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Brief Communication

A CRISPR/Cas9-based genome-editing system for yam (*Dioscorea* spp.)

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To Editor

Yam (*Dioscorea* spp.) is a multi-species tuber crop providing food and income to millions of people worldwide, particularly in Africa (Price *et al*., 2016). The 'yam belt' in West Africa, including Nigeria, Benin, Togo, Ghana, and Côte d'Ivoire, accounts for 92% of 72.6 million tons of global yam production (FAOSTAT, 2018). Despite the economic importance, yam cultivation is plagued by several biotic and abiotic factors. Yam genetic improvement via conventional breeding has not achieved substantial progress mainly due to the dioecy nature, long breeding-cycle, polyploidy, heterozygosity, poor seed set, and non-synchronous flowering (Mignouna *et al*., 2008). A precise genome-engineering holds the potential to overcome some of these limitations. CRISPR/Cas9 is the most popular genome-editing system applied extensively for crop improvement, wherein yam is lagging far behind other crop species. The genetic transformation technologies and genome sequences, only recently available, made it possible to realize the potential of CRISPR-based genome editing for basic and applied research in yam (Manoharan *et al*., 2016; Nyaboga *et al*., 2014; Tamiru *et al*., 2017). Here, we report, for the first time, the successful establishment of a CRISPR/Cas9-based genome-editing system and validation of its efficacy by targeting the *phytoene desaturase* gene (*DrPDS*) in a West African farmer-preferred *D. rotundata* accession Amola. The *PDS* gene is involved in converting phytoene into carotenoid precursors phytofluene and ζ-carotene (Mann *et al*., 1994). It is commonly used as a visual marker to validate genome editing in plants, as disruption of its function causes albinism.

We first sought to identify the promoters for expressing guide RNAs (gRNAs) in yam. Five U6 genes from *D. alata* were identified, and the respective promoters (~300 bp) were synthesized. To identify the best DaU6 promoters, a gRNA targeting a mutated green fluorescence protein gene (*GFP + 1*) was constructed under each DaU6 promoter. Individual gRNAs and Cas9 (pCas9-DaU6::gGFP + 1) and p35S::GFP + 1 were mixed equally and introduced into the yam mesophyll protoplasts, while pUbi::GFP and p35::GFP + 1 were used as positive and negative control, respectively, using the method described previously for assessing the efficacy of wheat U6 promoters (Zhang *et al*., 2019). Protoplasts transfected with pUbi::GFP showed strong fluorescence 40 h post-transfection (Figure 1a). In contrast, no fluorescence was observed in protoplasts with non-functional GFP + 1. Some protoplasts transfected with pCas9-DaU6::gGFP + 1 and p35S::GFP + 1 together showed GFP fluorescence, indicating the GFP + 1 was correctly edited into the functional *gfp* gene (Figure 1a). A comparison of the efficacy of different yam U6 promotors (DaU6.1 to DaU6.5) using the protoplast transfection assay showed variation in the number of GFP-fluorescing protoplasts and their intensity. Promoter DaU6.5 performed best, while DaU6.2 and DaU6.3 yielded similar fluorescence scores (Figure 1b). Consequently, we selected promoters DaU6.3 and DaU6.5 to direct the gRNA expression for stable transformation of yam. The complete sequence of *DrPDS* was identified by Blast searching the NCBI database using Arabidopsis phytoene desaturase 3 protein (NP_193157.1) (Figure 1c). A plasmid construct, pCas9-gRNA-PDS, was built. This construct carries a *Cas9-gfp* fusion gene driven by maize ubiquitin promoter (Zhang *et al*., 2019), two gRNAs targeting exon 2 of *DrPDS* (Figure 1c) under DaU6.3 and DaU6.5 promoters individually, and a plant selectable marker *nptII* gene under CaMV35S promoter (Figure 1d).

We next evaluated the efficacy of the *Cas9-gfp* gene expression in yam using agroinfiltration. The agroinfiltration-based system was established through the infiltration of young leaves of two months old potted plants with *Agrobacterium* harbouring the construct pCas9-gRNA-PDS (Figure 1d). The effect of various factors, including the age of leaves (young, unopened; young, fully expanded; and mature), infiltration buffer, *Agrobacterium* strain (EHA105, LBA4404), and cell density (OD_{600} = 0.05–2.0), supplementation of acetosyringone (200, 400 μM), and applica-

