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MATURE SEED-DERIVED REGENERATION AND IN PLANTA TRANSFORMATION OF TROPICAL MAIZE (ZEA MAYS L.)

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ABSTRACT
Maize (Zea mays L.) is an important cereal crop, but the application of genetic transformation in tropical germplasm is constrained by genotype-dependent regeneration and the limited availability of suitable explants. The present study evaluated mature seed-derived split internodes as an alternative explant source for plant regeneration and investigated Agrobacterium tumefaciens-mediated transformation in tropical maize genotypes. Six maize genotypes were evaluated for regeneration efficiency based on plantlet production, shoot and root development, and survival after acclimatization. Agrobacterium-mediated transformation was evaluated using sonication-assisted and conventional infection treatments, while transformation was assessed by histochemical GUS staining and PCR amplification of the bar and gus genes. Significant variation in regeneration response was observed among genotypes. HKI-161 showed the highest regeneration efficiency, producing 4.4 ± 0.3 plantlets per explant, whereas Pusa HQPM1 showed the highest survival rate (92%). Sonication-assisted Agrobacterium infection enhanced transient GUS expression compared with conventional infection, with 45 s sonication producing the highest response in most genotypes. HKI-161 showed the highest GUS-positive frequency (26.7%), while PCR analysis detected the expected bar and gus amplification products in selected seedlings, with a maximum putative transformation frequency of 6.7%. Overall, the results demonstrate that mature seed-derived tissues can provide a practical alternative to immature embryos for regeneration of tropical maize and indicate that sonication-assisted Agrobacterium infection can improve gene delivery into germinating seedlings. HKI-161 was identified as the most responsive genotype and may provide useful material for further transformation and genome-editing studies in tropical maize.

KEYWORDS: maize; mature seed; regeneration; Agrobacterium tumefaciens; in planta transformation; sonication

INTRODUCTION
Maize (Zea mays L.) is one of the world's most important cereal crops, serving as a staple food, livestock feed, and a major raw material for food, feed, and bio-based industries (Klopfenstein et al., 2013). Owing to its high yield potential, broad adaptability, and economic significance, maize plays a pivotal role in ensuring global food and nutritional security (Shiferaw et al., 2011). However, increasing demand for maize production is challenged by climate change, diminishing arable land, and the growing incidence of biotic and abiotic stresses (Badu-Apraku and Fakorede, 2017). Conventional breeding has substantially improved maize productivity; nevertheless, the development of superior cultivars with enhanced tolerance to multiple stresses remains time-consuming and is constrained by the available genetic diversity (Singh et al., 2024).
Genetic transformation has emerged as a powerful approach for introducing novel traits that are difficult or impossible to achieve through conventional breeding (Dhugga, 2022). It has enabled the development of maize cultivars with improved resistance to insects and diseases, enhanced tolerance to herbicides and environmental stresses, and improved grain quality (Lorenzo et al., 2023). The successful application of genome-editing technologies, including CRISPR/Cas systems, has further increased the importance of efficient transformation platforms for functional genomics and precision crop improvement (Yassitepe et al., 2021). Despite these advances, the widespread application of genetic transformation in maize is limited by the availability of efficient, reproducible, and genotype-independent regeneration systems (Martínez-Fortún et al., 2022).
Plant regeneration is a critical prerequisite for stable genetic transformation because transformed cells must regenerate into fertile plants (Xu et al., 2024). In maize, regeneration efficiency is highly genotype dependent and is influenced by the choice of explant, culture medium composition, and growth regulator combinations (Jiang et al., 2024). Immature embryos remain the preferred explant for most transformation protocols because of their high embryogenic potential and regeneration capacity (Frame et al., 2010). However, their use presents several practical limitations, including seasonal availability, the requirement for controlled greenhouse facilities, labor-intensive isolation, and poor applicability to many tropical germplasm (Que et al., 2014). These limitations have restricted the routine application of genetic transformation in breeding programs, particularly in

developing countries.

