# Generation of genome edited banana by knocking out of pathogen susceptibility genes

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## Introduction

Targeted genome-editing technology such as clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas9) can be used efficiently to induce targeted mutations in the genomes of many plants species to produce improved varieties.

Banana (*Musa* spp.) is an important fruit as well as staple food in many regions of the world. Unfortunately, banana production is seriously constrained by several diseases which are present in the immediate environment of the plant. Pests and diseases caused by insects, nematodes, fungi, bacteria and viruses, singly or in combination are adversely affecting the yield and productivity of the crop. Fusarium wilt (or Panama Disease), caused by the fungus *Fusarium oxysporum cubense* (Foc) and banana Xanthomonas wilt caused by *Xanthomonas campestris* pv. *musacearum* are among the most devastating disease of banana, which is one of the most important food crops particularly in sub-Saharan Africa where millions of farmers depend on bananas as a staple and cash crop. The production of resistant banana varieties is one of the most effective solutions to mitigate the negative effects of pathogens in banana production. As the improvement of banana through traditional breeding is very challenging, we would like to explore the potential of new breeding tool such as CRISPR/Cas9 mediated genome editing for controlling the economically important diseases. There are reports that resistance to bacterial and fungal diseases can be provided by the loss-of-function of a susceptibility gene (*S*-gene). Once S-genes become dysfunctional, pathogens are impeded from colonizing the plant, thus providing resistance to pathogen. For example, loss-of-function of the gene *Mildew resistance locus o* (*Mlo*) and *downy mildew resistance* (*DMR6*) was found to confer recessively inherited broad-spectrum resistance against the bacterial and/or fungal pathogen in a broad range of plant species.

Based on the literature and information from comparative transcriptome analysis of resistant wild type banana *Musa balbisiana* and susceptible banana and, we are testing the potential of knocking down of single susceptibility (*S*) genes such as *Mildew resistance locus O*13 (*Mlo*13), *downy*

*mildew resistance* (*DMR6*), and transporter genes such as *SWEET14* and *early nodulin* (*ENOD*) for resistance to bacterial wilt and Fusasium wilt diseases.

**Progress**

**1. Design of CRISPR/Cas9 constructs for editing of banana**

Following constructs were designed

1. pMDC32_Cas9_MuDMR6
2. pMDC32_Cas9_MuADH1
3. pMDC32_Cas9_MuMLO
4. pMDC32_Cas9_MuSWEET14
5. pMDC32_Cas9_MuENOD

**1.1 Design of gRNA**

The sequences of DMR6, MLO, ADH1, SWEET 14 and ENOD genes were downloaded from banana genome A (*Musa accuminata*) and genome B (*Musa balbisiana*) from the Banana Genome Hub (http://banana-genome-hub.southgreen.fr). The gene sequences from both genomes were aligned using Multalin (http://multalin.toulouse.inra.fr/multalin/) to identify conserved regions. Two gRNAs for each gene were designed from the conserved region using ATUM CRISPR DNA Design Tool (https://www.atum.bio). The gRNAs were selected 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 appropriate adaptors were added at the 5' end to enable cloning in the gRNA expression plasmids.

**1.2 Construction of CRISPR/Cas9 plasmid**

Guide RNA (gRNA) expression vectors pYPQ131 (for gRNA1) and pYPQ132 (for gRNA2) were linearized with *BsMB*I to produce 4 bp overhang for ligation of the gRNA oligos. Forward and reverse oligos of each gRNA were phosphorylated and annealed using $T_4$ polynucleotide kinase. The phosphorylated and annealed oligos were ligated to the linearized expression vectors using T4

ligase at room temperature (RT) for 2 hours. The ligated products were transformed to DH5α *E. coli* cells and selected on LB plates containing tetracycline. The plasmid DNA were isolated from the selected colonies and inserts were verified by Sanger sequencing.

Clones with the correct insert were assembled into the Golden Gate recipient and Gateway vector pYPQ142 by digestion with *Bsa*1 and then ligated with $T_4$ DNA ligase. The products were transformed to DH5α *E. coli* cells and selected on LB plates containing Spectinomycin. The plasmid DNA were isolated from the selected colonies and inserts were verified by restriction digestion with *Eco*RI and *Nco*I.

Plasmids resulting from the Golden Gate assembly above together with the Cas9 entry vector pYPQ150 were cloned into the Gateway binary vector pMDC32 by LR clonase™ (Invitrogen, New Zealand) recombination reaction. After selection on LB plates containing kanamycin, the clones were verified by digestion with *Kpn*I (Fig. 1). The clones with the correct insert were mobilized to *Agrobacterium* strain EHA105 by electroporation. Colonies were confirmed by PCR using Cas9 primers (Fig. 2).

