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REPORT

Cassava for the future: embryogenic liquid cultures suitable for new biotech techniques

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ABSTRACT

Cassava, a crop of importance for subsistence farming in Africa, Asia, and Latin America, has the potential to benefit from global economic integration as a versatile industrial resource. Enhancing cassava productivity is not just a matter of agricultural competitiveness but a crucial step toward ensuring many communities' food security and livelihoods. Given its high performance in marginal environments, where climate change poses threats, ensuring food security and livelihoods relies on rapidly adapting cassava. This study aimed to develop a protocol that swiftly transitions cassava embryogenic short-period liquid suspension cultures, facilitating the regeneration of genetically stable *in vitro* plants. The resulting protocol, with its potential to be a foundational component in future technologies employing various genome editing or genetic modification techniques, holds promise for the advancement of cassava biotechnology.

METHOD SUMMARY

The method combines the two major players in this protocol: Casava's short suspension culture and an alternative bacterial strain that shows the potential to recognize these cells as a target for genetic modification. The method exhibits a high potential for developing future editing protocols for cassava.

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AFLP; *ensifer adhaerens (OV14)*; genetic stability; genotyping; gus; *manihote esculenta*; plants; SNP

1. Introduction

Cassava, a crucial source of calories and income, particularly among low-income families in the tropics, notably Sub-Saharan Africa [1], faces significant challenges. It holds a top-tier position, often the first or second crop in terms of land and resource allocation, within numerous tropical farming systems, particularly in Africa. These systems, characterized by small-scale farmers averaging 0.5 hectares cultivating multiple crops concurrently, rely on cassava to safeguard the rural population in sub-Saharan Africa against hunger and malnutrition [2]. However, despite its importance, cassava has yet to fully harness emerging technologies' benefits [3,4]. These revolutionary techniques, which have the potential to fast-track plant science and innovation, propelling plant breeding and germplasm development [5], still need to be fully utilized by cassava. This research aims to address this gap by developing a protocol that swiftly transitions cassava embryogenic short-period liquid suspension cultures, facilitating the regeneration of genetically stable *in vitro* plants. The urgency of this research, underscored by the pressing need to adapt cassava to climate change rapidly, highlights the potential of this protocol in enhancing cassava productivity and competitiveness, making it a crucial and timely contribution to the field.

Regrettably, Africa's cassava production and millions of small-scale farmers confront significant challenges, hindering productivity and sustainability. One of the constraints for farmers is the Cassava Mosaic Disease (CMD), which causes severe damage to cassava production. CMD, transmitted via whiteflies, propagates primarily through infected cuttings, imposing significant constraints on cassava production [6]. The complexity deepens as farmers predominantly cultivate preferred cultivars selected based on economic value and suitability for diverse geographical and climatic zones [7]. Developing cassava cultivars resistant to Cassava Mosaic Disease (CMD) and Cassava Brown Streak Disease (CBSD) remains a critical priority, in tandem with ensuring high productivity and tolerance to post-harvest physiological deterioration (PPD) [8,9]. Traditional breeding methods

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for cassava can extend beyond a decade to release a new variety, often involving compromises between desirable and less desirable traits. To overcome these challenges, next-generation breeding programs increasingly turn to biotechnological approaches such as genomic selection (including marker-assisted selection) and transgenics (gene transfer). These advanced methods offer significant opportunities to enhance the suite of cassava varieties developed by CGIAR institutions like CIAT and IITA. Additionally, established tissue culture techniques such as embryogenic suspension cultures, alongside exploration of alternative bacterial strains [10], show great potential in advancing the development and distribution of disease-free cassava with enhanced traits like whitefly resistance, high yield and PPD tolerance.

Our research presented here aimed to simplify the protocol for cassava transformation by using embryogenic suspension cultures in combination with an alternative bacterial strain.

2. Materials & methods

2.1. Cassava in vitro donor material

Three cassava clones (*Manitoh esculenta* Crantz) Kasetsart (KU 50), SM1219-9, and TMS60444 as *in vitro* plants have been obtained from CIAT Gene Bank, Colombia collection, on medium Murashige-Skoog [11] hormone-free, supplemented with 2% sucrose and 0.5% agar, pH 5.6–5.8. All proliferated shoots were subcultured as node cuttings every 3–4 weeks on the same type of medium and cultivated at 28°C with a 12-hour photoperiod.

