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Agrobacterium-Mediated Transformation of Selected Kenyan Maize (*Zea mays* L.) Genotypes by Introgression of *Nicotiana* Protein Kinase (*npk*1) to Enhance Drought Tolerance

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Abstract

Currently maize production in the East and Central Africa (ECA) region is at 1.3 tonnes per hectare compared to the potential of up to 7.0 tonnes per hectare because of biotic and abiotic constraints, and this has resulted in prevalence of famine in sub-Saharan Africa. Drought is the most important abiotic stress affecting productivity of maize in Sub Saharan Africa leading to up to 70% crop loss and in certain cases total crop loss. Previous work has shown that Mitogenic Activated Protein Kinase (*MAPKKK*) gene activated an oxidative signal cascade, which led to tolerance to adverse condition. To analyze the role of the oxidative signal cascades on tropical maize, 4 transgenic tropical maize plants were developed through an *Agrobacterium*-mediated transformation with a *MAPKKK* homology from tobacco Nicotiana Protein Kinase 1 (*npk*1) and the insert was confirmed using Southern and Northern blot hybridization analysis. Fertile T_o maize plants were obtained which were planted to generate T_1 plants, which were used for comparison with non-transgenic plants. The T_1 plantlets of tropical inbred TL08-(2)4, single hybrid cross of a PTL001, a multiple cross hybrid DH01 and a dry land cultivar DLC1 genotypes were planted in the greenhouse and assessed for morphological and physiological changes associated with increase in drought stress tolerance when under water stress condition. The results showed that *npk*1 effectively enhanced drought tolerance in TL08-(2)4 and PTL001, and there was no significant morphological difference between transgenic controls (well watered) and transgenic tests (subjected to moderate drought stress). Overall, there were between 20% - 35% enhancements of yield of the transgenic stressed

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events compared with non-transgenic stressed control.

Keywords

Agrobacterium tumefaciens*; Drought Tolerance; *Nicotiana* Protein Kinase; Southern Blot Hybridization; *Zea mays

1. Introduction

Despite maize being the staple food for most of the population in the ECA region and an important crop as a source of income, animal feed, manure and industrial uses, maize production has often been inadequate. In Kenya for example, maize yield and production are 1.7 tons/ha and 2.7 million tons annually which are not adequate to meet its demand due to rapid population growth, making it a net importer of 427,000 tons of maize per annum [1] [2] This inadequacy is due to abiotic stress, such as drought, aluminum toxicity, or scarcity of nutrients; biotic stress being mainly pests and diseases [2]-[5].

Drought is a major abiotic stress affecting productivity of maize in Africa leading to up to 70% crop loss and in certain cases total crop loss [6]. It causes up to 24 million tons of yield loss in maize worldwide annually with the greatest impact being in Sub Saharan Africa (SSA) where erratic rain pattern and poor farming methods are common [7]. Drought reduces productivity by inhibiting plant growth and photosynthesis [8]. A correlation between agronomical traits and the rate of photosynthesis has been reported in several studies [9]-[11].

Studies by Shou *et al*. [12], have shown that a mitogen activated protein kinase kinase kinase (*MAPKKK*) gene activated an oxidative signal cascade that led to freezing, heat and salinity stress tolerance in transgenic tobacco. Nicotiana Protein Kinase (*npk*1) a tobacco *MAPKKK* gene has been isolated, characterized and expressed constitutively in temperate maize lines and the transgenic products able to greatly withstand adverse drought condition [12]. The *npk*1 is located upstream of the oxidative pathway and can induce expression of HSPs and GST1 in Arabidopsis and maize [13]. Activation of these stress genes could protect the photosynthesis machinery of plants from damage caused by drought, thereby improving the yield potential of the major cereal crop such as maize.

Transgenic events have been achieved via *Agrobacterium tumefaciens* and using gene gun [12]. The use of *Agrobacterium* as a vector of introgression of foreign genes has been favoured due to the ability to transfer large DNA fragments in low copy numbers [14] [15]. However, similar studies have not been carried out in tropical maize, which is the basis of this study. Thus the objective of this study was to transform tropical maize with *npk*1 gene, a drought tolerant gene according to the procedure described by Ombori *et al*. [16]. The selected *Agrobacterium*-mediated transgenic maize events were subjected to drought stress and the impact of *npk*1 drought gene on the morphological and physiological changes associated with drought stress in tropical maize was assessed.

2. Materials and Methods

2.1. Plant Materials

Seeds of TL08, KAT, PTL001, DH01 and DLC1 maize genotypes were planted at weekly intervals beginning 12 September 2008 (total of four plantings) in the screen house of the Biosafety Level II at Kenyatta University. Plants were either selfed or crossed to generate inbred, hybrid, hybrid selfed (F_2) or backcross-derived embryos or mature seeds. Ears were harvested when the immature zygotic embryos were 1 - 2 mm for excision.

2.2. DNA Constructs and Maize Transformation

Agrobacterium strain EHA101 harboring a binary vector pSHX004 [17] was used to transform the selected maize immature zygotic embryos as described by Ombori *et al*. [16]. T_o transgenic plants were then selfed to produce T_1 seeds.

2.3. DNA Extraction and Polymerase Chain Reaction Analysis

PCR analysis was performed on DNA extracted using the CTAB and salt extraction method [18] and its modifi-

cation [19].

2.4. Southern Blot Hybridization Analysis

Forty microliters of maize DNA samples were digested with *Stu1* and *EcoR*1 (NEB, Beverly, Mass. USA). The digested DNA was loaded onto 0.8% agarose gel (SeaKem LE FMC, Rockland, Me. USA) and electrophoresed for 12 hrs at 20 V and 15 mA. The gel was blotted onto a nylon membrane (Bedford, Mass., USA) overnight in 2X SSC. Traces of the gel were then removed from the nylon membrane, air dried at room temperature and cross-linked for 30 - 45 seconds.

Hybridization was preceded by a pre-hybridization for 1hr at a temperature of 68˚C using pre-hybridization buffer (30 ml of 20x SCC, 10 ml of 50x Denhardts reagent and 1ml of 20% SDS and dilute to 100 ml with dH_2O). Hybridization step involved addition of 10 µl denatured biotinylated *npk*1 probe to the pre-hybridization buffer and left in the hybridization chamber for 12 hrs.

After hybridization the membrane was washed in 0.1x SSC and 0.1% SDS for 30 min at a temperature of 60˚C and then incubated in blocking solution (5% SDS; 125 mMNaCl; 25 mM sodium phosphate, pH 7.2) for 5 min at room temperature with moderate shaking. This was followed by incubation for 5 min in streptividin and biotinylated alkaline phosphatase in blocking buffer. Each of the above steps was preceded and ended by a washing step before and after incubation. Two and a half mM CDP-star stock diluted with 1x CDP-star diluent was then used to detect DNA. The membrane was then exposed to X-ray film in the dark room for 5 - 10 seconds. The X-ray film was then developed for 5 - 15 min for the detection of the probe hybrid signal.

2.5. RNA Extraction and RT-PCR

Approximately 100 mg of fresh young maize leaves harvested from greenhouse-grown plants were used to extract total RNA using Plant RNeasymini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Extracted total RNA was treated with RNase-free DNase (1 unit for 30 min at 37˚C; Invitrogen) to ensure complete removal of genomic DNA. Reverse transcription PCR (RT-PCR) amplification was performed with the SuperScript First-Strand Synthesis System kit (Invitrogen, San Diego, CA) according to manufacturer's instructions with oligo (dT) 12 - 18 primers. One µg of purified total RNA was used as a template for the synthesis of cDNA. Twenty-five µl limited cycle PCR reactions were set up as follows: 2.5 µl of 10× PCR buffer (Invitrogen), 0.75 µl of 50 mM $MgCl_2$ (Invitrogen), 0.5 µl of 10 mMdNTP mix (Invitrogen), 1.25 µl of 10 µM forward and reverse primers, 0.15 µl of 5 u/µlTaq DNA Polymerase (Invitrogen); 1.5 µl of cDNA template and 18.35 µl of sterile distilled water. The primers used to amplify a 646 bp *npk*1 transcript: forward $^{5'}$GGC TGC AGG AAT TCT CAC ATGT-$^{3'}$ and reverse $^{5'}$GCT CCC GAA GTC ATT CTG CA-^{3}. PCR reactions were performed using the following conditions: initial DNA denaturation for 3 min at temperature of 94˚C, followed by 30 amplification cycles (denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s and extension at 72˚C for1 min 15 s) and final extension at 72˚C for 2 min.

