Ethiop. J. Agric. Sci. 28(2) 1-22 (2018)

Tropical Maize (*Zea mays* L.) Genotypes Respond Differently to *Agrobacterium*-mediated Genetic Transformation

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በጂኔቲክስ ምህንድስና (Genetic engineering) ዝርያዎችን የማሻሻል ሥራዎችን የበለጠ ስኬታማ ለማድረግ የትሮፕካል በቆሎ ዝርያዎች አግሮባክቲሪዬም ቱሚፋሲንስ ባክቴሪያን መሰረት ላደረገ የጂኔቲክስ ለውጥ (Agrobacterium-mediated Genetic transformation) ተስማሚነታቸውና በቲሹካልቸር የመባዛት አቅማቸው በቅድሚያ መገምገም ይኖርበታል። የዚህም ጥናት ዋና ዓላማ ከዚህ በፊት በቲሹካልቸር ለማባዛት የተገመገሙና ምቹ በመሆናቸው የተለዩትን ስድስት የትሮፕካል አፍሪካ የበቆሎ ዝርያዎችን አግሮባክቲሪዬም ቱሚፋሲንስ ባክቴሪያን መሰረት ላደረገ የጂኔቲክስ ለውጥ (Agrobacterium-mediated Genetic transformation) ተስማሚነታቸው መገምገምና ወደፊት በጂኔቲክስ ምህንድስና ዘረ-መሎችን ለመቀበል ምቹ የሆኑ ዝርያዎችን መለየት ነው። በጥናቱም ኢ. ኤች. ኤ. 101 (EHA 101) የተባለ የአግሮባክቶሪየም ቱሚፋሲንስ ባክቴሪያ ዝርያ (Agrobacterium tumefaciens strain) በተሸጋጋሪ የዲ. ኤን. ኤ. ክልል (T-DNA region) መጠኑ 4 ኪሎ ቤዝ ጥንድ (4 kilo base pair) የሚሆን P ::IPT::NOST and P ::PMI::NOST የያዘ pNOVIPT1 vector ተሸክሞ እናት በቆሎ ተክሎች ከተዳቀሉ ከ16 ቀናት በኋላ የተገኙ ፅንስ ተክሎችን (Immature zygotic embryos) ለማጥቃት ጥቅም ላይ ውሏል። በዚህ የጂኔቲክስ ምህንድስና የተለወጡ ፅንስ ተክሎችን ለመለየት የፎስፎማናስ አይሶሞራስ (Phosphomanose isomerase enzyme) አመንጪ ዘረ-መል እና ማኖስ የሚባል የስኳር ውሑድ በሚዲያ ውስጥ በመጨመር ተሞክሯል። በዚህ ጥናት የጂኔቲክስ ለውጥ (Genetic transformation) ስለመከሰቱ ከመሠረቱ ለማወቅ የማናስ ውሑድ በተጨመረበት ሚዲያ ላይ ያደጉ ተክሎች በዲ. ኤን. ኤ ደረጃ በፒ. ሲ. አር (PCR)፡ ሳዉዘርን ብሎት (Southern blot) እና አር. ቲ. ፒ. ሲ. አር (RT-PCR) በመጠቀም ትንተና ተደረጎ የተሸጋጋሪ ዘረ-መል መኖር፤ የተሸጋጋሪ ዘረ-መል ከበቆሎ ዲ.ኤን.ኤ (DNA) ጋር መወሃዱንና ራሱን መባለው ተረጋግጧል። የዚህ ጥናት ውጤት እንዳመለከተው በቲሹካልቸር ለመራባት ተስማሚ የሆኑ የትሮፕካል በቆሎ ዝርያዎች አግሮባክቲርየም ባክተርያን መሠረት በማድረግ የጂኔቲክስ ምህንድስናን (Agrobacterium-mediated Genetic transformation) ምቹነታቸው እንደየበቆሎ ዝርያዎች ባህርይ ይለያያል። በጥናቱ ከታዩት ስድስት ዝርያዎች ከዓለም ዓቀፍ የስንዴና በቆሎ ምርምር ማዕከል (CIMMYT) የተገኘ ሲ. ኤም. ኤል. 216 (CML216) እና ከኢትዮጵያ የግብርና ምርምር ኢንስቲትዩት የተገኘ መልካሳ-2 (Melkassa-2) የተባሉ ዝርያዎች ወደፊት በኢትዮጵያም ሆነ በመካከለኛውና ምስራቃዊ አፍሪካ በቆሎ አብቃይ አከባቢዎች በቆሎን የሚያጠቁ ህያውና ህያው ያልሆኑ የምርት ተግዳሮቶችን ለመቋቋም የሚረዱ ዘረ-መሎችን አግሮባክቲሪዬም ቱሚፋሲንስ ባክቴሪያን መሰረት ባደረገ የጂኔቲክስ ለውጥ (Agrobacterium-mediated Genetic transformation) በጂኔቲክስ ምህንድስና ለማሻሻል አመቺ መሆናቸው ታውቋል።

Abstract

The current study was designed to evaluate and ascertain genetic transformability of regenerable genotypes using Agrobacterium-mediated transformation method and to identify genotype(s) which can be used as better transgene recipient in future research. The super virulent Agrobacterium strain EHA 101 harbouring the binary vector pNOVIPT1 carrying the 4 Kbp T-DNA region, which included the P_{SARK}::IPT::NOST and P_{CMPS}::PMI::NOST expression cassettes, was used to infect immature zygotic embryos harvested 16 days after pollination. The phosphomannose-isomerase gene was used as a marker to select transgenic events on Linsmaier and Skoog selection medium having 5 g/l mannose as a selective agent. Molecular analyses of transgenic plants were carried out using polymerase chain reaction, Southern blot and semi-quantitative reverse transcription polymerase chain reaction which, respectively, indicated the presence, stable integration and expression of the transgene. The study indicated genotype dependent

response of tissue culture, proficient elite African tropical maize to Agrobacterium-mediated genetic transformation and possibility of enhancing the genetic basis of tropical maize through genetic engineering using Agrobacterium. Among the six maize genotypes tested, the CIMMYT inbred line CML216 and the Ethiopian open-pollinated variety Melkassa-2 produced normal and fertile transgenic plants and were identified for future use in genetic transformation aiming to overcome biotic and/or abiotic stresses of high priority in affecting maize production in the East and Central African region.

Introduction

The Portuguese brought maize (*Zea mays* L.) to Africa at the beginning of the 16th century (McCann, 2005). It has since gained tremendous importance and currently stands as one of the major crops, playing a significant role in African economy. Despite the potential yield of more than 10 t/ha, the actual average maize yield harvested by African farmers has been stagnating at less than 2.0 t/ha (FAO STAT, 2010). Several constraints, including biotic, abiotic and socio-economic factors contribute to this low yield (Bekele Shiferaw *et al*., 2011). Diseases (Ward et al., 1997), insects (De Groote, 2001; Demissew Kemal et al., 2004), weeds (Rezene Fessehaie, 1985; Parker, 1991; Kassa Yihun et al., 2007), poor soil fertility (Odendo et al., 2001), and drought are the major yield limiting factors. Drought alone is a major abiotic stress affecting productivity of maize in Africa with damages of up to complete yield losses (Edmeades et al.1994).

