Research Article

Optimization of Different Parameters for Transformation of Elite Maize Inbred Lines

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Abstract | Four elite corn inbred lines were evaluated for susceptibility to *Agrobacterium* infection by investigating different parameters such as embryo physiological age, co-cultivation period, addition of L-cysteine in co-cultivation medium, heat shock to immature embryos prior to infection, resting stage and *Agrobacterium* strains that deliver T-DNA into plant cell from which whole plant can be regenerated. The results indicated that optimal embryo physiological age is 15 and 18 days after pollination and transient transformation efficiency decreased at 22 days after pollination. Enhanced transient beta glucuronidase (*GUS)* expression with average frequency of 40.56% was noticed with three days of co-cultivation period while two days of co-cultivation period resulted 24.07% transient *GUS* expression. Addition of 100 mgL^{-1} L-cysteine in co-cultivation medium increased the transient transformation by 33.9% compared to control treatment. It has also been observed that cysteine enhanced the expression of *GUS* in the basal scutellar regions of immature embryos compared to apical and embryo axis region. Heat shock of 46 °C for 3 minutes increased the transformation efficiency of immature embryos by 26.96%. Growth of infected immature embryos on resting medium after co-cultivation period enhanced the survival of kanamycin resistant calli on selection medium by 93.97%. Agropine strain (EHA101) of *Agrobacterium* was found 25.67% more effective than octopine strain (LBA4404). The intensity of *GUS* expression was also enhanced with EHA101 compared to LBA4404. The maize genetic transformation protocol developed in this study will possibly improve the efficiency to produce new transgenic maize lines expressing desirable agronomic characteristics.

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Keywords | Immature embryos, Maize inbred lines, T-DNA delivery, Transformation efficiency, Transient *GUS* expression.

Introduction

Maize is the highest yielding cereal crop in the world (USDA, 2016). United States, Brazil and China are the leading countries in global maize production and alone produced 661.74 million metric tons out of total 1014.02 million metric tons during 2014-15 (USDA, 2016). Maize has great importance for Pakistan where increasing population requires more food supplies. Currently it is planted on 1140 thousand hectares which produce 4900 thousand tonnes with average national yield of 4290 kg ha^{-1} (USDA, 2016). Maize contributes 2.1% to the value added in agriculture and 0.4% to Gross Domestic Product (GDP) of Pakistan (GoP, 2015). The demand of maize is increasing day by day and it is estimated that demand of maize would be double in 2050 in the developing world (Chaudhary et al., 2014). Yield gap between maize national average i.e. 4290 kg ha^{-1} (USDA, 2016) and that of potential yield

i.e. 9200 kg ha^{-1} (Aslam, 2016) is nearly 2.14 times. Plant transformation is an excellent technology to improve the existing cultivars of crops and to minimize the yield gap. Hence, development of an efficient transformation method is mandatory. Genetic transformation method based on the *Agrobacterium tumefaciens* is dominant and powerful technology to genetically alter the crop in beneficial way. Extensive research has been conducted to improve the molecular machinery of *A. tumefaciens* to transport T-DNA into host plant cells. The factors which influence the transformation efficiency have been optimized for different crop species. All these factors need to be optimized before attempting any genetic transformation experiment with new genotype as the influence of these factors varies with the genotypes and explant used (Tzfira and Citovsky, 2006). *Agrobacterium* mediated transformation is influenced by many factors including *Agrobacterium* strain, *Agrobacterium* cell density, plant genotypes, plant growth hormones, antibiotics, use of antioxidant in co-cultivation medium and explant (Olhoft et al., 2003; Cheng et al., 2004; Tzfira and Citovsky, 2006). The identification of new factors and optimization of existing factors would increase the host range of *Agrobacterium* and will improve the transformation efficiency. Several researchers identified that different tissues and cell types within same plant differ in their susceptibility to *Agrobacterium* infection (Ritchie et al., 1993; Wang et al., 2009). Immature embryos are the most suitable explant for genetic transformation of cereals due to their morphogenetic competencey (Cheng et al., 2004; Shrawat et al., 2007).

