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Vol. 15(43), pp. 2447-2464, 26 October, 2016
DOI: 10.5897/AJB2016.15228
Article Number: FDDCDB061322
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Drought tolerant tropical maize (*Zea mays* L.) developed through genetic transformation with isopentenyltransferase gene

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Received 18 January, 2016; Accepted 21 July, 2016

Maize is a staple food crop for millions of Africans. Despite this fact, African farmers have been harvesting average grain yield of not more than 2 t/ha while there is a potential of producing more than 10 t/ha. Drought is one of the major abiotic constraints contributing to this low productivity. Drought diminishes crop productivity mainly by causing premature leaf senescence. The *ipt* gene codes for isopentenyltransferase (IPT) enzyme which catalyzes the rate limiting step in the biosynthesis of cytokinin and has been shown to enhance tolerance to drought in transgenic crops by delaying drought-induced leaf senescence. This created interest to investigate if *ipt* gene can be useful in enhancing drought tolerance in locally adapted African tropical maize genotypes. The tropical maize inbred line CML216 was transformed with *ipt* gene using Agrobacterium-mediated transformation method. Five transgenic lines which were proved to be stably transformed through Southern blot analysis with copy number of 2 to 4 per event were developed. In drought assay carried out in the glass house, transgenic lines expressing the *ipt* gene showed tolerance to drought as revealed by delayed leaf senescence compared to the wild type plants. Transgenic plants maintained higher relative water content and total chlorophyll during the drought period and produced significantly higher mean grain yield of 44.3 g/plant while the wild type plants produced mean grain yield of 1.43 g/plant. It is proposed that the transgenic lines developed in this study can be further tested for tolerance to drought under contained field trials. Furthermore, transgenic lines developed can be used in breeding programs to improve drought tolerance in other commercial tropical maize genotypes through conventional breeding.

Key words: Cytokinin, delayed leaf senescence, drought inducible, CML216, *ipt* gene.

INTRODUCTION

Drought is a major limitation to crop production world-wide. It is now a decade and a half since drought was reported to affect maize production in about 20 to 25% of the global maize area (Heisey and Edmeades, 1999).

Two decades have been counted since yield losses of more than 70% was reported as a result of drought affecting tropical maize in over 60 million hectares in the tropics (Edmeades et al., 1994). With the forthcoming global climate change (Battisti and Naylor, 2009), more yield losses are expected with consequences of affecting 140 million people each year (Jones and Thornton, 2003). By 2050, crop yields are expected to diminish further by 10 to 20% as a result of higher temperature and reduced rainfall (Jones and Thornton, 2003). To reverse this problem, it is mandatory to develop agricultural technologies adapted to such changing environment (Rivero et al., 2007). New maize varieties having improved tolerance to drought stress rank higher in the list of such technologies. The contribution of conventional breeding towards this goal has become insufficient because of limited genetic diversity in the maize gene pool (Hardy, 2010; Shiferaw et al., 2011) and lack of suitable selection criteria for tolerance to drought stress (Nigussie et al., 2002). This brought the need to diversify the genetic basis of locally adapted tropical maize germplasm by introgressing genes responsible for improving tolerance to drought stress.

The *ipt* gene from *Agrobacterium tumefaciens* codes for isopentenyltransferase (IPT) enzyme which catalyzes the rate limiting step in the cytokinin biosynthesis pathway (Akiyoshi et al., 1984). Crops genetically engineered with this gene showed increased level of cytokinin and enhanced tolerance to drought stress as a result of delayed leaf senescence (Rivero et al., 2007; Rivero et al., 2009; Peleg et al., 2011). In maize, the upper eight to nine leaves form major sources contributing 75 to 90% of the assimilate to the grain (Allison and Watson, 1996). Maize responds to drought by launching leaf senescence as a strategy to avoid drought by reducing canopy size and to mobilize nutrients to support the growth of the upper younger leaves and grains (Grabau, 1995). This regulation of leaf senescence has an obvious adaptive value in wild plants allowing them to complete their life cycle even under stressful conditions. In crop plants, drought induced leaf senescence is often associated with reduced grain yield (Gan and Amasino, 1996; Gungula et al., 2005) causing premature death of photosynthetically active leaves. Drought induced production of cytokinin has been shown to play a major role in changing this source/sink relationships and was applied as an important component for the development of drought-tolerant rice (Peleg et al., 2011). It is hypothesized that the same strategy can be applied to tropical maize, which is also a monocot, ending with improved drought tolerance.

Transgenic crops developed with *ipt* gene manifest different morphological and physiological abnormalities depending on the promoter driving the gene. Such abnormalities occur in condition where constitutive promoters like the cauliflower mosaic virus promoter CaMV35S or the ubiquitin maize promoter is used to drive the gene to the extent of preventing studies on cytokinin overproduction in normal plant tissues (McKenzie et al., 1998). In maize, the *ipt* gene has been expressed using senescence-activated promoters (Young et al., 2004; Robson et al., 2004). In P*SAG12:IPT* maize, senescence was not delayed despite the observed expression of *ipt* in senescing leaves (Young et al., 2004). Extended greenness accompanied by a delay in senescence induced by nitrogen stress has been reported in temperate maize transformed with *ipt* gene driven by native promoter of the senescence enhanced (*SEE)* gene (Robson et al., 2004). Similar to previous report by Jordi et al. (2000), transgenic lines failed to recycle internal nitrogen from senescing lower leaves which has accounted for significant chlorosis in emerging younger leaves when plants were grown in low nitrogen stress condition (Robson et al., 2004). Hence, these drawbacks limited the use of the senescence enhanced promoter to drive *ipt* gene to delay leaf senescence in maize.

The other option was, therefore, to resort to drought inducible expression of the *ipt* gene as a strategy for maize transformation with the objective of delaying drought induced leaf senescence. This strategy could also delay drought induced leaf senescence by synchronizing cytokinin production with the onset of drought stress in the plant (Rivero et al., 2007). A drought inducible senescence-associated receptor kinase (*SARK*) promoter was identified from a gene of which expression is up-regulated at the earliest stage of leaf senescence before any visible sign like leaf yellowing in haricot bean (*Phaseolus vulgaris* L.) (Hajouj et al., 2000). This was linked to the *ipt* gene and used to transform tobacco (Rivero et al., 2007) and rice (Peleg et al., 2011), using *A. tumefaciens*. In both crops, transgenic lines expressing $P_{SARK::}$*IPT* did not differ in appearance from the wild type. They further exhibited extreme tolerance to drought by delaying senescence compared to wild type plants. Unlike the previous report of Gan and Amasino (1996) on P_{SAG}*::IPT* transgenic lines, the expression of $P_{SARK::}$*IPT* during drought was not only enhanced in basal leaves but also in the middle and apical leaves, confirming the drought responsiveness of the *SARK* promoter. Their work shows that nitrogen mobilization was not affected in the $P_{SARK::}$*IPT* plants, because the basal leaves displayed chlorophyll degradation during drought.

The performance of the P_{SARK}*::IPT* rice lines (Peleg et al., 2011) created interest that the *ipt* gene has the potential to bring improved tolerance to drought in tropical

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maize which is also a monocot crop. This study was therefore designed to investigate if *ipt* gene driven by the drought inducible SARK promoter can be useful in enhancing tolerance to drought stress in maize genotypes adapted to tropical African environment. For this purpose, a construct carrying P_{SARK}::*IPT::NOST* cassette was obtained from the Department of Plant Sciences, University of California, Davis, CA, USA. We sub-cloned the expression cassette to the binary vector, pNOV2819, to avoid use of antibiotic resistance marker gene and to take advantage of the *pmi* gene as plant selectable marker and mannose as selective agent (Negrotto et al., 2000) to develop a product, which is safe to the environment and the consumers. The tropical maize inbred line CML216 was transformed using *Agrobacterium*-mediated transformation technique and successfully regenerated normal and fertile transgenic plants. Though *ipt* gene has already been used to transform temperate maize under the promoter of *SAG12* gene and *SEE* gene of *Arabidopsis* and maize, respectively, we are reporting for the first time on successful enhancement of tolerance to drought stress in tropical African maize by delaying drought induced leaf senescence through inducible expression of the *ipt* gene under the SARK promoter.

MATERIALS AND METHODS

Construct preparation

The AF234301 (pCAMBIA1380::pSARK:IPT) (10.4 kbp) construct (Figure 1A) carrying the P_{SARK}::*IPT::NOST* expression cassette was received from the Department of Plant Sciences, University of California, Davis, CA, USA having the hygromycine phosphotransferase gene (*hptII)* as a plant selectable marker. The expression cassette was sub-cloned to the pNOV2819 binary vector provided by Syngenta (Figure 1B), to avoid use of *hptII* gene as a marker and to take advantage of the *pmi* gene in pNOV2819 vector as a plant selectable marker and mannose as selective agent (Negrotto et al., 2000).