Mature seeds represent an attractive alternative explant because they are readily available throughout the year, easy to handle, inexpensive, and suitable for large-scale experimentation (Sudheer et al., 2026). Nevertheless, regeneration from mature seed-derived tissues is generally less efficient than from immature embryos and often exhibits strong genotype dependence (Shrawat and Good, 2010). Recent studies have demonstrated that organogenesis from split internodes derived from mature seeds can provide an effective regeneration route, offering a practical alternative for maize improvement (Pathi et al., 2013). However, systematic evaluation of this approach in tropical maize germplasm, especially Indian elite genotypes, remains limited. Among the available transformation methods, Agrobacterium tumefaciens-mediated transformation is widely preferred because it typically results in low transgene copy number, stable gene integration, and reduced genome rearrangements (Azizi-Dargahlou and Pouresmaeil, 2024). Conventional Agrobacterium-mediated protocols, however, generally rely on tissue culture and regeneration from immature embryos, making them laborious and genotype dependent (Huang and Wei, 2005). In planta transformation has emerged as an attractive alternative by directly targeting actively dividing meristematic tissues, thereby reducing the time, cost, and somaclonal variation associated with prolonged in vitro culture (Bélanger et al., 2024). Furthermore, sonication-assisted Agrobacterium infection has been reported to enhance transformation efficiency by facilitating bacterial access to internal plant tissues through micro-wounding (Bakshi et al., 2011).

Indian tropical maize germplasm has received comparatively less attention in regeneration and transformation studies than temperate germplasm, despite its importance in breeding programs (Yadav et al., 2016). The development of robust regeneration and transformation protocols that utilize mature seeds would eliminate the dependence on seasonally available immature embryos and facilitate routine genetic manipulation of elite tropical maize lines (Ye et al., 2022). Therefore, the present study aimed to evaluate the regeneration potential of selected tropical maize genotypes using mature seed-derived split internodes and to assess the efficiency of Agrobacterium-mediated in planta transformation. The study further sought to identify maize genotypes with superior regeneration and transformation competence and to establish an efficient platform that can support future functional genomics studies and genetic improvement of tropical maize.

MATERIALS AND METHODS

Plant materials and seed sterilization

Mature seeds of tropical maize (Zea mays L.) genotypes were obtained from Division of Genetics, IARI, New Delhi.

Thirty seeds of each maize variety were soaked in distilled water overnight, followed by immersion in 1% Bavistin supplemented with 1 drop of Tween-20 for 10 min. Seeds were rinsed three times with distilled water followed by a 70% ethanol wash for 5 min. Seeds were then surface sterilized in 2% sodium hypochlorite for 15 min. Sterilized seeds were again rinsed with distilled water five to six times and dried on sterilized tissue paper. All steps were performed in laminar airflow.

Seed germination and explant preparation

Surface-sterilized seeds were inoculated on Murashige and Skoog (MS) medium supplemented with 5.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 3.0 mg L^{-1} 6-benzylaminopurine (BAP) (Carvalho et al., 1997). Cultures were maintained at 25 ± 1°C under a 16-h light/8-h dark photoperiod. After approximately two weeks, swollen internodes were excised from the germinated seedlings, longitudinally split under aseptic conditions, and used as explants for callus induction.

Callus induction and plant regeneration

Split internodes were cultured on callus induction medium consisting of MS basal medium supplemented with 2,4-D (1.0–3.5 mg L^{-1}) and BAP (0.1–2.5 mg L^{-1}). Cultures were incubated at 25 ± 1°C under a 16-h light/8-h dark photoperiod with a light intensity of approximately 4000 lux. Callus formation was monitored after two weeks.

Regeneration was induced by transferring the calli to MS regeneration medium containing 2.0 mg L^{-1} BAP, 1.0 mg L^{-1} kinetin, and 0.5 mg L^{-1} NAA. Adventitious shoot formation was recorded after 1–2 weeks. Individual shoots were separated from multiple shoot clusters under aseptic conditions and transferred to rooting medium consisting of MS medium supplemented with 1.0 mg L^{-1} NAA. Rooted plantlets were maintained for 2–3 weeks before evaluation.

Acclimatization and evaluation of regeneration efficiency

Rooted plantlets were gently removed from the culture medium, washed thoroughly to remove residual agar, and transplanted into plastic pots containing a sterile mixture of vermiculite, sand, and peat moss (1:1:1). High humidity was maintained initially by covering the pots with transparent polyethylene bags. The humidity was gradually reduced by perforating the covers before transferring the hardened plants to normal greenhouse conditions.