2.6. Northern Blot Hybridization Analysis

Ten microlitres of extracted total RNA was pipetted and 5 µl of sterile water was added to increase its total volume to 15 µl. The RNA was then denatured by heating the samples at a temperature of 60˚C for 20 min, then snap cooling on ice. Samples were then loaded into the wells and run at 100 volts for 2 hrs. The gel was stained with ethidium bromide, viewed and photographed to determine the quality. This was used later in matching the position of the ribosomal markers on the photographed with the final X-ray film of the hybridized mRNA. This step was followed by blotting of the gel onto a nylon membrane (Bedford, Mass., USA) overnight in 2x SSC. The rest of the hybridization and detection steps were as per southern blot hybridization analysis. The gel was probed for *npk*1 gene with a biotin labeled *npk*1 probe for the four transgenic events DH01-1, TL08-(2)4, DLC1-8 and PTL001-17. RNA from the respective non-transgenic genotypes was run beside the transgenic events and used as control.

2.7. Progeny Test

Seeds of T_1 plants from *Agrobacterium* derived events of TL08, single cross hybrid PTL001, hybrids DH01 and cultivar DLC1, were germinated in potted soil and grown for 14 days in the glasshouse. DNA was isolated from

the leaves of the plants and screened for the presence of the transgene by PCR analysis. DNA extraction protocol, PCR primers and reaction conditions were as described previously. Southern blot hybridization analysis was carried out on PCR positive events to confirm stable integration of the *npk*1 gene.

2.8. Evaluation of Phenotypic Adaptation and Biomass of the Seedling Subjected to Drought Stress

Transgenic maize seedlings from each of the transgenic events along with non-transgenic control plants of the same seeds were selected and planted into pots containing loam soil. The plants were well watered daily for germination to take place. Two week-old seedlings were grown for 2 weeks at a temperature of 28˚C during day time and 21˚C at night with water supply before the assessment of the effect of water stress. Evaluation of tolerance against drought stress was performed by assessing the phenotypic changes of a seedling in which water supply was withheld for 2 days in the 1st week and 3 days in the 2nd week and the control were subjected to the same condition. The plants were then grown with a water supply for 2 weeks. All drought-stress evaluation experiments were conducted in a glasshouse with 16 hrs photoperiod and temperatures of 28˚C during day time and 21˚C at night. After 28 days, plant height, length from the first basal leaf to the one immediately above, and leaf width were measured. This was carried out for both the transgenic and the non-transgenic plants. Survival of plants was observed and recorded after the 1st week of recovery. After the 1st week of recovery, the aerial parts of transgenic plants and the surviving controls were detached and weighed immediately and then placed on a laboratory bench at a temperature of 25˚C.

Detached aerial parts were removed and then dried at a temperature of 80˚C for 24 hr to determine final dry weight (DW). Water content was standardized as a percentage relative to the initial water content of aerial parts of the plant and calculated as follows: $[(FW_i - DW)/(FW_0 - DW)] \times 100$, where FW_i and FW_0 are fresh weight for any given interval and original fresh weight, respectively. Four T_1 plants per line were used for each test. All tests were set up in a batch of three experiments.

2.9. Evaluation of Drought Stress

Plants were divided into two groups just before the drought stress (DS) period. The drought stress period was timed so that the stress period was predicted just before flowering and no further watering was done [20]. This was carried out on the transgenic events of the selected genotypes while the non-transgenic group was maintained under well-watered (WW) conditions in which water was added daily and the soil kept moist.

Plant height and leaf numbers/width of maize plants were measured at the tassel stage and repeated after one week until there was no change in plant height, when the silk had dried up and ear filling was complete (full maturity). Seed weights from each of the treated plants were measured after harvesting.

2.10. Data Analysis

Student T-test was then used to compare mean of the transgenic and the non-transgenic seedling of the selected genotypes so as to determine the effect of *npk*1 gene under drought stress condition.

3. Results

3.1. Transformation with npk1 for Drought Tolerance

Callus from the four genotypes (PTL001, DLC1, DHO1 and TL08) had different survival rates on bialaphos selection media with DH01 having the highest at 11.88% and TL08 with 2.24% as the lowest (**Table 1**). The total number of the putative transformants of the four genotypes formed plants at different efficiencies in which the highest was DH01 with the 81.81% and TL08 the lowest at 18.18%.

3.2. PCR analysis of Putative Transformed Events

*Assessment of the npk*1 *gene in putatively transformed plants*

The presence of *bar* gene in the genomic DNA of the putative transformed plantlets was confirmed by PCR amplification of a 646bp *npk*1 gene fragmentin the T_1 plants (**Figure 1**). The plants labeled TL08-3, TL08-4, DHO1-1, DH01-2, DH01-4, DLC1-6, DLC1-8 and PTL001-17 were positive for *npk*1 gene.

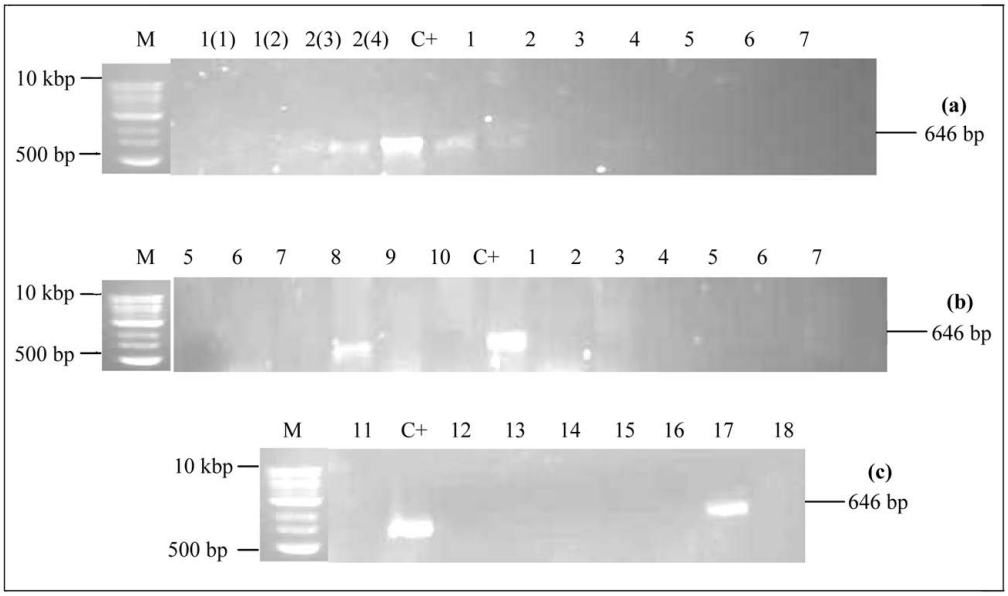


Figure 1. Amplification of *npk*1 gene in T_1 plants. M, 1Kb Ladder; C+, pSHX004 plasmid DNA. (a), Lanes 1(1), 1(2), 2(3), 2(4), DNA samples of TL08 event; Lanes 1 - 7, DNA samples of DH01 events, (b), Lane 5 - 9, DNA samples of DLC1 events; Lanes 1 - 7, DNA samples of PTL001 events. (c), Lanes 12 - 18, DNA samples of PTL001 event.

Table 1. Regeneration of putative transgenic events from the four maize genotypes.

Genotype	Total number of embryos inoculated	Number (%) of surviving callus	Number of callus forming plants	Total number of putative transformants T_0
PTL001	509	33 (6.54)	11	9 (81.81)
DLC1	401	10 (4.01)	14	10 (71.42)
DHO1	542	59 (11.88)	20	16 (80.00)
TL08	442	9 (2.24)	11	2 (18.18)

Value in paranthesis shows the percentage.

3.3. Progeny Test of Selected Four Transgenic Plants

Out of the twenty seeds of each of the four selected genotypes planted, 76.46% of PTL001-17, 55.55% of DLC1-4, 71.42% of DH01-1 and 80.00% of TL08-(2)4)4 of the plantlets that survived had the *npk*1 gene (**Table 2**). The percentage of plants, which were positive for the *npk*1 gene in the T_1 progeny of PTL001-17 and DH01 indicated that the gene was inherited and segregated in the 3:1 Mendelian ratio for single locus integration. This was not true to the transgenic events from DLC1 and TL08.

3.4. Southern Blot Hybridization Analysis

Southern blot hybridization using biotin labeled PCR *npk*1 probe confirmed the integration of *npk*1 gene into the genome of DH01-1, TL08-(2)4, PTL001-17 and DLC1-8 T_1 maize plants obtained from immature zygotic embryos infected with EHA101 (pSHX004) (**Figure 2**). The four events had between 1 - 5 copies of the *npk*1 transgene when digested with *Stu1*, which has a single restriction site on the *npk*1 gene. When digested with *EcoR*I, which had two restriction sites within the left and right border, it resulted to 1 - 8 copies and 1.8 kb fragment was distinct.

3.5. Northern Blot Hybridization Analysis

The *npk*1 gene was found to be stably intergraded into the four transgenic events, DH01-1, TL08-(2)4, DLC1-8 and PTL001-17 (**Figure 3**). The signal was not dectected in the control plants.

