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RESEARCH ARTICLE

A quick method of *Agrobacterium*-mediated transformation protocol from immature embryos of tropical Indian maize genotypes

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Abstract

The principal obstacle in transferring desirable genes is the absence of an efficient regeneration and transformation protocd. In our study, we achieved successful plant regeneration and transformation by employing immature embryos derived from five tropical inbred lines of *Zea mays*, namely, V390, HK193-2, PV5, PV6 and HK1-163. Notably, the formation of embryogenic calli was observed within a brief 7-day period and subsequent plant regeneration was accomplished within 30 days from the embryogenic callus on a culture medium containing MS supplemented with 1mg/L 6-Benzylaminopurine (BAP) and 0.1 mg/L Thidiazuron (TDZ). For the transformation experiments, we employed *Agrobacterium tumefaciens* EHA105 carrying the binary vector pCAMBIA 3301, which includes the bialophos/ phosphinothricin resistance (*bar*) gene and the GUS reporter gene. After successful transformation, the regenerated plants underwent a hardening process and exhibited standard growth patterns. Among the five inbred lines, HKI 163 demonstrated the highest transformation efficiency (10.6 %). The highlight of this protocol was the development of transgenic plants accomplished in a remarkably short period of approximately two months. This advancement sets the stage for future genome editing experiments within these inbred lines.

Keywords: *Agrobacterium*; immature embryos; transformation; transgenic; tropical inbreds

Introduction

Maize is a highly adaptable cereal crop, boasting the most significant genetic potential, production output and productivity among its peers. Over recent decades, plant tissue culture and transformation techniques have emerged as a pivotal factor in advancing agriculture, facilitating the introduction of advantageous transgenes and the modulation of endogenous gene expression, among other applications. Nevertheless, it's crucial to acknowledge that the capacity for *in vitro* regeneration is profoundly influenced by factors such as genotypic variation, the choice of explants and the composition of the growth medium, among others (1). Recent reports indicate that genetically modified maize varieties have demonstrated yield increases of up to 10 % compared to their conventional counterparts (2). Establishing a dependable genetic transformation technology in maize has played a pivotal role in advancing agronomy and it will continue to be a critical component for the future development of significant maize cultivars. Furthermore, it is integral to fundamental scientific investigations encompassing functional genomics, phenotypic trait analysis and plant science (3-5).

The success of genetic transformation experiments hinges on a reliable plant regeneration mechanism and the identification of responsive genotypes. In maize, using immature embryos has conventionally been the preferred method for *in vitro* culture and subsequent plant regeneration. A successful and reproducible method for *Agrobacterium*-mediated maize transformation, challenging the previous belief that monocots like maize were resistant to transformation, was reported in 1996 (6). The approach involved using immature embryos from the maize inbred A188, an *Agrobacterium* "super-binary" vector system and the *bar* selectable marker gene. Transformation rates varied between 5 and 30 %, with more than 70 % of the T0 plants being fertile and morphologically normal. Several transformation reports were made after that, but high dependence on genotype was reported simultaneously. Hybrid lines Hi II, a high type II callus containing A188 and B73 inbreds, became one of the most widely used hybrids in maize transformation in academia and industry. Generally,

for maize transformation, embryogenic cultures starting from immature embryos and cell suspension cultures of embryogenic callus are used (7, 8). However, it is essential to note that most studies in this context have primarily focused on highly responsive temperate genotypes, such as the Hi-II hybrid and A188, with limited exploration into tropical maize genotypes' regeneration and transformation potential. This study investigated the regeneration and transformation efficiency of five prominent inbred lines of tropical maize genotypes.

Materials and Methods

Plant materials

This study employed five inbred lines (HK193-2, HK1-163, PV5, PV6 and V390) of *Zea mays* provided by the Indian Agricultural Research Institute (IARI), New Delhi. Ears of maize were harvested 10 to 15 days after pollination (DAP) and subjected to subsequent processing. The outer sheaths of the ears were carefully removed (Fig. 2a) and surface sterilized using Tween 20 surfactant and 1.67 % sodium hypochlorite for 20 min, followed by thorough rinsing with distilled water three times and air drying. Subsequently, immature embryos measuring 1.5-2.0 mm in size (Fig. 2b) were aseptically dissected from the ears of all five *Zea mays* inbred lines. These isolated embryos were then subjected to genetic transformation.