Regeneration efficiency was assessed in six genotypes: HKI-161, PMI-PV2, PMI-PV6, PMI-PV7, Pusa HQPM 1 Improved, and Pusa Vivek 27 Improved, by recording the number of regenerated plantlets per explant, average shoot length, average root length, and survival percentage after acclimatization. Thirty explants per genotype, arranged in three biological replicates of ten explants each, were used for regeneration experiments.

Agrobacterium-mediated in planta transformation

Transformation experiments were performed using Agrobacterium tumefaciens harboring the binary vector pCAMBIA3301 (Fig. 1) containing the gus reporter gene and the bar selectable marker gene. Sixty mature seeds of four genotypes (PMI-PV2, HKI-161, Pusa Vivek 27 Improved, and PMI-PV6) were germinated on sterile substrate for three days prior to transformation.

Overnight-grown bacterial culture was centrifuged at 13,000 rpm for 10 min, and the pellet was resuspended in liquid MS medium at a ratio of 1:15 (v/v) supplemented with 200 µM acetosyringone to induce virulence gene expression. The bacterial suspension was incubated at 29°C with shaking (200 rpm) for 2–3 h before infection. Three-day-old germinated seedlings were wounded by making 10–14 punctures at the junction of the plumule and radicle using a sterile syringe needle to facilitate bacterial entry. The wounded seedlings were immersed in the Agrobacterium suspension and subjected to two infection treatments. For the conventional treatment, infected seedlings were incubated at 28°C with shaking (180 rpm) for 1.0, 1.5, or 2.0 h. For sonication-assisted transformation, seedlings immersed in the bacterial suspension were exposed to ultrasonic treatment at 50 Hz for 15, 45, or 60 s using an ultrasonic cleaner. Following infection, seedlings were air-dried overnight under sterile conditions and transferred to pots containing sterile substrate for further growth.

Confirmation of putative transformants by PCR

Genomic DNA was extracted from 18-day-old seedlings using the CTAB method. PCR amplification was performed using gene-specific primers targeting the bar (440 bp) and gus (1032 bp) genes (Table 1). PCR products were separated by agarose gel electrophoresis and visualized under UV illumination to detect the target sequences.

Histochemical GUS assay

Transient expression of the gus reporter gene was evaluated by histochemical staining of transformed seedlings using X-Gluc substrate. Seedlings expressing β-glucuronidase activity developed blue coloration, whereas non-transformed control plants remained unstained. Transient gene expression was calculated as the percentage of GUS-positive seedlings.

Experimental design and statistical analysis

Regeneration experiments were conducted using a completely randomized design with three biological replicates per genotype. Data were expressed as mean ± standard error. Regeneration efficiency, shoot length, root length, survival percentage, and transformation frequency were calculated for each genotype and treatment. Mean comparisons were performed using the least significant difference (LSD) test at the 5% probability level.

RESULTS

Regeneration efficiency from mature seed-derived split internodes

Split internodes obtained from germinated mature seeds exhibited progressive swelling within two weeks of culture, followed by the initiation of organogenic callus along the cut surfaces. The induced calli were compact, light yellow, and capable of producing multiple shoots after transfer to regeneration medium (Fig. 2).

Marked differences in regeneration capacity were observed among the six maize genotypes (Table 2). HKI-161 produced the highest number of regenerated plantlets (4.4 ± 0.3 plantlets per explant), followed by Pusa HQPM1 (3.4 ± 0.3 plantlets per explant). Pusa Vivek 27 exhibited the lowest regeneration efficiency (1.4 ± 0.2 plantlets per explant). Although PMI-PV6 produced the longest shoots (14.0 cm), Pusa HQPM1 recorded the highest survival rate (92%) after rooting and acclimatization. Root development was most vigorous in HKI-161, with an average root length of 8.7 cm. These findings demonstrate genotype-dependent variation in regeneration efficiency, with HKI-161 showing superior overall regeneration performance.

