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Agrobacterium Mediated Transformation of Selected Maize Inbred Lines with pPZP200 towards Enhancement of Lysine and Methionine Content

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Abstract
Maize (*Zea mays* (L.) is one of the most important cereals used both for human and animal consumption in the world. Despite its importance, maize is not a suitable single source of nutrition because it does not provide the essential amino acids lysine and methionine in sufficient quantities to meet the nutritional needs of humans and other animals. Lysine is a necessary building block for protein in the body while methionine is the body's primary source of sulphur. Strategies to improve the nutritional quality of maize for high lysine and methionine have involved both genetic engineering (GE) and non-genetic engineering approaches such as marker assisted selection. Breeding is however laborious, lengthy and carries along undesired alleles. The objective of this work was to manipulate maize inbred lines towards enhancement of lysine and methionine content in the endosperms through *Agrobacterium* mediated transformation. Maize kernels mainly store proteins as α, β, γ and δ zeins. The immature embryos of three tropical maize inbred lines (TL18, CML216 and CML144) and a temperate line (A188) were transformed using *Agrobacterium tumefaciens* strain EHA101 carrying an expression cassette designed to up-regulate the Z10 protein for methionine enhancement as well as down-regulate the α zein storage protein by RNAi. The T-DNA also contained P-zp22/6 as the promoter and the phosphinothricin acetyltransfarase gene (bar) used for selection of transformed tissue. Putative transformants were tested for presence of the transgene by PCR designed to amplify the P-zp22/6 promoter sequence. Calli survival frequencies were calculated as a percentage number of surviving calli in relation to the total number of embryos infected. These ranged from 2.89 % for TL18 to 9.11 % for A188. This data did not detect any significant difference ($p>0.05$) among the genotypes on the percentage of calli which survived. Transformation efficiency was calculated as a percentage of the number of PCR positive plants divided by the total number of embryos infected. This ranged from 0% for TL18 to 1.83% for A188. The data suggest the possibility of manipulating storage proteins and regenerating normal transgenic maize with normal kernels. Further work should involve gene expression assays for accumulation of β, γ and δ prolamins in the kernels and southern blot analysis to confirm stable integration and the copy numbers of P-zp22/6 gene in the PCR positive plants.
Keywords: RNAi, Z10, Lysine, Methionine, pPZP200

1.Introduction

Maize or corn (*Zea mays* L.) is an important crop in the world. It is a warm weather crop and is not grown in areas where the mean daily temperature is less than 19 ºC or where the mean of the summer months is less than 23 ºC (Jean, 2003). The genus *Zea* consists of four species of which *Zea mays* L. is economically important. The other *Zea* species, referred to as teosintes, are largely wild grasses native to Mexico and Central America (Doebley, 1990).

It is a versatile crop grown over a range of agro climatic zones. It is grown from 58^{o}N to 40^{o}S, from below sea level to altitudes higher than 3000 m, and in areas with 250 mm to more than 5000 mm of rainfall per year and with a growing cycle ranging from 3 to 13 months (Shaw, 1988; Dowswell *et. al.,* 1996).

Maize is the world's third most important cereal crop after rice and wheat. It is is high yielding, easy to process, readily digested and costs less than other cereals (IITA, 2002). It occupies an important position in world economy and trade as food, feed and an industrial grain crop. It is an important source of carbohydrate. It is one of the most important cereals both for human and animal consumption and is grown for grain and forage (Doorenbos and Kassam, 1988). The importance of maize has preferentially edged it over other food staples, particularly sorghum and millet in sub Saharan Africa (Smith *et al*., 1994). As human feed it can be used in many forms such as porridge, popcorn, baked bread and can be cooked or roasted for consumption in the unripe state, when the kernels are fully grown but still soft. The cooked unripe kernels may also be consumed as vegetables, salads and garnishes. Maize is also a major source of cash for smallholder farmers (Smith *et al*., 1997).

While maize is a major source of food and feed worldwide, it is not a suitable single source of nutrition. Maize alone does not provide the essential amino acids, lysine and methionine, in sufficient quantities for the nutritional needs of humans and farm animals that feed on these seeds. The major storage proteins of maize called prolamins are stored in the endosperm of a maize seed. The prolamins of maize also called zeins are

comprised of α, β, γ and δ-zeins (Gibbon and Larkins 2005). Alpha (α) zeins which are deficient in lysine and methionine constitute more than 70% of the total proteins in the prolamins. The others (β, γ, δ-zeins) are minor groups which are rich in lysine and methionine. This results in nutritional deficiencies in these amino acids when the whole grain is fed to monogastric livestock such as pigs and poultry (Shewry and Niger, 2002).

Efforts to increase lysine levels in maize using conventional breeding have been pursued for many years. In 1964 the opaque2 mutants in maize were found to produce elevated levels of lysine. The high lysine content of opaque*2* is thought to be caused by the reduction of lysine-poor α zeins and a pleiotropic increase in the lysine-rich storage proteins (Damerval and Devienne, 1993; Habben *et al*., 1993; Lopez-Valenzuela *et al*., 2004). However, conventional methods are costly, time consuming, difficult and require long-term investments, sustained research efforts and patience. In addition they require continuous administrative, financial and scientific support.

There is therefore need to complement conventional breeding with other techniques such as genetic engineering to address the constraints encountered to increase lysine and methionine levels in the maize kernels.

