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Maize bioengineering with c-repeat binding factor 1 (*CBF1*) as a technique for drought tolerance

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Abstract. *Kuria EK, Machuka J, Runo S. 2019. Maize bioengineering with c-repeat binding factor 1 (CBF1) as a technique for drought tolerance. Trop Drylands 3: 1-10.* Africa is a drought-prone continent leading to failure in cultivation particularly to small-scale farmers who rely on rain-fed agriculture. Maize is the most widely cultivated main crop in Africa with more than 300 million people relying on it as their major staple food. Drought causes crop failure, famine and poverty and this is being aggravated by climate change. Therefore, it is necessary to develop drought tolerant maize. Since traditional propagation techniques are laborious and lengthy, bioengineering becomes a viable option. In this study, three maize inbred lines and one hybrid were altered with *CBF1* gene and appointed with mannose utilizing the Phosphomannose isomerase (*PMI)* gene. Genetic alteration was conducted through *Agrobacterium tumefaciens* and PCR was utilized to ascertain altered plants. Alteration frequency, alteration effectiveness and regeneration effectiveness were equated among the distinct genotypes altered. There were no remarkable dissimilarities in alteration frequency among the four maize genotypes. CML216 had the highest alteration effectiveness and regeneration effectiveness, followed by A188. No alleged transgenic plants were regenerated from TL27 and A188×TL18 under the circumstances implemented on account of their low regenerability. Further molecular analysis and drought stress tentatives on the expanded transgenic maize are significant prior to commercial release. Availability of drought tolerant maize would bring positive outcomes to fight famine, particularly in Africa.

Keywords: Bioengineering, corn, c-repeat binding factor 1, CBF1, desiccation toleration

INTRODUCTION

Maize is the third most important cereal in the world after wheat and rice. Based on endosperm and kernel proportion, McCann (2001) stated that there are five maize phenotypes, namely sweet, pop, floury, dent and flint. All of these phenotypes are originated from a single ancestor, i.e. teosente, that occurred in Mexico. Teosente is a wild grass native to Mexico and Central America (Doebly 2004). In general, its appearance is like maize but bears much longer lateral branches and produces only 5 to 12 kernels each secured tightly in a stony wrapper. Maize ear can bear about 500 or more naked kernels attached to the central axis of the ear called cob. The morphological dissimilarities between maize and teosente were hypothesized to have emerged from human screening during the domestication process (Doebly 2004). This hypothesis was verified to be true by Doebly (2004) who, through the utilization of quantitative trait loci (QTL) mapping, examined that five or six genes scattered throughout the genome were included in the evolution. These genes had quantitative impacts rather than distinct Mendelian impacts. It is the distinctive and epistatic impacts of these genes that altered teosente into maize.

Maize was brought into Africa by the Portuguese at the beginning of the 16th century (McCann 2001). It is important material for diet, fiber, oil, biofuel source and animal feed source, and is the main staple food for more than 300 million people in sub-Saharan Africa. Because of the combination of population growth, temperature increase due to global warming and successive decline of water table, there has been deficiency of maize supply while the demand for maize and its products is escalating. Between 2003 and 2005, the world food program spent US$ 1.5 billion to fight food deficiency due to drought and crop failure in sub-Saharan Africa alone (Edmeades 2008).

In both temperate and tropical regions, drought is the most significant abiotic stress, limiting maize cultivation. Therefore, it is necessary to develop drought-tolerant maize to fulfill the rising demand. This will increase land productivity capacity, price stability and may facilitate agricultural extensification which provides farmers with more cultivating options (Al-Abed et al. 2007). Drought is estimated to cause an annual loss in maize production of about 17% in the tropics and about 60% in regions such as southern Africa (Monneveux et al. 2006). Edmeades (2008) suggests that drought-tolerant maize will adapt to the variety in rainfall and temperatures which tend to be hotter and erratic. About 25% of crop losses caused by drought can be mitigated by genetic enhancement of drought tolerant genotypes (Edmeades 2008).

Traditional propagation practices have been greatly utilized to grow varieties accustomed to survive under abiotic and biotic stress. It is however laborious, lengthy and needs a lot of space. It is also hindered by the limited genetic variability within the genotypes (Ombori et al. 2008). Gene transition technologies are thus being utilized to complement conventional propagation practices. In

maize genetic modification, these technologies involve *Agrobacterium* mediated gene transition (Ishida et al. 1996), Polyethylene glycol (PEG) mediated alteration of protoplasts (Armstrong et al. 1990), microprojectile bombardment (Golovkin et al. 1993), and alteration via pollen tube pathway using a microsyringe (Zhang et al. 2005).

Though no drought tolerant transgenic maize has been commercialized so far, a lot has been conducted towards its establishment. Monsanto, an American-based multinational agricultural biotechnology corporation, is scheduled to start commercial sales of transgenic drought tolerant maize in the year 2012 (Edmeades 2008). The maize is expected to yield an average harvest enhancement of 15% under drought stress. Various studies on alteration of maize for drought tolerant types have been carried out. Shou et al. (2004) developed drought tolerant maize through expression of *Nicotiana* protein kinase 1 (*NPK1*), a tobacco mitogen activated protein kinase (MAPK) that activates genes securing photosynthesis process from breakage induced by drought and thus increasing harvest potential. Jeanneau et al. (2002) examined drought tolerant maize through overexpression of abscisic acid stress maturing protein (*ASRI*) and C4-phosphoenolpyruvate carboxylase (C4-PEPC), an enzyme that catalyzes the early fixation of atmospheric CO_2 even in high concentration of O_2 in relation to CO_2 due to the closing of stomata during drought. ASRI is an alleged transcription factor that has been suggested to govern transcription of genes that are regulated by sugar and abiotic stress in fruit and vegetative tissues (Shkolnik and Bar-Zvi 2008). Transgenic maize with raised expression of *Zea mays* nuclear factor Y B subunit (ZmNFYB2), a maize transcription factor analogous to *Arabidopsis thaliana* nuclear factor Y (NF-Y) that confers improved performance under drought circumstances, indicated tolerance to drought based on the reactions of stress-related parameters, such as chlorophyll content, stomatal conductance, leaf temperature, reduced wilting and maintenance of photosynthesis (Nelson et al. 2007).

