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Agrobacterium mediated transformation of leaf derived callus of tropical maize (*Zea mays* L.) inbred lines

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Genetic transformation of maize has been a challenge due to the genetic variability of the genotypes and differences in their responses to the *in vitro* culture and *Agrobacterium*-mediated transformation procedures which has resulted to low transformation frequencies. In this study embryogenic callus was initiated from young leaves of six tropical maize inbred lines (CML216, CML331, TL09, TL18, TL27, MU25). Embryogenic callus were immersed in the infection medium containing N6 macro and micro salts and modified KT vitamins supplemented with 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 2.88 g l^{-1} proline, 100 mg l^{-1} casein hydrolysate and 2.2 mg l^{-1} 2,4-D, 68.4 g l^{-1} sucrose, 36 g l^{-1} glucose and 200 µM AS (acetosyringone). The callus was then immersed in EHA101(pTF102) *A. tumefaciens* suspension supplemented with 200 µM AS and then incubated in the dark for 60 minutes. The infected callus were transferred onto the co-cultivation medium containing N6 macro and micro salts and modified KT vitamins supplemented with 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 2.88 mg l^{-1} proline, 100 mg l^{-1} casein hydrolysate, 2.2 mg l^{-1} 2,4-D, 800 mg l^{-1} silver nitrate and 20 g l^{-1} sucrose, 200 µM AS (acetosyringone) and 0.3 % gerlite and incubated in the dark at 20 ^{o}C for three days. Callus was transferred onto selection medium I containing N6 macro and micro salts and modified KT vitamins supplemented with 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 2.88 mg l^{-1} proline, 100 mg l^{-1} casein hydrolysate, 2.2 mg l^{-1} 2,4-D, 800 mg l^{-1} silver nitrate and 20 g l^{-1} sucrose, 200 µM AS (acetosyringone), 1.5 mg l^{-1} bialaphos and 0.3 % gerlite. Surviving callus was transferred onto selection medium II containing the same composition as selection medium I except 1.5 mg l^{-1} bialaphos which was replaced with 3 mg l^{-1} bialaphos. Actively growing callus on selection medium II was transferred onto the regeneration medium containing N6 macro and micro elements and modified KT vitamins supplemented with 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 2.88 g l^{-1} proline, 100 mg l^{-1} casein hydrolysate, 2% sucrose, 0.3% gerlite and 0.5 mg l^{-1} benzylaminopurine (BAP).

All the non-infected callus (control) turned brown, necrotic and died when they were grown on selection medium II after four weeks of culture. Actively growing resistant embryogenic callus were observed in CML216, TL18 and MU25 maize callus lines on the 4th week on selection medium II. Infected callus of CML331, TL09 and TL27 did not survive on bialaphos (Table 1). The frequency of callus which survived on selection medium containing bialaphos was very low (Table 1). The difference between the maize lines on the percentage of callus which survived was statistically significant ($p<0.05$). Transformation frequency of the resistant surviving callus selected on bialaphos (3 mg l^{-1}) containing medium ranged between 0 to 1.8 % (Table 1). Plants were regenerated on a medium containing 0.5 mg l^{-1} BAP from putative transformed callus (surviving callus) of

CML216 and TL18 and none from CML331, TL09, TL27 and MU25 (Table 1). The number of plants regenerated was low ranging from 1 to 3 among the genotypes.

Table 1. Selection of a leaf-derived callus on bialaphos containing culture medium

Genotype	Number of callus inoculated	Number of callus surviving	Transformation frequency (%)	Number of putative transformed plants regenerated
CML216	59	1	1.7	1
CML331	18	0	0	0
TL09	47	0	0	0
TL18	227	4	1.8	3
TL27	15	0	0	0
MU25	204	2	0.9	0

Transient *gus* activity was used as an initial step to assess if the transfer of the transgene had taken place. Transient *gus* expression was confirmed by histochemical β-glucuronidase (*GUS*) activity. Transient g*us* expression was observed on the 3rd day of co-cultivation of the leaf derived embryogenic callus after infection with EHA101(pTF102) *A. tumefaciens*. Transient *gus* expression was detected in the infected leaf derived callus of CML216, CML331 and TL18 and maize lines tested. Transient *gus* activity was not detected in non-transformed callus (control) and infected callus of TL09, TL27 and MU25 maize lines (Table 2). The frequency of callus expressing *gus* activity ranged from 0 % to 60 %. Significant differences ($p<0.05$) were detected among the maize lines tested on percentage area of callus expressing the *gus* activity. TL18 had the highest mean percentage area of callus showing *gus* activity (13%) followed, CML331 (8 %); CML216 (4 %), TL27, MU25 (0 %). Transformation frequency of CML331 embryogenic callus on the selection medium did not correspond to the transient *gus* expression. This could be probably due to transient expression when the transgene is transferred into the cytoplasm of the plant cell but stable integration into the maize genome does not occur. Transient *gus* expression assays have proved to be a useful tool which is routinely used as an initial step to demonstrate *gus* expression in cells and tissues of *Agrobacterium*-mediated transformed plants prior to stable integration of the transgene.

In conclusion, results from this study show that the transfer of the transgene into the infected callus was genotype dependent. There is need to assess if there is stable integration and inheritance of the transgene in the plants that were regenerated in this study.

Table 2. Transient *gus* expression of a leaf derived callus of six tropical maize inbred lines infected with EHA101(pTF102) *A. tumefaciens*

Genotype	**Total number of callus tested**	**Number of callus showing *gus* activity**	**Frequency of callus showing *gus* activity**	**Mean percentage area of callus showing *gus* activity (%)**
CML216	5	1	20	4.0±1.0a[+]
CML331	5	3	60	8.00±4.00a
TL09	20	0	0	0a
TL18	52	21	40.3	13.00±2.00b
TL27	50	0	0	0a
MU25	46	0	0	0a

[+]Means followed by the same letters within columns are not significantly different according to Tukey`s Honest Significant Difference at 5 % level.