The forthcoming global climate change is expected to exacerbate the situation as a result of increased temperature and rainfall variability affecting crop performance (Battisti and Naylor, 2009). The prevalence and severity of diseases and insect pests are also expected to increase with changes in climate having a more pronounced effect on crop yield. It was earlier reported that crop yields are expected to fall further by 10-20% as a result of higher temperature and reduced rainfall in Africa by 2050 (Jones and Thornton, 2003). By this time the world and African population are expected to exceed 9.7 and 2.5 billion, respectively (UN population growth forecast, 2017), and food production is projected to increase by more than 70% (FAO, 2009). Considering its higher yield potential and adaptation to diverse environment maize is expected to contribute substantially to the projected food production. It has, therefore, been identified as a priority crop that deserves an investment in enhancing adaptation to this changing environment (Lobell et al., 2008; Lobell *et al*., 2011).

It is a consensus that maize varieties having improved tolerance to the various biotic and abiotic constraints must be developed and made available for commercial production. The contribution of conventional breeding towards this goal has, become insufficient because of limited genetic diversity in the maize gene pool (Hardy, 2010) for tolerance to stresses such as drought. It is true that conventional breeding has played significant role in developing improved crop

varieties, which have contributed to better food production. We, however, cannot totally rely on conventional breeding to feed the ever-increasing population, as it cannot yield faster solutions to the problems ahead. It has long been concluded that maize has to undergo genetic modification to adapt to the changing environment (Bekele Shiferaw et al., 2011). It is high time for Africa that conventional breeding has to be combined with molecular and transgenic breeding to develop improved germplasm that overcome the upcoming challenges emanating from climate change.

This calls for efficient and cost effective transformation techniques and transformation competent germplasm, which can lead to regeneration of normal and fertile transgenic counterpart in the shortest time possible. *Agrobacterium*-mediated transformation method has been advocated superior to other direct transformation methods as a tool in crop genetic engineering (Koncz et al., 1989; Komari et al., 1996; Hamilton, 1997; Lee et al., 2001). This technique has the advantage of yielding fertile transgenic plants in which foreign genes are inherited in a Mendelian manner (Rhodora and Thomas, 1996) and greater proportion of stable, low copy number of transgenic events (Ishida et al., 1996) which leads to fewer problems with transgene silencing.

The major successes in maize genetic transformation using *Agrobacterium* were obtained with temperate germplasm (Negrotto et al., 2000 and Frame et al., 2006) chiefly because of their good responses to *in vitro* regeneration. Genetic improvement in tropical maize through genetic engineering lagged behind for decades mainly because tropical maize is recalcitrant to *in vitro* regeneration and resistant to *Agrobacterium*. To alleviate this problem one has to evaluate the *in vitro* behavior of agronomically desirable elite African tropical germplasm developed through conventional breeding. This study evaluated responses of commercial and elite tropical maize genotypes to *in vitro* regeneration and *Agrobacterium*-mediated genetic transformation and identified genotypes which can be used in future genetic transformation with the aim of overcoming challenges to maize production in the East and Central African (ECA) region.

This particular work is targeting commercial and elite Ethiopian maize genotypes and CIMMYT inbred lines adapted to Ethiopian and other ECA countries` maize environment. In the first phase of this work we optimized *in vitro* regeneration of six maize genotypes which have hence shown potential for use in genetic transformation (Leta Tulu Bedada et al., 2011 and Leta Tulu Bedada et al., 2012). This study was designed to further ascertain transformability of these six tissue culture responsive genotypes using *Agrobacterium*-mediated transformation method. It was specifically intended to identify locally adapted tropical maize genotypes that combine desirable agronomic values with better *in vitro* culture response and competence for *Agrobacterium*-mediated genetic improvement. The

six maize genotypes were subjected to infection by the *Agrobacterium* strain EHA 101 harbouring the binary vector pNOVIPT1 plant expression vector carrying the P_{SARK}*::IPT::NOST* and P_{CMPS}*::PMI::NOST* expression cassettes in the T-DNA region. Putative transgenic callus events were identified on Linsmaier and Skoog (LS) selection medium having 5 g/l mannose as selective agent. Normal and fertile transgenic plants were successfully recovered in the Ethiopian open-pollinated maize variety; Melkassa-2, and CIMMYT maize inbred line, CML216. Molecular analyses of transgenic plants were carried out using polymerase chain reaction, Southern blot and semi-quantitative reverse transcription polymerase chain reaction which, respectively, indicated the presence, stable integration and expression of the transgene. The study indicated genotype dependent response of tissue culture proficient elite African tropical maize to *Agrobacterium*-mediated genetic transformation and identified maize genotypes which can be used in future genetic transformation aiming to overcome biotic and/or abiotic stresses of high priority in affecting maize production in the ECA region.

Materials and Methods

Agrobacterium strain and vector

The pNOVIPT1 binary vector, cloned as indicated in Leta Tulu Bedada et al. (2016), carrying the 4 Kbp T-DNA region, encompassing P_{SARK}*::IPT::NOST* and P_{CMPS}*::PMI::NOST* expression cassettes (Fig. 1) was inserted in the super virulent *Agrobacterium* strain EHA 101 (Hood et al., 1986) and used for infecting immature zygotic embryos collected from glasshouse grown plants 16 days after pollination.

Pre-induction of *Agrobacterium* for infecting immature zygotic embryos

In preparation for infecting immature zygotic embryos the Agrobacterium strain EHA 101 carrying the gene construct pNOVIPT1 was grown in LBA medium supplemented with 100 mg/l spectinomycin and 100 mg/l kanamycin at 28°C for 2 days in the dark. This plate was kept at 4°C as a source of inoculums for experiments up to one month after which it was regularly refreshed from long term glycerol stock kept at -80°C.

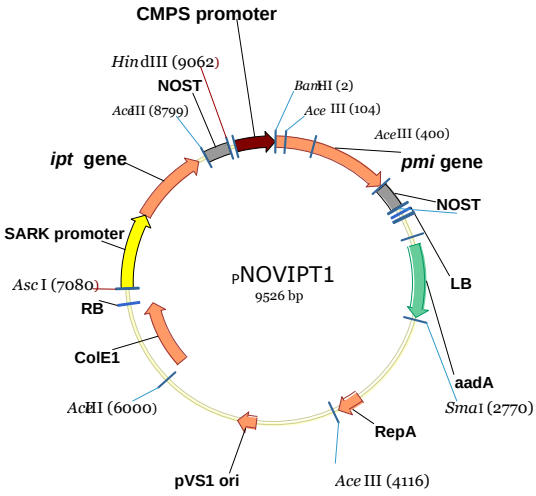


Figure 1. The pNOVIPT1 binary vector carrying the *P*$_{SARK}$*::IPT::NOST* and the *P*$_{CMPS}$*::PMI::NOST* expression cassettes in the T-DNA region. IPT is a gene of interest having an agronomic value of enhancing drought tolerance by delaying drought-induced leaf senescence; PMI is a selectable marker gene enabling transformed cells to solubilise mannose and use it as a source of carbon. **SARK promoter**: Drought inducible promoter identified from SARK (Senescence-Associated Receptor Kinase) gene in haricot bean (*Phaseolus vulgaris* L.). **NOST**: Termination sequence of the *nopaline synthase* gene, isolated from *Agrobacterium tumefaciens*. The function of this sequence is to signal the termination of the gene expression. **CMPS promoter**: A strong constitutive promoter from cestrum yellow leaf curling virus which can be used for regulating transgene expression in a wide variety of plant species. Here, it was used to drive the PMI gene, **RB**; Right border, **LB**: Left border