Currently, a study is being carried out by a partnership called Water Effective Maize for Africa (WEMA) to develop drought tolerant maize through conventional plant propagation and genetic engineering. This project is predicted to increase maize productivity by more than 15% under moderate drought stress by the year 2017 (Edmeades 2008).

Arabidopsis CBF transcription factors interact with the drought responsive element (DRE), a *cis*-acting proponent element that governs gene expression in reaction to drought, brine and freezing stress (Al-Abed et al. 2007). In *Arabidopsis thaliana*, there are three *CBF* genes namely *CBF1, CBF2* and *CBF3* (Medina et al. 1999). These genes are also recognized as *DREB1b*, *DREB1c* and *DREB1a*, respectively. Members of this family of transcription factors hold a single APETALA2/ Ethylene responsive factor (AP2/ERF) DNA binding domain which strongly and specifically interacts with the DRE (Morran et al. 2007). Overexpression of these transcriptional factors, led by cauliflower mosaic virus 35S proponent, escalates stress toleration to freezing, drought and high brininess (Maruyama et al. 2009).

The objectives of this research was (i) To establish a gene construct comprising both *CBF1* and *PMI* genes; (ii) To alter Kenyan, tropical, temperate, and hybrid maize lines with *CBF1* gene through *Agrobacterium* mediated gene transition; (iii) To ascertain dissimilarities in alteration frequency, alteration effectiveness and regeneration effectiveness of the utilized distinct maize genotypes.

MATERIALS AND METHODS

Plant materials and explant source

One Kenyan line (TL27), one tropical line (CML 216), one temperate line (A188) and one hybrid (A188 ×TL18) were utilized for this study. The grains were cultivated in the field at the Plant Transformation Laboratory at Kenyatta University. To achieve fertilization phase, the plants were self-bred and unripe zygotic embryos excised 12-16 days after fertilization based on achievement of convenient embryo size. These unripe embryos were utilized as explants.

A188 was utilized due to its high alterability as informed by Ishida et al. (1996), Ishida et al. (2007), and Negrotto et al. (2000). It is a good administration for the alteration procedure and transgenic A188 can be crossed with other genotypes of preferable agronomic characteristics to treat drought tolerance to them. CML216 bears good combining capability for hybrid composition. It is immune to maize streak virus, *Erwinia turcicum* and *Puccinia sorgi*. It is late maturing and accustomed to tropical Africa (CIMMYT). This variety is highly regenerable as informed by Ombori et al. (2008). TL18 and TL27 are Kenyan lines accustomed to maize growing areas in the country. These genotypes were propagated at Kenya Agricultural Research Institute (KARI) and are tolerant to maize streak virus. Protocol for regeneration of these genotypes was explained by Ombori et al. (2008).

Establishment of gene construct

The plasmid pCAMBIA-CBF1 (Figure 1.A), acquired from Dr. Ron Mitler (University of Nevada), and pNOV 2819 (Figure 1.B) from Syngenta were utilized. The plasmids were altered into capable *E. coli DH5α* cells. Competent *E. coli DH5α* cells were arranged using calcium chloride technique as explained by Tu et al. (2005). For cloning, the two plasmids were refined from the *E. coli DH5α* cells using Qiagen plasmid miniprep kit (Hilden, Germany). Primers for amplification of *CBF1* gene cassette in pCAMBIA-CBF1 plasmid were developed with *SalI* and *Hin*dIII impediment sites integrated at the ends of the forward and reverse primers respectively (Table 1). *CBF1* gene cassette was then amplified using high fidelity taq DNA Polymerase (Fermentas, Austria). The PCR product was refined using Qiagen PCR refinement kit (Hilden, Germany) and absorbed sequentially with *SalI* and *Hin*dIII impediment enzymes (New England Biolabs, Ipswich, USA) to generate sticky ends. This insert was ligated to

pNOV 2819 vector which had been linearized using the same two enzymes used on the insert. pNOV 2819 carries the *PMI* gene that is significant for picking up altered plants on mannose comprising media. The resultant plasmid, named pNOV 2819-CBF1 (Figure 1.C), was altered into *Agrobacterium tumefaciens* strain *EHA101* which was used to infect the explant material. *CBF1* and *PMI* gene-specific primers (Table 1) were developed and utilized to ascertain the existence of the genes after alteration of *E. coli*, *Agrobacterium* and maize.

