GENETIC TRANSFORMATION OF FARMER PREFERRED TROPICAL MAIZE VARIETIES AND INBRED LINES USING DROUGHT TOLERANCE CONFERRING GENES ISOLATED FROM *XEROPHYTA VISCOSA*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other award.

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DEDICATION

I dedicate this work to my Almighty God who gave me life, strength and opportunity to serve my people through scientific research

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ABBREVIATIONS AND ACRONYMS

AS	Acetosyringone
ASARECA	Association for Strengthening Agricultural Research Eastern and Central Africa
BME	β-Mercaptoethanol
BSA	Bovine serum albumin
Bp	Base pair
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
CIF	Callus induction frequency
CIM	Callus induction medium
CIMMYT	International maize and wheat improvement centre
CML144	CIMMYT maize line 144
CML395	CIMMYT maize line 395
CML443	CIMMYT maize line 443
CML488	CIMMYT maize line 488
CMPS	Cetrium yellow leaf curling virus promoter
CMV35S	35S cauliflower mosaic virus promoter
CRD	Complete randomised design
CTAB	Cetyltrimethyl ammonium bromide
2,4-D	2,4-dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EC	Embryogenic calli
EC A	Eastern and Central Africa
ECF	Embryogenic calli frequency
EtOH	Ethanol
FAO	Food and Agriculture Organisation
FSD	Food security department
Inf	Infection medium
Kb	Kilo base
LB	Left border
LBA	Luria-Bertan agar medium
LBB	Luria-Bertan broth medium
LS	Linsmaier and Skoog
MAB	Marker assisted breeding
MAS	Marker assisted selection
MES	2-N-Morpholinoethane sulfonic acid
$MgCl_2$	Magnesium chloride
MS	Murashige and Skoog
NaCl	Sodium chloride
NaEDTA	Sodium ethylenediaminetetraacetic acid

NaOAc	Sodium acetate
NARES	National agricultural research systems
OD	Optical density
OPV	Open pollinated varieties
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMI	Phosphomanose Isomerase
Psi	Pounds per square inch
RB	Right border
REG I	Regeneration I medium
REG II	Regeneration II medium
REG III	Regeneration III medium
RF	Regeneration frequencies
RH	Relative humidity
RM	Resting medium
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
Rpm	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcriptase- polymerase chain reaction
RWC	Relative water content
SEM	Selection medium
Sec	Seconds
SdH_2O	Sterile distilled water
SSA	Sub-Saharan Africa
T_0	Seeds generated from first plants of tissue culture
T_1	Seeds/plants generated from T_0 seeds
Tris-EDTA	Tris- ethylenediaminetetraacetic acid
TBE	Tris boric EDTA
TAE	Tris acetate EDTA
TE	Transformation Efficiencies
T-DNA	Transfer DNA
TF	Transformation Frequencies
Ti	Tumour inducing
XvPrx2	*Xerophyta viscosa Peroxiredoxin2* gene
XVSAP1	*Xerophyta viscose sap1* gene
YEP	Yeast extract peptone

ABSTRACT

Maize supports lives of more than half of the population in Africa yet its production is extremely affected by drought. Yield losses of up to 70% are frequently attributed to drought stress. However, in extreme cases, total yield loss is often experienced. Low maize productivity may persist given the worsening situation of changing climate, coupled with erratic rainfall, prolonged drought spells and rising temperatures. Narrow genetic base of drought tolerant traits in available germplasm is another existing limitation in breeding for drought tolerance. Existing breeding strategies to develop maize capable of surviving drought can be complemented with recent biotechnology tools including genetic engineering. Plants such as *Xerophyta viscosa* Baker that have unique ability to survive extreme drought conditions have in the past provided useful genes and promoters that may find application in crop improvement against drought stress. For example, *Xerophyta viscosa peroxiredoxin 2,* (*XvPrx2*) and *Xerophyta viscosa SAP1,* (*XVSAP1*) genes have previously been isolated and characterised from *Xerophyta viscosa*. The *XvPrx2* gene encodes a type II peroxiredoxin that scavenges for excess reactive oxygen species whereas the *XVSAP1* gene encodes for an integral membrane protein that plays a role in stabilizing membrane integrity during dehydration stress. It was hypothesized that since ROS sequestration and maintenance of membrane intergrity are critical during drought stress, *XvPrx2* and *XVSAP1* genes could be useful in developing transgenic drought tolerant maize. The present study assessed the regeneration ability of selected maize germplasms adapted to Eastern and Central African region and the most regenerable ones were targeted for transformation. Plant expression vectors for genetic transformation contained phosphomannose isomerase gene that allowed the use of mannose as selective agent. One inbred line maize (CML144) and one open pollinated maize variety (Staha) were transformed using *Agrobacterium*-mediated transformation. Ten and 6 transgenic lines were recovered from CML144 and Staha maize, respectively using *XvPrx2* gene and six transgenic lines were recovered from CML144 maize using *XVSAP1* gene. To confirm the success in genetic transformation, polymerase chain reaction, Southern blotting and reverse transcription PCR were used to analyse transgenic maize. Transgenic maize plants were then subjected to drought stress assays that compared the performance of both transgenic and non-transgenic plants under dehydration. Transformation frequencies for CML144 and Staha transformed with *XvPrx2* gene averaged at 12.9% and 23.9%, respectively. Transformation frequency for CML144 transformed with *XVSAP1* gene was 45.2%. Stable transgene integration was revealed in transgenic T_1 plants with low copy numbers ranging from 1 to 3. RT-PCR in T_1 plants confirmed the expression of gene transcripts in transgenic maize plants under drought stress. Under drought stress, relative water content in transgenic CML144 and Staha plants were significantly higher than in non-transgenic maize. Analysis of chlorophyll contents under drought stress revealed relative stability of chlorophylls in transgenic CML144 and Staha compared to non transgenic maize plants. The transgenic plants designated CMl144-XvPrx2, Staha-XvPrx2 and CML144-XVSAP1generated in this study demonstrate great ability to tolerate drought stress under controlled laboratory conditions. However, further assessement of these transgenic plants under confined field conditions need to be conducted prior to availing the seeds to national agricultural research systems for integration into the breeding programs. The transgenic drought tolerant maize developed from this work can be grown in drought prone areas of Eastern and Central Africa.

CHAPTER ONE

INTRODUCTION

1.1 Background

Climate change will have major impacts on the availability of water for growing food and on crop productivity in the decades to come. In addition, the world's population is predicted to rise from the current 6.7 billion to more than 9 billion by 2050 (Edmeades, 2008). As the global population grows, the number of people subjected to hunger continues to increase. It is estimated that 850 million people worldwide are undernourished, with many suffering chronic hunger and unable to feed themselves or their families (Edmeades *et al.,* 1997).

In sub-Saharan Africa, the populations of some countries are doubling or tripling every 50 years, thereby intensifying food insecurity. Rapid population growth obstructs efforts to raise income, protect livelihoods and reduce food shortages, particularly in rural areas where food insecurity is often most terrifying. For instance, in Tanzania and other East African countries, food prices often become highly explosive due to drought thus overwhelming the capacity of national Strategic Grain Reserves (FAO, 2010). Similar to many African countries, agriculture in Tanzania is mostly rain fed, thus crop yields are very vulnerable to environmental constraints including rainfall variation and prolonged drought. Currently, only about 150 thousand hectares are under irrigation, accounting for

approximately 1.6 percent of cultivated land (GoT, 2007). Estimated irrigation potential is about 29.4 million hectares with varying potential levels.

1.2 Production and consumption of maize

Maize (*Zea mays* L.) is the world's third most important cereal crop (after rice and wheat) (Kange *et al.*, 2013). In sub-Saharan Africa, maize is grown by both large and small-scale farmers (Smale and Jayne, 2004) and its shortage has become synonymous to famine. Maize is the main staple grain consumed in Eastern Africa region (Tanzania, Kenya, and Ethiopia). In Uganda, maize is grown mainly as a commodity for regional trade whereas in Tanzania maize production is generally around 3-4 million metric tons per year (NBS, 2007) and is mainly targeted for local consumption. According to the 2002-03 National Sample Census of Agriculture (NSCA), maize is the most widely grown crop in Tanzania, produced by 4.5 million farm households representing about 82% of all Tanzanian farmers. Unlike paddy and sorghum whose production is concentrated in a handful of regions, maize production is geographically spread throughout the country.

In Tanzania, maize is the second most important staple foods after cassava with a per capita consumption of 73 Kg (Minot, 2010). Because of its greater caloric density, maize is more important as a source of calories, contributing 33% of the total compared to 15% for cassava (Minot, 2010). This means that Tanzania is

more dependent on maize than Uganda and Ethiopia that rely on banana and teff, repectively.

1.3 Constraints to maize production

Although maize is a crucial staple food crop, the average yield per hectare in Africa is the lowest in the world (ISAAA and KARI, 1999). Furthermore, the annual yield increase in Africa has not kept pace with the rising population growth. This low production has largely been affected by environmental stresses such as drought, salinity and extreme temperatures (de Vries, 2000; Wani *et al.,* 2003; Rockström *et al.,* 2007). These constraints in the face of global warming have left most countries in the Eastern and Central African (ECA) with recurrent food shortages.

The challenges to increased maize yields are varied. The problems of agricultural productivity in sub-Saharan Africa (SSA) are compounded by the nature of the environment, crops, pests and diseases, farming systems, climate change, low irrigation capacity and unfavourable policy regimes. The productivity of smallholder farmers in SSA remains low due to numerous biotic and abiotic production constraints. Technological innovations that could address such constraints may exist and those that are being developed are increasingly coming with patents that control their commercialisation thereby limiting wide-scale

dissemination of such technologies for the benefit of smallholder farmers in SSA (AATF, 2010).

Although pests and diseases have been mentioned as biotic constraints to maize production, some studies in sub-Saharan Africa have shown that drought is more critical than even nutrient required for yield production (Bray *et al.,* 2000). Across the ecological regions of sub-Saharan Africa, abiotic constraints are attributed to the frequent erratic amount and distribution of rains and poor soil conditions for plant growth (Wani *et al.,* 2009). Drought-induced crop losses result when water deficit occurs during sensitive phases of crop growth notably during crop establishment and flowering (Bolaños and Edmeades, 1996; Chapman and Edmeades, 1999; Wani *et al.,* 2009). Drought-induced yield losses are estimated at 2.4 million tons per year (Edmeades *et al.,* 1994). For instance, Nkonya *et al.* (1998) reported that more than 25% of the land in Tanzania is threatened by drought either annually or once in every four years thereby affecting yields of maize and other crops. Similarly over, 80% of the total land in Kenya is under arid and semi-arid agro-ecology (Survey of Kenya, 2003) thus making these countries food insecure (Figure 1.1).

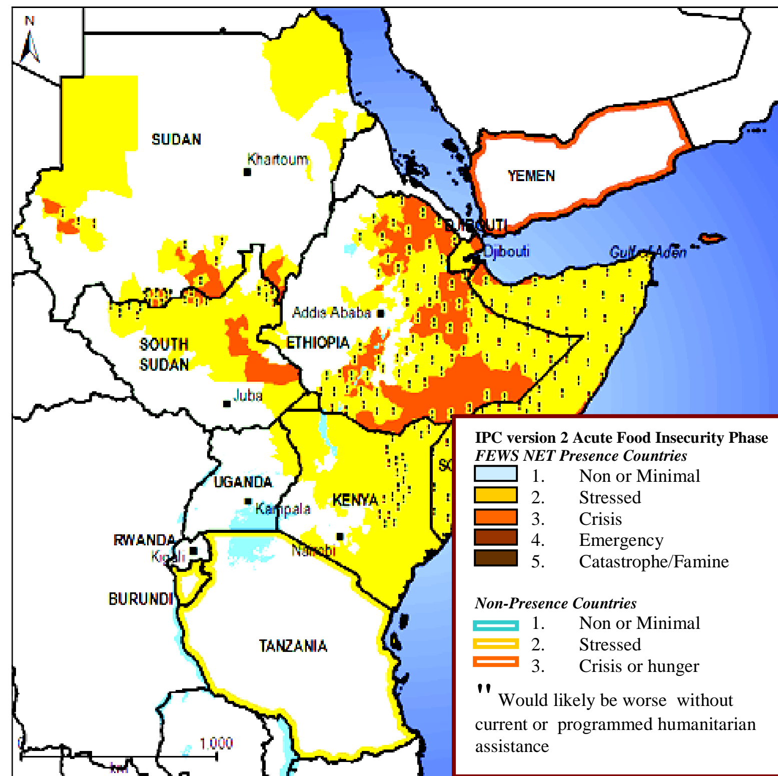

Figure 1.1: Severity map of food insecurity in Eastern Africa
Source: FEWS NET Jan-June 2013

As the world population continues to grow and water resources for crop production decline, the development of drought-tolerant cultivars and water-use-efficient crops is a global concern (Barnabas *et al.,* 2008). In the context of current and predicted water scarcity, increasing irrigation is generally not a viable option for alleviating drought problems in rainfed maize-growing systems. It is therefore critical that genetic management strategies for drought focus on maximum extraction of available soil moisture and its efficient use in crop establishment and growth to maximize yield. Extensive genetic variation for drought tolerance exists in maize germplasm. However, the current challenge is to translate the

complexities of drought tolerance in maize and exploit all available genetic resources to produce maize varieties combining drought adaptation with high yield potential, quality and adaptable to biotic stresses. The strategy described here aimed at developing a pipeline for elite breeding lines and open pollinated maize genotypes that can be integrated with efficient management practices and delivered to maize farmers. This involves the development of drought tolerant maize genotypes through genetic engineering strategy that can allow genes for yield components under stress to be efficiently expressed and their effects assessed on a range of drought-related environments.

While conventional plant breeding has succeeded in producing a wide variety of commercial plants and crops with a range of important agronomic traits, it is however, to a large extent an unpredictable process. Conventional breeding involve combining large parts of parental genomes in a rather uncontrolled manner, although this is currently being improved due to integration with the modern technique of marker assisted breeding (MAB). However, use of MAB by introgression of genomic potions referred to as quantitative trait loci (QTLs) involved in stress tolerance often brings along undesirable agronomic characteristics from the donor parents (Bhatnagar-Mathur *et al.,* 2008). In view of shortcomings of marker assisted selection (MAS) and/or MAB brought by lack of a precise knowledge of the key genes underlying the QTLs, the deployment of genetic engineering to introduce and/or over-express genes confering agronomic

value is currently viewed to be a viable option to speed up the breeding process of crops plants against drought and other agronomic traits. The use of *Agrobacterium tumefaciens* as a mode of introgressing genes has been increasingly used as a method of choice due to low copy number of transgenes, well defined borders of T-DNA and high transformation efficiencies (Negrotto *et al.,* 2000; Frame *et al.,* 2002).

Target transgenes and stress-inducible promoters needed for crop improvement against drought can be obtained from unique angiosperms such as the 'resurrection plant (*Xerophyta viscosa*) that possess the ability to withstand desiccation of their vegetative tissues and revive from air dry state (Gaff, 1987). As such, drought tolerant confering *XvPrx2* (Govendor, 2006) and *XVSAP1* (Garwe *et al.,* 2003) genes previously isolated from the resurrection plant (*X. viscosa*) may be used in crop improvement to confer drought tolerance. In the current study, the two genes (*XvPrx2* and *XVSAP1*) were used to genetically transform selected tropical maize varieties and inbred lines for drought stress adaptability.

1.4 Problem statement and justfication

The socio-economic significance of maize in the ECA sub-region has been well documented. In the ECA, maize has become the number one staple food crop, with about 90% used as food (ECAMAW, 2005). However, the productivity of this crop is very low with yields not exceeding 1.2t/ha. This is predominantly due to

low farm investiment, biotic and abiotic constraints, especially drought and low soil fertility (CGIAR, 2003).

Drought has emerged to be one of the major abiotic factor that contributes to reduced maize productivity. This is worsened by lack of suitable varieties that perform well under insufficient and erratic rainfall. As a result, maize productivity has significantly reduced, with grain yield not exceeding 1.3 t/ha, compared to the potential of over 10 t/ha.

The contribution of classical breeding to solve drought stress problem has been very limited. Breeding work done for the past many years emphasized on the high yield potential areas. As a result, many improved maize varieties/hybrids have been made available to farmers in those areas. In contrast, few improved maize varieties have been released and being grown with low levels of management in drought affected areas. The reason is that maize research for drought stress tolerance has proceeded slowly through convetional breeding. The breeding activities are also handicapped by a narrow genetic base of available germplasm, and the selection criteria for drought tolerant maize varieties are marginally established (Mandefro *et al.,* 2001). The potential of modern biotechnology tools (MAS and genetic engineering) to overcome impediment of conventional breeding is a better option. This can only be achieved by the improvement of water use

efficiency of the regional germplasm using the available biotechnological tools such as genetic engineering.

The present study assessed the potential for regeneration of four open pollinated maize varieties (OPVs) accessed from Tanzania and three international maize and wheat improvement centre (CIMMYT) maize inbred lines. The best regenerating genotypes were transformed using two binary vector single gene constructs harbouring drought tolerance confering *XvPrx2* and *XVSAP1* genes through *Agrobacterium tumefaciens*-mediated transformation. The performance of the resulting transgenic maize was compared to non-transformed controls under drought stress.

1.5 Hypothesis

Transgene expression of *XvPrx2* and *XVSAP1* gene transcripts can confer drought stress tolerance in tropical maize genotypes.

1.6 Objectives

1.6.1 Broad objective

To evaluate the regeneration ability and genetically engineer selected tropical maize varieties and inbred lines with genes enhancing drought adaptability.

1.6.2 Specific objectives

i. To evaluate the regeneration ability of selected tropical maize genotypes using immature zygotic embryo as explants.

ii. To develop transgenic maize through transformation of immature zygotic embryos using *XvPrx2* and *XVSAP1* single gene constructs.

iii. To evaluate the performance of transgenic maize plants under drought stress.

CHAPTER TWO

LITERATURE REVIEW

2.1 Plant responses to dehydration stress

Plant tolerance to drought can be classified into escapism, avoidance and tolerance strategies (Chaves *et al.,* 2003). These responses can as well be categorised as morphological, physiological, developmental and or genetic responses.

2.1.1 Morphological responses

Avoiding dehydration depends on maintenance of a high plant water status during stress. Avoiding dehydration may be a result of minimized water loss (due to stomata closure, trichome formation, and reduced leaf area senescence of older leaves) or maximized water uptake by increased root growth (Barnabas *et al.,* 2008).

2.1.2 Developmental responses

Escape strategy relies on successful reproduction by means of completing the life cycle early before the onset of severe drought stress, or by high rate of growth or the efficient storage and use of reserves for seed production.

In cereals, apical morphogenesis is sensitive to water deficit. Water stress during flowering and inflorescence development leads to delay in flowering (anthesis), or

complete inhibition (Wopereis *et al.,* 1996; Winkel *et al.,* 1997). Other studies have reported a delay in flower initiation caused by water stress in *Pennisetum* and *Sorghum* (Mahalakshmi and Bidinger, 1985; Craufurd and Peacock, 1993).

2.1.3 Physiological responses

Plant tolerance to to drought stress is based on maintenance of plant function under limited water availability and/or the recovery of plant water status and plant function after stress, which may involve osmotic adjustment, but may also be the result of rigid cell walls or small cells. Drought tolerance may as well be associated with the efficient scavenging of reactive oxygen species (ROS) formed as a result of disturbed metabolism (Sairam and Saxena, 2000).

At molecular level, drought stress induces various biochemical and physiological responses in plants. According to Dat *et al.* (2000) these responses include, but not limited to generating reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide or the hydroxyl radical. The radicals are by-products of electron transport chains in chloroplast and mitochondria, photorespiration in the peroxisome, and activity of cell wall oxidases and peroxidises.