1.2 Lysine

Lysine is the most limited amino acid in cereal seeds. It is synthesized in plants through the aspartate metabolic pathway, which also leads to methionine and threonine production (Galili, 1995). It is not produced by the human body. Lysine is the essential amino acid found in the smallest quantity in all cereal grains but is plentiful in all legumes (Shotwell and Larkins, 1989). Without enough lysine or any other of the eight essential amino acids the body cannot build protein to sustain muscle tissue functioning. It promotes the body's absorption of calcium, slows the damage to the eye caused by diabetes and it may help cure atherosclerosis. It is a necessary building block for all protein in the body. It plays a major role in calcium absorption, tissue repair and growth, building muscle protein; recovering from surgery or sports injuries and the body's production of hormones, enzymes and antibodies (Keli, 2008). Its proportion in the proteins of some important food crop such as maize is so low that populations dependent on these grains as the sole source of dietary protein suffer from lysine deficiency, affecting growth in children and adults (Keli, 2008).

1.3 Methionine

Methionine is an essential amino acid required in the diet of non-ruminant animals mostly human and monogastric mammals. It is an essential sulphur-containing amino acid that can be obtained only through food. It is the body's primary source of sulphur. The body uses sulphur to influence hair follicles and promote healthy hair, skin and nail growth. Sulphur increases the production of lecithin in the liver, which reduces cholesterol, protects the kidneys and reduces bladder irritation by regulating the formation of ammonia in the urine (Bentley, 2005).

Methionine is used to treat acetaminophen poisoning to prevent liver damage (Ellis, 2008). It also assists in the breakdown of fats and thus prevents the buildup of fat in the arteries. It also assists with the digestive system and removes heavy metals from the body since this sulphur containing amino acid can be converted to cysteine (Yan *et al*., 2001) which is very important nutrient in detoxifying the liver. Methionine is also a great antioxidant because the sulphur it supplies to the body inactivates free radicals. It is also one of the three amino acids that is needed by the body to manufacture a compound called monohydrate, which is very essential for energy production and muscle building (Bentley, 2005).

1.4 Genetic transformation to manipulate seed proteins

The protein composition of several cereals has been altered by the introduction of seed storage protein genes from other species. The entire heterologous gene cluster of a class of sorghum prolamins the α-kafirins, has been incorporated into maize using particle bombardment (Song et al., 2004).

Efforts to improve the amino acid balance in non-cereal crops have also used cereal-derived storage protein genes. The maize γ-zein gene has been expressed in soybean to significantly increase the cysteine and methionine content of the seed protein (Li et al., 2005). Various approaches to improve lysine content in maize have been investigated (Mazur et al., 1999; Ferreira et al., 2005). Bacterial-derived DHDPS has been used to increase seed lysine levels (Galili and Höfgen, 2002). RNA interference (RNAi) technology has been used to specifically reduce the 22-kDa α-zeins in maize leading to increased lysine content and reduced levels of leucine, alanine and glutamine (Segal et al., 2003). In maize sense and antisense silencing of 19-kDa α-zeins has been used to increase levels of lysine, tryptophan and methionine (Huang et al., 2004). Endosperm-specific expression of Cordap A combined with reduced zein accumulation increased lysine content in more than two-fold (Huang et al., 2005).

2. Materials and methods

2.1 Plant material

Four maize inbred lines (CML144, CML216, TL18 and A188) were used in this study. CML216 and CML144 are tropical lines which were obtained from International Centre for Maize and Wheat improvement, Mexico (CIMMYT). TL18 is a Kenyan line while A188 is a temperate line which was also obtained from CIMMYT. The regeneration protocol of these lines is already documented and inbred lines A188 and CML 216 are known to transform very well. Seeds were planted in pots at the Plant Transformation Laboratory garden at Kenyatta University. On maturing, plants were self pollinated.

2.2 Gene construct

The transformation construct pPZP200 was obtained from Dr. Joachin Messing of Rutgers State University. It harboured *Z10* gene coding for methionine enhancement and an RNAi cassette for silencing α zein storage proteins. The construct contained a *bar* (phosphinothricin acetyltransferase) gene encoding resistance to bialaphos. The selectable marker cassette was driven by ubiquitin promoter (Alan *et al*., 1996) and a nopaline synthase terminator sequence (T-NOS) (Sivamani and Qu, 2006).

The gene of interest was driven by α-zein P-zp22/6 promoter (Song *et al*., 2001) and terminated by cauliflower mosaic virus terminator sequences (T-35S) (Figure 1).

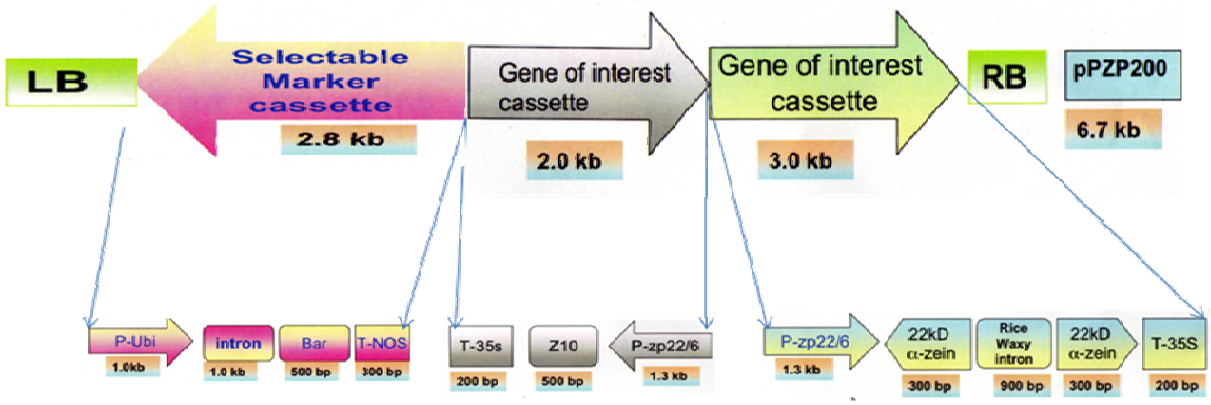

Figure 1: The T-DNA region of pPZP200 gene construct used for transformation. This contained *bar* selectable marker cassette that confers resistance to bialaphos driven by ubiquitin promoter, the Z10 gene and RNAi cassette which were driven by α-zein P-zp22/6 promoter terminated by cauliflower mosaic virus terminator sequencies (T-35S). LB - Left Border, RB - Right Border, P-Ubi - Ubiquitin promoter, Bar - Bialaphos, T-NOS - Nopaline synthase terminator, T-35S - Cauliflower mosaic virus terminator, P-zp22/6-Zein proteins promoter, 22KD- 22 kilodaltons.