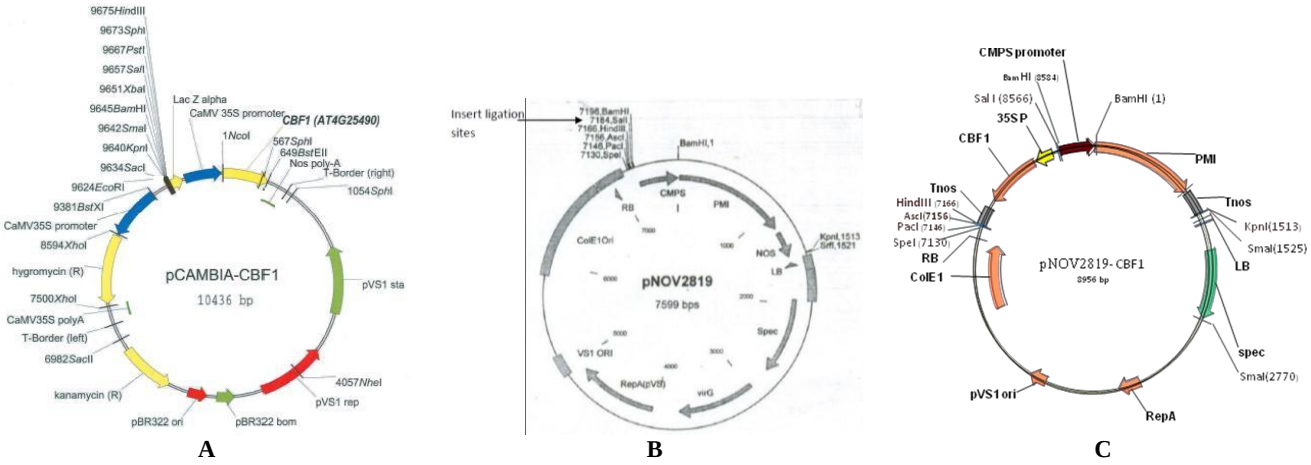
Alteration of *Agrobacterium tumefaciens*

The *Agrobacterium* strain *EHA101* was initiated from stock plates stockpiled for less than two weeks at 4°C and preserved on LB medium (Duchefa, Netherlands) enhanced with 100 mgl^{-1} kanamycin. The *Agrobacterium* became capable to accept DNA through calcium chloride technique as defined by Tu et al. (2005), with the following modifications: (i) The cells were evolved at 28° C for all processes that needed brooding for accretion. (ii) The cells were evolved for 5 h after refreshing contrary to 2 h. (iii) After reaping, the cells were first resuspended in 0.15M $NaCl_2$ and hatched on ice for 15 min before 0.1M $CaCl_2$ was appended as in alteration of *E. coli* cells. (iv) After alteration process the cells were hatched for 1.5 h on LB medium before plating on selective plates comprising 100 mgl^{-1} kanamycin, and 100 mgl^{-1} spectinomycin. (v) Positive colonies were sifted after three days contrary to the 12 h needed for *E. coli* cells.

The process of maize alteration

Between 12-16 days after fertilization, distinct maize ears with unripe embryos of adequate size were reaped. The size of embryos was ascertained while the plant was still on the field. The maize ear was opened up a little to reveal the kernels. A spatula was utilized to detach an embryo and if the size was 1-1.2 mm in length along embryo axis, the ear was reaped and used quickly for alteration (Ishida et al. 2007).

In detaching the husk, the ears were surface sterilized by immersing in 2.5% sodium hypochlorite comprising two drops of tween 20 for 20 min. The cobs were then cleansed 3 times with hygienic refined water under aseptic circumstances in a tissue culture hood. A forceps speared the end of the ear on the silk scar side of the kernel to act as a handle while isolating the embryos. The top half of kernels was detached with a hygienic scalpel while the kernels were still embedded to the cob. The embryos were then pulled out with a spatula aseptically (Negrotto et al. 2000) and put in infection media.



A **B** **C**

Figure 1. A. pCAMBIA-CBF1 with *CBF1* gene and hygromycin opposition gene for picking up in plants; B. Vector (pNOV2819) with *PMI* gene and cloning sites for insertion of *CBF1* gene; C. Construct pNOV 2819-CBF1 carrying *PMI* and *CBF1* gene cassettes between the right and the left border

Table 1. Primers for amplifying *CBF1* gene cassette, *CBF1* and *PMI*

Primer	**Sequence**
35S forward (with *salI* site)	5’ TATCACA**GTCGAC**TGAGACTT 3’
Tnos reverse (with *Hin*dIII site)	5’ GCC**AAGCTT**TCCCGATCTAGTAACATAGAT 3’
CBF1 forward	5’ ATGAACTCATTTTCAGCTTTTTCTG 3’
CBF1 reverse	5’ TTAGTAACTCCAAAGCGACACG 3’
PMI forward	5’ ACAGCCACTCTCCATTCA 3’
PMI reverse	5’ GTTTGCCATCACTTCCAG 3’

Agrobacterium cultures were evolved for three days at 28° C on LB solid medium enhanced with 100 mgl^{-1} kanamycin, and 100 mgl^{-1} spectinomycin. One full loop of bacteria culture was scraped from the plate and dispersed in 5 ml of liquid infection medium in a 50 ml falcon tube. The tube was fixed horizontally on a bench-top shaker and shaken at 200 rpm for four h at 28° C. The unripe zygotic embryos from one ear were dissected and placed in bacteria-free LS infection media enhanced with 100 µM acetosyringone in hygienic petri dish. This media was then aspirated out and 20 ml of *Agrobacterium* suspension (OD_{550}= 0.3-0.4) was appended to the embryos. This plate was shielded with aluminum foil to allow darkness for effective *Agrobacterium* infection and left for infection for 10 min.

