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Research

An *Agrobacterium*-mediated base editing approach generates transgene-free edited banana

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Summary

- Genome editing for the development of improved varieties is supported by the possibility of segregating out the editor T-DNA cassette after genome editing in many crop species. Removal of the T-DNA cassette prevents potential continuous editing activity in the transformed plant and furthermore facilitates regulatory approval. While transgene outcrossing of exogenous sequences is possible for many crops, this is not the case for vegetatively propagated and sterile crops, such as Cavendish bananas. Therefore, gene editing techniques leading to transgene-free edited plants are essential to untap the potential of genome editing for those crops. Here, we present a method for transgene-free gene editing in sterile banana (*Musa* spp.) through a co-editing strategy.
- A novel *Agrobacterium tumefaciens*-mediated transgene-free gene editing approach combining embryogenesis and chlorsulfuron selection was established in sterile banana and validated through whole genome sequencing.
- Editing of the *acetolactate synthase* (*MaALS*) genes in banana using a plant base editor allows effective selection of edited plants. Moreover, transgene-free plantlets were regenerated with mutations at two target sites, indicating that the strategy can be used to target multiple genomic sites.
- The presented method allows for efficient transgene-free gene editing and represents the first report of a co-editing strategy in sterile crop species.

Introduction

In the last decade, genome editing has revolutionized crop breeding by significantly reducing the time required for the introduction of improved traits in elite germplasms (Razzaq *et al.*, 2021). The clustered regularly interspaced short palindromic repeat (CRISPR)-/CRISPR-associated nuclease 9 (Cas9) system facilitates the rapid and cost-effective targeting of plant genome sequences to introduce mutations, with the possibility to increase their resilience to pests and diseases, abiotic stress factors or improving nutritional traits (Li *et al.*, 2024). The initial development of CRISPR/Cas9 in plants utilized *Agrobacterium tumefaciens* for the genomic integration of a transfer DNA (T-DNA) expressing the guide RNA (gRNA) and accompanying protein (Li *et al.*, 2013; Nekrasov *et al.*, 2013; Shan *et al.*, 2013). However, the *Agrobacterium*-mediated introduction of transgenes into plant genomes is random and can result in unpredictable and unstable outcomes (Jaganathan *et al.*, 2018). Moreover, the use of genetically modified organisms remains controversial in public opinion and is subject to costly approval procedures in many parts of the world (Kennedy & Thigpen, 2020; Levi, 2022; Vanderschuren *et al.*, 2023). Hence, transgenesis still limits the commercialization of edited crops (Turnbull *et al.*, 2021; Ahmad *et al.*, 2023). However, removal of the transgene can be achieved for most crops through transgene outcrossing (Gao, 2021) and facilitates the generation of transgene-free edited plants in the T1 generation. Removal of the T-DNA cassette, moreover, prevents potential continuous editing activity in the transformed plant as well. Because outcrossing of transgenes remains challenging for vegetatively propagated and sterile crops, methodological development has to be focussed on approaches circumventing foreign DNA integration to generate transgene-free gene-edited plants immediately in the T0 generation (X. Huang *et al.*, 2023).

For example, DNA-free gene editing methods using mRNA transcripts (Zhang *et al.*, 2016; Liang *et al.*, 2018) or ribonucleoprotein complexes, consisting of preformed Cas9 and single-guide RNAs (sgRNAs) (Svitashev *et al.*, 2016; Liang *et al.*, 2017, 2018; Zong *et al.*, 2018; Li *et al.*, 2022), have been established to edit plant genomes. These can be delivered through a variety of DNA-free gene editing techniques, such as particle bombardment, often of immature embryos, or protoplast electroporation or polyethylene glycol transformation (Woo *et al.*, 2015; H. Kim *et al.*, 2017; Andersson *et al.*, 2018; Banakar *et al.*, 2022). Another approach consists of using transient

exogenous DNA expression. Exogenous DNA is delivered through particle bombardment (Zhang *et al.*, 2016; Hamada *et al.*, 2018; Zong *et al.*, 2018) or protoplast transformation (Andersson *et al.*, 2017; Lin *et al.*, 2018; Hsu *et al.*, 2024), and the subsequent transient expression allows editing of the genome. In addition to the above-mentioned delivery methods, transient expression can also occur from T-DNA delivered through *A. tumefaciens*. After T-DNA delivery into the host cell as a single-stranded molecule (Albright *et al.*, 1987), double-stranded DNA is generated independently of T-DNA integration, enabling transcription of encoding genes (Zhuobin Liang & Tzfira, 2013). It has been shown that mutations can also be introduced by transiently expressed T-DNA (Chen *et al.*, 2018; Zong *et al.*, 2018; R. Zhang *et al.*, 2019; Bánfalvi *et al.*, 2020; X. Huang *et al.*, 2022, 2023; Jia *et al.*, 2024), without integration in the host genome.

However, as no transgenic DNA is integrated in the above-mentioned methods, only the selection of edited cells is extremely difficult, laborious and expensive (Kocsisova & Coneva, 2023). While plants derived from protoplasts are mostly the result of a single-cell event (Reed & Bargmann, 2021), this is not the case for plants derived from other, multicellular, tissues, often resulting in chimerism. Methods allowing the selection of edited cells and tissues are required to avoid the regeneration of chimeric plants that are partially edited. Moreover, contrary to DNA-free genome-editing techniques, *Agrobacterium*-based transient expression of *CRISPR/Cas9* not only involves the selection of those plants harbouring mutations but also has the extra criterion that selected regenerants need to be transgene-free. One of the promising selection markers to select transformed and mutated cells and tissues is *acetolactate synthase* (*ALS*) or *acetohydroxyacid synthase* (*AHAS*), an essential gene in the synthesis of branched amino acids (Yu & Powles, 2014). *Acetolactate synthase* has been used extensively as a proof-of-concept target site for genome editing, given its conservation in the plant kingdom, as well as an easily observable phenotype upon mutation (Hussain *et al.*, 2021). Indeed, *ALS* can result in sulfonylurea herbicide resistance by mutations at various distinct target sites in its coding sequence, with the most frequently observed and used mutation being Pro197 (*Arabidopsis thaliana* residues), which can be modified into at least 11 other amino acids to result in chlorsulfuron resistance (Heap, 2024). It is therefore a suitable target to be used as an immediate selection marker to confirm the presence of edits in proof-of-concept studies, as previously demonstrated in tomato, tobacco, potato, citrus and maize (Svitashev *et al.*, 2016; Danilo *et al.*, 2019; Veillet *et al.*, 2019; Alquézar *et al.*, 2022; X. Huang *et al.*, 2022, 2023; Jia *et al.*, 2024). Evidently, the most desired mutations are not easily selectable, requiring vast screening methods to identify plants containing the correct and desired mutation(s). However, it has recently been shown that using edits in *ALS*, other secondary mutations at desired target sites can be enriched, thereby allowing efficient co-editing of your required alleles (X. Huang *et al.*, 2023; Jia *et al.*, 2024). Nevertheless, regeneration through organogenesis from explants such as leaf often results in chimeric plants. In contrast to shoot organogenesis, embryogenesis from embryogenic cell suspensions (ECS) has a lower chance of resulting in chimerism as individual clumps are mostly seen as a single entity (Bertsch *et al.*, 2005; Bhatia & Bera, 2015).