2.2. Liquid embryogenic cultures & cassava in vitro plant regeneration

For somatic embryogenesis induction, axillary buds from *in vitro* plants were collected in filters sterilized liquid MS medium (micro, macro elements) in combination with Gamborg B5 vitamins [12] supplemented with 50 mg/l picloram, 0.5 mg/l $CuSO_4.5H_2O$, 150 mg/l casein hydrolysate, 20 g/l sucrose, pH 5.6–5.8. Usually, cultures are kept in the dark at 24°C until embryogenic tissue and somatic embryos are formed [12]. On the 10th day, the liquid medium with the released cells was pipetted into a sterile tube, and the cultivation continued for 3–5 days on a shaker, shaking at 50 rpm in the dark at 24°C.

Cultures were filtrated through the Büchner funnel following the cell suspension with sterile filter paper inside. The filter was placed into Petri dishes with solid MS medium supplemented with 5 mg/l 2,4-D, 150 mg/l casein hydrolysate, 450 mg/l L-glutamine, 20 g/l sucrose, and 2.7 g/l gelrite, pH 5.6–5.8. Cultures were grown in the dark at 24°C in a growth chamber.

Subsequently, proliferating embryogenic cultures were transferred to shoots induction medium MS supplemented with 3 mg/l BAP, 1 mg/l NAA, 150 mg/l casein hydrolysate, 450 mg/l L-glutamine, 30 g/l sucrose and 2.7 g/l gelrite, pH 5.6–5.8. Cultures were grown in light intensity (75 μmol/m^2/sec) for a week following transfer to light (120 μmol/m^2/sec) with a photoperiod of 12 h at 24°C.

Within 3 weeks, induced shoots were transferred to MS medium supplemented with 0.5 g/l gibberellic acid and 100 mg/l edamin, pH 5.6–5.8, for shoot elongation. Plants have been growing at 28°C with the full light regime (120 μmol/m^2/sec) and a photoperiod of 12 h. Plant rooting took place on an MS hormone-free medium supplemented with 20 g/l sucrose and 3 g/l gelrite, pH 5.6–5.8, and plants have been growing at the same culture conditions.

2.3. Bacterium: mediate cassava suspension culture transformation

For the *Agrobacterium*-mediated transformation experiments, we used two bacterial strains: Agrobacterium tumefaciens EHA 105 harboring pCAMBIA 1305.2 and *Ensifer adhaerens* (OV14) harboring the super binary vector pCAMBIA 5105. Both bacterial strains were handled according to [10] and carried the gusInt or *gus* Plus gene, respectively.

2.4. Inoculation & co-culture of suspension cultures

Cassava cell suspension cultures KU 50, SM1219-9, and TMS60444, were supplemented with 200 μM acetosyringone and shaken for approximately 4 hours at 100–200 rpm in the dark at 24°. This was followed by bacteria inoculation using *E. adhaerens* (OV14) or *A. tumefaciens* (OD 0.1), respectively, in volume 1:1. Co-culture with bacteria took place for approximately 50 min at 21°C without shaking.

Subsequently, the cassava suspensions were filtrated through a Büchner funnel with sterile filter paper. The filter was placed into a Petri dish with Co-culture MS medium supplemented with 5 mg/l 2,4-D, 150 mg/l casein hydrolysate, 450 mg/l L-glutamine, 200 μM Acetosyringone, 500 mg/l MES, 20 g/l sucrose and 2.7 g/l gelrite, pH 5.6–5.8. Cultures were grown for 3 days at 21°C in the dark.

After 3 days of co-culture with bacteria, the filter paper with cell cultures was transferred to fresh MS medium supplemented additionally with 160 mg/l timentin and 3 mg/l BAP, 150 mg/l casein hydrolysate, 450 mg/l L-glutamine, 500 mg/l MES, 20 g/l sucrose, 2.7 g/l gelrite,