Infected embryos were moved to the surface of co-farming medium and excess *Agrobacterium* suspension was aspirated off the medium surface. Embryos were oriented with the embryo-axis side in contact with the medium and scutellum side up as recommended by Negrotto et al. (2000). These embryos with *Agrobacterium* were stored in the dark for three days at 18° C and then moved to LS resting medium enhanced with 1.8 mgl^{-1} silver nitrates and 250 mgl^{-1} carbenicillin and kept at 28° C in the dark for 10 days to establish callus (Negrotto et al. 2000).

The generated embryogenic tissues established unripe embryos and were moved to mannose comprising LS picking up media and cultured for 4 weeks with a subculture after 2 weeks. For A188, the first two weeks of picking up were conducted on media comprising 10 gl^{-1} mannose and 5 gl^{-1} in the subsequent two weeks as proposed by Negrotto et al. (2000). For the other genotypes, picking up was conducted at 5 gl^{-1} for the entire picking up period.

Surviving calli from picking up media were moved into regeneration media enhanced with 2.5 gl^{-1} mannose and placed under light at 28° C for 2 weeks. The generated green tissues were then moved to regeneration 2 medium without accretion regulators for two weeks and then to regeneration 3 medium comprising half strength of macro and micronutrients. The regenerated plants were acclimatized to soil and evolved in the greenhouse in accordance with the protocol by Ishida et al. (2007).

Analysis of transgenic plants

Putative transgenic plants were sifted for the existence of *CBFI* and *PMI* genes through PCR analysis. Leaf genomic DNA was isolated in accordance with CTAB extraction technique as defined by Zidani et al. (2005) with various modifications as defined below: About 5 g of leaf sample was cut from the alleged transgenic plant, immediately covered with aluminum foil and quickly chilled in liquid nitrogen in cryogenic tank where they remained until DNA isolation was conducted. Leaf tissues were detached from the tank and quickly crushed with a pestle in mortar comprising 4 ml of CTAB extraction buffer (100 mM Tris-HCL, 1.4 M NaCl, 20 mM EDTA) at 65°C. The liquid mixture was moved to a 10 ml falcon tube and hatched at 65° C in a water bath for 30 min. As much as 4ml of chloroform isoamyl alcohol (24:1) was appended to the samples and inverted twice to mix. The mixture was centrifuged at 5500 rpm for 10 min after which 1 ml of the aqueous layer was moved to 2 ml Eppendorf tube carefully. To precipitate DNA, 0.7 ml of isopropanol (stored at-20° C) was appended to each sample and inverted twice to mix. The tubes were chilled at -20°C for 20 min and then centrifuged at 5500 rpm for 15 min. The supernatant was discarded, 1 ml of 70% ethanol appended to rinse the DNA pellet and centrifuged at 12000 rpm. The supernatant was discarded and the DNA pellet air was desiccated for 30 min after which 100 µl of water was appended to liquefy the pellet and the liquefied DNA was stored at 4°C.

The result of the PCR was ascertained through agarose gel electrophoresis where 5 µL of the DNA was blended with 1 µL of ×6 gel loading dye (New England Biolabs, Ipswich, USA) and 1 µL of Sybr green gel loading dye (Invitrogen, USA) before loading. Electrophoresis was conducted at 100 volts for 40 min utilizing 0.8% agarose in ×1 TAE buffer and 1kb ladder (New England Biolabs) as the standard. Gel documentation equipment was utilized to visualize and record the gel.

Data analysis

Dissimilarities in alteration frequency, alteration effectiveness and regeneration effectiveness between genotypes were analyzed using ANOVA at 95% confidence interval. Alteration frequency was enumerated as the percentage number of mannose resistant callus events per number of embryos infected. Alteration effectiveness was enumerated as the percentage number of PCR positive events per total number of alleged transgenic events acquired and tested. Regeneration effectiveness was enumerated as the percentage number of shoots established per number of calli put on regeneration media.

RESULTS AND DISCUSSION

Preparation of plasmid construct

The awaited fragment containing the *CBF1* gene cassette was amplified from the plasmid pCAMBIA-CBF1. This insert was refined, absorbed with *SalI* and *Hin*dIII impediment enzymes to generate sticky ends and utilized for ligation. This insert was 1675 bp long as ascertained through agarose gel electrophoresis as indicated in Figure 2.A. The vector pNOV 2819 was linearised using *SalI* and *Hin*dIII impediment enzymes. Linearised plasmid generated a sharp band after electrophoresis while circular plasmid generated a thick band as indicated in Figure 2.B.

Proper ligation of the *CBF1* gene cassette insert to pNOV2819 vector was obtained through PCR and impediment digestion. These results were ascertained through agarose gel electrophoresis. On double digestion with *Hin*dIII and *KpnI* impediment enzymes, the awaited fragments of 3468 bp (consisting of the *PMI* and *CBF1* gene cassettes or the T-DNA) and 5671 bp (consisting of the remaining part of pNOV-2819 plasmid) were released as indicated on the gel picture in Figure 2.C.

Induction of callus from infected maize unripe embryos

Only slight increase in size was discovered on infected unripe zygotic embryos after being on co-farming media for 3 days. However, while on resting media, callus establishment happened. The embryos swell within the first two days before proliferation of cells to establish some calli by the fourth day. Both the compact type I and friable type II calli were established from the distinct maize genotypes. It was discovered that TL27, and A188×TL18 and had more cases of poor callus establishment. A188×TL18 established more watery non-embryogenic calli while TL27 established more rhizogenic calli (Figure 3).

Selection of transformants on mannose comprising media

Selection of altered calli from unaltered ones was clearly discovered after the first two weeks of picking up media. Transformed calli expanded vigorously on picking up media while unaltered calli remained small, turned brown and eventually dying by the fourth week of picking up. Putatively altered callus (x) grew vigorously while unaltered callus (y) remained small and brown (Figure 4).