Generating transgene-free gene-edited plants is especially important in vegetatively propagated and sterile crops, such as banana. Banana is the most important fruit crop world-wide, with an annual global production of *c.* 135 million tonnes (FAO, 2023). A single variety, Cavendish, represents over 40% of global banana production, and due to the widespread monocropping practices, it suffers from extreme levels of genetic vulnerability (Drenth & Kema, 2021). As a result, several major diseases have emerged and have spread globally, with *Fusarium oxysporum f. sp. cubense* tropical race 4 now also reaching the status of pandemic (Zorrilla-Fontanesi *et al.*, 2020; Turrell, 2024). Other major challenges include banana bunchy top virus, black sigatoka, nematodes and Xanthomonas wilt (Drenth & Kema, 2021; Justine *et al.*, 2022; Tripathi *et al.*, 2024). Despite some successes in generating improved cultivars through classical breeding for smallholder farmers (Ortiz, 2013; Tushemereirwe *et al.*, 2015), the sheer scale and duration (up to 10–20 yr) of such a project make it an undesirable pathway for cultivar innovation. New traits are mostly introduced through *A. tumefaciens*-mediated transformation (Pérez Hernández *et al.*, 2006; Remy *et al.*, 2013; Dale *et al.*, 2017; Tripathi *et al.*, 2019; Zorrilla-Fontanesi *et al.*, 2020), although transgenic bananas have also been regenerated through particle bombardment (Sági *et al.*, 1995; Matsumoto *et al.*, 2002; Dong *et al.*, 2020). However, these techniques have in common the integration of exogenous DNA as the main bottleneck. While strategies have been established that allow for recombination-induced deletion of either the selectable marker (Kleidon *et al.*, 2020) or all transgenes (Hu *et al.*, 2023) integrated into the banana genome, they inevitably leave scars at the integration site, which could represent a major burden for their regulation in different parts of the world, including the European Union. The possibility to utilize protoplasts for CRISPR/Cas9-mediated genome editing of desired target sites has been investigated (Wu *et al.*, 2020; Zhao *et al.*, 2022), but regeneration of banana plants from protoplasts remains challenging, and there is so far no report of successful production of edited banana plants using protoplast systems.

Here, we present a simple and efficient method for the generation of transgene-free gene-edited banana plants (*Musa acuminata* cultivar (cv) Williams). We show that editing both of the *ALS* genes in banana is a viable strategy for the selection of edited plants. Moreover, edits in two distinct genes in a transgene-free way can be achieved following this strategy.

Materials and Methods

Plant material

ECS of the *Musa acuminata* cv 'Williams' (AAA) (subgroup Cavendish) were initiated from ITC0570 (International Musa Transit Centre) and maintained in liquid ZZ medium as described previously (Strosse *et al.*, 2003). Suspensions were

cultured on a rotary shaker (70 rpm) at 26 ± 2°C under continuous light of 50 μE m^{-2} s^{-1} and subcultured at 2-wk intervals.

Acetolactate synthase sequence analysis

Acetolactate synthase genes in banana were identified through blast with the *Arabidopsis thaliana* (L.) Heynh. ortholog *CSR1* (*AT3G48560*), also known as *AHAS* or *ALS*. Genes identified in the reference genome *Musa acuminata* DH Pahang (v.4.3) (Belser *et al.* (2021), Banana Genome Hub) were used as a starting prompt for BLASTN (Camacho *et al.*, 2009) for further phylogenetic studies in other *Musaceae* species with available fully sequenced genomes (Supporting Information Table S1) (Zorrilla-Fontanesi *et al.*, 2016; Rouard *et al.*, 2018; Wang *et al.*, 2019; Belser *et al.*, 2021; Wang *et al.*, 2022; H.-R. Huang *et al.*, 2023; Dussert *et al.*, unpublished; Ziwei *et al.*, unpublished). Haplotypes for Williams were resolved by cloning (CloneJet PCR Cloning Kit, Cat. no. K1231; Thermofisher Scientific, Waltham, MA, USA) and colony sequencing. Phylogeny mapping was performed through MUSCLE (Edgar, 2004) and PHYLIP Neighbour Joining (Felsenstein, 1989).

Arabidopsis transformation with banana *MaALS* genes

To validate the functionality of the banana *MaALS* genes, *Macma4_06_g18410* and *Macma4_10_g15750* were codon-optimized for *A. thaliana* (Twist Bioscience, South San Fransisco, CA, USA). The proline 186 or 181 residue was adapted to either a leucine or phenylalanine. Sequences were hybridized into *pGGC000* (Lampropoulos *et al.*, 2013) between *Kpn*I and *BamH*I restriction sites through Gibson assembly using the GenBuilder™ Cloning Kit (Genscript, Piscataway, NJ, USA) to form a functional C-cassette for Greengate assembly. Codon-optimized genes were placed under a CaMV35S promoter and terminator through Greengate assembly together with plasmids *pGGA004*, *pGGB003*, *pGGD002*, *pGGE001*, *pGGF007* and *pGGZ003* according to Lampropoulos *et al.* (2013). Plasmids were electroporated into *A. tumefaciens* GV3101, and *A. thaliana* Col-0 was transformed through floral dip.

Plasmid generation

For banana, a cloning vector was designed containing a cytosine base editor (addgene #98160; Zong *et al.*, 2017), with the possibility of integrating two sgRNAs. The base editor was amplified with primers PBE_pETKUL21_F and PBE_pETKUL21_R (Table S2) to integrate *Spe*I and *BamH*I restriction sites, after which the fragment was integrated into *pETKUL21* (pCambia1380+maize Ubiquitin promoter, unpublished results) through restriction-ligation at mentioned sites, generating plasmid *pETKUL21_PBE*. On *pYPQ131C* (addgene #69284; Lowder *et al.*, 2015), *Bbs*I overhangs were introduced bordering the sgRNA module to generate two independent sgRNA entry modules, with primers pENTR1_F1 and pENTR1_R1, and pENTR_F2 and pENTR_R2, for *pENTR1* and *pENTR2*, respectively. Fragments were incorporated into *TOPO* plasmids using Zero Blunt™ TOPO™ PCR Cloning Kit (Cat. no. 451245; Thermofisher Scientific).

sgRNAs were designed using CRISPOR (Concordet & Haeussler, 2018). An extra G was incorporated at the 5′ end of the sgRNAs to increase efficiency (Belhaj *et al.*, 2013). sgRNAs were integrated into entry modules by annealing two complementary oligos, each harbouring a 4-bp overhang mimicking *Esp3I* overhangs in *pENTR1* and *pENTR2*. 100 ng of annealed oligos was mixed with 50 ng of *pENTR1* or *pENTR2*, together with 10 U Esp3I (Cat. no. R0734S; New England Biolabs (NEB), Ipswich, MA, USA), 400 U T4 DNA ligase (Cat. no. M0202S; NEB), 10× CutSmart buffer and 1.5 μl 10 mM ATP in a 15 μl reaction. The reaction was incubated for 30 cycles at 37°C for 3 min and 16°C for 3 min, followed by 5 min at 50°C, 5 min at 80°C and an infinite hold at 12°C. Ligation products were heat-shock-transformed in *E. coli*, and positive clones were selected with PCR through the reverse sgRNA primer, together with M13 reverse primer.

For the integration of the sgRNAs in destination vector *pETKUL21_PBE*, *pETKUL21_PBE* was cut with BamHI-HF (Cat. no. R3136S; NEB), HindIII-HF (Cat. no. R3104S; NEB) and the addition of Quick CIP (Cat. no. M0525S; NEB) and purified using the GeneJET PCR purification kit (K0702; Thermofisher Scientific). *pENTR1* and *pENTR2* (containing the sgRNAs) were cut with BbsI-HF (Cat. no. R3539S; NEB) and purified using the GeneJET gel extraction kit (Cat. no. K0692; Thermofisher Scientific). Components were ligated with T4 DNA ligase, and the ligation products were heat-shock-transformed into *Escherichia coli*. Positive colonies were selected by PCR with primers pDEST_F and the reverse sgRNA primer.

In vitro cleavage assay

To validate the functionality of the designed sgRNAs, an *in vitro* cleavage assay was performed. Designed sgRNAs were cloned under a T7 promoter in vector *pT7-gRNA* (addgene #46759; Jao *et al.*, 2013) for *in vitro* transcription. *In vitro* transcription was performed as in Zhen Liang *et al.* (2018), with an initial 3-h incubation step at 37°C for RNA production. sgRNAs were mixed with purified Cas9 protein (Cat. no. M0386T; NEB) according to the manufacturer's protocol and incubated for 10 min at 25°C, before adding substrate DNA. Substrate DNA was amplified with primers bALS6_F1 and bALS6_R4 for *MaALS6* and bALS10_F3 and bALS10_R4 for *MaALS10*, respectively. After cleavage for 15 min at 37°C, 1 μl Proteinase K (Cat. no. 8107S; NEB) was added, and the samples were incubated at room temperature for 10 min, after which the samples were loaded on gel.

