

Full Length Research Paper

Drought tolerance in transgenic tropical maize (*Zea mays* L.) by heterologous expression of *peroxiredoxin2* gene-*XvPrx2*

Micah S. Seth^{1,3*}, Leta T. Bedada¹, Steven M. Runo¹, Mathew P. Ngugi¹, Revel Iyer², Jennifer A. Thomson², Emmarold E. Mneney³ and Richard O. Oduor¹

¹Plant Transformation Laboratory, Department of Biochemistry and Biotechnology, Kenyatta University, P. O. Box 43844, Nairobi, Kenya.

²Plant Stress Laboratory, Department of Cell and Molecular Biology, University of Cape Town, Private Bag, Cape Town, South Africa.

³Mikocheni Agricultural Research Institute, P. O. Box 6226, Dar Es Salaam, Tanzania.

Received 30 March, 2016; Accepted 26 May, 2016

Transformation of a tropical maize inbred genotype (CML144) with the *Xerophyta viscosa* peroxiredoxin2 (*XvPrx2*) gene was reported. The protective role of peroxiredoxin2 against the damage resulting from reactive oxygen species (ROS) under dehydration stress was further determined. Successful integration of *XvPrx2* gene into maize we achieved and recovered 10 independent transgenic events. Transformation and regeneration frequencies were 12.9 and 31.3%, respectively. Reverse transcription polymerase chain reaction (PCR) revealed the expression of the *XvPrx2* gene in transformed plants under dehydration. Stressed transgenic plants had higher relative water content (RWC) as compared to the conventional plants. Recovery irrigation showed higher RWC in transgenics than in conventional plants. Unlike in conventional plants, rapid morphogenic recovery was observed in transgenics within 24 h. Chlorophyll contents decreased faster in conventional plants than in transgenics with prolonged drought. Generally, transgenic plants were more tolerant to dehydration stress than conventional plants. This tolerance may be associated with the over expression of peroxiredoxin2 playing a role in managing ROS generated in plant cells.

Key words: *Xerophyta viscosa*, reactive oxygen species (ROS), transgenic drought tolerant maize, plant breeding.

INTRODUCTION

Drought is one of the leading environmental stresses that accounts for severe losses of grain yield in the Eastern

and Central Africa sub regions. Relative water content (RWC) has been reported to be a relevant physiological

*Corresponding author. E-mail: micahgunah@yahoo.co.uk.

measure for plant water loss due to drought (Blum et al., 1998; Rodriguez-Maribona et al., 1992). RWC gives the current status of water content in the tissue of a leaf relative to its optimum amount of water that can be held at full turgidity. Ideal values of RWC when the cells are turgid have been estimated to range between 98% in transpiring leaves to about 40% in severely desiccated and dying leaves. Further, RWCs of most crop species at wilting have been estimated to be about 60 to 70% (Lugojan and Ciulca, 2011).

Under mild desiccations, the internal leaf CO₂ concentration is reduced due to stomatal closure, which results in reduction in photosynthetic activity (Cornic, 2000; Flexas et al., 2004). Severe drought on the other hand results in reduction of chlorophyll content and thereby destruction of the photosynthetic apparatus.

Chlorophyll content is one of the key indicators of the availability of nitrogen in crop plants and plays an important role as light harvesting machinery essential for carbon assimilation. Severe dehydration decreases photosynthetic pigments resulting in increased levels of carotenoids (xanthophylls and carotene). Carotenoids are important in plants because they tend to reflect back the heavy surge of light thereby protecting chlorophylls from further destruction. The reduction in chlorophylls (*a*, *b* and *total*) is reported to be caused mainly by oxidative stress due to excessive accumulation of reactive oxygen species (ROS) under drought stress (Smirnov, 1995).

Plants can protect themselves against harmful ROS by undergoing a variety of biochemical and physiological responses. One of such response is the generation of antioxidant enzymes, such as peroxiredoxin2, which plays a fundamental role in scavenging ROS and converting them to harmless molecules, thereby protecting plant cell membranes and DNA from damage. Peroxiredoxins are post-translationally targeted to chloroplasts where they protect the photosynthetic membrane from photo-oxidative destruction (Baier and Dietz, 1997, 1999).

Xerophyta viscosa is a resurrection plant, native to South Africa, which has evolved mechanisms of withstanding prolonged drought stresses. This plant can be dehydrated to an air dry state, but upon rewatering, can rehydrate within 24 to 80 h (Mundree et al., 2002). This phenomenon has been predicted to be controlled by a number of genes, including the *XvPrx2* which encodes peroxiredoxin2 (Govender, 2006).

A type II peroxiredoxin gene (*XvPrx2*) polypeptide has been shown to display significant similarity with other plant type II peroxiredoxins, with the conserved amino acid motif (PGAFTPTCS) proposed to constitute the active site of the enzyme (Govender et al., 2016). The *XvPrx2* gene is stress-inducible in response to abiotic stresses and it has been revealed that *XvPrx2* homologues exist within the *X. viscosa* proteome (Govender et al., 2016). *In vitro* DNA protection assay has shown that, in the presence of *XvPrx2*, protection of

DNA occurs. *In vitro* assays have also revealed maximum activity of the *XvPrx2* with DTT as electron donor and H₂O₂ as substrate (Govender et al., 2016), implying that the gene is responsible for managing the ROS generated by plants under stress.

Thus, the objective of the present study was to transform CML 144 inbred maize lines using *XvPrx2* gene construct and evaluate the performance of transformed maize lines against their non-transgenic counterparts under drought stress condition. Hence, the relative water content (RWC), chlorophylls (*a*, *b* and *total* chlorophyll) contents, and the chlorophyll *a/b* ratio in the transgenic and conventional maize plants were measured to determine the ability of the former to overcome the effects of dehydration stress.

MATERIALS AND METHODS

Vector development and *Agrobacterium* strain

The *XvPrx2* gene and the drought inducible promoter *XvPsap1* were kindly obtained from the Department of Molecular and Cell Biology, UCT, Cape Town. The gene was previously isolated and cloned into the pTF101.1 vector. The construct harbours an herbicide resistant *bar* gene for selection of transformed plant cells. Given the rising concern about the use of antibiotics and herbicides to generate transgenics, the expression cassette of the gene was sub-cloned into the plant expression vector, pNOV2819 to take advantage of the *manA* gene as a plant selectable marker and allow the use of mannose as a selective agent. The construct contains the right and left T-DNA borders from *nopaline* strains of the *A. tumefaciens*, a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aadA*) for bacterial selection (Figure 1). The *XvPsap1* promoter (Oduor et al., 2009, WIPO patent No.WO/2009/060402) was used to drive the expression of the *XvPrx2* and terminated by the *nopaline synthase* (*nosT*), whereas the expression of the *PMI* (*manA*) selectable marker gene was under the CMPS promoter from cestrum yellow leaf curling virus and terminated by the *nosT*. The resulting gene construct was transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1986) and used to transform immature zygotic embryos of tropical CML 144 maize adapted to East and Central African countries.

Bacterial growth and maintenance media

The EHA101 carrying the gene construct was maintained in Luria-Bertani, LB (10 g Bacto-Tryptone; 5 g yeast extract; 5 g sodium chloride NaCl and 10 g Bacto-Agar) medium supplemented with 100 mg/L spectinomycin (for plasmid), 100 mg/L kanamycin and 25 mg/L chloramphenicol (for EHA101) [pH 6.8].

