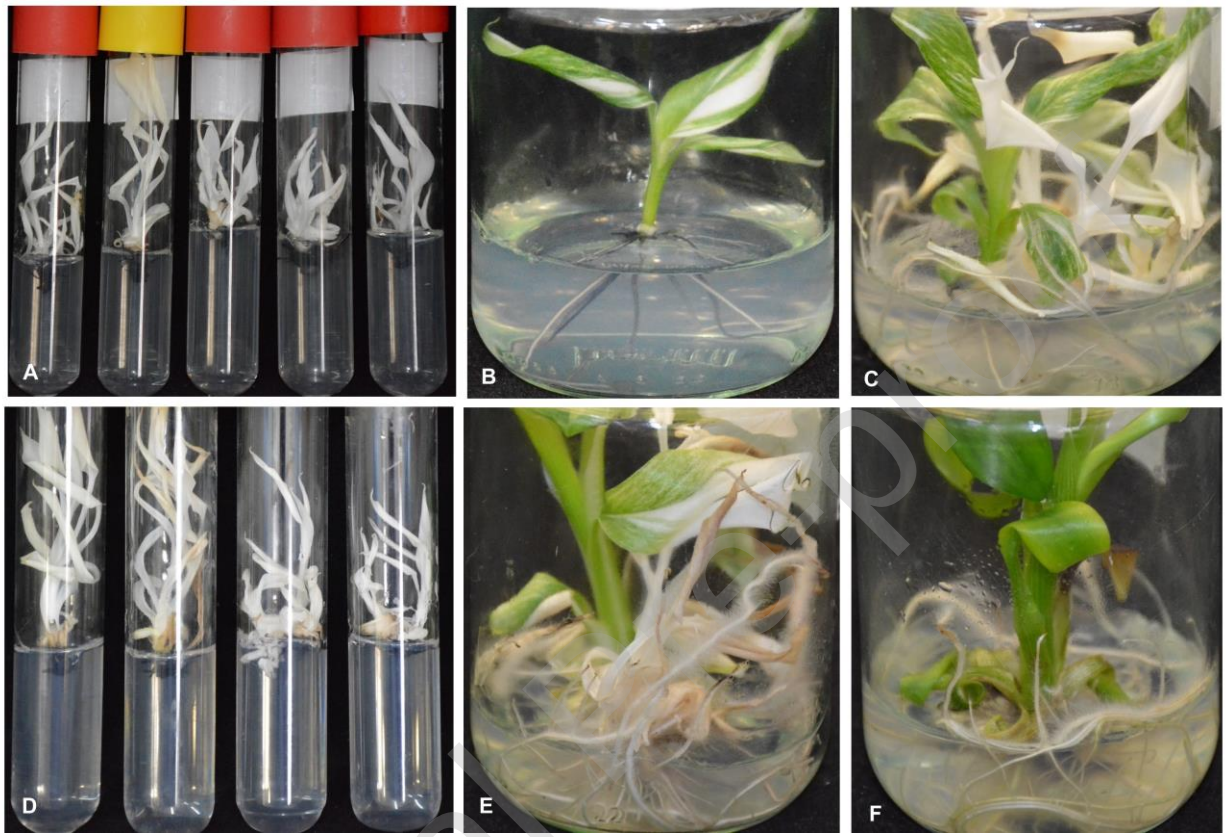


Fig. 5. Molecular characterization of genome edited events of 'Sukali Ndiizi' (upper panel) and 'Gonja Manjaya' (Lower panel). A-B) PCR amplification to confirm the presence of Cas9 gene, C-D) PCR analysis to detect band shift in edited events. If Cas9 cleaved the two gRNAs simultaneously, mutant events were supposed to have band shift of 723 bp compared to the wild type control plant. M, molecular marker; P, positive control plasmid pMDC32_Cas9_MaPDS; 1-50, independent edited events; ES19 and EG13-escape plants; WT, wild type non-edited control plant; NTC, no template control.