Banana transformation and selection on chlorsulfuron

Agrobacterium tumefaciens EHA105 was used for the transformation of banana cultivar Williams. Before transformation, *A. tumefaciens* was plated on YM medium (0.1 g l^{-1} NaCl, 0.2 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.5 g l^{-1} $K_2HPO_4{\cdot}3H_2O$, 0.4 g l^{-1} yeast extract, 10 g l^{-1} mannitol, 13 g l^{-1} bacto agar, pH 7.0) at

28°C for 2 d, after which single colonies were inoculated in YEP medium (10 g l^{-1} yeast extract, 10 g l^{-1} peptone, 5 g l^{-1} NaCl) and incubated at 28°C and 210 rpm for 30 h. Banana transformation was performed as published previously (Pérez Hernández *et al.*, 2006; Kovács *et al.*, 2013). After 6 d on nonselective ZZ semisolid medium, cocultivated samples were transferred to ZZ containing 200 μg ml^{-1} timentin supplemented with either 50 μg ml^{-1} hygromycin or 40 μg l^{-1} chlorsulfuron. Clumps were grown for 8–14 wk with refreshment of the medium every 2 wk. After 8 to 14 wk, clumps growing on both hygromycin and chlorsulfuron were transferred to RD1 regeneration medium (Strosse *et al.*, 2003) containing 40 μg l^{-1} chlorsulfuron and 200 μg ml^{-1} timentin and subcultured every month for 1–3 months, after which they were transferred to RD2 (Strosse *et al.*, 2003) without antibiotics. After subculturing for 1–3 months, lines were transferred to Reg medium (Pérez Hernández *et al.*, 2006) and finally placed in the light at 100 μmol m^{-2} s^{-1}.

DNA purification

DNA was purified through cetyltrimethylammonium bromide (CTAB) extraction. A small piece of cell clump or leaf (for whole genome sequencing (WGS) analysis) was crushed and resuspended in 400 μl of cetyltrimethylammonium bromide buffer (10 mM Tris–HCl pH 7.5, 0.7 M NaCl, 10 mM EDTA, 1 g l^{-1} CTAB). Samples were incubated at 60°C for 1 h with intermittent inversion, after which 200 μl of ice-cold chloroform was added and mixed for 1 min after the samples had returned to room temperature. After centrifugation at 1500 ***g*** for 10 min, supernatant was transferred to a new tube and 440 μl of ice-cold isopropanol was added. Samples were incubated at −20°C for 20 min, followed by centrifugation at 1500 ***g*** for 15 min. Supernatant was discarded, and the pellet was resuspended in 70% ethanol, followed by another identical centrifugation step. Ethanol was removed and evaporated, after which the genomic DNA was resuspended in MQ water.

WGS and data analysis

To confirm the absence of T-DNA in regenerated lines, purified DNA samples were first subjected to a PCR targeting the T-DNA. Samples were verified for the presence of the T-DNA with primers T-DNA-check_2F/R (PP1) and T-DNA-check_5F/R (PP2) (Table S3). Primer pairs Sanger_ALS6_F/R and Sanger_ALS10_F/R served as a control to warrant the quality of the purified DNA. Moreover, for 10 samples per transformed plasmid, one amplification with the latter amplicons was sent for Sanger sequencing to verify mutation ratios at on-target sites. Allelic variability was always interpreted conservatively, to avoid overestimating mutation rates. Where the same chromatogram could be the result of multiple mutation profiles on all alleles, allelic variability was interpreted to contain the least amount of edited alleles.

Six randomly selected putative transgene-free lines were sent for WGS at 10× genome coverage per line to verify the absence of T-DNA (Suzuki *et al.*, 2008; Willems *et al.*, 2016). Additionally, a control plant from the cultivar Williams from the *in vitro* stock at the International Musa Transit Centre (KU Leuven, Belgium) and one regenerated from cell suspension but without transformation were sent along.

The 150-bp paired-end reads WGS data were generated using the Illumina NovaSeq 6000 platform by GenomeScan (Leiden, the Netherlands). Image analysis, base calling and quality check were performed with the Illumina data analysis pipeline RTA v.3.4.4 (Illumina Inc, 2024) and BclConvert v.4.2.4 (Illumina Inc, 2023). The sequences of the eight samples were checked with FastQC v.0.11.9 for final quality inspection. Subsequently, the high-quality paired-end short genomic reads (≥ Q30) were mapped with Bwa-Mem v.0.7.17 (Li & Durbin, 2009) to the reference genome of *Musa acuminata* (DH Pahang v.4; https://banana-genome-hub.southgreen.fr/filebrowser/download/522). The alignment files were sorted with Samtools v.1.13 (Li *et al.*, 2009), and duplicates were marked with Picard v.2.18.23 (Broad Institute, 2009). The target site mutations were visually inspected using Igv v.2.17.4 (Robinson *et al.*, 2011).

To determine whether the edited lines contain fragments from the plasmids, the high-quality paired-end short genomic reads were mapped to the reference plasmid sequences (*pETKUL21-PBE-ALS6* or *pETKUL21-PBE-ALS6+10*) using Bwa-Mem v.0.7.17 (Li & Durbin, 2009) and visualized with Igv v.2.17.4 (Robinson *et al.*, 2011) (Table S4). Using Blastn (Camacho *et al.*, 2009), putative integration sites for the WGS reads were verified by blasting the WGS reads mapping to the plasmid sequences *pETKUL21-PBE-ALS6* or *pETKUL21-PBE-ALS6+10* back to the *Musa acuminata* genome (DH Pahang v4). For the transgenic sample ALS6:21-3, integration in the genome was checked using TC-hunter (Börjesson *et al.*, 2022), which utilizes read alignment information to detect breakpoints in the plasmid and integration sites in the genome.

Potential off-target sites were determined using CRISPOR and CasOFFinder (Bae *et al.*, 2014; Concordet & Haeussler, 2018) and were investigated in WGS samples for off-target mutations (Table S5). The target sites were screened for C-to-D mutations within the editing window of the base editor (Bases 3–10) or frameshift mutations in the entire protospacer. Off-target mutations were considered valid if present in at least two independent reads.

Results

To utilise chlorsulfuron as a selective agent for gene editing (Veillet *et al.*, 2019), it is essential to first investigate whether banana cv Williams is naturally resistant. ECS of Williams were used to investigate its natural resistance, as this is the target tissue of choice for *Agrobacterium*-mediated transformation. The natural resistance to chlorsulfuron was first profiled using a range of chlorsulfuron concentrations (Figs 1a, S1). While cell weight did increase slightly for all chlorsulfuron concentrations after 2 wk, growth stagnated shortly after, even for concentrations as low as 10 μg l^{-1}. After 8 wk on selective medium, weight had increased between two- and threefold for

