Transformation and Regeneration Protocol for Two Farmer Preferred Open Pollinated Tropical Maize (Zea Mays) Varieties

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Abstract: In vitro regeneration of open pollinated varieties (OPVs) Kakamega Striga Tolerant Population 94 (KSTP’94) and ‘Namba Nane’ alongside a tropical inbred line (CML144) was evaluated using immature zygotic embryos as explants. Four callus induction media (CIM) regimes; Murashige and Skoog (MS), Linsmaier and Skoog (LS), Chu (N6) and N6*(N6 medium fortified with 0.35 g L⁻¹ L-proline and 0.8 mg L⁻¹ AgNO₃) were evaluated for their potential to induce callus in the three genotypes. All the media were supplemented with sucrose and five levels of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹) while the subsequent embryogenic calli were regenerated on hormone-free media. Transformability of these varieties was assessed via histochemical analysis of β-glucuronidase (GUS) reporter gene following Agrobacterium-mediated transformation. Statistical analyses were done using Statistical Analysis Software (SAS) and Graphpad Prism softwares with mean separations achieved at 95% confidence intervals. Of the 2 OPVs, KSTP’94 recorded the highest callus induction frequency (84.4%) while Namba Nane (45.6%) had the lowest. Similarly, KSTP, 94 had the highest mean of mature somatic embryos (59.7%) while Namba Nane recorded the lowest (16.4%). Assessment of regeneration frequencies from embryogenic calli revealed no significant differences among the 3 lines although CML 144 had the highest mean number of juvenile plantlets (36.7%). Analysis of transformation frequency (upon selection of calli on media with basta) showed that Namba Nane recorded the lowest transformation frequency (average 13.5%) some words missing. Transformation frequency (based on GUS positive calli) of these varieties ranged from 0.8 to 2.1%. This work therefore provides an empirical platform for potential introduction of useful genes into these varieties.

Keywords: Callus Induction, Embryogenesis, Plant Regeneration, Open Pollinated Varieties, GUS

1. Introduction

Maize is an important food crop and in Sub Saharan Africa (SSA), majority of resource-poor small-scale farmers largely depend on open-pollinated maize varieties (OPVs) for subsistence [1]. These varieties offer the economic advantage of seed recycling without the risk of a decline in yield as is the case with hybrid maize [1, 2]. Given the high price of certified maize seed, high input level and longer maturity period expected of hybrids, small-scale farmers in western Kenya predominantly cultivate OPVs especially during the short rains. Also, some of these varieties have Striga
tolerance attributes that are useful in Striga-infested regions [3, 4]. Furthermore, OPVs have been reported to have superior taste and better keeping quality than hybrids [4, 5]. Demand for maize in the region outstrips supply with farmers insisting on cultivating OPVs such as Kakamega Striga Tolerant Population 94 (KSTP’94) and ‘Namba Nane’. The risk of declining seed quality and farm yield due to on-farm seed production of these varieties calls for the need to use biotechnological approaches to improve these varieties for the benefit of resource poor rural famers. It is therefore important to set up platform for genetic improvement of the OPVs.

One way of achieving this is by developing a reliable in vitro regeneration and genetic transformation platform to enable rapid improvement of the farmer preferred OPVs [6, 7]. In vitro establishment of maize callus cultures through somatic embryogenesis has been reported for various maize lines but it is genotype dependent [8]. Currently, a variety of basal media have been used to initiate maize callus cultures and subsequent plant regeneration [9]. Generally, the incorporation of an auxin such as 2, 4-dichlorophenoxyacetic acid (2, 4-D) or dicamba (3, 6-dichloro-o-anisic acid) in callus induction media enhances callus formation [10]. However, the concentration levels of these auxins are reported to vary in maize culture media depending on genotype and type of explant used [10, 11]. Furthermore, it is reported that addition of silver nitrate (AgNO₃), and amino acids such as L-proline and casein hydrolysate enhances induction of regenerable type II embryogenic callus in maize [10].

In setting up a transformation protocol it is important that rapidly verifiable markers are used to distinguish transformed from non-transformed tissues. The β-glucuronidase (GUS) gene has been widely reported to be an important marker in evaluating transformation systems among various plants [12, 13]. The aim of this study therefore was to develop and optimize an in vitro regeneration and transformation protocol for KSTP’94 and Namba Nane alongside a commonly transformed tropical inbred line CML144 as a check.