Agrobacterium-mediated in planta transformation and histochemical GUS assay

Transient gene expression was evaluated by histochemical GUS staining following Agrobacterium tumefaciens-mediated transformation. Blue staining was observed exclusively in treated seedlings, whereas control plants showed no detectable GUS activity (Fig. 3).

Sonication markedly enhanced transient gene expression compared with conventional infection (Fig. 4). Among the sonication treatments, 45 s produced the highest GUS expression in three of the four evaluated genotypes. HKI-161 exhibited the greatest transient gene expression, with 26.7% GUS-positive seedlings, followed by PMI-PV6 (20.0%), PMI-PV2 (16.7%), and Pusa Vivek 27 (16.7%).

In contrast, transformation without sonication resulted in lower GUS expression across all genotypes (Fig. 4). The highest transformation frequency obtained under this treatment was 13.3%, observed in HKI-161 and PMI-PV2 after 1.5 h of bacterial incubation. Overall, sonication approximately doubled transient gene expression relative to conventional infection, indicating that sonication-assisted Agrobacterium infection can improve DNA delivery into germinating maize seedlings.

PCR detection of bar and gus sequences

Transformation events were further evaluated by PCR amplification using gene-specific primers targeting the bar and gus genes. Gradient PCR established an optimal annealing temperature of 59°C for both primer pairs.

PCR analysis detected the expected amplification products in selected treated seedlings, whereas no amplification was detected in non-transformed controls (Fig. 5). Among the sonication treatments, HKI-161 and Pusa Vivek 27 exhibited the highest PCR-positive putative transformation frequency (6.7%) following 45 s of sonication (Fig. 6). In the absence of sonication, HKI-161 again showed the highest PCR-positive putative

transformation frequency (6.7%) after 1.5 h of bacterial incubation (Fig. 6). The PCR results corroborated the GUS assay and provided molecular evidence for the presence of the target sequences in selected seedlings.

Overall performance of maize genotypes

Among the evaluated genotypes, HKI-161 consistently exhibited superior performance throughout the study. It showed rapid seed germination, the highest regeneration efficiency, vigorous root development, and the greatest transient gene expression in both GUS and PCR analyses. Pusa HQPM1 displayed excellent plant survival during regeneration, whereas Pusa Vivek 27 showed moderate regeneration capacity but responded well to Agrobacterium-mediated transformation. Collectively, these findings identify HKI-161 as the most responsive genotype among the tropical maize germplasm evaluated in this study.

DISCUSSION

The development of efficient regeneration and transformation systems remains a major challenge for the genetic improvement of maize because both processes are highly genotype dependent. In the present study, a mature seed-based regeneration protocol coupled with an Agrobacterium-mediated transformation approach was evaluated in tropical maize genotypes. The study demonstrates that mature seeds can serve as a practical alternative to immature embryos for regeneration and transformation, thereby addressing one of the constraints associated with conventional maize transformation systems.

Considerable variation in regeneration efficiency was observed among the evaluated maize genotypes, confirming the strong influence of genotype on in vitro culture response. HKI-161 consistently exhibited superior regeneration efficiency, while Pusa HQPM1 showed the highest survival after acclimatization. Similar genotype-dependent responses have been reported in maize regeneration studies, where the genetic background significantly influences organogenic and embryogenic competence (Bohorova et al., 1995; Pathi et al., 2015). The superior performance of HKI-161 suggests that this genotype possesses greater morphogenic competence, making it a useful candidate for transformation-based breeding and functional genomics studies. Immature embryos have traditionally been considered the preferred explant for maize regeneration because of their high regenerative capacity (Duncan et al., 1985; Ishida et al., 1996). However, their seasonal availability, labor-intensive isolation, and dependence on synchronized plant growth restrict their routine use. The present study successfully regenerated plants from mature seed-derived split internodes through organogenic callus formation, demonstrating that mature seeds can provide a practical and year-round explant source for tropical maize. Although the regeneration frequency may be lower than that reported for some immature embryo-based systems, the use of mature seeds simplifies experimental procedures and broadens the applicability of regeneration protocols to breeding programs where immature embryos are not readily available.