Under ideal environmental condition, ROS are necessary for plants because they participate in signal transduction (Orozco-Cardenas *et al.,* 2001; Mullineaux and Karpinski, 2002) and play a role in response to pathogen attack and regulation of

plant development (Bolwell, 1999; Dat *et al.,* 2000; Kwak *et al.,* 2003; McInnis *et al.,* 2006; Potocky *et al.,* 2007). Under unfavorable environmental conditions, such as extreme temperature, drought, or salt stress, the rate of carbon fixation is limited, causing an increase in photoinhibition potentially steering the photosystem toward overproduction of superoxide radicals and H_2O_2 (Foyer and Noctor, 2005). The biotic or abiotic stress may further promote excessive generation of ROS which inturn leads to lipid peroxidation (causing breakage of the redox balance of the cell), damaged DNA and hampered protein sysnthesis. Metabolic changes in response to water stress include accumulation of organic acids such as malate, citrate and lactate accompanied by accumulation of proline, sugars (trehalose) sugars alcohols (manitol and sorbitol) and betaine in the cell (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997; Tabaeizadeh, 1998), and an overall reduction in protein synthesis (Ritchie *et al.,* 1990).

However, some studies have shown that a combination of drought and heat stress on growth and productivity of maize, barley, sorghum and various grasses have a significantly greater detrimental effect compared to single stress effect applied individually (Savin and Nicolas, 1996; Wang and Huang, 2004). For instance, in a previous study conducted by Xu and Zhou (2006) on perennial grass *Leymus chinensis*, high temperature with severe soil drought, reduce the function of PSII by destroying the photosynthetic pigments, weakening nitrogen utilization, elevating protein catabolism and hightening lipid peroxidation. Metabolic profiling

has also revealed that plants subjected to a combination of drought and heat stresses accumulate sugars such as sucrose, maltose and glucose. In contrast, proline has been shown to accumulate in plants subjected to drought alone, whereas during a combination of drought and heat stresses no accumulation of proline has been observed in plants (Xu and Zhou, 2006). Heat stress has been found to mitigate the toxicity of proline to cells, suggesting that during a combination of drought and heat stress sucrose replaces proline as the major osmoprotectant (Rizhsky *et al.,* 2004) or it is more expensive to make. Based on physiological and molecular characterizations, many similarities between the responses of *Arabidopsis* and *Nicotiana* to drought and heat stress combination have been reported, suggesting that this mode of defense response is conserved among different plant species (Rizhsky *et al.,* 2004).

However, to protect macromolecules such as lipids, proteins, or nucleic acids from damage caused by ROS produced by cells due to drought stress, cells contain a large variety of antioxidant enzymes that include catalase, superoxide dismutase, ascorbate and glutathione dependent peroxidases, and peroxiredoxin (Bre´he´lin *et al.,* 2003).

2.1.4 Genetic responses

At genetic level, plants respond by down or up regulation of some of their genes under drought stress. Genes responsive to dehydration and cold, such as

RESPONSIVE TO DEHYDRATION/Dehydrin COR47 (*RD17/COR47*), *RD29A/COR78/Iti78* and *NICOTIANA PROTEIN KINASE 1* (*NPK1*) have been reported to be up-regulated during drought (Seki *et al.,* 2002; Machuka, 2004; Shou *et al.,* 2004a; Shinonzaki and Yamaguchi-Shinozaki, 2007). Such genes can be used to improve drought stress tolerance of plants by gene transfer (Bajaji *et al.,* 1999; Roy and Basu, 2009). Previously, drought stress tolerance has been engineered in temperate maize and other crops such as rice, tobacco and wheat using various genes, such as dehydration responsive element and C-repeat binding factors which are transcriptional factors (DREB1/CBF4), *RESPONSIVE TO DEHYDRATION* gene (*RD29A*), *NICOTIANA PROTEIN KINASE 1* (*NPK1*) and *MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE* (*MAPKKK*) with varying success (Ishida *et al.,* 1996; Seki *et al.,* 2002; Kasuga *et al.,* 2004; Shou *et al.,* 2004a).

2.2 *Xerophyta viscosa* and the resurrection hope

Recent studies have revealed that unique resurrection plants such as *Xerophyta viscosa* Baker [family Velloziaceae] (Figure 2.1) have evolved ability to tolerate prolonged drought condition. Studies have also shown that this plant can be dehydrated up to 5% relative water content (RWC) and upon rewatering the desiccated plant rehydrates completely within 80 h, resuming full physiological activities (Sherwin and Farrant, 1998). A number of genes that are up regulated during drought stress from *X. viscosa* Baker have been identified (Mundree *et al.,*

2002), some of which code for antioxidant enzymes postulated to protect DNA against free radicals. Similarly, other genes are thought to be involved in stabilizing the cell membrane leading to stability of osmotic balance and reduced water loss during periods of abiotic stress. Such genes include *XvPrx2* which encode peroxiredoxin2 an antioxidant enzyme that manage ROS (Govendor, 2006) and *XVSAP1* which encode an integral membrane protein that is expressed in response to low temperature and dehydration stress (Garwe *et al.,* 2003).

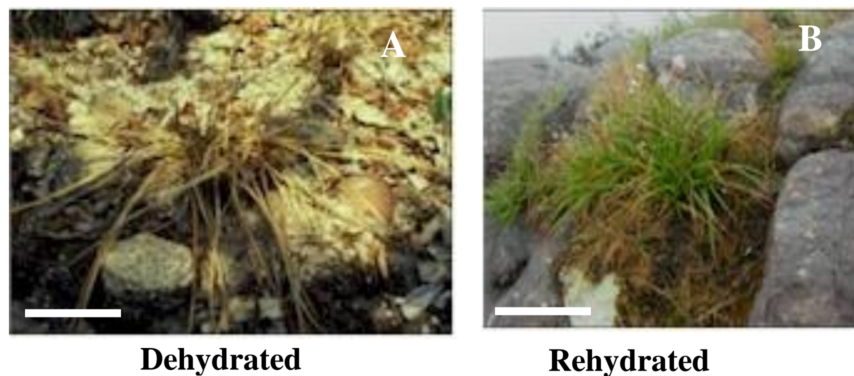

Figure 2.1: *Xerophyta viscosa* plants in their natural habitat
A: Resurrection plant, *Xerophyta viscosa* in their dehydrated state on rock crevices, **B:** *Xerophyta viscosa* resurrecting after rewatering, *bar* = 100 cm.

The presence of cysteine (Cys) residues in peroxiredoxin2 gene family plays an important role in ROS scavenging in that the peroxide reacts with the *N*-terminally located peroxidatic thiol group, oxidising it to sulphonic acid, releasing H_2O, alcohol and nitrite from hydrogen peroxide (H_2O_2) and nitric oxide (NO) substrates, respectively. The sulphonic acid group of peroxidatic Cys reacts with

the second Cys residue to form an intra-molecular or intermolecular disulphide bridge and water (Figure 2.2).

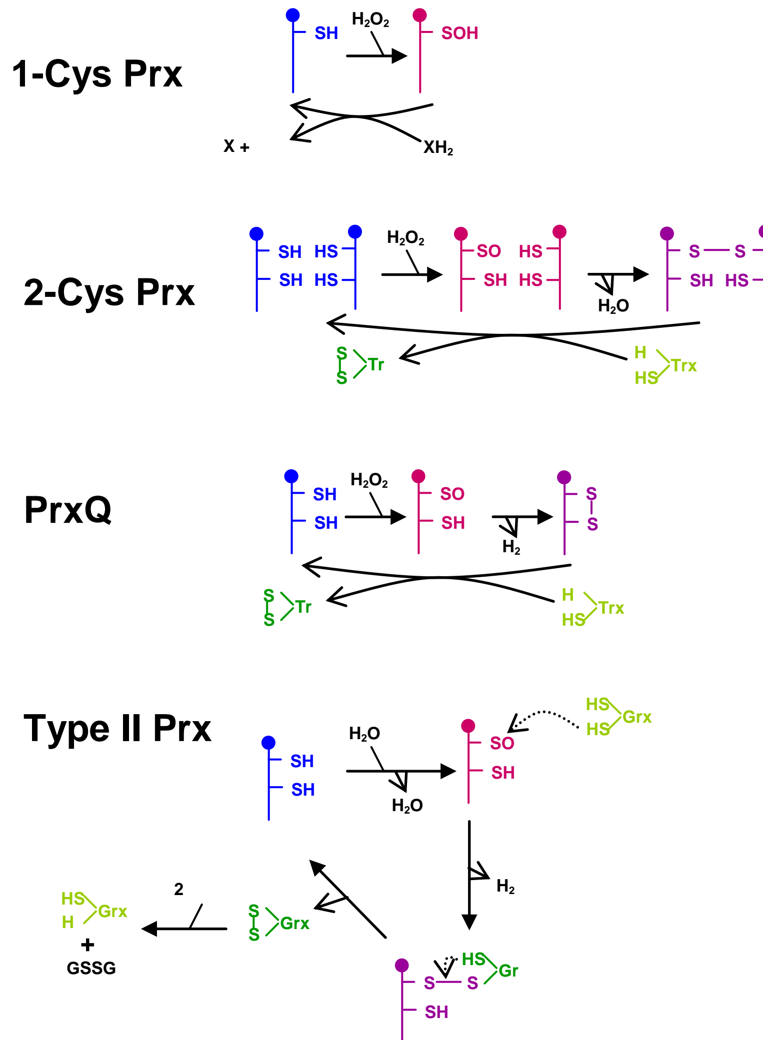

Figure 2.2: Illustration of the proposed catalytic mechanisms of H_2O_2 reduction and Prx regeneration for the four Prx groups.
The proteins are represented by pins with the knob being the N-terminus. Source: Rouhier and Jacquot (2002).

Similarly, integral membrane proteins that are highly hydrophobic and anchored on a lipid of plasma membrane and are predicted to have a role to play in protecting the plant against environmental stresses (Garwe *et al.,* 2003). It is

therefore postulated that *XVSAP1* plays an important role in response to water deficit stress by maintaining integrity of membrane proteins during stressful conditions that result in protein denaturation. One of the roles that *XVSAP1* could play is the stabilization of membranes during the dehydration process (Garwe *et al.,* 2003).

According to Garwe *et al.* (2003), examination of the expression of *XVSAP1* during dehydration and rehydration of *X. viscosa,* showed that only dehydration and not rehydration induces the expression of the gene. Interestingly, Garwe *et al,* (2003) indicated that *XVSAP1* expression was strongly induced at relative water contents (RWC) of 51% and 44% and not at any other stage. This indicates that *XVSAP1* is not required in the initial stages of dehydration but is only expressed when dehydration becomes severe and the plant has dried down to approximately 51% RWC. Furthermore, no expression of *XVSAP1* was observed during rehydration indicating that *XVSAP1* has no role to play during this process.

The *XvPrx2, XVSAP1* and *XvPer* genes have been tested in drought sensitive species of plants such as *Digitaria sanguinalis, Nicotiana tabacum* and *Arabidopsis thaliana* and have shown to improve tolerance to drought, cold, salt and high temperature stresses (Mundree *et al.,* 2002; Garwe *et al.,* 2006; Govendor, 2006; Peters, 2006).

2.3 Maize improvement strategies for drought tolerance

2.3.1 Conventional breeding for drought stress tolerance of maize

For a long time, breeding efforts placed yield rather than survival into the focus of crop, especially cereal, improvement. Consequently, high yielding cereal cultivars became widespread in agriculture and yield safety gained more importance. Abiotic stresses such as extreme temperatures and low water supply frequently limit the growth and productivity of major crop species including cereals. The process of grain filling, the accumulation of reserve nutrient in the developing and maturing grain, is also sensitive to environmental conditions strongly affecting final yield both quantitatively and qualitatively (Barnabas *et al.,* 2008).

Strategies to improve maize for drought stress tolerance through conventional breeding have been to extract inbred lines from improved populations using pedigree selection and test-cross evaluation. Researchers at Institutions such as the Applied Biotechnology Centre at CIMMYT, Mexico (Bruce *et al.,* 2002) and several national agricultural research establishments (NAREs) in the Eastern and Central Africa (ECA) region have combined molecular markers with conventional breeding tools to improve tolerance to drought (Bruce *et al.,* 2002). Despite all these efforts, limited success has been achieved. The shortfall of conventional approach has been due to the fact that drought tolerance is a complex trait, thus the breeding activities are handicapped by narrow genetic base of available germplasm and unestablished selection criteria for drought tolerance trait and existence of

biochemical and physiological knowledge gap at gene level (Seth, 2007). With this understanding, there is an urgent need to compliment conventional breeding with genetic engineering approaches.

2.3.2 Genetic engineering approach

Genetic engineering is the genetic alteration of a cell resulting from introduction, uptake and expression of foreign genetic material such as DNA or RNA (Avery *et al.,* 1944). Various transformation techniques have been employed in delivering genes to plants ranging from direct to indirect. Direct methods include biolistic or particle bombardment, electroporation and microinjection.

Of the three direct methods, particle bombardment is the most popular. The method uses rapidly propelled tungsten or gold microprojectiles coated with DNA. These DNA coated particles are 'fired' into plant cells and the DNA of interest is often incorporated at random into the target plant genome. Besides being very costly, the method is unable to transfer large DNA segments (Hamilton *et al.,* 1996) and produces high copy numbers of the transgenes (Ishida *et al.,* 1996; Zhao *et al.,* 1998). Electroporation employs electrical impulses to create transient pores in the cell membrane and cell walls increasing their permeability to DNA contained in the surrounding solution. Microinjection involves injection of DNA directly into the cell nucleus using an ultrafine needle and poly-ethelyne-glycol (PEG) in which plant cell protoplasts are treated with PEG making them

momentarily permeable to allow uptake of DNA from the surrounding solution (Datta *et al.,* 1992; Peng *et al.,* 1992; Fujimoto *et al.,* 1993). These methods have been employed but with low efficiency and the fertility of the transgenic plants have always been poor suggesting the need to explore other potential indirect methods such as *Agrobacterium*-mediated transformation.

2.3.2.1 *Agrobacterium*-mediated transformation

More recently, *Agrobacterium*-mediated transformation was developed for 'recalcitrant' species, including cereals like maize (Ombori *et al.,* 2013). These advances have been enhanced by the development of proper gene constructs for *Agrobacterium* based on binary vector concept (Hoekema *et al.,* 1983). The binary vector must include marker gene for efficient selection of transformed plant cells while facilitating ligation strategies for insertion of target genes which are to be transferred. The method has been increasingly used as the method of choice for reasons such as the ability to develop transformants having low copy number, well-defined borders, and high transformation frequency (Negrotto *et al.,* 2000; Frame *et al.,* 2002). In addition, *Agrobacterium*-mediated transformation generates transgenic plants with intact foreign gene, better fertility and stable gene expression (Dai *et al.,* 2001).

Natural living soil bacteria, such as *A. tumefaciens* and *A. rhizogenes*, are examples of natural plant transformation systems, causing crown gall disease and 'hairy root syndrome' respectively. These bacteria have natural transformation ability that can be exploited in plant biotechnology. During infection of plant cell, *Agrobacterium* can transfer a section of its plasmid DNA, known as the 'transfer DNA' (T-DNA) into plant cells carried on its Ti (Tumour Inducing) plasmid. The T-DNA is flanked by two 25 base pair indirect repeats and in principle any DNA contained within the borders can be transferred to the host cell (Zupan and Zambryiski, 1995; Gelvin, 2003; Lee and Gelvin, 2008). Besides causing a gall, the *Agrobacterium* harnesses the plant's machinery to produce unique sugars called opines as a source of nutrients. The common methods for introducing DNA into plant cells is by use of 'disarmed' *A. tumefaciens* in which the Ti plasmid gene in the T-DNA regions are replaced with target genes such as those capable of conferring drought tolerance in higher plant.

2.4 Plant promoters, gene expression and safety concerns of selectable markers

Genetic transformation requires a transgene (gene cassette) constituting the gene of interest and a selectable marker gene that should be integrated in the plant genome and expressed in the transgenic plant. The selectable marker genes are usually included to serve as indicators to identify transgenic cells/plants.

2.4.1 Promoters

The expression of the transgenes and marker genes only occur in the presence of promoters. A promoter is a regulatory region of DNA located upstream (towards the 5' region) of a gene, providing a control point for regulated gene transcription. The promoters of the two genes may or may not be different. The promoters may be constitutively expressed such as cauliflower mosaic virus (*CaMV35S*), cestrium yellow leaf curling virus (*CMPS)*, *actin* and maize *ubiquitin* promoter or may be inducible promoter or specific tissue promoter such as *SARK, Rab21* and *Wsi18* promoters (Bhatnagar-Mathur *et al.,* 2008). Transgenic plant developed with the gene of interest expressed only in certain tissues or under certain conditions is more beneficial than one that is expressed throughout. This is because constitutive expression of genes tends to aggregate gene products throughout even when they are not required; as a result, they become deleterious to plants. Stunted growth, decreased fertilization and germination are a few adverse effects caused by accumulation of secondary metabolites or other molecules such as trehalose or H_2O_2 in transgenic plants constitutively expressing drought responsive associated transcription factors (Bhatnagar-Mathur *et al.,* 2008; Ergen *et al.,* 2009).

To down regulate the gene products in time that they are not required in transgenic plants is therefore important. This can be achieved by over-expressing the gene of interest only in the targeted tissues or only after imposing an environmental stress such as drought stress or pathogenic infection. For example, using root specific

promoter *RCc3* (Jeong *et al.,* 2010) with nematode resistance genes can be targeted to the roots, delayed fruit ripening to the fruit or stalk borer resistance to the stalk. This may as well assist safety and regulatory aspects if, for example, nematode resistance is not expressed in edible fruits.

Recently a novel stress inducible XvPsap1 promoter from a resurrection plant, *Xerophyta viscosa* was characterised (Oduor *et al.,* 2009). Analysis of this promoter fragment in transgenic Black Mexcan Sweet (BMS) cells, tobacco and maize indicated that luciferase activity peaked on the third day of dehydration stress and was followed by a significant decline after rehydration. The resulting transgenic plants were observed to display normal growth with insignificant phenotypic variations (Oduor *et al.,* 2009). These results demonstrated that the XvPsap1 promoter is both active and stress-inducible. The XvPsap1 promoter being stress inducible could therefore be used for generation of transgenic drought tolerant monocot plants like maize.

2.4.2 Selectable marker genes and safety concerns

Visible reporter genes have been used to detect or track whether a particular site directed promoter is able to target the gene of interest to the desired tissues in transgenic plants. This is achieved by fusing the gene of interest to a reporter gene that is readily visualized in a manner that is not destructive to the plant. Such genes include the luciferase gene (*luc*) and the green fluorescent protein (*gfp*),

which originates from the bacterium, *Vibrio harveyi* or the firefly (*Photinus pyralis*) and from the jellyfish, *Aequorea Victoria,* respectively (Greer III and Szalay, 2002). Light emission can be monitored visually, photographically or electronically for expression of luciferase gene and in order to visualize the fluorescence in green tissue the mutant form of the *gfp* gene with improved emission must be used and driven by a very strong promoter.

For successful plant transformation the issue of developing a reliable and efficient system for transgenic tissue selection is of paramount importance. Selectable marker genes can be divided into several categories depending on whether they confer positive or negative selection and whether selection is conditional or non-conditional on the presence of external substrates. Positive selectable marker genes are those whose effect would favour growth of transformed tissues while non-transformed tissues would suffer growth or developmental disadvantages imposed by the selective substrate or agent. On the other hand negative selectable marker genes result in the death of the untransformed tissue. Selectable marker genes that are conditional on the use of toxic agents, such as antibiotics, herbicides or drugs were the first to be developed and exploited (De Block *et al.,* 1987; Simpson and Filipowicz, 1996; Russell and Fromm, 1997; Wang *et al.,* 1997; Limanton-Grevet and Jullien, 2001; Howe *et al.,* 2002). Unfortunately, antibiotic and herbicide resistance marker genes are not appropriate for all plant species, for example, for monocots (Wilmink and Dons, 1993), and they are not favourably accepted by the

public despite the fact that the safety of these markers has been thoroughly tested and proven over several years (Williams *et al.,* 2000). In addition, the selective agents used may adversely affect the transformed plant cells bringing about a decrease in the regeneration of transformed cells by accumulation of toxic compounds from dead non-transformed cells (Hansen and Wright, 1999). More recent developments include positive selectable marker genes that are conditional on non-toxic agents that may be substrates for growth or that induce growth and differentiation of the transformed tissues. Recent strategies of positive selectable marker genes include those that are not conditional on external substrates but which alter the physiological processes that govern plant development (Miki and McHugh, 2004).