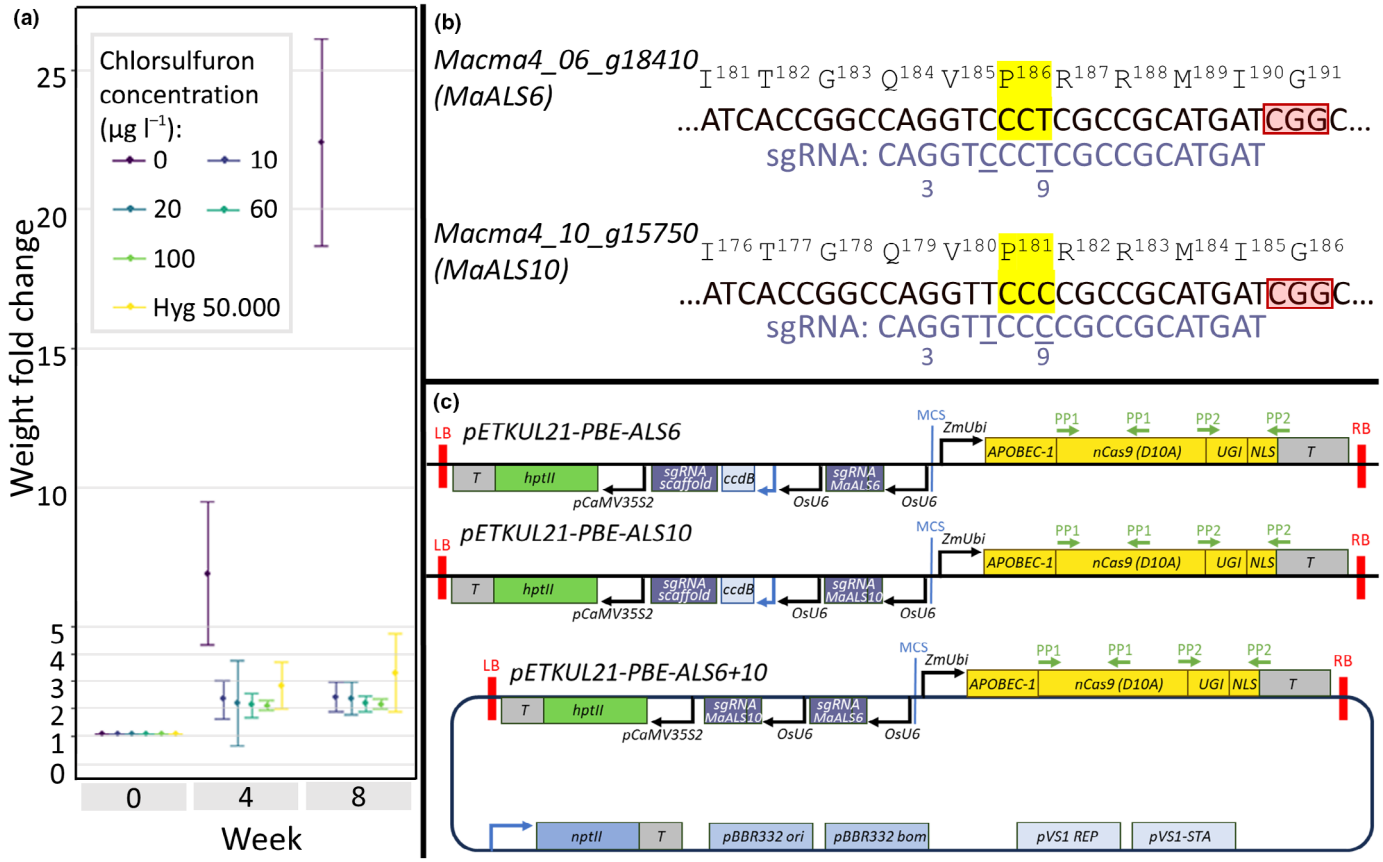


Fig. 1 Construct overview for cytosine base editing in banana cultivar 'Williams' (*Musa* spp.). (a) Resistance of nonedited embryogenic cell suspensions of the cultivar Williams towards different concentrations of chlorsulfuron compared with 50 µg ml^{-1} hygromycin. Whiskers represent SE. (b) Single-guide RNAs (sgRNAs) designed to target *Macma4_06_g18410* (*MaALS6*) and *Macma4_10_g15750* (*MaALS10*) are indicated in purple beneath the gene sequence. The targeted proline residue is indicated in yellow. Protospacer adjacent motif is indicated in red. Nucleotide differences between the sgRNA targeting *MaALS6* and *MaALS10* are underlined. (c) Overview of plasmids *pETKUL21-PBE-ALS6*, *pETKUL21-PBE-ALS10* and *pETKUL21-PBE-ALS6+10* designed for cytosine base editing. The plant base editor consists of rat cytidine deaminase APOBEC-1, nCas9 (D10A), uracil glycosylase inhibitor (UGI) and nuclear localization signal (NLS), preceded by *Zea mays* (maize) ubiquitin promoter (*ZmUbi*) and terminator (T); *hptII*, hygromycin resistance gene; *ntpII*, kanamycin resistance gene; *ccdB*, bacterial cytotoxicity gene; *OsU6*, *Oryza sativa* (rice) U6 promoter; LB, left border; RB, right border; PP1, primer pair 1; PP2, primer pair 2.

all chlorsulfuron concentrations, while weight increased more than 20-fold for cells plated on nonselective medium. A concentration of 10 µg l^{-1} of chlorsulfuron performed similar to the positive control hygromycin at a concentration of 50 µg ml^{-1}.

Two banana *MaALS* genes were identified in the reference genome of *Musa acuminata* DH Pahang (v.4.3) (Banana Genome Hub) (Droc *et al.*, 2013; Belser *et al.*, 2021), *Macma4_06_g18410* and *Macma4_10_g15750*, and they were abbreviated to *MaALS6* and *MaALS10*, based on their respective chromosomes. This duplication event is conserved among the *Musaceae* family, and their genomic locations are conserved among the *Musa* genus (Fig. S2). *MaALS6* and *MaALS10* showed 71% and 77% protein identity, respectively, to their *Arabidopsis thaliana* ortholog *CSR1* (*AT3G48560*), also known as *AHAS* or *ALS*. Transcriptomic analysis indicated that both genes are active, with *MaALS6* consistently transcribed at a higher level in comparison with *MaALS10* (Fig. S3).

The genes *MaALS6* and *MaALS10* were amplified from ECS of cv Williams and mapped to the reference genome. Allelic differences at both gene and protein levels were identified (Genbank PQ304390–PQ304395) but were not present in the target region, that is the protospacer, confirming the absence of natural resistance in Williams ECS. Residues Pro186 and Pro181 were identified as homologous to Pro197 in *A. thaliana*, for *MaALS6* and *MaALS10*, respectively. Analysis of the targeted region surrounding these proline residues showed that it is conserved among *Musaceae* (Fig. S2). *MaALS6* and *MaALS10* were codon-optimized for *A. thaliana*, and three versions of each gene were generated. *MaALS6* or *MaALS10* was either introduced into *A. thaliana* as is, or the above-mentioned proline residues were altered to either leucine or phenylalanine (Pro186Leu and Pro186Phe for *MaALS6*, Pro181Leu and Pro181Phe for *MaALS10*). In other species, these mutations were shown to be a gain-of-function, resulting in chlorsulfuron resistance while maintaining the enzyme function.

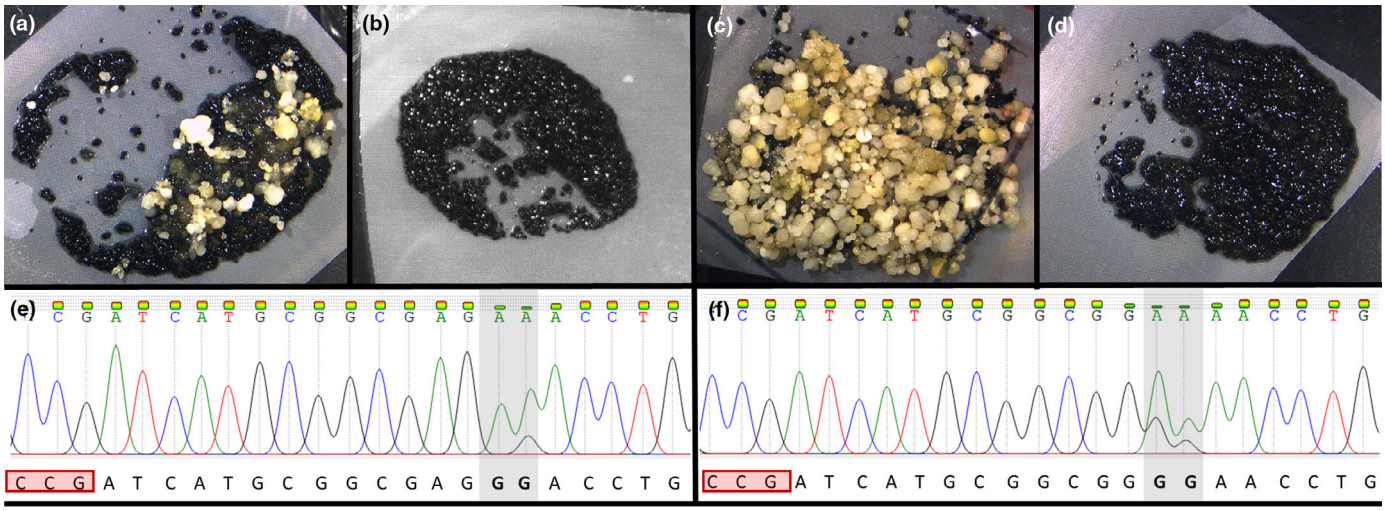
Transformation of *A. thaliana* with any of the unmodified versions of the banana *ALS* genes did not result in chlorsulfuron resistance. However, editing Pro186 of *MaALS6* or Pro181 of *MaALS10* in either leucine or phenylalanine did result in transgenic *Arabidopsis* resistant to chlorsulfuron (40 μg l^{-1}), indicating that both *MaALS6* and *MaALS10* function as *bona fide ALS* genes (Fig. S4).