Plant transformation and regeneration media

All media and vitamin composition used in this study are shown in Table 1. Infection medium (LS-Inf), co-cultivation (LSAc), resting (RM) and selection (SEM) were prepared based on Linsmaier and Skoog (1965) salts and regeneration (REGI) for embryo maturation, REGII for shoot induction and REGIII (for root induction) media were prepared based on Murashige and Skoog (1962) basal salts. Except for infection medium whose pH was adjusted to 5.2 and

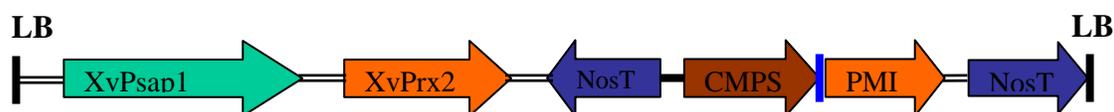


Figure 1. A T-DNA region of pNOV2819 vector harbouring *XvPrx2* gene; LB: Left border, RB: Right border, NosT: Termination sequence of the *nopaline synthase* gene, isolated from *Agrobacterium tumefaciens*. The function of this sequence is to signal the termination of the gene expression. CMPS: A strong constitutive promoter from cestrum yellow leaf curling virus which can be used for regulating transgene expression in a wide variety of plant species. Here, it was used to drive the PMI gene, *Xvpsap1* a promoter sequence native to *Xerophyta viscosa* used to drive the *XvPrx2* gene.

Table 1. Media composition for transformation and regeneration.

Medium	Composition
LS-Inf	Macro- and micro-salts (Linsmaier and Skoog, 1965), LS-vitamins ^a , 1.5 mg/L 2,4-D, 1 g/L casein hydrolysate, 34.25 g/L sucrose, 18 g/L glucose, 100 µM acetosyringone (As), pH 5.2
LSAc	Macro- and micro-salts, LS-vitamins ^a , 1.5 mg/L 2, 4-D, 700 mg/L proline, 500 mg/L MES, 100 µM As, 30 g/L sucrose, 10g/L glucose, 8 g/L agar, pH 5.8
RM	Macro- and micro-salts, LS vitamins ^a , 2 mg/L 2, 4-D, 700 mg/L praline, 500 mg/L MES, 30 g/L sucrose, 1.6 mg/L silver nitrate, 8 g/L agar, 250 mg/L Carbenicillin, pH 5.8
SEM	RM with 2 mg/L 2, 4-D, 5 g/L mannose, 25 g/L sucrose, no silver nitrate
REG I	MS macro- and micro- salts, vitamins ^b , 0.5 mg/L kinetin, 700 mg/L proline, 500 mg/L MES, 25 g/L sucrose, 2.5 g/L mannose, 8 g/L agar, 250 mg/L carbenicillin, pH 5.8
REG II	MS macro- and micro- salts, vitamins ^b , 25 g/L sucrose, 2.5 g/L mannose, 8 g/L agar, 250 mg/L carbenicillin, pH 5.8
REG III	½ strength MS macro- and micro- salts, vitamins ^b , 20 g/L sucrose, 5 g/L mannose, 8 g/L agar, 250 mg/L carbenicillin, pH 5.8

^aLS Vitamins contains: 100 mg myo-Inositol and 0.1 mg Thiamine HCl; ^bVitamins: 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 100 mg/L mayo-inostol, 2 mg/L glycine†; †Glycine is an amino acid but it was included in vitamin preparations.

filter sterilized, the pH of all media were adjusted to 5.8 before autoclaving.

Source of seeds and plant establishment

Maize seeds of CML144 (QPM) were provided by Dr. Dan Makumbi (CIMMYT, Nairobi). CML 144 was chosen based on consultation with Kenya Agricultural Research Institute (KARI) and CIMMYT breeders where it was agreed that besides being quality protein maize (QPM), it is also sensitive to drought, therefore it should be easy to see the improvement. Maize plants were grown in pots (150 mm radius with 330 mm depth) containing sterile forest loam soils mixed with manure and sands in the ratio of 2:2:1 and bulked in the greenhouse at Plant Transformation Laboratory (PTL), Kenyatta University, Nairobi, Kenya.

Surface sterilization and immature embryo excision

Ears harvested 12-15 days post pollination were surface sterilized in 3% (v/v) commercial bleach (JIK) with 2 drops of Tween[®]20 for 15 to 20 min and rinsed three times with sterile distilled water. Immature zygotic embryos (1-1.5 mm) were aseptically excised from kernels and placed in Petri-plates containing infection medium

Agrobacterium pre-induction procedures

A. tumefaciens (EHA 101) containing the single gene construct pNOV2819-*XvPsap1*-*XvPrx2*-*nosT* was cultured at 28°C for 2 days in darkness on LB agar supplemented with the same antibiotics used for bacterial maintenance. One loop of bacteria was scooped from a freshly grown plate and suspended in 10 mL LB broth supplemented with 100 mg/L spectinomycin, 100 mg/L kanamycin and 25 mg/L chloramphenicol and shaken at 75 rpm overnight at 28°C. The overnight grown culture was centrifuged and the medium discarded. *Agrobacterium* cells were then resuspended in a fresh LB broth (without antibiotics) and centrifuged to remove any remaining antibiotics. Thereafter, the *Agrobacterium* cells were resuspended in 5 mL of liquid infection medium (LS-Inf) supplemented with 100 µM acetosyringone, AS (LS-Inf + AS) in sterile 50 ml falcon tube. The bacterial cell densities were then adjusted to an optical density (OD₅₅₀) of 0.3 to 0.4 using 722 N Visible spectrophotometer (EVERICH MEDCARE IMPORT AND EXPORT Co. LTD, Nanjing, China) before infecting embryos. The pre-induction step was carried out for all experiments.

Infection, co-cultivation and resting

The infection medium (LS-Inf) was drained off from the Petri-plates

before the pre-induced *A. tumefaciens* was poured onto the plates containing excised embryos and incubated for 5 min in darkness. The infected embryos were then poured onto the co-cultivation medium (LSAc) and the excess infection medium drained off using a micropipette. Embryos were then aligned in such a way that the axis faced the medium. The plates were wrapped with parafilm and incubated at 20±2°C for 3 days in darkness. Thereafter, embryos were transferred to resting medium (RM) and incubated at 28±2°C in the dark for 10 days.

Selection for transformed events

From RM, all the embryos were transferred to selection medium (SEM) containing 5 g/L mannose. Embryos (35 embryos per plate) were incubated on this medium for 4 weeks in darkness with fortnightly sub-culturing onto fresh medium.

Regeneration of shoots from calli

Embryogenic calli that survived selection were regenerated by maturing them for 1-2 weeks on REGI medium in darkness followed by transfer to light for shoot induction on REGII as described by Negrotto et al. (2000). Putative transgenic maize plantlets with well-developed root systems were removed from culture bottles and washed with sterile distilled water to remove adhering agar from the roots. The plantlets were then hardened in small pots (100 x 100 x 100 mm) containing peat moss (KEKKILA Co. Ltd, Tuusula, Finland). Plantlets were covered with transparent polyethylene bags for 2 - 3 days and allowed to grow in these pots to about 3 leaves prior to transplanting to bigger pots (150 mm radius with 330 mm depth) containing sterile forest loam soils mixed with manure and sands in the ratio of 2:2:1. The putative transgenic maize plants were allowed to grow to full maturity and self-pollinated at the flowering stage to give T₀ seeds.

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was extracted from the transgenic CML144 and non-transformed control maize lines as per the CTAB method (Allen et al., 2006). The PCR amplification was performed in 50 µL reaction volume containing 100 ng of genomic DNA, 1X PCR buffer (supplied with the Taq polymerase enzyme) with 1.5 mM MgCl₂, 20 pmoles primers each of either (*PMI-U-1*: 5'-ACA GCC ACT CTC CAT TCA-3'; *PMI-L*: 5' GTT TGC CAT CAC TTC CAG-3') or promoter specific (*XvPsap1-F1* 5'-GGA CTT CAT GGC ATC CAT GTG C-3'; *XvPsap1-R1* 5'-ATT TGC CCC ATG GAA AGT GAC G-3'), 200 µM each of dNTPs, and 1 unit of Taq DNA polymerase enzyme. The reaction was carried out using Eppendorf Vapo Protect thermal cycler (EPPENDORF AG 22331 Hamburg Germany). The following reaction condition was used: initial denaturation step for 10 min at 95°C followed by 45 cycles of 95°C for 30 s; 55°C for 45 s; 72°C for 50 s, and a final elongation step at 72°C for 7 min. The amplification product (550 bp for PMI and 395 bp for *XvPsap1* promoter) was resolved on 1% TAE agarose gel and photo was captured (SYNGENE BIOIMAGING model No. 55000, SYNOPTICS LTD, Cambridge, UK).