Table 2. Progeny test of T_1 plants.

Genotype	Number of T_0 seeds PCR +ve for *bar* gene that survived	Number of T_1 plants +ve for the *gene*	% of plants that survived and +ve for the *npk*1 gene	Segregation ratio
PTL001-17	17	13	76.46	3:1
DLC1-8	18	10	55.55	1:1
DH01-1	14	10	71.42	3:1
TL08-(2)4	10	8	80	4:1

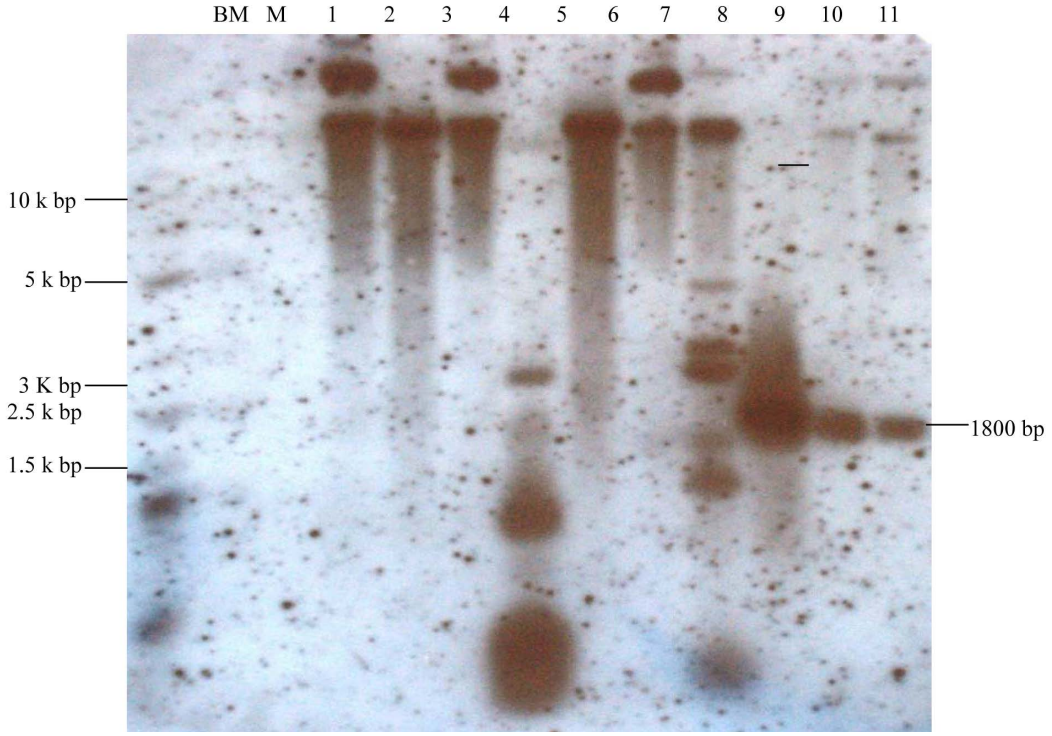


Figure 2. Southern blot hybridization analysis with *npk*1 probes, of transgenic maize events DH01-1, TL08-(2)4, DLC1-8 and PTL001-17. BM, 1 kb biotinylated marker; M, 1 kb Ladder. The DNA was digested with *Stu1*: Lane 1, NT; Lane 2, DH01-1; Lane 3, TL08-(2)4; Lane 4, DLC1-8; Lane 5, PTL001-17 and Lane 6, NT spiked with pSHX004). DNA digested with *EcoR*I: Lane 7, DH01-1; Lane 8, PTL001-17; Lane 9, DLC1-8; Lane 10, TL08-(2)4 and Lane 11, NT spiked with pSHX004 plasmid). The selected genotypes showed integration of the *npk*1 gene into their genome.

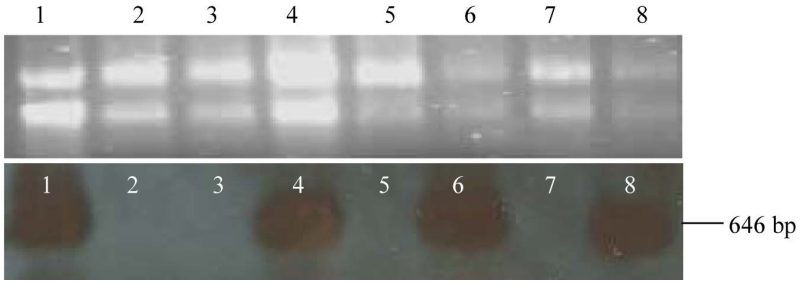


Figure 3. Northern blot hybridization analysis below the ethidium bromide gel of RNA quality. Lane 1, DH01-1; Lane 2, DH01 (NT); Lane 3, PTL001 (NT); Lane 4, PTL001-17; Lane 5, DLC1 (NT); Lane 6, DLC1-8; Lane 7, TL08 (NT); Lane 8 TL08-(2)4. The RNA gel was probed with biotin labeled 646 bp *npk*1 probe.

3.6. Transgene Detection by RT-PCR

The cDNA synthesized from total plant RNA from selected four genotypes showed the presence of *npk*1 transcript as a result of PCR amplification of 646 bp (**Figures 4(a)** and **4(b)**). Different intensities of the transcription levels were observed in the four transgenic events DH01-1, TL08-(2)4, DLC1-8 and PTL001-17.

3.7. Drought Tolerance of Seedlings

The drought stressed transgenic (T_1) seedling showed different phenotypic adaptations associated with drought tolerance when compared with the non-transgenic seedling (**Figure 5**). Among the DH01-1 event, the non-trangenic (control) plants had pale green almost yellow shrivel leaves as compared to the transgenic plants which had green leaves. There was also retarded growth among the control plants while the transgenic plants were relatively taller and exhibited leaf rolling pattern.

The significant morphological feature among the DLC1 plants was logging due to poor rooting and weak stem in non-transgenic controls as compared with transgenic (T_1) DLC1-8 seedlings (**Figure 6**). The non-transgenic plants had drooping leaves and on re-hydration most of the stressed non-transgenic plants did not survive.

Overall the transgenic plants of the four genotypes were resilience to drought stress condition compared to the controls. The difference in the phenotypic characteristics of the experiment and the control was clear as seen among DH01-1 (**Figure 7**).

3.8. Evaluation of TL08-(2)4 Seedlings

Significant differences were detected in the seedling height of eight TL08-(2)4 transgenic (T_1) plants when

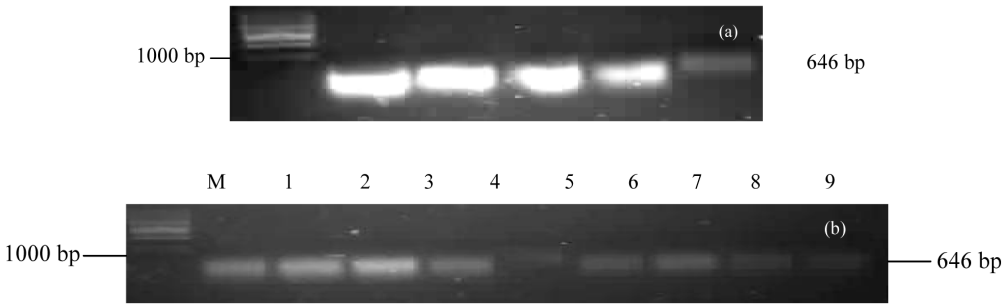


Figure 4. RT-PCR resolution of amplified *npk*1 gene in transgenic event. a: M, 1 kb Ladder; Lanes 1 - 4, DH01-1 and Lane 5, pSHX004 plasmid (+ve control). b: Lanes 1 - 2, TL08-(2)4; Lanes 3 - 4, DLC1 - 8; Lane 5, pSHX004 plasmid (control) and Lanes 6 - 9 PTL001-17.