sgRNAs were designed to specifically target the proline residue (Fig. 1b) for both *MaALS6* and *MaALS10*, as C-to-T conversion at the proline residue in both genes has been shown to result in chlorsulfuron resistance in other species (Svitashev *et al.*, 2016; Danilo *et al.*, 2019; Veillet *et al.*, 2019). sgRNAs were first tested *in vitro*, and both sgRNAs displayed similar efficiency (Fig. S5). We assembled a construct containing the cytosine base editor consisting of a rat cytidine deaminase APOBEC1 fused to an nCas9(D10A) and a uracil glycosylase inhibitor under the control of a maize ubiquitin promoter (Fig. 1c). The constructs *pETKUL21-PBE-ALS6*, *pETKUL21-PBE-ALS10* and *pETKUL21-PBE-ALS6+10* thus included a sgRNA targeting either *MaALS6*, *MaALS10* or both sgRNAs together, respectively (Fig. 1c). An ECS of the banana cultivar Williams was transformed with the above-mentioned constructs, and after 6 d, placed on selective medium. After 8 to 12 wk of selection on chlorsulfuron, 104 clumps could be regenerated from ECS transformed with *pETKUL21-PBE-ALS6*, 158 clumps with *pETKUL21-PBE-ALS10* and 234 clumps with *pETKUL21-PBE-ALS6+10*. In total, 496 independent cell clumps grew on chlorsulfuron from *c.* 2.5 ml settled cell volume (SCV). These individual clumps are expected to be derived from a single cell (Bertsch *et al.*, 2005; Bhatia & Bera, 2015). Differences could be observed in the amount of growing clumps from various cell suspensions ($P = 0.0074$, *t*-test, df = 34), highlighting the variability that can occur at any point in the transformation process (Fig. S6). As the construct also harboured a hygromycin resistance gene (*hptII*, Fig. 1c), it was possible to compare selection using hygromycin resistance occurring through the stable T-DNA integration in the banana genome with selection using chlorsulfuron resistance, which would only occur with the C-to-T conversion in at least one of the *MaALS* genes. For this, an identical amount of SCV was incubated on selective medium containing 50 μg ml^{-1} hygromycin and 200 mg l^{-1} timentin. The amount of clumps regenerated on the hygromycin medium was 10- to 30-fold higher in comparison with regeneration on chlorsulfuron (Fig. 2a–d). This difference is expected, as stable transformation events are more likely to directly result in resistant cells in comparison with base edits, which require an extra step post-T-DNA integration and therefore occur more rarely. In comparison with previous work reporting genetic transformation and regeneration of Williams cv (Pérez Hernández *et al.*, 2006), we are here reporting regeneration levels that are substantially higher. However, regeneration capacities of different banana cell suspensions have been shown to fluctuate, while the biological features behind this variation have remained elusive (Strosse *et al.*, 2003, 2006). While clumps regenerated on hygromycin are transgenic, they are not necessarily edited and thus resistant to chlorsulfuron.

Therefore, a selection of clumps regenerated on hygromycin was transferred to chlorsulfuron-selective plates after 8 to 12 wk, as to make an estimation of the editing rate. For banana transformants with *pETKUL21-PBE-ALS6*, only nine out of 23 survived, while for banana transformants with *pETKUL21-PBE-ALS10*, 19 out of 30 survived. Noticeably, 27 out of 34 transformants with *pETKUL21-PBE-ALS6+10* could grow on chlorsulfuron. No clumps could regenerate from untransformed cell suspensions on selective medium containing either chlorsulfuron or hygromycin (Fig. 2b,d).

Clumps grown on chlorsulfuron-selective plates were further regenerated, and a total of 411 clumps out of 496 regenerated into independent plantlet lines. Each clump mostly resulted in the regeneration of one plant line, with an overall regeneration efficiency of 82.8% (411/496). Lines transformed with plasmids *pETKUL21-PBE-ALS6*, *pETKUL21-PBE-ALS10* and *pETKUL21-PBE-ALS6+10* and regenerating on chlorsulfuron were randomly selected in order to profile their edits generated by the base editor at the *MaALS6* and *MaALS10* target sites. Sanger sequencing indicated that all selected lines harboured at least one edited *MaALS* allele. Derived allelic variability for selected lines transformed with plasmids *pETKUL21-PBE-ALS6*, *pETKUL21-PBE-ALS10* and *pETKUL21-PBE-ALS6+10* was calculated (Figs S7–S9). However, as one chromatogram can sometimes be the result of different mutations, which cannot be distinguished, allelic variability was always interpreted conservatively to avoid overestimating mutation rates. One single mutated allele was sufficient to result in chlorsulfuron resistance, as such lines were also retrieved (Fig. 2h). Base edits resulted in nonsynonymous mutations replacing Pro186 and Pro181 for *MaALS6* and *MaALS10*, respectively, with amino acids serine, leucine and phenylalanine, as expected (Fig. 2h). Four lines out of 10 targeted with both sgRNAs showed mutations at both target sites. Furthermore, additional mutations were detected in 10 out of 30 lines, including nonsynonymous mutations towards valine, cysteine, arginine, threonine and alanine, as well as frameshift mutations (Figs 2h, S7–S9). Importantly, these nonsynonymous mutations have also been reported to generate chlorsulfuron resistance in other plant species (Heap, 2024). Because the sgRNA targeting *MaALS6* or *MaALS10* only differs by two nucleotides, the sgRNA targeting *MaALS6* has a CFD off-target score (Doench *et al.*, 2016) of 0.85 for *MaALS10*, and the sgRNA targeting *MaALS10* has a CFD off-target score of 0.79 for *MaALS6*. As a consequence, two lines out of 10 targeted with *pETKUL21-PBE-ALS6* showed edited alleles of *MaALS10*, and one line out of 10 targeted with *pETKUL21-PBE-ALS10* showed edited alleles of *MaALS6* (Fig. 2h). In total, 63 mutant alleles were observed across all samples for *MaALS6* and *MaALS10* combined, out of 180. The overall on-target mutation ratio was 27/60 for *MaALS6* and 32/60 for *MaALS10*.

Subsequently, chlorsulfuron-resistant lines were screened for the absence of T-DNA. A total of 444 lines were analysed. A PCR-based approach was developed to identify transgene-free lines. Twenty-two lines did not show PCR amplification with a first primer pair targeted at the T-DNA (PP1, Fig. 1c). Lines that appeared negative for the T-DNA PCR1 were subsequently



(g)

Line	*MaALS6*	AA	*MaALS10*	AA
ALS6+10:21-3	CAGGTTTCTCGCCGCATGATCGG	Phe	CAGGTTTTCCGCCGCATGATCGG	Phe
	CAGGTTTCTCGCCGCATGATCGG	Phe	CAGGTTTTCCGCCGCATGATCGG	Phe
	CAGGTCTCTCGCCGCATGATCGG	Leu	CAGGTTCCCCGCCGCATGATCGG	Pro

(h)

pETKUL21-PBE-ALS6

	MaALS6			*MaALS10*		
ALS6:8-3	FRM			Ser	Ser	
ALS6:10-2	Cys	Cys				
ALS6:12-1	Ser					
ALS6:12-3	Arg	FRM				
ALS6:19-2	Ser	Ser				
ALS6:20-1	Phe					
ALS6:21-1	Phe					
ALS6:22-3				Thr		
ALS6:23-4	Phe	Phe				
ALS6:24-1	FRM	Cys	Arg			

pETKUL21-PBE-ALS10

	MaALS6			*MaALS10*		
ALS10:7-1				Ser	Ser	
ALS10:7-5				Ser	Ser	Ala
ALS10:8-18				Phe	Ser	
ALS10:9-1				Ser	Ser	Ser
ALS10:9-5	Phe			Ser		
ALS10:9-6				Phe	Phe	
ALS10:10-1				Ser		
ALS10:10-4				Phe	Ser	
ALS10:10-7				Phe	Ser	
ALS10:20-2				Ala	Ala	

pETKUL21-PBE-ALS6+10

	MaALS6			*MaALS10*		
ALS6+10:8-4	Ser			Ser		
ALS6+10:8-5	Val					
ALS6+10:9-3	Ser	Ser		FRM	Ser	
ALS6+10:9-12				Phe	Ser	
ALS6+10:10-6	Ser	Ser		Ser		
ALS6+10:10-7				Phe	Ser	
ALS6+10:11-3	Phe	Phe				
ALS6+10:12-2				Ala	Ala	
ALS6+10:20-1	Phe					
ALS6+10:21-3	Phe	Phe	Leu	Phe	Phe	