Regeneration of callus into plants

Matured somatic embryos established on calli turned green within the first two days of exposure to light and shoots were established within a week (Figure 5). This was followed by root composition on regeneration 3 media though some plants established roots while still on regeneration 2 media.

Hardening and acclimatization of alleged transgenic plants

The alleged transgenic plants were well hardened and acclimatized on autoclaved peat moss. They were first carefully removed from the media taking care not to break the roots. The roots were washed with hygienic water to detach any media attached to them, planted on peat moss and shielded with polythene bag for 3 days to prevent death from excessive evapotranspiration as indicated in Figure 6.A. These plants were then moved to soil after two weeks and evolved to maturity in the greenhouse (Figures 6.B and 7).

Molecular analysis of alleged transgenic maize

As ascertained through agarose gel electrophoresis on isolated maize genomic DNA, high concentration of good quality DNA for further molecular analysis was acquired (Figure 8). PCR analysis utilizing *PMI* gene specific primers uncovered the existence of the *PMI* gene on most of the transgenic plants (Figure 9). DNA from the alleged transgenic plants was also subjected to PCR utilizing *CBF1* gene specific primers to ascertain the existence of *CBF1* gene and most were positive.

Gel picture in Figure 10 shows *CBF1* PCR for some seven T_0 transgenic plants and an unaltered plant as a negative control. pNOV-CBF1 plasmid was utilized as a positive control.

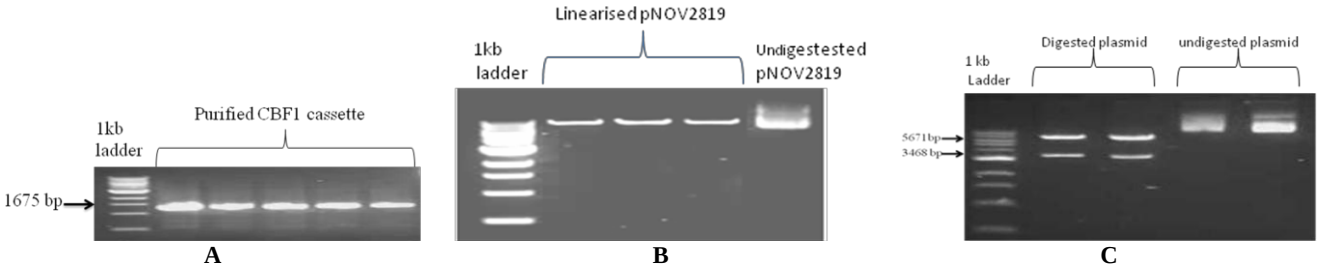


Figure 2. A. *CBF1* gene cassette amplified from pCAMBIA-CBF1 plasmid; B. linearised pNOV2819 after digestion with *SalI* and *Hin*dIII in comparison to undigested vector; C. Validation of pNOV-CBF1 plasmid through impediment digestion with *Hin*dIII and *KpnI*

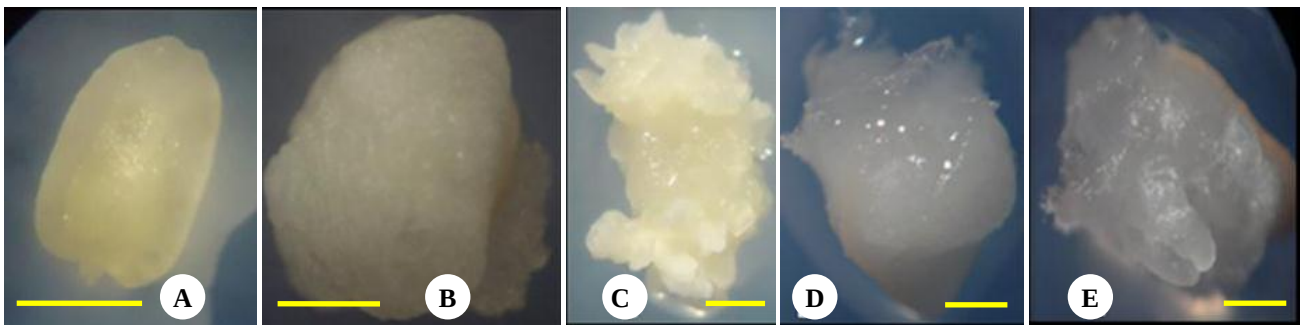


Figure 3. Callus composition from CML 216 maize embryos. Immature zygotic embryo excised from a cob (A). Type I callus (B). Type II friable callus (C). Watery non-embryogenic callus (D). Rhizogenic callus forming root-like structures (E) (bar = 1 mm)

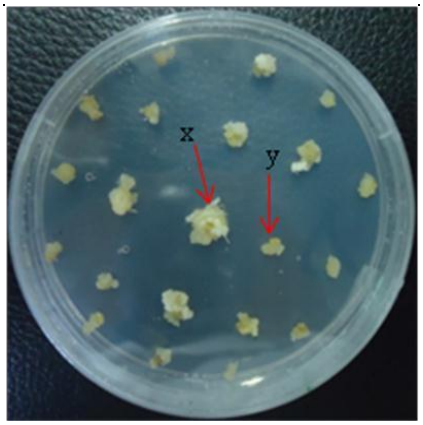


Figure 4. A188 calli on picking up media presenting putatively altered callus (x). Untransformed callus (y) after three weeks on picking up media. (bar = 1 cm)