2.3 Preparation of yeast extract peptone media for bacterial culture

Media for bacteria culture consisted of Yeast extract peptone (YEP) broth (5 g/l yeast extract, 10 g/l peptone and 5 g/l NaCl, pH 6.8). Bacteriological Agar (15 g/l) was added in case of solid YEP before autoclaving. Relevant antibiotics were added after autoclaving. The chemicals used were obtained from Duchefa company (Haarlem Netherlands).

2.4 Transformation of *Escherichia coli* with pPZP200 plasmid

The bacterial *Escherichia coli* strain DH5α cells were grown in liquid (YEP) media for 2 hrs. They were chilled at 4 ^{o}C for 1 hr and then centrifugation was done at 4000 rpm for 10 mins. The supernatant was discarded and the cells re-suspended in 1 ml of 0.1 M prechilled $CaCl_2$. One µl of pPZP200 plasmid DNA was added to 100 µl DH5α competent cells. The cells were thawed by placing the tubes in a water bath at 37 ^{o}C. They were then put in ice for 1 hr. One millitre of YEP media was added to the tube and incubated at 37 ^{o}C with shaking at 100 rpm for 1-2 hrs. Centrifugation was done for 30 secs at 6000 rpm. The supernatant was discarded and the cell pellets were plated on YEP plates supplemented with 100 mg/l spectinomycin.

2.5 Transformation of *Agrobacterium* strain EHA101 with pPZP200 plasmid

Agrobacterium tumefaciens strain EHA101 was grown overnight on a shaker in 10 ml of liquid YEP media supplemented with 50 mg/l kanamycin. One millitre of overnight culture was added to 100 ml of YEP supplemented with 50 mg/l kanamycin. This was incubated for 4 hrs on a shaker, then the cells were centrifuged for 10 mins at 4000 rpm. The supernatant was discarded and the harvested cells were suspended in 1 ml of 0.15 M NaCl and then centrifuged at 4000 rpm for 10 mins. The supernatant was discarded and the cells suspended in 1 ml of 0.1 M ice cold $CaCl_2$ to form competent cells. Plasmid pPZP200 DNA (2 µl) was added into 100 µl of

the competent cells and incubated at 0 °C on ice for 30 mins. They were heat shocked at 42 °C for 2 mins followed by resting on ice for 1 min. Liquid YEP (900 µl) was added and the cells incubated for 1 hr with continuous shaking at 37 °C. The cells were then plated on solid YEP medium supplemented with 100 mg/l spectinomycin and 50 mg/l kanamycin. Positive colonies that emerged were streaked onto fresh solid YEP containing plates and incubated at 28 °C in the dark.

Transformed *Agrobacterium* strain EHA101 was maintained on solid YEP medium supplemented with 100 mg/l spectinomycin and 50 mg/l kanamycin. *Agrobacterium* strains EHA101 carries a gene coding for resistance to kanamycin. Kanamycin hence selected *Agrobacterium* while spectinomycin selected the plasmid pPZP200 which was used in this study. Bacterial cultures for weekly experiments were initiated from stock plates stored for up to two weeks at 4 °C.

2.6 Media used in transformation

The media used for *Agrobacterium*-mediated transformation is shown in Table 1. Antibiotics, infection medium, bialaphos, acetosyringone (AS), kanamycin and silver nitrate were filter sterilized while all the other media were autoclaved at 121°C for 30 mins. The vitamin ingredients of LS and MS basal media as used in the media preparation are given in Table 2.

Table 1: Composition of transformation media

Type of media	Composition of media
Infection	LS* micro- and macro- salts, LS vitamins, 2 mg/l 2,4-D, 1 g/l casein hydrolysate, 68.5 g/l sucrose, 36 g/l glucose, 36.7 mg/l FeNaEDTA, 100 mM Acetosyringone, (pH 5.2)
Co-cultivation	LS macro- and micro- salts, LS vitamins, 2 mg/l 2,4-D, 0.7 g/l proline, 0.6 g MES, 20 g/l sucrose, 10 g/l glucose, 10 mg/l silver nitrate, 100 mM AS, 36.7 mg/l FeNaEDTA, 8 g/l agar, (pH 5.8)
Resting	LS macro- and micro- salts, LS vitamins, 2 mg/l 2,4-D, 0.7 g/l proline, 30 g/l sucrose, 10 mg/l silver nitrate, 250 mg/l cefotaxime, 0.6 g/l MES, 8 g/l Agar, (pH 5.8)
Selection	LS macro- and micro- salts, LS vitamins, 2 mg/l 2,4-D, 0.7 g/l proline, 30 g/l sucrose, 250 mg/l cefotaxime , 1.5 mg/l or 3 mg/l bialaphos, 0.6 g/l MES, 8 g/l Agar, (pH 5.8)
Maturation	LS macro- and micro- salts, LS vitamins, 2 mg/l 2,4-D, 0.7 g/l proline, 60 g/l sucrose, 1 mg/l NAA, 250 mg/l cefotaxime, 3 mg/l bialaphos, 0.6 g/l MES, 8 g/l agar, (pH 5.8)
Regeneration	4.43 g/l MS medium + vitamins, 30 g/l sucrose, 250 mg/l cefotaxime, 0.6 g/l MES, 8 g/l agar (pH 5.8)