Agrobacterium strain and vector

The binary vector pCAMBIA 3301 (GenBank Accession No. AF234297) was used for genetic transformation in this study, utilizing *Agrobacterium tumefaciens* strain EHA105, procured from the National Institute for Plant Biotechnology (NIPB), New Delhi, for subsequent transformation experiments. This vector harbours a P35S-bar selectable marker gene cassette (phosphinothricin acetyltransferase gene driven by the cauliflower mosaic virus [CaMV35S] promoter) and a P35S-gus-int reporter gene cassette (β-glucuronidase [GUS] gene with an intron driven by the CaMV 35S promoter) within the T-DNA region (Fig. 1). Additionally, it confers two selectable markers for antibiotic resistance, namely Rifampicin and kanamycin, used at concentrations of 25 mg/L and 50 mg/L respectively.

Embryo transformation

The immature embryos were incubated in maize infection medium (MIM) containing *Agrobacterium tumefaciens* in 2 ml centrifuge tubes. All the media component details are presented in Table 1. For all media, the pH was maintained at 5.8. These embryos in MIM were gently vortexed for 30 seconds and then allowed to stand for 5 minutes. Later, the tubes containing embryos and *Agrobacterium* were placed on plates of maize co-cultivation media (MCM) containing 0.8 % agar. Excess *Agrobacterium* suspension was carefully removed via pipetting and the embryos were placed on MCM with the scutellum (round) side facing

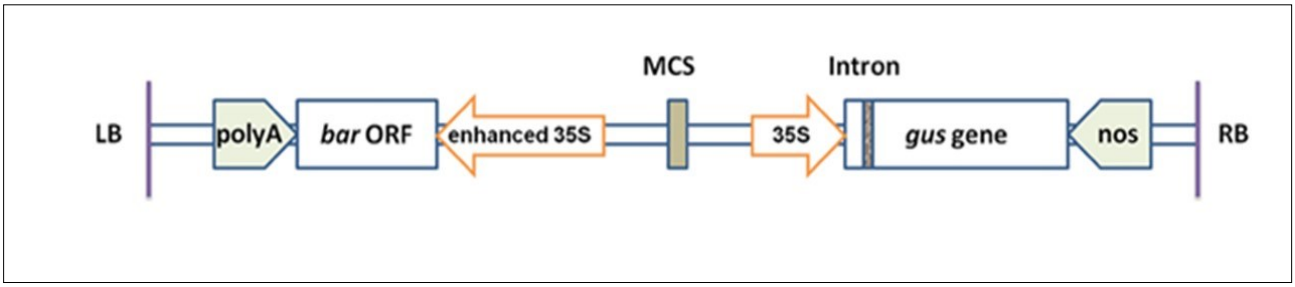

Fig. 1. Schematic structure of the T-DNA region of the binary vector pCAMBIA3301. LB, left border; RB, right border; bar, phosphinothricin acetyltransferase gene; gus-int, β-glucuronidase gene containing an intron; P35S, CaMV 35S promoter; T35S, nos terminator; MCS: multiple cloning sites.