Fig. 2 Regeneration of chlorsulfuron-resistant banana calli depends on *MaALS* mutation. (a) Clumps regenerating on selective medium containing chlorsulfuron and timentin and (b) negative control. (c) Clumps regenerating on selective medium containing hygromycin and timentin and (d) negative control. (e) Chromatogram of clump ALS6+10:21:3 showing the targeted sequence of *MaALS6* and (f) *MaALS10*. Original sequence can be seen below the chromatogram, and the protospacer adjacent motif site is indicated in red. (g) Edited alleles for line ALS6+10:21-3. Expanded figures can be found in Supporting Information Figs S7–S9. (h) Overview of obtained mutations for clumps targeted with different constructs at their Pro186 and Pro181 sites for *MaALS6* and *MaALS10*, respectively. Desired mutations are indicated in green, and unexpected mutations in grey. Ten transgenic lines per construct were genotyped. AA, amino acid with appropriate three-letter abbreviation; FRM, frameshift.

analysed with a second primer pair (PP2, Fig. 1c). Of these, 17 did not show amplification with both primer pairs (Fig. S10). A full overview of the transgenic state of individual lines can be found in Table S3. Six lines were randomly selected for WGS. No plasmid DNA could be identified in two lines (Fig. 3a,b; Table S4). In another three lines, tiny snippets of reads were present across the plasmid T-DNA and backbone. Surprisingly, however, this pattern of coverage was also present in both negative controls. Given that this pattern is also in these negative controls, it is therefore assumed that this is the result of contamination across samples on either the DNA extraction or library preparation for WGS.

Indeed, many of the reads mapping partially to the negative control also mapped partially to coronavirus sequences (Fig. S11). To investigate whether the coverage on these negative controls was similar to those of the edited samples, the coverage by the amount of reads was calculated for each individual base along the plasmid backbone (total of 17 529 and 16 891 bases for *pETKUL21-PBE-ALS6* and *pETKUL21-PBE-ALS6+10*, respectively). Subsequently, the inverted distribution was plotted, showing the amount of bases of the plasmid backbones that were covered by a given amount of reads (Fig. 3). Bases covered by zero reads were omitted from the figure for clarity. Here, five edited lines showed lower or similar distributions compared with

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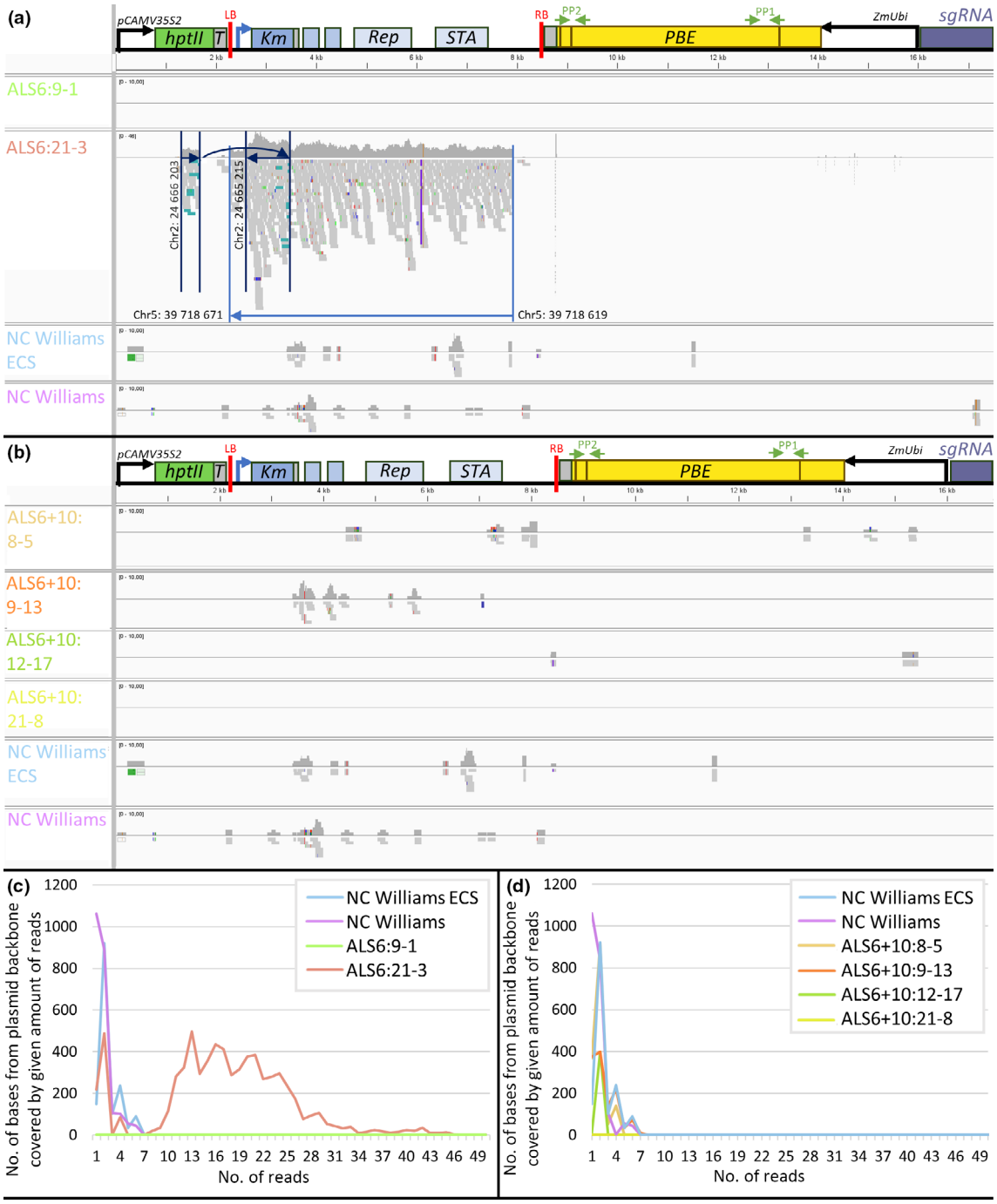

Fig. 3 Mapping of whole genome sequencing Illumina reads from transformed banana lines of the cultivar 'Williams' to the reference plasmid for (a) *pETKUL21-PBE-ALS6* and (b) *pETKUL21-PBE-ALS6+10*. Low coverage is present among all but two samples (ALS6:9-1 and ALS6+10:21-8). Negative controls (NC), either regenerated from embryogenic cell suspension (ECS), namely NC Williams ECS, or from *in vitro* plantlet, namely NC Williams, show low coverage on both plasmids. ALS6:21-3 contains two independent integration sites of a large part of the plasmid backbone on Chromosome 5 and a fusion of a partial hygromycin gene with additional backbone on Chromosome 2. (c) Line plot showing the number of bases of the plasmid that are covered by a given amount of reads on template *pETKUL21-PBE-ALS6* and (d) *pETKUL21-PBE-ALS6+10*, showing lower coverage for edited samples in comparison with wild-type, except for ALS6:21-3.

the negative controls (Fig. 3). One line (ALS6:21-3) did show substantially higher coverage. Further analysis showed two independent integration events. A large part of the plasmid backbone of *c.* 5.5 kb integrated in an intergenic region in Chromosome 5. A second event of *c.* 1.2 kb integrated in an intron of *Macma4_02_g10980*, a conserved hypothetical protein on Chromosome 2, and consisted of a partial hygromycin gene, linked to an inverted fragment of plasmid backbone (Fig. 3a). Both regions

(a)

	MaALS6			*MaALS10*		
ALS6:9-1	Phe	Val				
ALS6:10-7	Phe	Phe	Leu			
ALS6:21-3	Phe	Phe				
ALS6:23-8	Cys	Arg				