One full loop (3 mm) of bacteria was scooped from this fresh culture and suspended in 10 ml of infection medium supplemented with 100 µM acetosyringone in a sterile 50 ml falcon tube. The tube was sealed with parafillm and covered with aluminium foil and fixed on a shaker in a horizontal position and the culture was left to grow for 3-4 hours at 250 rpm and temperature of 28^{o}C until OD of 0.4 to 0.6 was attained at A260nm. This procedure called pre-induction step was routinely carried out before all transformation experiments.

Preparation of plant material

Immature zygotic embryos of 1.2-1.7 mm size were obtained from glasshouse grown plants of six maize genotypes (Table 1) 16 days post pollination. Pollination and determination of the right size of immature zygotic embryos and sterilization techniques were accomplished as described by Leta Tulu Bedada et al. (2016).

Table 1. Origin and important distinguishing agronomic characters of the six maize genotypes used in the study.

Genotype	Origin	Desirable traits	
		Response to drought	Nutritional quality
Melkassa-2*	Ethiopia	Tolerant	Normal
Melkassa-6Q*	Ethiopia	Tolerant	QPM[a]
[CML387/CML176]-B-B-2-3-2-B[QPM]) **	Ethiopia	Tolerant	QPM
CML442**	CIMMYT-Kenya	Tolerant	Normal
CML395**	CIMMYT-Kenya	Susceptible	Normal
CML216**	CIMMYT-Kenya	Susceptible	Normal

[a]*QPM: Quality protein maize, having a higher level of lysine and tryptophane, compared to normal maize, *Open-pollinated varieties, **Inbred lines*

Media for maize transformation

Infection, co-cultivation, resting, selection and maturation media were based on LS (Linsmaier and Skoog, 1965) salts with specific formulations modified from Negrotto et al. (2000) and contained LS modified vitamins and 1.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (Table 2). The pH of the infection medium was adjusted to 5.2 using 1N NaOH and/or 1N HCl and then filter sterilized using 0.2 µm pore size filter. It was then aliquated in to volume of 50 ml and kept at -20^{o}C until it was used. Carbenicillin (250 mg/l) was added to the resting medium for counter selecting *Agrobacterium*. Selection medium contained 5 g/l D-Mannose as a selective agent for selecting transformed cells. Regeneration medium was based on MS (Murashige and Skoog, 1962) and was hormone free. Except infection medium all media were solidified with the addition of 0.8% (w/v) agar and sterilized by autoclaving at 121^{o}C and 15 Psi after adjusting pH to 5.8. Acetosyringone, LS vitamins, silver nitrate, and carbenicillin were added to the respective media after autoclaving and cooling to 40-50 ^{o}C.

Table 2. Media used for infection, callus induction, selection and regeneration of transgenic maize plants.

Media	Composition
LS infection	LS macro-and micro-salts, 1.5 mg/l 2,4-D, 1 g/l casein hydrolysate, 68.5 g/l sucrose, 36 g/l glucose, modified LS vitamins*, 100 µM/l Acetosyringone, pH=5.2, filter sterilized
LS cocultivation	LS macro-and micro-salts, 1.5 mg/l 2,4-D, 0.7 g/l proline , 0.5 g/l MES, 30 g/l sucrose, 10 g/l glucose, 8 g/l agar, pH=5.8, autoclave, LS vitamins, 100 µM/l Acetosyringone
LS resting	LS macro-and micro-salts, 1.5 mg/l 2,4-D, 0.7 g/l proline, 0.5 g/l MES, 30 g/l sucrose, 8 g/l agar, pH=5.8, autoclave, 1.6 g/l silver nitrate, LS vitamins, 250 mg/l carbenicillin
LS selection	LS macro-and micro-salts, 1.5 mg/l 2,4-D, 0.7 g/l proline, 0.5 g/l MES, 25 g/l sucrose, 5 g/l D-mannose, 8 g/l Agar, pH=5.8, autoclave, LS vitamines, 250 mg/l carbenicillin
LS maturation	LS macro-and micro-salts, 0.5 mg/l kinetin, 0.7 g/l proline, 0.5 g/l MES, 25 g/l sucrose, 2.5 g/l D-mannose, 8 g/l Agar, pH=5.8, autoclave, LS vitamins, 250 mg/l carbenicillin
MS Regeneration	4.43 g/l MS premix, 30 g/l sucrose, 2.5 g/l D-mannose, 0.7 g/l proline, 0.5 g/l MES, 8g/l agar pH=5.8 autoclave , 250 mg/l carbenicillin

**LS Vitamins contained: 100 mg/l myo-Inositol, 0.1 mg/l Thiamine HCl; βVitamins: 0.5 mg/l nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/l thiamine HCl, 2 mg/l glycine†; †Glycine is an amino acid but it was included in vitamin preparations.*

Infection and co-cultivation

After being aseptically removed from the cob, the immature zygotic embryos were placed in 90 x 15-mm Petri plates containing infection medium. Infection was carried out by removing part of the infection medium from these Petri-plates to which pre-induced *Agrobacterium* culture was introduced and mixed by slightly swirling the plate. The plates were then covered with aluminium foil and incubated in the dark for 5 min to encourage attachment of the *Agrobacterium* cells to the immature zygotic embryos. The infected immature zygotic embryos while still in infection medium was transferred to co-cultivation medium and the entire infection medium was carefully drained off by using sterile pipette tips. All the immature zygotic embryos were then rearranged ensuring direct contact with the medium, embryo axis down and scutellum side up. The plates were sealed with parafilm and incubated at 23°C for three days in the dark. After three days of co-cultivation, the embryos were transferred to resting medium for callus induction. Each infected embryo was picked carefully and put on resting medium with 20 to 25 embryos in 90 × 15-mm plate still ensuring contact of the embryos with the medium. The culture was incubated at 27±1°C in the dark for 10 to 15 days with occasional observation on the process of callus induction.

Selection of putatively transformed events and plant regeneration

Immature zygotic embryos which did and did not produce callus were transferred to selection medium having 5 g/l D-mannose, for selection of transformed events and incubated at 27±1°C in dark for four weeks with sub-culturing onto fresh selection medium every 15 days. Healthy embryogenic type I and type II calli were transferred to maturation medium to mature the somatic embryos and incubated at 27±1°C in dark for 15 days. Type I calli was identified by their compact nature and white to creamy color, while type II were friable and light yellow in color growing faster than type I calli. After 15 days of culture in LS maturation medium, embryogenic calli was transferred to MS regeneration medium to regenerate putative transgenic maize plants. The culture was incubated at temperature of 27±1°C and 16/8 h light/dark photoperiod until plantlets have grown fully with well-developed shoots and roots. Transformation frequency was determined as the ratio of the total number of mannose resistant independent callus events that regenerated transgenic plants to the total number of immature zygotic embryos infected and expressed in percentage.