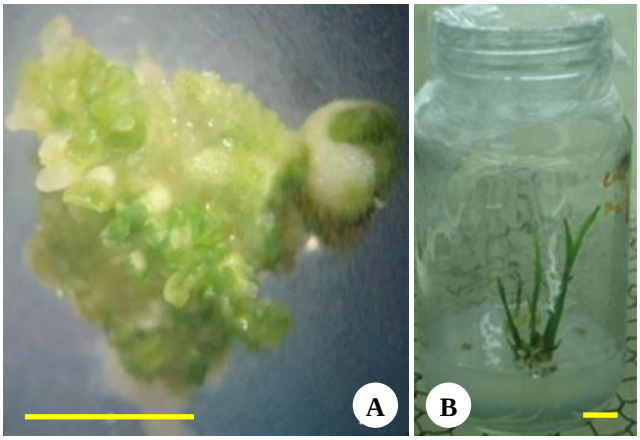


Figure 5. CML 216 Callus on regeneration media turning green and forming shoots on exposure to light (A). Established shoots and roots on regeneration media (B) (bar = 1 cm)

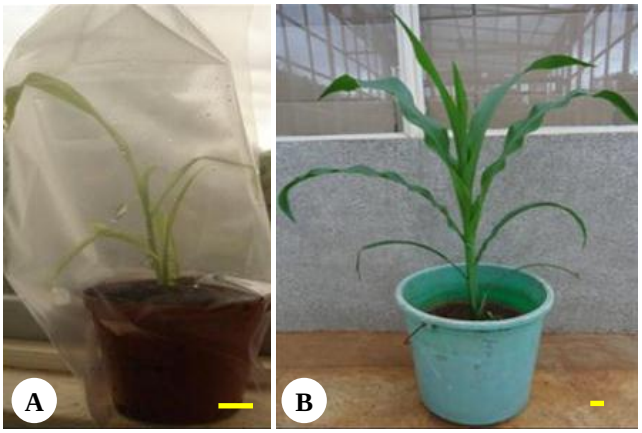


Figure 6. Hardening and acclimatization of alleged transgenic CML216 plants: Hardening a plant on peat moss (A). Hardened plant on soil in the glasshouse (B) (bar = 2 cm).

Figure 7. Mature CML 216 transgenic plant (line 3) with normal tassel and ears. (bar = 10 cm)

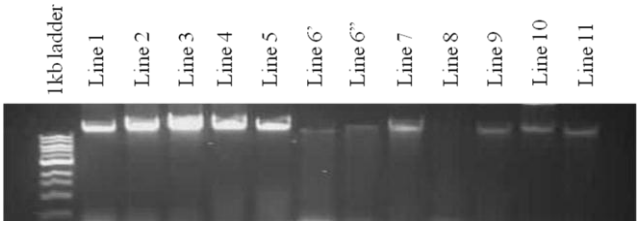


Figure 8. Gel electrophoresis of refined maize genomic DNA, presenting high concentration of good quality DNA acquired from putatively transgenic plants

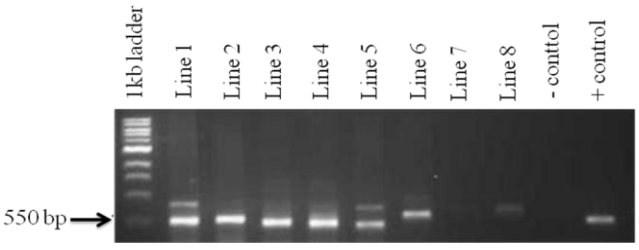


Figure 9. PCR utilizing PMI gene specific primer on maize genomic DNA; lines 1 and 2; putatively transgenic A188, lines 3-8; putatively transgenic CML 216; Negative control was unaltered maize DNA and Positive control was PNOV 2819 plasmid DNA

Exhibition of somaclonal variety by alleged transgenic plants

Though most T_0 plants indicated normal establishment, a few abnormal characteristics were discovered most probably as a result of somaclonal variety. These characteristics involved dwarf plants, outturn of silk and setting of grains at the tassel, folding/drooping downwards during flowering phase and outturn of multiple cobs on one node (Figure 11).

Alteration and regeneration

Though mannose tolerablish calli were discovered in all the genotypes, representing triumphant alteration, only A188 and CML216 regenerated to give putatively transgenic plants (Table 2). There was no remarkable dissimilarity discovered in alteration frequency among the four maize genotypes utilized in this study after one way ANOVA at 95% confidence interval of the mean.

An analysis of variance (ANOVA) uncovered remarkable dissimilarity in the regeneration effectiveness (P=0.007) at 95% confidence interval of the mean (Table 3). Tukey's HSD post-hoc test indicated remarkable dissimilarity in the regeneration effectiveness of CML216 equated to TL27 (P=0.010) and A188×TL18 (P=0.010).

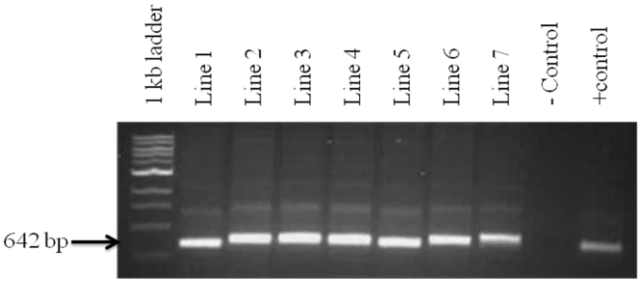


Figure 10. PCR utilizing CBF1 gene specific primers on maize genomic DNA; lines 1 and 2; alleged transgenic A188. Lines 3-7; alleged transgenic CML 216.-control was unaltered maize. +control was PNOV 2819-CBF1 plasmid

One way ANOVA indicated a remarkable dissimilarity in alteration effectiveness in the four maize genotypes at 95% confidence interval of the mean. Tukey's HSD post-hoc test indicated that CML216 and A188 had remarkable dissimilarity in alteration effectiveness at P=0.05 with TL27 and A188×TL18.