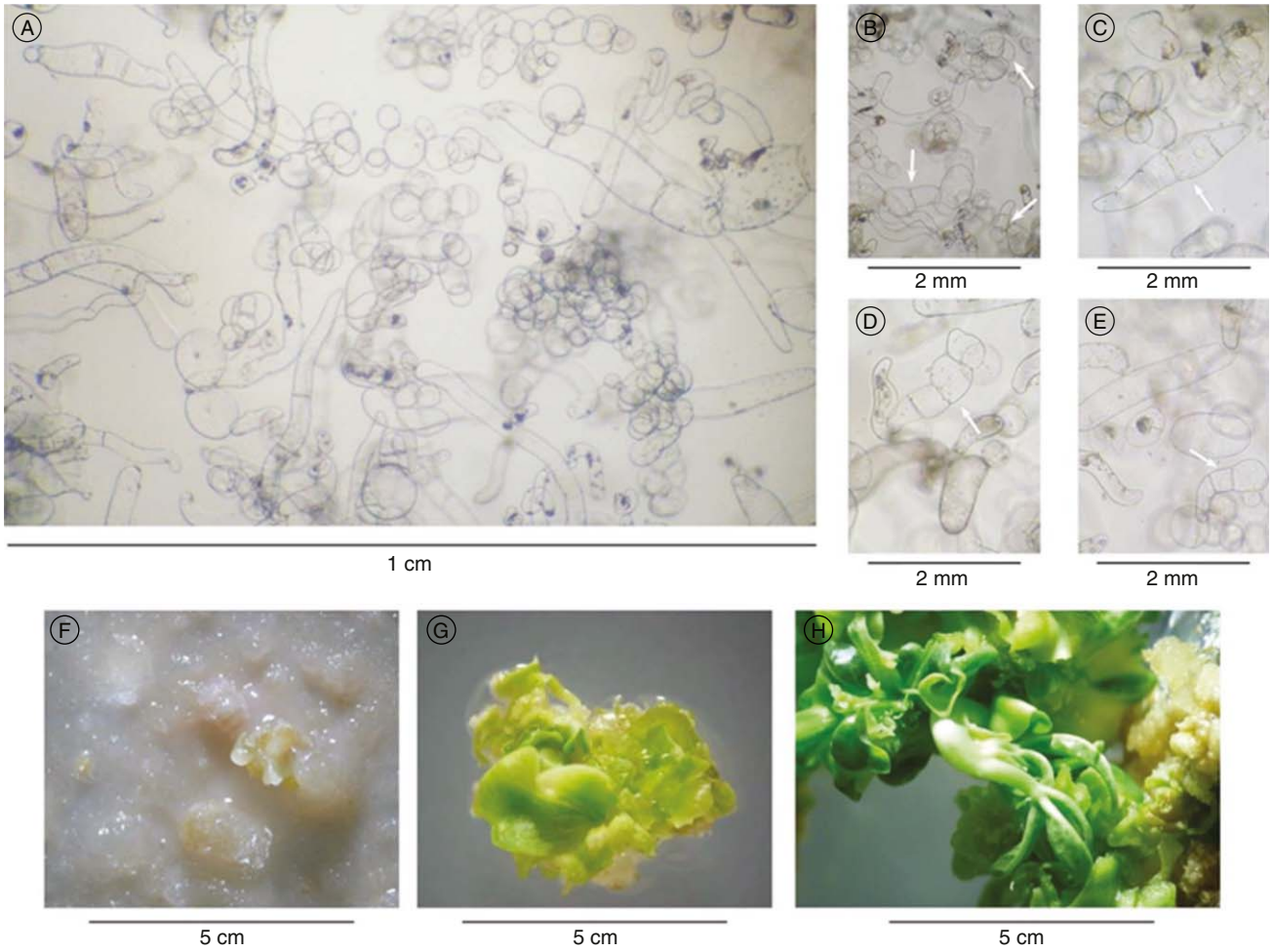

Figure 1. Cassava suspension cultures **(A–E)** magnification 20× arrows showing an early somatic embryo development, **(F)** embryogenic callus and somatic embryos growing on solid medium with filter paper, **(G & H)** TMS60444 shoots induction and elongation.

pH 5.6–5.8. Cassava suspension culture recovery took place for 7–10 days. Cultures grew at 24°C with light (75 μmol/m^2/sec) with a 12 h photoperiod. On the 10th day, embryonic suspension cultures were carefully collected from the filter paper disk and were assayed for *gus* gene expression according to [13].

2.5. Molecular analysis of Cassava in vitro plantlets

Leaf samples were collected from 40 *in vitro*-grown cassava plantlets. The plantlets were generated from the embryogenic suspension of the genotype TMS60444, which is cataloged as NGA11 in the CIAT gene bank. For DNA extraction, we utilized a modified CTAB (Cetyltrimethylammonium bromide) based protocol described by [14]. Modifications included DNA extraction from leaf tissues that had been ground to powder. This tissue was processed using a Qiagen Tissue Lyser system (Venlo, Netherlands).