Acclimatization and glasshouse growth of putative transgenic plants

Putative transgenic maize plants with well-developed shoots and roots were transferred to small pots filled with sterile peat moss (Kekkila Co. Ltd, Tuusula, Finland) for acclimatization and hardening. Acclimatization was carried out following procedure described by Leta Tulu Bedada et al. (2012). After 7 to 10 days the plantlets were transferred to nursery pots containing sandy loam soil mixed with manure and sand at a ratio of 2:2:1, and kept in containment in the glasshouse till they grew to maturity. Individual transgenic plants were self-pollinated to give T0 seeds for further analysis.

PCR analysis of transgenic plants

Polymerase chain reaction analyses of putative transgenic plants of CML216 were carried out using forward 5'-ATAGGCGCGCCGAATTCTTCTTCCTTA-3' and reverse 5'-GCCAAGCTTTCCCGATCTAGTAACATAGAT-3' primers flanking the SARK promoter and the *NOST* region targeting the whole expression cassette of 2 Kbp size. All PCR reactions were carried out using Eppendorf Vapour Protect thermal cycler (EPPENDORF AG 22331 Hamburg, Germany). The PCR program included: initial denaturation for 10 min at 98°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min and final extension of 15 min at 72°C. Transgenic plants generated in Melkassa-2 were analyzed using *pmi* gene specific forward: 5'-ACAGCCACTCTCCATTCA-3' and reverse: 5'-GTTTGCCATCACTTCCAG-3' primers with the same PCR condition indicated in Negrotto et al. (2000) targeting a region of 550 bp size. In both cases 25 µl volumes of PCR reaction were used containing 0.5 µl of forward and reverse primers each (10 pmol µl/1), 2.5 µl PCR buffer (×10), 0.5 µl dNTPs (10 mM), 1.25 µl $MgCl_2$ (25 mM), 0.5 µl *Taq* polymerase (5 U/µl) and 1 µl template (20 ng). The final volume was brought up to 25 µl by adding 18.5 µl nuclease free sterile water. Transformation efficiency was determined as the ratio of total number of PCR positive plant events to the total number of plant events regenerated and expressed in percentage (Leta Tulu Bedada *et al*., 2016).

Southern blot analysis

Total genomic DNA was extracted from 2 to 3 g of young T1 and wild type (WT) plant leaves using the cetyltrimethylammonium bromide (CTAB) method (Allen et al., 2006). Ten micrograms of genomic DNA were digested per sample completely with HindIII restriction enzyme (New England Biolabs, UK) overnight with incubation at 37°C. Genomic DNA obtained from WT plants (CML216) was included as a negative control. For positive control, the pNOVIPT1 vector plasmid DNA was diluted to 20 ng/µl, and 1 µl was used. Digested products of each sample were loaded per lane and subjected to gel electrophoresis on 1% (w/v) agarose gel at 30 v overnight. The DNA was then transferred overnight to HybondTM-N+ membrane optimized for nucleic acid transfer (Amersham) in 20× standard sodium citrate (3 M NaCl, 0.3 M Na-citrate, pH 7.0) following the procedure described in Sambrook et al. (1989). Hybridization probes were prepared by PCR amplification of specific region of the pmi gene using forward 5'-ACAGCCACTCTCCATTCA-3' and reverse 5'-GTTTGCCATCACTTCCAG-3' primers with the same PCR condition indicated in Negrotto et al. (2000). The probes were labelled with alkaline phosphatase and used to hybridize the blots using Gene Images Alkaphose direct DNA labelling and detection kit supplied by Amersham (GE Healthcare UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

For total RNA extraction, leaf samples of about 100 mg were collected from drought stressed transgenic and WT plants and immediately frozen in liquid nitrogen. The leaf samples were then crushed into powder using mortar and pestle under liquid nitrogen. Subsequent RNA extraction steps were carried out using Qiagen RNeasy® Plant Mini Kit (Qiagen N. V. Valencia, USA) following the manufacturer's instruction. Extracted RNA was re-suspended in 50 µl of RNase free water and kept at -70°C. Complementary DNA (cDNA) was synthesized following SuperScript IIITM first-strand synthesis system for RT-PCR (Invitrogen Corp. Carlsbad CA, USA). Forward 5'-CCAACTTGCACAGGAAAGACGACG-3' and reverse 5'-TCCAGATGAAGACAGGTGCGAC-3'

primers were used to amplify 0.69 Kbp of the *ipt* gene transcripts. The PCR programme was based on initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min and final extension of 15 min at 72°C. Each PCR reaction contained 0.5 µl each of forward and reverse primers (10 pmol.µl/1 each), 2.5 µl PCR buffer (×10), 0.5 µl dNTPs (10 mM), 1.25 µl $MgCl_2$ (25 mM) and 0.5 µl *Taq* polymerase (5 U/µl) and 2 µl (10 ng/µl) of cDNA as a template. The final volume was brought up to 25 µl by adding 17.5 µl RNase free water. The PCR products were loaded in a 1% (w/v) agarose gel and electrophoresis was carried out for 1h.

Result and Discussion

Response of tropical maize genotypes to *Agrobacterium*-mediated transformation

Callus induction and survival on mannose selection

For successful transformation and regeneration of transgenic plants it is not just enough that tissues convert to calli. Formation of embryogenic calli and their further development to plantlets are equally essential. The results from the six genotypes evaluated in this study for their responses to *Agrobacterium*-mediated transformation, however, showed that the flow of these sequential events has been affected in one or more step(s) in the process. The callus induction frequency of the immature zygotic embryos, pooled across experiments, varied from 0 to 100 per cent (Table 3). This was in contrary to the previous *in vitro* regeneration studies which reported primary and embryogenic callus frequencies that varied from 87 to 100% and 67 to 97%, respectively, from the same embryo explants without infection with *Agrobacterium* (Leta Tulu Bedada et al., 2011 and Leta Tulu Bedada et al., 2012). The differences could be due to sensitivity of the genotypes to infection by *Agrobacterium* in the current study. More differences that are obvious were observed among the genotypes with respect to the survival ability of the calli on selection medium containing mannose after the first and second two-week time. Generally, most of the calli could not stand selection pressure on medium containing mannose during the first two-week time.

As a category, the three Ethiopian maize genotypes showed better average performance than the three CIMMYT maize inbred lines in terms of the callus induction capacity of their immature zygotic embryos on resting medium post co-cultivation with *Agrobacterium* and in terms of the surviving ability of the callus on selection. Within the group, the open-pollinated variety, Melkassa-2, and the inbred line, [CML387/CML176]-B-B-2-3-2-B [QPM], were superior to the second open-pollinate variety, Melkassa-6Q, having corresponding post *Agrobacterium* infection callus induction frequency of 99.6 and 95.9 per cent on resting medium.