Agrobacterium mediated transformation is well established for dicots as they are natural hosts of *A. tumefaciens*. The *Agrobacterium* mediated transformation has also been extended to monots. Efficient genetic transformation through *Agrobacterium* was reported in rice (Hiei et al., 1994), maize (Ishida et al., 1996), wheat (Cheng et al., 1997), barley (Tingay et al., 1997) and sorghum (Zhao et al., 2000). Only few model cultivars of maize have been successfully transformed with *Agrobacterium* efficiently such as A188 or its hybrids in maize. Although elite cultivars were also transformed with *Agrobacterium* but the number is limited and lower transformation efficiency is always noticed (Gordon-Kamm et al., 2002). The genetic transformation studies with elite cultivars of maize revealed genotype dependent response of *Agrobacterium* which is the major hindrance in extending the *Agrobacterium* mediated transformation to economically important elite cultivars (Karami, 2008) and thus genetic transformation of elite inbred lines remained a challenging task (Frame et al., 2006). Therefore it becomes imperative to make elite cultivars responsive to genetic transformation by investigating and optimizing different parameters affectting transformation efficiency.

In the present study thorough and detailed investigation were carried out to specify the chemical, physical and biological conditions for genetic transformation of indigenously developed elite maize inbred lines.

Material and Methods

Plant material

Four different Maize Inbred lines (MIBL) were selected from Maize hybrid program, Crop Science Institute (CSI), National Agricultural Research Centre (NARC) Islamabad. The genetic background of MIBL-4 and MIBL-5 inbred lines were from subtropical material while genetic background of MIBL-2 and MIBL-6 were from temperate material. The source materials of these inbred lines were obtained from CIMMYT. Inbred lines were selected based on good agronomic characters, best combining ability and high regeneration efficiency. The inbred lines were regularly sown throughout the year in green house facility of National Institute for Genomics and Advanced Biotechnology (NIGAB) on different dates to obtain the immature embryos for experimentation.

Effect of genotype, co-cultivation period and embryo physiological age on transient transformation efficiency

In order to determine the effect of genotype, co-cultivation period and embryo physiological age on transient transformation efficiency, a tri-factorial experiment was designed. The immature embryos were isolated after 15, 18 and 22 days of pollination from green house grown self-pollinated maize inbred lines. Fresh single colony of *Agrobacterium tumefaciens* strain LBA4404 harbouring pCAMBIA 1301 having *GUS* reporter gene under the transcriptional control of constitutive 35 S promoter from cauliflower mosaic virus and Neomycin phosphotransferase (*NPT-II*) gene as a plant selectable marker under the control of NOS promoter was taken and suspended in 15 ml liquid LB media supplemented with 50

mgL^{-1} kanamycin and 100 µM acetosyringone and were incubated at 28 ^{o}C for 48 hours at 250 rpm in dark. The culture was centrifuged at 4000 rpm for 10-15 minutes at room temperature. The supernatant was removed and the cells pellet was resuspended in liquid infection media (LIM) containing 3.99 gL^{-1} N6 basal medium w/ vitamins (Phytotechnology laboratories, USA), 30 gL^{-1} sucrose, 2.8 gL^{-1} proline, 4 mgL^{-1} 2, 4-D, 0.5 gL^{-1} 2-N-morpholino ethanesulfonic acid (MES) and 100 µM acetosyringone. The pH of LIM was adjusted to 5.8. Immature embryos were incubated twice with *Agrobacterium* suspension (OD 660 = 0.5) in LIM for 5 minutes. During each incubation the tubes were inverted 20 times. LIM was removed and the immature embryos were transferred to sterilized whatman filter papers placed in glass plates in laminar air flow cabinet. The immature embryos were transferred to co-cultivation media with the help of fine forcep. The composition of co- cultivation media was 3.99 gL^{-1} Chu's N6 basal medium w/ vitamins (Phytotechnology laboratories, USA), 30 gL^{-1} sucrose, 0.7 gL^{-1} proline, 4 mgL^{-1} 2, 4-D, 100 µM acetosyringone, 5 µM each of silver nitrate and copper sulphate, gellan gum agar 0.3% and pH was adjusted to 5.8. The immature embryos were allowed for either two or three days on co-cultivation media at 28 ^{o}C in dark and then *GUS* assay was performed following the protocol of Jefferson et al. (1987) to determine the transformation efficiency of each inbred line.

Effect of L- cysteine on transient transformation efficiency

In order to evaluate the effect of L-cysteine on transient transformation, the two inbred lines (MIBL-4 and MIBL-5) were selected as these lines exhibited enhanced transient expression in our previous experiment. The immature embryos from both inbred lines were harvested after 18 days of pollination and infected with transformed *Agrobacterium tumefaciens* using already described procedure. The composition of co-cultivation media was kept same except the addition of L-cysteine. The co-cultivation media without L-cysteine was designated as Treatment-I (T1) and co-cultivation media with 100 mgL^{-1} L-cysteine was designated as Treatment- II (T2). The immature embryos were subjected to *GUS* assay after 3 days of co- cultivation period and data was recorded and analyzed statistically.