RNA isolation from drought stressed plants

Total RNA was isolated using the Trizol reagent (Gibco-BRL). Maize leaves (200 mg) from stressed plants were ground in liquid nitrogen and homogenized in 0.75 mL of the reagent. Following incubation for 5 min at room temperature, 0.2 mL chloroform was added followed by a further incubation at room temperature for 10

min. Samples were centrifuged at 12000 rpm for 10 min at 4°C and the RNA was precipitated using isopropanol. The RNA was treated with RNaseA free-DNase I (Invitrogen, USA). RNA was separated on a 1.2% agarose formaldehyde gel and stained with cyber green to verify the quantity.

cDNA synthesis using RT-PCR

cDNA was synthesized using a cDNA synthesis kit (Invitrogen, USA) according to the manufacturer's instruction. Standard PCR was carried out using either *XvPrx2* gene (*XvPrx2-F* 5'-ACG ATC CCA GAC GGA ACG CT-3' and *XvPrx2-R* 5'-CTT CAA GAT CTC ATC GGC ACC-3') along with an internal control maize actin (*Zm-actin*) gene primers (GenBank accession no: AY107106) (*ZmAct-F* 5'-ACC CAA AGG CTA ACC GTG AG-3' and *ZmAct-R* 5'-TAG TCC AGG GCA ATG TAG GC-3'). The PCR machine was programmed as follows, initial denaturation step of 5 min at 94°C followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, elongation at 72°C for 50 s, and a final elongation step at 72°C for 7 min. The *XvPrx2* gene and maize *actin* gene transcripts were detected by amplifying a 458 and 426 bp fragment, respectively.

Drought stress experiments

From single transformation event T₀ maize seeds (2 seeds per pot) were planted in pots (17 cm diameter and 21 cm height) containing 3 kg of forest soil mixed with manure and sand at the ratio of 2:2:1. Although, the study was in response to drought, it was envisaged that in nature, plants face multiple stresses. Hence the glasshouse temperature was maintained at 43±2°C and relative humidity of 55%. After getting T₁ seeds, they were planted in pots as described earlier. Germinated T₁ plants were thinned and transplanted into other pots such that the number remaining one plant per pot. Eight transgenic T₁CML144 maize plants and 8 conventional controls at the age of 6 weeks were selected for the experiment. From germination, plants were watered with half a litre of water once (every morning) daily before dehydration stress was imposed. From each group (transgenic and conventional plants) of maize plants, 4 maize plants were used for dehydration stress treatment while 4 were watered with half a litre once daily throughout the experiment. The experiment was arranged in 2 x 4 x 3 factorial (2 genotypes (transgenic and conventional maize) with 4 observation points under drought stress (days and recovery) and 3 replications) in a complete randomised design.

Determination of watering regime for plants during drought stress assays

Plant water requirement for daily irrigation was determined empirically as the difference between the wet soil and dry soil. This was done by first weighing the empty pot (W₁). The pot was filled with 3 kg of oven dry soil and weighed again (W₂). The soil was watered slowly until the first drip of water was seen at the bottom of the pot and then the soil was left until there was no more dripping. Thereafter, the weight of the pot containing wet soil (W₃) was recorded. The difference between the weight of wet soil (W₃-W₁) and the weight of oven dry soil (W₂-W₁) was used as the volume of water required to water plants considering the density of water to be 1 g/cm³.

Drought stress assay procedures

Before commencement of dehydration stress experiment, plants were equally watered at mid-day (at around 12 noon) to standardize

on a particular point the water content and metabolic fluctuation on their circadian rhythm. The following morning at 6 am, pieces of leaf samples representing day 0 were taken in triplicate for RWC and chlorophylls content determination from both transgenic and conventional plants. Plant photos were taken to mark as a reference for comparison of maize responses to dehydration stress. Thereafter, sampling was done from same plants at mid-day on days 7, 14 and 21 after withholding water in transgenic and conventional plants. Sampling was also done after 24 h following one time re-watering of plants at the end of dehydration treatment to determine the rate of plant recovery. Plant photos were also taken in each sampling day to compare the morphogenic response between the transgenic and the conventional plants.

Determination of leaf relative water content

To determine leaf relative water content (RWC), 3 pieces of leaf samples (3 x 4 cm) were cut from each experimental plant and weighed to obtain leaf fresh weight (FW). The leaves were immediately immersed in sterile distilled water and incubated overnight at 4°C to re-hydrate to full turgor. The following morning, leaves were drained and weighed to obtain saturation or leaf turgor weight (SW). The leaves were then dried at 80°C in the oven for 24 h or until a constant weight was repeatedly recorded. This weight was recorded as leaf dry weight (DW). The RWC was then calculated using the following formula by Turner (1981):

$$\text{RWC} = (\text{FW} - \text{DW} / \text{SW} - \text{DW}) \times 100$$

Determination of total chlorophyll, chlorophyll a, b and chlorophyll ratios

Photosynthetic pigments were extracted using 2 ml of 100% (v/v) of acetone per sample as described by Lichtenthaler and Wellburn (1983). Ten leaf discs of paper punch size from the upper part of same leaf were prepared from each plant in triplicate and crushed separately with pestle in a mortar. The 2 ml acetone leaf extract was placed into 2 ml Eppendorf tubes and centrifuged for 10 min at 14000 rpm. Then the supernatant (1.5 ml) was transferred to a new clean 2 ml Eppendorf tubes. Chlorophyll extracts were transferred into cuvettes for OD reading at $A_{662\text{nm}}$ and $A_{645\text{nm}}$. One cuvette with acetone served as a blank. Maximum absorbance for chlorophylls (Chl) a and b were recorded at $A_{662\text{nm}}$ and $A_{645\text{nm}}$, respectively. The respective pigments were calculated using the formula by Lichtenthaler and Wellburn (1983):

$$\text{Chla } (\mu\text{g/gfw}) = 11.75A_{662} - 2.350A_{645}; \text{ Chlb } (\mu\text{g/gfw}) = 18.61A_{645} - 3.960A_{662}; \text{ Total Chl } (\mu\text{g/gfw}) = \text{Chla}(\mu\text{g/gfw}) + \text{Chlb}(\mu\text{g/gfw}).$$

Chlorophyll ratio was simply obtained by dividing Chla ($\mu\text{g/gfw}$) by Chlb ($\mu\text{g/gfw}$).

Data management and analysis

Transformation frequencies (TF%) was calculated as number of mannose resistant calli events recovered per 100 embryos infected and transformation efficiencies (TE%) as number of PCR positive plant events per total numbers of embryos co-cultivated. The regeneration frequency (RF%) was computed as number of shoots regenerated per 100 embryogenic calli transferred to REGII medium. Data recorded on the leaf fresh weight, leaf turgor/saturated weight, and leaf dry weight were used to compute leaf relative water content (RWC) on each sampling days. Optical density (OD) of chlorophyll extract determined by reading absorbance at $A_{662\text{nm}}$ and $A_{645\text{nm}}$, were used to compute the amount/content of chlorophyll a, and chlorophyll b, respectively.

Analysis of variance (ANOVA) was carried out using GenStat Discovery Edition 4 (VSN International software for biosciences, (www.vsn.co.uk/software/genstat/) to test the statistical significance of differences among the transgenic and conventional plants and the days of exposure to dehydration stress. Pair wise comparison of means (for RWC, chlorophylls a content, chlorophylls b content, total chlorophylls content and chlorophyll a/b ratio) was carried out using least significance difference (LSD) test at 5% probability level.

RESULTS

Transformation of CML144 maize

The infected but untransformed cells (Figure 2A) grew slowly before turning necrotic and finally died. In contrast to this, putatively transformed cells grew quickly and increased in size forming white or creamy white friable embryogenic calli, which developed somatic embryos (Figure 2A).