Forward 5'-GTGCTCCACCATGTTGGGCCCGGCGCGCCGA-3' and reverse 5'-GCCAAGCTTTCCCGATCTAGTAACATAGAT-3' primers were designed on the sequences of the *PUC* multiple cloning site and SARK promoter, and nos terminator (*NOST)*, respectively, using FastPCR software. The forward primer incorporated restriction sites for *Asc*I and *Eco*RI from the *PUC* multiple cloning site available in AF234301 (pCAMBIA1380:: pSARK:IPT) while *Hind*III site was engineered to the 5` of the reverse primer. The PCR reaction was carried out in Eppendorf (Eppendorf AG, Hamburg, Germany) PCR machine using *Pfu* DNA polymerase (5 U/µl) (Fermentas Inc, Maryland, USA). The PCR programme was composed of initial denaturation at 98°C for 5 min, followed by 35 cycles of denaturation at 98°C for 30 s, annealing at 45°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 15 min. The PCR product was inserted in the multiple cloning site of pNOV2819 vector as *Asc*I/*Hind*III fragment. The P_{SARK}::*IPT::NOST* cassette was then sequenced to check the originality of the sequences. The result indicated complete consensus of the sequences with the original sequences of the SARK promoter, *ipt* gene and nopaline synthase terminator. The map of this construct drawn using vector NTI software is shown in Figure 1B. The pNOV2819 vector carrying the P_{SARK}::*IPT::NOST* expression cassette (PNOVIPT1) was then inserted in to the *Agrobacterium* strain EHA 101 for subsequent use in maize transformation.

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 on LBA (Luria Bertani Agar) medium supplemented with 100 $mg.l^{-1}$ spectinomycin and 100 $mg.l^{-1}$ kanamycin at 28°C for two days in dark. This plate was kept at 4°C as a source of inoculum for experiments up to one month after which it was regularly refreshed from long term glycerol stock kept at -80°C. One full loop (3 mm) of bacteria was scooped from this fresh culture and suspended in 10 ml of Linsmaier and Skoog (LS), (1965) infection medium (Table 1) supplemented with 100 µM acetosyringone in a sterile 50 ml falcon tube. The tube was sealed with parafilm and covered with aluminium foil and fixed on a shaker in a horizontal position and the culture was left to grow for 3 to 4 h at 250 rpm and temperature of 28°C until optical density (OD) of 0.4 to 0.6 was attained at A260 nm. This procedure called pre-induction step was routinely carried out before all transformation experiments.

Plant material

Seeds of the tropical maize inbred line CML216 were obtained from the International Maize and Wheat Improvement Centre (CIMMYT-Nairobi). Twenty-five (25) plants were grown in the glasshouse at the Biosafety Level II Plant Transformation Laboratory (PTL) of Kenyatta University to supply immature zygotic embryos for *in vitro* culture. Glasshouse conditions were temperature of 30/25°C (day/night) and relative humidity of 30% and 16/8 h (light/dark) photoperiod. Soils for growing maize were prepared by mixing sandy loam soil with compost in the ratio of 2:1 and about 15 kg was filled into buckets having diameter of 3 mm and height of 3.1 mm. Each bucket was planted with one seed and then fertilized with 10 g di-ammonium phosphate (DAP) having 18% (w/w) ammonical nitrogen and 46% (w/w) available P_2O_5 avoiding contact with the seed. The soil was then watered fully up to complete saturation with 2 L of water for the first time. Subsequent watering was done by applying 1 L of water daily. The plants were top dressed with 7 g of urea having 46% (w/w) nitrogen when they had grown to a height of 10 cm and this top dressing was repeated depending on plant demand. All the 25 plants were self-pollinated to produce genetically and true-to-type immature zygotic embryo explants. Production, collection and sterilization of maize cobs and excision of immature zygotic embryos were carried out following protocol described by Seth et al. (2012).

Media for maize transformation

Infection, co-cultivation, resting, selection and maturation media were based on LS salts with specific formulations modified from Negrotto et al. (2000) and contained LS modified vitamins and 1.5 $mg.l^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) (Table 1). Infection medium contained 1 $g.l^{-1}$ casein hydrolysate, 68.5 $g.l^{-1}$ sucrose and 36 $g.l^{-1}$ glucose and 100 µM acetosyringone. The pH of this medium was adjusted to 5.2 using 1 N NaOH and/or 1 N HCl and then filter sterilized using 0.2 µm pore size filter. It was then aliquated into volume of 50 ml and kept at -20°C until it was used. Both co-cultivation and resting media contained 0.7 $g.l^{-1}$ L-proline, 0.5 $g.l^{-1}$ 2-(N-morpholino) ethanesulfonic acid (MES), and 30 $g.l^{-1}$ sucrose. Co-cultivation differs from resting medium as it contained 10 $g.l^{-1}$ glucose and 100 µM acetosyringone while resting medium also

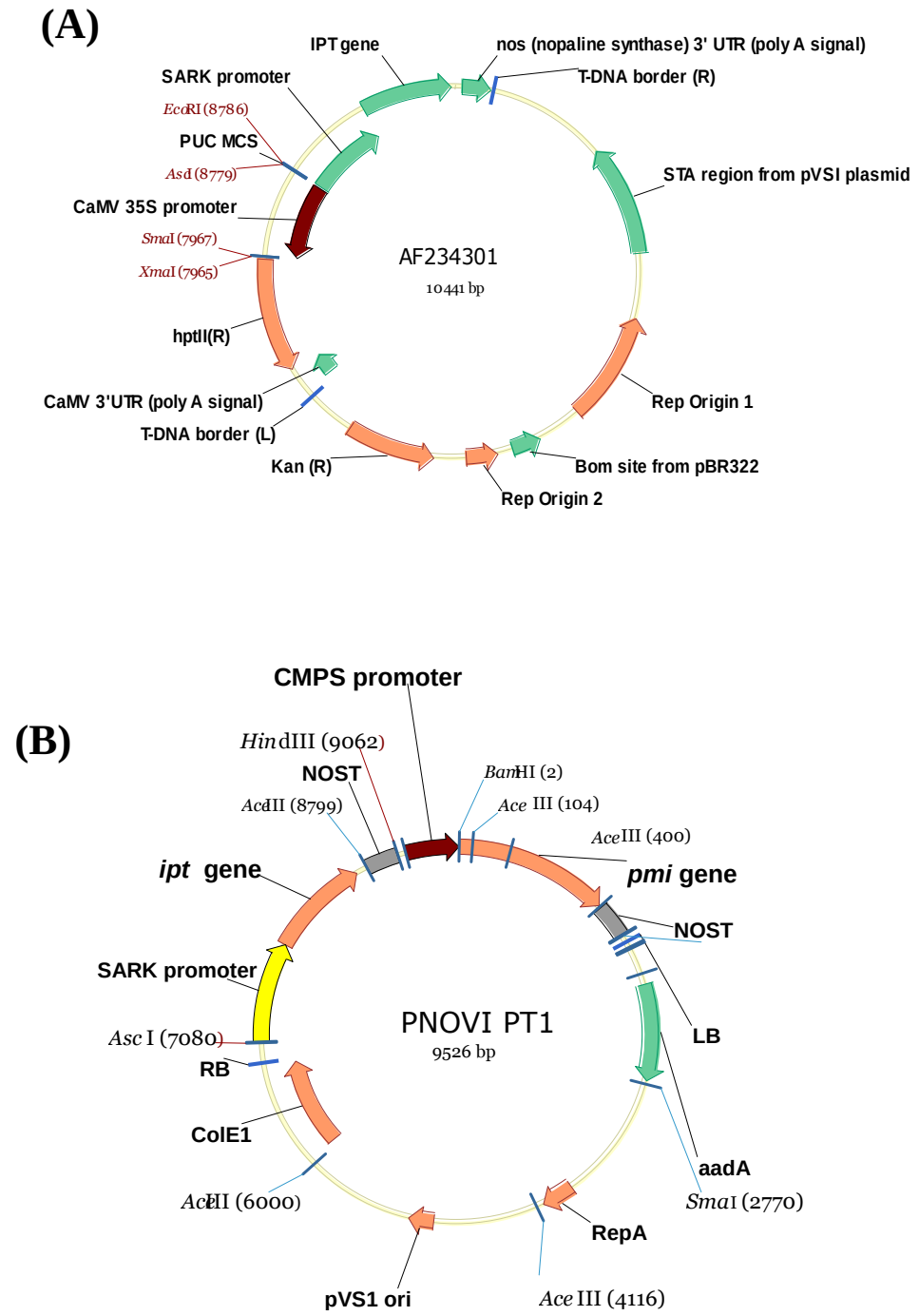

Figure 1. AF234301 construct carrying the P_{SARK}::*IPT*::*NOST* expression cassette (A) and pNOV2819 binary vector carrying the CMPS (cestrium yellow leaf curling virus promoter short version), the selectable marker *pmi* gene with NOST and the P_{SARK}:: *IPT*::*NOST* expression cassette (B).

differs as it contained 1.6 mg.l^{-1} silver nitrate and 250 mg.l^{-1} carbenicillin for counter selecting *Agrobacterium*. Selection medium was similar to resting medium except that it contains 25 g.l^{-1} sucrose and 5 g.l^{-1} D-mannose and it lacks silver nitrate. Maturation medium is similar to selection medium except that the amount of mannose was reduced by half and it was supplemented with 0.5 mg.l^{-1} kinetin instead of 2,4-D. Regeneration medium was based on Murashige and Skoog (MS), (1962) and hormone free. Except infection medium, all media were solidified with addition of 0.8% (w/v) agar and sterilized by autoclaving at 121°C and 15 Psi after adjusting pH to 5.8. Acetosyringone, LS vitamins, silver nitrate and carbenicillin were added to the respective medium after autoclaving

Table 1. Media used for infection, callus induction, selection and regeneration of transgenic maize plants.