2. Material and Methods

2.1. Plant Material and Explant Preparation

Seeds of OPVs ‘Namba Nane’ and KSTP’94 were acquired from Kenya Agricultural and Livestock Organization (KALRO) while tropical maize inbred line CML144 was acquired from International Maize and Wheat Improvement Center (commonly called by its Spanish acronym CIMMYT for Centro Internacional de Mejoramiento de Maiz y Trigo) and grown in a research field at Jomo Kenyatta University of Agriculture and Technology (JKUAT). Upon silking, ears were self-pollinated and then harvested between 9th and 11th day after pollination (DAP) to obtain immature embryos. Harvested ears were taken to Plant transformation Laboratory (PTL) at Kenyatta University where they were carefully de-husked. To sterilize the maize, de-husked ears were first dipped in 70% ethanol for two minutes and rinsed thrice using double-distilled water, followed by dipping for 30 minutes in sodium hypochlorite solution containing two drops of Tween 20. The ears were then rinsed three times with double-distilled water. The kernel crowns were aseptically excised and zygotic embryos (0.8 mm - 1 mm in size) of every variety isolated independently using a sterile blunt spatula into a sterile petri plate containing 10 ml of embryo suspension media (ESM).

2.2. Media Preparation

Excised embryos were separately cultured on four different callus induction media (CIM) denoted MS; comprising Murashige and Skoog (MS), Linsmaier and Skoog (LS); Chu (N6); and N6*(N6 medium fortified with 0.35 g L⁻¹ L-proline and 0.8 mg L⁻¹ AgNO₃ i.e silver nitrate). The four media were fortified with 30 g L⁻¹ sucrose, 2, 4-D at five concentration levels (0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹) and solidified using 3 g/L gelrite (Duchefa Biochemie, Haarlem, Netherlands, cat no. 004016). MS and N6 media were used for callus maturation (CMM) and were supplemented with five concentration levels of 2, 4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹), 60 g L⁻¹ sucrose and 3 g L⁻¹ gelrite (Duchefa Biochemie, Haarlem, The Netherlands, cat no. 004016). Regeneration media (RM) were similar to the callus maturation media but lacked 2, 4-D while the sucrose was reduced to 30 g L⁻¹ and were solidified using 8 g L⁻¹ phytoagar (Duchefa Biochemie Haarlem, The Netherlands, cat no. 025113). The pH of all the media was adjusted to 5.8 before autoclaving at 121°C for 15 min. AgNO₃ was filter-sterilized and added to autoclaved CIM and CMM media.

2.3. Callus Induction

To initiate callus, a method by Omer et al., [14] was used. Summariy, the embryos of the three varieties were separately cultured on four CIM fortified with five concentration levels (0.5, 1.0, 1.5, 2.0 and 2.5 mg L⁻¹) of 2, 4-D and replicated three times. In total One thousand, four hundred and forty embryos were used whereby four hundred and eighty immature embryos of each individual variety were used to set up primary callus induction experiment (40 embryos per treatment). Immature embryos with undamaged scutellar tissues were cultured with the embryo axis in contact with the medium and incubated in darkness at 25 ± 2°C. After two weeks of incubation, the number of immature embryos producing primary callus was recorded in each variety before subculturing onto new media.

2.4. Callus Maturation

For maturation of somatic embryos, primary calli from the setup above were sub cultured onto two CMM media (described earlier) fortified with five concentration levels of
2.4-D (0.5, 1.0, 1.5, 2.0 and 2.5 mgL$^{-1}$). A total of 24 calli sets (36 embryos per set) were distributed in an experimental design defined as 2 CMM X 3 maize varieties (X 4 replicates (calli sets) were setup with an individual set consisting of 36 calli. They were incubated in darkness at 25±2°C for two weeks, subcultured once and incubated for another two weeks. The calli were regularly screened (at three days interval) under microscope (Leica MZ10F/CLS150LED installed with Leica application suite V4) for embryogenic calli formation (globular and coleoptilar shapes) and the number of observed embryogenic calli recorded.