Regeneration of putative transgenic plants

On regeneration I (REG I) medium, embryogenic calli were seen to develop somatic embryos (Figure 2B). Upon transfer to regeneration II (REG II) medium under 16 h light and 8 h dark photoperiods, the embryogenic calli turned green and formed shoot buds (Figure 2C), which continued photosynthesizing and grew into distinct shoots, and later developed roots. Shoots which did not form roots in REGII medium were transferred to regeneration III (REG III) for rooting (Figure 2D). Shoots with good root systems were hardened successfully in peat moss (Figure 2E). Plants transplanted into soil in buckets grew further developing to maturity (Figure 2F). Pollinated T_0 plants formed cobs with T_0 seed (Figure 2G). All putative transgenic T_0 events presented in (Table 2) were screened by PCR using PMI specific primers and results were presented in Figure 3. Transgenic (T_1) plants were also screened by PCR using the Psp1 specific primers and results were presented in Figure 4. Primers for XvPrx2 gene specific was not used for PCR to avoid possible amplifying a native Prx2. RT-PCR (Figure 5) revealed the expression of the XvPrx2 gene transcripts in transgenic maize.

Effect of drought stress on morphological response of CML144 maize

Upon commencement of drought stress, leaf wilting was observed within three to five days in conventional and transgenic plants, respectively. However, photo documentation was taken on the seventh day after withholding water (Figure 6A and B). On day 14, transgenic maize plants were still morphologically active and upright whereas conventional maize plants had folded their leaves (Figure 6C). On day 21, both transgenic and

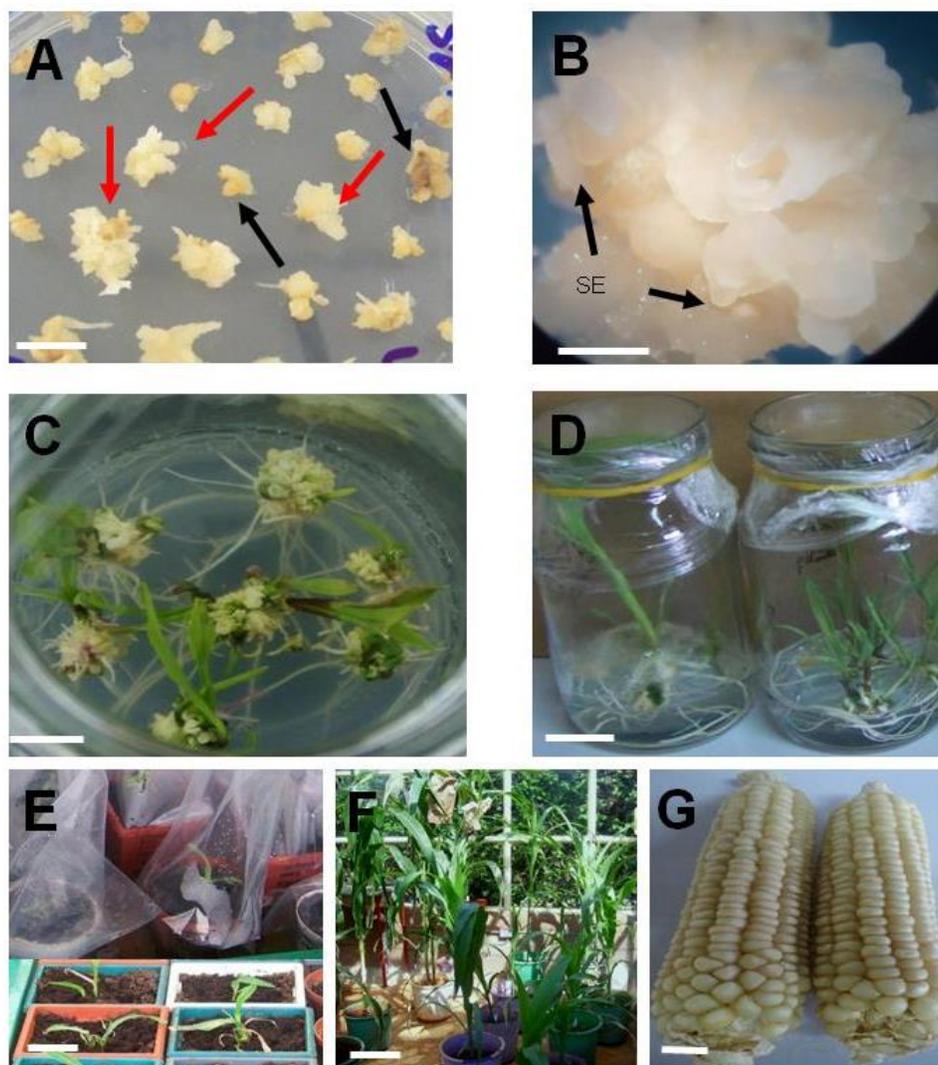


Figure 2. Transformation and regeneration profile of putative transgenic maize. A: Transformed embryos 6 weeks on SEM medium, (red arrows indicate transformed calli surviving on SEM and black arrows indicates untransformed calli dying on SEM), bar = 10 mm; B: Embryogenic callus with somatic embryos 7 days on REG I ready to be transferred to REG II, bar = 10 mm; C: Shooting transformed calli on REG II, bar = 10 mm; D: Putatively transformed T₀ plantlets with good root system ready for hardening, bar = 20 mm; E: Putative transformants undergoing hardening process, bar = 40 mm; F: Putative T₀ regenerants growing in the glass house, bar = 300 mm; G: Harvested T₀ cobs with seeds bar = 10 mm.

Table 2. Putative CML144 transformed with *XvPrx2* gene and recovered T₀ events

Exp	E.I	MRC	Event	T ₀ Plants **	PCR+ plants **	No. Cobs (seeds)	TF (%)	RF (%)	TE (%)
1	224	22	22	5(3)	2(2)	3(~200)	9.8	22.7	0.89
2	295	45	45	16(8)	16(8)	22(~2000)	15.3	35.5	5.42
Total	519	67	67	21(11)	18(10)	25(~2200)	12.9	31.3	3.46

**In brackets represent number of events, Exp: Independent experiment; E. I: Embryos infected and co-cultivated; MRC: mannose resistant callus; T₀ PCR+: number of putative maize plants tested positive by PCR, TF (%): transformation frequency-obtained as a percentage of mannose resistant calli over the total number of immature zygotic embryos infected and co-cultivated, RF (%): Regeneration frequency-obtained as a percentage of number of T₀ plantlets per number of total number of calli events transferred to REG II medium, TE (%): Transformation efficiency-obtained as a percentage of PCR positive plants over the total number of immature zygotic embryos infected and co-cultivated.

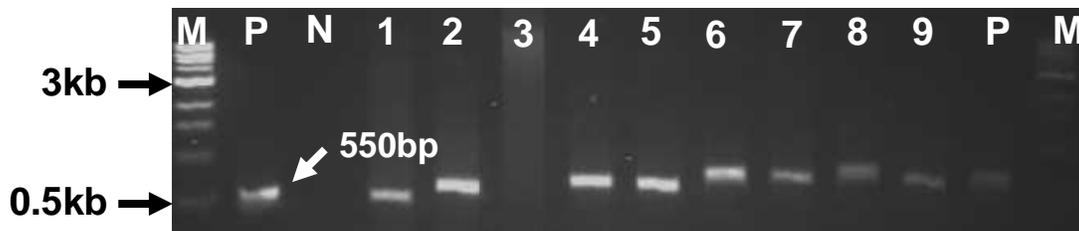


Figure 3. PCR detection of transgenic CML144 maize plants using *PMI* gene specific primers. M: 1 kb ladder, P: positive control (Plasmid construct used for transformation), N: negative control (DNA from conventional CML144 maize), Lanes 1-9 PCR product from DNA of putative transgenic CML144 maize. Expected band size for *manA* gene (positive control) was 0.550 kb.