Differences were also obvious among these genotypes in terms of number of mannose resistant calli recovered after the first and second two-week time of

selection on mannose containing medium. Melkassa-6Q suffered most from this mannose-based selection where it lost 91.3 and 99.1 per cent of the calli originally formed on resting medium during the first and second two-week selection time, respectively. With this genotype, no callus could stay healthy enough to survive on maturation medium, which showed resistance of the variety to *Agrobacterium*-mediated transformation.

On the contrary substantial number of the primary callus of Melkassa-2 and [CML387/CML176]-B-B-2-3-2-B [QPM] could tolerate selection pressure from mannose during the 2 two-weeks time interval selection period and could reach maturation stage where they were further subjected to selection on medium having a half dose of mannose. However, only Melkassa-2 could give plantlets and these were obtained from a single experiment.

Among the inbred lines of CIMMYT origin, CML442 showed the complete failure of its immature zygotic embryos to form callus from the scutellum cells in all of its nine sets of experiments. This was evidenced by brownish and the necrotic appearance of the embryos plated on resting medium, which led to complete death. Hence, for this line no immature zygotic embryos were healthy enough to yield callus viable to be advanced to selection on medium containing mannose. Similar necrotic responses were reported in maize embryogenic calli and immature zygotic embryos infected with *Agrobacterium* causing poor survival of the target plant tissues (Lupotto et al., 1999; Lupotto, 2000). This is ascribed to the plants hypersensitive reaction to *Agrobacterium* infection (Goodman and Novacky, 1994) which is one of the plants defence mechanisms and it is the sequence of events during this reaction that subsequently lead to necrosis and death of cells. In a system where mannose was used as a selective agent, Stein and Hansen (1999) reported, that mannose 6-phosphate itself induced apoptosis in non-transformed cells. The authors further discovered that a mannose 6-phosphate induced nuclease is responsible for the development of the laddering of DNA, a characteristic of apoptosis.

Table 3. Post *Agrobacterium* infection callus induction of tropical African maize immature zygotic embryos and selection and regeneration of transgenic plants using *pmi* gene and mannose as selectable marker and selective agent, respectively.

Genotype	Experiments	No. of immature zygotic embryos infected	No. of callus formed	No. of mannose resistant events recovered		No. of embryogenic callus events on LS maturation media	No. of events regenerated to plants on MS regeneration media	No. of shoots	Regeneration efficiency (%)[a]	Transformation frequency (%)[b]	Transformation efficiency (%)[c]
				2 weeks	4 weeks						
[CML387/CML176]-B-B-2-3-2-B[QPM]	7	546	426(95.9)*	105	83	32	0	0	0	0	0
Melkassa-2	7	1279	1214(99.6)	644	172	19	19	28	147.5	1.5	94.7
Melkassa-6Q	7	1559	1496(78)	130	13	0	0	0	0	0	0
CML395	3	186	186(100)	23	1	1	1	1	100	0.53	100
CML216	8	537	511(95.2)	58	50	37	9	22	244.4	2.05	100
CML442	9	894	0	0	0	0	0	0	0	0	0

**Values in parentheses indicate callus induction frequency= (No. of immature zygotic embryos forming callus/No. of immature zygotic embryos infected) x 100. [a]Regeneration efficiency= (No. of shoots regenerated on MS regeneration medium/No. of independent embryogenic callus events transferred to the medium) x 100. [b]Transformation frequency = (No. of transgenic independent events/No. of embryos infected) x 100. [c]Transformation efficiency= (No. of PCR positive events/No. of events regenerated to plants) x 100*

On the other hand, the two CIMMYT lines, CML216 and CML395 performed well with callus induction capacity of 95.2 and 100 per cent, respectively. Nevertheless only 23 calli, which accounted for 12.3% of the initially infected immature embryos of CML395 survived the first two weeks selection. With further selection for the second two-week time almost all the calli died and only one embryogenic callus was taken to maturation medium for further selection in the third two-week time. The major drawback of CML395 is that all calli surviving selection were watery and hence had no embryogenic potential which disqualified them from recovering transgenic plants. Such non-embryogenic calli were not considered surviving selection as the case of embryogenic calli in other genotypes. As a result, only one transgenic event has been recovered from this line.

CML216 appeared to be a genotype of which immature zygotic embryo derived calli tolerated selection on medium containing mannose. About 60% of the calli from this line survived the first two-week time selection on medium containing mannose and almost the same calli continued callusing further on selection medium in the second two-week time. Transformation ability of CML216 was further noted by very distinct differences among the transformed embryogenic calli and untransformed calli of which growth was significantly hampered on mannose selection ending in death. The transgenic plants were normal and fertile to set seeds. Across both categories, CML216 and Melkassa-2 were the best genotypes suitable for *Agrobacterium*-mediated transformation.

Somatic embryo maturation and regeneration of transgenic plants

Somatic embryos were observed on embryogenic calli after two weeks of selection on medium containing mannose (Table 2). Despite their previous survival on selection medium containing 5 g/l mannose, all embryogenic calli of [CML387/CML176]-B-B-2-3-2-B [QPM] and the majority of Melkassa-2 lost their embryogenic potential on MS regeneration medium in the light. Because of this phenomenon, no transgenic plants were regenerated from [CML387/CML176]-B-B-2-3-2-B [QPM]. Regeneration efficiency ranged from zero to 244.4 per cent (Table 3). Regeneration efficiency of 100 per cent registered with CML395 was misleading because it was based on only one shoot regenerated from one embryogenic callus. Melkassa-2 ranked second with regeneration efficiency of 147.5 because of 19 independent events that regenerated plants in one experiment while six experiments did not yield any plant, indicating interference of external factors in influencing the regeneration capacity of this genotype. Very low proportion of the immature zygotic embryos infected regenerated plants as indicated by low transformation frequency. The highest transformation frequency of 2.05 % was obtained for CML216. Across all the genotypes that yielded plantlets, twenty-nine independent putative transgenic plants were

regenerated. All the plantlets produced shoots and roots in the regeneration medium and it was not necessary to transfer to rooting medium (Fig. 2 D).

The study revealed genotype dependent response of the maize genotypes to *Agrobacterium* infection in terms of callus induction, survival on mannose selection and potential of regenerating transgenic plants. Such differences among maize genotypes for competence to *Agrobacterium* infection have already been noted in other studies (Neff and Binns, 1985; Gurlitz et al., 1987). Genotype-specific availability of *vir* gene-inducing substances, endogenous hormone levels at the time of immature zygotic embryo excision, embryo size and the availability of receptors for a productive attachment of *Agrobacterium* to the surface of meristematic cells are some of the factors contributing to these differences. Plants produce different inducer molecules that vary in inducing ability and cellular concentration leading to differences in the level of *vir* gene expression in different hosts, thereby affecting their sensitivity to infection by *Agrobacterium* (Karami, 2008). Of the six genotypes tested only two, Melkassa-2 and CML216, performed better yielding transgenic plants with regeneration efficiency of 147.5 and 244.4 %, respectively, on MS regeneration medium indicating regeneration of reasonably better proportion of mannose resistant embryogenic calli to plants even after infection with *Agrobacterium*. These figures are much better than regeneration efficiency of 0.6 shoots/callus reported by Mgutu, (2011) for the tropical inbred line CML216 using the same selection system and regeneration efficiency of 0.045 to 0.06 shoots/callus reported from the same inbred line using the phosphinothricine acetayltransferase (*bar)* gene as a selectable marker reported by Ombori et al. (2008). Regeneration efficiency of tropical maize has been reported several times as a problem in transformation experiments when compared to its temperate counterpart (Anami et al., 2010).