2.6. Amplified fragment length polymorphism genotyping

Amplified fragment length polymorphism (AFLP) was performed essentially as described by Vos et al. (1995) [15], with the following modifications for cassava as reported in [16]: The AFLP templates were prepared by digesting 1 μg of genomic DNA with the restriction enzymes *Eco*RI & *Mse*I, and the resulting AFLP fragments were visualized using 2 *Eco*RI/*Mse*I primer combinations (PC) (PC-1: ACT/CAT and PC-2: ACA/CAC). The AFLP fragments were resolved on 6% denaturing polyacrylamide gels and labeled by silver staining [17]. The presence/absence of scorable AFLP fragments was captured and evaluated in the 40 cassava accessions.

2.7. AFLP identification of duplicate individuals

To identify duplicate individuals within the AFLP dataset, we employed a genetic distance approach using the Hamming distance. This method quantifies the number

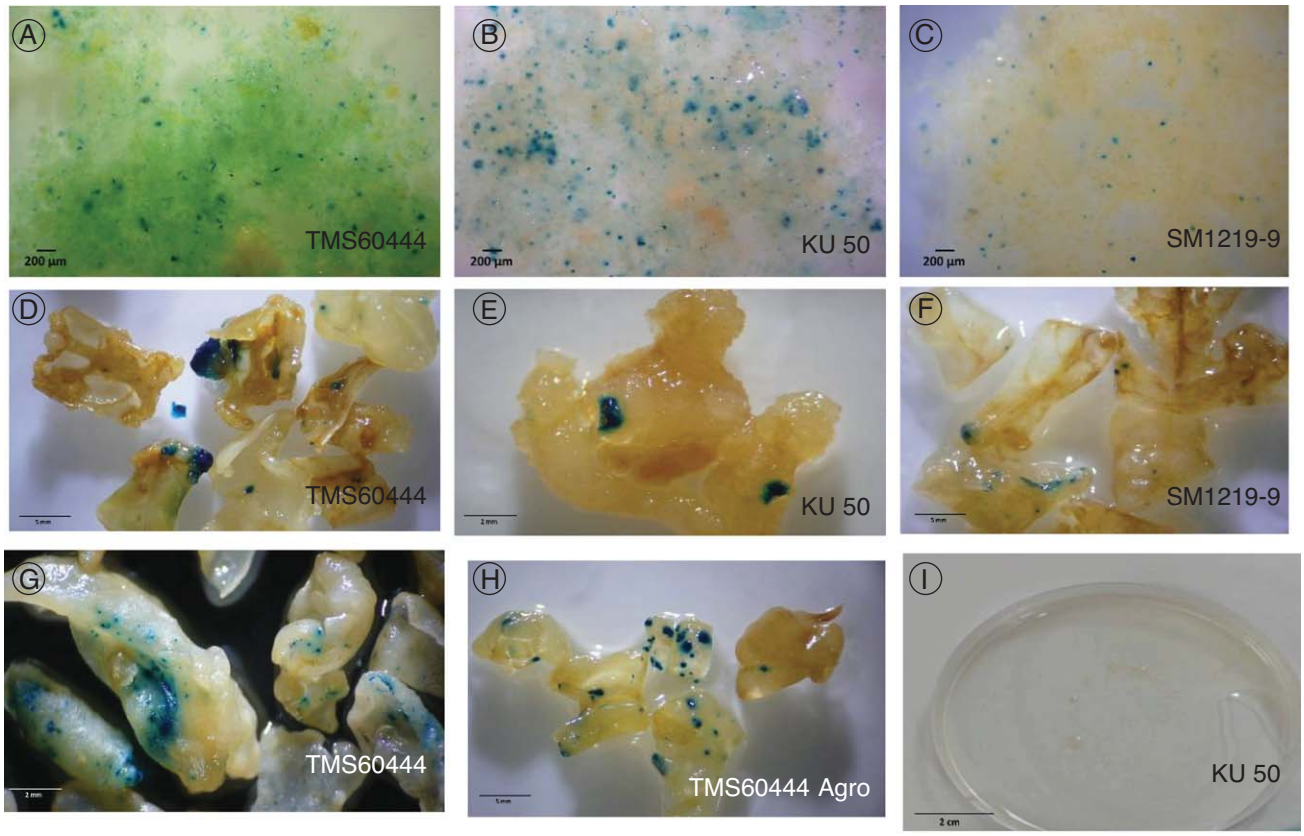

Figure 2. Shows Gus-Plus expression in cassava tissue transformed with *E. adhaerens* (OV14): **(A–C)** suspension cultures, **(D–F)** leaves from *in vitro* plants, **(G)** cotyledons from *in vitro* cultures, **(H)** cotyledons *A. tumefacient* EHA 105 harboring pCAMBIA 1305.2 (positive control), and **(I)** a negative control test for endogenous gus expression in *E. adhaerens* (OV14).

of differing markers between pairs of individuals. For this analysis, we utilized the **stringdist** package in R, known for its robust computation of Hamming distances for binary data, according to van der Loo, 2014 [18]. Initially, AFLP data were prepared by encoding them into binary matrices, with rows representing individual samples and columns representing markers that indicate the presence or absence of AFLP bands. This data was loaded into R using standard methods [e.g., read.csv [18] (Version 12, R Foundation for Statistical Computing, Vienna, Austria).