To date, a number of marker genes have been employed for the development of alternative selection methods. Such selection systems include benzyladenine-N-3-glucuronide (Joersbo and Okkels, 1996), phosphomannose isomerase (PMI) (Joersbo *et al*., 1998), D-xylose (Haldrup *et al.,* 1998), 2-deoxyglucose (Kunze *et al.,* 2001), D-amino acid (Erikson *et al.,* 2004), D-serine (Erikson *et al.,* 2005), ferredoxin-like protein gene (You *et al.,* 2003), or *E. coli* threonine deaminse gene (Ebmeier *et al.,* 2004) as selectable markers. As the production of transgenic plants is labour intensive, expensive and difficult for most species, practical issues govern the choice of selectable marker genes that are used. Many of the genes have specific limitations or have not been sufficiently tested to merit their

widespread use. For research, a variety of selection systems are essential, as no single selectable marker gene has been found to be sufficient for all circumstances.

2.4.2.1 Phosphomannose isomerase selection system

Joersbo *et al.* (1998) first developed the phosphomannose isomerase (*PMI*) gene as a selectable marker for the transformation of sugar beet. Since then *PMI* gene has been succesifully used as marker in the *Agrobacterium*-mediated transformation of a number of plant species such as cassava (Zhang *et al.,* 2000), maize (Negrotto *et al.,* 2000; Reed *et al.,* 2001; Wright *et al.,* 2001), arabidopsis (Todd and Tague, 2001), wheat (Wright *et al.,* 2001; Gadaleta *et al.,* 2006), rice (Lucca *et al.,* 2001), sweet orange (Boscariol *et al.,* 2003), hemp (Feeney and Punja, 2003), pearl millet (O'Kennedy *et al.,* 2004), bentgrass (Fu *et al.,* 2005), papaya (Zhu *et al.,* 2005), sorghum (Gao *et al.,* 2005), almond (Ramesh *et al.,* 2006), onion (Aswath *et al.,* 2006), cucumber (He *et al.,* 2006), chinese cabbage (Ku *et al.,* 2006) and tomato and potato (Briza *et al.,* 2008).

The PMI is common in bacteria, yeast and mammals including humans (Lee and Matheson, 1984). However, in plant kingdom, there are only a few species like soybeans and several other legumes where the enzyme has been found (Lee and Matheson, 1984). Therefore the cells of majority of plant species including maize take up mannose and convert it by endogenous hexokinase to mannose-6-

phosphate. The accumulation of mannose-6-phospate in plant cells inhibits glycolysis, depletes the cell of inorganic phosphate and induces endonucleases to degrade DNA (Stein and Hansen, 1999). So the addition of sucrose or glucose could alleviate the effect of mannose on growth and germination (Joersbo *et al.,* 1998).

Mannose and its derivatives are common constituents of living cells and are key components of intermediary metabolism. Mannose is also the major sugar residue in the carbohydrate portion of glycoproteins. PMI catalyses the reversible inter-conversion of mannose-6-phosphate to fructose-6-phosphate which is an intermediate product of glycolysis and positively influences the growth of transformed cells, whereas in its absence, the mannose-6-phosphate accumulates in cells and block the glycolytic pathway thereby cells are deprived of energy and stop growing. The selection system employing the *manA* gene (Miles and Guest, 1984) coding for PMI is very handy in transgenic research because it is considered safe than antibiotics and herbicides. The safety assessment for PMI (Reed *et al.,* 2001) revealed that purified PMI protein has no evidence of adverse effects in mice and does not change glycoprotein profiles in PMI-transformed plants. PMI posses no sequence homology to any known allergens and contains no *N*-glycosylation consensus sequence (Reed *et al.,* 2001). Toxicity assessment on mice has shown no clinical signs of toxicity or any negative effect on body weight gain following acute oral administration of *E. coli* purified PMI (for more review

on safety of mannose see Privalle *et al.,* 2002). Comparing the agronomic characteristics of non-transgenic and transgenic maize plants transformed with *PMI* gene, no statistically significant differences in growth or other agronomic parameters have been found (Reeds *et al.,* 2001; Mburu, 2007).

2.5 *In vitro* regeneration of tropical maize

Genetic transformation of plants through *Agrobacterium*-mediated and other related techniques seems to be a promising means in crop improvement. However, the major drawback to genetic transformation of recalcitrant cereal crops such as maize is lack of reliable and efficient *in vitro* regeneration system (El-Itriby *et al.,* 2003; Satyavathi *et al.,* 2004; Oduor *et al.,* 2006). To achieve optimal plant transformation, a good and efficient regeneration system must be established for recalcitrant crops like tropical maize genotypes. It is often desirable that the regeneration protocols for each selected genotype for transformation be optimised as genotype dependent responses have previously been reported (Tomes and Smith 1985; Hodges *et al.,* 1986; Willman *et al.,* 1989; Bohorova *et al.,* 1995). Except for a few reports (El-Itriby *et al,*. 2003; Oduor *et al.,* 2006; Binott *et al.,* 2008; Ombori *et al.,* 2008), most studies on regeneration of maize have utilized genotypes adapted to temperate environments with little or no focus on assessing regeneration potential of tropically adapted maize germplasm. In addition, limited work has been carried out to genetically improve tropically adapted maize germplasm for drought tolerance (Ombori *et al.,* 2013).

In vitro regeneration represents an important step for maize genetic transformation. From the successful regeneration of maize by Green and Phillip (1975) through somatic embryogenesis, regeneration has developed and maize genotypes used expanded to elite inbreds among several responding lines such as A188, A619, A634, Wf9, W64A, H99 (Duncan *et al.,* 1985; Hodges *et al.,* 1986). Even with all these efforts, the number of genotypes suitable for efficient plant regeneration is still limited (Duncan *et al.,* 1985; Hodges *et al.,* 1986; Bohorova *et al.,* 1995). In view of this, it is important to establish efficient regeneration procedures for particular maize OPVs and inbred lines adapted to ECA region with the purpose of identifying their amicability for genetic transformation.

2.6 Somaclonal variation

When tissue culture driven plants are potted or transplanted into soils and grown up under normal environmental conditions, the regenerants may exhibit transient, non-genetic or epigenetic changes as well as heritable, genetic variation (Karp 1995; Vasil, 2005). Epigenetic changes appear to be a direct effect of the culture process, being physiological in origin and perhaps resulting from effects of the growth regulators in the medium (Fluminhan *et al.,* 1996). Such epigenetic changes are of no value for crop improvement, as they are not expressed in sexual progeny although may have tremendous effect on the development such as impaired fertility and growth of R_0 or T_0 plants. However, studies have shown that alteration in chromosome number and structure can occur on *in vitro* regenerated

plants and that chromosome instability can be induced by media components, culture age, explant tissue and even by plant genotype (Peschke and Phillips, 1992; Jain, 2001). Chromosome variations in regenerable maize callus cultures have been investigated in few studies and only changes in chromosome number have been reported (Fluminhan *et al.,* 1996).

The fact that somaclonal variation can occur in high frequency in regenerants, and that heritable single gene changes can occur in nuclear and organelle genomes, it presents an attractive avenue to breeders only if the variation can occur in subsequent generations . The most convenient way would be to introduce the best available varieties into culture and then select for those somaclones that display positive improvements as a result of somaclonal variation. This may purpose at retaining all the favourable qualities of the variety as well as to affix an additional trait such as drought tolerance, disease resistance or herbicide tolerance (Lindsey and Jones, 1989). However, long time scores of over two decades have revealed that much of the observed variations are neither novel nor useful. To date, no important new crop plant including maize germplasm has been developed as a result of somaclonal variation, and this is no more an active field of research (Vasil, 2005).

CHAPTER THREE

REGENERATION OF SELECTED TROPICAL MAIZE GENOTYPES USING IMMATURE ZYGOTIC EMBRYO EXPLANTS

3.1 INTRODUCTION

This chapter describes development of regeneration protocol for selected commercially important Tanzanian maize OPVs and CIMMYT inbred lines using immature zygotic embryos as source of explants through somatic embryogenesis. The objective of this chapter was to identify the most suitable genotypes for establishment of a highly efficient regeneration system in maize genetic transformation. As such, three CIMMYT inbered lines namely CML395, CML443 and CML488 and four open pollinated maize varieties namely Kito, Situka M-1, Staha and TMV-1 from Tanzania were regenerated *in vitro* using immature zygotic embryos as source of explant. The 2, 4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 1, 1.5, 2 or 2.5 mg/l were used to induce calli in maize immature zygotic embryos. Induction of calli and regeneration of plantlets from embryogenic calli was carried out as described by Ombori *et al.* (2008). With this regeneration system Kito, Situka M-1 and Staha were found to be the most regenerable genotypes among the open pollinated maize genotypes whereas CML395 was the most regenerable genotype among the inbred lines studied. However, Kito had high rate of somatic embryo abortion whereas Situka M-1 was

observed later to be prone to somaclonal variations. TMV-1 was the least performer in terms of calli induction although it demonstrated to have high regeneration ability. CML443 was only responsive under low levels of 2,4-D (optimally at 1mg/l) whereas CML488 failed to respond completely and was not regenerable in all levels of 2,4-D tested. Generally, low concentrations of 2,4 D (1-1.5 mg/l) were observed to be the optmal levels for calli induction and subsequent plant regeneration. These results further underscore the fact that calli induction and regenerability of various lines are largely genotypic dependent.

3.2 MATERIALS AND METHODS

3.2.1 Source of explants, plant establishment and seed bulking

Maize seeds of Tanzanian commercial OPV; Kito, Situka M-1, Staha and TMV-1 were collected from East African Seed (T) Ltd (EASEED), Arusha, Tanzania. The CIMMYT inbred lines; CML488, CML443 and CML395 were kindly provided by Dr. Dan Makumbi of CIMMYT, Nairobi, Kenya. The OPVs were selected based on farmers' preference for yield, maturity, and tolerance to diseases, pounding quality and the inbred lines by consultation with CIMMYT breeders on the potential for genetic improvement. Maize plants were grown in pots and bulked in the greenhouse at the Plant Transformation Laboratory (PTL), Kenyatta University, Nairobi, Kenya. The planting time and days to silking and tasseling of the varieties were recorded to determine the anthesis-silking intervals.

3.2.2 Controlled pollination of maize

Growth and development of maize plants was closely monitored to mark the emergence of ears. The ear bags were tightly secured on the emerging top ear of the plant to cover them before the silk emerged to prevent contamination from foreign pollen. A day before pollination, ears were cut to enable fresh silk emergence and efficient trapping of pollen. The ear bags were punctually placed over the freshly cut ears to protect silks. The plant's tassels were routinely checked to ensure that the plants shed enough pollen for pollination. After identifying the mature tassels, pollination bags were carefully placed over the plant's tassels and

the tassels grasped by holding the bag and making a central ridge for better support. The pollination bags were then secured with paper clip.

Pollination was carried out mid day. Pollen was tapped into pollen bag by shaking the tassel five to seven times to release pollen. The bags were carefully taken down to avoid contamination from other pollen sources by keeping shut the opening of the bag. The ear bags were pulled out and the pollination bags were straightened and tapped slightly to allow the pollen to be introduced to the fresh silk. Inbred lines were self pollinated whereas open pollinated varieties were sib-crossed to maintain their genetic stabilities. All the four flaps of the bags were finally tightly stapled against the stalk and the ear. Kernels were allowed to develop for 12 to 16 days before harvesting for subsequent experiments.

3.2.3 Surface sterilization and embryo excision

Ears were harvested between 12-16 days post pollination when the immature embryos attained a size of 1-1.5 mm. Ears were dehusked and surface sterilized by 3% (v/v) commercial bleach (Jik Bleach Regular; RECKITT BENCKISER East Africa) supplemented with 1-2 drops of Tween 20® for 20 min under sterile conditions prior to rinsing 3 times with sterile distilled water. Embryos were aseptically removed using a spatula after trimming the top edge of kernels superficially with scapel blade.

3.2.4 Callus induction, maintenance and maturation

Calli were initiated by placing 35 embryos per plate in triplicates on callus induction media (CIM) amended with varying concentrations (1, 1.5, 2 or 2.5 mg/l) of 2, 4-D (Appendix I). The embryos were incubated on CIM for 14 days in the dark at a temperature of 26±2℃. Primary calli were transferred to callus maintenance medium (CMM) and further incubated for 28 days in the dark at a temperature of 26±2℃ following a fortnight sub-culturing regime (Ombori *et al.,* 2008). Calli from CMM were transferred to embryo maturation medium (EMM) (Binott *et al.,* 2008). All media were prepared using MS premix basal salts (Murashige and Skoog, 1962) enriched with relevant supplements as presented in Appendix I. The pH of all media used in this study was adjusted to 5.8 before autoclaving at a temperature of 121℃ for 20 min with a pressure of 15 psi.

3.2.5 Regeneration and hardening of plantlets

Embryogenic calli (EC) with mature somatic embryos were transferred from EMM medium to shoot induction medium (SM) and incubated under cool white fluorescent tubes (60-90 µmol photons $m^{-2}s^{-1}$) photoperiod of 16 h light and 8 h darkness at a temperature of 26±2℃. Sprouted shoots with well developed root system were directly hardened whereas those with no or few roots were transferred first to half strength MS medium (RM) to enhance rooting under similar conditions as those for shooting. Hardening of plantlets was carried out in pots (100 mm x 100 mm x 100 mm depth) containing peat moss (KEKKILA Co. Ltd, Tuusula,

Finland) and covered with white transparent polythene bags to maintain relative humidity of 80% for 3 days. After 5-7 days, surviving regenerants were transplanted to plastic pots (150 mm radius with 330 mm depth) containing forest soils for development and maturity.

3.2.6 Experimental design and data management

Experiments for calli initiation and embryogenic calli formation were designed as a 7x4 factorial (7 genotypes treated with 4 concentration levels of 2, 4-D) arranged in a completely randomized design (CRD) with each experiment conducted in triplicates. Callus induction frequency (CIF) was scored as the percentage of the total number of immature embryos, which formed embryogenic calli in CMM per total number (35 embryos per plate) of initial immature zygotic embryos cultured. Embryogenic calli formation frequency (ECF) was determined as the percentage number of calli containing at least a single somatic embryo in EMM per number of the initial immature embryos cultured. Regeneration frequency (RF) was calculated as the percentage number of shoots proliferated per total number of EC transferred to SM. The analysis of variance (ANOVA) for CIF, ECF and RF data was done using GenStat Discovery computer software Edition 4 (VSN International software for biosciences, www.vsni.co.uk/software/genstat/). Means were separated using least significant difference (LSD) at 5% level of significance. Graphics were prepared using excel software (Microsoft).

3.3 RESULTS

3.3.1 Source of explants and determination of anthesis-silking interval

Determination of anthesis-silking interval (ASI) was carried out only in four varieties (Kito, Situka M-1, Staha and TMV-1). The ASI was not determined in inbred lines (CML395, CML443 and CML488) because their information was available. Although days to silk and anthesis varied among the varieties (Table 3.1), the ASI was found to be six days for all the varieties except for Kito, which was two days. ASI is an important parameter that is useful in determining the right time for pollination and eventual collection of embryos at the right age and size. In the present study, immature zygotic embryos of 1-1.5 mm obtained at the age of 12 to 16 days after pollination were found to be the best sources of explant

Table 3. 1: Developmental features and attributes of Tanzanian OPV maize varieties used for tissue culture

Variety	Type	Year released	Recommended altitudes (M)	Days to Anthesis *	Days to Silking *	ASI (Days) *	Yield (t/ha)		Agronomic attributes
							Potential	Expected	
Kito	OPV	1983	0-1,500 Low to mid altitude	92	94	2	6.0	2.50-3.5	Small stature varieties, early maturing variety
Situka M-1	OPV	-	0-1,500 Low to mid altitude	99	105	6	-	-	Drought tolerant
Staha	OPV	1983	0-1,500 Low to mid altitude	92	98	6	6.5	4.0-4.5	Tolerance to maize streak virus disease
TMV-1	OPV	1987	0-1,500 Low to mid altitude	98	104	6	4.25	-	Has white, flinty grain, is streak resistant, and has intermediate maturity

ASI: Anthesis-silking interval.
*These developmental features were obtained from the current study, whereas other data were obtained from East African Seed (T) Ltd (EASEED Arusha, Tanzania).

3.3.2 Callus induction, maintenance, maturation and regeneration

Maize embryos formed primary calli within 4 to 5 days on CIM. These calli were observed to originate at the scutellum of the zygotic embryo (Figure 3.1A). After 28 days of culture on CMM, a mass of dedifferentiated cells was visible (Figure 3.1B). Two types of calli were observed which were classified as non embryogenic, *NE* (Type I) and embryogenic, *EC* (Type II) calli (Figure 3.1C). Type I calli were characterized as white, compact, organized and slow growing, whereas type II calli looked creamy yellow, brittle or friable and fast growing. Type II embryogenic calli are the most preferred as they often regenerate to plantlets upon exposure to SM (Oduor *et al*., 2006). Explants on MS medium amended with 2,4-D resulted into calli production whereas those on control plate (devoid of 2, 4-D) germinated instead of forming calli. Shoot formation was observed 3 to 4 days after transferring EC to hormone free SM. Upon exposure to light, EC greened in 2-3 days (Figure 3.1D) followed by sprouting of shoot which occured 3-4 days thereafter (Figure 3.1E). In this study, all shoots developed roots on SM (Figure 3.1F). Shoots with stable root systems were hardened in peat moss for 3 to 5 days (Figure 3.1G). Hardened plantlets grew to maturity when they were transplanted in the forest soil (Figure 3.1H). During plant development in soil, no abnormal phenotypes were observed (Figure 3.1I).

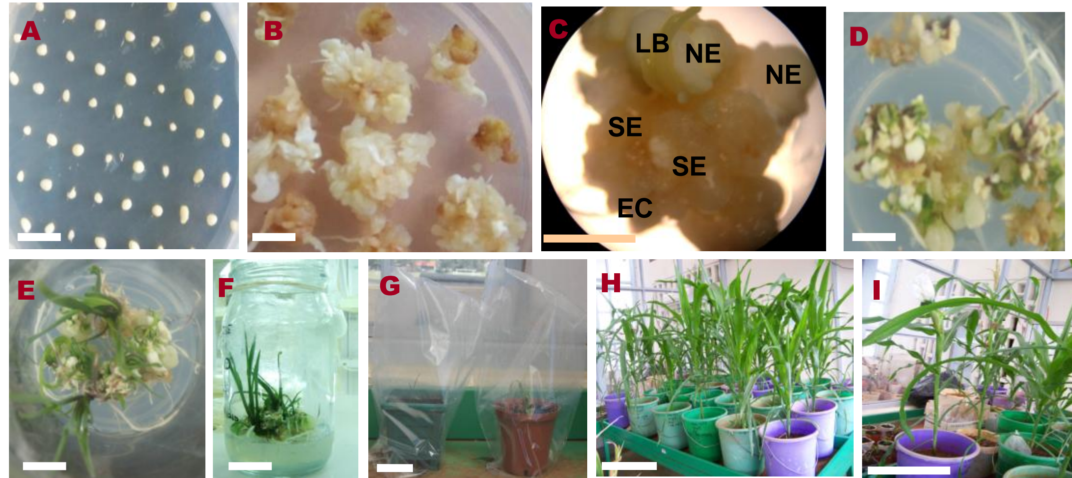

Figure 3.1: *In vitro* regeneration of tropical maize genotypes
A: Immature embryos bulging 1-3 days of culture on CIM, *bar* = 10 mm; **B:** Embryos forming calli 14 days on CMM, *bar* = 10 mm; **C:** Callus displaying both type I, *NE* and type II, *EC*, somatic embryos *SE*, leaf bud, *LB* emerging from mature somatic embryo, *bar* = 1 mm; **D:** Somatic embryos greening 2-5 days on SM upon exposure to light, *bar* = 10 mm; **E:** Leaf buds emerging from mature somatic embryos on SM, *bar* = 10 mm; **F:** Two weeks growing maize shoots on SM, *bar* = 10 mm; **G:** Hardening of plantlets in peat moss in the glass house, *bar* = 10 mm; **H:** Regenerated maize plants growing in forest soils in the glass house, *bar* = 300 mm; **I:** *In vitro* maize regenerants tasselling and silking at Kenyatta University PTL glass house, *bar* = 300 mm.