Effect of heat shock on transient *GUS* expression of embryos

Immature embryos of MIBL-4 and MIBL-5 were subjected to different heat shock times (0, 2, 3 and 5 minutes) prior to infection in order to evaluate the effect of heat shock (46 ^{o}C) on transient transformation efficiency.

Effect of resting phase on survival of infected explants

The effect of screening agent at two different time periods was analyzed. In one experiment the immature embryos of both inbred lines were incubated on selection media immediately after co-cultivation period and in another experiment the embryos were cultured on resting media initially for one week and then incubated on selection media. The composition of selection media was 3.99 gL^{-1} Chu's N6 basal media w/ vitamins (Phytotechnology laboratories, USA), sucrose 3%, myoinositol 0.1 gL^{-1}, casein hydrolysate 0.3 gL^{-1}, proline 0.7 gL^{-1}, 2,4-D 4 mgL^{-1}, kanamycin 50 mgL^{-1}, carbenicillin 250 mgL^{-1}, gellan gum agar 0.3%. The composition of resting media was same as selection media except inclusion minus kanamycin. The data was recorded on 4 weeks o1d kanamycin resistant calli for both methods of selection.

Effect of different bacterial strains on transient transformation efficiency

Immature embryos were infected with two different *Agrobacterium tumefaciens* strains EHA101 and LBA4404 containing *GUS* construct. After three days of co-cultivation, the immature embryos were subjected to *GUS* assay. The embryos were counted for *GUS* expression and data was analyzed using Statistix 8.1.

Results

Effect of genotype, co-cultivation period and embryo physiological age on transient *GUS* expression

Immature embryos harvested after 15 and 18 days of pollination were non-significantly different from each other for transient *GUS* expression (Table 1). The im mature embryos harvested after 22 days of pollination showed less transient *GUS* expression. The results indicated that optimal embryo physiological age is 15 to 18 days after pollination and transient transformation decreased at 22 days after pollination. Enhanced transient *GUS* expression with average frequency of 40.56% was noticed with three days of co-cultivation period compared to 24.07% with two days of co-cultivation period and hence three days of co-cultivation period was found more suitable for transformation experiments (Table 1). Among inbred

Table 1: *Effect of genotype, co-cultivation period and embryo physiological age on transient GUS expression*

Inbred Lines	Co-cultivation (days)	Days after pollination			Means of Inbred lines
		15	18	22	
MIBL-2	2	33.3	33.3	22.2	**38.1 C**
	3	51.1	53.3	35.6	
MIBL-4	2	35.6	33.3	26.7	**42.6 B**
	3	55.6	57.8	46.7	
MIBL-5	2	31.1	35.6	37.8	**48.5 A**
	3	68.9	62.2	55.6	
MIBL-6	2	0.0	0.0	0.0	**0.0**
	3	0.0	0.0	0.0	
Treat. Mean	**2 days: 24.07 B** **3 days: 40.56 A**	**34.4 A**	**34.4 A**	**28.1 B**	

LSD 0.01 for inbred lines =3.84, days after pollination = 3.33 and co-cultivation days = 2.72; Each calculated value without alphabet represents the percentage of total 45 immature embryos in three replicates and each replicate was consisted of 15 immature embryos; Means followed by different alphabets are significantly different at 1% level of significance

lines, maximum *GUS* expression (48.5%) was noticed in MIBL-5 followed by MIBL-4 (42.6%) and MIBL-2 (38.1). The MIBL-6 was found completely recalcitrant to *Agrobacterium* infection and transient *GUS* expression (Table 1). In short, the designed experiment recommends three days of co-cultivation period for maximum transient transformation in immature embryos harvested after 15 to 18 days of pollination in all inbred lines except MIBL-6.