2.5. Plant Regeneration, Acclimatization and Growth of Regenerants to Maturity

Embryogenic calli were further subcultured to hormone-free regeneration media (RM) namely MS and N6 supplemented with 30 gL$^{-1}$ sucrose and 8 gL$^{-1}$ phytagar (Duchefa Biochemie Haarlem, The Netherlands, cat no. 025113) for shoot regeneration. After CMM incubation period, All the calli were subculture on the two regeneration media (RM) in the same callus maturation experimental design. The cultures were maintained at 25 ± 2°C under a 16-h photoperiod using cool white fluorescent lights (40 mmol m$^{-2}$s$^{-1}$) for four weeks with one subculture (after two weeks of incubation). The number of embryogenic calli pieces that spontaneously produced shoots and roots was recorded. The plantlets were grown until they were about 10 cm tall and those with fully developed roots removed from culture, rinsed in water to remove media, and transplanted into a mixture of autoclaved peat moss and vermiculite (ratio of 1:1). They were then grown under humid conditions in a glasshouse, for acclimatization, for two weeks. Surviving plantlets were then transplanted into potted soil in the glasshouse where they were grown with regular watering until maturity.

2.6. Assessment of Transformability of the Maize Genotypes

To determine whether the maize genotypes under study are transformable, binary vector pTF 102 containing the Gus gene was used [15]. The binary vector, harbored in Agrobacterium tumefaciens, EHA 101, contains a Gus reporter gene, driven by 3SS promoter and terminator. It also contains the Bar gene that confers resistance to the herbicide Basta. The bacterial culture was maintained on solid Yeast extract peptone (YEP) medium at 28°C supplemented with 50 mgL$^{-1}$ kanamycin sulfate and 100 mgL$^{-1}$ spectinomycin. Agrobacterium cultures on solid YEP media were grown at 28°C for 2 days prior to transformation experiments. Liquid infection media (MS, LS, N6 and N6*) were used for pre-culture, washing and Agrobacterium infection steps of embryos according to Frame et al. [15]. For transformation, 740 embryos of each individual maize variety prepared as earlier described were infected with A. tumefaciens harboring the binary vector and cocultivated on MS media according to Frame et al. [15]. Induced calli were transferred onto selection media supplemented with 1.5 mg/L Basta and later subjected to histochemical Gus analysis as described by Jefferson et al. [12]. Staining of the calli following Gus action was confirmed by microscopic observation of the stained calli and recorded as Gus transformants using Leica MZ10F/CLS150LED microscope (Leica application suite V4).

3. Statistical Analysis

Statistical analysis for determination of frequencies (%) of callus induction, somatic embryogenesis, regeneration and transient Gus expression was performed using SAS version 9.2. Tukey’s HSD test at 95% confidence interval was used to determine significant differences among the parameters under study. Graphpad prism version 6.0 was used to generate the graphs.

4. Results

4.1. In Vitro Regeneration of Namba Nane, KSTP’94 and CML144

In this study, immature zygotic embryos (Fig. 1A) of CML144, KSTP’94 and Namba Nane, cultured on callus induction media showed primary callus formation by swelling of scutellum within 10 days of culture (Fig. 1B). Miniature globular somatic embryos were then observed at the surface of the friable callus in all genotypes by the 16th day of incubation on callus induction media. Transfer of these calli to maturation media resulted into two types of calli; non-embryogenic tissues that were soft, organized and slow-growing and embryogenic ones that were compact, friable and fast growing (Fig. 1C). Upon transfer of the coleoptilar-like embryogenic calli to hormone free regeneration media, juvenile shoots and roots emerged spontaneously after two weeks of culture (Fig. 1D). Well-rooted plantlets formed within two weeks of subculture on fresh regeneration media. They were transferred to vermiculite and peat moss mixture and hardened for one week (Fig. 1E). These plantlets were later transplanted in potted soil in the glasshouse (Fig. 1F), and allowed to develop to maturity. Some somaclonal variation was observed as evidenced by some of the harvested cobs (Fig. 1H).

Frequencies of callus induction in the three maize genotypes cultured on media supplemented with varying concentrations of 2, 4-D are shown in Figure 2. Generally, the frequency ranged from 45.6±2.1 to 85.6±2.0%. In Namba Nane, the highest callus induction frequency (83.9 ± 3.8%) was recorded on N6* supplemented with 2.5 mg/L 2, 4-D while the lowest (45.6±2.0%) was recorded on MS at 0.5 mg/L 2, 4-D (Fig. 2b). On the other hand, CML144 cultured on LS media recorded the highest callus induction frequency (85.6
± 2.1%) at 2 mg/L 2, 4-D while the lowest frequency (48.9±2.2%) was recorded on MS at 0.5 mg/L 2, 4-D (Fig. 2c). There were no significant differences (P≤0.05) in the 3 genotypes across all the media regimes used in this study (Fig. 2).