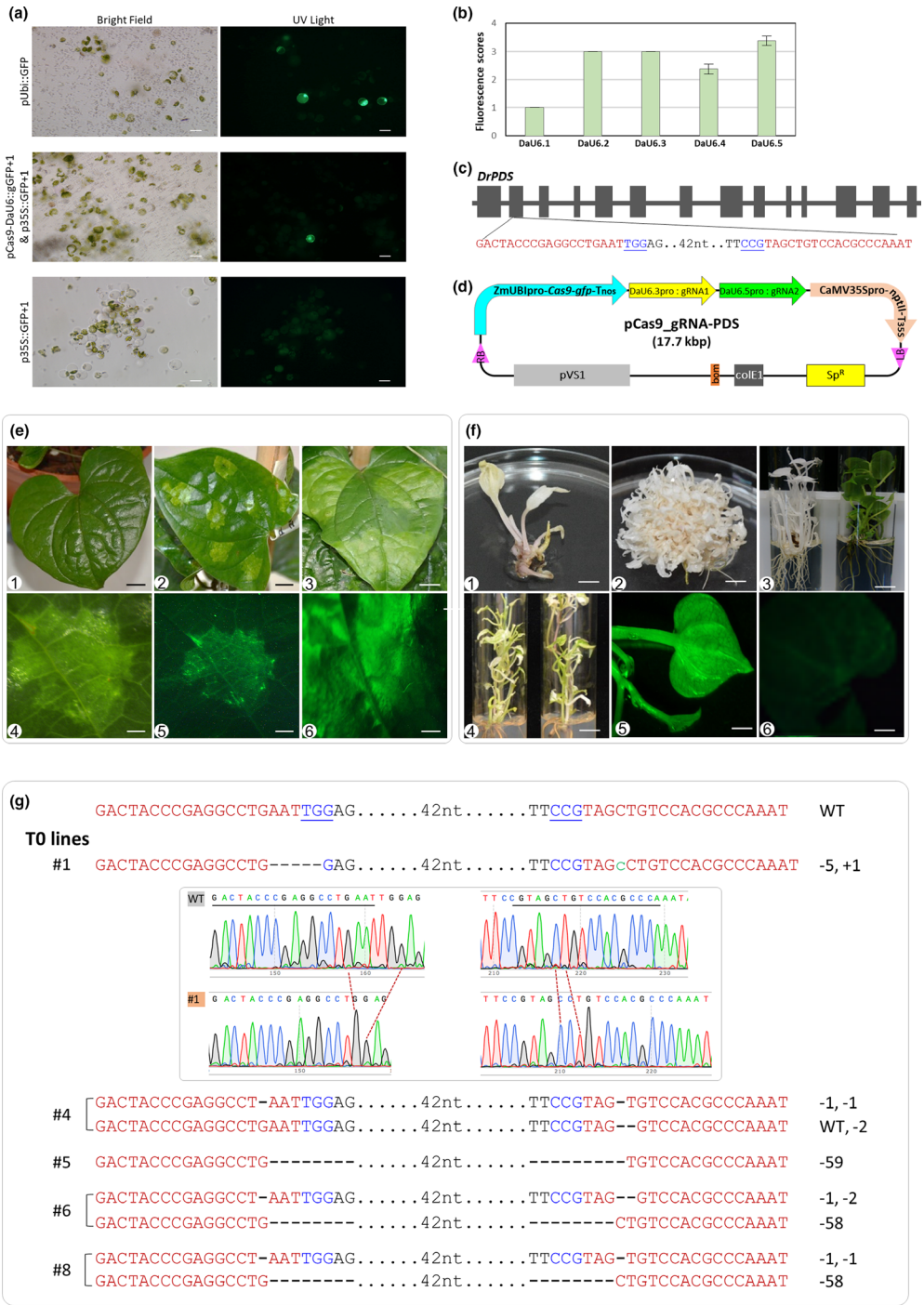

Figure 1 CRISPR/Cas9-mediated genome-editing of yam targeting *phytoene desaturase* (*DrPDS*) gene. (a) Expression of GFP in protoplasts transfected with pCas9-DaU6::gGFP + 1 and non-functional p35S::GFP + 1. Scale bars, 25 μm. (b) Editing efficiency of Cas9 and DaU6 promoters in yam protoplasts. Fluorescence was scored as scale from 1 to 10, with no tracked fluorescence scored as 0 for p35S::GFP + 1, scored as 10 for pUbi::GFP. (c) Gene structure of *DrPDS* showing the gRNA targets. Black bars indicate exons of the gene, PAM sequences in blue and underlined, and protospacer sequences in red. (d) Schematic presentation of pCas9_gRNA-PDS used to generate genome-edited events. (e) Transient gene expression in yam leaves agroinfiltrated with *Agrobacterium* harbouring pCas9_gRNA-PDS. (e-1) Leaf infiltrated with infection medium only, (e-2&e-3) leaf infiltrated with *Agrobacterium* showing bleached patches, (e-4) microscopic examination of an infiltrated leaf section, (e-5) green fluorescent micrograph of the infiltrated section, (e-6) green fluorescent micrograph of an infiltrated section heat treated at 2 dpi and photographed at 4 dpi. Scale bar: e-1, e-2 and e-3, 1 cm, e-4, e-5, and e-6, 0.25 mm. (f) Genome-edited events of yam accession Amola. (f-1) Albino shoot (#5) on the selective medium, (f-2) Albino shoots (#1) with a bushy phenotype, (f-3) complete albino (left, #5) and wild-type (right, #7) plantlet, (f-4) variegated albino plantlets (#6&8), (f-5) genome-edited leaf of #5 under UV light, (f-6) wild-type leaf under UV light. Scale bar: f-1, f-2 and f-3, 1 cm; f-4, f-5, and f-6, 0.25 mm. (g) Site-specific mutations of *DrPDS* induced by two gRNAs. PAM is in blue and protospacers in red. Deletions are denoted by black dashes and insertion by green.