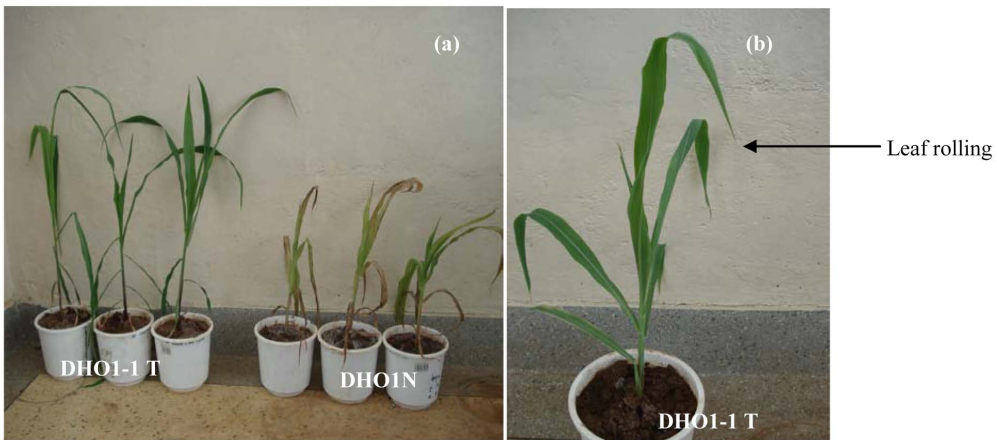


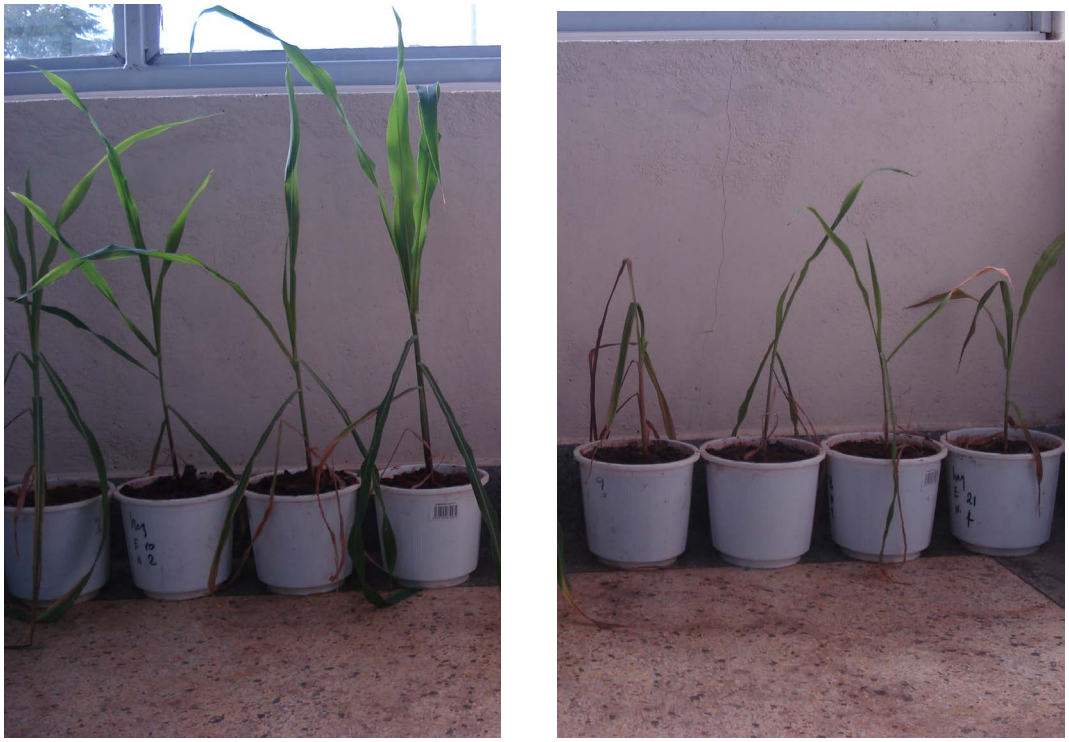
Figure 5. Plants subjected to water stress. (a), Green transgenic T_1 and pale green non-transgenic (control) seedlings with shrivel leaves and retarded growth. (b), Leaf rolling in drought stressed transgenic seedling.

compared to the non-transgenic (control) ($p < 0.05$). The average height of transgenic plants was 39.4 cm and the non-transgenic plants average was 31.2 cm (**Figure 8**). There was no significant difference ($p < 0.05$) in the leaf width and internode length among the transgenic and the non transgenic control of TL08.

3.9. Evaluation of PTL001-17 Seedlings

The pattern exhibited by TL08-(2)4 T_1 plants was repeated in thirteen of the single cross PTL001-17 T_1 plants as there was a significant difference in the seedling height between the non-transgenic (control) and transgenic (T_1)

Figure 6. Logging due to poor rooting and weak stem in non transgenic controls compared with transgenic seedlings subject to drought stress.



(a) (b)

Figure 7. Seedlings subjected to drought stress. (a),Transgenic(DH01-1 T); (b), Non transgenic (DHO1 NT).

plants ($p < 0.05$). The transgenic plants had an average of 32.2 cm and the non-transgenic 21.4 cm (**Figure 9**). There was no significant difference ($p < 0.05$) in the leaf width and internode length among the plants, which survived drought stress.

3.10. Evaluation of DH01-1 Seedlings

Of the ten DH01-1 transgenic (T_1) plants, there was no significant difference ($p > 0.05$) in leaf width within the transgenic plants and non-transgenic controls. There was a significant difference in height ($p < 0.05$) between the non transgenic and transgenic plants. The transgenic plants had an average height of 42 cm and the non transgenic plants 33.5 cm (**Figure 10**). There was also a significant difference ($p < 0.05$) in the seedlings internode between the transgenic and non transgenic plant. The average length of the transgenic seedling almost doubled the non transgenic control plants.

3.11. Evaluation of DLC1-8 Seedlings

Among the ten DLC1-8 transgenic (T_1) seedling which survived, there was no significant difference ($p < 0.05$)

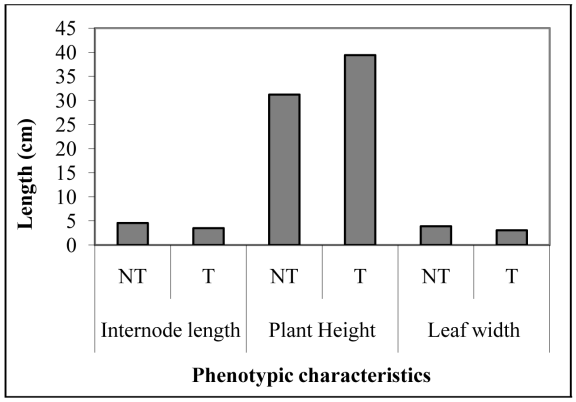


Figure 8. Average inter-node length, plant height and leaf width of selected TL08-(2)4 transgenic and non-transgenic maize seedling under drought stress. T, Transgenic (T_1) plants; NT, Non transgenic (control) plants.

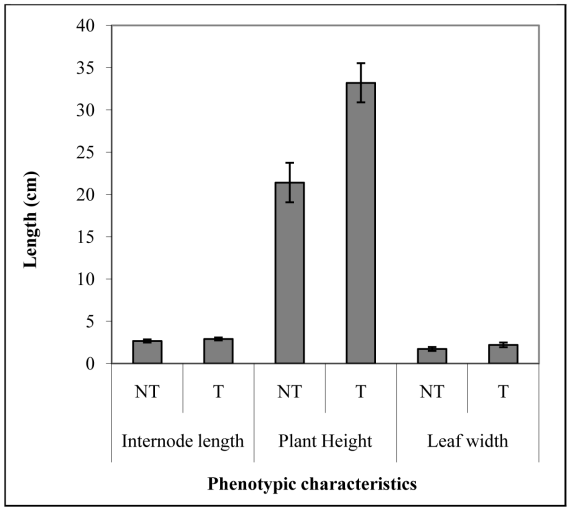


Figure 9. Average inter-node length, plant height and leaf width of selected PTL001-17 transgenic and non-transgenic maize seedling under drought stress. T, Transgenic (T_1) plants; NT-Non transgenic (control) plants.

in the three phenotypic features internode length, plant height and leaf width when compared with the non transgenic controls. The transgenic plants were though taller compared to the controls as the transgenic plants had an average length of 31.4 cm while the non-transgenic plants had an average length of 29 cm (**Figure 11**).

3.12. Evaluation of Water Loss in Detached Aerial Parts of Seedlings

Evaluation of water loss in TL08-(2)4

The average water retaining capacity of transgenic (T_1) TL08-(2)4 was higher compared to the non-transgenic (control). The rate of water loss in the transgenic plants was slower compared to the control. At 120 min post detachment, the control had lost about 62% of its water content while the transgenic had lost less than 30% of its water content (**Figure 12**). There was a drastic increase in the % of water loss after 320 minutes in the transgenic plants.