Table 2. Alteration and regeneration data

Genotype	Exp.	Number of embryos	TF (%)*	RE (%)*	TE (%)*
A188	1	280	36	0	0
	2	151	35	18	100
	3	187	69	61	90
CML216	1	175	19	91	87
	2	148	20	50	88
	3	179	39	129	87
TL27	1	50	12	0	0
	2	205	66	0	0
	3	125	8	0	0
A188×TL18	1	62	37	0	0
	2	200	31	0	0
	3	75	13	0	0

Note: TF: Alteration frequency RE: Regeneration Efficiency TE: Alteration effectiveness

Table 3. Analysis of variance for alteration frequency, regeneration effectiveness and alteration effectiveness

Para-meter	Genotypes A188	CML216	A188×TL18	TL27	P-value
TF*	46.67±11.17	26.00±6.51	27.00±7.21	28.7±18.7	0.592
RE*	26.3±18.1	90.0±22.8	0±0	0±0	0.007
TE*	63±31.8	87.33±0.33	0±0	0±0	0.009

Note: TF: Alteration frequency RE: Regeneration Efficiency TE: Alteration effectiveness

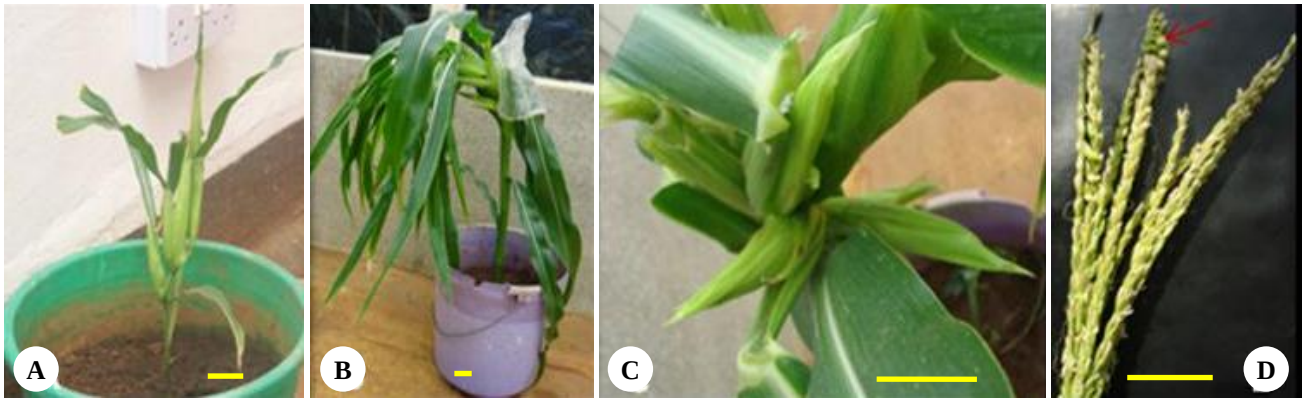


Figure 11. Somaclonal variety discovered on T_0 plants: A. Dwarf phenotype discovered in line 1. B. Drooping of shoot during the flowering phase (line 10). C. Multiple cobs generated on a single node (line 10). D. Setting of tassel grains (line 16). (bar = 5 cm)

Discussion

Maize alteration and regeneration

Transgenic maize was acquired through *Agrobacterium* mediated gene transition. Only two genotypes of maize, i.e. A188 and CML216, gave transgenic plants. No plants were regenerated from TL27 and A188×TL18. In spite of observation of altered callus from these genotypes that did well on mannose comprising picking up media, these genotypes were unable to regenerate under the utilized circumstances. This is ascribed to their poor regenerability.

Regeneration of TL27 and TL18 was informed by Ombori et al. (2008) at lower mean number of shoots per culture equated to CML216. It was predicted that a hybrid between the highly regenerable A188 as defined by Negrotto et al. (2000) and the lowly regenerable TL18 as defined by Ombori et al. (2008) would bear a higher regeneration frequency. This is based on findings of Bronsema et al. (1997) who informed that embryogenic capacity of maize unripe zygotic embryos is maternally heired. This was however not the case and probably this does not take place with the composite of the two genotypes. Failure to regenerate this hybrid and TL27 could also be on account of a genotype dependent interaction with *Agrobacterium* inherent in TL18 and TL27 alike to the hypersensitivity to *Agrobacterium* defined by Kuta and Tripathy (2005).

Determination of the right time to harvest the unripe zygotic embryos was complicated by their accretion being highly dependent on environmental circumstances and the seasonal nature of availability of quality unripe embryos. Once embryos achieved the right size, they could only be stored for a maximum of two days. In accordance with Ishida (2007), triumphant maize alteration is primarily dependent on the reaction of unripe embryos in tissue culture, the type of cells that grow from them and subsequent characteristics in accretion and regeneration. Quality of unripe embryos, unripe embryos establishment phase and circumstances utilized during alteration are also critical factors for triumphant alteration. To achieve these critical factors and to overcome the above mentioned challenges, only vigorously growing plants were utilized. Embryo size was ascertained while the cob was still attached to the plant and cultivating was conducted at regular intervals for continuous supply of embryos.