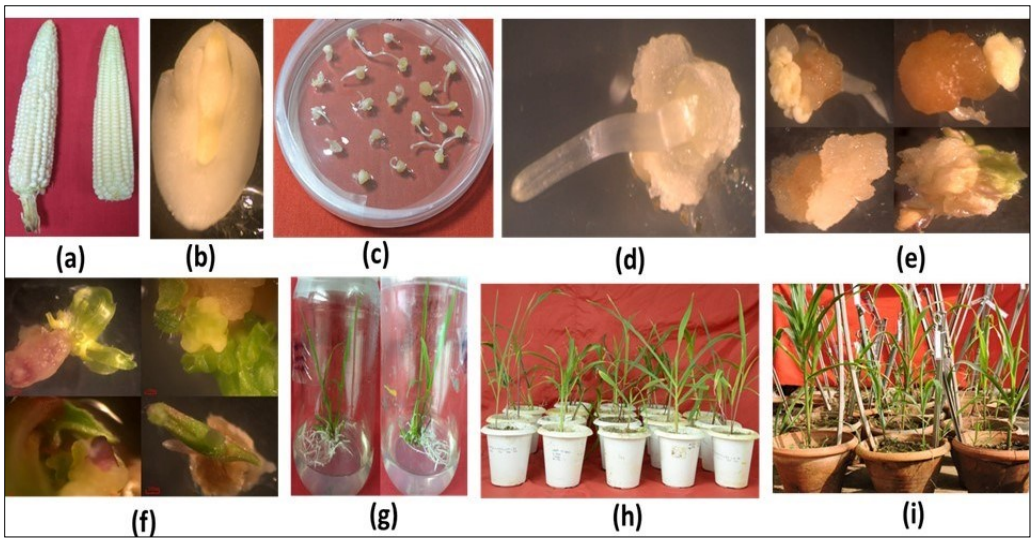

Fig. 2. Different stages of embryogenic callus induction from immature zygotic embryos and plant regeneration in inbred lines of maize. a. Ears harvested 10-15 days after pollination; b. Immature embryo; c-d. Callus induction after 7 days of infection; e. Embryogenic callus development after 2 weeks; f. Developing tissues after 4 weeks; g. development of multiple shoots; h. Primary hardening; i. Plants were grown to maturity in the net house.

Table 1. Media compositions for immature embryo transformation

Maize infection media (MIM) (Liquid media)	Maize co-cultivation media (MCM)	Maize resting media (MRM)	Maize shoot formation media (MSM)	Rooting media (RM)
MS Basal Medium, 4.4 g/L 2,4-D, 1.5 mg/L Sucrose, 68.5 g/L Glucose, 36 g/L	N6 Basal Salt Mixture, 4.0 g/L Sucrose, 30 g/L	MS Basal Salt Mixture (1x), 4.4 g/L N6 Macro Salts (0.6x), 60 mL/L B5 Micro Salts (0.6x), 0.6 mL/L Eriksson's Vitamins (0.4x), 0.4 mL/L S&H Vitamins (0.6x), 0.6 g/L Ferrous Sodium stock (0.6x), 6 mL/L KNO_3, 1.68 g/L Casein Hydrolysate, 0.3 g/L L-proline, 2 g/L Sucrose, 20 g/L Glucose, 0.6 g/L	MS Basal Medium, 4.4 g/L Sucrose, 60 g/L	MS Basal Medium, 4.4 g/L Sucrose, 40 g/L
	Silver Nitrate, 1 mg/L Acetosyringone, 100 µM 2,4-D, 2.0 mg/L	Dicamba, 1.2 mg/L Cefotaxime, 100 mg/L Timentin, 150 mg/L Silver Nitrate, 3.4 mg/L Thiamine HCl, 0.2 mg/L 2,4-D, 0.8 mg/L	Thidiazuron, 0.1 mg/L BAP, 1 mg/L Carbenicillin, 100 mg/L Kinetin, 0.5 mg/L	NAA, 0.5 mg/L

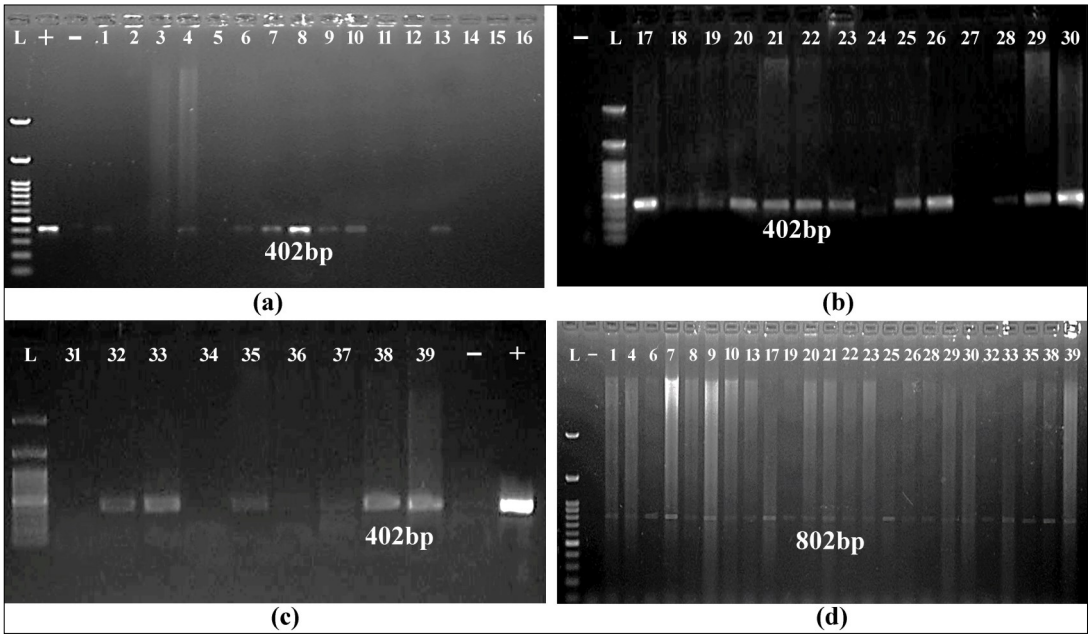

Fig. 3. PCR screening for transformants in *Zea mays*. a-c. *Bar* gene showing an amplicon size of 402 bp; d. GUS gene showing an amplicon of 802 bp; (L)- Ladder; (+) - positive control; (-) -negative control.