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

*LS: Linsmaier and Skoog, ** Murashige and Skoog

and cooling to 40 to 50°C.

Infection and co-cultivation

After being aseptically removed from the cob, the immature zygotic embryos were placed in 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 dark for 5 min to encourage attachment of the *Agrobacterium* cells to the immature zygotic embryos. The infected zygotic embryos while still in infection medium were 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 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 dark for 10 to 15 days with occasional observation on the process of callus induction.

Selection of putatively transformed events and plant regeneration

The immature zygotic embryos which did and did not produce callus were transferred to selection medium having 5 g.l^{-1} 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 after transfer to selection medium. 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 were 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 on LS maturation medium, embryogenic calli were 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 Seth 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 T_0 seeds for further analysis.

PCR analyses of transgenic plants

Polymerase chain reaction analyses of putative transgenic plants 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. The PCR programme 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. Each of 25 µl PCR reaction contained 0.5 µl 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), 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 per centage.

Southern blot analysis

Total genomic DNA was extracted from 2 to 3 g of young T_1 and wild type (WT) plant leaves using the cetyltrimethylammonium bromide (CTAB) method (Allen et al., 2006). Ten micrograms of genomic DNA per sample were digested completely with *Hind*III 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 pNOV2819 vector carrying the P_{SARK}::*IPT::NOST* cassette (PNOVIPT1) plasmid DNA was diluted to 20 ng.µl^{-1}, 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 over night to Hybond^{TM}-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 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 III^{TM} 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^{-1}) and 2 µl (10 ng.µl^{-1}) of cDNA as template. The final volume was brought up to 25 µl by adding 17.5 µl RNase free water.

The ZmAct forward 5'-ACCCAAAGGCTAACCGTGAG-3' and ZmAct reverse 5'-TAGTCCAGGGCAATGTAGGC-3' primers were used to amplify 0.426 kbp of the *ACTIN* gene transcripts as internal control. The PCR reaction of 25 µl was set up with the same PCR condition indicated for the *ipt* gene specific primers. The PCR programme was based on initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and final extension of 72°C for 2 min. The PCR products were loaded in a 1% (w/v) agarose gel and electrophoresis was carried out at 70 v for 1 h

Glasshouse drought experiment

Growth condition and plant establishment

Seeds obtained from selfed T_1 generation plants of stable transgenic events of P_{SARK}*::IPTCML216* and non-transformed (wild type, CML216) plants were planted in the glasshouse at Kenyatta University Plant Transformation Laboratory. Glasshouse conditions were temperature of 40/35°C (day/night) and relative humidity of 35% and 16/8 h (light/dark) photoperiod. Both the transgenic and wild type plants were treated alike in terms fertilizer application and watering frequency. The amount of water needed to keep soil moisture near the field capacity was determined following the procedure described by Dastane (1967). Plants were watered with 1 l of water, as established through this method, daily (every 24 h) from planting to physiological maturity whenever they are grown under optimal moisture condition. Drought was imposed by withdrawing watering from the transgenic and wild type plants for three weeks when they were at the age of eight weeks (six fully grown leaf stage). Watering was restored in all stressed plants after these three weeks of drought, giving 1 l of water daily (every 24 h) up to physiological maturity (black layer formation).

Evaluation for physiological parameters

Plants' physiological responses to drought stress was monitored by measuring relative water content, chlorophyll a, b and total carotenoid levels before dehydration, at weekly intervals during the drought period, and 24 h after one time re-watering with 1 l of water and a week after re-watering daily with the same volume of water.

Relative water content

Relative water content (RWC) was measured in leaf samples collected at 2 to 3 pm, when plants were expected to experience the most drought stress as result of high temperature and soil moisture deficit, and fresh weight was recorded immediately using sensitive balance. The samples were then immersed in distilled water and kept in darkness at 4°C overnight to minimize respiration losses and turgid weight was recorded after blotting the adhering water using blotting paper. The samples were then dried in an oven (Combi-H12, FINEPCR, Korea) set at 70°C for 24 h and dry weight was recorded. RWC was calculated according to Rivero et al. (2007) as:

$$RWC = (FW-DW/TW-DW) \times 100 \quad (1)$$

Where, FW is fresh weight, DW is dry weight and TW is turgid weight of leaf samples.

Chlorophyll extraction and quantification

For chlorophyll pigment analyses, leaf samples were collected at the same time point as for relative water content and punctured into uniform circular sizes using paper puncture. Ten punctured pieces were then transferred to a mortar and were thoroughly homogenized under 4 ml of 100% acetone using a mortar and pestle in the dark. The homogenates were centrifuged at 12,000 rpm for 10 min. The supernatants were then collected and the absorbencies were measured at 662, 645 and 470 nm using an ultraviolet (UV)-visible spectrophotometer (722 Visible

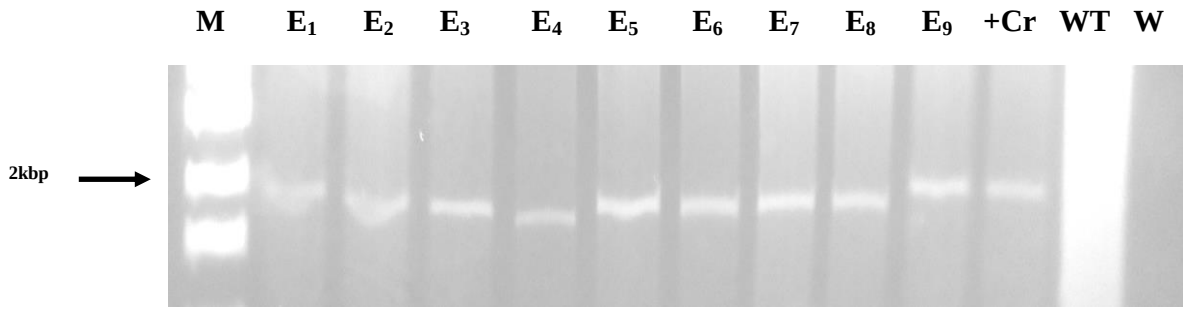

Figure 2. PCR analysis of putative T_0 *P_{SARK}::IPTCML216* plants using primers specific to the *P_{SARK}::IPT::NOST* expression cassette. M, 1 kbp marker; E_1-E_9, PCR products from nine independent T_0 events; +Cr, positive control, plasmid DNA; WT, DNA from non-transformed CML216 plant; W: water.

Spectrophotometer, China). Then chlorophyll a, chlorophyll b, total chlorophylls, and total carotenoids (xanthophyll + ß-carotene) content were calculated applying the following equations (Lichtenthaler and Wellburn, 1983) as:

$$\text{Chlorophyll a } (\mu g/gfw) = 11.75A_{662} - 2.35A_{645} \quad (2)$$

$$\text{Chlorophyll b } (\mu g/gfw) = 18.61A_{645} - 3.96A_{662} \quad (3)$$

$$\text{Total carotenoid } (\mu g/gfw) = 1000(A_{470} - 2.27) \text{ chlorophyll a } (\mu g/gfw) - [81.4 \text{ chlorophyll b } (\mu g/gfw)]/227 \quad (4)$$

Induction of leaf senescence in dark

To investigate on the occurrence of leaf senescence in transgenic and WT plants in the dark, senescence was induced in young leaf tissues collected from the drought stressed WT and transgenic plants at intervals of four days over 12 days time. The leaves from the WT plants were kept in Petri plates filled with sterile distilled water and kinetin and 6-Benzylaminopurine (BAP) each at concentration of 5 $mg.l^{-1}$ while leaves from transgenic plants were exclusively kept in sterile distilled water (Peleg et al., 2011). All samples were kept in dark at 30°C with frequent supervision for development of senescence. On the 12^{th} day, all the leaves were taken from the dark and picture was taken. Chlorophyll a and b were extracted from the leaf tissues before dark assay and on the 12^{th} day of dark treatment and quantified using the method described in Lichtenthaler and Wellburn (1983).