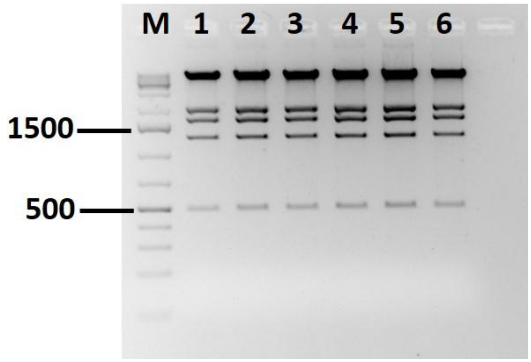


**Figure 1:** Gel electrophoresis of pMDC32–Cas9_MuDMR6 gRNA restriction with *Kpn*I; M: Generuler 1kb plus DNA ladder (Fischer scientific), 1-6: DNA from individual colonies.

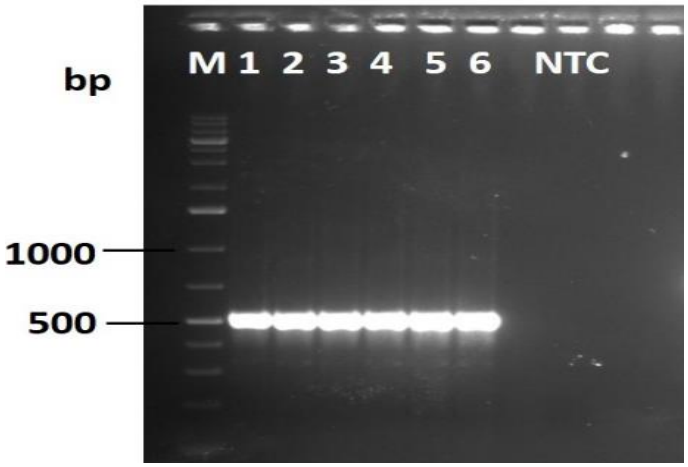


**Figure 2:** Colony PCR analysis of EHA105pMDC32_Cas9_BaDMR6 individual colonies; M: Generuler 1kp plus DNA ladder (Fischer scientific), 1-6: DNA from individual colonies, NTC: No template control.

## 2. Generation of edited banana plants using DMR-6 construct

### 2.1 *Agrobacterium* mediated transformation

The embryogenic cells of "Sukali Ndiizi" were transformed with *Agrobacterium* and regenerated on hygromycin B (25mg/L) selective media (Fig. 3). In total 30 transgenic events were generated in two experiments. Each transgenic event was maintained and multiplied by regular 6-8 weekly subculture in proliferation media.

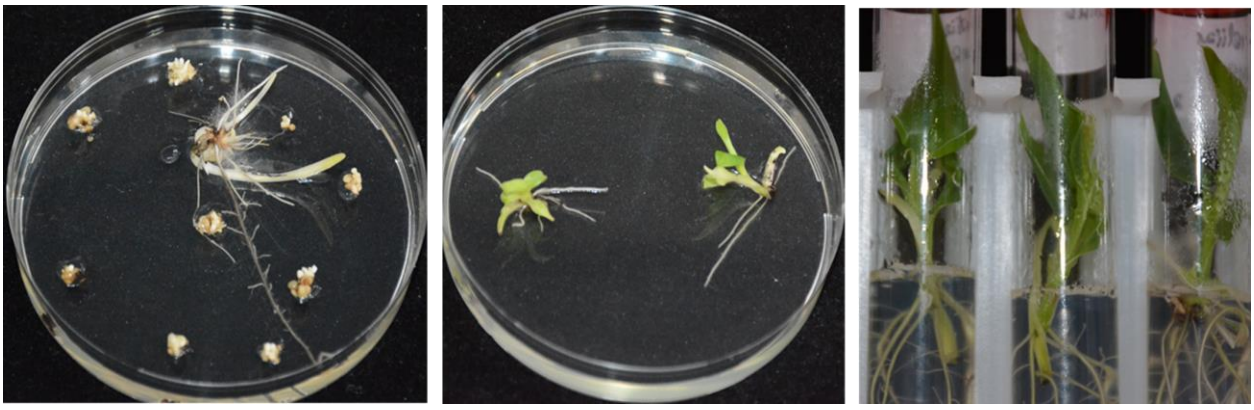

**Figure 3:** Various stages of generation of transgenic events. A) Germinating embryos in selection medium, B) small green shoots regenerated on selective medium, C) rooted genome edited plants.

## 2.2 Molecular analysis of mutated events

Plant DNA was extracted from all the 30 mutated events using modified CTAB method. DNA quality and quantity were checked by nanodrop and gel electrophoresis. Regenerated events were analysed by PCR, and sequencing to confirm mutation at targeted site. All the events showed positive amplified product of expected size on gel electrophoresis (Fig. 4A). In the sequencing results, although all the events showed indels, however, in some events, there were large indels (deletion/addition) ranging between 30-182 bp (Fig. 4B).