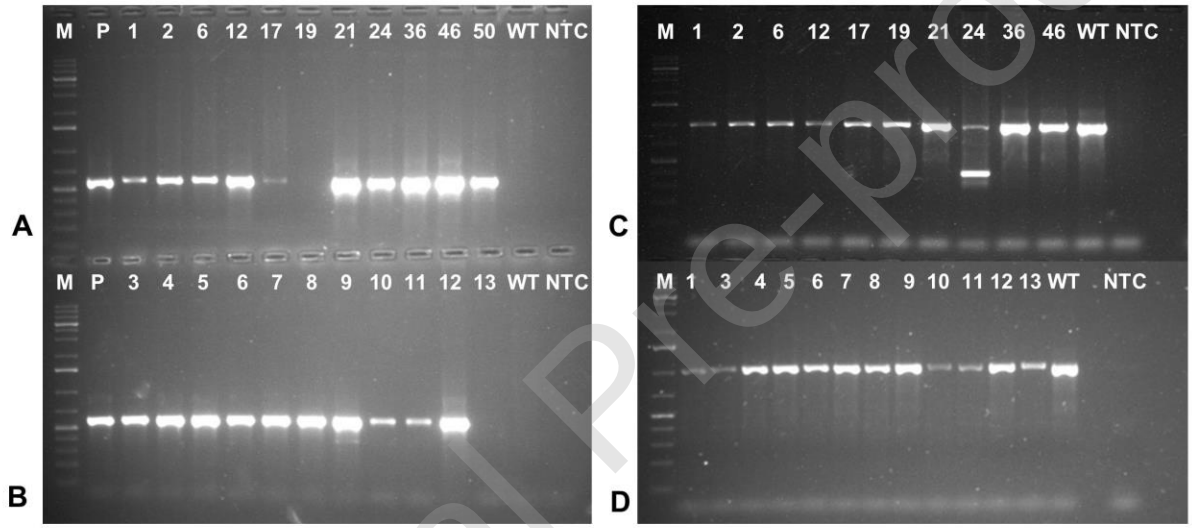
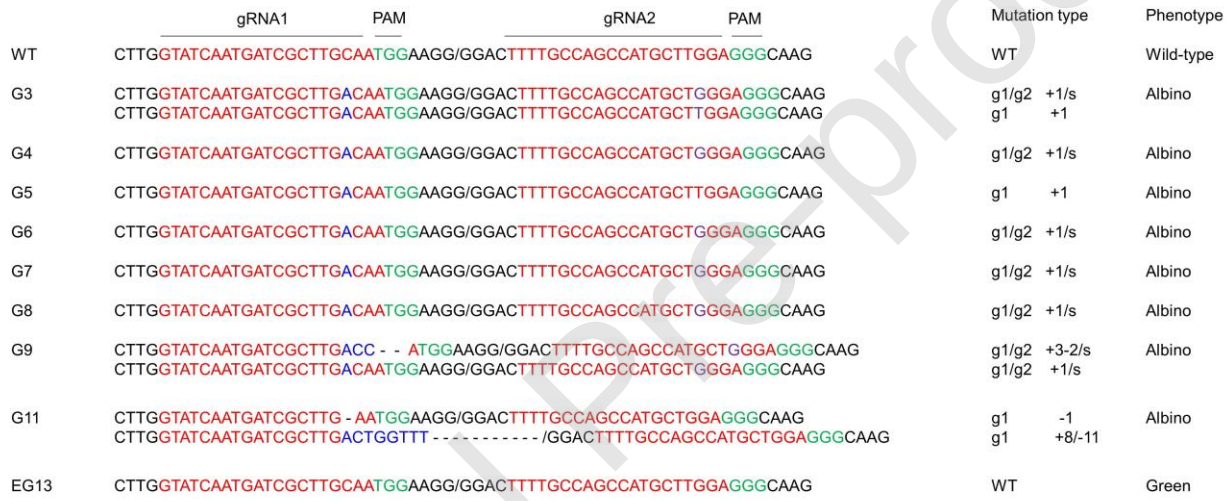


Fig. 6. Sequence analysis of PDS edited events of 'Sukali Ndiizi'. Red nucleotides indicate the gRNAs and green nucleotides indicate Protospacer Adjacent Motif (PAM). Black dashes (---) represent deletions, blue nucleotides indicate insertion. WT, wild-type control sequences; S1-S50, independent edited events; ES19- escape plant. The type of mutations at target site g1 (gRNA1), g2 (gRNA2), or g1/g2 (gRNA1/gRNA2), number of indels as deletions (–), insertions (+), and the phenotype of the edited event are indicated on the right panel.

Fig. 7. Sequence analysis of PDS edited events of 'Gonja Manjaya'. Red nucleotides indicate the gRNAs and green nucleotides indicate Protospacer Adjacent Motif (PAM). Black dashes (---) represent deletions, blue nucleotides indicate insertion and purple nucleotide indicate substitution (s). WT, wild-type control sequences; G3-G11, independent edited events; EG13- escape plant. The mutations in g1 (gRNA1), g2 (gRNA2), or g1/g2 (gRNA1/gRNA2), number of indels as deletions (–), insertions (+) or substitution (s), and the phenotype of the edit event are indicated on the right panel.



Tables

Table 1. Phenotype of the mutants generated for banana cultivar 'Sukali Ndiizi' and plantain 'Gonja Manjaya'.

S/N	Cultivar	Mutants regenerated	Albino %	Variegated %	Albino and variegated %	Mutation frequency %
1	Sukali Ndiizi	77	67.5	26	6.5	100
2	Gonja Manjaya	17	94.1	5.9	-	100

Table 2. Number of potential off-targets for the gRNAs in A and B genomes.

gRNA sequence from PDS + PAM	**Number of potential off-targets**	
	A genome	**B genome**
GTATCAATGATCGCTTGCAA**TGG**	3	5
TTTTGCCAGCCATGCTTGGA**GGG**	7	12