Evaluation of important agronomic parameters

Important agronomic parameters were evaluated both in the transgenic and wild type plants. Phenological traits were evaluated in terms of days to anthesis and silking. Days to anthesis were determined as the number of days a plant has taken from germination to the day half of the tassel has started pollen shading. Days to silking was also determined as the number of days a plant has taken from germination to the day silks have emerged at least 2 cm above the ear sheath. Anthesis-silking interval (ASI) was recorded as the difference between days to silking and days to anthesis. Plant and ear heights were, respectively, measured as the height from the soil level to the base of the tassel and to the node bearing the uppermost ear. The whole plant was cut at the soil level and the weight was recorded as biological yield in grams per plant. After recording the biological yield the whole plant was divided in to leaf, stalk and ear which were dried separately in the oven at 70°C for 24 h after removing the cob bearing the kernels. The cob was dried separately after removing the kernels, which were air dried to uniform moisture of 13% before recording dry weight. Dry weight of the different separate parts was then added to give total plant dry weight.

The weight of the seed obtained after drying in the sun to uniform moisture content of 13% was recorded as seed yield per plant. Thousand seeds were counted and weighed on sensitive balance and the value was recorded as thousand seed weight in grams.

Statistical analysis of physiological and agronomic data

Analysis of variance (ANOVA) was carried out using genstat discovery edition 4 (VSN international software for biosciences, www.vsn.co.uk/software/genestat) to test the statistical significance of genotypes and drought on RWC and chlorophyll concentration at different time points and growth, seed yield and agronomic characters. Whenever the ANOVA revealed significant differences, the means were separated using the least significant difference (LSD) test at 5% probability level.

RESULTS

Nine independent putative transgenic events were generated through *Agrobacterium*-mediated transformation of the tropical maize inbred line CML216 with the SARK promoter linked to the *ipt* gene. All plants were normal and fertile despite phenotypic aberrations that appeared as a result of tissue culture induced variation (Larkin and Scowcroft, 1981). Polymerase chain reaction analyses indicated the presence of the transgene in all events (Figure 2) indicating transformation efficiency of 100% in transforming tropical maize following the *pmi*/mannose based selection system. Southern blot analysis revealed stable integration of the transgene in T_1 generation of five independent events with copy number of 2 to 4 per event (Figure 3) indicating stable transformation efficiency of 55% as opposed to transformation efficiency of 100% obtained with PCR analysis in the T_0 generation. Individual plants of the stably transformed events were then advanced to T_2 generation through self-pollination. Reverse transcription PCR also indicated presence of the *ipt* mRNA transcript in transgenic plants. The result is shown only for four

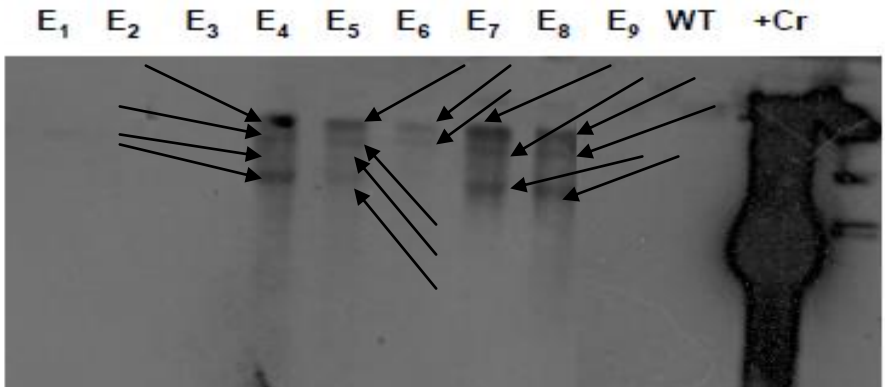

Figure 3. Southern blot analysis of nine independent events of P_{SARK}*::IPTCML216.* Ten micrograms of genomic DNA extracted from young leaf tissues of T_1 plants (E_1-E_9) was digested with *Hind*III restriction enzyme and hybridized with the *pmi* probe. WT, Genomic DNA from non-transformed maize inbred line CML216 plants taken as a negative control; +Cr, 20 ng of plasmid DNA digested with *Hind*III used as a positive control.

Figure 4. P_{SARK}*::IPTCML216* transgenic and wild type plants growing in the glasshouse. Delayed leaf senescence contributed to strong source/sink relationship, which led to better ear development and enhanced grain productivity under drought in transgenic plants expressing *ipt* gene (middle and right). Wild type plants (left) suffered loss of green leaf area, which affected ear development and grain yield under drought.

plants (Figure 4) from two independent events grown under drought (Figure 5).

Development of stress and leaf senescence

Drought stress symptoms were observed in the WT plants faster than in the transgenic plants as revealed by leaf wilting on the fifth day after withdrawing watering. The transgenic plants did not show any sign of wilting during this time. One week of drought could induce senescence in the lower leaves in the WT plants and this progressed further to the second leaves within the second week of drought. Senescence began on leaf tips and margins and progressed to the centre of the leaves as drought intensified further. By the end of the second week of drought, two to three leaves died from senescence in the WT plants. The transgenic plants did not show any sign of senescence during this time though symptom of drought stress was observed by wilting and rolling of few leaves temporarily in the hottest part of the day. These plants could show temporary recovery from drought stress in the morning hours as indicated by leaf unrolling and achieving fully turgid condition. On the contrary, the wild type plants could not show any sign of recovery from drought stress. Rather leaf rolling, wilting and senescence continued at much magnified level up to the third week of drought. No recovery was observed even 24 h after one time rewatering with 1 l of water.

The strongest drought stress developed in the transgenic plants at the end of the third week of drought when leaf wilting and rolling combined with development of dark green color was observed. At this time, point only the lowest leaves could show complete death from senescence. These plants could recover completely from this drought stress within 24 h after 1 time re-watering with 1 l of water. The relative water content measured after 24 h of one time re-watering with 1 l of water was almost equivalent to the amount measured at pre-dehydration indicating faster recovery of the transgenic plants from drought stress.

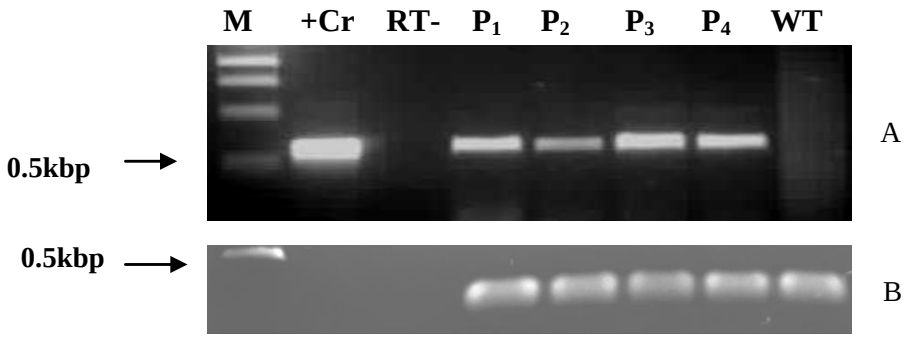

Figure 5. RT-PCR analysis of *ipt* gene expression in drought stressed P_{SARK}*::IPTCML216* transgenic plants (P_1-P_4). A, *ipt* gene specific primers amplified 0.69 kbp of the gene transcripts; M, 1 kbp Molecular marker; +Cr, Plasmid DNA used as positive control; RT-, cDNA synthesis mix with DNAs I treated RNA but without Superscript III; WT, cDNA from WT plant exposed to drought stress at the age of 8 weeks; B, Actin primers amplified 0.426 kbp of the *ACTIN* gene transcripts as internal control.

The wild type plants showed deficit of 20% tissue water content compared to their pre-dehydration relative water content even after a week of re-watering with 1 L of water daily.

Despite restoration of optimal soil moisture, senescence was more accelerated in the wild type plants as consequence of previous drought experienced at the age of 8 to 11 weeks. Figure 6 shows both transgenic and wild type plants 20 days after pollination (one month after drought). By this time, more than 50% of the photosynthetically active green leaf area was lost in the wild type plants. On the contrary, leaf senescence was delayed significantly and was confined to the margins and tips of leaves below the ears in the transgenic plants. Only the lower most two leaves died per plant as result of senescence, which should be expected in maize even under normal production circumstances. Within one month from this time (at the age of four months and two months after drought) 80% of the WT leaves died as a result of the previous stress (Figure 6D).