To facilitate the analysis, we converted the binary matrices into string formats for each individual, enabling the use of the stringdistmatrix function from the stringdist package. We computed the Hamming distance matrix to identify duplicate pairs, explicitly excluding self-comparisons. We identified duplicates as pairs of individuals with a Hamming distance of zero.

2.8. AFLP assessment of genetic diversity

Genetic diversity within the cassava variants was quantified using the diversity function from the vegan [19] package, focusing on Shannon's index as a measure of diversity. Furthermore, we evaluated the genetic relationships among the variants through hierarchical clustering, employing the Jaccard distance measure to determine the linkage.

2.9. Visualization of AFLP results

A heatmap of the distance matrix was generated using the ggplot2 and reshape2 packages, highlighting the clustering of duplicate samples. Additionally, the frequency of identified duplicates was illustrated through a bar plot, which employed the barplot function to quantify and visually represent the redundancy within the dataset.

2.10. Single nucleotide polymorphisms genotyping

This study's standard DNA amount of 60 ng per sample was meticulously processed using a protocol for genotyping 96 single nucleotide polymorphism (SNP) genotyping in cassava. This genotyping was performed utilizing the EP1™ system and 96.96 SNP type assays of Fluidigm® version S.01, SNPY-Chip, which facilitates the concurrent collection of both end-point and real-time data from a single chip cell. This technique