The regeneration protocol developed in this study produced multiple shoots from organogenic calli within approximately eight weeks. Although the average number of regenerated shoots per explant was lower than that reported by Pathi et al. (2015), successful regeneration was achieved across several tropical maize genotypes using a relatively simple protocol. The rapid development of rooted plantlets and high survival rates further demonstrate the practical utility of the system. The high survival observed in Pusa HQPM1 indicates that regeneration efficiency alone does not necessarily predict acclimatization success, emphasizing the importance of evaluating multiple regeneration parameters when selecting genotypes for transformation studies. Efficient transformation depends not only on DNA delivery but also on the accessibility of target cells during infection. In the present study, sonication-assisted Agrobacterium tumefaciens-mediated treatment increased transient GUS expression compared with conventional infection. The highest GUS expression was obtained following 45 s of sonication, suggesting that controlled ultrasonic treatment may facilitate bacterial penetration through temporary micro-wounding. Similar improvements in transformation efficiency following sonication have been reported in soybean (Trick and Finer, 1998), winter cherry (Sivanandhan et al., 2015), and cowpea (Bett et al., 2019), where sonication facilitated bacterial access to internal tissues and increased transient gene expression.

Among the four maize genotypes evaluated for transformation, HKI-161 consistently produced the highest GUS positivity and PCR-positive putative transformation frequency under both sonication-assisted and conventional treatments. This observation suggests that genotype-dependent morphogenic competence may influence transformation response. However, the present data demonstrate transient gene expression and PCR detection of target sequences in selected seedlings; stable genomic integration and heritable transmission were not evaluated. Therefore, further molecular and progeny-based validation is required before these seedlings can be classified as stable transgenic events.

A major strength of the present study is the integration of mature seed-derived regeneration with in planta Agrobacterium-mediated transformation. Most published maize transformation systems rely on immature embryos, which limits their practical application because of seasonal constraints and intensive tissue-culture requirements. By contrast, the approach evaluated here utilizes mature seeds that are readily available throughout the year and reduces dependence on prolonged callus culture. This may improve the feasibility of routine transformation in tropical maize, although additional optimization and validation across a larger set of genotypes will be necessary.

Overall, the study provides evidence that mature seed-derived split internodes can support regeneration of several tropical maize genotypes and that sonication-assisted Agrobacterium treatment can enhance transient

gene expression in germinating seedlings. HKI-161 showed the most consistent response across the regeneration and transformation-related assays. These findings provide a useful basis for further optimization of transformation and genome-editing protocols in tropical maize.

Conclusion

Mature seed-derived split internodes provided a practical explant source for regeneration of the tropical maize genotypes evaluated in this study. HKI-161 showed the highest regeneration efficiency and the strongest response in the transformation-related assays, while Pusa HQPM1 exhibited the highest survival after acclimatization. Sonication-assisted Agrobacterium treatment, particularly 45 s sonication, enhanced transient GUS expression compared with conventional infection. PCR detection of bar and gus sequences provided molecular evidence in selected seedlings, but stable integration and inheritance require further validation. The optimized mature seed-based approach therefore provides a useful platform for subsequent transformation and genome-editing studies in tropical maize.

ACKNOWLEDGMENTS

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Table 1. Primer sequences used for PCR amplification of bar and gus genes

Primer	**Sequence**	**Primer**	**Sequence**
BAR(F)	ATCTCGGTGACGGGCAGGA	GUS(F)	GAAACCCCAACCCGTGAAA
BAR(R)	CGTCAACCACTACATCGAGACA A	GUS(R)	AGCTGAAAGCCGACAGCAG

Figure legends and locations

Figure 1 near here

Figure 1. Plant transformation vector used in this study. (A) pCAMBIA3301 vector used for in planta transformation.

Figure 2 near here

Figure 2. Different stages of regeneration from maize seed. (A) Seeds from each variety on germination medium; (B, C) swollen internodes; (D) separated internode; (E, F) split internodes on callus induction medium; (G) proliferating callus; (H) individual shoot transferred to rooting medium; (I–K) rooting; (L) complete plantlet; (M) enlarged image of a separated plantlet.

Figure 3 near here

Figure 3. Histochemical GUS assay. (A) WT; (B) GUS-positive seedlings in solution; (C, D) GUS-positive Agrobacterium-mediated transformed seedlings; (E–G) microscopic images of GUS-positive samples.