Effect of drought stress on leaf relative water content

Both the wild type and transgenic plants had RWC of 95% before drought (Figure 7). The wild type plants showed drastic reduction of tissue water content down to 70% (25% loss) within the first 1 week of drought stress. The transgenic plants showed loss of only 10% and maintained that level until the end of the third week when it further lost 10% of the tissue water content. By this time, the wild type plants lost 40% and RWC stood at 57.9%. One time re-watering with 1 l of water did not improve the RWC in these plants while it replenished to 88.7% in the transgenic plants, which maintained the same level after one week of re-watering with the same volume of water daily. At this time point, the RWC of the

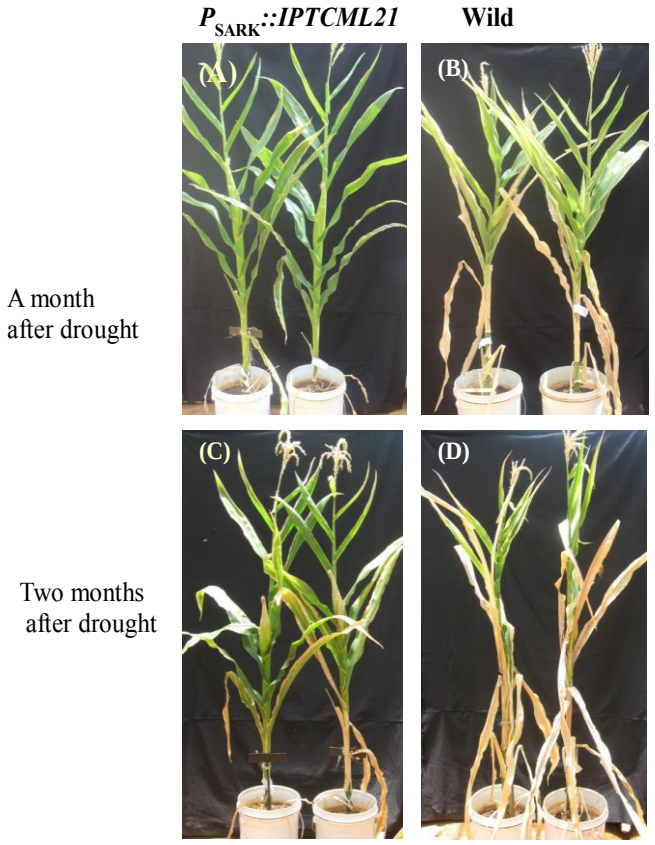

Figure 6. Drought induced leaf senescence was delayed in P_{SARK}*::IPTCML216* transgenic plants (A & C) compared to wild type plants (B and D). The P_{SARK}*::IPTCML216* transgenic plants at the age of four months (A) did not show sign of senescence compared to the wild type plants of equivalent age (B) which had lost 50% of the green leaf area to drought stress imposed for three weeks at the age of 8 weeks. At the age of five months the P_{SARK}*::IPTCML216* transgenic plants (C) still maintained green leaf area compared to the equivalent age wild type plants (D) which lost 80% of the green leaf area as a result of drought.

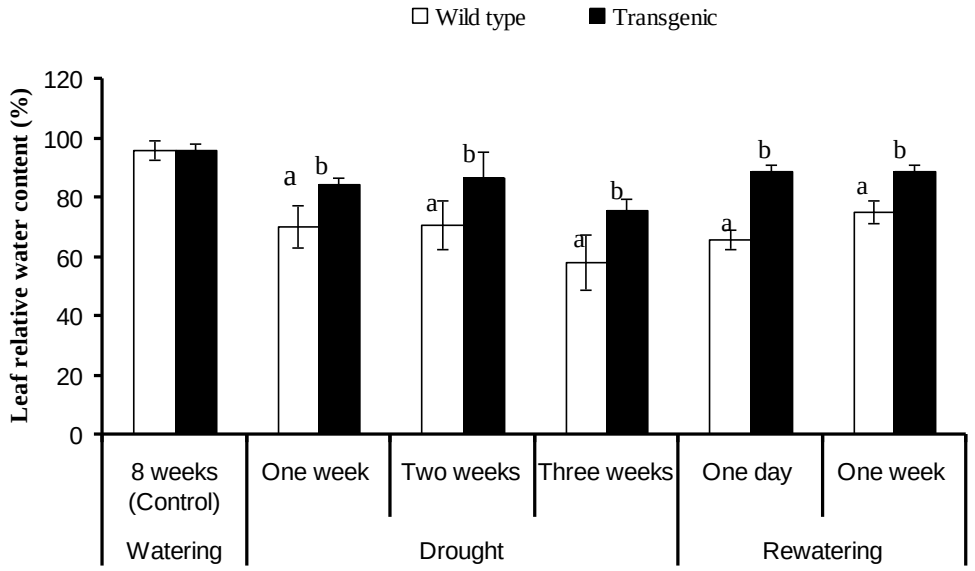

Figure 7. Relative water content measured in 8 weeks old transgenic and wild type plants at different time points during three weeks of drought stress assay in the glass house. Each data point represents mean of four replications ±SE. Mean values followed by different letters at specific time points are significantly different from each other according to LSD test at P<0.05.

wild type plants refilled to 75% only still showing deficit of 20% compared to the values before drought.

Effect of drought stress on total chlorophyll

Total chlorophyll declined with increasing time of drought stress in both the wild type and transgenic plants (Figure 8A). However, the transgenic plants had higher total chlorophyll compared to the wild types at all-time points. The wild type plants experienced 35.6% loss in total leaf chlorophyll content during the third week of drought stress compared to the level recorded at pre-drought period. During this time the transgenic plants lost only 4.3% of the total leaf chlorophyll content. In both cases total chlorophyll continued to decline despite restoration of moisture in the soil.

Effect of drought stress on chlorophyll a and b

Chlorophyll a was almost stable in both the wild type and transgenic plants during drought except where it declined in the wild type after the third week of drought (Figure 8B). In general, changes in leaf chlorophyll b content followed the same trend as the total chlorophyll with time of drought stress in both the wild type and transgenic plants (Figure 8C). Both had almost the same level of chlorophyll b content before drought. However, as drought progressed, the wild type plants manifested substantial reduction as a result of which the transgenic plants had relatively higher level throughout the drought period. The wild type plants experienced loss of 54 and 70% during the third week of drought and after one week of rewatering, respectively, while the transgenic plants manifested loss of 11.9 and 33.3% during the same time points compared to their respective values recorded before drought. This drastic reduction was equally observed in all the wild type plants while there was variation among the transgenic ones.

Effect of drought stress on total carotenoid content

Contrary to total chlorophyll content, total carotenoid content increased progressively with increase in drought intensity in both the wild type and transgenic plants. However, the wild type plants showed substantial increment compared to the transgenic counterparts over the three weeks of drought period. This increment was mild during the first week of drought both in the wild type and transgenic plants though it was still higher in the wild type plants (Figure 8D). It then peaked up exponentially in the wild type plants during the third week of drought stress recording 75% increment compared to the level at the end of the second week. It followed the same trend in the transgenic plants but at lower magnitude compared to the wild type plants. The exponential phase lagged behind by one week in the transgenic plants. This started with re-watering at the end of the third week and continued one week after re-watering when transgenic plants recorded 97% increment compared to the level before re-watering. During this time, the increment in the wild type plants has already stabilized.