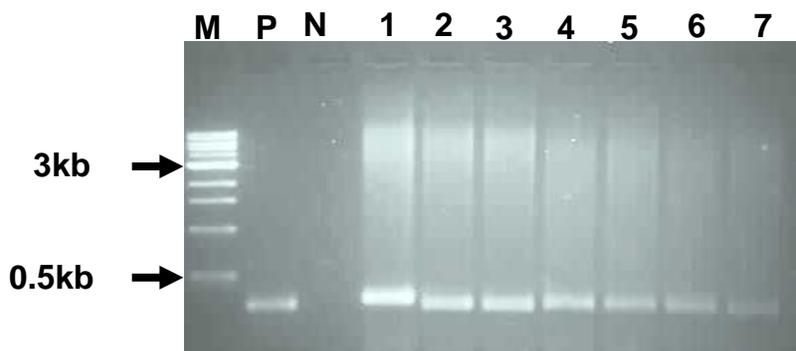


Figure 4. PCR detection of transgenic T₁ CML144 plants using *XvPsp1* promoter specific primers. M: 1 kb ladder, P: Positive control (plasmid construct used for transformation), N: negative control (DNA of conventional CML144 maize), 1-7: PCR product from T₁ transgenic CML144 plants transformed with *XvPrx2* gene construct. Expected band size of 0.395 kb was observed (positive control).

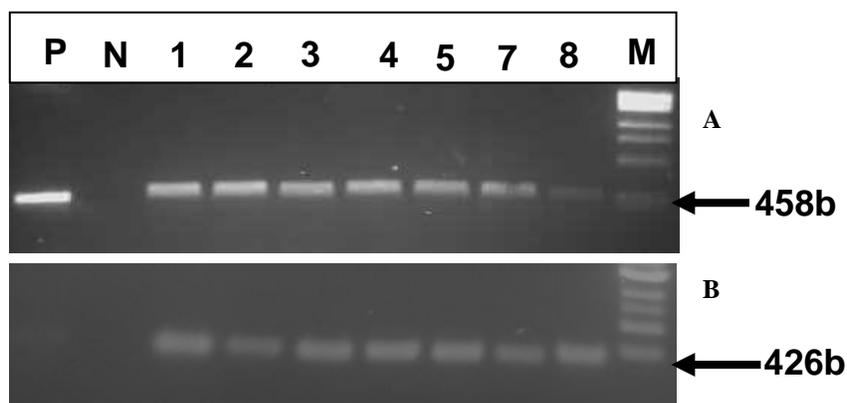


Figure 5. RT-PCR on transgenic and conventional CML144 maize. Panel A: Results obtained using *XvPrx2* specific primers, Panel B: The same substrates amplified with maize actin gene primers for loading control. M: 1 Kb ladder (New England Biolab, UK); P: Positive control (PCR on plasmid of the construct used for maize transformation), N: Negative control (RNA untreated with superscript reverse transcriptase), Lanes 1-7: RT-PCR product from transgenic maize plants cDNA under dehydration, 8: cDNA from conventional maize under dehydration. Expected band size for *XvPrx2* and *Zm-actin* gene was 458 and 426 bp, respectively.

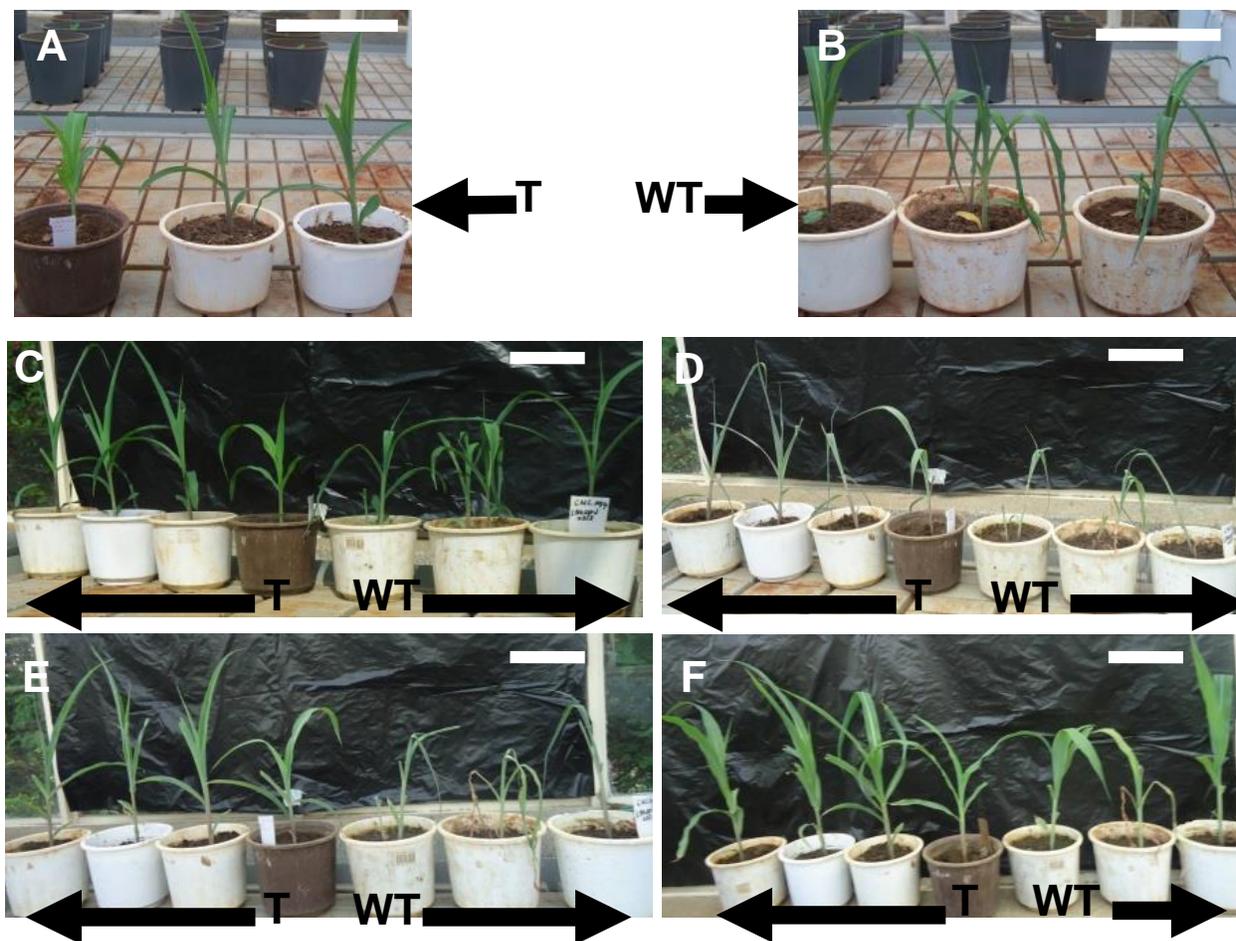


Figure 6. Six week old transgenic and conventional CML144 maize genotypes under different stages of drought stress. A: Transgenic maize after 7 days of drought stress, bar = 150 mm, B: Non transgenic maize after 7 days of drought stress, bar = 150 mm, C: Transgenic (T) and non-transgenic (WT) 14 days after drought stress, bar = 150 mm, D: Transgenic and non-transgenic maize plants 21 days after stress just before re-watering, bar = 150 mm, E: Recovery irrigation of transgenic and non-transgenic plants 6 h after re-watering, bar = 150 mm, F: Recovery irrigation of transgenic and non-transgenic maize plants after 4 days of re-watering, bar = 150 mm. T: Transgenic (CML144-XvPrx2), WT: Conventional CML144.

conventional maize plants had wilted. However, conventional maize showed bleached leaf colour and almost collapsing (Figure 6D). Upon rewatering, recovery in transgenic CML144 maize genotype was noted within 6 to 24 h (Figure 6E) as compared to over 4 days in the conventional CML144 maize counterparts (Figure 6F).