*Linsmaier and Skoog (1965)

Table 2: Vitamin ingredients of LS and MS basal media as used in the maize transformation

Vitamin	LS medium (mg/l)	MS medium (mg/l)
Thiamine-HCL	0.4	0.1
Nicotinic acid	-	0.5
Pyridoxine-HCL	-	0.5
Myo-inositol	100	100

2.7 Preparation of immature zygotic embryos

Immature embryos were harvested from self pollinated plants as explained in section 3.1, after attaining 1-1.5 mm in length after pollination and either used immediately or refrigerated for 1-2 days at 4 °C while still in the husk. Dehusked ears were surface sterilized for 3 mins in 70% (v/v) alcohol. They were rinsed three times with distilled water and then soaked in 3% (v/v) commercial Jik (sodium hypochlorite) containing two drops of wetting agent (Tween 80) for 1 hr. Rinsing was done three times with sterile distilled water to remove Jik. The top half of kernels were cut off with a sterile scalpel. Immature embryos were aseptically removed with a sterile pointed spatula in a laminar flow cabinet and precultured in infection medium (Table 1) supplemented with 100 mM Acetosyringone in petri plates.

2.8 Infection of immature embryos with EHA101 harbouring pPZP200 plasmid

A loop of bacteria culture (*Agrobacterium tumefaciens* strain EHA101 harbouring pPZP200 plasmid) were scraped from YEP plates stored at 4 °C and suspended in 10 ml of liquid infection medium (IM) (Table 1) supplemented with filter sterilized AS (100 mM). The tube was fixed horizontally to a bench-top shaker and shaken at 200 rpm for 4 hrs at a temperature of 28 °C. Precultured immature zygotic embryos in liquid infection medium supplemented with AS were infected with *Agrobacterium* suspension for 5 mins.

2.9 Co-cultivation

After infection embryos were transferred onto the co-cultivation medium (CCM) (Table 1) and excess *Agrobacterium* suspension was pipetted off the surface of the medium. Embryos were oriented with the embryo-axis side in contact with the medium (scutellum side up). Plates were wrapped with aluminium foil and incubated in the dark at 20 ^{o}C for 3 days. The number of embryos in each plate was recorded.

2.10 Resting

After 3 days of co-cultivation, all embryos were transferred to resting medium (RM) (Table 1). The plates were sealed with parafilm, wrapped with aluminium foil and incubated in the dark at 28 ^{o}C for 14 days. The number of embryos forming primary calli in each plate was recorded.

2.11 Selection and maturation of transformed calli

Selection of transformed calli was done in two stages, each containing different concentrations of bialaphos. The first selection medium was LS medium containing 1.5 mg/l bialaphos (Table 1) for 14 days. The surviving embryos or calli were transferred onto selection medium containing 3 mg/l bialaphos (Table 1) for 14 days followed by the second subculture on the same fresh medium for the same number of days. All these stages were carried out in the dark at 28 ^{o}C. The number of surviving calli was recorded. Surviving embryogenic calli were transferred to maturation media (Table 1). The calli were incubated for two weeks in the dark at 28 ^{o}C.

2.12 Regeneration of putative transgenic plants and hardening

Embryogenic calli were put onto the regeneration media containing MS basal salts (Murashige and Skoog, 1962) supplemented with 3% sucrose (Table 1). Putative transformants were washed to remove agar and then transferred into pots containing autoclaved peatmoss. Each pot was covered with a clear transparent polythene bags for 3-5 days during acclimatization to prevent excessive evapotranspiration. Polythene bags were cut at the corners and opened gradually. The putative transformants were removed from peatmoss after 7 days and then transplanted into pots containing soil mixed with humus (1:1). They were maintained in a containment glass house.

2.13 Plasmid DNA extraction

A bacterial colony from a freshly streaked EHA101 plate was inoculated in a starter culture of 20 ml YEP medium supplemented with 50 mg/l Kanamycin and 100 mg/l spectinomycin. It was incubated for 12 hrs at 37 ^{o}C with vigorous shaking at 3000 rpm. The bacterial cells were harvested by centrifugation at 6000 rpm for 15 mins and the supernatant discarded. The bacterial pellets were resuspended in 300 µl of P1 buffer (Table 3) in which 100 µg/ml RNAse had been added. Three hundred µl of P2 buffer (Table 3) was added and mixing was thoroughly done by inverting the sealed tubes 4-6 times. The tubes were then incubated at room temperature for 5 mins. Three hundred µl of chilled buffer P3 (Table 3) was added and immediately mixing was done by inverting the tubes 4-6 times. The tubes were then incubated on ice for 5 mins. Centrifugation was done at 3000 rpm for 10 mins. The clear supernatant was removed promptly. Isopropanol (500 µl) was added to the supernatant and incubated at -20 ^{o}C for 20 mins. Centrifugation was done at 3000 rpm for 10 mins and the supernatant was poured. One ml of 70% ethanol was added to the pellet for washing and centrifuged at 3000 rpm for 10 mins. The pellet was dried by inverting the tubes upside down on a filter paper. The pellet was dissolved in 20 µl distilled water and stored at 4 ^{o}C.