In this study, only 2, 4-D was used because it is by far the most efficient auxin among the different analogues used to induce somatic embryogenesis. Analysis of variance (Table 3.2) revealed that varying 2,4-D concentrations had no significant effect on CIF within genotypes. However, significant ($p \leq 0.05$) differences with respect to CIF and ECF were observed among the genotypes. Similarly, ECF were significantly ($p \leq 0.05$) influenced by 2,4-D and the effect of interaction of 2, 4-D by genotype.

Table 3. 2: Analysis of variance for callus induction frequency, embryogenic callus formation frequency and regeneration frequency in seven genotypes at four 2, 4-D concentrations

Sources of variation	Df	Mean Square		
		CIF	ECF	RF
Genotypes (G)	6	13488.6**	6142.2**	24892.
2, 4-D (mg/l) (GR)	3	65.8	618.4*	27063.
G x GR	18	172.2	655.6**	14722.
Residual (E)	56	149.9	210.9	13794.
CV (%)		19.5	43.0	396.6

* Indicates statistical significant differences at $p \leq 0.05$; ** Indicates statistical significant differences at $p \leq 0.01$; **Df:** Degree of freedom, **G:** Genotype, **GR:** Growth regulator (2, 4-D), **E:** Residual (Error), **CIF:** Callus induction frequency, **ECF:** Embryogenic callus formation frequency, **RF:** Regeneration frequency, **CV (%):** Coefficient of variation.

Among the maize varieties tested, Situka M-1, Staha, Kito and CML395 had significantly higher CIF of 93, 89, 84 and 79%, respectively (Table 3.3), compared to CML443 (51%) and TMV-1 (43%). CML488 failed completely to induce callus at all concentrations of 2, 4-D studied.

Table 3. 3: Callus induction frequencies as influenced by genotype and 2, 4-D

Genotypes	2, 4-D (mg/l)				Genotype *
	1.00	1.50	2.00	2.50	
CML395	93.7±1.9†	74.7±3.5	81.3±18.7	65.3±3.5	79±5.0[ab]
CML443	40.7±5.2	60.0±8.3	52.0±6.9	52.0±6.1	51±4.0[b]
CML488	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0±0.0[c]
KITO	73.3±7.0	90.7±3.5	87.6±13.2	86.2±10.0	84±4.0[a]
SITUKAM-1	96.0±2.3	94.8±5.2	89.3±10.7	90.7±3.5	93±3.0[a]
STAHA	89.3±1.3	84.0±4.6	92.0±6.1	89.3±10.7	89±3.0[a]
TMV-1	40.0±4.6	33.3±6.7	54.7±9.6	45.3±3.5	43±4.0[b]
2, 4-D (mg/l)*	62.0±8.0[a]	62.0±7.0[a]	65.0±8.0[a]	61.0±7.0[a]	

†Values=Mean±SE,
*Means followed by different letters indicates statistically different from each other according to LSD at $p \leq 0.05$.

On the other hand, Kito demonstrated the highest ECF (69%) followed by Situka M-1 (50%), CML395 (39%), Staha (35%), CML443 (27%) and TMV-1 (15.7%). The highest ECF for Kito, Situka M-1, CML395, Staha, CML443 and TMV-1 were optimally obtained at 2.5, 1, 2, 1, 1.5 and 1.5 mg/l 2, 4-D, respectively (Table 3.4). ECF were significantly higher when lower concentrations (1-1.5 mg/l) of 2, 4-D were used, implying that these concentrations are sufficiently optimal for embryogenic callus induction in the genotypes used in this study. In this study, formation of somatic embryos (SE) on calli was observed while on CMM. However, SE proliferation and growth appeared to be slow on EMM. Among the varieties tested, Kito was observed to abort SE on EMM.

Table 3. 4: Embryogenic callus induction frequencies as influenced by genotypes and 2, 4-D

Genotypes	2, 4-D (mg/l)				Genotype*
	1.00	1.50	2.00	2.50	
CML395	34.3±6.9^{b} †	41.3±8.7^{ab}	52.4±18.2^{ab}	26.7±4.8^{b}	39±5^{b}
CML443	30.0±1.2^{b}	34.7±3.5^{b}	25.3±3.5^{b}	18.7±4.8^{b}	27±2^{c}
CML488	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0.0±0.0^{c}	0±0^{e}
KITO	61.3±7.1^{ab}	64.0±8.3^{a}	75.3±14.4^{a}	77.3±6.6^{a}	69±5^{a}
SITUKA M-1	85.9±4.1^{a}	64.9±28.3^{a}	8.3±1.9^{c}	40.0±4.6^{ab}	50±11^{b}
STAHA	52.0±6.1^{ab}	36.0±2.3^{b}	29.3±2.7^{b}	24.4±8.4^{b}	35±4^{bc}
TMV-1	10.7±5.8^{bc}	22.7±7.4^{b}	13.3±3.5^{b}	16.0±2.3^{b}	15.7±3^{d}
2, 4-D (mg/l)*	39.0±6.0^{a}	38.0±6.0^{a}	29.0±6.0^{b}	29.0±5.0^{b}	

†Values=Mean±SE, Values followed by different letters in column and rows indicate statistically different from each other according to LSD at $p \leq 0.05$
*Means followed by different letters indicates statistically different from each other according to LSD at $p \leq 0.05$.

3.3.3 Effect of genotype and 2,4-D on regeneration frequency

Means of RF for CML395 was observed to be 120%, possibly because some calli produced more than one shoot. The mean regeneration frequency for CML443 was very small (8%) and it was also observed that response to CIF, ECF and subsequent regeneration of this genotype was only possible with lower concentrations (1-1.5 mg/l) of 2, 4-D. On the other hand Staha was comparatively the most regenerable among the OPVs with RF of 32.6% compared to Kito (Figure 3.2) which had the highest callus induction and embryogenic callus formation. TMV-1 that was less responsive to calli formation showed high regeneration ability compared to Situka M-1 that had good performance on CIM.

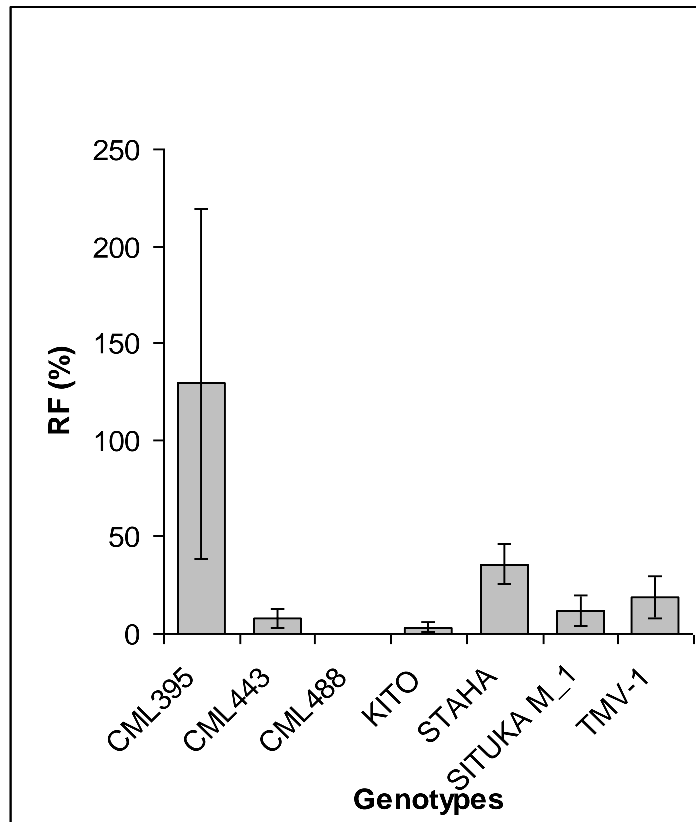

Figure 3.2: Influence of maize genotype on cumulative regeneration frequencies

Cummulative regeneration frequency was high in CML395, followed by Staha, TMV-1 and Situka M-1. CML443 was the least regenerable inbred line whereas CML488 failed completely to respond on regeneration.

In this study, high shoot induction for the genotypes studied were otained from calli that were initially cultured on MS media amended with low 2, 4-D concentrations compared to those previously cultured on higher 2, 4-D concentrations (Figure 3.3).

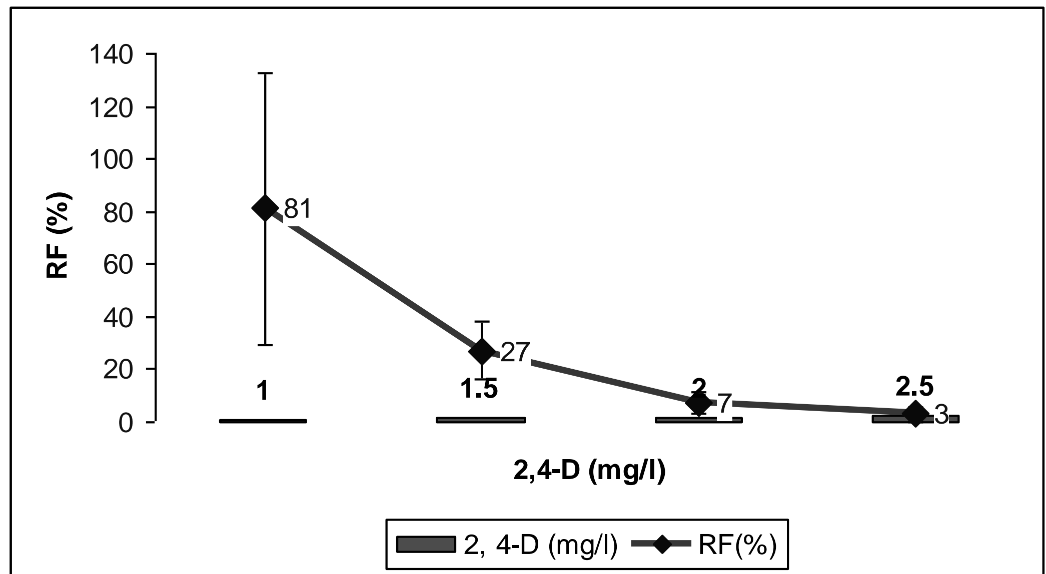

Figure 3.3: Effect of 2, 4-D on cumulative regeneration of maize genotypes
Cummulative regeneration frequency was affected by 2, 4-D used initially for calli induction in the genotypes studied. Calli initiated on 1-1.5 mg/l had high cumulative regeneration frequency than calli initiated on high concentrations of 2,4-D (2-2.5 mg/l).

3.4 DISCUSSION

Maize regeneration through somatic embryogenesis is influenced by several factors. Inclusion of $AgNO_3$ in CIM has been reported to influence type II embryogenic calli proliferation (for review see Vain *et al*., 1989; El-Itriby *et al*., 2003). Growth regulators such as auxin are normally included in culture media and play an important role in somatic embryogenesis. Frequently used auxins are 2, 4-D, picloram and dicamba. Although they perform similar functions, these auxins differ significantly in their effectiveness when used in various plant species at various concentrations (Satyavathi *et al*., 2004). In this study, embryos cultured on 2,4-D (1, 1.5, 2 or 2.5 mg/l) amended medium expanded and formed calli whereas those on 2,4-D free medium (control) germinated. The ability of immature zygotic embryos to form calli may probably be influenced by the presence of auxin (2,4-D). While working with temperate maize embryos of A188, Bronsema *et al*. (2001) observed a similar trend where EC formation was only achieved at 0.2 up to 2 mg/l of 2, 4-D, but below 0.02 mg/l of 2, 4-D, embryos did not form calli at all instead they followed an organogenesis path way.

The fact that there was significant difference in CIF and ECF observed among the genotypes, indicating that the genetic differences existing in the genotypes under study were influencing callus induction and embryogenic callus formation. This also suggests that there was an independent and combined effect of 2, 4-D and maize varieties on embryogenic calli formation. These results are not surprising as

genotype dependency of plant regeneration have been reported particularly in cereals (Przetakiewicz *et al*., 2003; Satyavathi *et al*., 2004; Oduor *et al*., 2006; Ombori *et al*., 2008). The variation in callus induction and embryogenic calli formation within genotypes is often attributed to many factors including but not limited to media composition, 2, 4-D concentration and duration of exposure to 2, 4-D, type of explant, size of zygotic embryos and age of the explant (Green and Phillip,1975; Slesak *et al*., 2005).

Somatic embryo (SE) proliferation from EC is an important developmental stage which is associated with shoot emergence. However, in EMM somatic embryos exhibited slow growth rate and in some genotypes notably Kito, somatic embryos aborted. The slowed growth rate and retarded proliferation of EC during somatic embryo maturation are often linked to gene regulation. The ability of 2, 4-D to trigger embryogenic pathway in calli cells may be related to its capacity to induce stress genes (*SOMATIC EMBRYOGENESIS RECEPTOR KINASE* (*SERK), HEAT SHOCK (HSP)*, genes encoding 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and ACC oxidase (ACO)), which have been shown to contribute to the cellular reprogramming of somatic cells toward embryogenesis (Kitamiya *et al.,* 2000). In this study, slow growth rate of embryos was observed during embryo maturation. Che *et al.* (2006) reported that reduced cell proliferation and slow growth during somatic embryo maturation is associated with down regulation of histone and ribosomal genes and up-regulation of genes such as those encoding

hydrolytic enzymes (nucleases, glucosidases and proteases) and a few storage genes (an α-zein and caleosin). However, down regulated genes recovered drastically during shoot emergence.

Although no observable morphological changes in growth and development of plants regenerated *in vitro* were recorded in this study, it has been shown that long duration of culture *in vitro* can impose some morphological changes in regenerated plants. Such variations are often attributed to somaclonal variations which usually are epigenetic (Ombori *et al*., 2008).

Embryogenic calli formation is one of the most important changes that may highly determine regeneration potential of a given variety. The results from the analysis of variance (Table 2) showed that regeneration frequencies (RF) were not correlated to CIF and ECF and the differences were statistically non significant ($p \leq 0.05$). Generally, the ability to form EC was not always comparable to regeneration as almost all varieties gave low regeneration frequency (RF). This low RF could have resulted from somatic embryo abortion as was demonstrated by Kito or due to morphological abnormalities of somatic embryos (Bedada *et al.,* 2012). These abnormalities have been associated with the use of 2,4-D in the induction medium (Rodriguez and Wetzstein, 1998). The morphological quality of somatic embryos affects the efficiency of conversion into plantlets. Other factors leading to low RF include high rate of cell/tissue necrosis and massive root

development without shoots. Furthermore, necrosis of EC on SM due to initial cell death of aborted somatic embryos highly contributed to low RF.

The failure in shoot regeneration and massive regeneration of roots from EC observed in this study may be linked to the effect of long tissue culture and the effect of 2, 4-D. Such massive root emergence is a response often associated with the type of auxin. Auxins can exert residual effect to regenerating tissues thereby causing *in vitro* epigenetic changes which affect regeneration. Leyser (2001) reported that the auxin signal is received by plant cells and rapidly transduced to a wide range of responses in the growth and development of plant organs, including changes in the direction of growth, shoot and root branching and vascular differentiation.

Although 2, 4-D is required initially for calli induction and somatic embryo formation, it is also important to reduce its concentration for further embryo development and sprouting (Bronsema *et al*., 2001). High rate of cell death could have probably been due to released phenol-like chemical compound by dying cells on the medium which may be toxic to surviving cells/tissues. These findings suggest that when this happens it is important to subculture the surviving calli in a fresh SM media as it was done in the current study. Zhao *et al*. (2010) while working on sweet sorghum (*Sorghum bicolor* Moench) observed that non embryogenic calli would turn brown and die during subsequent culture and

regeneration. Also, calli often exuded a reddish orange pigment which inhibited callus growth. However, these authors minimized the effect of pigments generated by the addition of 10 mg/l vitamin C and polyvinylpyrrolidone (PVP). Overcoming the hurdle of a reproducible regeneration protocol, the next chapter demonstrates the application of the protocol in transforming selected regenerable maize genotypes using gene constructs containing drought tolerant genes.

CHAPTER FOUR

TRANSFORMATION OF CML144 AND STAHA MAIZE WITH DROUGHT TOLERANCE CONFERING GENES FROM RESURRECTION PLANT *XEROPHYTA VISCOSA*

4.1 INTRODUCTION

In this chapter, two single gene constructs each with *XvPrx2* and *XVSAP1* genes were separately used to transform CML144 (QPM inbred line) germplasm. In addition, Staha (MSV-resistant OPV) maize germplasm from Tanzania was genetically modified for drought stress adaptability using *XvPrx2* gene. Both genes are from resurrection plant *Xerophyta viscosa* (Govendor, 2006; Garwe *et al*., 2003). Gene delivery to immature zygotic embryos of maize was carried out using *A. tumefaciens*-mediated transformation. Mannose selection system was used for the selection of tranformants. Polymerase chain reaction and Southern blot hybridization indicated that the *XvPrx2* and *XVSAP1* genes were in plants and stably integrated, thereby giving indicative achievement for successful transformation. Reverse transcription polymerase chain reaction (RT-PCR) revealed that *XvPrx2* and *XVSAP1* gene transcripts were expressed under drought stress. The results reported in this chapter, reveal significant progress towards developing transgenic drought tolerant maize. The transgenic plants should be subjected to varying drought stress conditions to evaluate their performance.

4.2 MATERIALS AND METHODS

4.2.1 Development of *XvPrx2* and *XVSAP1* gene constructs

The *XvPrx2* and *XVSAP1* genes previously isolated from *Xerophyta viscosa* were initially cloned into pZY101.1 (pTF101.1) vector (Appendix II). The pTF101.1 vector uses a herbicide resistance (*bar*) as a selectable marker gene. To avoid use of *bar* gene in selecting for transformed plants, the *XvPrx2* and *XVSAP1* genes were sub-cloned into plant expression pNOV2819 vector (Appendix II). The pNOV2819 vector contains *manA* gene which, encodes for phosphomannose isomerase, PMI an enzyme that metabolizes mannose sugar into usable source of carbon. Most organisms including maize lack this enzyme and thus naturally unable to metabolize mannose.

4.2.1.1 Screening for the available restriction sites in *XvPrx2* and *XVSAP1* gene sequences

The *XvPrx2* and *XVSAP1* gene sequences were screened for restriction sites to ensure that the restriction enzymes selected for cloning could not cut the target transgene. This was done by using DNaman software (version 5.2.10, Lynnon BioSoft). The two genes (XvPrx2 and XVSAP1) were then engineered with Pac1 restriction site to allow ligation between *Hind*III and *Pac*1 sites in pNOV2819 plant transformation vector. To achieve this *sacB* gene from *Bacillus subtilis* was used for amplification by PCR to engineer the *Not*I, *Xba*I, *Pac*I and *Eco*RI.

The *Bacillus subtilis sacB* gene coding for levansucrase is a popular counterselectable marker. In its natural gram-positive environment its expression is harmless to the bacterium. However, cloning of *sacB* in *E. coli* and other gram-negative bacteria leads to the death of the transformed bacteria when they are plated in the presence of sucrose.

4.2.1.2 Primer design to engineer *Pac*1 restriction site in *sacB* gene sequence

Using DNaman software, a set of primers was designed using *sacB* gene sequence to engineer a range of restriction sites as indicated in Table 4.1. *sacB* gene has no association with the drought conferring (*XvPrx2* and *XVSAP1*) genes sought for sub-cloning. The *sacB* gene was only used as a template in intermediate vectors to carry the engineered restriction sites and serve as couterselectable marker. The *sacB* gene fragment was removed later in the process to allow ligation of genes of interest (*XvPrx2* and *XVSAP1*). The idea was to avoid introduction of errors in sequences of *XvPrx2* and *XVSAP1* by direct amplification to engineering restriction sites through PCR.