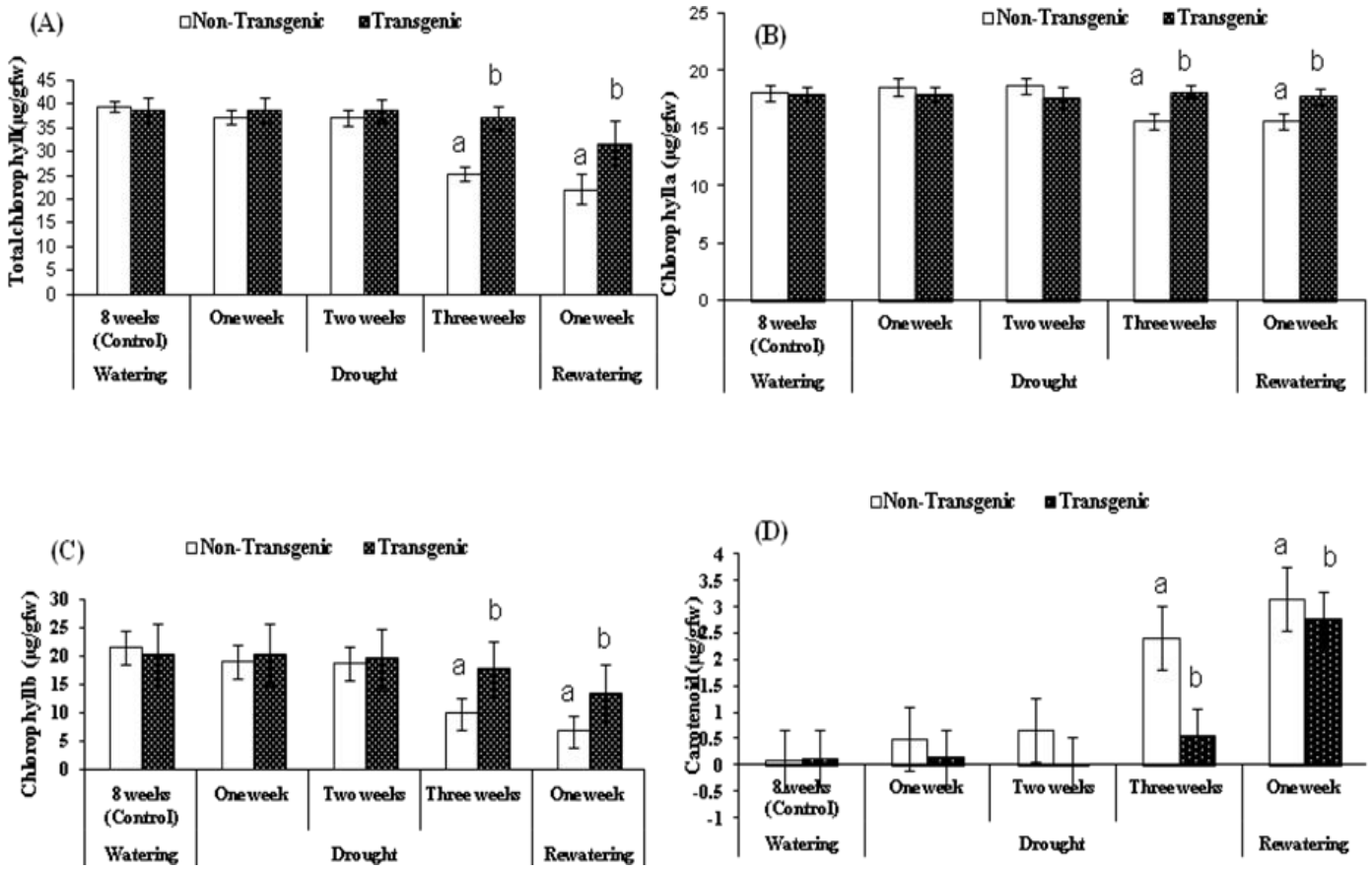

Figure 8. Chlorophyll data measured during watering, drought and rewatering experiments in the glass house, A: Total chlorophyll, B: Chlorophyll a, C: Chlorophyll b, D: Total carotenoids. Values are mean±SE (n=12).

Dark assay of leaf senescence

Dark assay of leaf senescence was carried out to compare the effect of cytokinin externally applied and endogenously produced by transgenic maize in delaying leaf senescence. Leaf segments taken from wild type drought stressed plants and kept in distilled water manifested senescence on the 8th day of time lapsed in darkness (Figure 9). When leaf segment from the same plant was kept in 5 mg l^{-1} cytokinin (BAP or kinetin) senescence was delayed up to the 12th day. Leaf segments detached from drought stressed transgenic maize plants and kept in distilled water in dark did not show sign of senescence at the end of the 12th day. This result showed that endogenously produced cytokinin is better off in delaying leaf senescence than externally applied ones even in detached leaves in maize. Under the dark assay leaf chlorophyll a and b, and hence total chlorophyll contents were reduced significantly in the wild type leaf tissues 12 days after the dark assay (Figure 10). Leaf tissues obtained from the transgenic plants maintained higher level of chlorophyll content even better than externally applied cytokinin supporting the result obtained with physical senescence of the leaf tissues after dark assay. In general, the dark assays were very much supportive of the senescence data obtained from *in vivo* evaluation made in the glass house drought assay.

Effect of drought stress on growth and agronomic performance of transgenic and wild type plants

The effect of drought on growth was monitored in terms of fresh and dry weight of plants at harvest. Transgenic plants produced significantly ($P<0.05$) higher root fresh and dry weight than the WT plants. They also produced higher above ground plant fresh and dry matter, and higher total plant fresh and total plant dry matter, both including the root fresh and dry matter, respectively, than the WT plants though the differences were not statistically significant. Higher root growth was due to massive fibrous roots produced in all the transgenic plants as opposed to the WT plants, which produced few taproots with a small number of secondary roots (Figure 11). Better cob growth and stay green character of the transgenic plants have also contributed to their higher

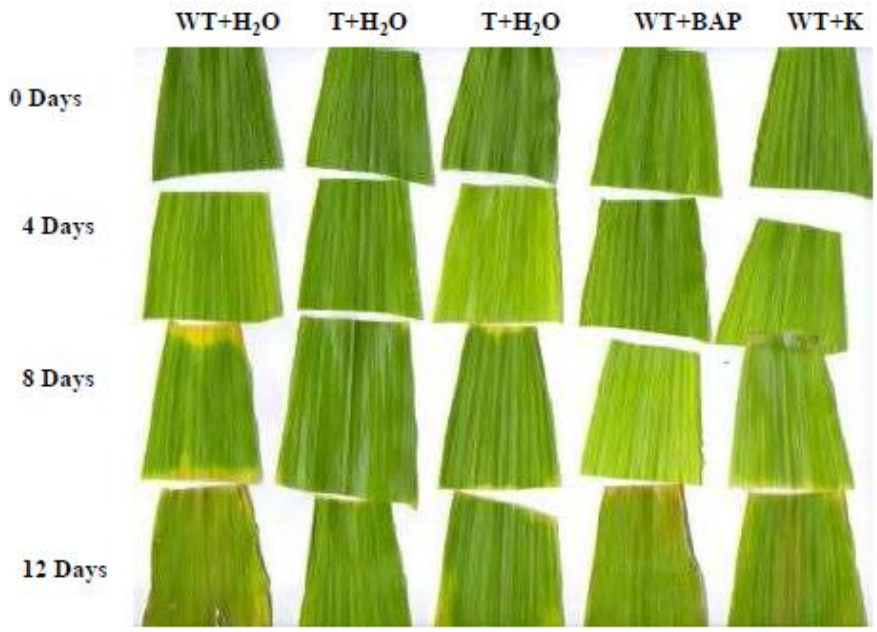

Figure 9. Dark assay of leaf senescence in leaves detached from drought stressed transgenic and wild type plants. W+H_2O: leaf segments detached from wild type plant and kept in water, T+H_2O: leaf segments detached from transgenic plants and kept in water, WT+BAP: leaf segments detached from wild type plants and kept in 5mg.l^{-1} 6-Benzylaminopurine, WT+K: leaf segments detached from wild type plants and kept in 5mg.l^{-1} kinetin.

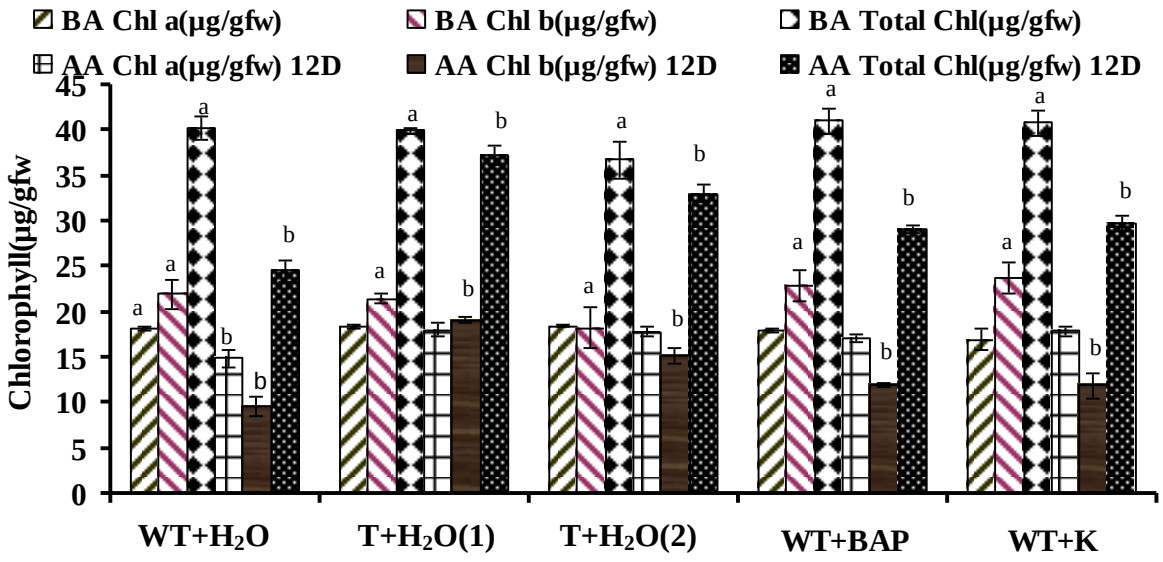

Figure 10. Concentration of chlorophyll a and b, and their total measured in wild type and transgenic leaves before senescence (BS) and 12 days after senescence (DAS) of dark assay. WT+H_2O: wild type leaves incubated in sterile distilled water, T+H_2O(1) and H_2O(2) leaves from transgenic event 1 and 2, respectively, incubated in sterile distilled water; WT+BAP and WT+K: leaves from wild type plants incubated in 5mg.l^{-1} Benzyleaminopurine and kinetin, respectively. Values are mean ±SE (n=3). BS: Before senescence; DAS: Days after senescence.

total shoot dry matter. Data recorded on important phenological and agronomic characters, and seed yield and major yield components in WT and transgenic plants are shown in Tables 2 and 3, respectively. Transgenic

Figure 11. Root architecture of P_{SARK}::*IPTCML216* transgenic (A) and wild type (B) plants after watering/drought/rewatering experiment.