Table 3: Plasmid DNA extraction buffers

Buffer	Composition in 1 L buffer solution
P1	6.06 g Tris base, 3.72 g NA_2EDTA, (pH 8.0), 100 µg/ml RNase
P2	8.0 g NaOH, 20% SDS
P3	294.5 g CH_3CO_2K, (pH 5.5)

2.14 Genomic DNA extraction

Genomic DNA was extracted from leaf samples of putatively transformed (after 2 weeks of transfer into pots containing soil) and untransformed (control) plants using the CTAB protocol (Allen *et al*., 2006). Plant leaves (200 mg) were harvested and put in 15 ml tubes and plunged into liquid nitrogen tanks. The leaves were then crushed into small pieces using a glass rod before two metal beads (2 mm thick) were added. The tubes were placed back into liquid nitrogen for 2 mins. They were then vortexed one at a time at full speed for 30 secs until the tissue was ground into fine powder.

Eight millilitres of a 65 ^{o}C pre-warmed extraction buffer (Table 4) was added and the tissue was mixed and incubated for 1 hr at 65 ^{o}C in a water bath with occasional mixing. The mixture was then extracted with 2/3 volume of chloroform/ isoamyl alcohol (24:1) which was mixed gently for 20 mins and centrifuged at 6000 rpm

for 30 mins at room temperature. The clear solution was collected and put into sterile 15 ml tubes. RNAse (2 µl) was added in each tube and the tubes were incubated at 37 ^{o}C for 20 mins. Ice cold isopropanol (2/3 of the volume) was added and the tubes were mixed gently by inverting several times to allow DNA to aggregate. The suspended DNA was hooked with a clean thin glass rod and immersed in 1.5 ml 70% (v/v) ethanol. The DNA pellet was then washed twice with 1 ml 70% (v/v) ethanol and air-dried. The pellets were dissolved in 100 µl of TE buffer (10 mM Tris-HCL, 1 mM sodium EDTA, pH 7.4) overnight and then stored at 4 ^{o}C.

Table 4: DNA extraction buffer

Reagents	Stock	Amount (g/l)	Final concentration	Volume used per 100 ml of the buffer
Trizma base pH 7.5 with HCL	1 M	121.2	100 mM	10 ml
NaCl	5 M	292.2	1.4 M	28 ml
EDTA pH 8.0 with NaOH	0.5 M	186.12	20 mM	4 ml
CTAB*	-	-	2%	2.0 g
PVP*	-	-	1%	1.0 g
-mercarptoethanol*	14 M	-	140 mM	1.0 ml

Trizma base, NaCl and EDTA stocks were autoclaved and stored at room temperature.
* Were added just before use.

2.15 DNA estimation

The concentration of the isolated plant genomic DNA was estimated by running it on a 0.8% (w/v) agarose gel. One microlitre of DNA was mixed with 2 µl of loading dye (New England Bio Labs., Ipswich USA) (NEB) and was loaded into wells. Five µl of 1kb ladder (NEB., Ipswich USA) was also loaded into one of the wells and electrophoresis was run at 100 volts for 30 mins and the gel was visualised using Gene Wizard (Syngene Bio, England). The level of the DNA bands against the 1kb ladder was used to determine the amount of the DNA.

2.16 Primer design and amplification parameters

Forward and reverse primers of P-zp22/6 were designed using primer 3 version 0.4.0 software (Steve and Helen, 2000). The expected amplified bands size was 222 base pairs (Appendix 1). The primers were synthesized at the International Livestock Research Institute (ILRI).

2.17 PCR amplification

Forward and reverse primers for P-zp22/6 which were used for PCR amplification in this study were 5'GGGCAATGCACCTACATACC3' and 5'TGTTGCTGCTGTAGGAATGC3' respectively. A working stock of 10 µM was prepared for both forward and reverse primers. The master mix of 25 µl in volume containing 1 µl genomic DNA was made as follows; 2.5 µl 10x Buffer, 0.5 µl of each primer (10 µM), 1.25 µl of 2.5 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.2 µl of 5 U/µl Taq polymerase and double distilled water for the PCR reaction. PCR amplification was performed in the following cycle: 94 ^{o}C for 10 mins initial denaturation, 94 ^{o}C for 30 secs, 50 ^{o}C for 30 secs, 72 ^{o}C for 1 min for 35 cycles and final elongation step at 72 ^{o}C for 5 mins. Two controls, one consisting of 25 µl of the master mix mixed with the genomic DNA from non transformed maize plant was used as a negative control while a positive control consisted of 1 µl plasmid DNA isolated from *A. tumefaciens* strain EHA101.

2.18 Agarose gel electrophoresis

PCR products 10 µl was mixed with 2 µl loading dye and loaded into wells in 0.8% agarose gel containing 5 µl ethidium bromide. Five micro litres (5 µl) of 1 kb DNA marker was also loaded as a standard into one of the wells. The electrophoresis gel tanks were buffered with 0.5X TBE. Electrophoresis was carried out at 100 volts for 30 mins. The gel was viewed using a Gene Wizard (Syngene Bio, England).

2.19 Experimental design

The transformation experiments involved four maize inbred lines, one plasmid (pPZP200) and one *Agrobacterium tumefaciens* strain EHA101. The data collected included the frequency of calli formation, calli survival, rate of regeneration and transformation efficiency. The experiments were arranged in a completely randomized design with three replicates.

2.20 Statistical analysis

The data from the experiments was used to calculate the frequency of callus initiation as the number of embryos forming callus divided by the total number of embryos infected X 100. Transformation frequency based on

callus survival was calculated as the number of surviving calli divided by the total number of embryos infected X 100. Regeneration frequency was calculated as the number of putative transformants divided by the total number of surviving calli X 100. Analysis of variance (ANOVA) with MINITAB version 23.22 statistical software was used to test significant effect on callus initiation, callus survival and regeneration frequency. Means were separated using Tukey's Honest Significant Difference (HSD) at 95% confidence interval. Transformation efficiency of the putative transformants of each maize line was calculated as the number of PCR positive plants divided by the total number of immature embryos inoculated X 100.