Effect of addition of L- cysteine in co-cultivation media on transient *GUS* expression

Statistical analysis revealed that significant differences exist ($p<0.01$) between two co-cultivation media one which contains L-cysteine and other which does not contain L-cysteine. Similarly the two inbred lines were also found significant ($p<0.01$) for transient *GUS* expression (Table 2). The designed experiment concluded that addition of 100 mgL^{-1} L-cysteine in co-cultivation media has increased the transient transformation efficiency by 33.9% compared to control treatment. It has also been observed that L-cysteine enhanced the expression of *GUS* in the basal scutellar regions of embryos compared to apical and embryo axis sides (Figure 1). Number of foci on each embryo was difficult to count as diffused transient expression was detected over the surface of the embryos. MIBL-5 showed 77.8% transient expression which is 14.7% higher than the MIBL-4.

Effect of heat shock on transient *GUS* expression of embryos

Analyzing the transient *GUS* expression in immature embryos, it was found that heat shock of 46 ^{o}C significantly increased the transformation efficiency. The three heat shock time periods i.e. 2, 3 and 5 minutes were not significantly different from each other and showed transformation efficiency of 71.67%, 78.3% and 75% respectively compared to 61.67% on control treatment (Table 3). The two inbred lines were also found significantly different from each other however the interaction between inbred lines and different heat shock time periods was found non-significant ($p=0.75$). The designed experiment concluded that heat shock of 46 ^{o}C for 3 minutes has the ability to increase the transformation efficiency by 26.96% compared to control treatment.

Table 2: *Effect of addition of L-cysteine in co-cultivation media on transient GUS expression*

Inbred lines	L- cysteine		Means of Inbred lines
	0 mg/l (T1)	100mg/l (T2)	
MIBL-4	57.8	77.8	**67.8 B**
MIBL-5	66.7	88.9	**77.8 A**
Treatment Means	**62.22 B**	**83.3 A**	

LSD 0.01 for inbred lines =9.13, L-cysteine = 9.13; Each numeric value without alphabet represents the percentage of total 45 immature embryos in three replicates and each replicate was consisted of 15 immature embryos; Means followed by different alphabets are significantly different at 1% level of significance.

Effect of resting phase on survival of infected explants

More kanamycin resistant calli were obtained with selection method involving resting media. The selection method involving resting media supported 80.83%

kanamycin resistant calli while selection method devoid of resting media supported 41.67% kanamycin resistant calli (Table 4). There was an increase of 93.97% resistant calli with selection method involving resting media compared to selection method lacking resting media. The two inbred lines were also found significantly different from each other and MIBL-5 produced 67.5% kanamycin resistant calli while MIBL-4 supported 55% kanamycin resistant calli. MIBL-5 produced 22.72% more kanamycin resistant calli compared to MIBL-4 (Table 4).

Table 3: *Effect of heat shock on transient GUS expression of embryos*

Inbred Lines	Heat shocks (Minutes)				Means of inbred lines
	0	2	3	5	
MIBL-4	56.67	66.67	76.67	73.3	**68.33 b**
MIBL-5	66.67	76.67	80.00	76.67	**75.00 a**
Treatment Means	**61.67 b**	**71.67 a**	**78.3 a**	**75.0 a**	

LSD 0.05 for inbred lines = 6.37, heat shock times = 9.01; Each numeric value without alphabet represents the percentage of total 30 immature embryos in three replicates and each replicate was consisted of 10 immature embryos; Means followed by different alphabets are significantly different at 5% level of significance

Effect of different bacterial strains on transient *GUS* expression

Table 4: *Effect of resting phase on survival of infected explants*

Inbred lines	Production of kanamycin resistant calli		Means of Inbred lines
	Resting	Without resting	
MIBL-4	76.67	33.3	**55.0 B**
MIBL-5	85.00	50.0	**67.5 A**
Treatment Means	**80.83 A**	**41.67 B**	

LSD 0.01 for inbred lines = 7.9, resting and without resting = 7.9; Each numeric value without alphabet represents the percentage of total 60 immature embryos in three replicates and each replicate was consisted of 20 immature embryos; Means followed by different alphabets are significantly different at 1% level of significance.

Transient *GUS* transformation efficiency of agropine strain (EHA101) was compared with octopine strain (LBA4404) of *Agrobacterium*. The two strains were significantly different from each other relevant to *GUS* expression in immature embryos. LBA4404 induced *GUS* expression in 61.67% immature embryos while EHA101 induced *GUS* expression in 77.50% immature embryos (Table 5). Agropine strain EHA101 was found 25.67% more effective compared to octopine strain. The intensity of *GUS* expression was also found more with EHA101 compared to LBA4404 (Figure 2). Similarly the two inbred lines were also found significantly different and MIBL-5 was found more susceptible to *Agrobacterium* infection (Table 5).