The frequencies of somatic embryo formation in the 3 maize genotypes using 2 media regimes supplemented with 2, 4-D are further shown in Figure 3. In Namba Nane, the highest frequency of embryogenic callus formation (54.7 ± 1.4%) was recorded on N6 media supplemented with 1.5 mg/L 2, 4-D while a significantly lower frequency (16.4±2.5%) was recorded on MS with 0.5 mg/L of the same auxin (Fig. 3a). In KSTP’94, the highest frequency of embryogenic callus formation (59.6 ± 1.0%) was recorded on N6 media enriched with 1.5 mg/L 2, 4-D while the lowest frequency (17.8±1.5%) was recorded on MS at 0.5 mg/L of the auxin (Fig. 3b). The highest frequency of embryogenic callus formation in CML 144 (59.7 ± 1.6%) was recorded on N6 at 1.5 mg/L concentration of 2, 4-D while the least (21.1±2.5%) was recorded on MS at 0.5 mg/L of 2.4-D (Fig. 3c). In general, there were significant differences (at P≤0.05) between 2, 4-D concentrations used however, there were no significant differences between the two media types used to induce embryogenic callus formation (Fig. 3).

An outline of shoot induction frequencies of the three genotypes cultured on MS and N6 media is presented in Figure 4. Overall, N6 medium induced higher numbers of plantlets than MS in all the 3 genotypes although these were not significantly different (P≤0.05). ‘Namba Nane’ recorded the lowest in vitro regeneration frequency. 28.2±4.8% of the embryogenic calli of ‘Namba nane’ on N6 resulted in shoot formation while a frequency of 24.5±4.2% was recorded on MS (Fig. 4a). In KSTP’94, 35.0±4.0% of embryogenic calli formed shoots on N6 medium while 29.6±6.4% was recorded when MS medium was used (Fig. 4b). Slightly similar results were observed for CML144 with an in vitro plant regeneration frequency of 36.7±5.9% and 34.3±4.3% recorded for N6 and MS respectively (Fig. 4c).
Fig. 2. Frequency of callus induction in three maize genotypes (a) Namba nane, (b) KSTP'94 and (c) CML144 using four media regimes. Vertical bars indicate standard errors of the means while letters represent mean separations at $P \leq 0.05$. Asterisks (*) indicate mean separations among the media regimes at $P \leq 0.05$.

Fig. 3. Frequency of embryogenic callus in the 3 maize genotypes (a) Namba nane, (b) KSTP'94 and (c) CML144 using two media regimes. Vertical bars indicate standard errors of the means while letters represent mean separations at $P \leq 0.05$. Asterisks (*) indicate mean separations between the media regimes at $P \leq 0.05$. 

4.2. Transient Transformation Analysis

In a separate experiment, immature embryos of CML144, KSTP’94 and ‘Namba Nane’ maize genotypes were co-cultivated with Agrobacterium tumefaciens harboring binary vector pTF102 with a GUS reporter gene according to Frame et al., (2002) [14]. Selection of putative transformed tissues was achieved using Basta (Fig. 5A) with the compact calli surviving herbicide action used for histochemical GUS assay to assess the expression of the uidA gene according to Jefferson et al., [12]. Untransformed callus used as a negative control showed no blue coloration characteristic of GUS expression (Fig. 5B). Putative transformed calli of KSTP’94 (Fig. 5C), Namba Nane (Fig. 5D) and CML144 (Fig. 5E) stained blue following histochemical assay. The frequency of transformation of maize (expressed as the percentage of transformed callus tissues surviving selection) significantly varied across the 3 maize genotypes. Particularly, CML144 recorded the highest frequency (18.2±0.9%) followed by KSTP’94 (14.15±1.3%) and Namba Nane (13.46±1.5%) and these were significantly different at P≤0.05 (Table 1). Based on expression of GUS (blue colouration) in putatively transformed tissues, CML144 once again recorded the highest frequency of transformation (2.14±0.2%). Namba Nane and KSTP’94 had significantly lower frequencies with 1.35±1.5% and 0.8±0.2% respectively (Table 1).