3. RESULTS

3.1 Plant transformation

Immature embryo between 1-1.5 mm in length (Figure 2 A) which were used as explants in all the transformation experiments initiated callus after the fourth day of culture on CIM medium occurring at the scutellum side (Figure 2 B). Most of the immature embryos which were infected from all the maize genotypes formed primary callus and the frequency of primary calli formation is shown in Table 5. The frequency of primary calli formed by immature embryos of TL18 maize lines (90.46%) was significantly different ($p<0.5$) from that formed by A188, CML216 and CML144 which were 96.43%, 95.16% and 96.47% respectively.

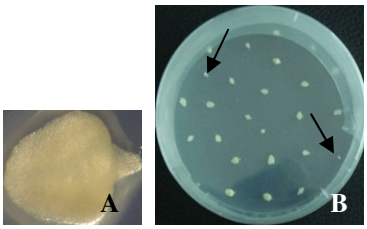

Figure 2: Immature embryo and calli formation. A, An embryo collected for infection. B, Embryos forming callus after infection. Embryos which are not forming callus (Shown by arrows).

Table 5: Calli formation on LS media

Genotype	Experiment	Cocultivation	Embryos forming calli*	Mean ± SE
A188	1	165	162(98.18)	
	2	131	125(95.41)	
	3	140	134(95.71)	
				96.43 ± 0.88b**
CML216	1	175	168(96)	
	2	65	60(92.30)	
	3	142	138(97.18)	
				95.16 ± 1.47b
CML144	1	150	146(97.33)	
	2	200	190(95)	
	3	206	200(97.08)	
				96.47 ± 0.74b
TL18	1	44	40(90.90)	
	2	60	54(90)	
	3	21	19(90.47)	
				90.46 ± 0.26a

*The number in the brackets show the percentage number of embryos forming primary calli in relation to the total number of embryos which were infected

**Means followed by different letters within the columns are significantly different from each other according to Tukey's HSD at 5% level.

Figure 3 A shows embryos in co-cultivation media immediately after infection. In all the four maize varieties, roots were observed in some of the calli during resting stage (Figure 3 B). Some of the calli started browning in selection one (Figure 3 C) and completely died in selection two while some of the calli survived (Figure 3 D).

All the four maize genotypes used in this study produced a mixture of hard and compact calli (Figure 4 A) and watery calli (Figure 4 B). Inbred line A188 produced more hard and compact calli as compared to the other lines while line TL18 produced more watery non-regenerable calli as compared to other lines.

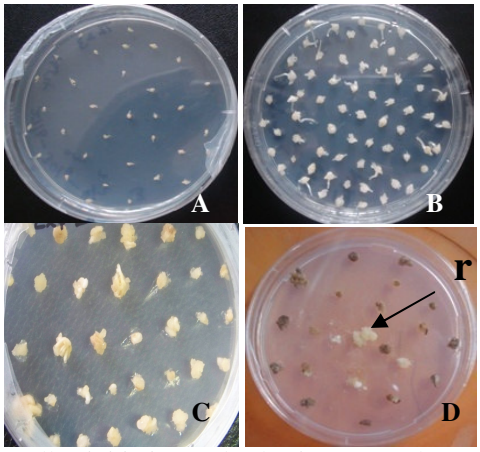

Figure 3: Callus initiation and selection. A, Embryos in co-cultivation media. B, Callus initiation from embryos in resting media. C, Callus on selection 1 medium. D, Calli after the second selection (r shows surviving calli).

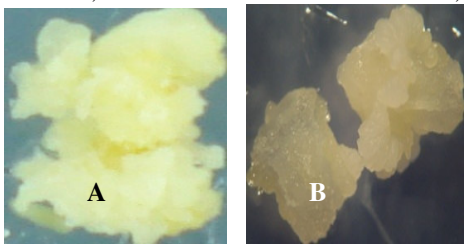

Figure 4: Type of calli formed. A, Hard and compact calli. B, Watery calli.

3.2 Callus survival after selection

Callus survival is shown in Table 6. Significant differences was not detected ($p>0.05$) among the genotypes on the percentage of calli which survived after selection. The mean numbers of calli which survived in TL18, A188, CML216 and CML144 were 2.89%, 9.11%, 5.87% and 4.57% respectively.

Table 6: Callus survival

Genotypes	Experiment	Co-cultivation	Selection 1	Selection 2	Surviving calli *	Mean±SE
TL18	1	44	43	5	1(2.27)	
	2	60	56	2	1(1.66)	
	3	21	19	3	1(4.76)	
						2.89±0.94a**
A188	1	165	161	45	21(12.72)	
	2	131	121	20	7(5.34)	
	3	140	139	19	13(9.28)	
						9.11±2.13a
CML216	1	175	170	53	8(4.57)	
	2	65	45	13	3(4.61)	
	3	142	130	21	12(8.45)	
						5.87±1.28a
CML144	1	150	147	32	11(7.33)	
	2	200	198	23	5(2.50)	
	3	206	205	25	8(3.88)	
						4.57±1.44a

*The numbers which are in the brackets indicates the percentages of the surviving calli in relation to the total embryos infected.

** Means followed with the same letters within the column are not significantly different from each other according to Tukeys Honest significance difference at 5% level.

3.3 Maturation

Figure 5 A shows embryogenic calli which survived selection on maturation media. This stage was meant to mature the calli before regenerating.

3.4 Regeneration of putative transformants

Regeneration of the three lines (A188, CML216 and CML144) from the surviving calli was not possible in the media containing bialaphos as a selective agent (Figure 5 B). When the surviving calli were transferred into the media without bialaphos several putative transformants emerged after two weeks (Figure 5 C). However there was no plant regenerated from the surviving calli of inbred line TL18.