Although 32% of the mannose resistant embryogenic calli of [CML387/CML176]-B-B-2-3-2-B [QPM] were successfully promoted to plant regeneration step in the light, no transgenic plant was regenerated from this inbred line. These calli turned green, but somatic embryos failed to germinate. Mgutu (2011) also reported similar result for the tropical inbred TL 23. Wright et al. (2001) also reported mannose resistant embryogenic calli failing to produce plants in temperate maize with the same mannose/sucrose combination even though the tissues turned green in a situation where particle bombardment transformation technique was used. Performance of CML442, CML395 and Melkassa-6Q was affected much at callus induction and selection on mannose indicating that these genotypes were not *Agrobacterium* friendly despite their shining responses to *in vitro* regeneration using immature zygotic embryos. No transgenic plant was regenerated from CML442, Melkassa-6Q and [CML387/CML176]-B-B-2-3-2-B[QPM] indicating regenerability does not always guarantee transformability in tropical maize at least using *Agrobacterium*-mediated transformation. Direct

transformation methods such as particle bombardment could be the right options to transform these genotypes targeting cell suspensions that can be established from their friable embryogenic calli.

CML216 and Melkassa-2 yielded transgenic plants at a transformation frequency of 2.01 and 1.5%, respectively. This is very low when compared to transformation frequencies of temperate maize. Wright et al., (2001) reported average transformation frequency of 45% for recovery of transgenic maize via bombardment using the *pmi* gene with mannose as a selective agent. Negrotto et al. (2000) also reported a transformation frequency of 32% with the temperate maize inbred A188 transformed through *Agrobacterium*-mediated transformation using the same selection system. While genotypic differences could be the dominant factor causing these differences, poor competence of the immature zygotic embryos has also been well known to contribute to low transformation frequency. Though donor plants were grown under controlled conditions in the glasshouse, minor inconsistencies in growth conditions might have still influenced the competence of immature zygotic embryos for transformation with *Agrobacterium*.

The Ethiopian open-pollinated quality protein maize (QPM) variety, Melkassa-6Q, proved to be completely resistant to *Agrobacterium* as indicated by death of the calli on selection medium having mannose. Genotype-strain specificity during *Agrobacterium*-mediated transformation of plants has been well reported (Owens and Cress, 1985; Kuta and Tripathi, 2005) indicating the presence of specific signals from specific *Agrobacterium* strain that could be recognized by specific plant genotype. Each *Agrobacterium*-susceptible plant cell (competent cell) has been shown to contain polysaccharide-polysaccharide binding sites recognizable by *Agrobacterium* (Sangwan et al., 1992). It has earlier been shown that the first step in the transfer of T-DNA molecule from *Agrobacterium* to plant is the recognition of a susceptible plant cell (Zambryski, 1988). Therefore, plant cell can be highly susceptible or non-susceptible to *Agrobacterium* infection, depending on the genotype of the host plant and the strain of the *Agrobacterium* (Jordan and Hobbs, 1994). Resistance of Melkassa-6Q could be due to lack of recognition by the *Agrobacterium* strain EHA 101 as a susceptible host. Differences in susceptibility of the genotypes to *Agrobacterium* infection may also be due to the presence or absence of inhibitors of *Agrobacterium* sensory machinery and their competence. Maize genotypes have been known to release organic exudates such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazine-3-one, commonly known as DIMBOA that inhibit the induction of the *vir* gene expression (Guo et al., 2016) and this can, in turn, render the plants recalcitrant by hindering the *Agrobacterium* to synthesize and transfer sufficient T-DNA strand essential for successful infection.

Acclimatization and growth of putative transgenic plants

The procedures followed in transformation and regeneration of transgenic tropical maize using *Agrobacterium* are indicated in Fig. 2 A-G. Twenty-nine putative transgenic plants, nineteen for Melkassa-2, nine for CML216 and one for CML395 were successfully acclimatized and transferred to soil where they grew to maturity (Fig. 2 G).

Most of the putative transgenic plants were normal and fertile to set seeds, despite aberrant phenotypes (Fig. 3 A-G) which appeared in some of them. Transgenic plants from Melkassa-2 were more affected by these phenotypes. These phenotypes appear usually as results of tissue culture induced somaclonal variations, which are either epigenetic or genetic in origin (Larkin and Scowcroft, 1981). Epigenetic changes are alterations in gene expression that are potentially reversible, and not due to genetic changes. They would involve a mechanism of gene silencing or gene activation that are not due to chromosomal aberrations or sequence changes (Kaeppler *et al.,* 2000). Second-generation (T_2) plants raised from seeds harvested from the somaclonally affected T_0 plants were normal indicating that the phenotypic abnormalities are not heritable (Vasil 2005; Anami *et al.,* 2010).