upwards. The plates were then incubated in the dark at 21 °C for 16-18 h of co-cultivation. Following this co-cultivation, the infected embryos were transferred onto the plates of maize resting media (MRM), which were incubated in the dark at 26 ± 2 °C for 1 week.

Regeneration and development of transgenic plants

After 1 week, immature embryos were carefully transferred from the MRM to maize shoot formation media (MSM) and incubated for 2-4 weeks at 26 ± 2 °C under dark conditions. The embryos were sub-cultured every 2 weeks onto fresh media. Afterwards, the resulting plantlets were transferred to rooting media (RM) and maintained at 26 ± 2 °C in the light (16 h light/ 8 h darkness) for 7-14 days. Robust plantlets with well-established root systems were carefully extracted from the RM and transplanted into 4-inch pots containing a mixture of autoclaved vermiculite and coco peat (1:1). To maintain optimal humidity, transparent polyethene bags were employed to encase the pots housing the plantlets. The hardened plants were transferred in identical plastic pots within a controlled greenhouse environment for 4-5 days before being transplanted into 12" x 10" pots filled with soil, where they were cultivated to attain maturity. A summarized workflow of the entire protocol is illustrated in Fig. 4.

Molecular confirmation of putative transgenic plants

The validation of transgenic plant lines was performed via PCR amplification targeting the GUS and *bar* genes present within the T-DNA region of the pCAMBIA 3301 vector. Genomic DNA was extracted from the leaf tissues of both putative transformants and wild-type (untransformed) plants, employing the CTAB extraction method. After the DNA extraction, PCR analysis was conducted using gene-specific primers designed with the IDT Primer Quest tool. The forward and reverse primer sequences utilized for the GUS gen were 5′TTCGATGCGGTCACTCATTAC3′ and 5′CAGCAGCAGTTTCATCAATCAC3′, respectively. Similarly, for the *bar* gene, the forward primer sequence utilized was 5'AAACCCACGTCATGCCAGTTC3' and the reverse primer sequence was 5'CGAGACAAGCACGGTCAACTTC3'. Following PCR amplification, the resulting amplicons were subjected to electrophoresis on a 1 % agarose gel containing ethidium bromide and visualization was performed using a gel documentation system.

Results

The present study's primary objective was to achieve regeneration using immature embryos as the preferred