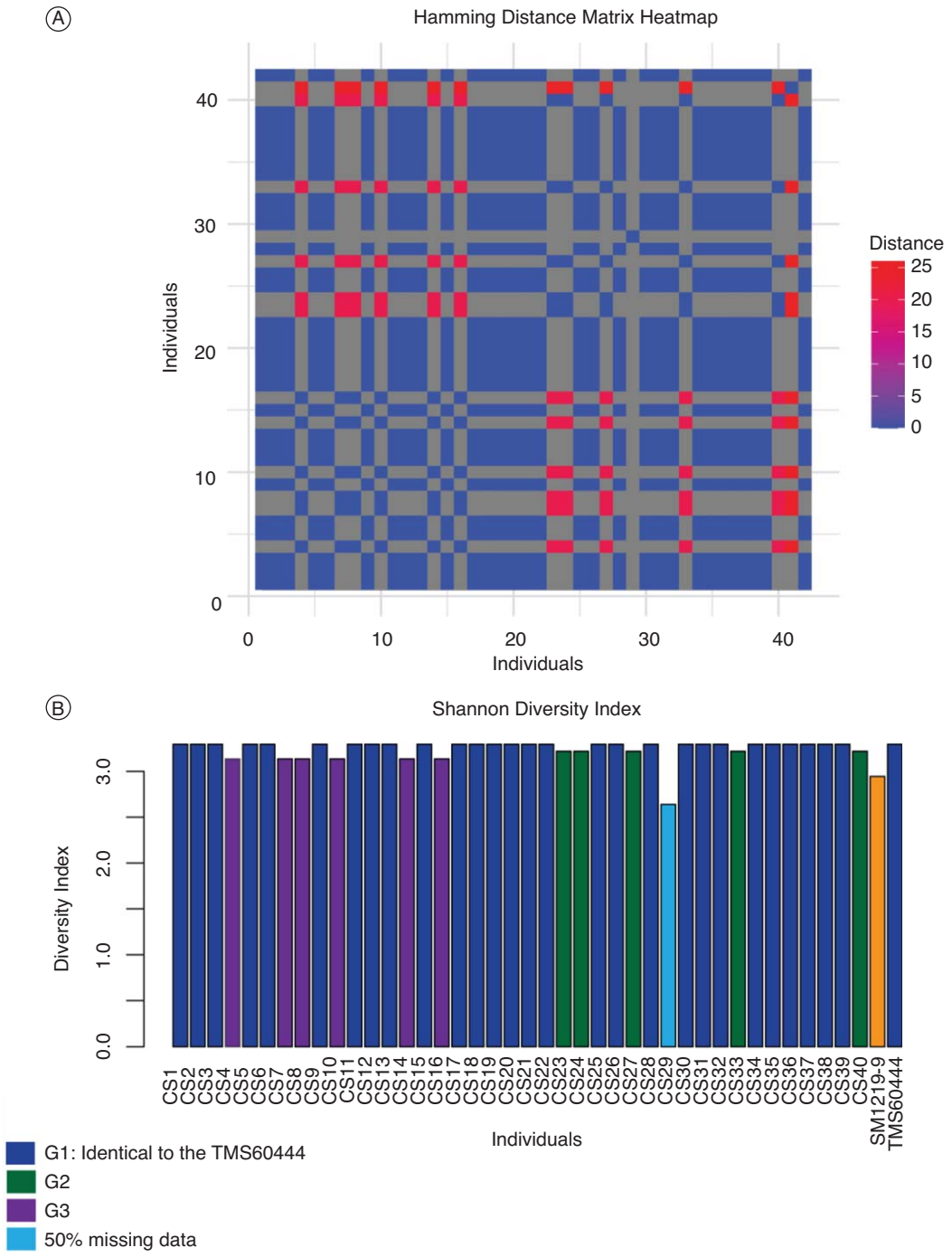

Figure 3. Genetic Diversity and Similarity Analysis, **(A)** Hamming Distance Matrix Heatmap: Displays pairwise Hamming distances among 44 individuals. Dark blue indicates lower distances (higher similarity), red signifies higher distances (lower similarity), and grey shows intermediate levels. Each axis represents individual comparisons, with the diagonal showing zero distance. **(B)** Shannon Diversity Index: This bar graph illustrates the Shannon Diversity Index for each individual, grouped into G1 (blue), G2 (green) and G3 (orange). G1 individuals are identical to the reference "TMS 604444". The index reflects genetic diversity, with most individuals displaying consistent levels except for a few outliers.

proves highly reliable, with a robust confidence level of 97%.

Notably, this protocol has demonstrated its efficacy across multiple prior studies, contributing to the analysis of cassava diversity and varietal identification [20,21]. Data was extracted using the Fluidigm SNP Genotyping Analysis software to obtain genotype calls. The resulting genotypic binary matrix was then integrated into a .vcf file format for further genetic analysis.