Table 2. Important phenological and agronomic characters of wild type and transgenic plants recorded after watering/drought/re-watering in the glasshouse.

Genotypes	Days to anthesis (No)	Days to silking (No)	ASI* (No)	Plant height (cm)	Ear Height (cm)	Leaf width (cm)	Leaf length (cm)	Total number of leaves/plant
Wild type	96±5.0	110±1.85[a]	14±4.0	145.8±3.9	78.3±3.7	6.5±1.0	54±5.6	15.5±0.53
Transgenic	103.3±6.4	113.8±1.7[b]	10±3.4	143±10.6	72.8±10.0	7.5±0.4	59±6.9	15.3±0.9

Mean values followed by different letter for a particular parameter are significantly different from each other according to LSD test at 5% probability level. Values are mean±SE(n=4), * ASI: Anthesis-silking interval.

Table 3. Seed yield and major yield components of wild type and transgenic plants recorded after watering/drought/re-watering in the glasshouse.

Genotypes	Ear length (cm)	Cob dry weight (g)	Seeds/plant (No.)	1000 seeds weight (g)	Seed yield (g/plant)
Wild type	10.5±2.2[a]	7.8±1.5[a]	8.0±6.9[a]	1.43±2.6[a*]	1.43±2.6[a]
Transgenic	12.13±2.2[b]	19.6 ±3.7[b]	145±4.7[b]	36.2±4.8[b]	44.3±2.9[b]

*Weight of 32 seeds produced per a single plant. Mean values followed by different letters for a parameter are significantly different from each other according to LSD test at 5% probability level. Values are mean±SE(n=4).

plants did not differ significantly from the WT plants in days to anthesis, ASI, plant and ear height, leaf size and number. They, however, had significantly ($p<0.05$) higher major yield components such as ear length, cob dry weight, seed number/plant, hundred seeds weight and seed yield/plant than the WT plants.

The WT plants came to anthesis within 96 days after germination while the transgenic plants extended this to 103 days. The WT plants also produced silks within 110 days that took the transgenic plants 113 days. Hence, in the WT and transgenic plants, there was ASI of 14 and 10 days, respectively. Wider ASI combined with poor receptivity of the late coming silks might have contributed to poor seed setting in the WT plants that produced seed yield of only 1.43 g/plant (Figure 12). On the other hand, the transgenic plants produced significantly ($p<0.05$)

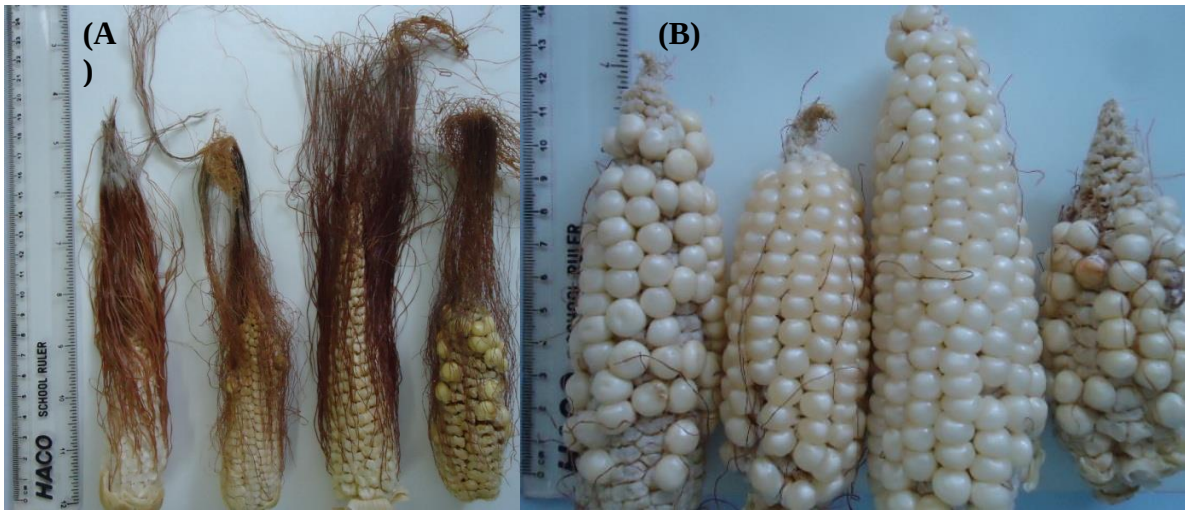

Figure 12. Ears harvested from the wild type (A) and P_{SARK}::*IPTCML216* transgenic plants (B) after watering/drought/rewatering treatments.

higher seed yield of 44.3 g/plant. Significant differences were also observed among the transgenic and WT plants in ear development that led to significantly ($p<0.05$) higher cob dry matter accumulation and ear size (length) in transgenic plants (Table 3).

DISCUSSION

Drought has more damaging effect on maize when it occurs at the most critical growth stages, which coincide with two weeks' time before and after anthesis (Bänziger et al., 2000). The three weeks drought applied in this study extended well to the pre-anthesis stage that marks the beginning of this susceptible growth stage in maize. This could, therefore, yield an insight into the role of *ipt* gene in saving plants and in securing reasonable crop productivity in tropical maize under drought. Glasshouse growth condition that combined sunny days with average day/night temperature of >40/25°C simulated the actual combination of stresses maize plants encounter under the real field condition in tropical environment.

The fact that drought stress inhibited grain formation in most of the WT plants confirms susceptibility of maize to the level of stress treatment applied at pre-anthesis growth stage. This might have happened because of previous morphological and physiological changes that had taken place in response to drought. Initially WT plants launched dehydration avoidance strategies such as leaf rolling and hence reduced leaf area, senescence of bottom leaves and possibly stomatal closure as well. Leaf senescence was much accelerated with crop developmental stages where at post-anthesis growth stage, out of the 16 leaves produced/plant, 14 were either fully or partially affected by senescence in the WT plants while the transgenic plants still had more than 14 leaves/plant fully functional.

Oneto et al. (2016) also transformed the temperate maize inbred line H-II with *ipt* and reported that transgenic plants treated with water deficit for two weeks around anthesis had prolonged total green leaf area, and maintained normal photosynthetic rate and stomatal conductance. With extended drought stress of three weeks imposed before anthesis, the current study yields added information that tropical maize transformed with IPT gene can still withstand drought for extended period of time. Prior to the onset of the senescence syndrome, the WT plants encountered massive chlorophyll degradation as a result of intensive dehydration at the end of the three-weeks drought period.

Gummuluru et al. (1989) reported higher total chlorophyll (chlorophyll a + chlorophyll b) as an indicator of drought tolerance in cereals. Chlorophyll b forms important component of the chlorophyll complex supporting more efficient energy conversion into ATP and NADPH which are then used as sources of energy to build carbohydrates from CO_2 (Bänziger et al., 2000). Hence, chlorophyll degradation in the WT plants can be an indication that photosynthesis might have been inhibited in these plants before the massive leaf senescence was observed. Consequently, the WT plants failed to recover from drought and continued to lose more green leaf area within a period of one month after anthesis/silking.

The P_{SARK}::*IPTCML216* transgenic plants demonstrated rapid recovery from dehydration may be as a result of total and chlorophyll b content that were maintained at higher levels during the drought period indicating chloroplast integrity even under dehydration. Peleg et al. (2011) reported similar results in rice. Faster replenishment of tissue water content after re-watering once with 1 L of water substantiated that carbon

assimilation was taking place in these plants in a manner similar to pre-dehydration. As a result, more grain yields were obtained in the P_{SARK}*::IPTCML216* plants.

Bolaños and Edmeades (1993) and Edmeades et al. (1999) reported narrow ASI as an indicator of tolerance to drought stress in maize. The relatively narrower ASI obtained with the P_{SARK}*::IPTCML216* transgenic maize plants could then indicate the role of *ipt* gene in enhancing tolerance to drought. Drought delays silk growth as opposed to the tassel, interfering with allocation of assimilates to ears, ovules and silks (Edmeades et al., 1993) and hence removes the synchrony of the male and female organs to the extent of inhibiting seed setting. To establish the damaging effect of drought on grain yield, all plants were self-pollinated except two WT plants that were sib-pollinated by fresh pollen from WT plants grown under optimal watering. Even then, seed setting did not improve showing that the late coming silks were not receptive enough for fertilization to take place. Otegui et al. (1995) reported that applying fresh pollen from unstressed plants did not improve seed setting in late coming silks in maize supporting lack of seed setting in the WT plants is related to poor receptivity of the late appearing silks instead of poor pollen viability.

Pospíšilová et al. (2005) reported that abscisic acid (ABA) and cytokinin (CK) contents have inverse relationship in plants under drought condition. Drought treatment reportedly encouraged increased ABA content in both WT and P_{SARK}*::IPT* tobacco plants without any effect on CK content indicating that changes in ABA was not related with drought tolerance displayed by transgenic tobacco plants (Rivero et al., 2007). In the context of maize, Liu et al. (2005) reported an increase in ABA content in the reproductive organs to play a role in yield reduction in response to drought stress. In former studies, the ABA concentration of the ovary was reported to increase substantially as the result of pre-flowering stress compared with irrigated maize plants (Ash et al., 2001) revealing the possibility that ABA may play a role in the abortion of female flowers.