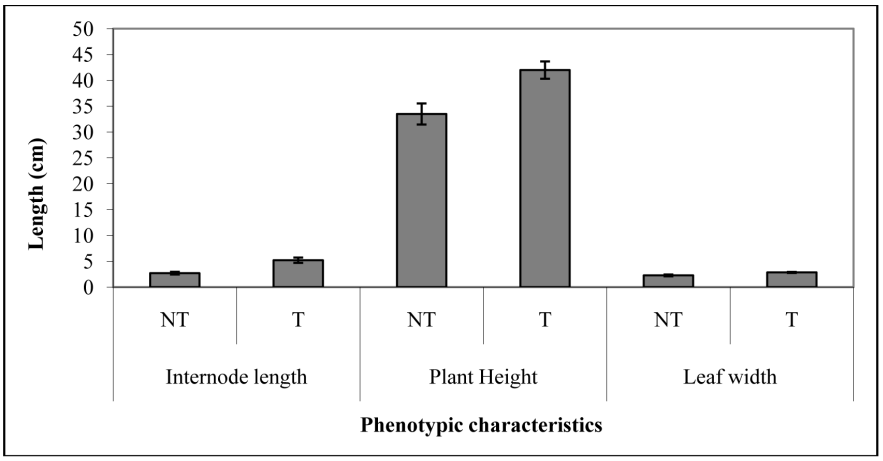


Figure 10. Average inter-node length, plant height and leaf width of selected DH01-1 transgenic and non-transgenic maize seedling under drought stress. T, Transgenic (T_1) plants; NT, Non transgenic (control) plants.

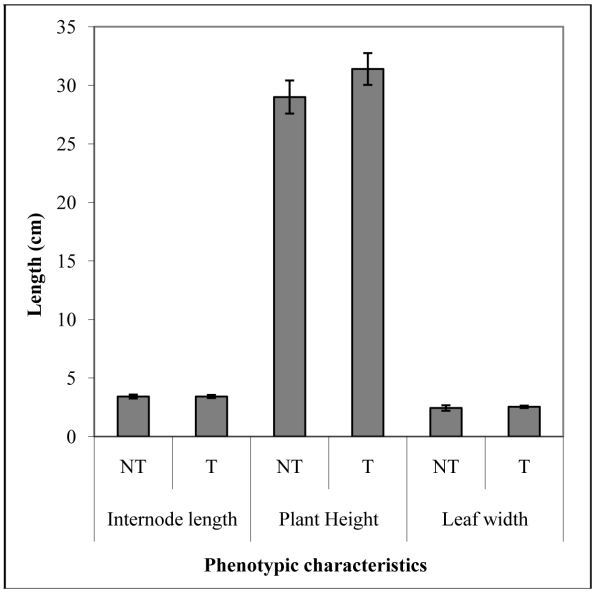


Figure 11. Average inter-node length, plant height and leaf width of selected DLC1-8 transgenic and non-transgenic maize seedling under drought stress. T, Transgenic (T_1) plants; NT, Non transgenic (control) plants.

Evaluation of water loss in PTL001-17

The relative percentage water loss was lower in transgenic PTL001-17 seedling compared with the non-transgenic (control). The non-transgenic seedling had lost about 50% of its water content after 80 min while the transgenic seedling had its water reduced to 50% after 3 hrs 20 min. The water loss after 320 min was much faster in transgenic seedling decreasing from 42.47 to 15.84 while in the non-transgenic seedling, it decreased from 29.24 to 16.76 after 1280 min under the same condition (**Figure 13**).

Evaluation of water loss in DHO1-1

Among the transgenic seedling, relative water loss was very slow at the beginning and a significant reduction ($p > 0.05$) in water content became visible after 2 hrs. Over the same period of time there was a significant decrease in the water content of the non transgenic seedling with a 20% water loss in the first 40 min. The transgenic genotype DHO1 seedling retained more than 50% of its water content for almost 3 hrs while the control had its water reduced to 50% in less than 2 hrs under the same condition (**Figure 14**).

Evaluation of water loss in DLC1-8

The relative percentage water loss in the transgenic seedling and the non-transgenic (control) was not significantly different ($p > 0.05$). There was no significant difference ($p > 0.05$) at 2 hr interval with the transgenic seedling retaining 61.27% water and the control retaining 54.98% water (**Figure 15**). This was consistent in the

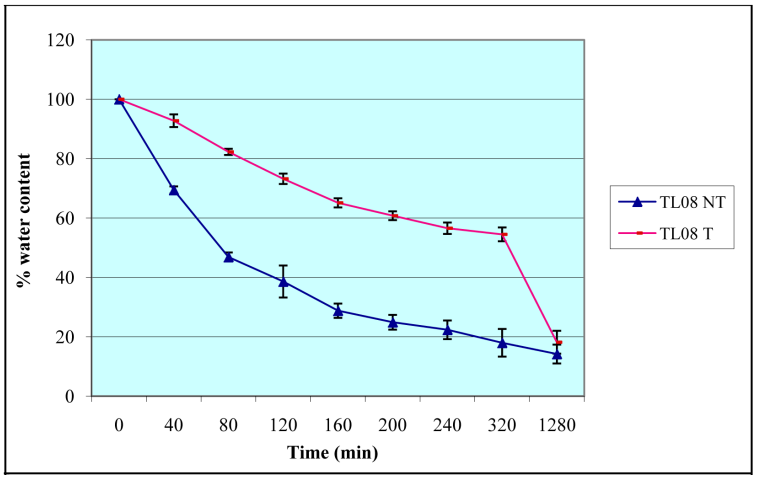


Figure 12. Average % water loss of selected detached aerial parts of TL08-(2) 4 transgenic and non-transgenic maize seedling under drought stress.

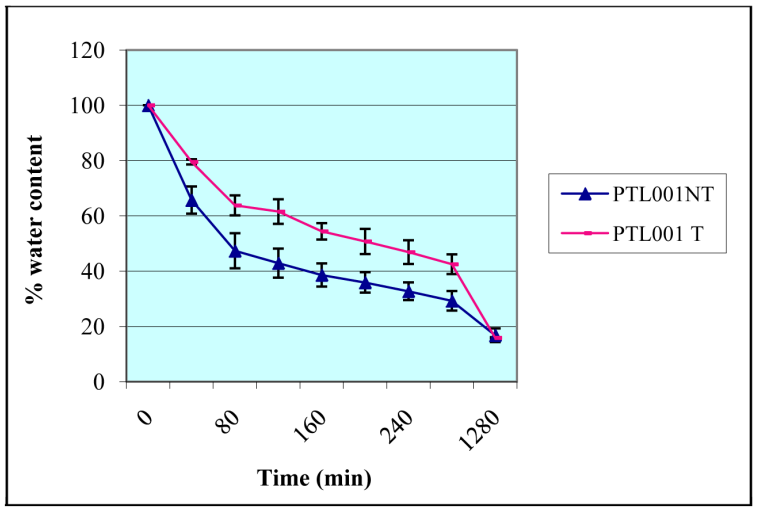


Figure 13. Average % water loss of selected detached aerial parts of PTL001-17 transgenic and non-transgenic maize seedling under drought stress.

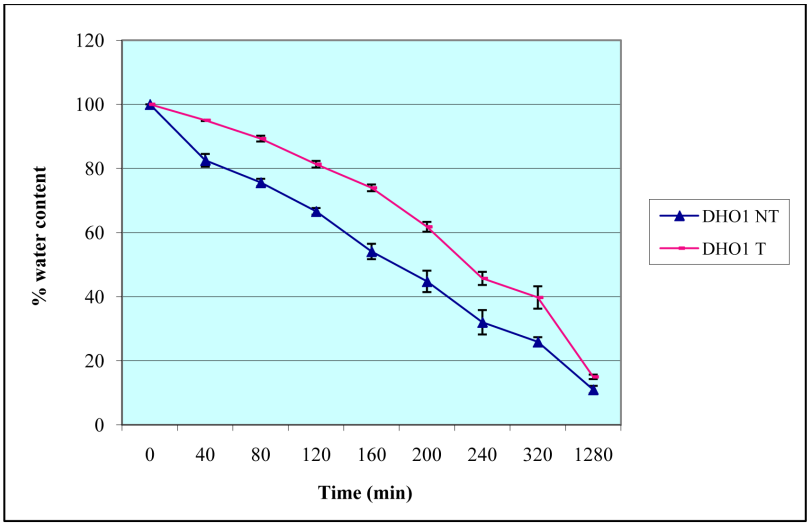


Figure 14. Average % water loss of selected detached aerial parts of DHO1-1 transgenic and non-transgenic maize seedling under drought stress.

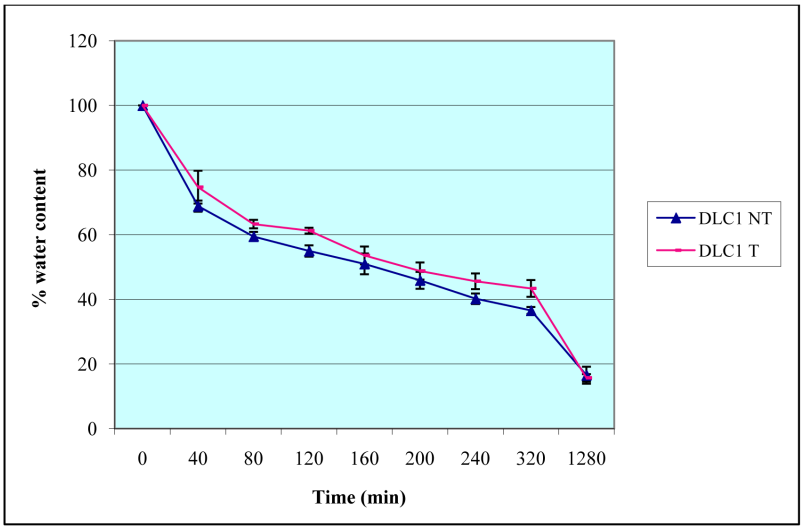


Figure 15. Average % water loss of selected detached aerial parts of DLC1-8 transgenic and non-transgenic maize seedling under drought stress.

entire 40 min interval for the experiment condition.