Table 5: *Effect of different bacterial strains on transient GUS expression*

Inbred lines	*Agrobacterium tumefaciens* Strains		Means of Inbred lines
	LBA4404	EHA101	
MIBL-4	55.00	71.67	**63.33 B**
MIBL-5	68.33	83.33	**75.83 A**
Treatment Means	**61.67 B**	**77.50 A**	

LSD 0.01 for inbred lines = 8.38, A. tumefaciens strains = 8.38; Each numeric value without alphabet represents the percentage of total 60 immature embryos in three replicates and each replicate was consisted of 20 immature embryos; Means followed by different alphabets are significantly different at 1% level of significance.

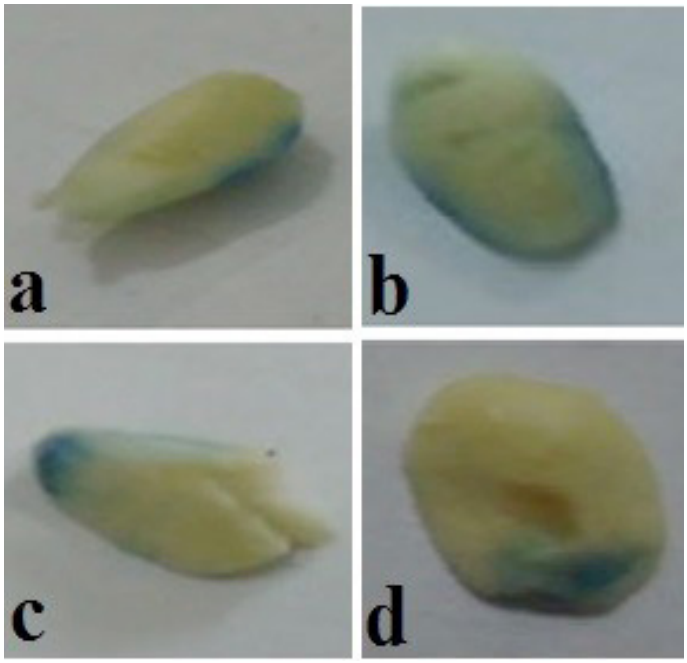

Figure 1: *Effect of addition of L-cysteine in co-cultivation media on transient GUS expression. a and b, 100 mgL^{-1} cysteine in co-cultivation media; c and d, no L-cysteine in co-cultivation media.*

Discussion

Meristematic tissues of embryos were investigated at different developmental stage for *Agrobacterium* mediated transformation. Direct correlation was found

between embryo physiological age and its compe -tency for *Agrobacterium tumefaciens* infection. The earlier developmental stages of embryos i.e. before 15 days of pollination were not investigated because apical meristem of embryos is usually undifferentiated at initial stages of embryo development and is not suitable for *Agrobacterium* infection while at lateral stages it develops two to three leaf initials and became competent for infection by *Agrobacterium*. Schalappi and Holn (1992) previously showed that transgene integration in the meristematic tissues of maize may require some enough specific plant receptors for *Agrobacterium* which may not be available in undifferentiated apical meristem of embryos and there is a window of competency for *Agrobacterium* infection in immature embryos of maize.

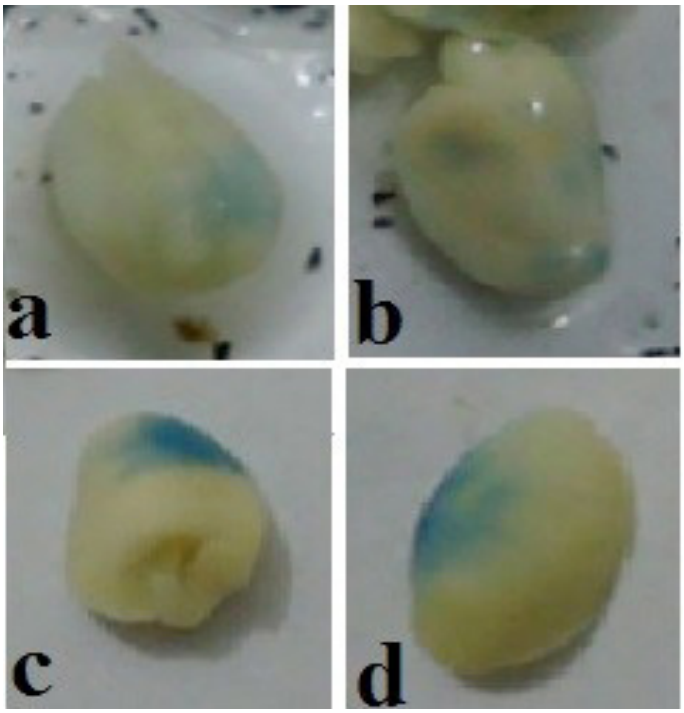

Figure 2: *Effect of different bacterial strains on transient GUS expression. a and b, transient GUS expression induced by LBA4404; c and d, transient GUS expression induced by EHA10l*