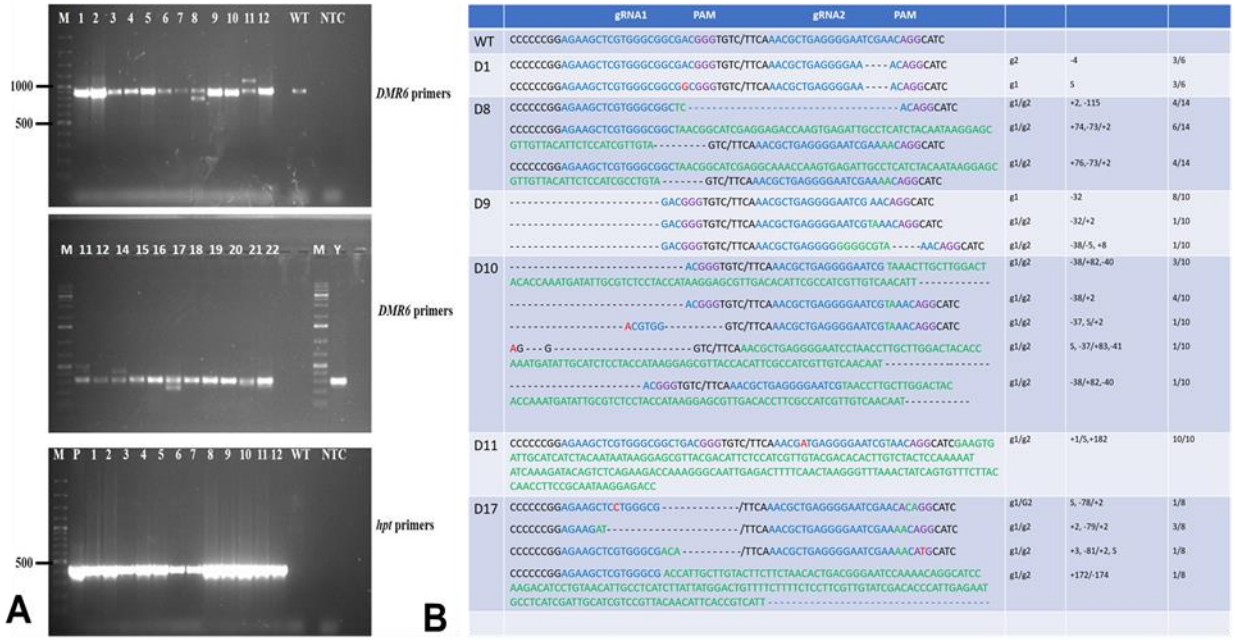


**Figure 4**: Molecular characterization of mutants. A) PCR analysis to detect band shift. B) DNA sequencing of edited lines. Up to 174 bp deletion/insertion was observed in few events.

## 2.3 *In-vitro* screening of mutated events against *Xanthomonas campestris* pv. *musacearum* (Xcm)

Eight -week -old *in vitro* plantlets of all the edited mutated events having fully develop rooting system with three replicates each were subjected to Xcm disease evaluation assay. Control plants were also included in the experiments. These events were inoculated with fresh inoculum of *Xanthomonas campestris* pv. *musacearum*. Several of the edited events showed enhanced

resistance to bacterial pathogen in comparison to wildtype plant (Fig. 5). This result needs to be confirmed by challenging the potted plants in the glasshouse.

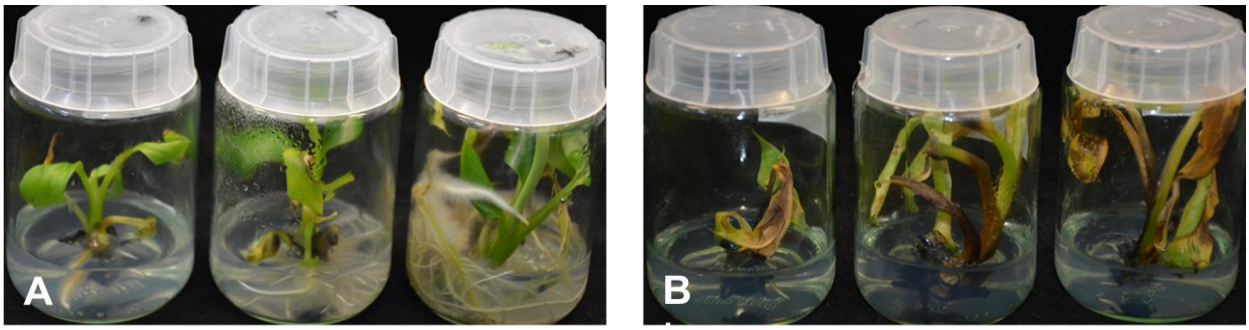


**Figure 5**: I*n-vitro* screening banana plantlets against *Xanthomonas campestris* pv. *musacearum*. A) Genome edited events showing enhanced resistance, B) Control plants showed disease symptoms wilting, browning and yellowing of leaves and complete wilting of plants.