Effect of drought stress on relative water content

RWC (%) of transgenic and conventional maize plants were found to be significantly different from each other at $p < 0.05$ (Table 3). Pairwise comparison of RWC between transgenic and conventional maize also revealed significant difference according to LSD at $p < 0.05$ in all the sampling points. Generally, there was a decrease in RWC in both conventional and transgenic maize plants upon exposure to drought stress. However, the effect

was more pronounced in conventional maize plants than in the transgenic plants (Figure 7). RWC decreased from 85.2 (day 0) to 78.2 (day 7), 73.1 (day 14) and 62.1% (day 21) in conventional maize plants, whereas in transgenic maize the RWC decreased from 85.5% on day 0 to 82.3% on day 7, 78.7% on day 14 and 72.3% on day 21, respectively. Conventional maize plants lost a total of 23.2% of their water within three weeks of drought stress as compared to transgenic maize plants, which lost only 13.3% under similar conditions. RWC determined 24 h after rewatering increased from 62.06 to 74.9% in conventional plants whereas in transgenic plants, the increase ranged from 72.3 to 82.4%.

Effect of drought stress on total chlorophyll content

According to analysis of variance (Table 3), total

Table 3. Analysis of variance for chlorophyll *a*, *b*, TChl, carotenoids content, Chl*a/b* ratio and RWC of transgenic CML144 and non transgenic CML144 maize plants as affected by drought stress.

Source of variation	Df	Mean square					
		Chla	Chlb	TChl	Chl a/b ratio	Carotenoids content	RWC
Genotypes (G)	1	5.01982**	15.9036**	38.7933**	0.244247**	0.63704**	228.682**
Days on stress (D)	4	11.90134**	96.4891**	171.7673**	0.528486**	2.20529**	270.197**
GxD	4	2.76271**	19.6947**	31.6174**	0.135938**	0.73507**	20.766*
Residual (E)	20	0.07617	0.3923	0.5760	0.002689	0.01977	5.250
CV (%)		1.6	4.2	2.4	4.0	8.6	3.0

*Significant differences and **highly significant differences at $p < 0.05$ and $p < 0.01$, respectively. Df: Degrees of freedom, GxD: Interaction of genotype by days under stress, E: Residual (Error), CV (%): Coefficient of variation, Chla: Chlorophyll *a*, Chlb: Chlorophyll *b*, TChl: Total chlorophyll, Chl *a/b*: The ratio of chlorophyll *a* to that of *b*, RWC (%): Leaf relative water content.

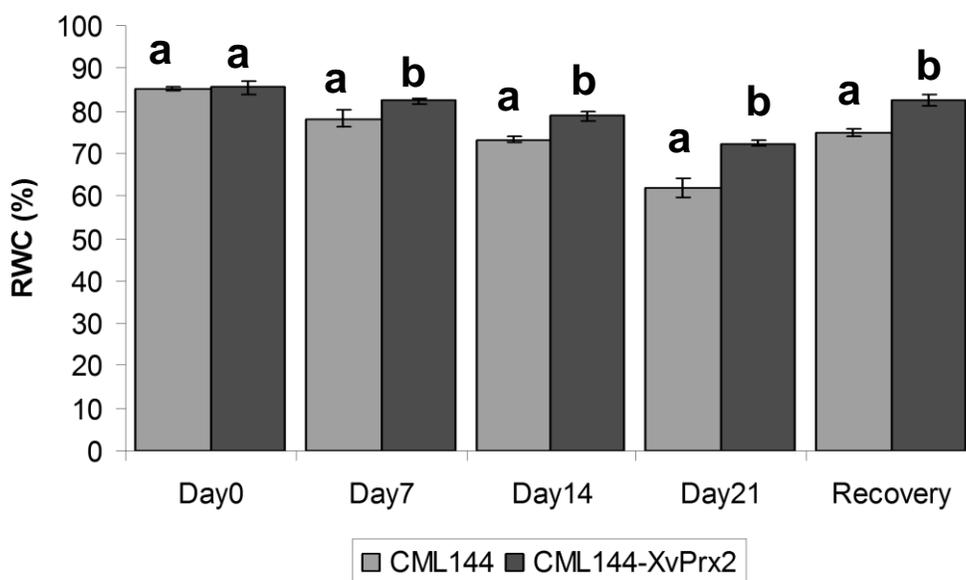


Figure 7. Leaf RWC as affected by drought stress and recovery after re-watering in transgenic maize and conventional CML144 maize plants. Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p < 0.05$.

chlorophyll (TChl) content was significantly different between the transgenic and conventional plants and between the days on which drought was imposed. Total chlorophyll was also significantly influenced by the interaction between the genotype and days of drought stress treatment ($p < 0.05$). Pairwise comparison of TChl between transgenic and conventional maize was significantly different according to LSD at $p < 0.05$ in all the sampling points. The TChl content in conventional plants decreased from 39.2 $\mu\text{g/gfw}$ on day 0 to 35.8 $\mu\text{g/gfw}$ on day 7, 28.9 $\mu\text{g/gfw}$ on day 14 and 22.1 $\mu\text{g/gfw}$ on day 21. In transgenics, the TChl content increased from 35.9 $\mu\text{g/gfw}$ on day 0 to 37.9 $\mu\text{g/gfw}$ on day 7 and thereafter it decreased to 35.7 $\mu\text{g/gfw}$ and 28.9 $\mu\text{g/gfw}$ on day 14 and day 21, respectively (Figure 8). Twenty four hours after rewatering, the total chlorophyll increased from 22.1 $\mu\text{g/gfw}$

to 28.4 $\mu\text{g/gfw}$ in conventional plants, whereas in transgenics, it decreased to 27.3 $\mu\text{g/gfw}$.

Effect of drought stress on chlorophyll *a* content

The Chla contents were significantly different ($p < 0.001$) among the genotypes, days of exposure to drought and the interaction between the genotypes by days of exposure to drought (Table 3). Pairwise comparison of Chla between transgenic and conventional maize also revealed significant difference according to LSD at $p < 0.05$ in all sampling points. Under drought stress, Chla in conventional plants decreased from 18.5 to 18.3, 17.2 and 13.6 $\mu\text{g/gfw}$ on day 0, day 7, day 14 and day 21, respectively. In transgenic plants, Chla decreased from

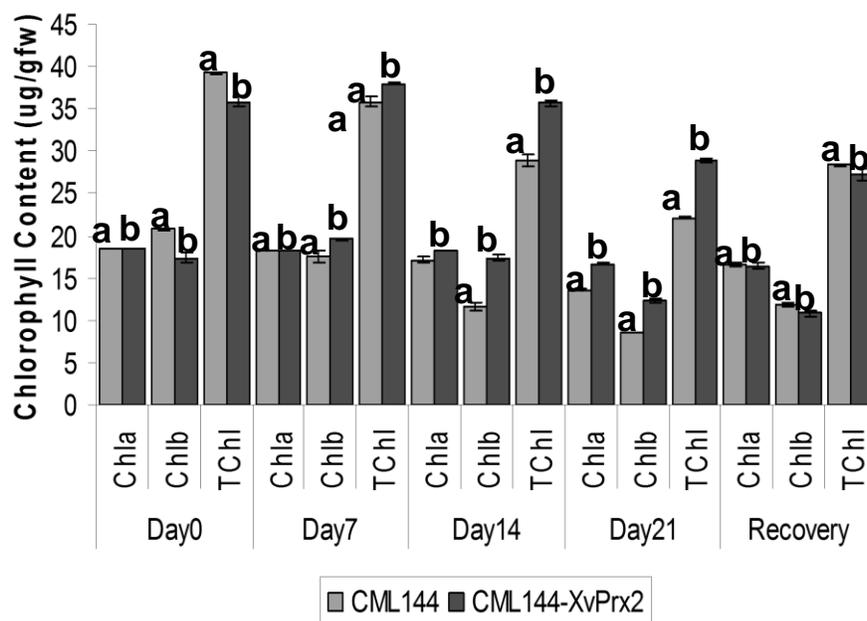


Figure 8. Effect of drought stress and recovery re-watering on chlorophyll content in transgenic and conventional CML144 maize plants. Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p < 0.05$.