Table 4. 1: Sequences of primers used for engineering of restriction site in *sacB* gene fragment

Primer	Sequence
SacB F-Primer	***Not*I *Xba*I** 5'-G**CGGCCG**C**TCTAGA**TTTTTATTTGTTAAC-3'
SacB R- Full Primer	***Eco*RI *Pac*I *Xba*I** 5'-**GAATTCTTAATTAATCTAGA**ACATATACCTGCCGTTCACTATTAT-3'
SacB R- short Primer	***Eco*RI *Pac*I *Xba*I** 5'-GAATTCTTAATTAATTCTAGAGCACAAGTTAACGTATCAGC-3'
M13 R-primer	5'-CAGGAAACAGCTATGACC-3'

Enzymes (*Not*I, *Xba*I, *Eco*RI and *Pac*I) and their corresponding restriction sites are colour coded

4.2.1.3 Polymerase chain reaction for amplification of *sacB* gene fragment

Synthesized primers were used to amplify *SacB* gene using the PCR master mix indicated in Table 4.2. PCR condition was programmed as follows; initial denaturation temperature of 94ºC for 5 min followed by 35 cycles each of 94ºC for 30 sec, annealing at 55ºC for 30 sec, initial elongation at 68ºC for 50 sec final extension at 72ºC for 7 min and held at 4ºC to infinite. The amplicons were resolved on 0.8% (w/v) TAE agarose gel run at 100 V for 30 min. The PCR product was visualized and photo taken for documentation.

Table 4. 2: PCR reagents and final volumes used in a standard PCR protocol

Components	Stock concentration	Final volume
dH_2O		18.4 µl
Buffer	(10X)	2.5 µl
$MgCl_2$	(25 mM)	1.5 µl
dNTPs	(10 mM)	0.5 µl
Forward primer	(10 µM)	0.5µl
Reverse primer	(10 µM)	0.5 µl
Standard Taq	(5U/µl)	0.1 µl
Template	(10 ng/µl)	1 µl
Total volume		25 µl

4.2.1.4 PCR product purification

Amplified DNA was purified using the EZ-10 Spin column PCR product purification Kit (Bio Basic, Canada). The PCR product was transferred to a 1.5 ml eppendorf tube and 3 volumes of binding buffer I added. After pulsing, the mixture was transferred to the column and let to stand for 2 min at room temperature before spinning for 1 min at 14000 rpm. The flow-through was discarded and the column placed back on the same collection tube. Five hundred microlitres of washing buffer was added to the column and centrifuged for 1 min at 14000 rpm. The flow-through was discarded and the column re-assembled with the collection tube. The washing step was repeated with centrifugation for 60 sec at 14000 rpm. To remove the residual wash solution, the centrifugation step was repeated. The collection tube was discarded and the column inserted into a sterile 1.5 ml eppendorf tube. To the center of the column, 40 µl elution buffer was added and incubated at room temperature for 2 min before centrifuging at 14000 rpm for

1 min. For DNA intended for cloning, water was used for elution. The purified DNA was stored at 4ºC for regular use or at −20ºC for long term storage.

4.2.1.5 Sub-cloning of *SacB* gene fragment into pTZ57R/T vector

4.2.1.5.1 Ligation of PCR product with pTZ57R/T vector

The PCR product was ligated into pTZ57R/T vector (Appendix II) as presented in Table 4.3 following manufacturer's instruction.

Table 4. 3: Standard ligation-reaction mixture using pTZ57R/T cloning vector

Component	Stock concentration	Volume per reaction (µl)
pTZ57R/T cloning vector	(50ng/µl)	3
PCR product	(24.6 ng/µl)	4
Distilled water		variable
5X Ligation buffer	(5X)	6
T4 DNA ligase	(5 U/ µl)	1
Total		20

4.2.1.5.2 Preparation of competent *E. coli* (DH5α) cells

E. coli strain DH5α cells were refreshed on LB solid (LBA) medium plate (Appendix III) without antibiotics two days before transformation. Then 1 mm thick of DH5α colonies was picked using a sterile wire loop and inoculated into 10 ml fresh LB broth (LBB) medium (Appendix III) without antibiotics and incubated overnight (O/N) on an oscillating horizontal mechanical shaker (250 rpm) at 37°C. Then, 2 ml of the O/N grown cells were inoculated in 10 ml of fresh LB broth

medium (without antibiotics) and grown for 2 h on an oscillating horizontal shaker at 37°C while checking the optical density (OD) of the medium at 550 nm. The OD_{550nm} should be 0.4-0.5 for appropriate culture. Thereafter, the cells were chilled on ice or at 4ºC for 1 h. The cells were aliquot in 1.5 ml tubes and centrifuged at 12000 rpm for 2 min at 4°C. The supernatant was carefully decanted off. This step was repeated until all the cell culture was finished. While making sure that all tubes were on ice, the cells were re-suspend in 1 ml ice cold 0.1 M $CaCl_2$ and incubated on ice for 30 - 40 min. Thereafter, the cells were centrifuged at 12000 rpm for 2 min at 4°C and the supernatant decanted off. Then the cells were re-suspended in 200 µl of ice cold 0.1M $CaCl_2$ and kept at 4°C or on ice. These cells were now competent to receive foreign DNA for at least 24 h if kept at -20°C or were used immediately for transformation. For long storage, competent cells were put in glycerol stock and kept at a temperature of -80°C.

4.2.1.5.3 Preparation of reagents for making bacteria cells competent

4.2.1.5.3.1 Calcium chloride unhydrate ($CaCl_2$,) [0.1 M]

A stock solution of 0.1 M $CaCl_2$ was prepared by dissolving 2.94 g of $CaCl_2$ unhydrous (Mwt 147.02) in distilled water to a final volume of 200 ml. The stock was then autoclaved for 20 min at a temperature of 121°C and pressure of 15 psi. The cooled solution was kept at 4 ^{o}C in a refrigerator.

4.2.1.5.3.2 Sodium chloride (NaCl) [0.15 M]

A stock solution of 0.15 M NaCl was prepared by dissolving 1.75 g of NaCl (Mwt 58.44) in distilled water to a final volume of 200 ml. The stock was then autoclaved for 20 min at a temperature of 121°C and pressure of 15 psi. The cooled solution was kept at 4 ^{o}C in a refrigerator.

4.2.1.5.4 Transformation of competent DH5α cells

The products of ligation were used to transform competent *E. coli* host strain DH5α cells using the following protocol:

Two microlitres of plasmid DNA (10 ng) was added into 0.5 ml eppendorf tube and 100 µl aliquots of competent cells were thawed on ice and added to the 2 µl of plasmid DNA in 0.5 ml eppendorff tube. The plasmid-competent cell mixture was incubated on ice for 30 - 40 min on the bench at room temperature (RT) without shaking. Thereafter, the mixture was slowly and carefully transferred to a water bath and heat shocked at exactly 42ºC for 60 - 90 sec without shaking. The tubes were then immediately cooled on ice for 2 min and 900 µl of room temperature equilibriated LB broth medium added and incubated at 37ºC for 1 h on 250 rpm horizontal oscillating mechanical shaker. The transforming mixture was centrifuged and supernatant discarded. The transforming cells were re-suspended in 100 µl LB broth medium and the 100 µl of transformation mixture was plated

on a pre-warmed (that is equilibriated at RT) selective LB solid medium plate and incubated at 37ºC overnight.

4.2.1.5.5 Selection of transformants carrying the *sacB* insert

Transformed *E. coli* cells were plated on LB medium supplemented with Amp^{100}/IPTG/X-gal for blue/white selections (Appendix III). White colonies containing vectors with an insert were picked and plated on the same medium and colony PCR was carried out on selected colonies using *SacB* gene F-primer and M13 R-primers (Table 4.1).

4.2.1.5.6 Selection of colonies with pTZ57R/T vector carrying *sacB* gene fragment with engineered *Pac*I site on sucrose medium

About 24 colonies were selected each from short and full length sequence insert of *SacB* gene and inoculated on both sucrose and non sucrose LB medium supplemented with Amp/IPTG/X-gal alone. These were incubated at 37ºC over night. The following day, the plates were examined for dead colonies.

4.2.1.5.7 Preparation of sucrose LB medium plate

LB medium was prepared and prior to adjustment of the pH, 7% (w/v) of sucrose were added. The pH was adjusted to 6.8 followed by addition of 15 g/l of

bacteriological agar. The medium was autoclaved for 20 min at 121ºC and 15 psi (longer autoclaving may cause sucrose to burn/degrade). Then the medium was cooled to 55ºC and Amp^{100}/IPTG/X-gal added and then dispensed into petri plates and left to solidify. Solidified media plates were inverted and stored at 4ºC until further use.

4.2.1.5.8 Confirmation of the presence of pTZ57RT vector carrying *sacB* fragment with engineered *Pac*1 restriction site in *E. coli* by PCR

From the five samples, colony PCR with the primer combination of either full length *sacB* forward and reverse or *sacB* forward and M13 reverse was conducted to ascertain the presence of pTZ57R/T vector 2.961 kb carrying *sacB* gene (1.886 kb) fragment with engineered *Pac*1 restriction site.

4.2.1.5.9 Plasmid DNA isolation from *E. coli* cells

Bacteria (DH5α cells) with pTZ57RT vector carrying *sacB* insert with *Pac*I restriction site were grown in 10 ml LB broth supplemented with Ampicillin overnight. Then the cells were harvested by centrifugation at 14000 rpm in 15 ml centrifuge tubes for 5 min in a table top centrifuge. The supernatant was discarded. Cells were then resuspend in 300 µl of P1 buffer, thoroughly vortexed and incubated at RT for 5 min. Four hundred microlitres of P2 buffer were added, mixed gently by inverting up and down without vortexing and then incubated for

exactly 5 min on ice. Thereafter, 300 µl of P3 buffer were added, mixed gently by inverting up and down without vortexing, and incubated for 15 min on ice. Then the mixture was centrifuged for 15 min at 14000 rpm in table-top centrifuge and 900 µl of supernatant pipetted off into a new 1.5 eppendorf tube. Then, 600 µl of isopropanol was added, mixed and incubated at -20ºC for 15 min and then centrifuged for 9 min at full speed in microfuge (approx. 13,000 rpm). The supernatant was discarded and 500 µl 70% (v/v) ethanol was added to wash the DNA pellet. The ethanol was decanted off and the plasmid DNA pellet dried for 15 to 30 min. Thereafter, the pellet was re-dissolved in 50 µl dH_2O. The purified plasmid DNA was stored at 4ºC for regular use or at −20ºC for long term storage.

4.2.1.5.10 Plasmid DNA extraction buffers

Buffers P1, P2 and P3 for plasmid DNA extraction were prepared as indicated in Table 4.4, 4.5 and 4.6.

Table 4. 4: Resuspension buffer

P1 buffers: 25 mM Tris-8.0, 10 mM EDTA, and 50 mM glucose

Stocks	**10 ml**	**20 ml**	**30 ml**	**40 ml**	**50 ml**
1.0 M Tris- 8.0	250 µl	500 µl	750 µl	1000 µl	1250 µl
0.5 M EDTA- 8.0	200 µl	400 µl	600 µl	800 µl	1000 µl
Glucose	90 mg	180 mg	270 mg	360 mg	450 mg

Add 1mg/ml RNase A before use (kept frozen at -20 ˚C)

Table 4. 5: Lysis buffer

P2 buffer: 0.2 M NaOH, 1.0% (w/v) SDS

Stocks	**100 ml**	**200 ml**	**300 ml**	**400ml**	**500 ml**
1.0 M NaOH	20 ml	40 ml	60 ml	80 ml	100 ml
20% SDS	5 ml	10 ml	15 ml	20 ml	25 ml

Table 4. 6: Neutralization buffer
P3 buffer*: 3.0 M KOAc, pH 5.5

Component	Amount
Potassium acetate	29.5g (dissolve it in 60 ml dH_2O)
Glacial acetic acid	Add enough to bring pH to 5.5 (approx.11 ml)
dH_2O	Bring final volume to 100 ml

*Keep on ice before use

4.2.1.5.11 Confirmation of the presence of pTZ57RT vector carrying the insert for *sacB* with *Pac*I restriction site by restriction digestion

The isolated plasmid was purified as earlier described (Section 4.2.1.4) and aliquots were digested with a series of enzymes, namely *Hin*dIII, *Pac*I (was not available so the content of this tube were just loaded undigested), *Bam*H1, *Xba*l, *Not*I and *Eco*RI for 3 h at 37ºC. The product of digestion were analyzed on 1% (w/v) agarose gel electrophoresis in 1X TAE buffer run at 90 V for 1 h and results documented using UV gel documentation machine.

4.2.1.6 Subcloning of *sacB* with *Pac*I fragment insert into pBluescript II SK (+/-) vector

4.2.1.6.1 Double digestion of pTZ57R/T vector carrying the insert for *sacB* with *Pac*I using *Not*1 and *Eco*R1 restriction enzymes

The remaining aliquot of positive DNA of pTZ57R/T vector with *sacB* gene fragment engineered with *Pac*I restriction site was double digested using *Not*I and *Eco*RI restriction enzymes as indicated in Table 4.7.

Table 4. 7: Reagents and final volumes used in a standard double digestion reaction

Composition	Stock concentration	Final volume (µl)
Plasmid DNA	(400 ng)	x
Enzyme I	(2U/µl)	1
Enzyme II	(2U/µl)	1
Appropriate Buffer	(10X)	2
dH2O		(16 - x)

The product of digestion was analyzed on 1% (w/v) agarose gel run in 1X TAE buffer. The *sacB* gene fragment with engineered *Pac*I restriction site was sliced from the gel and gel purified as described in section 4.2.1.6.2.

4.2.1.6.2 DNA purification from agarose gels

Excised DNA fragments from agarose gels were purified using the wizard SV Gel and PCR clean-up system (Promega). After excision of the band of interest from the agarose gel, the gel slice was placed in a sterile preweighed 1.5 ml Eppendorf tube and the mass estimated. For every 10 mg of excised agarose, 10 µl of membrane binding bufer was added, vortexed and the mixture incubated at 50 - 65ºC until gel slice was completely dissolved. The dissolved gel mixture was then transferred to SV mincolumn inserted into a collection tube and incubated at room temperature for 1 min before centrifuging for 1 min at 14000 rpm. The flow-through was discarded and the column placed back on the same collection tube. Seven hundred microlitres of membrane wash solution (with added ethanol) was added to the column and centrifuged for 1 min at 14000 rpm. The flow-through was discarded and the column was reassembled again into the collection tube. Five

hundred microlitres of membrane wash solution was added and the sample centrifuged for 5 min at 14000 rpm. The collection tube was empted and centrifugation step was repeated for 1 min more with microcentrifuge lid open to allow evaporation of any residual ethanol. The collection tube was discarded and the column transferred to a clean sterile 1.5 ml microcentrifuge tube. To the centre of the mincolumn, 50 µl of nuclease free water was added and incubated for 1 min at room temperature before centrifuging for 1 min at 14000 rpm. The purified DNA was stored at 4ºC for regular use or at −20ºC for long term storage.

4.2.1.6.3 Ligation of *sacB* gene fragment carrying the *Pac*1 restriction site into pBluescript II SK

The purified insert (*sacB* gene fragment carrying the *Pac*1 restriction site) was ligated into an intermediate pBluescript II SK (+/-) phagemid vector and the product of ligation were used to transform competent *E. coli* (DH5α). The pBluescript II SK (+/-) with *sacB* gene fragment was extracted from DH5α cells as previously described (Section 4.2.1.5.9).

4.2.1.6.4 Eliminating *sacB* gene in pBluescript II SK and recovery of *Pac*1 restriction site

The pBluescript $II_{sacBPac1}$ was digested overnight with *Xba*I and fractionated on 0.8% (w/v) agarose gel run in 1X TAE buffer at 90 V for 1 h to eliminate the *sacB*

gene fragment leaving only the engineered sites in the pBluescript II SK (+/-). The pBluescript II SK (+/-) vector with *Pac*I and other introduced restriction sites (***Not*1, *Xba*1** and ***Eco*RI**) was sliced from the gel and gel purified as described before (Section 4.2.1.6.2). The purified vector was then religated and used for transformation of competent *E. coli*.

4.2.1.7 Sub-cloning of expression cassettes for *XvPrx2* and *XVSAPI* genes into *Pac*1-engineered pBluescript II SK (+/-)

The pBluescript II SK (+/-) vector with *Pac*1 restriction site was extracted from *E. coli* and sequentially digested using *Hin*dIII and *Eco*R1 restriction enzymes. Likewise, the pTF101.1 (9.189 kb) carrying either the *XvPrx2* or *XVSAP1* expression cassettes were digested using the same restriction enzymes used for pBluescript II SK (+/-) vector in the same manner. The expression cassettes for either *XvPrx2* or *XVSAP1* were recovered through gel slicing followed by gel purification (Section 4.2.1.6.2) after being fractionated on 1% (w/v) agarose gel electrophoresis, whereas the digested pBluescript II SK (+/-) vector was PCR purified (Section 4.2.1.4). The expression cassette fragments were then singly ligated (Section 4.2.1.5.1) and the product of ligation used to transform competent *E. coli* (*DH5α*) cells.

4.2.1.8 Sub-cloning of *XvPrx2* and *XVSAP1* expression cassettes into plant expression pNOV2819 vector

The pBluescript II SK (+/-) vector (2.961 kb) harbouring either *XvPrx2* or *XVSAP1* expression cassettes were extracted from *E. coli* (Section 4.2.1.5.9) and the plasmid was double digested using *Pac*I and *Hin*dIII restriction enzymes (Section 4.2.1.6.1) and the expression cassettes were recovered after electrophoresis, from gel slicing followed by gel purification (Section 4.2.1.6.2). Likewise, the pNOV2819 vector (7.599 kb) was double digested and PCR purified before ligating with either XvPsap1::XvPrx2::NosT or XvPsap1::XVSAP1::NosT expression cassettes. The products of ligation were used to transform *E. coli* cells.

4.2.1.8.1 Screening of colonies carrying pNOV2819 vector with *XvPrx2* or *XVSAP1* gene construct by PCR

The selected transformants were screened by colony PCR using either gene specific primers for *XvPrx2* gene (expected band size 0.505 kb) or promoter specific XvPsap1 forward primer and *XvPrx2* gene specific reverse primer (expected band size (2.588 kb). The controls used for the colony PCR included the no template control (NTC) consisting of water and the positive control was a colony of the pBluescript II SK::XvPsap1::XvPrx2::NosT construct. In addition, the negative controls included a colony of pNOV2819 transformed into *E.coli* (DH5α) and a colony of *E.coli* (DH5α).

E.coli transformants habouring the construct carrying *XVSAP1* gene were screened by colony PCR using promoter specific XvPsap1 forward primer and *XVSAP1* gene specific reverse primer (expected band size was 2.468 kb). The controls used for the colony PCR included the no template control (NTC) consisting of water as the template and the positive control was a colony of the pGEM-Teasy_XVSAP1 construct or a colony of the pBluescript II SK carrying the *XVSAP1* gene construct. The negative controls were a colony of pNOV2819 transformed into *E.coli* (DH5α) and a colony of *E.coli* (DH5α).

4.2.1.8.2 Confirmation of the right *XvPrx2* and *XVSAP1* gene constructs by double digestion with *Pac*I and *Hin*dIII

To confirm whether the constructs made were as expected a double digestion experiment using *Pac*I and *Hin*dIII restriction enzymes was carried out. The products of digestion (7.599 kb of pNOV2819, 2.999 kb of *XVSAP1* expression cassette and 2.844 kb of *XvPrx2* expression cassette) were resolved on 1% (w/v) agarose gel electrophoresis in 1X TAE buffer run at 90 V for 1 h and results were documented using UV gel documentation machine.