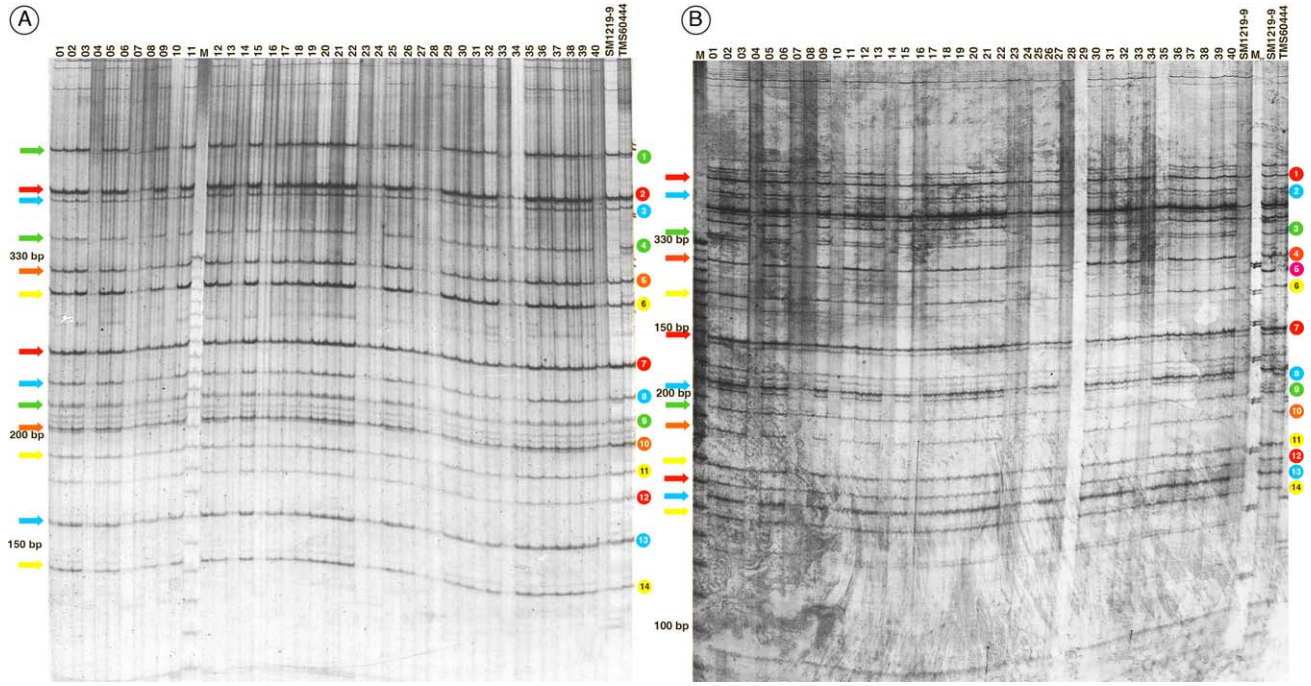

Figure 4. AFLP Marker Analysis with Two Primer Combinations. This figure presents AFLP marker analysis for TMS 60444 and SM 1219-9 genotypes alongside 40 plantlets derived from cell-suspension cultures of donor cassava plant TMS60444 (*M. esculenta* Crantz). Analyses utilized PC-1 (EACT/MCAT) and PC-2 (EACA/MCAC) primer combinations. Fourteen scorable AFLP fragments in Panel A and 14 in Panel B are indicated with arrows. Lanes 12 in Panel A and lanes 1 and 43 in Panel B display the molecular weight markers (30-330-bp AFLP® DNA Ladder; Invitrogen, USA). On lane 43, the size molecular weight markers failed preparation (M_{FP}). Lanes 2-40 represent the derived cassava plantlets from TMS 60444. Precisely, lane 41 in Panel A and lanes 41 and 43 in Panel B correspond to cassava genotype SM 1219-9. Lane 42 in both panels identifies the reference transformation cassava genotype TMS 60444.

2.11. SNP genotype profiling & clonality assessment

The analysis of SNP data was conducted using the VCF generated from the fluidity data. The study was performed on the NGSEP platform [22] along with CIAT's 12,000 SNPY-Chip database to determine genetic duplication accurately. During this process, samples were considered accurate genetic duplicates if they displayed 3% or fewer total differences across both homozygous and heterozygous loci, ascertained through a rigorous evaluation process.

3. Results & discussion

Several protocols based on FEC (friable embryogenic calli) solid cultures were developed and used in several laboratories in connection with *Agrobacterium*-mediated transformation [23] or genome editing [24]; however, this approach is still recognized as laborious and time-consuming, genotype-depending, and it can lead to changes in plant morphology [25]. This study developed a robust and user-friendly method for the induction of embryogenic cassava suspension cultures. We observed the early stages of somatic embryogenesis in suspension cultures (Figure 1A–E) very similar to the one described by [26] following the embryogenic callus and somatic embryo formation (Figure 1F) continued with the regeneration of *in vitro* plants (Figure 1G & H). The method worked well for all our cassava genotypes tested, which is valuable from a research and breeding perspective.

We performed transformation experiments with *E. adhaerens* (OV14) and the suspension cultures from three cassava genotypes. According to our results, cassava suspension cultures can be effectively transformed with this alternative bacterial strain (Figure 2A–C). *Gus* gene expression was visible in many cells; the best was cv. KU 50. If cassava *in vitro* leaves were used as a target tissue for transformation, the *gus* gene expression was detected in this tissue as well (Figure 2D–F), and cotyledons of TMS60444 can express the *GUS* gene, too (Figure 2G). Transformation experiments with *A. tumefacient* strain EHA 105 harboring pCAMBIA 1305.2 in combination with cassava TMS60444 *in vitro* leaves and cotyledons were performed as a control for the transformation parameters tested (Figure 2H). An empty *E. adhaerens* (OV14) bacterial culture was verified for the potential endogenous *gus* gene expression in suspension (Figure 2I), and we have seen no expression there.

In some *in vitro* cultures, somaclonal variation can occur due to recurring propagation in the medium, and regenerated plants can show phenotypic and genotypic variation [27,28]. We conducted a comprehensive genetic analysis of 40 *in vitro*-derived plantlets from the cell suspension cultures of the TMS60444 genotype using AFLP and SNP marker systems. The AFLP analysis revealed 28 scorable fragments and no polymorphic bands were observed within the groups, indicating a high level of genetic fidelity among the regenerated plants. Only 27% of the regenerated plants showed two to four polymorphic bands, suggesting minimal genetic drift from the original genotype (Figure 3). Only 27% of the regenerated plants showed two to four polymorphic bands, suggesting minimal genetic drift from the original genotype (Figure 4).