5. Discussion

The findings of this study indicate that open pollinated maize varieties (Namba Nane and KSTP’94) are regenerable and amenable to Agrobacterium-mediated transformation. Several studies have reported in vitro regeneration of maize genotypes important in agriculture that aimed at creating platforms for the genetic improvement of the maize germplasm [14, 16]. In the present study, CML144 significantly differed from KSTP’94 and Namba Nane in

Table 1. Transformation and transient GUS frequency (%) in maize.

<table>
<thead>
<tr>
<th>Maize Genotype</th>
<th>No. of embryos infected</th>
<th>Bar-resistant calli</th>
<th>GUS positive calli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Namba Nane</td>
<td>727</td>
<td>13.46±1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KSTP 94</td>
<td>730</td>
<td>14.15±1.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.37±0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CML 144</td>
<td>739</td>
<td>18.18±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.14±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means and their standard errors. Means in each column followed by the same letter are not significantly different according to Turkey’s test (P≤0.05).
terms of callus induction and maturation frequencies. Maize has been reported to be recalcitrant to in vitro regeneration [17] and it has been demonstrated that in vitro regeneration of maize is genotype-dependent, therefore, requiring optimization of protocols for efficient regeneration of each variety [17, 18, 19]. In the current study, this was clearly demonstrated by significant differences observed across the maize genotypes.

In vitro regeneration of maize has also been shown to be dependent on various factors such as the type of media, plant growth regulators (PGRs) and polyamines (e.g. L-proline) as well culture conditions. These parameters have been shown to significantly influence the in vitro regeneration process of maize [20]. Among the four types of media used, N6* medium not only recorded the highest primary callus induction frequency but also had a high frequency of the embryogenic calli formed. Addition of L-proline and silver nitrate (AgNO₃) to the nutrient medium has been found to increase the formation of Type II callus, ideal for transformation [6, 10]. Immature zygotic embryos used as explants in this study are vital to any cereal regeneration exercise since numerous competent cells for somatic embryogenesis with high potential of producing fertile plantlets are present at this stage compared to any other explant material [18]. The immature embryos were cultured with their axis in contact with the medium and the scutellum exposed. This placement technique together with the high sucrose levels aided high callus induction frequencies with limited direct precocious embryo germination previously reported [21].

Plant growth regulators such as 2, 4-D and Dicamba play a critical role in maize callus cultures, and their effect on in vitro regeneration of maize is well documented [19, 22]. Particularly, earlier studies revealed that the use of 2, 4-D to induce callus from immature maize embryos is a critical factor [23]. Other plant growth regulators such as kinetin and Abscisic acid have on contrary been reported to inhibit regeneration in maize callus cultures [24]. This study established that 1.5 mgL⁻¹ 2, 4-D was the best concentration level for induction of primary and embryogenic calli in the 3 maize genotypes. Higher concentrations beyond this level had negative effect on induction of embryogenic calli. Bronsema et al. [24] reported that at least 0.2 mgL⁻¹ of 2, 4-D is needed to cross the threshold level for the transition from germination to callus induction while a concentration range of 0.994 mg L⁻¹ to 3.006 mg L⁻¹ (4.5-13.6 řM) has been shown to be essential for the formation of embryogenic callus in cereals [11]. The mature maize kernels from in vitro regenerated maize that grew in the glasshouse showed somaclonal variation that was possibly due to the tissue culture conditions and the use of 2, 4-D. Similar findings have been observed in maize and flowering stage of groundnuts during regeneration [19, 25].

To evaluate transformation potential of the three maize genotypes, immature embryos were co-cultivated with Agrobacterium tumefaciens harboring the β-glucuronidase gene. This reporter system has widely been used for evaluating transformability potential of plants [12]. Expression of the GUS gene as detected by the colour-staining assay is a reliable indicator of transformability of the maize genotypes under this study. The GUS reporter system can only be expressed efficiently in plant cells but not in Agrobacterium [12]. Results of transformation efficiency of the tested maize genotypes provide a statistical estimate of the potential of introducing useful genes into these maize genotypes.

6. Conclusion

Derivative medium N6 (N6* medium) was the best medium for in vitro regeneration of the three maize genotypes. The best 2, 4-D concentration level for indirect organogenesis in the selected open pollinated tropical maize genotypes was 1.5 mgL⁻¹. Further, assessment of transformation of these genotypes revealed that they are amenable to transformation therefore setting up a platform for their potential improvement with novel genes. This developed regeneration and transformation protocol when optimized will provide a critical foundation for future genetic improvement of these genotypes.

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References