4.2.1.9 Transformation of final constructs into *Agrobacterium tumefaciens* (EHA101)

4.2.1.9.1 Preparation of *Agrobacterium* competent cells

Agrobacterium colonies (1 mm thick) were inoculated in 5 ml LB broth medium supplemented with appropriate antibiotics (50 mg/l Kanamycin) and incubated over night at 28ºC on a mechanical shaker. Then 2 ml of the over night grown culture were diluted into 40 ml LB broth in a sterile 50 ml falcon tube and shaken at 250 rpm for 4 - 5 h while checking the optical density ($OD_{550\ nm}$) not to go beyond the range of 0.3 - 0.4. The cells were then chilled on ice for 20 min to 1 h before harvesting them by centrifugation at 12000 rpm for 10 min at 4ºC. The supernatant was carefully discarded. The tubes were checked to make sure the cells were pelleted, if not the step was repeated at higher speed. The tubes were maintained on ice throughout. The cells were re-suspended in 1 ml ice cold 0.15 M $NaCl_2$ and incubated for 15 min before they were centrifuged at 12000 rpm for 5 min at 4ºC. The supernatant was then discarded and cells re-suspend in 1 ml of 0.1M $CaCl_2$ and incubated on ice for 40 min. The cells were centrifuged at 12000 rpm for 5 min at 4ºC followed by re-suspension of the pellet (cells) in 200 µl of ice cold 0.1M $CaCl_2$. The cells were then kept at 4ºC or on ice. These cells were competent to receive DNA for at least 24 h if kept at -20ºC. For long storage, competent cells were put in glycerol stock and kept at a temperature of -80°C.

4.2.1.9.2 Transformation of competent *A. tumefaciens* cells

The final constructs (Appendix II) were extracted from *E. coli* as described earlier and transformed into competent *A. tumefaciens* strain EHA101 before being used for transforming maize immature zygotic embryos. The procedure for transformation of competent EHA101 cells was as follows:

Two to 3 µl (2053 ng/µl) of plasmid DNA was added into 0.5 ml eppendorf tube. Then 100 µl aliquots of competent *A. tumefaciens* cells were thawed on ice and added to 2 - 3 µl of plasmid DNA in 0.5 ml eppendorf tube. The mixture of competent cells and plasmid was mixed by pipetting up and down twice and incubated on ice for 30 - 40 min on flat bench at room temperature (RT) without shaking. Thereafter, slowly and carefully without shaking the mixture was transferred to a water bath and heat shocked the mixture at exactly 42ºC for 60 - 90 sec. Then the tubes were taken out of the water bath and immediately cooled on ice for 2 min. Nine hundred (900 µl) microlitre of LB broth medium were added to cooled cells and incubated at 28ºC for 1.5 h on horizontal oscillating mechanical shaker. Then 100 µl of transformation mixture were plated on a pre-warmed (that is equilibrated at RT) selective medium plate and incubated at 28ºC for 2 - 3 days in the dark. Single transformed bacterial colonies were selected and streaked on a flesh solid LB plate with appropriate antibiotics (100 mg/l Kan and 100 mg/l Spec or Strep) and incubated at 28ºC for 2 - 3 days in the dark. Then the transformed

Agrobacterium cell cultures were stored at 4ºC for monthly use whereas long term storage required preparation of glycerol stocks and keeping at -80ºC.

4.2.1.9.3 Glycerol stock preparation for long term storage of cells

After an overnight culture of bacterial cell in LB broth medium, equivolume of cells and autoclaved 80% (v/v) glycerol were mixed gently then chilled in liquid nitrogen and immediately the stocks were stored at −80ºC. Stocks of constructs both in *E. coli* and *A. tumefaciens* were stored in glycerol stock.

4.2.2 Bacterial growth and maintenance media

The EHA101 with gene constructs were maintained in Luria-Bertani, LB (pH 6.8) medium supplemented with 100 mg/l spectinomycin, 100 mg/l kanamycin and 25 mg/l chloramphenicol, (Appendix III). Bacterial culture for weekly experiment was initiated from stock plates that had been stored for up to 1 month at 4ºC after being refreshed from long-term cultures kept at -80°C as glycerol stocks (Section 4.2.1.9.3).

4.2.3 Media for transformation, selection and regeneration of maize immature zygotic embryos

In this study, infection medium (LS-Inf), co-cultivation medium (LSAc) both supplemented with acetosyringone (As), resting medium (RM), selection medium

(SEM) and regeneration media were used for transformation experiments. Regeneration media were classified into three namely, embryo maturation medium (REG I), shooting medium (REG II) and rooting medium (REG III). All media were prepared as indicated in Appendix IV. Except for regeneration media that was based on MS salts (Murashige and Skoog, 1962), all other medium were prepared using LS salts (Linsmaier and Skoog, 1965) and amended as indicated in Appendix IV. All media were autoclaved except LS-Inf, which was filter sterilized.

4.2.4 *Agrobacterium* growth and pre-induction procedure

Three to four days prior to infection, the *Agrobacterium* cells were streaked onto Luria- Bertani with agar (LBA) plates amended with 100 mg/l kanamycin and 100 mg/l spectomycin and grown for two days at 28ºC in darkness. Then about 1 mm thick colony was inoculated into 10 ml of Luria-Bertani broth (LBB) amended with same antibiotics and grown overnight at 28ºC in darkness. The following day, cells were harvested in micro-centrifuge, re-suspended in fresh LBB without antibiotics to wash the cells and thereafter the cells were harvested and re-suspended in LS-inf supplemented with acetosyringone (LS-inf+As). Before infection of maize immature zygotic embryos the concentration of bacterial suspension was adjusted to an optical density (OD_{550nm}) of 0.3 - 0.4 using spectrophotometer.

4.2.5 Inoculation of *Agrobacterium tumefaciens* onto maize immature embryos

Harvested maize ears were surface sterilized as described in chapter three, section 3.2.3. Excised embryos (1-1.5 mm) were rinsed with LS-Inf+As, then *Agrobacterium* suspension was added to the petri plates containing embryos. The embryos in *Agrobacterium* suspension were incubated for 5 min in the dark at 19±1ºC. Thereafter, the *Agrobacterium* suspension was drained off using a pipette before the embryos were plated on LSAc medium with their embryo axis facing the medium. The embryos were incubated in the dark for 3 days at a temperature of 19 ±1ºC. The embryos were then transferred to RM and incubated for 10 days in darkness at a temperature of 28ºC for calli induction.

4.2.6 Selection, regeneration and hardening of putatively transformed plantlets

After 10 days on RM, embryos were transferred onto SEM and incubated for 4 weeks with subculture every two weeks. Embryogenic calli that survived selection were transferred onto REG I and incubated for two weeks at a temperature of 28ºC in darkness for somatic embryo maturation before shoot induction on REG II. Proliferation of T_0 putative transgenic maize plantlets and hardening was achieved as described in chapter three, section 3.2.5.

4.2.7 Molecular analyses of putative transgenic maize plants

4.2.7.1 DNA extraction from putative transgenic plants

Genomic DNA was extracted from young leaf samples of transgenic and non-transgenic (control) maize lines following the CTAB (cetyl tri-methyl ammonium bromide, Appendix V) method as described by Allen *et al.* (2007).

4.2.7.1.1 Procedures for DNA extraction from plant leaf tissues

Leaf samples (about 100-400g depending on the amount of DNA required) were harvested and chilled in liquid nitrogen and stored at -80ºC in a freezer until they were required for use. Samples were taken and pulverized slightly into powdery form using motor and pestle. Optimal force was used during crashing to avoid shearing DNA. Pulverized samples in liquid nitrogen were poured into the 15 ml tube and left to evaporate. Quickly 3 ml of pre-warmed CTAB buffer (60-65ºC) was added and vortexed vigorously until the entire sample were completely mixed with the buffer. Then the samples were incubated in water bath set at 65ºC for 30 min to 1 h. This process was repeated until all samples were done. After 30 min to 1 h of incubation an equal volume of chloroform: iso-amyl alcohol (24:1) was added and mixed gently by inverting the tube up and down. The samples were then centrifuged for 10 min at a speed of 14000 rpm. About 3 ml of the clear supernatant solution were transferred into a fresh 15 ml tubes containing 0.7 volumes of ice-cold propanol-2 and mixed gently by turning the tubes up and

down. Then the mixture was incubated for 5 to 20 min at -20ºC before they were centrifuged at 14000 rpm in a bench top micro-centrifuge for 9 min. Thereafter, the supernatant was discarded by decanting leaving the DNA pellet at the bottom of the tube. Then 70% (v/v) ethanol was added to wash the pellet and discarded off by decanting (This could be briefly centrifuged before decanting off the ethanol). Then the DNA pellet was air dried for 15 to 30 min before adding 100 µl of sterile distilled water to dissolve the DNA pellet. Two microlitres of 10 mg/ml RNase A was added to the dissolved DNA and incubated for 30 min at 37ºC. Alternatevely, the incubation step was allowed to run overnight at room temperature.

4.2.7.1.2 Precipitation of DNA with ethanol

About 50 µl of Tris EDTA (TE) or water was added to make the total volume of DNA to 100 ul in 1.5 µl eppendorf tube (if the starting DNA was 50 µl). Then 400 µl of ice cold absolute ethanol and 50 µl of 3M NaOAc were added and mixed gently. The sample mixture was incubated at a temperature of -20ºC for 5 - 10 min (optional) to aid precipitation. This was followed by centrifugation at a speed of 14000 rpm for 5 min. Then carefully the supernatant was decanted off and 500 µl of cold 70% (v/v) ethanol was added straight away to wash the pellet. A brief centrifugation of about 3 min was done to settle the DNA pellet before decanting off the ethanol. The DNA was air dried for 30 min to 1 h on a clean blotting paper placed on clean bench (or until there was no more ethanol left in the DNA pellet

and in an eppendorf tube). Thereafter, 50 - 200 µl (depending on the amount of DNA) of a suitable solvent (such as water) was added.

4.2.7.2 Detection of transgenes in putative transgenic plants by PCR

The PCR amplification was performed in 25 µl reaction volume containing 10 ng/µl of genomic DNA, 10x PCR buffer (supplied with Taq DNA polymerase enzyme), 25 mM $MgCl_2$, 10 µM primers each of either *PMI*, *XvPrx2* gene specific, or XvPsap1 promoter specific (Table 4.8), (Use of genes specific primer pairs was not encouraged because of possible amplification of the endogenous sequences which are conserved across species), 10 mM each of dNTPs and 1 unit of Taq DNA polymerase enzyme. The PCR master mix and working concentrations are indicated in Table 4.2. The reaction was carried out using eppendorf vapo-protect thermal cycler (EPPENDORF AG 22331, Hamburg, Germany) with the following reaction conditions: initial denaturation step for 10 min at 95ºC followed by 45 cycles of denaturation at 95ºC for 30 sec; annealing at 55ºC for 45 sec; elongation at 72ºC for 50 sec, and a final extension step at 72ºC for 7 min. In addition, *VirG* gene primer pair (Table 4.8) were used to determine whether the putative transgenic maize integrated the T-DNA along with the vector backbone or not. The amplification products (550 bp for *PMI,* 458 bp of internal sequence for *XvPrx2*, 395 bp internal sequence for XvPsap1 promoter and 390 bp for *VirG)* was run on 1% (w/v) agarose gel in 1x TAE buffer and photo documented using gel

documentation machine (SYNGENE BIOIMAGING model No. 55000, Synoptics Ltd, Cambridge, UK).

Table 4. 8: Primers used for screening of transgenic plants and their amplicon sizes

Primer ID	Internal Oligo sequences	Amplicon size
PMI-U	5'-ACA GCC ACT CTC CAT TCA-3'	550 bp
PMI-L	5' GTT TGC CAT CAC TTC CAG-3'	
XvPrx2-F	5′-ACG ATC CCA GAC GGA ACG CT-3′	458 bp
XvPrx2-R	5′-CTT CAA GAT CTC ATC GGC ACC-3′	
XvPsap1-F1	5’-GGA CTT CAT GGC ATC CAT GTG C-3’	395 bp
XvPsap1-R1	5’-ATT TGC CCC ATG GAA AGT GAC G-3’	
VirG-F	5’-CTG GCG GCA AAG TCT GAT-3’	390 bp
VirG-R	5’-TGT CGT AAA CCT CCT CGT-3’	
ZmAct-F	5’-ACC CAA AGG CTA ACC GTG AG-3’	426 bp
ZmAct-R	5’-TAG TCC AGG GCA ATG TAG GC-3’	

4.2.7.3 Southern blot hybridization analysis of transgenic maize plants

Seeds from T_0 plants were planted to obtain T_1 plants. Genomic DNA was extracted from young leaves of T_1 plants. About 5-10 µg of genomic DNA of CML144 transformed with *XVSAP1* gene was digested with *Eco*R1 at a temperature of 37°C overnight. Equal amount of genomic DNA of CML144 and Staha transformed with *XvPrx2* genes construct were digested with *Hin*dIII at 37°C overnight. Digested products were resolved on 1% (w/v) agarose gels in 1x TAE buffer and subsequently transferred to nylon membranes (Hybond

N+Amersham Pharmacia) as described by Sambrook *et al.* (1989). The DNA was fixed to the membrane by cross-linking of DNA and the membrane for 90 sec under ultra violet rays. The PCR purified PMI and XvPsap1 probes were separately alkaline phosphate-labelled and subsequently hybridized using a kit from amersham according to manufacturer's instruction (AMERSHAM GE HEALTHCARE, Europe GmbH, Freiburg, Germany).

4.2.7.4 RNA extraction from drought stressed transgenic plants

Because XvPsap1 promoter is stress inducible, maize leave samples (100 mg) from drought stressed T_1 plants were harvested and ground in liquid nitrogen. Samples were obtained from drought stressed plants because RNA was expected to be transcribed from the *XvPrx2* and *XVSAP1* genes largely under drought stress. Total RNA was isolated using the Qiagen kit (Qiagen, Germany) following manufacturers instruction. RNA was resolved in a 1.2% (w/v) agarose formaldehyde gel and stained with cyber green to verify the quality.

4.2.7.5 cDNA synthesis using RT-PCR

To determine transcribed *XvPrx2* and *XVSAP1* genes, cDNA was synthesized using a cDNA synthesis kit (Invitrogen, USA) according to manufacturer's instruction. Standard PCR was carried out using either *XvPrx2* gene or XvPsap1 promoter specific internal primers along with an internal control maize actin (*Zm-*

actin) gene primers (GenBank accession no: AY107106) (Table 12). The PCR machine was programmed as follows, initial denaturation step of 5 min at 94ºC followed by 45 cycles of denaturation at 94ºC for 30 sec, anneling at 55ºC for 45 sec, elongation at 72ºC for 50 sec, and a final elongation step at 72ºC for 7 min. The *XvPrx2* or *XVSAP1* and maize *actin* transcripts were detected by amplifying a 458 or 395 and 426 bp fragments, respectively.

4.2.8 Management of transformation data and analysis

Transformation frequencies, TF (%) was calculated as number of mannose resistant calli event recovered per 100 embryos infected and transformation efficiencies, TE (%) as number of PCR positive plant events per total numbers of putative transgenic events tested. The regeneration frequency, RF (%) was computed as number of shoots regenerated per 100 embryogenic calli transferred to REGII medium.

4.3 RESULTS

4.3.1 Engineering of *Pac*1 site on *sacB* gene fragment

4.3.1.1 PCR for amplification of *sacB* gene fragment

Result for PCR amplification of *sacB* gene engineered with *Pac*1 site is presented in Figure 4.1. The expected band sizes for the primer sets, 1.886 kb, for sacB-F and sacB-R full lenght primers, and 0.859 kb, for sacB-F and sacB-R short primer combinations were observed.

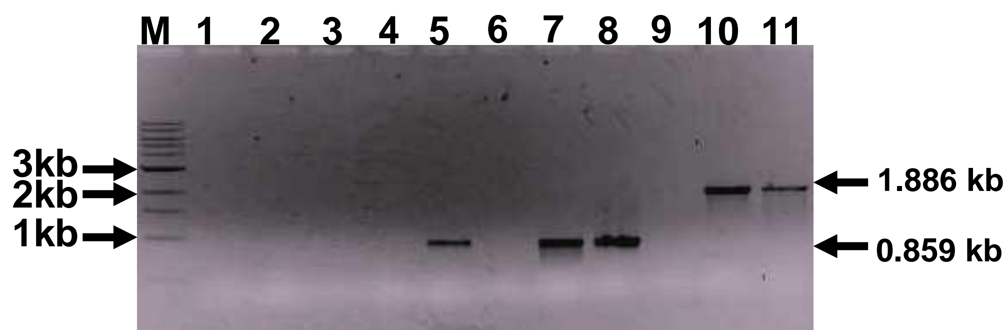

Figure 4.1: PCR product of *SacB* gene with *Pac*1 site resolved using various primer combinations.
M: 1 kb molecular marker, **Lanes 1 and 2:** without forward primer, **Lanes 3 and 4:** without reverse primer, **5:** Postive control (primers for *SacB* gene), **Lanes 6 and 9:** without template DNA, **Lanes 7 and 8:** PCR product of *sacB* gene using short primers of *SacB* gene, expected band size was 0.859 kb, **Lanes 10 and 11:** PCR product of *sacB* gene using full length primers for *SacB* gene, expected band size was 1.886 kb.

4.3.1.2 Selection of transformed colonies of *E. coli* cells carrying the *sacB*$_{Pac1}$ insert

White colonies that contained vectors with inserts (Figure 4.2) were picked and plated on the same medium and colony PCR result on selected colonies using *SacB* gene F-primer and M13 R-primer was presented in Figure 4.3.

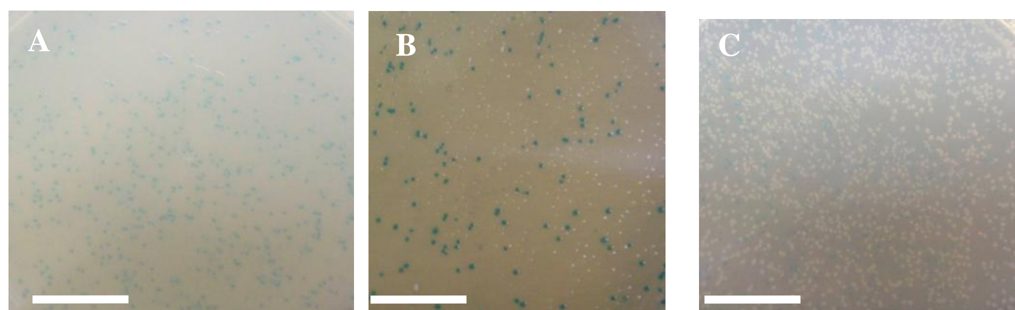

Figure 4.2: Blue white screening of transformed colonies with insert
A: Background control is colonies containing vector without insert, **B:** Experiment refers to colonies transformed with the vector carrying *sacB*$_{Pac1}$ insert (pTZ57RT$_{sacBPac1}$), and **C:** Control insert is colonies containing vector (pTZ57RT) with the control insert from manufacturers, *bars* = 10 mm. In white colonies the insert interfered with the *Lac Z* gene and could not display blue coloration whereas in blue colonies the vector was not affected by integrating the insert and rendered *Lac Z* gene active.

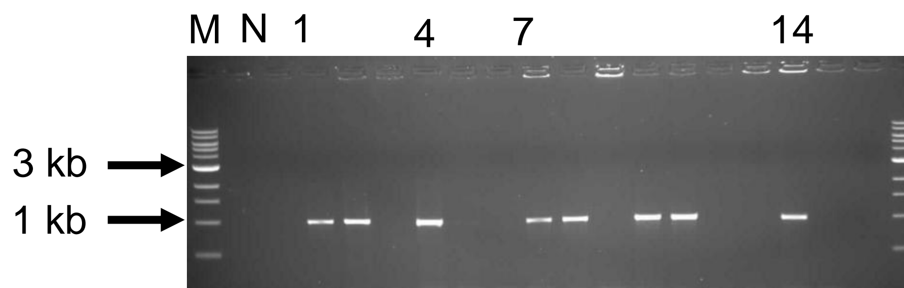

Figure 4.3: Colony PCR for pTZ5R/T$_{SacBPacI}$ using short *SacB* primer pairs.
M: 1 kb ladder, **N:** Negative control (PCR water), **Lanes 1-14:** PCR product from different pTZ5R/T$_{SacBPacI}$ colonies. The expected band size was 0.859 kb.