Figure 5: Calli on maturation and regeneration media. A, Calli on maturation media. B, Regeneration of putative transformants on the regeneration medium containing 3 mg/l bialaphos. C, Regeneration on the medium without bialaphos.

Regeneration frequency is shown in Table 7. The mean percentage of putative transformants which regenerated from surviving calli in A188, CML216, CML144 and TL18 were 98.41, 12.5, 9.09 and 0 respectively.

3.5 Hardening of putative transformants and transfer into the soil in the greenhouse

Putative transformants were successfully hardened in pots containing autoclaved peat moss covered with a polythene bag (Figure 6 A and 6 B). Surviving putative transformants were later successfully transferred into the soil in pots (Figure 6 C), grew to maturity to form tassels (Figure 6 D) and seeds (Figure 6 E). There were 40, 4 and 3 putative transformants from A188, CML 216 and CML 144 respectively which grew into maturity. However plants that were regenerated from the inbred lines A188 and CML144 were the ones which produced seeds.

3.6 Somaclonal variation

Plants with abnormal phenotypes were observed in inbred line A188 and CML144. Out of the 40 plants from A188 and 3 plants from CML 144 which grew to maturity, 3 and 1 plant respectively had abnormal phenotypes. These changes included dwarf plants, twisted stems, unbranched tassel and occurrence of silk and seeds on the tassel stalk (Figure 7 B, C, E and F) as compared to the control (Figure 7 A and D).

Table 7: Regeneration of putative transgenic events from surviving calli

Genotypes	Experiment	Co-cultivation	Surviving calli	Total no. of putative transformants*	Mean ± SE
A188	1	165	21	20(95.2)	
	2	131	7	7(100)	
	3	140	13	13(100)	
					98.41 ± 1.59b**
CML216	1	175	8	1(12.5)	
	2	65	3	0(0)	
	3	142	12	3(25)	
					12.5 ± 7.22a
CML144	1	150	11	3(27.3)	
	2	200	5	0(0)	
	3	206	8	0(0)	
					9.09 ± 9.09a
TL18	1	44	1	0(0)	
	2	60	1	0(0)	
	3	21	1	0(0)	
					0a

*The numbers in the brackets shows the regeneration frequency in relation to surviving calli.

**Values followed by different letters within the columns are significantly different from each other according to Tukey HSD at 5% level.

Figure 6: Acclimatization of putative transformants of A188, tasseling and mature seeds. A, First day of acclimatization. B, Three days in peat moss. C, Putative transformant growing in the pots containing soil one week after transfer. D, Putative transformant (three months old) during tasseling. E, Mature cobs with seeds.

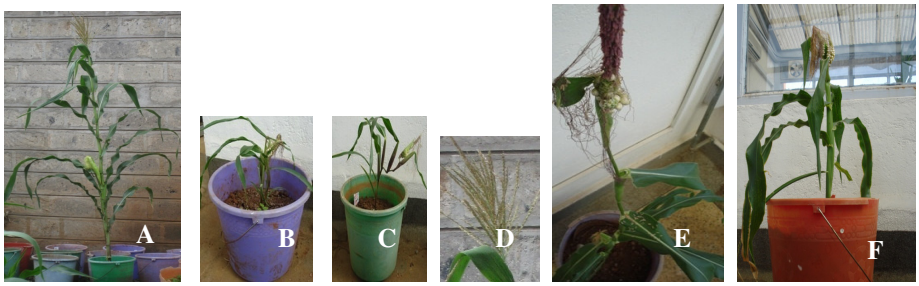

Figure 7: Somaclonal variations in T_o regenerants. A, Normal plant from A188 seed (control). B, Regenerated A188 dwarf plants. C, Regenerated A188 with twisted stems. D, Branched tassel from A188 seed (control). E, Regenerated A188 plant with unbranched tassel. F, Regenerated CML144 plant showing emergent of silk and seeds on the tassel stalk.

3.7 DNA extraction and PCR analysis

The results on gel electrophoresis (Figure 8) showed that the DNA was present in all the samples extracted and was of good quality to use for PCR analysis.

PCR amplification was done with P-zp22/6 primers, The expected band of approximately 222 base pairs was observed in lanes 2, 5, 6 and 7 (Figure 9).

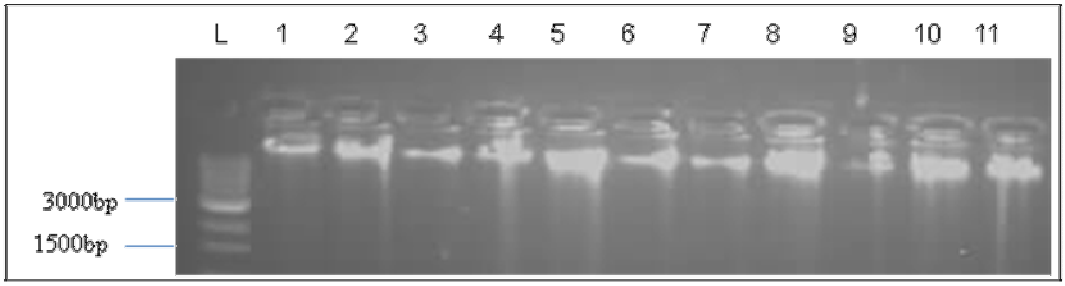

Figure 8: Gel electrophoresis of genomic DNA extracted from 10 putatively transformed plants and one non transformed plant. L, 1 kb molecular weight ladder (Biolabs inc.). Genomic DNA from putative transformants; Lane 1 & 2, CML216; lane 3, CML144; lanes 4 to 10, A188 and lane 11, non transformed plant.