18.5 µg/gfw on day 0 to 18.3 and 16.65 µg/gfw on day 7, and day 21, respectively (Figure 8). During recovery watering, Chla increased from 13.6 to 16.6 µg/gfw after 24 h in conventional plants, whereas in transgenics, Chla slightly decreased further from 16.7 to 16.5 µg/gfw.

Effect of drought stress on chlorophyll *b* content

Pairwise comparison of chlorophyll *b* (Chlb) between transgenic and conventional maize revealed significant difference according to LSD at $p < 0.05$ in all the sampling points. The Chlb content in conventional CML144 maize plants decreased from 20.7 µg/gfw on day 0 to 17.5 µg/gfw on day 7, 11.7 µg/gfw on day 14 and 8.5 µg/gfw on day 21. For transformed (CML144-XvPrx2) plants, the content of Chlb increased from 17.4 µg/gfw on day 0 to 19.6 µg/gfw on day 7, followed by a steady decrease to 17.4 and 12.3 µg/gfw on D14 and day 21, respectively. After 24 h of rewatering, the content of Chlb in conventional plants increased from 8.5 to 11.8 µg/gfw, whereas in transgenic maize, Chlb content slightly decreased from 12.3 to 10.8 µg/gfw (Figure 8).

Effect of drought stress to Chlorophyll *a/b* ratio

Chlorophyll (Chla/*b*) ratios were significantly different ($p < 0.05$) between the transgenic and conventional maize genotypes and days under drought stress. There was

also a significant interaction ($p < 0.05$) between genotypes and days under exposure to drought stress (Table 3). Pairwise comparison of Chla/*b* ratio between transgenic and conventional maize also revealed significant difference according to LSD at $p < 0.05$ in all sampling points. The Chla/*b* ratios increased with increase in severity of drought stress across the days for conventional plants. The transgenic plants also showed a relative increase in the Chla/*b* ratios as compared to conventional plants (Figure 9). Upon rewatering, the chlorophyll ratio decreased in conventional plants whereas a slight increase was observed in the transgenic plants (Figure 9).

DISCUSSION

Genetic engineering has become an integral strategy in crop improvement particularly for polygenic traits like drought stress tolerance. In this study, the authors' successfully introgressed *XvPrx2* gene into CML144 maize, a tropical breeding line. Successful over expression of *XvPrx2* gene transcripts was revealed by RT-PCR. However, conventional control also gave a faint signal revealing that *Prx2* is conserved in maize. Hence, the reason to use PMI and *XvPsap1* primers is to confirm the integration of the transgenes in the maize plant genome.

The transgenic plants showed higher RWC than the conventional plants. The amount of water lost by

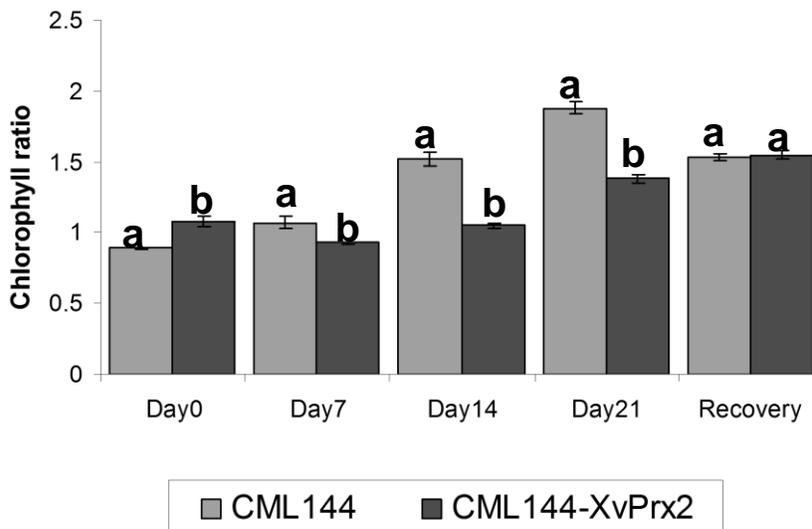


Figure 9. Effect of drought stress on chlorophyll *a/b* ratio in transgenic and conventional CML144 maize plants. Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p < 0.05$.

conventional plants after 21 days of stress was twice that lost by transgenic plants. The latter showed considerable ability to conserve cell water content which sustained the plants to conduct normal physiological activities efficiently under dehydration stress for long time.

The high RWC observed in transgenic maize plants under dehydration stress might have been due to a build up of peroxiredoxin2, the antioxidant product of the *XvPrx2* gene. Peroxiredoxin2 might be involved in ROS scavenging thereby maintaining integrity of the cell membrane by preventing shrinkage of the plasma membrane away from the cell wall and subsequent cytorrhesis. Reduced RWC in drought stressed plants has also been reported by other researchers (Arjenaki et al., 2012; Najafinezhad et al., 2014). The more rapid recovery of transgenic plants than in their conventional counterparts in the present study might have been as a result of the higher RWC delaying the onset of wilting. Several authors have reported similar findings in which drought tolerant genotypes exhibit higher RWC in different species such as *Macrotyloma uniflorum* (Bhadwaj and Yadav, 2012), *Phaseolus vulgaris* (Turkan et al., 2005), barley (Kocheva and Georgiev, 2003), *triticum* (Sairam and Srivastava, 2001) and *Vicia faba* (El-Tyeb, 2006).

Peroxiredoxin2 is likely to be involved in the detoxification of free radicals. The excessive accumulation of ROS under drought stress might have caused injury to cells in drought sensitive conventional plants. Injuries caused by ROS include lipid peroxidation, degradation of membrane protein and inactivation of enzymes such as those responsible for photorespiration (example RUBiSco, PEP carboxylase) which are important components in the

electron transport chain (Sairam et al., 2005; Zlatev and Lidon, 2012).

Under dehydration stress stomatal conductance is reduced to allow more water conservation which results in reduced CO_2 fixation thereby decreasing the rate of photosynthesis as noted by Flexas et al. (2004). Inflicting dehydration stress during vegetative growth stage, decreased substantially the content of TChl, Chla and *b* in both the transgenic and conventional plants. This reduction in photosynthetic pigments might have resulted due to reduced RWC. These findings are similar to report by Terzi and Kadioglu (2006) who noted reduction in photosynthetic pigments while studying drought stress tolerance and the antioxidant enzyme system in *Ctenanthesetosa*. However, the reduction of TChl, Chla and Chlb was less in transgenics than in conventional maize. Transgenic maize maintained a relatively higher content of chlorophylls than the conventional plants. These results complement the reports by Pastori and Trippi (1992) and Zaeifyzadeh and Goliov (2009) who revealed that resistant genotypes of wheat and corn had higher chlorophyll content than sensitive genotypes under the oxidative stress. Lamkemeyer et al. (2006) reported that the absence or presence of *Prx Q* gene (member of peroxiredoxin gene family) in transgenic *A. thaliana* affected chlorophyll *a* fluorescence parameters suggesting a role in maintaining photosynthesis.

Energy absorption in the photosynthetic apparatus acts as the main cause of excessive generation of ROS and plants tend to avoid this through degradation of photosynthetic pigments (Herbinger et al., 2002). The high resistance in degrading chlorophylls observed in transgenic plants in the present study might be attributed

to the presence of peroxiredoxin 2 which protects chlorophylls by quenching of ROS.

The increase in Chla/b ratio in the dehydrated transgenic plants in this study suggests that Chla was relatively stable. These results are in agreement with Ashraf et al. (1994) who reported that drought stress reduced the concentration of chlorophyll *b* more than chlorophyll *a*. This means that the Chla/b ratio may be used as an indicator of plant response to environmental stress such as drought. Therefore, lower Chla/b ratio observed in transgenic maize plants in the present study may indicate better adaptation to drought stress. However, different results were reported by Mafakheri et al. (2010) who worked on chickpea and observed that there was no effect on the chlorophyll *a/b* ratio.