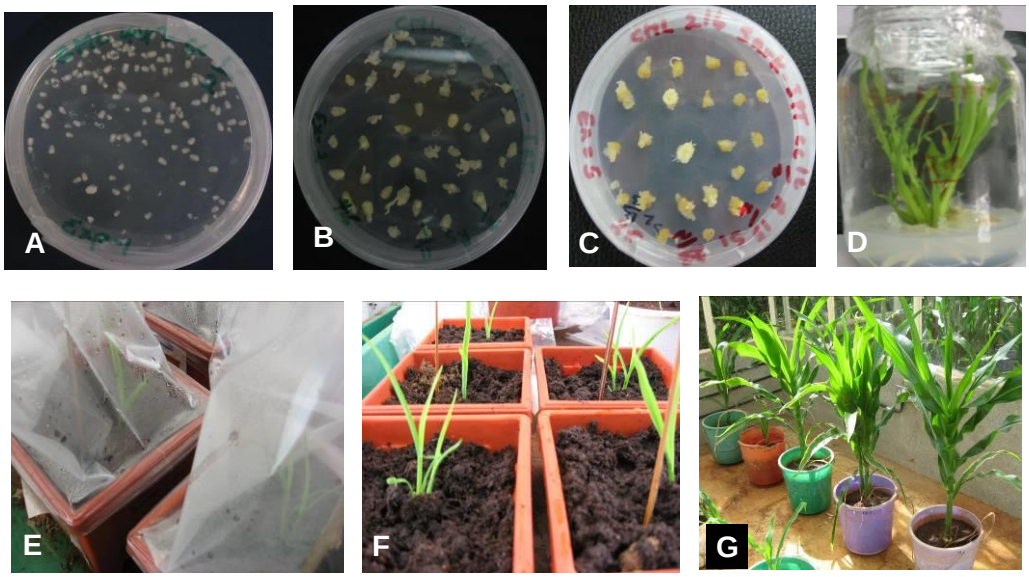
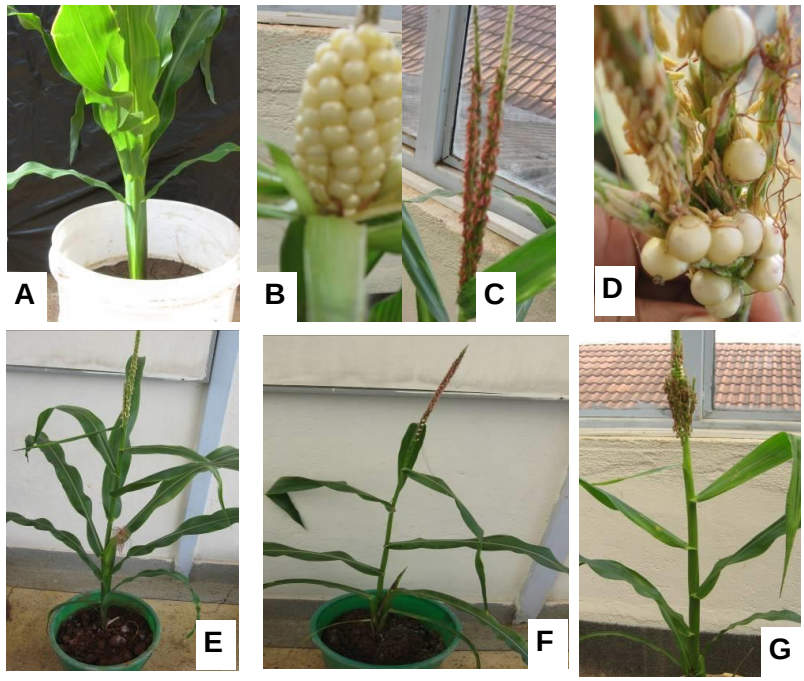


Figure 2. Procedures in transformation and regeneration of transgenic tropical maize using *Agrobacterium*, **A**: Post infection cocultivation of immature zygotic embryos with *Agrobacterium*, **B**: Immature zygotic embryos forming callus on resting medium, **C**: Putatively transformed calli (inside red circle) being selected on selection medium having 5 g/l mannose, **D**: Putative transgenic plants being regenerated on MS medium in the light, **E**: Putative transgenic plants being acclimatized in peat moss covered with polythene bags, **F**: Putative transgenic plants post acclimatization and before transfer to soil in the glasshouse. **G**: PCR positive transgenic P_{SARK}::*IPT* CML216 plants growing in soil in the glasshouse.



Molecular analyses of transgenic plants

Figure 3. Phenotypes observed among T_0 transgenic plants. **A**: Opposite leaves born on the same node instead of alternate leaves born on separate nodes; **B**: Seed on ear born on top of plant instead of tassel; **C**: Twin tassel instead of tassel with central axis and side branches; **D**: Seed on tassel producing pollen; **E**: Normal plant having ear and tassel on the right position on the plant; **F**: An ear born on the extreme lower node and seed born on tassel; **G**: Ear less plant with tassel seed.

PCR analysis of T_0 putative transgenic plants

Putative transgenic plants were analyzed through PCR using primers targeting different regions of the T-DNA. Results obtained using primers specific to the *whole* P_{SARK}*::IPT::NOST* expression cassette and the *pmi* marker gene are presented in Fig. 4 and 5. The first two primers amplified a fragment size of 2 Kbp in 9 independent transgenic events of CML216 and the positive control (plasmid DNA) indicating insertion of the intact P_{SARK}*::IPT::NOST* expression cassette into the maize genome (Fig. 4). Analyses of nineteen independent putative transgenic events generated from Melkassa-2 using primers specific to the *pmi* gene amplified the expected fragment size of 550 bps in eighteen independent events and the positive control indicating transfer of the gene to the genome of this genotype (Fig. 5). Transformation efficiency computed considering PCR positive, independent events for each genotype ranged from zero to 100% (Table 3). Transformation efficiencies of 97.4 and 100 per cent for Melkassa-2 and CML216, respectively, indicated stringency of the *pmi*/mannose based selection system. Melkassa-2 scored transformation efficiency of 94.7% as a result of eighteen independent transgenic events which proved positive for

transformation through PCR with primers specific to *pmi* gene. The two CIMMYT lines, CML395 and CML216, scored transformation efficiency of 100% each being tested positive for the presence of the transgene in all events.

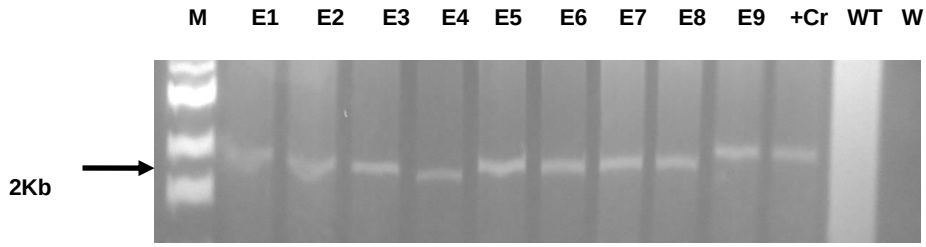


Figure 4. PCR analysis of CML216*P$_{SARK}$::IPT* T$_0$ plants using primers specific to *P$_{SARK}$::IPT::NOST* expression cassette; M: 1 Kbp ladder, **E1-E9**: 1-9 events of T$_0$ plants, **+Cr:** positive control, plasmid DNA, **WT**: DNA from non-transformed plant, **W**: water

Southern blot analysis

Digestion of the genomic DNA obtained from five independent transgenic lines released fragment sizes heavier than the expected 2Kbp size of the *P$_{SARK}$::IPT::NOST* expression cassette carrying the *ipt* gene (Fig. 6). Since these fragments were hybridized with the *pmi* probe, they indicated that the selectable marker gene stably integrated at different sites in the genome with copy number of 2-4 in five of the independent transgenic lines. No stable gene integration was observed in four of the lines (E1, E2, E3 and E9) as indicated by the absence of hybridization of any fragment with the probe. Similarly, lack of hybridization of the probe with any fragment in the negative control indicated absence of any fragment having sequence similar to the *pmi* gene.