3.13. Drought-Stress and Agronomical Trait Tests

Timing of drought stress by induction of drought stress (DS) 2 weeks after planting resulted in very poor growth relative to well watered (WW) plant (**Figures 16(a)** and **(b)**). The stunted plant developed to maturity but with poor ear filling and several abortions hence for this study; DS was timed after tassel formation (**Figure 17**) and left to grow to full maturity. The features adapted by Transgenic (T_1) plants during drought stress are shown in **Figure 18**.

3.14. Days to Maturity

There was a delay in the days to maturity of up to 14 days in the transgenic (T_1) inbred TL08-(2)4 and delay to maturity was consisted with all the other transgenic plants (**Table 3**). There was a delay in the maturity of TL08, PTL001, DH01 and DLC1 non-transgenic with the exception of DLC1 genotype that matured about 4 days early.

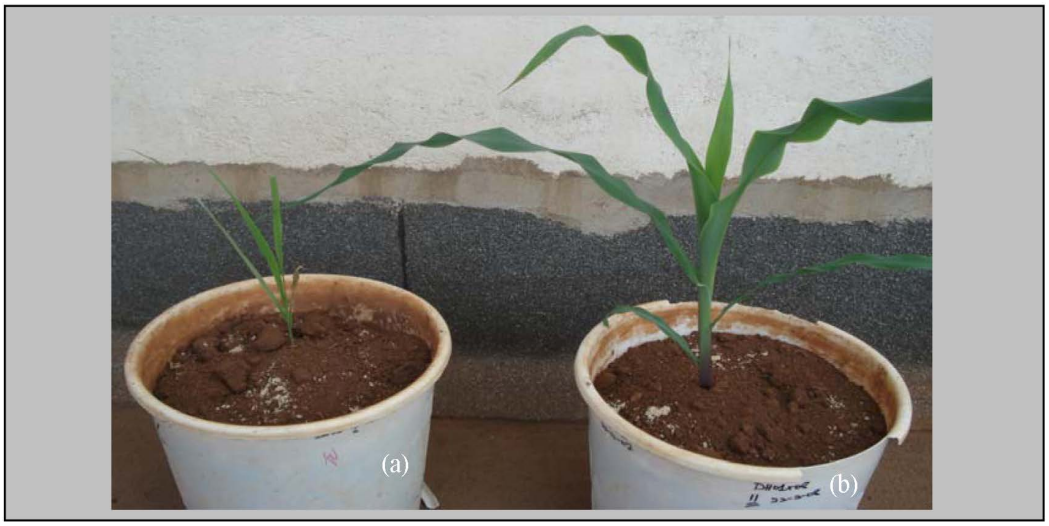


Figure 16. Drought stress. (a), One month old transgenic (DH01-1) T_1 plant when drought stress was started from the 2nd week. (b), One month old transgenic (DH01-1) T_1 plant well watered after top dressing with calcium ammonium nitrate.

Figure 17. Water stress experiment. (a), Stress experiment commenced just after tassel formation. (b), Plants watered once after pollination.

There was a significant difference in the effect of drought on the days to maturity on all the transgenic (T_1) plants and the non-transgenic control plants under study. There was no significant difference ($p < 0.05$) between the well watered transgenic (T_1) and well watered non transgenic controls. This trend was exhibited in the stressed transgenic and non-transgenic plants genotypes when subjected to drought stress.

3.15. Effect of Drought on Leaf Width of Maize Plant

An overall decrease in the leaf width of both transgenic (T_1) and non-transgenic plants: [TL08-(2)4 (1.08%),

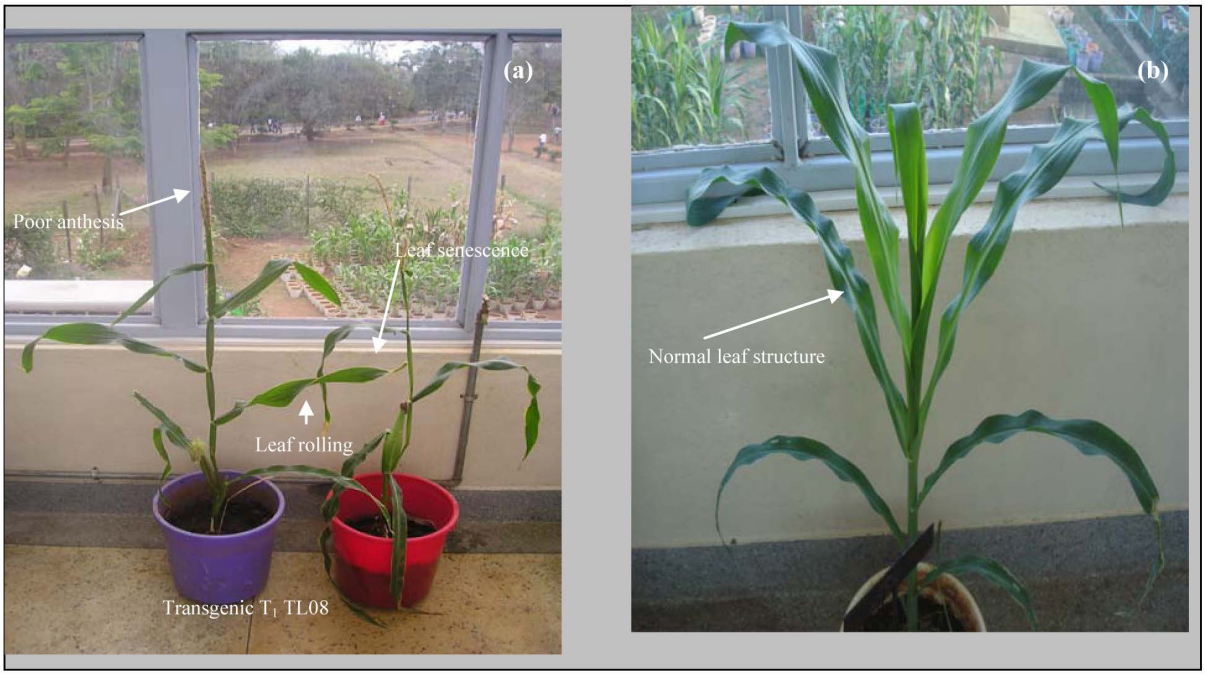


Figure 18. Features adapted by Transgenic (T_1) plants during drought stress, (a), Poor anthesis, leaf senescence. (b), Control.

Table 3. Effect of drought stress on days to maturity.

Events	Effects of drought on days to maturity (Mean ± SE)		Delay days
	Well watered	Moderate drought	
Transgenic (T_1) events			
TL08-(2)4	115.50 ± 5.77 bc[x]	129.25 ± 6.38 d[x]	13.7
PTL001-17	110.75 ± 5.45 b	119.75 ± 7.30 c	9.00
DH01-1	99.50 ± 8.90 a	107.25 ± 6.02 b	7.75
DLC1-8	99.75 ± 7.46 a	103.75 ± 5.31 ab	4.00
Non transgenic events (Checks)			
TL08	114.00 ± 1.87 bc	120.25 ± 6.30 c	6.25
PTL001	110.25 ± 0.83 bc	114.25 ± 2.87 bc	4.00
DH01	96.25 ± 9.63 a	103.50 ± 9.23 ab	7.25
DLC1	102.50 ± 7.92 ab	98.75 ± 7.60 a	−3.75

[x]Means with the same letters within the column are not significantly different at ($p < 0.05$).

PTL001-17 (1.39%), DH01-1 (2.86%) and DLC1-8 (3.03%)] among the transgenic plants and a double digit decrease among the non-transgenic [TL08 (18.18%), PTL001 (26.67%), DH01 (20%) and DLC1 (13.30%)] controls when the plants were subjected to DS was noted. There was no significant difference in leaf width among the well watered (transgenic and non-transgenic plants) when compared to the drought stressed transgenic plants. This was contrary to the non-transgenic plants when subjected to drought stressed, which had a significant difference ($p < 0.05$) in the leaf width when compared with the transgenic drought stressed plants.