4.3.1.3 PCR screening of the pTZ57R/T$_{sacBPac1}$ carrying colonies

Colony PCR with the combination of primer set of both full length sacB-F and sacB-R or sacB-F and M13-R primers did not amplify except one colony that amplified by sacB-F and M13-R primers (Figure 4.4).

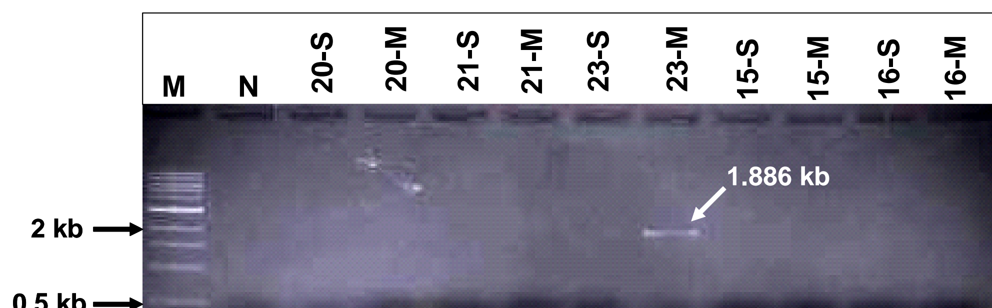

Figure 4.4: Colony PCR confirming the presence of pTZ57RT$_{sacBPac1}$.
M: 1 kb molecular marker, **N:** Negative control (PCR water), **Number with suffix-S:** Colony PCR product of samples amplified with SacB primers, **Number with suffix-M:** Colony PCR product of samples amplified with SacB Forward and M13 Reverse primers. The expected band size of 1.886 kb was observed.

4.3.1.4 Restriction digestion to confirm the presence of pTZ57R/T$_{sacBPac1}$

The restriction digestion on purified pTZ57R/T$_{sacBPac1}$ plasmid DNA using a series of enzymes indicated the expected band size for the vector (2.886 kb) and the *SacB* insert (1.886kb). The linearlized pTZ57R/T$_{SacBPacI}$ is therefore 4.772 kb as reflected by the product of digestion with *Hin*dIII, *Bam* H1, and *Not*I enzymes (Figure 4.5). These result confidently confirmed the presence of engineered sites as *Not*I was able to cut pTZ57R/T$_{SacBPacI}$ and it was not observed in the original pTZ57R/T vector sequence.

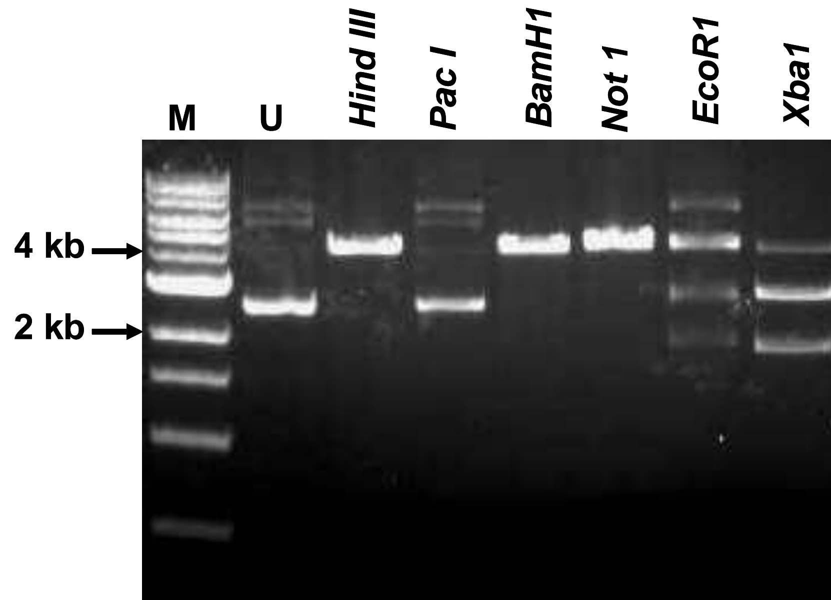

Figure 4.5: Gel picture of linearlized pTZ57R/T$_{\text{SacBPacI}}$ DNA with various restriction enzymes.

M: 1 kb molecular weight marker, **U:** Uncut pTZ57R/T$_{\text{SacBPacI}}$, **Lanes 3 to 8:** pTZ57R/T$_{\text{SacBPacI}}$ DNA digested with enzymes indicated on the picture. **Note:** No restriction enzyme was included in sample labelled *Pac*1 because the enzyme was out of stock

4.3.1.5 Cloning of *SacB-Pac*I insert into pBluescript II SK (+/-) vector

4.3.1.5.1 Double digestion of pTZ57R/T$_{\text{SacBPacI}}$ with *Not*1 and *Eco*R1

Double digestion of pTZ57R/T$_{sacBPac1}$ released two fragments. The high molecular weight fragment was the plasmid pTZ57R/T and the smaller fragment was *sacB* gene engineered with *Pac*1 site (*sacBPac*1) (Figure 4.6). The resulting *sacB-Pac*1 fragment was required for subsequent sub-cloning.

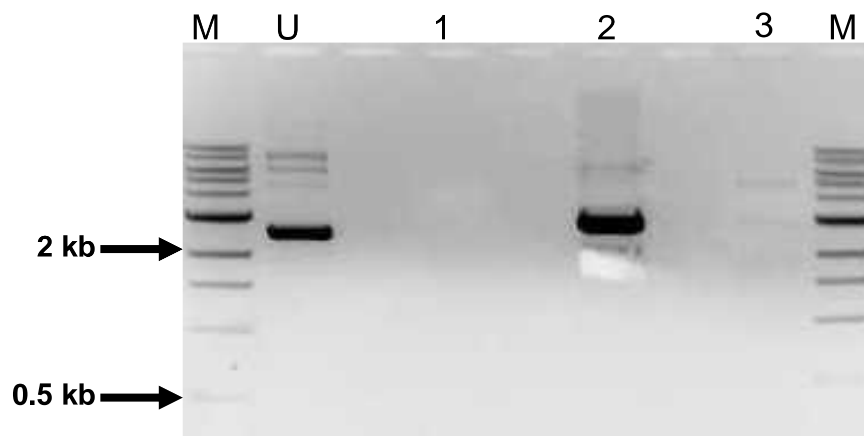

Figure 4.6: DNA samples of pTZ57R/T$_{SacBPacI}$ double digested by *Not*1 and *Eco*R1

M: 1 kb ladder, **U:** Undigested pTZ57R/T$_{SacBPacI}$ DNA, **Lanes 1-3:** Double digested DNA samples of pTZ57R/T$_{SacBPacI.}$ Only the sample on lane 2 had substantial DNA and the lower band with characteristic size of *SacB-Pac*1 fragment was sliced, purified and used for sub-cloning into pBluescript II SK.

4.3.1.6 PCR screening of pNOV2819::XvPsap1::XvPrx2::NosT transformants

Selected transformants of pNOV2819::XvPsap1::XvPrx2::NosT screened by PCR using either gene specific primers for *XvPrx2* gene or promoter specific XvPsap1 forward primer and XvPrx2 gene specific reverse primer revealed the following expected amplicon sizes:

The positive control and a positive transformant was 505 bp (Figure 4.7) or 2588 bp (Figure 4.8) for *XvPrx2* gene and XvPsap1 promoter specific primers respectively.

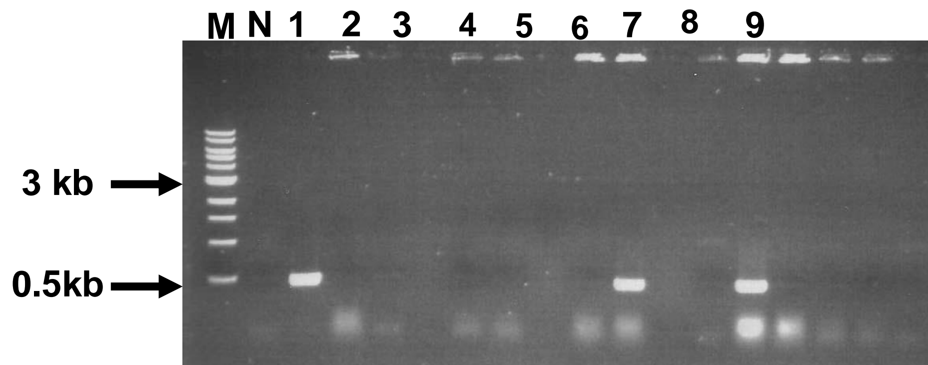

Figure 4.7: PCR screening of colonies with pNOV2819 habouring *XvPrx2* gene using *XvPrx2* gene specific primers

M: 1 kb marker, **N:** No template control (NTC), **Lane 1:** Positive control (pGEM-Teasy::XvPrx2 construct); **Lane 2:** Negative control (pNOV2819 in *E.coli* DH5α); **Lane 3:** Negative control (PCR water); **Lanes 4 to 12:** pNOV2819::XvPsap1::XvPrx2::NosT transformants. The expected band size of 0.505 kb was observed.

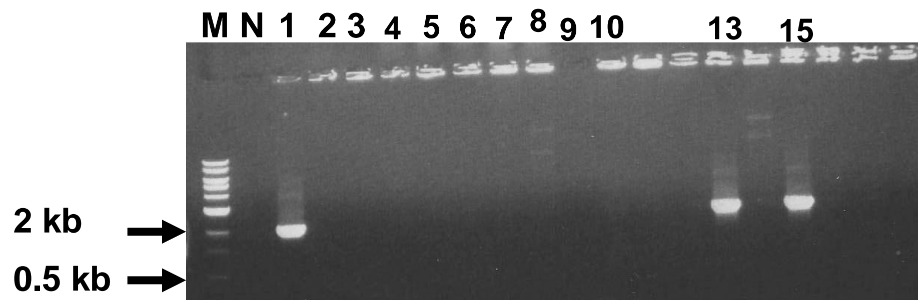

Figure 4.8: PCR screening of colonies with pNOV2819 habouring *XvPrx2* gene using XvPsap1-promoter specific primers.

M: 1 kb marker, **N:** No template control (NTC), **Lane 1:** Positive control (colony of pBluescript II SK::Psap1::XvPrx2::NosT), **Lane 2:** Negative control (pNOV2819 in *E.coli* DH5α), **Lane 3:** Negative control (PCR water), **Lanes 4 to 18:** pNOV2819::Psap1::XvPrx2::NosT transformants. The expected band size of 2.588 kb was observed.

4.3.1.7 Colony screening for pNOV2819::XvPsap1::XVSAP1::NosT transformants by PCR

Colony PCR for screening of pNOV2819::XvPsap1::*XVSAP1*::NosT gene construct with primers specific to promoter XvPsap1 forward primer and *XVSAP1* gene specific reverse primer revealed the expected amplicon sizes of 2468 bp for the positive control as well as positive transformant (Figure 4.9).

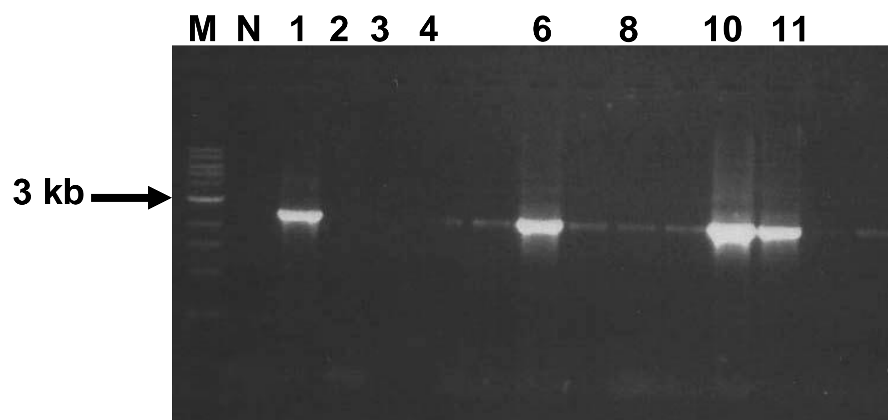

Figure 4.9: Screening of colonies with pNOV2819 habouring *XVSAP1* gene by PCR using XvPsap1-promoter specific primers.
M: 1 kb marker, **N:** No template control (NTC), **Lane 1:** Positive control (colony of pBluescript II SK::Psap1::XvPrx2::NosT), **Lane 2:** Negative control (pNOV2819 in *E.coli* DH5α), **Lane 3:** Negative control (PCR water), **Lanes 4 to 13:** pNOV2819::XvPsap1::XVSAP1::NosT transformants. The expected amplicon size of 2.468 kb was observed.

4.3.1.8 Confirmation of the *XvPrx2* and *XVSAP1* gene constructs by double digestion with *Pac*1 and *Hin*dIII

Double digestion experiment using *Pac*1 and *Hin*dIII restriction enzymes revealed the expected fragments from the double digested DNA. The fragment sizes were 7599 bp and 2844 bp for pNOV2819::XvPsap1::XvPrx2::NosT construct and 7599 bp and 2999 bp for pNOV2819::XvPsap1::XSAP1::NosT construct (Figure 4.10).

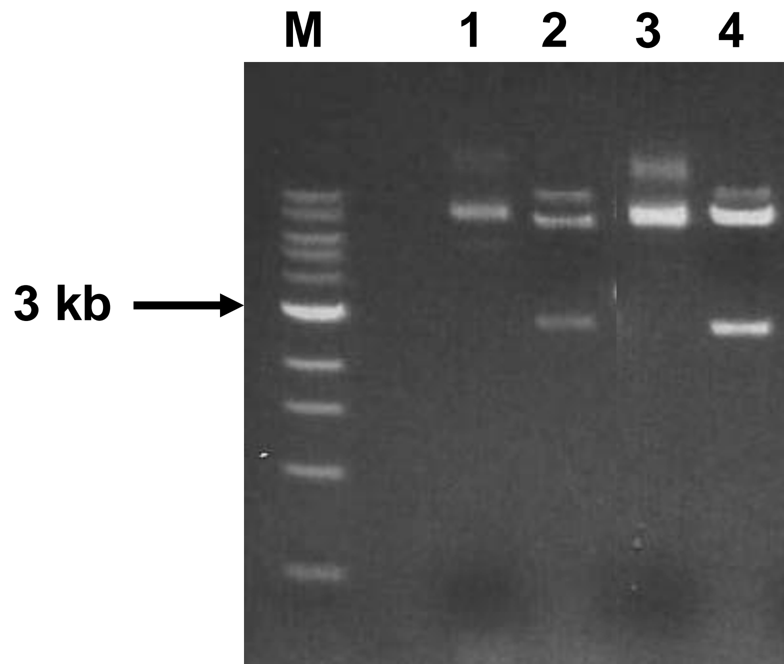

Figure 4.10: Double digestion of pNOV2819 vector habouring *X. viscosa* gene constructs with *Pac*I and *Hin*dIII.
M: 1 kb marker, **Lane 1:** pNOV2819::XvPsap1::XVSAP1::NosT undigested plasmid DNA, **Lane 2:** pNOV2819::XvPsap1::XVSAP1::NosT digested plasmid with *Pac*1 and *Hin*dIII, **Lane 3:** pNOV2819::XvPsap1::XvPrx2::NosT undigested plasmid DNA, **Lane 4:** pNOV2819::XvPsap1::XvPrx2::NosT plasmid construct digested with *Pac*1 and *Hin*dIII.

4.3.2 Transformation of maize immature zygotic embryos and plant regeneration

4.3.2.1 Co-cultivation and resting of infected maize immature zygotic embryos

During co-cultivation, both infected and non infected embryos had no observable difference. After 7 - 10 days on resting medium (RM), embryos formed primary calli originating from the embryo scutellum side (Figure 4.11A).

4.3.2.2 Selection of putatively transformed calli events

In the first two weeks of selection on SEM, embryos were observed to form more calli and increased in size compared to those on RM. Uninfected control embryos (Figure 4.11B) and the infected but untransformed calli (Figure 4.11C) grew slowly before turning necrotic and finally dying. In contrast, putatively transformed embryos grew quickly forming white friable embryogenic calli which developed somatic embryos (Figure 4.11C).

4.3.2.3 Regeneration of putative transgenic T_0 maize plantlets from calli events

On regeneration I medium (REG I), calli were seen to develop somatic embryos (Figure 4.11D). Upon transfer to regeneration II medium (REG II) with exposure to 16 h light and 8 h dark photoperiod, embryogenic calli greened and shoot buds

sprouted (Figure 4.11E). Shoot buds continued to green and grew into distinct shoots which later developed roots. Shoots which could not form roots in REGII medium were transferred to regeneration III medium (REG III) for rooting (Figure 4.11F). Not all embryogenic calli with somatic embryos were able to regenerate into plantlets on REGII medium. Some somatic embryos aborted leading to embryos death. The dead tissues exuded chemical substances. These chemical substances affected the survival of other healthier calli. Plantlets hardened in peat moss (Figure 4.11G) sprouted well and developed good root system. Maize plants that were transplanted into forest soil in buckets developed and grew to maturity (Figure 4.11H). Self pollination of T_0 maize plants resulted in good yield of T_0 seed (Figure 4.11I).

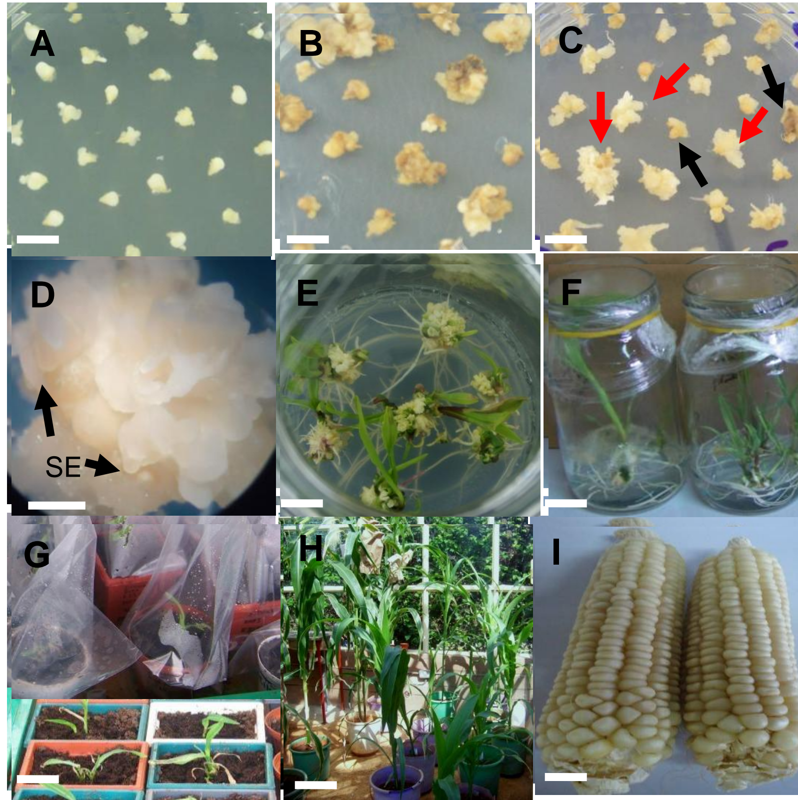

Figure 4.11: Transformation and regeneration profile of putative transgenic maize

A: Infected embryos forming calli after 10 days on RM, *bar* = 3 mm; **B:** Untransformed embryos 6 weeks on SEM (control), *bar* = 10 mm; **C:** 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; **D:** Embryogenic callus with somatic embryos 7 days on REGI ready to be transferred to REGII, *bar* = 10 mm; **E:** Shooting transformed calli on REG II, *bar* = 10 mm; **F:** Putatively transformed T_0 plantlets with good root system ready for hardening, *bar* = 20 mm; **G:** Putative transformants undergoing hardening process, *bar* = 40 mm; **H:** Putative T_0 regenerants growing in the glass house, *bar* = 300 mm; **I:** Harvested T_0 cobs with seeds *bar* = 10 mm.