tion of heat shock (Norkunas *et al*., 2018) to infiltrated plants were evaluated for protocol optimization. The optimal period for maximum infection was assessed by determining the GFP fluorescence intensities of leaves assessed at 0, 2, 4, 6, 8, and 10 days post-infiltration (dpi). The optimized agroinfiltration system with *Agrobacterium* strain EHA105 harbouring pCas9-gRNA-PDS (OD_{600} = 0.75) suspended in infiltration buffer (Murashige and Skoog medium salts and vitamins, 20 g/L sucrose, 1 mg/L 6-benzylaminopurine, 0.2 μM $CuSO_4$, pH 5.7) supplemented with 400 μM acetosyringone, infiltrated in the fully expanded young leaves and heat shock treatment at 37 °C for 30 min at 2 dpi showed the highest level of transient gene expression as bleached patches and a bright GFP fluorescence at 4 dpi (Figure 1e). The observed bleached patches could be the results from transient knockout of the *PDS* gene and *Agrobacterium* infection.

To validate the efficiency of CRISPR/Cas9 for targeted mutagenesis in stable transgenic plants, the construct pCas9-gRNA-PDS was introduced into nodal explants of Amola using the *Agrobacterium*-mediated transformation method developed by Nyaboga *et al*. (2014). A total of eight plants, representing 6 independent transgenic events, were regenerated from a total of 300 nodal explants over three transformation experiments. Seven plants except one (#7, green) showed phenotypes of variegated to complete albinism (Figure 1f). The variegated plants with a mosaic pattern of albinism suggest a high level of chimerism with mutations happening at different stages of plant regeneration. Some of the albino plants exhibited bushy phenotype and inadequate response to micropropagation (Figure 1f-1, 2). However, some of the albino and variegated events produced complete plants with well-developed roots similar to the wild-type plants (Figure 1f-3-4). The expression of the transgene in these events was further confirmed by GFP fluorescence under stereomicroscope with fluorescence illuminator (Filter GFP-B, Ex 570/40, and Em 525/50). Leaves of transgenic plants emitted a bright fluorescence (Figure 1f-5), while wild-type plants did not emit any fluorescence (Figure 1f-6). All putative transgenic plants contained *Cas9* as confirmed by PCR analysis.

The target region (300-bp) of *DrPDS* from individual plants, with 4 leaves per plant separately sampled for DNA, was amplified by PCR using gene-specific primers and the amplicons were subjected directly to Sanger sequencing. Plants #1 to #3 showed identical chromatographs with indels at the same locations (5-bp deletion for gRNA1 and 1-bp insertion for gRNA2), confirming the clonal nature of their same origin. Sequencing of other four transgenic plants (#4, 5, 6, and 8) showed various deletions (Figure 1g). All five mutant events (#1, 4, 5, 6, and 8) showed different indels proving to be independent. The indels were observed at both target sites for gRNA1 and gRNA2 in all the events within 3–4 bp upstream of the PAM sequences. Events #5, 6, and 8 carried the large deletions of 58–59 bp with sequences deleted between the cleavage sites of two gRNAs. As expected, the green plant (#7) showed no mutation at either target site. The genome-editing efficiency in yam accession Amola was 83.3% (5 mutant events out of 6 transgenic events). These results demonstrated that the CRISPR/Cas9 could induce site-specific disruption of the *PDS* gene and produced stable phenotypical changes in yam. And we expect the established CRISPR/Cas9 system, with improved genetic transformation, will enable function genomics and trait improvement in yam.

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Conflict of interest

Authors declare no conflict of interest.

Authors' contributions

KW, LT, and BY conceived and managed the project; LT, EDS, and JNT designed the experiments; EDS generated the edited events; MK maintained yam materials and assisted in protoplast experiments; ZZ identified yam promoters and made CRISPR constructs; EDS, JNT, and VON performed molecular characterization; VON and BY analysed the sequencing data; OOG and NKE participated in project discussion; EDS, JNT, LT, KW, and BY wrote and revised the manuscript with contribution from other authors.

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