Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2023), pp. 1–3 doi: 10.1111/pbi.14100

Brief Communication

A new and novel high-fidelity genome editing tool for banana using Cas-CLOVER

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Received 8 February 2023;
revised 29 May 2023;
accepted 5 June 2023.
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Keywords: Banana, genome editing, Cas-CLOVER.

To Editor,

Genome editing with engineered nucleases has developed rapidly in the last decade and has transformed researchers' ability to manipulate various organisms' genomes. To date, three main types of engineered nucleases have been customized for genome editing: zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas). Among all the genome editing technologies, CRISPR/Cas9, which is based on bacterial immunity against viral invasion and phage, has been adopted as the most popular technique for functional genomics and crop improvement due to its ease of use, design flexibility, high efficiency and multiplexing (Tripathi *et al*., 2020). It has become an essential tool for genome editing and has the potential to revolutionize agriculture, addressing climate resilience and food and nutrition security. It has been applied in crop improvement for more than 40 plant species in over 25 countries (Pixley *et al*., 2022). However, researchers need to address the challenges of intellectual property (IP) protection and licensing to release genome-edited crops for use by growers. Obtaining a licence is often tricky due to high demand and complexity. The availability of alternative genome editing approaches to CRISPR/Cas9 system with clear IP issues could facilitate the development of genome-edited crops.

We established an alternative genome-editing tool for banana using the Cas-CLOVER system (Madison *et al*., 2022). Cas-CLOVER is a dual-guided system that creates double-strand cuts upon dimerization with nuclease Clo051 (Figure 1a). It is a fusion protein that comprises a nuclease inactivated or dead Cas9 (dCas9) protein fused to the Clo051 endonuclease as a binding protein on the target site in the genome of any organism. As opposed to using a single guide RNA (gRNA) like in CRISPR, the Cas-CLOVER endonuclease system utilizes two gRNAs in addition to the Clo051 nuclease activity that requires dimerization of subunits associated with each guide RNA (Figure 1a). Using two gRNA makes the Cas-CLOVER genome editing system highly targeted and precise, as Clo051 makes a double-stranded break only upon dimerization when both gRNAs localize it at the correct target site in the plant genome. Dual gRNA localization of Clo051 occurs within a flexible optimal spacer length of 11–31 nt enabling flexibility of design. This technique allows for a more specific DNA cut with minimal chances of off-target effect and results in larger, sticky-ended overhangs compared to CRISPR/Cas9 with smaller blunt-ended deletions. The Cas-CLOVER gene editing technology is revolutionary, highly precise and off-target mutations are often undetectable (Madison *et al*., 2022).

Here, we demonstrate the use of the Cas-CLOVER system for editing the genome of a banana (*Musa* spp.) for the first time. Banana is a valuable staple food and cash crop cultivated in about 136 nations in the tropics, and subtropics, feeding millions of people with annual global production of 170 million tons (FAOSTAT, 2022). Africa produces one-third of the world's bananas, with East Africa being the leading producer, accounting for about 40% of the total production. Banana production is severely affected by several pathogens and pests, particularly in regions where some of them co-exist (Tripathi *et al*., 2020). There is a critical need to develop broad-spectrum disease-resistant varieties to minimize the yield gap in banana. The traditional breeding of banana is challenging because of the low genetic variability in germplasm, polyploidy, lengthy production cycle and sterility of many cultivars (Tripathi *et al*., 2020). There is, therefore, the need to use new breeding tools, such as genome editing, to develop improved varieties with multiple disease resistance.

We designed a Cas-CLOVER editing system for banana expressing two gRNAs targeting the banana *phytoene desaturase* (*MusaPDS*) as a visual marker gene (Figure 1b,c). PDS is a crucial enzyme in the carotenoid biosynthesis pathway, catalysing the breakdown of colourless phytoene into ζ-carotene, which is further converted into lycopene, a colourful compound in the pathway (Ntui *et al*., 2020).

The Cas-CLOVER plasmid construct pDMT_Cas-CLOVER_*MusaPDS* (Figure 1c) was delivered into the embryogenic cell suspensions (ECS) of banana cultivar 'Sukali Ndiizi' (AAB genome) through *Agrobacterium*-mediated transformation as previously described (Tripathi *et al*., 2015). The detailed procedure for preparing plasmid construct pDMT_Cas-CLOVER_*MusaPDS* (Figure S1) is presented in the supplementary file. Nine edited events were regenerated in about 8 months after the *Agrobacterium* infection of ESC (Figure 1d) and validated for the presence of Cas9 by PCR analysis. The efficiency for generating edited events was low as only nine events were generated from 1 mL of settled cell volume of ECS transformed. However, all the

Please cite this article as: Tripathi, L., Ntui, V.O., Tripathi, J.N., Norman, D. and Crawford, J. (2023) A new and novel high-fidelity genome editing tool for banana using Cas-CLOVER. *Plant Biotechnol. J*., https://doi.org/10.1111/pbi.14100.