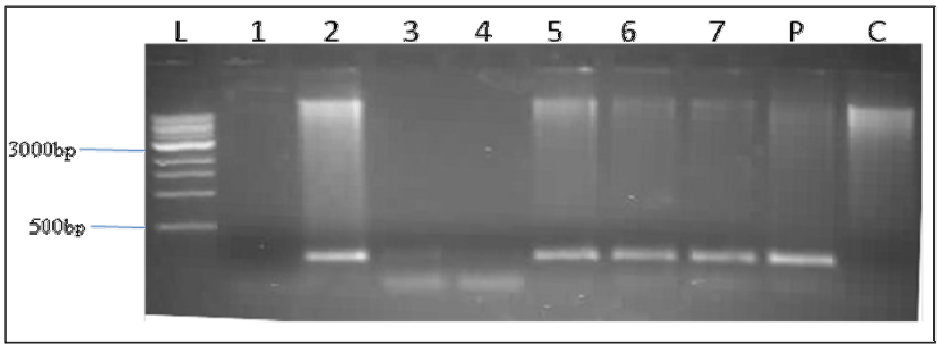

Figure 9: Gel electrophoresis showing the results of PCR amplification with P-zp22/6 primers. L, 1 kb molecular weight ladder, Lanes 2, 5, 6 and 7 gave the expected band of 222 base pairs which is absent in the negative

control, lane C. P, is the plasmid. Lane 5, inbred line CML216, lane 6, CML144 while lanes 2 and 7 A188. Lane 1, inbred line CML216 while lanes 3 and 4 inbred line A188.

3.8 Transformation efficiency

Transformation efficiency is shown in table 8. Inbred line A188 had the highest transformation efficiency of 1.83 followed by CML216, CML 144 and TL18 with transformation efficiencies of 0.26, 0.18 and 0 respectively.

Table 8: Transformation efficiency

Genotypes	Total No. of embryos infected	Total No. of putative transformants	PCR +VE	T E(%)
A188	436	40	8	1.83
CML216	382	4	1	0.26
CML144	556	3	1	0.18
TL18	126	0	0	0

4. Discussion

Most of the work in maize tissue culture and transformation involves the use of immature zygotic embryos as an explant source (Danson *et al.,* 2006). However, immature embryos are seasonally available and have strictly limited suitable duration of culture, 12-19 days after pollination (DAP) (Odour *et al.*, 2006). This imposed tedious routine tissue culture activities within the specified time frame and continuous planting for continuous supply of the immature embryos which was done in this study.

Callus initiation frequency, analysed using MINITAB at 95 % confidence interval, revealed that there was no significance difference in primary calli formation between the inbred lines A188, CML216 and CML144. However there was a significant difference between these three lines and the inbred line TL18 (Table 6). This difference may be attributed to the genotype as healthy embryos which were within the same range in size (1-1.5 mm in length) were infected in all the experiments.

There were two types of calli, hard and compact and watery calli. Hard and compact calli was the one which regenerated into shoots while watery calli did not. This was in agreement with (Ishida *et al*., 2007). In this study however, all the hard and compact calli which formed from inbred line TL18 died during selection. These calli were probably untransformed and hence could not resist the selectable agent bialaphos. Among the four inbred lines used, A188 had more hard and compact calli yield followed by CML216, CML144 and TL18 in that order. Therefore the type of callus is genotype dependent, as reported previously (Ishida *et al.,* 2007).

All the media from selection one to maturation contained bialaphos to ensure that the untransformed calli did not survive. However regeneration of shoots from regeneration media containing bialaphos was not possible, but was achieved when the calli was transferred to MS regeneration media without bialaphos. This was in agreement with the report by Lu *et al*. (2009) who observed that elimination of bialaphos from shooting media led to survival and regeneration of transgenic plants. This may be attributed to the effect of bialaphos on auxins which stimulate initiation and growth of shoots and roots.

There was no significant difference in regeneration response between the inbred lines TL18, CML216 and CML144. However the three lines had a high significant difference with the inbred line A188. This shows that regeneration is genotype dependent. Similar results have been reported by others (El–itriby *et al*., 2003, Huang and Wei, 2004).

Regenerated plants appeared fertile but in some phenotypic variations were observed. These abnormalities are known as somaclonal variations. These abnormalities are typical of plants regenerated through callus phase in tissue-culture. They have been described as either epigenetic or genetic in occurrence (Larkin and Scowcroft, 1981). Epigenetic changes cannot be passed on from one generation to another. On the other hand, genetic changes are heritable and arise as a result of changes in genetic constitution, the chromosome structure and number. The observation of phenotypic aberrations in regenerated plants has been reported in other local maize genotypes (Oduor *et al*., 2006). Despite the presence of somaclonal variations, plants formed seeds, majority of which were established from the tassels of some mature plants.

The transfer of T-DNA into the genome may be influenced by several factors. These include plant genotype, type of explant, vector-plasmids, bacteria strain, culture media composition, tissue damage, suppression and elimination of *Agrobacterium tumefaciens* infection after co-cultivation. Some other factors have found importance in the transfer of T-DNA into the crops (Komari *et al*., 1996, Klee, 2000 and Opabode, 2006). The genotypes used in this study were not found to influence the transfer of T-DNA into the genome as the frequency of callus survival (table 6) was not significantly different ($p>0.05$) between the genotypes. This may be attributed to the use of similar conditions for all the experiments.

PCR analysis revealed the presence of P-zp22/6 in the inbred lines A188, CML216 and CML144 as illustrated in Figure 9. Out of the 40, 4 and 3 regenerated plants from A188, CML216 and CML144, the plants that tested positive with PCR were 8, 1 and 1 respectively. As expected there was no band observed for the non-

transformed plant. This shows that bands observed for plants recovered from infected embryo was as a result of transfer of the gene construct into the plant tissues. Therefore there was a transfer of the gene of interest.

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