Embryogenic type I, type II and non embryogenic calli were established from the distinct maize genotypes utilized in this study. The composition of these embryo types has been informed by McCain (1988), Songstad et al. (1992), and Bronsema et al. (1997). Type I callus is compact on account of the somatic embryos being morphologically complex and organized while type II callus is friable on account of the somatic embryos being discretely organized throughout the cultures (Songstad et al. 1992). In accordance with McCain (1988), type I callus grows from fusion of aborted somatic embryos that arise directly from the scatellum base of the zygotic embryos or from continued accretion of the scatellum itself forming a callus. Inheritance of somatic embryogenesis and regeneration in maize includes two genes expressed in the abaxial region of the middle and basal side of the scutellum of unripe zygotic embryos. If these genes fail to proliferate only non embryogenic callus is established (Bronsema et al. 1997). Thus the composition of distinct types of calli in distinct maize genotypes is on account of their genetic properties.

The established non embryogenic calli involved the soft watery callus and the rhizogenic calli. The former changed into brown during culture and finally perished without establishing somatic embryos. Rhizogenic callus was generally discovered in TL27. This callus established many root-like structures and established no somatic embryos. Rhizogenic callus takes place when the callus meristem is established endogenously and its establishment is ascribed to genetic properties of distinct genotypes (Bronsema et al. (1997).

The Polymerase Chain Reaction (PCR) was effectively utilized to ascertain the alteration of *E. coli* cells, *Agrobacterium* cells and maize. Utilizing *PMI* and *CBF1* gene specific primers, the predicted fragment of 550 and 642 base pairs respectively were amplified from transgenic maize (Figure 9 and 10). Bands that were a little heavier than predicted were discovered in some events. Shou et al. (2004) ascribed this to some inefficiency in *Agrobacterium* mediated gene transition that brings about the unification of part of non T-DNA vector backbone along with the T-DNA. The level of this co-transition relies on the plant species altered, *Agrobacterium* strain and utilized alteration technique.

The terms alteration frequency and alteration effectiveness are utilized to delineate the triumph of a particular alteration tentative as a result of unification of the DNA into plants genome, propagation of altered cells and regeneration and description of alterants. Triumph of alteration is then ascertained by alteration capability of host cells, selectable marker utilized as some impede accretion of altered cells and the capability of a plant to regenerate from altered cells (Zuo et al. 2002). Alteration frequency mean of between 46% and 27% was discovered for the distinct genotypes (Table 3). For A188 the mean acquired (46%) was higher than that informed in Negrotto et al. (2000) of 30%. This dissimilarity could be ascribed to the time of infection of 10 min contrary to the 5 min utilized by Negrotto et al. (2000). Such high alteration frequency (45%) was achieved utilizing biolistics by Wright et al. (2001) when selecting maize on mannose.

A188 held a greater alteration frequency than the other genotypes, representing that it was more alterable. However, CML 216 indicated greater regeneration effectiveness than A188 in spite of its lower alteration frequency. The high alteration frequency of A188×TL18 and TL27 as equated to CML216 was not of considerable value as these genotypes had very low regeneration effectiveness and failed to give alleged transgenic plants. This agrees with Ombori et al. (2008) that the *in vitro* regeneration system is often the restricting factor in the practice of genetic alteration methods for crop enhancement of most maize genotypes.

The various abnormal phenotypes discovered in transgenic plants acquired such as dwarfism, drooping of shoot, composition of multiple ears in one node and tassel

grains (Figure 9) are primarily ascribed to somaclonal variety. This refers to genetic and phenotypic variety among clonally propagated plants of a single donor that occurs as a result of epigenetic changes (Kaeppler et al. 2000). Dwarf and plants of normal height that were positive for *CBF1* gene were acquired.

The dwarf phenotype could have been on account of somaclonal varieties as a result of tissue culture or overexpression of *CBF1* as informed by Hsieh et al. (2002) who recommended that heterologous expression of the gene affects establishment process in transgenic tomato plants. Similar impacts were informed in *Arabidopsis* by Kasuga et al. (1999) on overexpression of *CBF3* and associated this with the utilization of 35S cauliflower mosaic virus promoter which was also utilized in this study. Composition of tassel grains and dwarfism as a result of somaclonal variety was also discovered by Omer et al. (2008) during regeneration of Sudanese inbred lines and open bred varieties.

Triumph in further molecular analysis, drought stress and toxicology studies on these acquired transgenic maize genotypes would set the phase for the release of drought tolerant maize for commercialization. Drought tolerant plants would bring about raised productivity and price stability. This would bear a very considerable impact in decreasing famine and poverty particularly in sub Saharan Africa where maize is the main crop.

Conclusion

A plant alteration vector comprising drought tolerance increasing CBF 1 gene and PMI gene for picking up on mannose was established and utilized in alteration of four maize genotypes. Out of these, two genotypes were triumphantly altered and the acquired grains were utilized for further analysis of transgenic plants. There were no remarkable dissimilarities in alteration frequency among the genotypes utilized in this study. CML 216 was more regenerable than other genotypes as it indicated higher regeneration effectiveness. It was therefore found to be more appropriate for alteration under the circumstances utilized in this study. This is because the ability to regenerate is critical in plant alteration. Triumph in alteration of TL27 and A188×TL18 was restricted by their inability to regenerate under the circumstances that were utilized in this study. This is because these genotypes gave alteration frequency mean that was not significantly distinct from the others but their calli could not regenerate to give whole plants.

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