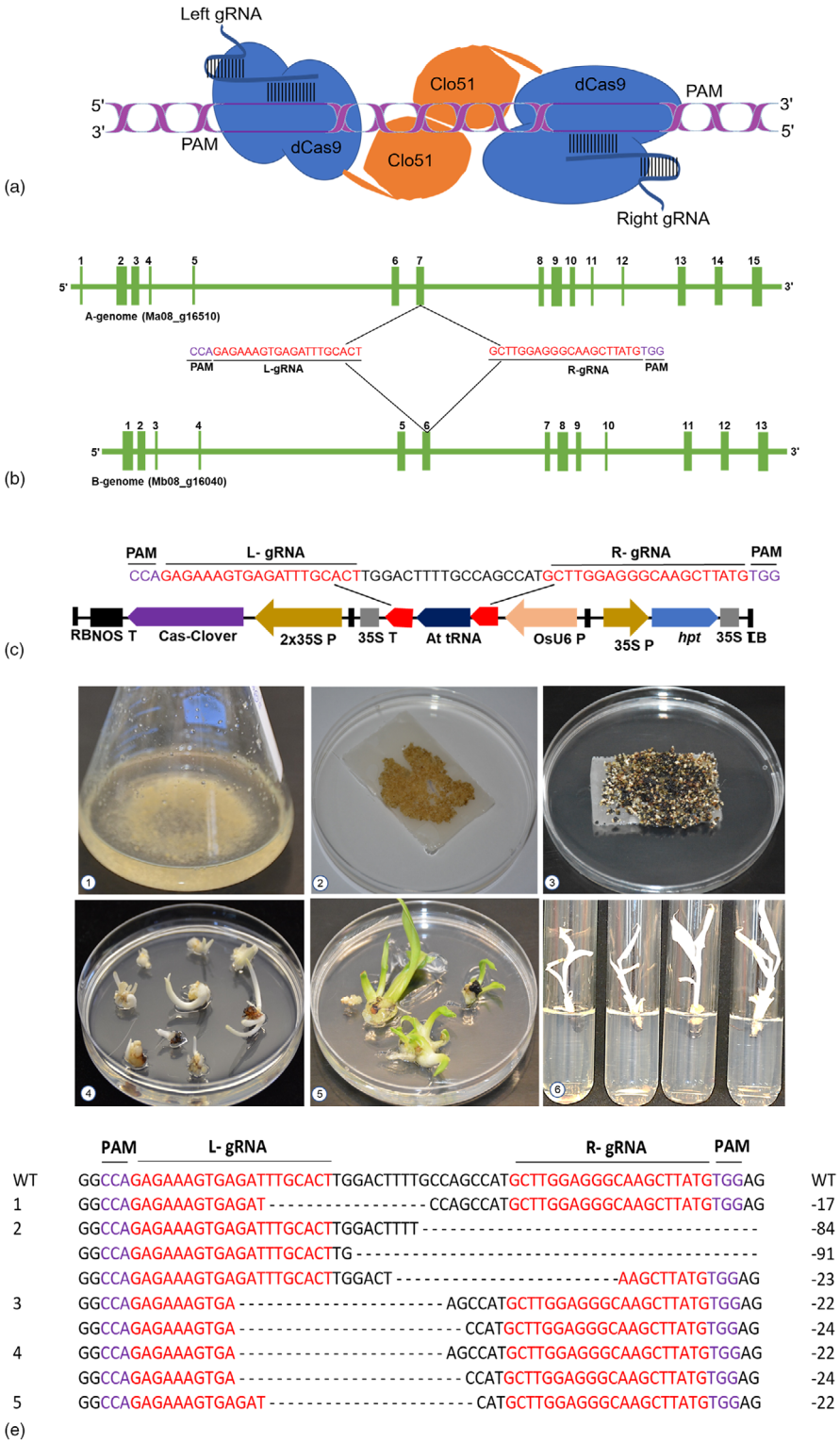

Figure 1 Cas-CLOVER based genome editing of banana targeting phytoene desaturase (*MusaPDS*) gene. (a) Schematic representation of the Cas-CLOVER complex. Cleavage by Clo051 nuclease requires dimerization of subunits associated with each gRNA. (b) Structure of *MusaPDS* genes showing the position of gRNAs targeting both A and B genomes of banana. Green bars represent exons; digits indicate exon numbers while introns are indicated by lines. gRNAs and PAM (Protospacer Adjacent Motif) sequences are indicated in red and purple, respectively. (c) Schematic diagram of Cas-CLOVER plasmid construct pDMT_Cas-CLOVER_*MusaPDS* used to generate genome-edited banana. (d) Regeneration of genome-edited banana events using the Cas-CLOVER technology targeting the *MusaPDS* gene. (1) Cas-CLOVER plasmid delivered to embryogenic cell suspension (ECS); (2) *Agrobacterium*-infected ECS proliferated on regeneration medium; (3) Embryo development from *Agrobacterium*-infected ECS; (4) Embryo germination from transformed ECS; (5) Control non-edited shoots; (6) Albino edited events. (e) Sequence analysis of *MusaPDS* edited events, 1–5, independent mutants, WT, Wild-type control, PAM is in purple and gRNAs in red. Black dashes denote deletions.

regenerated events had albino phenotypes, different from our previous results using CRISPR/Cas9, where the regenerated edited events were either complete albino or a mixture of albino and variegated (Ntui *et al*., 2020). The albino plants did not develop proper leaves and roots and grew very slowly and turned brown after a few sub-culturing required to maintain banana plantlets in tissue culture.

The target region of *MusaPDS* from five individual albino events was amplified by PCR using primers flanking the target region (L-site and the R-site) (Figure 1c), and the amplicons were subjected directly to Sanger sequencing to confirm the targeted mutations. All the albino events showed the desired mutations within the target site (Figure 1e). All the mutations observed in the albino events were deletions ranging from Δ17 to Δ91 bp (Figure 1e). The deletions are bigger compared to CRISPR/Cas9 system using single gRNA. These results indicate that the albino phenotype of the events was due to mutations disrupting the function of the *MusaPDS* gene through the action of the Cas-CLOVER system. In the Cas-CLOVER system, mutations usually occur between the L-site and the R-site of target sites, instead at 3–5 bp upstream of the PAM segment, as with the CRISPR/Cas9 technology. In this system, dCas only served as a fusion protein and did not cut DNA as it is mutated. This phenomenon was observed in our work, where all the mutations occurred between the L-site and the R-site target region (Figure 1d). In event 2, where up to 91 nucleotides were deleted, the mutation occurred between the two sites but proceeded beyond the R-site, where most of the nucleotides were deleted (Figure 1d). Out of five edited events sequenced, two showed homogenous mutations, whereas the remaining three showed allelic variations. The results of this study indicate that Cas-CLOVER is an efficient genome editing technology in banana. So far, the Cas-CLOVER system has only been demonstrated in tobacco (Demeetra, 2023).