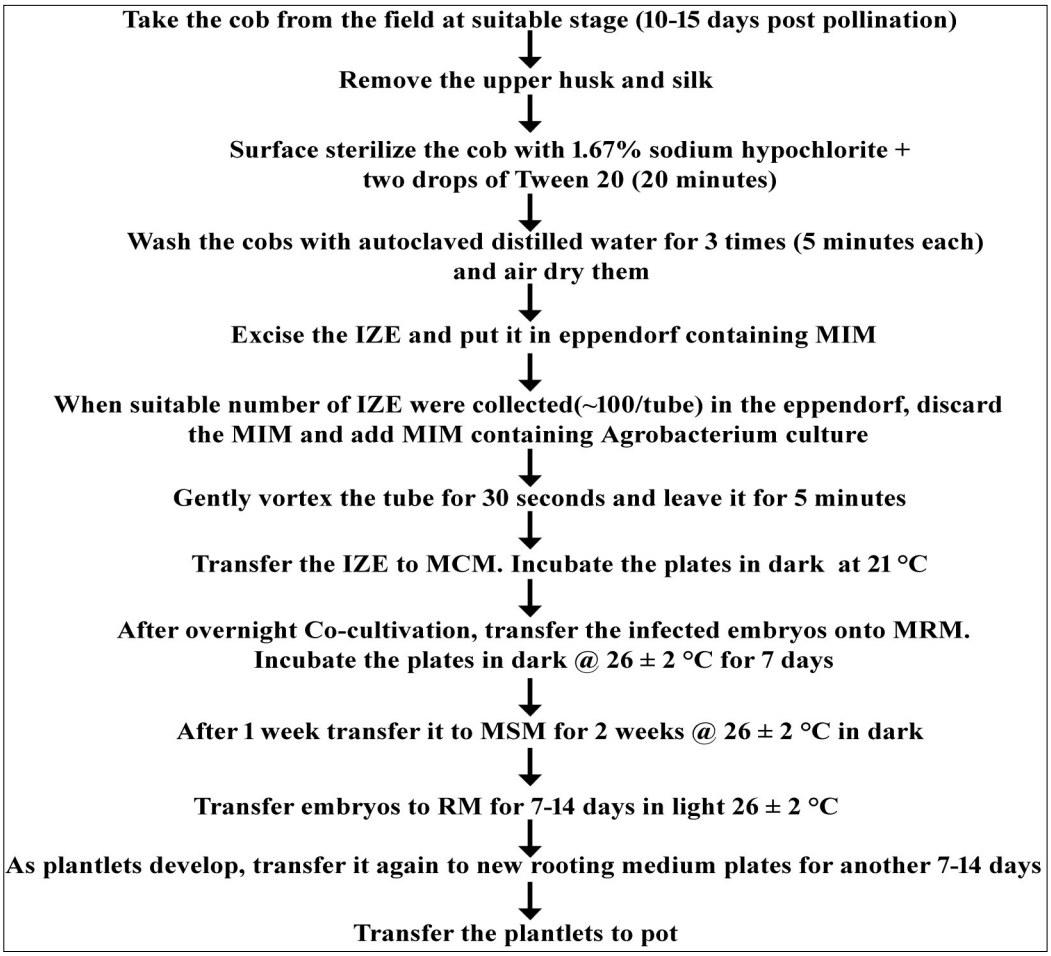

Fig. 4. Schematic workflow of transformation protocol in tropical maize using immature embryos as starting material.

explants in the different tropical maize genotypes. In pursuit of this goal, we predominantly adhered to the protocol that reported the effectiveness of N_6 medium supplemented with Dicamba and silver nitrate ($AgNO_3$) for inducing callus formation and promoting plant regeneration (9).

Embryogenic callus induction

Callus initiation from cultured embryos was observed within one week of culture initiation on MRM, characterized by scutellum swelling, a mass on the scutellum's surface and coleoptile development (Fig. 2c). Immature embryos have the potential to initiate two distinct types of callus cultures on their scutellar surfaces, namely Type I and Type II callus. Type I callus is compact and organogenic and readily derived from immature embryos. In contrast, Type II callus is friable and embryogenic, but its initiation occurs at a lower frequency than Type I (10). Only a limited number of tropical genotypes have been observed to possess the ability to initiate Type II callus (11). Type II callus has been observed to exhibit more significant regenerative potential compared to Type I callus (12). A combination of Type I and Type II calli was observed in all the inbred lines investigated in this study. The callus induction efficiency (%) was determined using the standard formula (13). Among the five maize inbred lines, V390 exhibited the highest callus formation frequency (93.3 %), followed by HKI 193-2 (53.3 %), PV6 (52 %), PV5 (51 %) and HKI 163 (50 %) (Table 2).

Shoot regeneration and rooting

After one week of incubation, the immature embryos were carefully transferred from the MRM to the MSM. Before transfer, coleoptiles were carefully excised using a scalpel blade (Fig. 2(d)). After the transfer of embryogenic calli to the MSM, the callus surface exhibited green pigmentation and shoot development was observed within 2-4 weeks (Fig. 2e-2f). The regenerated plantlets were then transferred to RM for the development of roots. Within two weeks on RM, healthy plantlets with well-developed roots were observed (Fig. 2g). The regenerated plantlets with developed roots were washed gently with sterile water to remove any traces of agar and then planted in soil rite mixture for primary hardening of plants (Fig. 2h) followed by secondary hardening, which is carried out in the net house (Fig. 2i). The

Table 2. Summary of immature embryo transformation

Variety	Total no. of embryos inoculated	No. of callus induced	Callus induction efficiency (%)	No. of plantlets	Regeneration frequency (%)	No. of trans-formed plants	Percentage of transformation frequency (%)
V390	150	140	93.3	6	4	6	4
HKI 193-2	150	80	53.3	13	8.6	7	4.6
HKI 163	150	75	50	25	16.6	16	10.6
PV5	150	76	51	10	6.6	8	5.3
PV6	150	78	52	8	5.3	8	5.3

regeneration frequency (%) was calculated using the standard formula (13). Among the five inbred lines studied, HKI 163 exhibited the highest rate of plantlet regeneration at 16.6 %, followed by HKI 193-2 at 8.66 % and the lowest was from V390 at 4 %, as presented in Table 2.