Figure 4 near here

Figure 4. Agrobacterium-mediated transient gene expression through GUS histochemical assay.
(A) With sonication; (B) without sonication. Figure 5 near here

Figure 5. PCR detection of bar and gus sequences in Agrobacterium-treated plants. (A) With sonication; (B) without sonication.

Figure 6 near here

Figure 6. Agrobacterium-mediated PCR-positive putative transformation frequency. (A) With sonication; (B) without sonication.

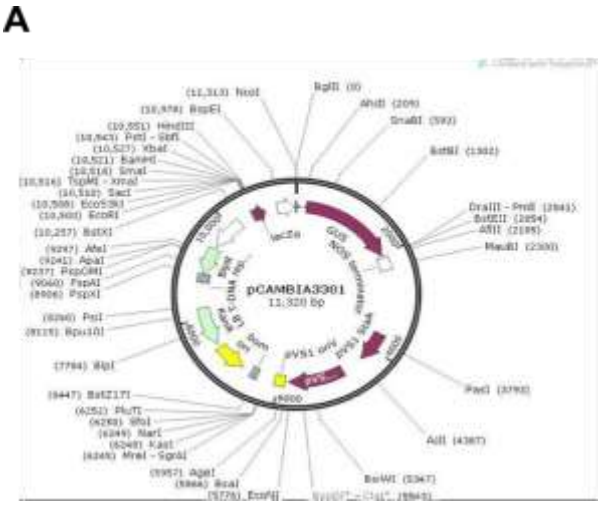


Fig. 1 Plant transformation vectors used in this study. (A) pCAMBIA 3301 vector used for *in planta* transformation method

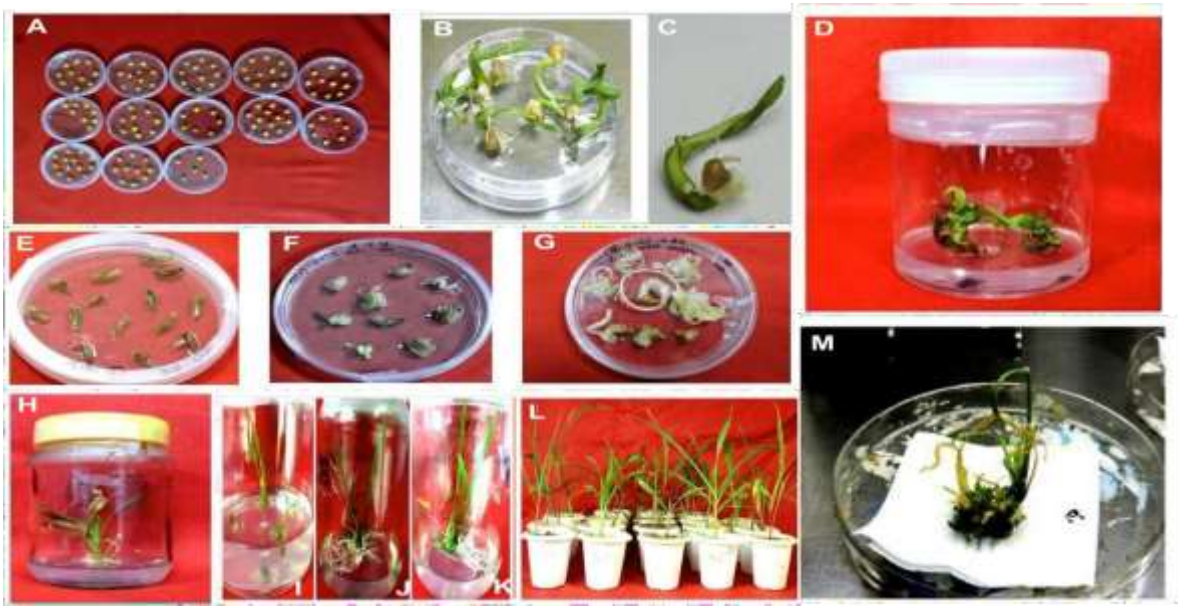


Fig 2. Different stages of regeneration from maize seed. (A) Seeds from each variety on germination media, (B) (C) Swelled internodes (D) Separated internode (E)(F) Split internodes on callus induction media (G) Proliferating callus (H) individual shoot transferred to rooting media (I)(J)(K) rooting observed (L) complete plantlet (M) zoomed image of separated plantlet