	MaALS6			*MaALS10*		
ALS10:10-3				Ser		
ALS10:21-6				Cys		

	MaALS6			*MaALS10*		
ALS6+10:8-5	Cys					
ALS6+10:8-15	Tyr					
ALS6+10:8-16	Phe					
ALS6+10:9-13	Phe			Ser		
ALS6+10:9-22	Phe					
ALS6+10:10-15	Cys					
ALS6+10:10-33				Cys		
ALS6+10:12-17				Ser		
ALS6+10:21-8	Phe					
ALS6+10:21-10	Phe	Phe	FRM	Phe	Phe	
ALS6+10:21-17	Phe					

(b)

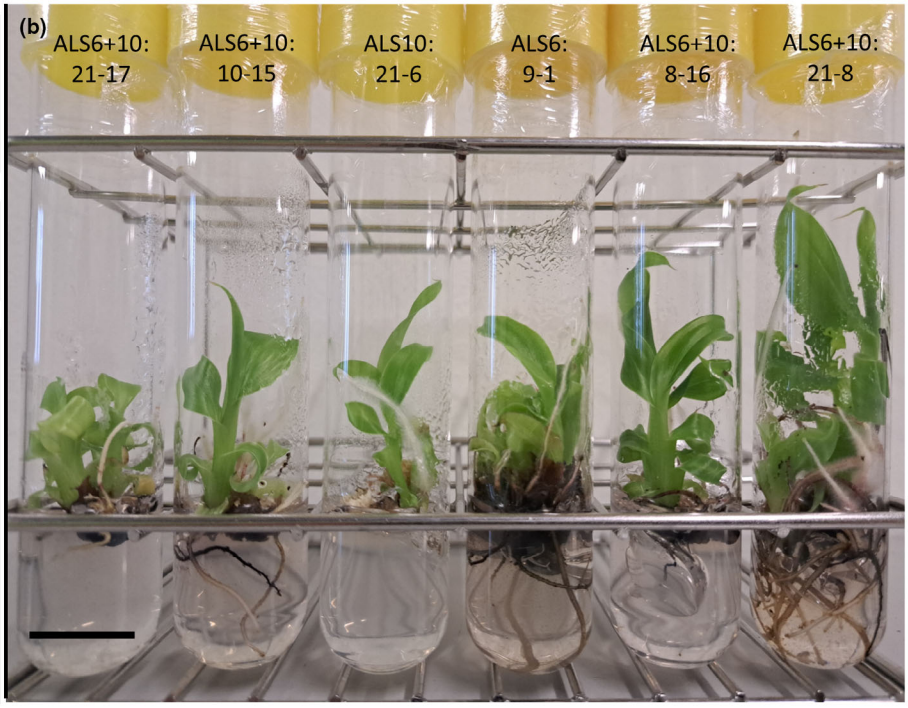

Fig. 4 Efficient and transgene-free editing of the *MaALS* genes in banana. (a) Mutations in *MaALS* genes of transgene-free lines. (b) Regenerating transgene-free plantlets. Amino acids are represented with the appropriate three-letter abbreviation. Bar, 2 cm.

were outside the amplified PCR fragments for T-DNA integration, confirming the results of the PCR-based detection of transgenes in edited banana lines. While the presence of partial T-DNA sequences cannot be excluded in lines identified as transgene-free with the PCR-based approach, the WGS data suggest that most of those lines could also be considered transgene-free. Therefore, the transgene-free editing rate could be estimated at *c.* 3.2% (5/6*17/444 lines) using the above-described method. The target sites in the *MaALS* genes of these transgene-free lines were investigated for mutations, and the result is visualized in Fig. 4. While most lines show only one edited allele, we were able to regenerate two lines with edits in both genes (ALS6+10:9-13 and ALS6+10:21-10), indicating the possibility of co-editing. In contrast to transgenic samples, no off-target editing could be identified on *MaALS6* when targeting *MaALS10*, or vice versa. The transgene-free gene editing rate was 5.4% (11/204 lines) for *pETKUL21-PBE-ALS6+10*. Eighteen per cent (2/11 transgene-free lines) harboured mutation at two target sites, indicating secondary mutations can be captured using our editing strategy. The overall efficiency to recover transgene-free gene-edited lines with edits in two distinct target genes is thus estimated at 1.0% (2/204) of all regenerating lines. Lines sent for WGS were screened for off-target mutations at off-target sites with a maximum of four mismatches. However, no C-to-D mutation nor frameshift could be identified within the editing window of the base editor at any of the putative off-target sites, indicating that no off-target effects were present in transgene-free gene-edited lines (Table S5). It should be noted that some off-target sites did already show some level of allelic differences in comparison with the reference genome in the negative controls, as can be seen in Table S5.

Discussion

As triploid banana contains two *bona fide ALS* genes, six alleles can be targeted to generate chlorsulfuron resistance. As the targeted region is conserved among *Musaceae* (Fig. S2), the used strategy can easily be expanded to other cultivars for which cell suspensions are already available (Strosse *et al.*, 2006). We observed that transformation with plasmid *pETKUL21-PBE-ALS10* appeared to generate a higher proportion of regenerants in comparison with *pETKUL21-PBE-ALS6*. Moreover, more transgenic clumps selected on hygromycin could survive after transfer to chlorsulfuron, when transformed with *pETKUL21-PBE-ALS10*. While transcriptomic data indicated that *MaALS6* is more actively transcribed (Fig. S3), this might indicate that targeted mutations are more easily achieved at the *MaALS10* locus in comparison with *MaALS6*. The sgRNA itself did not seem to effect Cas9 cleavage, as they had similar efficiencies *in vitro* (Fig. S5). Nevertheless, lines with a single edited allele could be regenerated for both *MaALS6* and *MaALS10*. Most of the transgenic lines showed at least two *MaALS* alleles edited. Editing of three alleles, however, appeared to be rare, even in transgenic lines after 12 wk. In wheat, rice and soybean, the level of sulfonylurea resistance has been shown to increase with the number of edited *ALS* alleles (Kawai *et al.*, 2007; R. Zhang *et al.*, 2019; Niu *et al.*, 2024). Most edits observed were clean C-to-T conversion, resulting in nonsynonymous mutations from proline to serine,

leucine or phenylalanine. Edited banana lines displaying a proline to either valine, cysteine, arginine, threonine or alanine conversion also displayed chlorsulfuron resistance. This is in line with observations made in other dicot and monocot species (Komor *et al.*, 2016; R. Zhang *et al.*, 2019; Heap, 2024). In addition, frameshift mutations were observed at low rates, as the use of the endogenous repair pathway for base excision repair sometimes results in indel mutations due to imprecise nonhomologous end-joining (Komor *et al.*, 2016; Ren *et al.*, 2021). Furthermore, one line showed mutations outside the editing window of Bases 3 to 9 distal to the protospacer adjacent motif (PAM) region, resulting in amino acid substitution R187C in *MaALS6*. This expanded editing window has been reported previously (Lv *et al.*, 2020; Jia *et al.*, 2024), indicating that the editing window for rAPOBEC1-BE3 should be reconsidered to include Base 10. While base editors with expanded editing windows and higher mutation efficiencies are readily available in plants (Shimatani *et al.*, 2017; Zong *et al.*, 2018; Jin *et al.*, 2020; Molla *et al.*, 2021; Ren *et al.*, 2021), they also increase the risk of undesired cytosine editing within the editing window, known as bystander editing. Furthermore, although base editors in general show greatly increased specificity in comparison with their Cas9-counterparts (D. Kim *et al.*, 2017), they can also show sgRNA-independent off-target deamination due to the high affinity of the deaminase for single-stranded DNA (Jin *et al.*, 2019; Zuo *et al.*, 2019; Randall *et al.*, 2021; Ren *et al.*, 2021). Notably, measuring such sgRNA-independent off-target effects would require sequencing of edited lines deep enough for unambiguous identification of such mutations. However, the introduction of sgRNA-independent off-target mutations is in the same order of magnitude as mutations arising from somaclonal variation (Ren *et al.*, 2021), and they can be further mitigated through various novel base editor strategies (Jin *et al.*, 2020; Ren *et al.*, 2021; Xiong *et al.*, 2023; He *et al.*, 2024). Because the transient delivery of our base editor system is likely to decrease the incubation time for the introduction of both sgRNA-dependent and sgRNA-independent off-target effects, the level of off-target effects is logically expected to be lower as compared to stable transformation with T-DNA carrying editor cassettes. The stable presence of the Cas9 editing machinery can indeed result in increased off-target effects and mosaicism (Goralogia *et al.*, 2024).