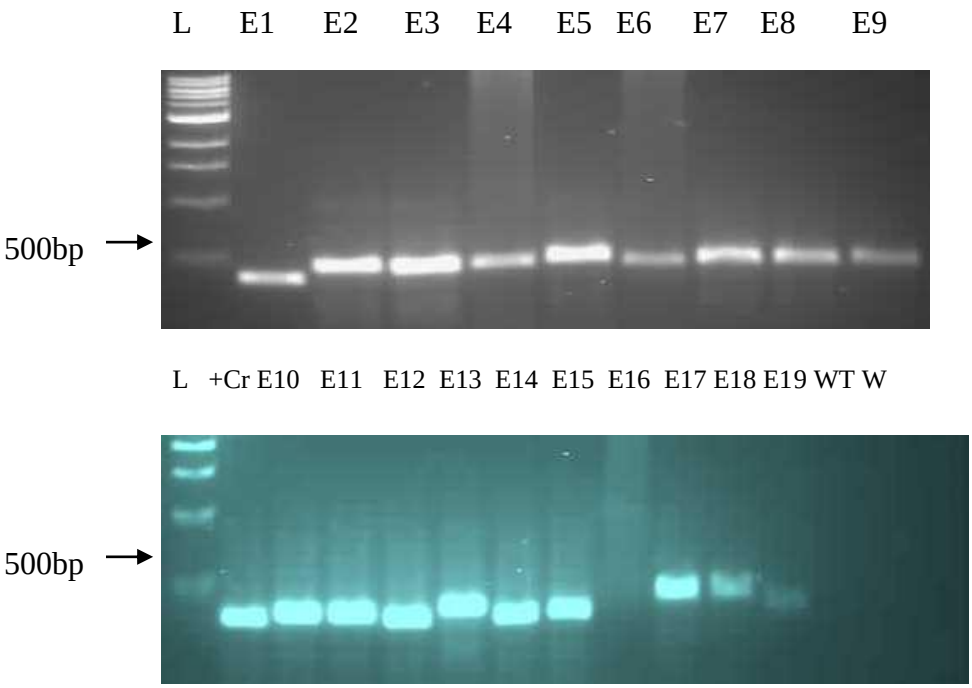


Figure 5. PCR analysis of putative T_0 *P_{SARK}::IPTMelkassa-2* plants using primers specific to the *pmi* gene. **M,** 1 Kbp marker**; E_1-E_{19}**, PCR product from 19 independent events; **+Cr**, Plasmid DNA used as a positive control, **WT**, DNA from non-transformed Melkassa-2 plant; **W**, water**.**

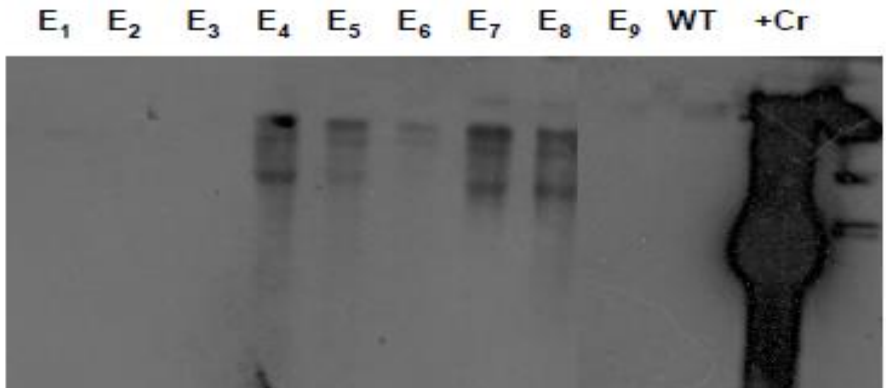


Figure 6. Southern blot analysis of seven independent events of *P_{SARK}::IPT* CML216. Ten micrograms of genomic DNA extracted from young leaf tissues of T_1 plants were digested with *Hin*dIII restriction enzyme and hybridized with the *ipt* probe. **M**: 1Kbp molecular ladder, **WT**: Genomic DNA from non-transformed maize inbred line CML216 taken as negative control. **+Cr**: 20 ng of plasmid DNA digested with *Hin*dIII taken as positive control.

Transgene expression analysis using RT- PCR

Transgene expression was analyzed in T_2 plants of two independent events (two plants from each event) exposed to drought stress for three weeks (21 days) after the age of 8 weeks. The presence of the agronomically important gene (*ipt)* in these plants was previously confirmed through PCR using primers specific to the gene. PCR amplification of the cDNA obtained from the four plants using the same primers indicated presence of the *ipt* gene transcript as indicated by amplification of the expected band size of 0.69 Kbp (Fig. 7).

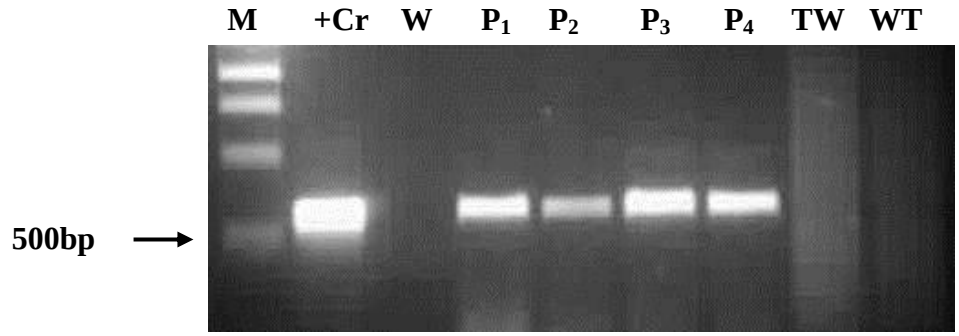


Figure 7. RT-PCR analyses of *P*$_{SARK}$*::IPT CML216* transgenic plants. M: 1 Kbp Molecular marker, **+Cr**: Plasmid DNA, **W**: Water, P1 to **P4** transgenic plants exposed to drought stress at the age of 8 weeks, **TW**: Transgenic plant well watered during the whole of the growth cycle, **WT**: Wild type plant exposed to drought stress at the age of 8 weeks

The SARK promoter driving the *ipt* gene was originally identified in haricot bean which is a dicot and has already been reported to be active in rice which is a monocot crop (Peleg et al., 2011). This study has shown that the drought inducible SARK promoter works correctly in maize as demonstrated by the expression of the *ipt* mRNA transcript. Furthermore, the phenotype of delayed leaf senescence observed in transgenic plants (Leta Tulu Bedada et al., 2016) is evidence corroborating that *ipt* gene produced functional isopentenyltransferase enzyme.

Conclusion

Lack of tissue culture responsive genotypes used to be the main obstacle to application of *Agrobacterium*-mediated transformation technique to genetic improvement of tropical maize. In this study, it was practically demonstrated that this technique could be extended even to elite tropical germplasm having agronomic traits desired for commercial production and breeding purposes. The finding from this study revealed that tissue culture responsiveness is not a general problem of tropical maize as such. It must be rather mentioned that, as per the results obtained in this study, even tissue culture responsive genotypes are not always positive respondent to *Agrobacterium*-mediated genetic transformation technique. This has been very well substantiated by the finding

from this study that indicated genotype dependent response of tissue culture proficient elite African tropical maize genotypes to *Agrobacterium*-mediated transformation and possibility of genetic engineering to enhance the genetic basis of tropical maize using *Agrobacterium.* The tropical maize inbred line, CML216, and the Ethiopian open-pollinated maize variety, Melkassa-2, can be used in future transformation studies targeting biotic and/or abiotic stresses of high priority in affecting maize production in the ECA region. We, however, recommend that future work should capitalize on selection of *Agrobacterium* friendly tropical maize genotypes in different to previous studies that focused on evaluating germplasm exclusively for *in vitro* regeneration potential.

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