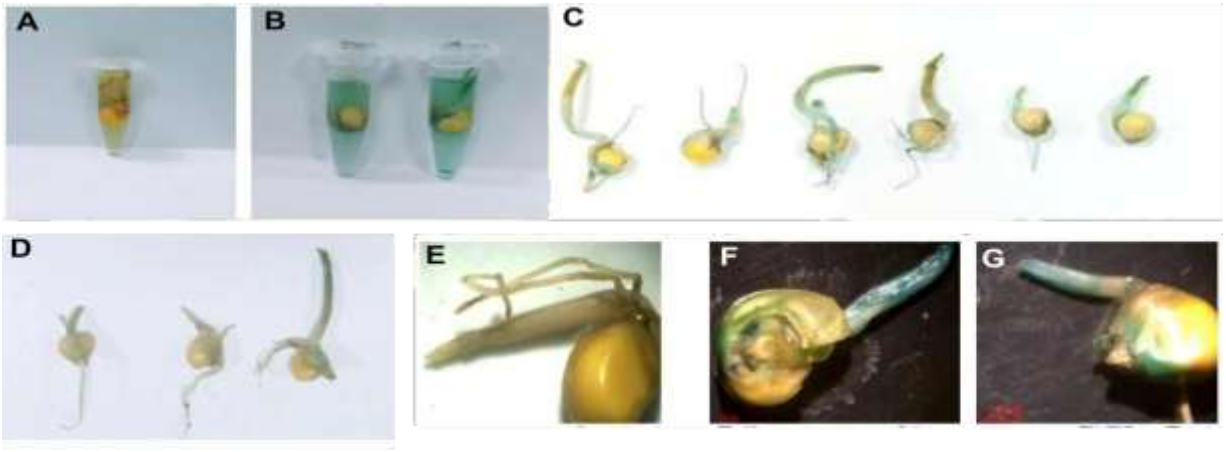


Fig 3. Histochemical GUS assay. (A) WT (B) GUS positive seedlings in solution (C) (D) GUS positive agrobacterium mediated transformed seedlings (E) (F) (G) microscopic image of GUS positive samples.