Cysteine is an antioxidant and its inclusion at co-cultivation stage during genetic transformation experiment may reduce the cell death initiated due to the hypersensitive reaction of scutellum cells against *A. tumefaciens* infection. Four hundred mg L^{-1} of cysteine in co-cultivation media is already recruited to enhance the overall stable genetic transformation (Frame et al., 2002). We obtained 33.9% enhanced transient expression with the addition of 100 mg L^{-1} L-cysteine in co-cultivation media compared to control treatment. This enhanced expression may be attributed to survival of scutellum cells competent to produce embryogenic calli. Gui- rong et al. (2013) also observed beneficial effect of 100 mgL^{-1} cysteine on transient gene expression. Our experiment with 100 mgL^{-1} of L-cysteine favored more transient expression in the basal scutellar regions of embryos compared to apical and embryo axis sides. Similar results were also obtained by Frame et al. (2002) with the inclusion of 100 and 200 mgL^{-1} cysteine in co-cultivation media.

Gui-rong et al. (2013) found that heat treatment is necessary to enhance the competency of cells to infection by *A. tumefaciens*. In our experiments with two inbred lines, the transformation frequency on average was improved by 26.96% with 3 minutes of 46 ^{o}C heat treatment. Hiei et al. (2006) improved transformation efficiency in immature embryos of maize and rice with heat treatment and centrifugation prior to infection. The beneficial effect of heat treatment of 41^{o}C for 5 minutes in transformation of *Withania somnifera* L. through *A. Rhizogenes* has also been recently recognized (Thilip et al., 2015).

Several protocols recommend the incubation of immature embryos of maize on non-selection media after co-cultivation for better transformation efficiency (Zhao et al., 2002). Gui-rong et al. (2013) suggested that low selection pressure at initial stage after co-cultivation is important for maximum transformation efficiency in certain genotypes of maize. Low selection pressure might improve the tolerance of embryos and calli to screening agent and result in better differentiation and sprouting in lateral stages of regeneration. It has also been reported that resting phase gives the opportunity to small and slow responding embryos to form callus (Frame et al., 2002). Similarly, delayed selection procedure was also applied by Mitic et al. (2014).

The variability in competency of different *Agrobacterium* strains to infect plant genotypes is a major drawback of *Agrobacterium* mediated transformation. Cao et al. (2014) concluded that agropine strains are more efficient in transforming multi shoots cultures and shoot tips of maize than nopaline and octopine strains. The present investigation showed that agropine strain is also more effective in transforming immature embryos of two locally developed inbred lines. Similarly Gui-rong et al. (2013) showed 65% more transformation efficiency in immature

embryos of maize with the EHA105 compared to LBA4404, while our results indicated that EHA101 which is also agropine strain is 25.67% more effective than LBA4404. Huang and Wei (2005) also identified that agropine strain of *agrobacterium* (EHA105) is better than nopaline (GV3101) and octopine strain (LBA4404) for efficient transformation of elite maize inbred lines.

Conclusions

Owing to the major role of crops in human diet, food security cannot be achieved without major increases in crop yield. Genetic transformation has become a major tool to improve the crop yield by inserting desirable traits in its genome. The main hindrance to genetic transformation system is the genotype specific responses of crop species. Major step in any genetic transformation experiment is to select the right combination of critical parameters that positively affect the transformation efficiency during infection and co-cultivation stages. Exploring and optimizing such parameters hold great promise to make the cells more responsive to *Agrobacterium* mediated transformation and to extend this technology to the elite inbred lines and cultivars. The effects of various parameters have been examined in the present study to extend the benefits of genetic transformation to economically important elite inbred lines and cultivars to achieve the future food security.

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Author's Contribution

ZA conducted experiments and wrote the manuscript. GMA conceived the idea and provided technical input at every step. SA mentioned the methodologies and designed the experiments. JD helped in data analysis.

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