### 2.4 *In-vitro* screening of mutated events against *Fusarium oxysporum* f. sp. *Cubense* race 1

Eight -week -old invitro plantlets of all the edited events having fully develop rooting system with five replicates each were inoculated with *Fusarium oxysporum* f. sp. *Cubense* (Foc) race 1. Five replicates of non-mutated inoculated and five replicate non-mutated non-inoculated plants were also included in the experiment. These plants were inoculated with $10^6$ fungal spores and observed up to 6 weeks for symptoms appearance. Disease symptoms appearance like yellowing and wilting of leaves and pseudostem were recorded. Internal symptoms of blackening of rhizome and pseudostem were also recorded and photograph at last of the experiment. Several of the edited events showed reduce severity of fusarium wilt disease with mild disease symptoms in comparison to control wildtype plants (Fig. 6). This result needs to be confirmed by challenging the potted plants in the glasshouse.

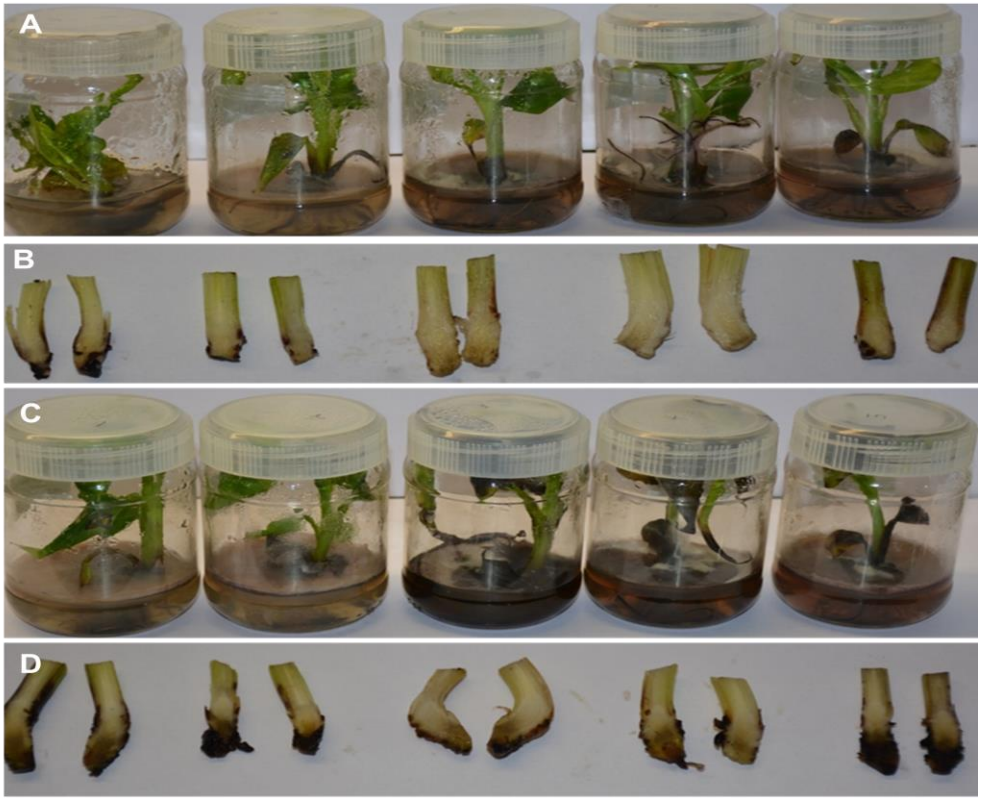


**Figure 6.** I*n-vitro* screening of banana plantlets against *Fusarium oxysporum* f. sp. *cubense* race 1. A) Genome edited events showing enhanced resistance. B) Cut section of pseudostem of edited events showing minimal internal symptoms. C) Control plants showing wilting symptoms. D) Cut section of pseudostem of control plants showing necrosis and blackening.

## 3. Conclusion

In this work, 100% mutation efficiency was obtained as al the edited events showed indels. The genome edited banana plants were phenotypically normal. These edited plants showed enhanced resistance to both Xcm as well as Foc under the *in vitro* rapid bioassay. We are planning to test these events in the glasshouse using potted plants to verify our result of *in-vitro* study. In coming year, edited plants will also be generated from the remaining construct and test them for disease resistance. This work will provide new breeding tools for genome editing in banana for disease resistance.