For Cas-Clover to edit a specific location in the genome, the L-gRNA and R-gRNA must be designed and recognized in a specific orientation and within a specified distance from each other for the system to dimerize and function successfully. This is the primary disadvantage of using Cas-CLOVER compared to CRISPR/Cas9 as it requires the design of two gRNAs in the target region instead of one, and in some limited cases, particularly in very small target regions, the design can be more challenging. However, due to the requirement of two 20 bp guides that must be oriented perfectly and appropriately spaced apart to cut the genome, the chances of targeting other locations in the genome that fulfil these specifications remain negligible. The 64 bp fragment containing PAM-L-gRNA, 18 nt spacer sequence, and R-gRNA-PAM was blasted to the banana genome to predict potential off-target mutations. This fragment did not hit any other position apart from the *MusaPDS* gene, confirming negligible chances of off-target mutations.

One of the significant challenges of genome editing in a banana is the inability to eliminate the transgenes after editing as the banana is vegetatively propagated, and transgene cannot be segregated out by backcrossing. The Cas-CLOVER system fused with *PiggyBac* has been designed to enhance genome editing in crops without integrating foreign DNA. Cas-CLOVER will serve as editor by introducing targeted double-strand breaks in genomic DNA, resulting in indels; the *piggyBac* then helps introduce small to huge genetic cargo and removes T-DNA in a scarless manner in the edits. This technology has the potential to develop transgene-free genome-edited banana.

Acknowledgement

This research was supported by the CGIAR program for roots, tubers and banana.

Conflict of interest

Authors declare no conflict of interest.

Authors' contributions

LT and JC conceived the idea; LT and DN planned experiments; JNT generated the mutants; VON analysed the mutants; DN prepared the Cas-CLOVER construct; all authors wrote the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary Material S1: Preparation of Cas-CLOVER construct.

Figure S1. Cas-CLOVER plasmid construct pDMT_Cas-CLOVER_-*MusaPDS* used to knockout *Phytoene desaturase* (*PDS*) gene in banana. The Cas-CLOVER is driven by CaMV35S promoter and a NOS terminator. The *hygromycin phosphotransferase (hpt)* gene was used as a plant selection marker. Guide expression is driven by the OsU6 promoter with a plant optimized tRNA linker between the guide complexes.