Conclusion

The expression of the *XvPrx2* gene has revealed considerable tolerance to drought stress in transgenic maize as compared to conventional plants. In transgenic maize, RWC, Chla, *b*, *a/b* ratios were higher than in conventional CML144 maize plants implying a functional role of *XvPrx2* gene. Thus, the *X. viscosa* peroxiredoxin2 gene could be used as a strategy for drought stress improvement of maize.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

- Allen GC, Vergara MAF, Krasnyanski S, Kumar S, Thompson WF (2006). A modified protocol for rapid DNA isolation from plant tissue using cetyltrimethylammonium bromide. *Nat. Protoc.* 1:2320-2325.
- Arjenaki FG, Jabbari R, Morshedi A (2012). Evaluation of Drought Stress on Relative Water Content, Chlorophyll Content and Mineral Elements of Wheat (*Triticum aestivum* L.) Varieties. *Int. J. Agric. Crop Sci.* 4(11):726-729.
- Ashraf MY, Azmi AR, Khan AH, Ala SA (1994). Effect of water stress on total phenols, Peroxidase activity and chlorophyll content in wheat. *Acta Physiol. Plant.* 16(3):185-191.
- Baier M, Dietz KJ (1997). The plant 2-Cys peroxiredoxin BAS1 is a nuclear encoded chloroplast protein. Its expressional regulation, phylogenetic origin, and implications for its specific physiological function in plants. *Plant J.* 12:179-190.
- Baier M, Dietz KJ (1999). Protective function of chloroplast 2-cysteine peroxiredoxin in photosynthesis. Evidence from transgenic *Arabidopsis*. *Plant Physiol.* 119(4):1407-1414.
- Bhadwaj J, Yadav SK (2012). Comparative study on biochemical parameters and antioxidant enzymes in a drought tolerant and a sensitive variety of Horsegram (*Macrotyloma uniflorum*) under drought stress. *Am. J. Plant Physiol.* 7(1):17-29.
- Blum A, Mayer J, Golan G, Sinmena B (1998). Drought resistance of a doubled-haploid line population of rice in the field. Workshop on Genetic Improvement of Rice for Water-Limited Environments. IRRRI, Los Baños, Philippines, 1-3 December, 1998.
- Cornic G (2000). Drought stress inhibits photosynthesis by decreasing stomatal aperture-not by affecting ATP synthesis. *Trends Plant Sci.* 5:187-188.
- El-Tyeb MA (2006). Differential responses of pigments, lipid peroxidation, organic solutes, catalase and peroxidase activity in the leaves of two *Vici faba* L. cultivars to drought. *Int. J. Agric. Biol.* 1:116-122.
- Flexas J, Bota J, Loreto F, Cornic G, Sharkey TD (2004). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. *Plant Biol.* 6:1-11.
- Govender K (2006). Characterisation of *XvPrx2*, a type II peroxiredoxin isolated from the resurrection plant *Xerophyta viscosa* (Baker). PhD Thesis, University of Cape Town, Cape Town, South Africa.
- Govender K, Thomson JA, Mundree S, ElSayed AI, Rafudeen MS (2016). Molecular and biochemical characterisation of a novel type II peroxiredoxin (*XvPrx2*) from the resurrection plant *Xerophyta viscosa*. *Functional Plant Biology.* 2016 Jan 12.
- Herbinger K, Tausz M, Wonisch A, Soja G, Sorger A, Grill D (2002). Complex interactive effects of drought and ozone stress on the antioxidant defense systems of two wheat cultivars. *Plant Physiol. Biochem.* 40:691-696.
- Hood EE, Helmer GL, Fraley RT, Chilton MD (1986). The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol.* 168:1291-1301.
- Kocheva K, Georgiev G (2003). Evaluation of the reaction of two contrasting barley (*Hordeum vulgare* L.) cultivars in response to osmotic stress with PEG 6000. *Bulg. J. Plant Physiol.* 49:290-294.
- Lamkemeyer P, Laxa M, Collin V, Li W, Finkemeier I, Schöttler MA, Holtkamp V, Tognetti VB, Issakidis-Bourguet E, Kandlbinder A, Weis E (2006). Peroxiredoxin Q of *Arabidopsis thaliana* is attached to the thylakoids and functions in context of photosynthesis. *Plant J.* 45(6):968-981.
- Lichtenthaler HK, Wellburn AR (1983). Determination of Total Carotenoids and Chlorophylls A and B of Leaf in Different Solvents. *Biol. Soc. Trans.* 11:591-592.
- Linsmaier EM, Skoog F (1965). Organic growth factor requirements of tobacco tissue culture. *Physiol. Plant.* 18:100-127.
- Lugoian C, Ciulca S (2011). Evaluation of relative water content in winter wheat. *J. Hortic. Forest. Biotechnol.* 15(2):173-177.
- Mafakheri A, Siosemardeh A, bahrmejad B, Struik PC, Sohrbi Y (2010). Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. *Aust. J. Crop Sci.* 4(8):580-585.
- Mundree SG, Baker B, Mowla S, Peters S, Marais S, Vander Willigen C, Govender K, Maredza A, Muyanga S, Farrant JM, Thomson JA (2002). Physiological and molecular insights into drought tolerance. *Afr. J. Biotechnol.* 1(2):28-38.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant* 15:473-497.
- Najafinezhad H, Sarvestani ZT, Sanavy SAMM, Naghavi H (2014). Effect of irrigation regimes and application of barley residue, zeolite and superabsorbent polymer on forage yield, cadmium, nitrogen and some physiological traits of corn and sorghum. *Int. J. Biosci.* 5(3):234-245.
- Negrotto D, Jolley M, Beer S, Wenck AR, Hansen G (2000). The use of phosphomannose-isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via *Agrobacterium* transformation. *Plant Cell Rep.* 19:798-803.
- Oduor RO, Iyer R, Machuka J, Thomson JA (2009). Plant promoters of the *XVSAP1* gene. World Intellectual Property Organization Patent no. Patent No. WO/2009/060402.
- Pastori GM, Trippi VS (1992). Oxidative stress induces high rate of glutathione reductase synthesis in drought-resistant maize strain. *Plant Cell Physiol.* 33:957-961.
- Rodriguez-Maribona B, Tenorio JL, Conde JR, Ayerbe L (1992). Correlation between yield and osmotic adjustment of peas (*Pisum sativum* L) under drought stress. *Field Crops Res.* 29:15-22.
- Sairam R, Srivastava G, Agarwal S, Meena RC (2005). Differences in antioxidant activity in response to salinity stress in tolerant and susceptible wheat genotypes. *Biol. Plant.* 49:85-91.
- Sairam RK, Srivastava GC (2001). Water stress tolerance of wheat (*Triticum aestivum* L.): Variations in hydrogen peroxide accumulation and antioxidant activity in tolerant and susceptible genotypes. *J. Agron. Crop Sci.* 186:63-70.
- Smirnoff N (1995). Antioxidant system and plant responses, In: Smirnoff N. (ed) *Environment and Plant metabolism: Flexibility and*

- Acclimation, Bios Scientific Publishers, Oxford. pp. 217-243.
- Terzi R, Kadioglu A (2006). Drought stress tolerance and the antioxidant enzyme system in *ctenanthse setosa*. Acta Biol. Crac. Ser. Bot. 48(2):89-96.
- Turkan I, Bor M, Ozdemir F, Koca H (2005). Differential response of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Sci. 168:223-231.
- Turner NC (1981). Techniques and experimental approaches for the measurement of plant water status. Plant Soil 58:339-366.
- Zaeifyzadeh M, Goliov R (2009). The Effect of the Interaction between Genotypes and Drought Stress on the Superoxide dismutase and Chlorophyll Content in Durum Wheat Landraces. Turk. J. Biol. 33:1-7.
- Zlatev Z, Lidon FC (2012). An overview on drought induced changes in plant growth, water relations and photosynthesis. Emir. J. Food Agric. 24(1):57-72.