There was no significant difference in leaf width ($p > 0.05$) among the transgenic and the non-transgenic plants when the plants were well watered but there was a significant difference ($p < 0.05$) between the drought stressed transgenic and drought stressed non-transgenic controls (**Table 4**).

Table 4. Effect of drought stress on leaf width.

Event	Effects of drought on leaf width in cms (Mean ± SE)		Leaf width increase (%)
	Well watered	Moderate drought	
Transgenic (T_1) events			
TL08-(2)4	9.38 ± 0.41 b[x]	9.28 ± 0.5 a[x]	−1.08
PTL001-17	9.13 ± 0.54 b	9.00 ± 1.06 b	−1.39
DH01-1	8.80 ± 0.21 ab	8.75 ± 1.09 ab	−2.86
DLC1-8	8.50 ± 0.5 ab	8.25 ± 0.83 ab	−3.03
Non transgenic (controls)			
TL08	9.75 ± 0.25 c	8.25 ± 1.03 ab	−18.18
PTL001	9.50 ± 0.5 b	7.50 ± 0.35 a	−26.67
DH01	9.00 ± 0.5 b	7.50 ± 0.5 a	−20.00
DLC1	8.50 ± 0.5 ab	7.50 ± 0.87 a	−13.30

[x]Means with the same letters within the column are not significantly different at $p < 0.05$.

Table 5. Effect drought stress on plant height.

Event	Height in centimeters (Mean ± SE)		Height change due to drought (%)
	Well watered	Drought stressed	
Transgenic events			
TL08-(2)4	180.00 ± 3.81 cd[x]	178.25 ± 6.10 cd[x]	−1.54
PTL001-17	171.00 ± 4.64 c	171.75 ± 7.69 c	0.44
DH01-1	165.50 ± 3.64 bc	166.38 ± 7.05 bc	0.53
DLC1-8	157.38 ± 2.16 ab	147.00 ± 4.30 a	−7.06
Non transgenic event			
TL08	179.25 ± 1.48 cd	172.75 ± 9.12 c	−3.76
PTL001	172.5 ± 13.04 c	163.63 ± 6.57 bc	−5.42
DH01	166.75 ± 2.05 bc	153.25 ± 2.38 ab	−8.81
DLC1	159 ± 2.55 ab	139.50 ± 17.10 a	−14

[x]Means with the same letters within the column are not significantly different at $p < 0.05$.

3.16. Height of Maize Plant

There was no significant difference in height in TL08-(2)4, PTL001-17 and DH01-1 transgenic (T_1) plants with the exception of DLC1-8 between the well watered control and drought stressed plants ($p > 0.05$). Among the non-transgenic plants there was a significant difference in height among the four genotypes when subjected to drought stress with 14% decrease in height in DLC1 and about 9% in DH01 being the greatest percentage decrease in height compared with the well watered plants (**Table 5**). There was also no significant difference in height among the well watered transgenic events and well watered non-transgenic events. There was a significant difference ($p < 0.05$) in height among the entire drought stressed transgenic and drought stressed non-transgenic with DLC1 as an exception. There was a significant difference ($p < 0.05$) between all the well watered non-transgenic events and the drought stressed non-transgenic events, a factor which was also observed in the leaf width, height and maturation period of the four selected genotypes.

3.17. Effect of Drought on Anthesis-Silking Interval

With the exception of DLC1-8, there was a significant difference in anthesis-silking interval ($p < 0.05$) between genotypes TL08-(2)4, PTL001-17 and DHO1-1 transgenic (T_1) drought stressed and transgenic watered plants (Table 6). A significant difference was also observed among the four genotypes when the non-transgenic control plants were well watered and compared to drought stressed plants. In TL08, PTL001, and DH01 there was up to 2 days difference when the plants were subjected to stress.

There was also a significant difference ($p < 0.05$) between the in the anthesis-silking interval of all the well watered transgenic and well watered non transgenic plants under study with the exception of DLC1. There was no exception as all the plants both the transgenic and the non-transgenic plants were significantly affected by drought stress.

3.18. Effect of Drought on Kernel Number

There was no significant increase in kernel number between the transgenic (T_1) well watered and the transgenic drought stressed single cross PTL001-17 and DLC1-8 ($p < 0.05$) (Table 7). There was a significant difference ($p < 0.05$) among the rest of the transgenic experiment with an increase in kernel number of up to 32.43% in in TL08-(2)4 and 28.12% in DHO1-1. There was no significant difference between the kernel number among the well watered transgenic and non-transgenic events. This was contrary to the drought stressed transgenic and non-transgenic events in which there was a significant difference in kernel number ($p < 0.05$). The same trend was exhibited between the well watered non-transgenic and the drought stressed non-transgenic with a significant decrease in kernel number of 11.9% in DLC1, 9.31% in PTL001, 4.75% in TL08 and an insignificant decrease of 2.36% in DHO1.

3.19. Effect of Drought Stress on Kernel Weight

There was no significant decrease ($p < 0.05$) in kernel weight between the well watered transgenic (T_1) events and the drought stressed transgenic event. The same trend was duplicated among the watered transgenic and non-transgenic events. There was though a significant difference among the transgenic and non-transgenic drought stressed events with a decrease of 14.45% in TL08, 5.72% in PTL001, 12.72% in DH01 with an insignificant 1.05% decrease in weight DLC1 (Table 8). There was also a significant difference at among the well watered and the drought stressed non-transgenic events with an average decrease of between 17.5% in TL08-(2)4, 9.12% in PTL001, 14.4% in DH01 and 4.01% in DLC1 in weight of 10 kernels selected randomly.

Table 6. Effect of drought stress on anthesis-silking interval.

Event	Anthesis-silking interval (Mean ± SE)		Anthesis-silking
	Well watered	Drought	
Transgenic events			
TL08-(2)4	5.75 ± 0.19 ab[x]	7.75 ± 0.19 c[x]	2.00
PTL001-17	5.25 ± 0.19 a	7.00 ± 0.32 bc	1.75
DH01-1	5.75 ± 0.37 ab	7.25 ± 0.37 bc	1.50
DLC1-8	6.75 ± 0.49 b	6.50 ± 0.39 b	−0.25
Non transgenic event			
TL08	5.25 ± 0.37 a	7.25 ± 0.19 bc	2.00
PTL001	6.50 ± 0.50 b	5.00 ± 0.32 a	−1.50
DH01	7.75 ± 0.19 c	5.50 ± 0.22 ab	−2.25
DLC1	6.00 ± 0.45 b	7.00 ± 0.32 bc	1.00

[x]Means with the same letters within the column are not significantly different at $p < 0.05$.

Table 7. Effect of drought stress on kernel number.

Event	Kernel number (Mean ± SE)		Kernel number increase (%)
	Well watered	Drought	
Transgenic events			
TL08-(2)4	175.75 ± 6.22 ab[x]	232.75 ± 19.56 c[x]	32.43
PTL001-17	200.00 ± 13.32 b	211.00 ± 23.37 b	5.5
DH01-1	156.50 ± 17.24 ab	200.50 ± 35.95 b	28.12
DLC1-8	161.75 ± 12.27 ab	176.50 ± 34.09 ab	9.11
Non-transgenic event			
TL08	184.00 ± 22.62 ab	175.25 ± 43.37 ab	−4.75
PTL001	196.50 ± 8.32 b	178.25 ± 20.80 ab	−9.31
DH01	158.50 ± 11.15 ab	154.75 ± 28.60 ab	−2.36
DLC1	159.75 ± 27.87 ab	140.75 ± 26.95 a	−11.89

[x]Means with the same letters within the column are not significantly different at $p < 0.05$.

Table 8. Effects of drought stress on kernel weight.

Event	Weight of 10 kernel in (g) (Mean ± SE)		Kernel weight decrease (%)
	Well watered	Drought	
Transgenic events			
TL08-(2)4	2.58 ± 0.05 b[x]	2.46 ± 0.067 b[x]	4.65
PTL001-17	2.65 ± 0.09 b	2.56 ± 0.09 b	3.40
DH01-1	2.82 ± 0.098 c	2.76 ± 0.06 c	2.13
DLC1-8	2.70 ± 0.065 b	2.62 ± 0.075 b	2.96
Non-transgenic event			
TL08	2.69 ± 0.05 b	2.22 ± 0.06 a	17.5
PTL001	2.63 ± 0.06 b	2.39 ± 0.08 ab	9.12
DH01	2.84 ± 0.08 c	2.43 ± 0.09 b	14.4
DLC1	2.74 ± 0.07 bc	2.63 ± 0.07 b	4.01

[x]Means with the same letters within the column are not significantly different at ($p < 0.05$).