WGS and further analysis indicated that five lines out of six were transgene-free. One line (ALS6:21-3) featured two independent transgene insertions, including a plasmid backbone. Integration of the plasmid backbone (Ming *et al.*, 2008) and the T-DNA/backbone conglomerations has both been shown previously (Jupe *et al.*, 2019). While backbone integration is relatively common, it further increases the risk for false positives when specifically looking for transgene-free plantlets. Backbone integration is mostly the result of a read-through at the LB sequences, and multiple LB sequences have been shown to reduce unwanted backbone integration (Kuraya *et al.*, 2004; Thole *et al.*, 2007). Moreover, introducing a marker gene just outside the T-DNA near the LB sequence could facilitate early screening for backbone integration (Thole *et al.*, 2007), although WGS would remain essential to verify the absence of any plasmid backbone DNA.

While we are certain of a success rate of at least 1.1%, extrapolation from 17 samples would allow us to place an expected efficiency for transgene-free editing at 3.2% of regenerated lines. Noticeably, the rate of multiple edited alleles was higher in stable transformants as compared to transgene-free edited lines. Nevertheless, we were able to regenerate at least two transgene-free lines (ALS6+10:9-13 and ALS6+10:21-10) that show mutations at both target sites. Selection on chlorsulfuron facilitates the exclusive regeneration of successfully edited lines. As a result, the probability of recovering lines harbouring additional desired secondary mutations is increased in comparison with regeneration on nonselective medium. This strategy can thus be used to enrich for secondary edits at target sites without clear phenotype or selection procedure, in line with recently published co-editing strategies (X. Huang *et al.*, 2023). In banana, this strategy could practically be expanded to perform 18 transformations within one year by one person, requiring the monthly observation and maintenance of *c.* 2700 clumps, extrapolating to *c.* 86 transgene-free plantlets, of which 16 would contain mutations at both target sites. Recent publications have reported the aforementioned strategy to enrich for secondary and tertiary mutations in potato, tobacco, tomato and citrus (X. Huang *et al.*, 2023; Jia *et al.*, 2024). The use of markers for the identification of transgenic individuals can further increase the efficiency of the strategy, as this would allow the immediate removal of transgenic individuals. Fluorescent reporters have been used extensively in the selection of both transgenic and transgene-free progeny, but are themselves unable to select only mutated transgene-free events (He *et al.*, 2022). Other selection procedures for the removal of transgenic events have been reported, including hypersensitivity reactions to bentazon in rice (Lu *et al.*, 2017) or hygromycin and mannose (Wu *et al.*, 2019; Li *et al.*, 2020). However, these techniques use selection based on susceptibility, with a lower resistance towards bentazon, hygromycin and/or mannose in transgene-free individuals, which unwillingly creates a stressful environment for regenerating plantlets. Therefore, positive selection strategies are usually preferred because they reduce the risk of losing valuable edited lines.

While immediate editing through *A. tumefaciens* in the T0 generation has been shown in multiple crops, including highly heterozygous crops and those with long juvenility, this is to the best of our knowledge the first report that shows such transgene-free gene editing in a sterile crop species. As the process of embryogenesis facilitates the regeneration of full plantlets from a single cell, chimerism is expected to be limited for transgene-free gene-edited lines (Bertsch *et al.*, 2005; Bhatia & Bera, 2015). In contrast to previous studies (Hamada *et al.*, 2018; Bánfalvi *et al.*, 2020; X. Huang *et al.*, 2023; Jia *et al.*, 2024), no chimeric regenerants were observed for transgene-free gene-edited lines based on the investigation of the on- and off-target sites. Chimeric regeneration cannot be ruled out for stable transformants, as continuous editing is likely to result in multiple mutations and mosaicism at both on- and off-target sites. Especially in vegetatively propagated crops, chimerism cannot be eliminated through breeding and selection of new planting material requires sequence verification.

With the establishment of a transgene-free gene editing platform in banana, it is now possible to develop banana varieties for commercialization with desired edits. The generation of edits in multiple genes in such a co-editing strategy paves the way for efficient selection of edited transformants. The combined targeting of *MaALS* and one or several candidate genes for improved traits will be instrumental in generating and selecting transgene-free bananas harbouring the edits of interest. Importantly, however, the proposed strategy, targeting *MaALS* to select for desired edits at secondary target sites, is a one-time solution, as selection for continual iterative editing in a single cultivar is not feasible once chlorsulfuron resistance is introduced. Strategies to increase the multiplex capacity during a single transformation event could therefore further increase the strength of the proposed system. Additionally, other target sites could expand the possibilities to introduce further edits in previously improved cultivars. Although not validated in banana, targeted mutations in, for example, *acetyl-coenzyme A carboxylase* (*ACCase*) (Liu *et al.*, 2020), *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) (Li *et al.*, 2016) and rice tubulin gene *OsTubA2* (Liu *et al.*, 2021) have resulted in resistance to aryloxyphenoxypropionate herbicides (including haloxyfop), glyphosate and dinitroaniline herbicides, respectively, in several crop species.

While banana streak virus sequences, integrated in the B genome of banana cultivars, appear as straightforward target sequences to generate virus-free germplasm for breeding purposes (Tripathi *et al.*, 2019), identification of mutations associated with resistance to stresses, improved nutritional composition or enhanced agronomic performance (Ortiz & Swennen, 2014; Zorrilla-Fontanesi *et al.*, 2020; Tripathi *et al.*, 2024) will help empower the transgene-free editing strategy by providing target genes for the generation of improved banana edited lines.

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Competing interests

None declared.

Author contributions

SVdB, BP and HV designed the experiments and contributed to the writing and editing of the manuscript. SVdB performed experiments and conducted phenotypic characterization and maintenance of cell lines. YN performed the WGS analysis. SVdB, YN, BP and HV contributed to the interpretation of the results.

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Data availability

The haplotype-resolved *MaALS* sequences of Williams are available at Genbank, PQ304390–PQ304395. WGS of transgene-free lines and Williams control is available at NCBI through Bioproject PRJNA1157598. Supporting Information (Figs S1–S11; Tables S1–S5) is provided together with the article.

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Supporting Information

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

Fig. S1 Natural resistance of banana embryogenic cell suspension of the cultivar 'Williams' to chlorsulfuron at different concentrations.

Fig. S2 Phylogeny of variants of the *acetolactate synthase* genes in the *Musaceae* family.

Fig. S3 Transcriptional regulation of *MaALS6* and *MaALS10* in different *Musa* species and cultivars.

Fig. S4 Chlorsulfuron resistance of transgene *Arabidopsis thaliana* plants harbouring codon-optimized banana *MaALS* genes with mutations conferring resistance to chlorsulfuron.

Fig. S5 Gel electrophoresis visualizing *in vitro* cleavage assay of *MaALS6* and *MaALS10* with designed single-guide RNAs.

Fig. S6 Variability between banana embryogenic cell suspension regeneration rates on chlorsulfuron.

Fig. S7 Allelic variability after transformation with *pETKUL21-PBE-ALS6* in banana.

Fig. S8 Allelic variability after transformation with *pETKUL21-PBE-ALS10* in banana.

Fig. S9 Allelic variability after transformation with *pETKUL21-PBE-ALS6+ 10* in banana.

Fig. S10 Gel electrophoresis visualizing amplification of T-DNA in chlorsulfuron-resistant banana clumps.

Fig. S11 Reads of negative control (NC) samples NC Williams embryogenic cell suspensions and NC Williams mapping (partially) to plasmid *pETKUL21-PBE-ALS6+ 10*.

Table S1 Genomic location of *MaALS* in *Musaceae*.

Table S2 Primers used in this study.

Table S3 Overview of T-DNA state of chlorsulfuron-resistant banana clumps.

Table S4 Plasmid coverage on banana edited samples sent for whole genome sequencing.

Table S5 Off-target homologous sites with up to four mismatches to the target sites in the *MaALS6* and *MaALS10* genes.

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