Molecular analysis

Confirmation of the T_0 putative transgenic plants was carried out by PCR analysis using two sets of primers specific for GUS and *bar* genes. Agarose gel electrophoresis of the PCR products revealed amplicons of sizes 402bp (Fig. 3a-c and 3d.) 802bp for the *bar* and GUS genes confirms *Zea mays'* successful transformation using this method. Among the five inbred lines used for the study, the most robust transformation efficiency of 10.6 % was observed in HKI 163, followed by PV5 and PV6 at 5.3 %, HKI 193-2 (4.6 %) and V390 (4 %) (Table 2).

Discussion

An efficient regeneration protocol is a fundamental requirement for the successful execution of genetic transformation in any crop plant. Maize genotypes exhibit significant variations in their suitability for *in vitro* culture. This study primarily focuses on developing a standard regeneration protocol for tropical Indian maize inbred lines using immature embryos as the preferred explants. The traditional approach for maize transformation involves the isolation of immature embryos, followed by generating transgenic callus tissue, which is subsequently regenerated into viable plants. Although effective, this method is characterized by time-intensive steps, ranging from three to six months to complete the tissue culture process, leading to plant generation (6). The technique presented herein offers a significant reduction from this conventional paradigm by providing an expeditious and efficient alternative, resulting in the regeneration of T_0 plants in approximately half the time. Here, we utilized the basic protocol reported in the research, with a key difference in the vector used (9). They have used the morphogenic genes to induce and regenerate somatic embryos. However, using their protocol without morphogenic genes, we obtained transformation frequencies ranging from 4-10.6 %, comparable to theirs. In recent years, to overcome the problem of genotype dependency, the use of morphogenic genes like BABY Boom (ZmBbm), WUSHEL (ZmWus2) and OVULE DEVELOPMENT PROTEIN 2 (ODP2) has been reported in maize transformation (14). Morphogenic Regulator-Mediated Transformation (MRMT) vectors containing these morphogenic genes can be introduced into *Agrobacterium* strains and used for transformation. Increased plant regeneration rates, recovery of transformed plants from recalcitrant genotypes and a shortening in the time needed for transformation by avoiding the callus culture step have been obtained using the MRMT strategy (14). However, the expression of morphological regulators has a negative pleiotropic effect on developmental traits. Therefore, expression must be restricted to the embryogenesis step, or their expression must be knocked out using a recombination system (15). However, we can get comparable transformation frequencies within a short time. Over the past three decades, maize tissue culture and transformation protocols have undergone continual refinement and our streamlined approach is poised to accelerate this evolutionary trajectory further. Integrating this technique with emerging tools, such as genome engineering, promises future maize research and breeding developments as technology advances.

Conclusion

This study establishes a highly efficient and rapid maize regeneration and genetic transformation protocol, significantly reducing the time required to generate transgenic plants. Transgenic (T_0) plantlets were obtained within 60 days post-infection, halving the duration typically required in conventional maize regeneration protocols by expediting callus selection and proliferation. The reproducibility and scalability of this protocol hold promise for accelerating the development of genetically modified and genome-edited maize varieties with improved agronomic traits, such as enhanced stress tolerance, disease resistance and nutritional value. Future research should focus on integrating genome-editing technologies like CRISPR-Cas9 and elucidating the molecular and epigenetic mechanisms underlying tissue culture responsiveness to enhance transformation efficiency further. This study provides a robust platform for advancing functional genomics, precision breeding and sustainable crop improvement by significantly improving transformation efficiency, contributing to global food security and agricultural resilience.

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Authors' contributions

JM conceptualized and planned the study; and drafted and edited the manuscript. PM conceptualized and planned the study. FH conceptualized and planned the study. VM maintained and grew the inbred lines in the field. RZ maintained and grew the inbred lines in the field. AK conducted the *in vitro* and molecular work and drafted and edited the manuscript. KM performed the *in vitro* and molecular work.

Compliance with ethical standards

Conflict of interest: The authors declare no competing financial or non-financial interests.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

While preparing this work, we used Quillbot to check grammar and paraphrase wherever required. After using this

tool/service, we reviewed and edited the content as needed and we take full responsibility for the publication content.

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