4. Discussion

4.1. Production of npk1 Transgenic Maize

Previous studies have shown that *Agrobacterium tumefaciens* transformed events had similar copy number of the *npk*1 gene and bar gene with a correlation coefficient of 0.9098 [12]. This correlation coefficient is due to the fact that the sequence between the left and the right border which include the *bar* gene and the *npk*1 gene are integrated in the maize genome simultaneously. Thus in this study, detection of *npk*1 gene by PCR was used to determine the presence of the *npk*1 gene in T_1 plants and southern blot was used to determine copy number.

According to Shou *et al*. [12], low copy numbers is defined as (<3 copies), medium (3 - 9 copies) to high (>10 copies) of the transgene. Of the four genotypes used in this study, none of the T_1 events had a high copy number a feature common with bombardment-derived events. The derived events had between 1 - 5 copy numbers and this were designated as low copy numbers and medium copy events [12]. The result from this study did conform

to previous finding that *Agrobacterium* mediated transformation results in fewer copy numbers of the transgene and transformants [16] [21].

Transgenic events PTL001-17, DHO1-1, DLC1-8 and TL08-(2)4 were selected for drought stress experiment. The inbred genotypes TL08-(2)4 and multiple cross hybrid DHO1-1 exhibited more resilience to drought stress relative to the cultivar DLC1-8. These observations were in agreement regarding *Agrobacterium* mediated transformation of HiII single cross hybrid [16] [21] with the exception of the cultivar. High segregation among the cultivar and the multiple cross with high copy made a direct correlation between the copy number and the expression of the transgene hard to detect. Significant morphological adaptations in resilient genotypes included long anthesis period increasing the chance of pollination of the plants that have formed their silk early and those that have formed their silk late and the compensation mechanism resulting in delayed maturity. These were positive adaptive phenomena for a plant to be able to survive drought stress.

The study indicated that *Agrobacterium*-mediated transformation events with low copy numbers had high expression levels and the results of the RT-PCR correlated with the northern and the southern blot hybridization analysis. The inverse correlation on the intensity of the RT-PCR and the copy number better termed as correlation between expression levels and copy numbers that we observed support the argument that multiple copies of transgene may lead to co-suppression and silencing [21] [22].

Despite the fact that no abnormal transgenic plants were observed among the T_1, the transformation efficiency in this experiment was reduced from between 5.98% - 13.03% when using pTF102 to 2.24% - 6.54% when using pSHX004 despite both the constructs having the same back bone. The reduced transformation efficiency could be due to death of the high *npk*1 expressers during the selection process [12]. This difference was relatively low compared to the experience by Shou *et al*. [12] where the transformation efficiency was between 4% - 8% for the pSHX004 *Agrobacterium*-derived events.

Previous work by Shou *et al*. [12] has shown that over expression of *npk*1 can lead to death of transgenic events. It is likely that the transgenic maize plants generated in this study had relatively low expression of the transgene and the *npk*1 gene was not optimally utilized and over-expressers were not able to survive. Incases where the *npk*1 signal was switched on and the over expressers were able to survive, a correlation between the copy number and the expression level is hard to determine and it's likely that additional factors influence transgenic silencing. These may include methylation, DNA rearrangement, and chromatin structure of the surrounding area of the transgene insertion [22]-[24]. Suppression and over-expression of *npk*1 has been shown to have detrimental effects on cell division, embryogenesis and seed development [13]. This was notable when studying seed weight of well-watered seeds of transgenic plants compared with well-watered non-transgenic plants where despite both seeds being provided with adequate water, the later had better average weight (**Table 8**).

4.2. Effect of npk1 Gene Expression on Agronomic Traits under Drought Stress

The agronomic traits assayed included days to maturity, and phenological characteristics (leaf width, height and anthesis-silking interval). There was a delay in the days to maturity of up to 14 days in the transgenic inbred TL08-(2)4 and delay to maturity was consisted with all the transgenic events. As for the non-transgenic, there was a delay in the TL08, PTL001 and DH01 with DLC1 as an exception for the genotype matured about 4 days early. In respect to the maturity period, the compensation mechanism by which plants adjusted to water stress may have been attributed to the prolonged days to maturity. Under drought condition, *npk*1 transgenic plants extended their maturity period by an average of 5 days with up to 8 days in TL08 and about 1 day in DH01 as compared with the non-transgenic control plants. It is possible that the transgene expression had increased the adaptation ability in transgenic maize plants through a concerted effort of several complex epigenetic factors including methylation, DNA arrangement and chromatin structure around the transgene a set of mechanism yet to be clearly understood [24] [25]. There was a significant difference in the effect of drought on the days to maturity on all the transgenic events and the non-transgenic events under study. There was though no significant difference ($p < 0.05$) on days to maturity between the well watered transgenic and well-watered non transgenic events. This trend was exhibited in the stressed transgenic and non-transgenic plants when subjected to drought stress.

In relation to plant height and leaf width, there was no significant percentage difference in leaf width among the (WW) well watered (transgenic and non-transgenic plants) when compared to the drought stressed (DS)

transgenic plants. This was contrary to the non-transgenic drought stressed plants, which had a significant difference ($p < 0.05$) in the percentage leaf width difference when compared with the transgenic drought stressed plants. This showed the effectiveness of *npk*1 gene in switching on the signal cascades, which facilitate expression of drought countering protein. The proteins in turn have effect on the rate of photosynthesis [17] and in the process affect the plant leaf growth. Transgenic plants used in this study when subjected to drought stress tend to roll their leaves. This phenomena tend to reduce the surface area with most of the stomata exposed to direct sunlight rays in the process reduces the rate of transpiration and in turn decrease in photosynthesis rate.

The decrease in surface area is expected to increase evaporation/transpiration ratio and affect the growth pattern. This was not the norm with the non-transgenic events as plants did not attain their normal height in stressed condition. The degree of leaf rolling was lower in non transgenic plants which did not maintain high turgor and thus had less leaves rolling this was very visible in the transgenic OPV DLC1 and both the hybrids. The significant decrease in leaf width resulted in a decrease surface area of the non transgenic commercial hybrid DH01 and the single cross hybrid PTL001. Decrease in surface area seems to have a significant effect on the respective plants evaporation/transpiration ratio leading to poor growth in height of the non-transgenic variety. Despite the PTL001 and DH01 being hybrids, difference in polymorphism within maize line suggest that even the same line may have unique arrangements of retro elements making room for a possible external stress factors eg disease susceptibility may affect the height and leaf width of the non transgenic drought stressed germplasm under study in relatively different ways [26]. Thenon-transgenic DLC1 exhibited a trend similar to the transgenic events as there was no significant difference ($p < 0.05$) when it came to height due to its polygenic nature as an open pollinated variety.

4.3. Effect of npk1 Gene Expression on Yield Components under Drought Stress

Apart from the transgenic open pollinated variety DLC1, there was a significant difference in the anthesis-silking interval among both the transgenic and non-transgenic events under well watered and drought stressed condition. The assumption was *npk*1 had complementary effect on drought affecting the anthesis-silking pattern of the four genotypes. A similar trend to the anthesis-silking pattern was exhibited in the kernel number among the transgenic stress experiment with significant increase in kernel number and a significant decrease ($p < 0.05$) among the non-transgenic stress observed. This correlation is as a result of lack of a consisted synchrony between pollen maturity and the appearance of silk resulting in poor seed filling and in extreme cases abortion of ears due to unavailability of viable pollen when the silk appears among the non-transgenic stressed events. The *npk*1 transformed events seem to have a relatively higher mean kernel number even when exposed to drought stress (**Table 7**). This may be due to better drought/heat tolerance of the transformed pollen making it viable for a longer period of time despite the longer anthesis-silking interval experienced under drought stress. The most significant effect of the *npk*1 gene ($p < 0.05$) was the notable increase in the kernel number in TL08-(2)4 and DH01-1 to 32.43 and 28.12 percent respectively.

There was no significant difference among the well watered and the drought stressed transgenic events while there was a significant difference among the non-transgenic events subjected to the same condition. The effects of *npk*1 gene enabled the transformed events withstand drought stress making no difference in average kernel weight among the transgenic group. The significant difference in average kernel weight in non transgenic events of up to 17.5% decrease in TL08-(2)4 kernel weight was as a result of the inability of the maize plants to adjust to water deficit condition. DLC1 an OPV with a mixed background may have provided the contrary results to the other non-transgenic events. The mixed background is as a result of segregation of genetic elements involved in yield potential or stress tolerance may have influenced their performance in tissue culture and transformation.

5. Conclusion

This study provides a platform for transformation of identified amenable Kenyan lines for drought tolerance. The stress experiments enable us to appreciate the extent of tolerance of the four lines as potential candidates for future drought tolerance experiments.

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Abbreviations

CaMV: Cauliflower mosaic virus
CTAB: Cetyltrimethylammonium bromide
bar: Phosphinothricinacetyltransfarase gene
IZEs: Immature zygotic embryos
*Npk*1: Nicotiana protein kinase
T_o: Primary transformants
T1: Secondary transformants