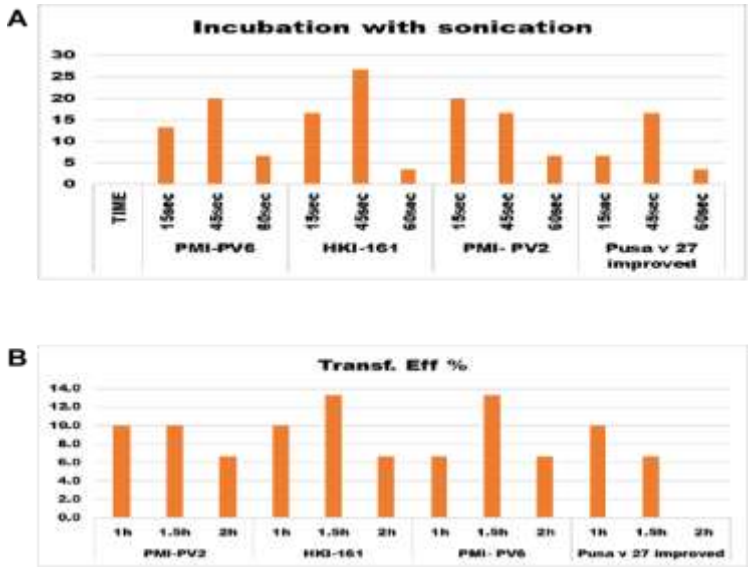
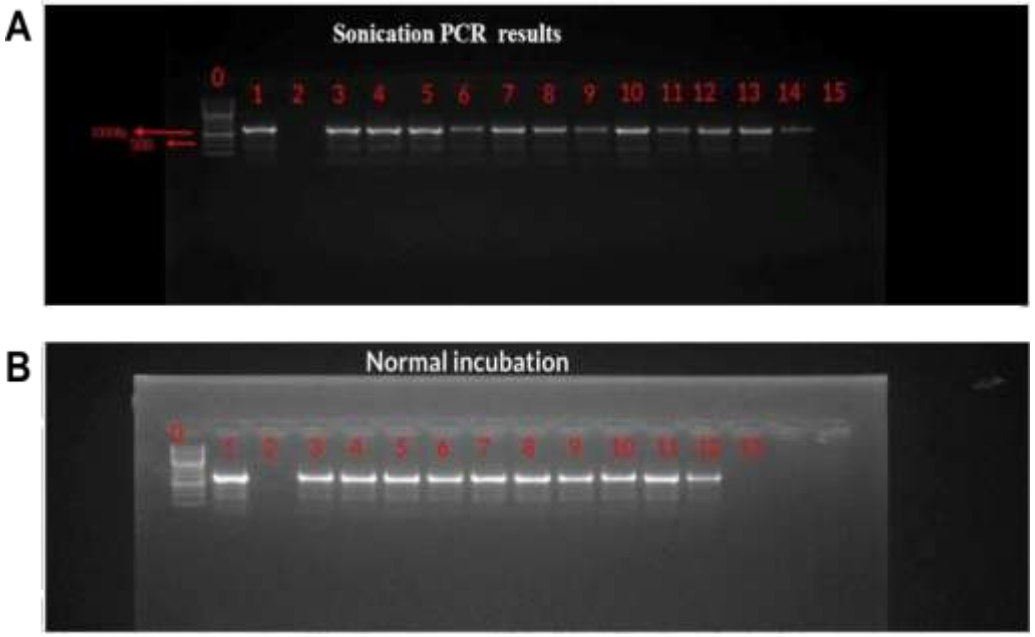


Fig. 4 Agrobacterium mediated transient gene expression through GUS histochemical assay (A) with sonication (B) without sonication

Fig. 5 PCR confirmation of Agrobacterium mediated transformed plants (A) with sonication (B) without sonication



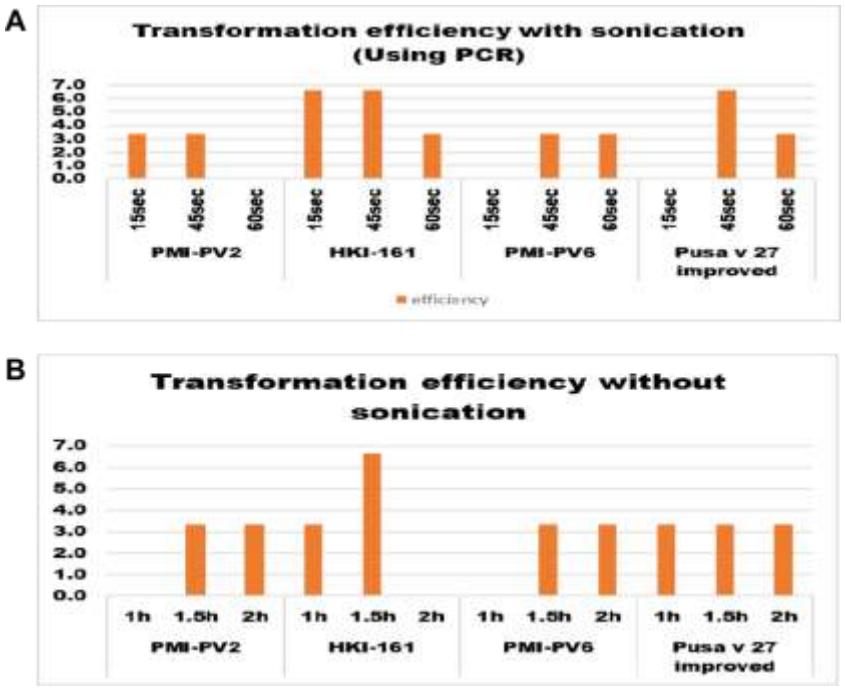


Fig. 6 Agrobacterium mediated transient gene expression through PCR confirmation (A) with sonication (B) without sonication