The SNP analysis was performed using CIAT's highly diagnostic SNP arrays on the Fluidigm platform. Each of the 40 embryogenic lines underwent SNP characterization using chip #1381900245 against CIAT's extensive cassava SNP database comprising over 12,000 genotypic entries. The SNP results confirmed that the genetic makeup of the cell-suspension-derived plantlets was identical to the original TMS60444 genotype. This was further supported by SNP analysis of additional TMS60444 samples run on different Fluidigm chips, all clustering within the same genetic group (Cluster-9). Similarly, SM1219-9 samples assessed on various Fluidigm chips formed a distinct genetic group (Cluster-352), underscoring the reliability of our SNP-based assessment.

AFLP technology, enhanced by the use of methylation-sensitive enzyme *Mse*I along with the methylation-insensitive *Eco*RI, provided insights into the epigenetic changes potentially influencing gene expression during regeneration. Notably, the loss of a few specific AFLP bands in groups of plants (G3 lost two bands; G2 lost four bands) indicated epigenetic modifications can be controlled by improving the tissue culture methodology. The epigenetic changes reported here are consistent with findings from previous studies suggesting that tissue micropropagation in cassava leads to genome-wide changes in DNA methylation [29] (Figure 4).

These findings collectively underscore the potency of the tissue culture protocol outlined in this study. By mitigating or even eliminating the potential for somaclonal variation both before and during the genetic transformation process in cassava, our results have far-reaching implications. They lay a robust foundation for the secure and productive utilization of *in vitro* technology in various applications, ranging from breeding programs to the broader distribution of cassava. Our work advances our understanding of molecular marker applications [14] and bolsters the reliability and viability of genetic manipulation strategies in cassava propagation.

4. Conclusion

This paper proposes inducing cassava embryogenic suspension cultures as a target tissue for *Agrobacterium*-mediated transformation. At the same time, the results presented here show that *E. adhaerens* (OV14) can be used as an alternative bacterial strain for cassava genetic modification. Through our molecular marker analysis, we could unequivocally establish the absence of interclonal polymorphism among the 40 embryogenic lines. These lines originated from diverse embryogenic cell types derived from the cassava genotype TMS 60444, and further comparison with the naturally propagated TMS 60444 genotype reinforced this conclusion. Remarkably, the regenerated cassava plantlets stemming from embryos sourced from the TMS 60444 cell suspension displayed fewer differences in DNA sequences when contrasted with the original TMS 60444 cassava clone.

5. Future perspective

Many efforts have been made in the past decades to simplify cassava transformation protocols. However, they must still be more complex, laborious, and reproducible for clones and cultivars. They contain several steps, where just producing the required amount of tissue, which bacteria can recognize as the target, is laborious and time-consuming. Exploiting embryogenic suspension cultures, which can be scaled up using bioreactor technology, can mean a real and significant break. It can also be done with the help of alternative bacteria. The low methylation rates observed in our experiments are particularly substantial, indicating that our tissue culture methods are less likely to induce unwanted genetic and epigenetic variations. These findings are supported by detailed AFLP and SNP analyses, which have shown high genetic fidelity among regenerated plants. Such stability is essential for maintaining the integrity of desirable traits across generations, thereby ensuring the effectiveness of subsequent breeding programs.

Article highlights

Cassava tissue cultures

- Successful establishment of friable embryogenic callus from axillary buds of cassava cv. KU 50, SM1219-9, and TMS60444.
- Successful establishment of cassava embryogenic suspension from three cultivars.

Cassava genetic modification

- Developing a method for *Agrobacterium* and an alternative *Encifer*-mediate transformation utilizing cassava suspension cultures.
- *Gus* gene expression was observed in different types of cassava tissue.

Molecular marker analyses & general contribution to the technology

- Confirmation of high genetic stability plants regenerated from embryogenic suspension cultures.
- Enhanced Protocols for Cassava Biotechnology, making them more user-friendly, less labor-intensive, and scalable.
- Potential to accelerate disease-resistant and high-yield cassava variety development and deployment.

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Author contributions

B. Dedicova planned and designed this study, performed the experiments, analyzed the data, and wrote the manuscript. LAB Lopez-Lavalle performed the molecular analyses for the AFLP and SNP genotyping analyses, analyzed the data of regenerated *in vitro* cassava plants, and wrote the molecular part of the manuscript.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, stock ownership or options, and expert testimony.

Writing disclosure

No writing assistance was utilized in the production of this manuscript.

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