In the current study, high ABA concentration might have weakened, the receptivity of the female flowers in the WT plants as silk growth was not affected. In the P_{SARK}*::IPTCML216* plants increased level of CK might have antagonized the negative effect of ABA. Pospíšilová et al. (2005) reported application of benzyladnine inhibits water stress induced accumulation of ABA in maize. Substantial seed setting achieved in the transgenic plants despite delayed anthesis could therefore be attributed to the protective role of CK under drought stress (Rivero et al., 2009).

In maize, the upper 8 to 9 leaves contribute the lion's share of the assimilate fed to the sink (Allison and Watson, 1996). Loss of these leaves had negative effect on productivity of WT plants. Drought inducible expression of *ipt* gene has sufficiently shown itself to improve drought tolerance by delaying loss of these leaves through senescence in tropical maize. *In vitro* dark treatment of leaf segments has yielded results supportive to this effect. Peleg et al. (2011) report delayed senescence in P_{SARK}*::IPT* transgenic rice plants under pre-anthesis drought for 15 days and in flag leaf sections treated in dark from the same plants. Contrary to this report, however, 12 days of incubation in externally applied CK did not keep WT leaves greener than leaves from the transgenic plants. This could be due to concentration effect of the externally applied CK or uptake of externally applied CK by maize leaf tissues that may not be as efficient as in rice leaf tissues (Gan and Amasino, 1996).

Cytokinin delays senescence through its diverse influences on many plant metabolic processes (Smart et al., 1991). During senescence, genes encoding enzymes such as RNases, proteinases and lipases that degrade RNA, proteins and lipids, respectively, are expressed (Gan and Amasino, 1997). Cytokinin delays senescence by inhibiting transcription of these genes (Buchanan-Wollaston, 1997). Rivero et al. (2007) reported that the protective role of CK under drought comes through enhancement of stress-related gene expressions encoding antioxidant enzymes playing role in ROS scavenging. They further elaborate that suppression of drought-induced leaf senescence in the transgenic plants is also accompanied by enhanced expression of stress-response transcripts, such as dehydrins and heat-shock proteins, possibly also contributing to the enhanced tolerance and suppressed ROS concentrations in the transgenic plants during drought.

In this study, total carotenoids content increased significantly in the WT plants following maximum dehydration (57% RWC) at the end of the three-weeks drought period. Munné-Bosch et al. (1999) reported an increase of 26% in leaf carotenoid content in rosemary plants grown under drought during hot summer season at midday when plants were dehydrated to less than 50% RWC. Carotenoids are non-enzymatic antioxidant quenching activated oxygen species. Its substantial increment in the WT plants under drought may indicate that the level of this antioxidant was insufficient before drought treatment to guarantee protection against oxidative stress by quenching reactive oxygen species.

Mannose based selection system increases transformation frequencies compared to selection system based on antibiotics like kanamycin (Joersbo et al., 1998) which may adversely affect the growth of transformed plant cells through release of toxic compounds from necrotic non-transformed tissues (Lindsey and Gallois, 1990). Higher transformation frequency observed with *pmi* selection could be attributed to the metabolic advantage with mannose selection and absence of any toxic metabolites released from the dying non-transformed cells. In wheat transformation, *pmi* is preferred to *bar* gene that confers resistance to the herbicide bialaphos (Gadaleta et al., 2006). Gadaleta et al. (2008) reported *pmi* as a useful marker even when used as minimal gene cassette in a linear DNA fragment.

In the current study, mannose based *Agrobacterium*-meditated transformation of the tropical maize inbred line CML216 yielded transgenic plants at transformation frequency of 1.7 per cent. This is very low when compared to transformation frequencies of temperate maize. Wright et al. (2001) report average transformation frequency of 45% for recovery of transgenic maize via particle bombardment using the *pmi* gene with mannose as a selective agent.

Negrotto et al. (2000) also reported transformation frequency of 32% through *Agrobacterium*-mediated transformation of the temperate inbred A188 using the same selection system. While genotypic differences could be one of the factors playing the leading role in causing these differences, poor competence of the immature zygotic embryos has also been well known to contribute to low transformation frequency (Negrotto et al., 2000). The desirable aspect of the *pmi*/mannose based selection system in the current study is its stringency in identifying transformed plants as revealed by transformation efficiency of 100%. Such high selection efficiency can be an advantage in saving time and resources that would otherwise be needed to handle plants that are not actually transformed or escapes.

The *pmi* gene is ubiquitous in nature and has been cloned from several bacteria and yeast species. Its use as selectable marker is, therefore, not expected to cause any hazard to environment. Furthermore, safety assessment of PMI reports no toxicity to mammals and no undesirable effects on the agronomic or nutritional composition of transgenic plants (Reed et al., 2001). Studies have proved that this gene causes no allerginicity to consumers and no undesirable agronomic traits in transgenic maize developed using *pmi* gene as plant selectable marker. Moreover, mannose is the cheapest and a 'user-friendly' selection agent for use in plant genetic transformation (Wright et al., 2001). Hence, commercial cultivation of the transgenic line developed in this study should not be suspected to bring negative effects to the environment or to the consumers.

The *ipt* gene has been used to develop transgenic temperate maize for delayed leaf senescence under the control of the senescence activated SAG promoter (Young et al., 2004) and the native promoter of the senescence enhanced (*SEE*) maize gene (Robson et al., 2004). Under the control of the SAG promoter, *ipt* gene could not delay leaf senescence in transgenic plants though RT-PCR showed expression of its transcript (Young et al., 2004). With the native, *SEE* maize promoter transgenic plants displayed delayed leaf senescence when grown under nitrogen stress condition. The major problem with this system was nitrogen deficiency in the upper younger leaves as a result of cytokinin accumulation in the lower senescing leaves that caused nitrogen immobilization (Robson et al., 2004). As a result, the transgenic plants could not be proposed for cultivation in soils poor in nitrogen. The drought inducible SARK promoter was found to be the appropriate promoter driving the *ipt* gene expression resulting in the expected delayed leaf senescence in the transgenic maize under drought condition. Nitrogen deficiency in the upper younger leaves was not a problem with this promoter, as the gene expression is not limited to lower leaves but in all plant tissues facing drought stress (Rivero et al., 2007). In addition, the older lower leaves showed senescence, which caused nitrogen mobilization to the younger leaves. Transgenic plants were normal and the source/sink relationship was not affected. As reported by Rivero et al. (2007) and Peleg et al. (2011) P_{SARK}*::IPT* transgenic tobacco and rice plants differed from their respective WT plants exclusively in delayed leaf senescence. In this study, transgenic and WT plants had distinct root morphological architecture in addition to differences in delayed leaf senescence, where the transgenic plants had multi-branched fibrous roots as opposed to the few long primary roots dominating the WT plants.

In transgenic *Arabidopsis* plants transformed with *ipt* gene under the control of the promoter region from a maize gene encoding a heat shock protein (HSP70), similar results were reported showing root hairs emerging closer to the root tip suggesting a reduction in elongation zone in the transgenic plants (Medford et al., 1989). Such increased root branching and enhanced fibrous root formation was reported under condition of low CK content in CK-deficient transgenic plants which over express the cytokinin oxidase/dehydrogenase (*CKX*) genes that result in an enlarged root meristem and formation of lateral roots closer to the root apical meristem (Schmülling et al., 1989). Hence, the factor that caused increased volume and growth of branched root in transgenic maize plants showing enhanced expression of CK could not be established in this study and can be considered as an area of interest for further investigation. However, whatever the cause may be, this root architecture might have contributed positively to the drought tolerance exhibited by the transgenic plants by enhancing nutrient mining and water absorption from the soil.

The *ipt* gene has shown itself to improve drought tolerance by delaying leaf senescence in tropical maize. This can be considered as a breakthrough in improving drought tolerance in much faster manner compared to conventional breeding that takes long time with unpredictable outcome. Maize produces CK naturally, and increasing the level of this hormone by over expressing the *ipt* gene ectopically is not expected to cause any safety problem to the consumers and the environment. Considering all the positive aspect of the transgenic maize developed in this study, future research should focus on further evaluation of transgenic maize in confined field-trials for tolerance to drought. The line can be used as a source of the transgene for improving drought tolerance in commercial and well-adapted maize genotypes through conventional breeding. The *ipt* gene

can be used further to improve drought tolerance in important tropical monocot and dicot crops.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work is part of the research project on Genetic Engineering of Maize for Drought Stress Tolerance in East and Central Africa funded by the United States Agency for International Development and the Multi-donor Trust Fund of the World Bank to the Association for Strengthening Agricultural Research in East and Central Africa. The tropical maize inbred line used in this study was kindly provided by the International Maize and Wheat Improvement Centre.

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