Regeneration frequencies for CML144 and Staha calli transformed with *XvPrx2* gene averaged at 31.3 and 5.6%, respectively (Table 4.9 and 4.10). Whereas the

RF for individual experiments for CML144 and Staha maize genotypes recorded up to 35.5 and 11.6% (Table 4.9 and 4.10), the TF values for CML144 and Staha transformed using *XvPrx2* gene construct averaged at 12.9 and 23.9%, respectively (Table 4.9 and 4.10). The TE valuaes of CML144 and Staha averaged at 3.46 and 1.35%, respectively. Using *XvPrx2* gene construct and mannose selection system a total of 10 (with 2200 seeds) and 6 (with 1850 seeds) T_0 events were recovered for CML144 and Staha, respectively.

Table 4. 9: Putative CML144 transformed with *XvPrx2* gene and recovered T_0 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; **T_0 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 T_0 plantlets per number of total number of calli events transferred to REGII medium, **TE (%):** Transformation efficiency-obtained as a percentage of PCR positive plants over the total number of immature zygotic embryos infected and co-cultivated.

Table 4. 10: Putative Staha maizetransformed with *XvPrx2* gene and recovered T_0 events

Exp	E.I	MRC	Event	No. T_0 Plants **	PCR+ plants **	No. Cobs (Seeds)	TF (%)	RF (%)	TE (%)
1	227	58	58	1(1)	1(1)	1(~200)	25.6	1.7	0.44
2	79	11	11	1(1)	1(1)	2(~300)	13.9	9.1	1.26
3	176	43	43	5(3)	5(3)	6(~1200)	24.4	11.6	2.84
4	110	30	30	1(1)	1(1)	1(~150)	27.3	3.3	0.90
Total	592	142	142	8(6)	8(6)	10(~1850)	23.9	5.6	1.35

****** In brackets represent number of events, **Exp:** Independent Experiment; **E. I:** Embryos infected and co-cultivated; **MRC:** Mannose resistant callus; **T_0 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 T_0 plantlets per number of total number of calli events transferred to REGII medium, **TE (%):** Transformation efficiency-obtained as a percentage of PCR positive plants over the total number of immature zygotic embryos infected and co-cultivated.

For CML144 transformed with *XVSAP1* gene construct, RF was observed to range from 15.6 to 66.7% with an overall RF of 32.8% (Table 4.11). The TF for CML144 maize transformed with *XVSAP1* gene construct ranged from 39.5 to 46.5% with an overall value of 45.2%, whereas the TE was 2.34% (Table 4.11). Using *XVSAP1* gene construct and mannose selection system, a total of 13 T_0 events were recovered for CML144. However, only 7 T_0 events survived and grew to maturity giving up to 1708 seeds.

Table 4. 11: Putative CML144 maize inbred line transformed with *XVSAP1* gene

Exp	EI	MRC	Event	Plants Hardened **	Dead	Live plant **	PCR+ plants**	No. Seeds	TF (%)	RF (%)	TE (%)
1	43	17	6	4(3)	3	1(1)	1(1)	292	39.5	66.7	2.33
2	243	108	32	5(3)	2	3(3)	3(3)	248	44.4	15.6	1.23
3	312	145	29	13(7)	4	9(3)	9(3)	1168	46.5	44.8	2.88
Total	598	270	67	22(13)	8	14(7)	14(7)	1708	45.2	32.8	2.34

****** Number in brackets represent transformation events, **Exp:** Experiment; **EI:** Embryos infected and co-cultivated; **MRC:** Mannose resistant callus; **T_0 PCR+:** Number of putative maize plants that tested positive by PCR; **TF (%):** Transformation frequency- obtained as percentage of mannose resistant calli over the total number of immature zygotic embryos infected and co-cultivated; **RF (%):** Regeneration frequency-obtained as percentage of number of T_0 plantlets per number of total number of calli events transferred to REGII medium; **TE (%):** Transformation efficiency-obtained as percentage of PCR positive plants over the total number of immature zygotic embryos infected and co-cultivated.

Transplanted T_0 transgenic plants grew normally as untransformed counterparts with all T_0 plants being fertile and able to produce anthers and silks. However, minimal somaclonal variants were observed in CML144 maize transformed with *XVSAP1* gene construct as a result of prolonged tissue culture (Figure 4.12).

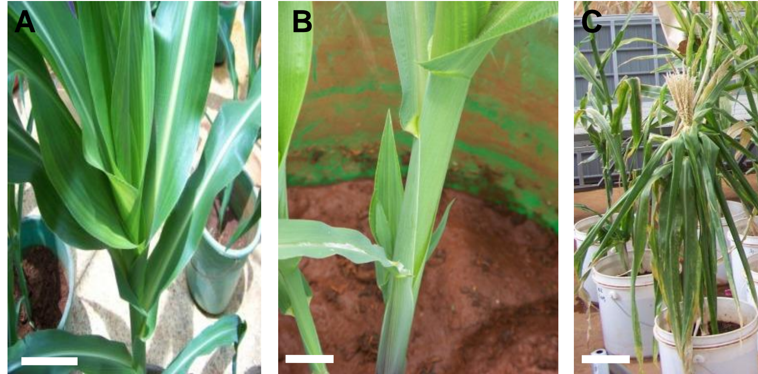

Figure 4.12: Transgenic T_0 maize plants transformed with *XVSAP1* gene showing different somaclonal variations.

A: Somaclonal variant with reduced internodes, *bar* = 300 mm. **B:** Somaclonal variant with shoot buds forming multiple shoots, *bar* = 40 mm **C:** Somaclonal variant with leaves originating from a single node and tassels without pollen, *bar* = 150 mm.

4.3.3 Molecular analysis of putative transgenic maize plants

4.3.3.1 Detection of transgenes in transgenic plants by PCR

Putative T_0 transgenic CML144 and Staha transformed with *XvPrx2* gene screened by PCR using PMI specific primers revealed expected band size of 0.550 kb (Figure 4.13).

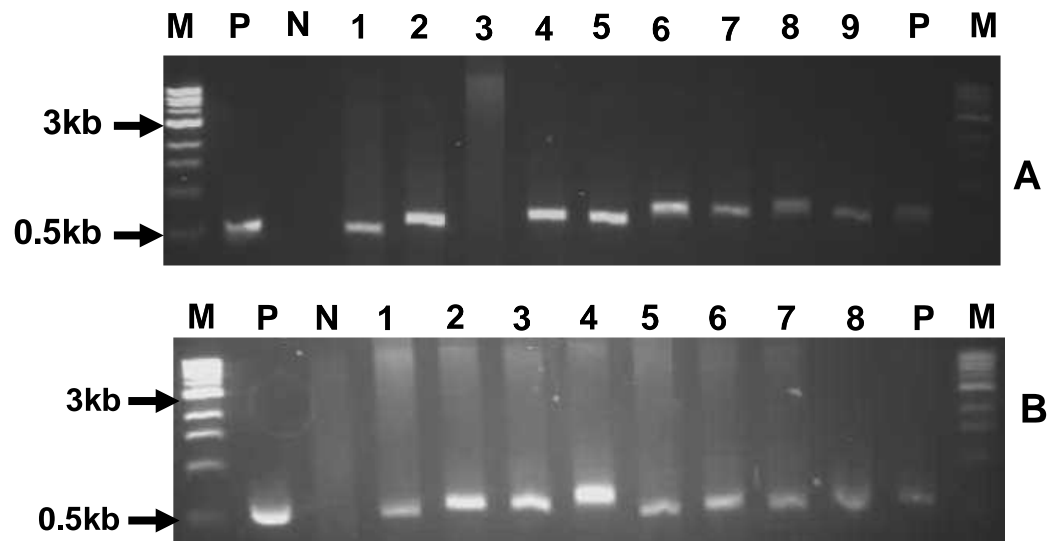

Figure 4.13: PCR detection of transgenic plants using *PMI* gene specific primers.

A) CML144 inbred maize line transformed with *XvPrx2* gene construct, **B)** Staha OPV maize variety transformed with *XvPrx2* gene construct. **M:** 1 kb ladder, **P:** Positive control (Plasmid construct used for transformation), **N:** Negative control (Untransformed maize), **Lanes 1-9 (Panel A)** and **Lanes 1-8 (panel B):** PCR product from putative transgenic CML144 and Staha, respectively. Expected band size for *manA* gene (positive control) was 0.550 kb.

To verify the presence of *XvPrx2* gene in putative T_0 transgenic CML144 and Staha, 458 bp band size of PCR product using *XvPrx2* gene specific primers was observed on PCR product resolved on 1% (w/v) agarose gel in 1x TAE buffer (Figure 4.14).

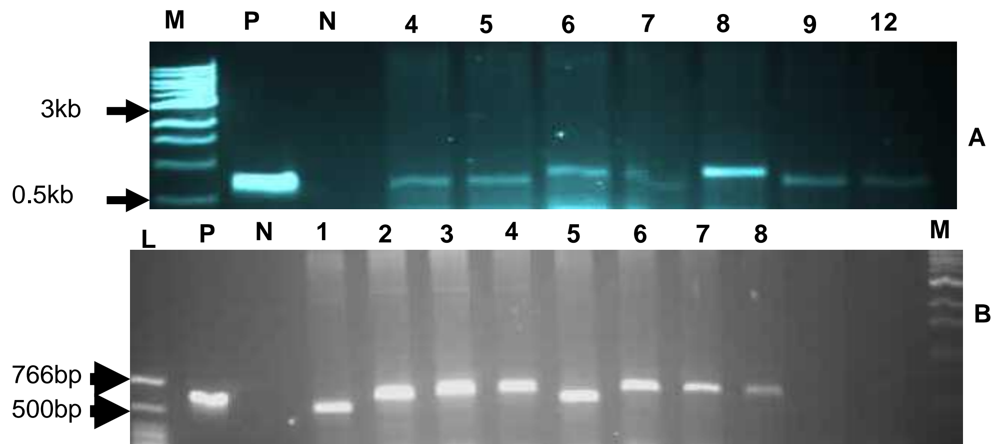

Figure 4.14: PCR detection of transgenic plants using *XvPrx2* gene specific primers.

A) CML144 inbred maize line transformed with XvPrx2 gene construct, **B)** Staha OPV maize variety transformed with XvPrx2 gene construct. **M:** 1 kb ladder, **P:** Positive control (Plasmid construct used for transformation, **N:** Negative control (Untransformed maize), **Lanes 4-1p2 (Panel A)** and **Lanes 1-8 (panel B):** PCR product from putative transgenic CML144 and Staha, respectively. Expected band size for *XvPrx2* gene (positive control) was 0.458 kb.

Further PCR screening results on T_1 transgenic plants verifying the presence of the promoter driving the *XvPrx2* gene using promoter specific primers (XvPsap1 primers) revealed expected band size of 395 bp (Figure 4.15).

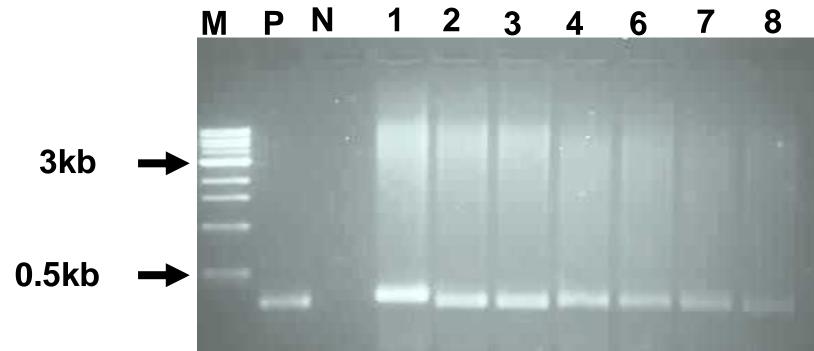

Figure 4.15: PCR detection of transgenic T_1 CML144 plants using XvPsap1 promoter specific primers.
M: 1 kb ladder, **P:** Positive control (Plasmid construct used for transformation, **N:** Negative control (Untransformed maize), **1-7:** PCR product from T_1 transgenic CML144 plants transformed with *XvPrx2* gene construct. Expected band size of 0.395 kb was observed (positive control).

For CML144 transformed with *XVSAP1* gene, PCR results revealing the presence of the transgene using XvPsap1 promoter specific primers indicating the expected band size of 395 bp (Figure 4.16).

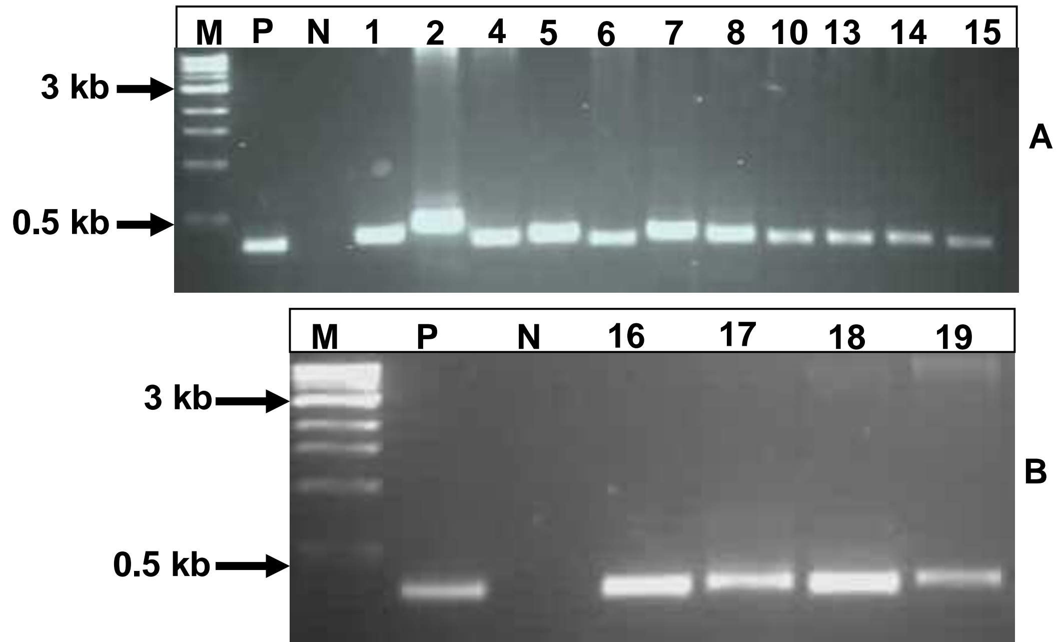

Figure 4.16: PCR detection of transgenic T_0 CML144 plants using *XvPsap1* gene specific primers for the promoter

M: 1 kb ladder, **P:** Positive control (Plasmid construct used for transformation), **N:** Negative control (Untransformed maize), **1-15 (Panel A) and 16-19 (panel B):** PCR product from putative transgenic CML144 transformed using *XVSAP1* gene construct. Band size for Psap1 gene (positive control) was 0.395 kb.

To determine whether the transgenic plants were contaminated by *Agrobacterium* and/or vector backbone, PCR amplification using *VirG* specific primers was done and detected no sequence in DNA of T_0 maize plants while detecting expected band size of 390 bp in positive control (Figure 4.17).

Figure 4.17: PCR analysis of T_0 maize for vector backbone contamination. **M:** 1 kb ladder, **P:** Positive control (Colony PCR of *A. tumefaciens* harbouring construct used for transformation), **N:** Negative control (DNA from non-transformed maize), **Lanes 1-10:** PCR product of DNA samples from transgenic maize plants amplified using VirG primers. No amplification was detected in all transgenic and negative control samples. Band size for VirG primers was 390 bp for positive control.

4.3.3.2 Southern blot hybridization analysis of transgenic T_1 plants

Southern blot analysis on genomic DNA of T_1 transgenic plants was carried out to determine stable integration and transgene copy numbers. Southern hybridization results for CML144 and Staha maize genotypes transformed with *XvPrx2* gene construct were presented in Figure 4.18. Southern blot result for CML144 transformed with *XVSAP1* gene construct is presented in Figure 4.19. Results indicate stable integration of the genes with low copy number ranging from 1 to 3 copies.

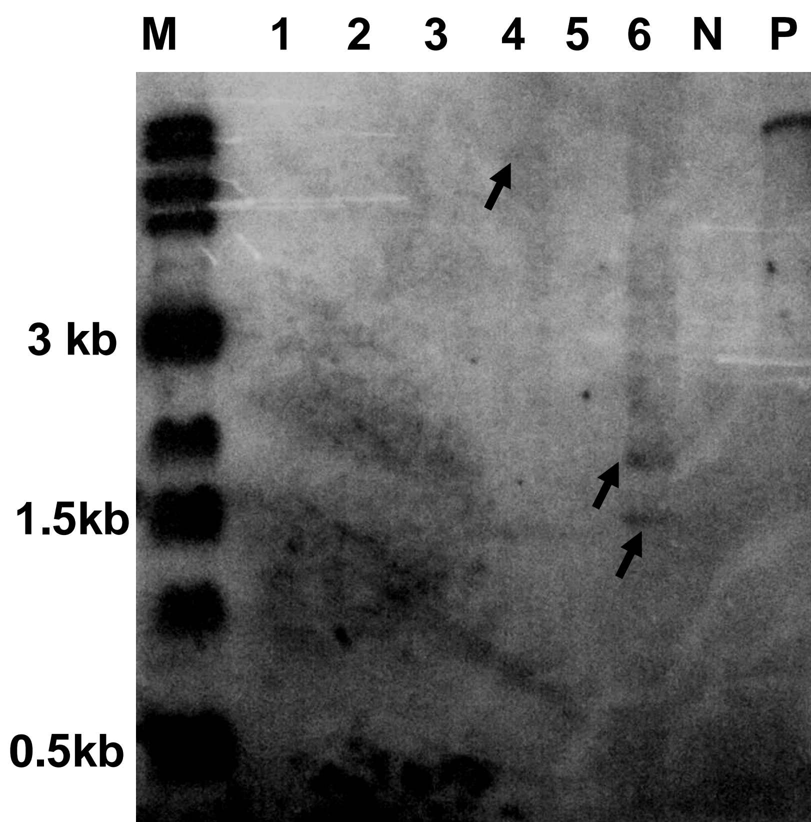

Figure 4.18: Southern blot analysis of six T_1 events from maize plants transformed with *XvPrx2* gene construct.

Plant genomic DNA was probed with promoter (XvPsap1) specific probes, **M:** 1 kb Ladder, **1-6:** genomic DNA of different transgenic maize events digested with *Hin*dIII, **N:** Negative control (genomic DNA of untransformed maize digested with *Hin*dIII), **P:** Positive control (Plasmid DNA linearised with *Hin*dIII).

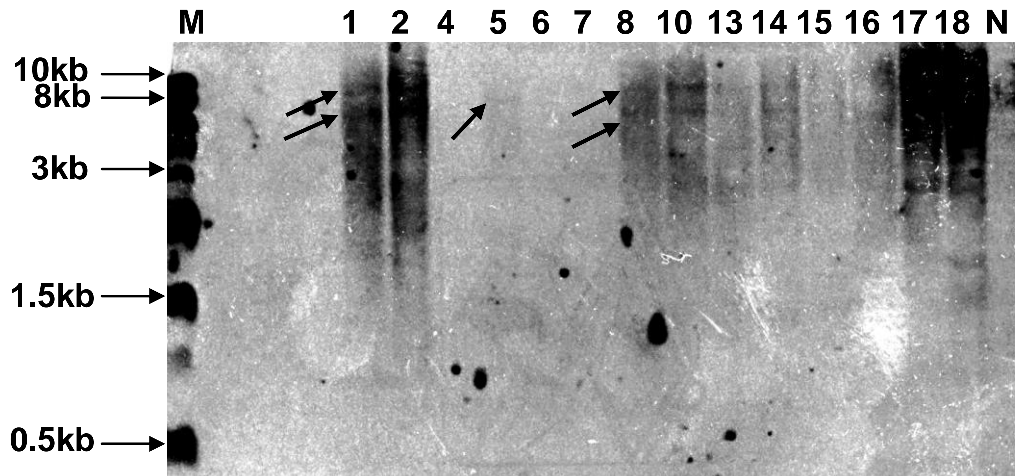

Figure 4.19: Southern blot analysis of six T_1 events from CML144 maize transformed with *XVSAP1* gene construct.

Plant genomic DNA was probed with promoter (XvPsap1) specific probes. **M:** 1 kb Ladder, **1-18:** genomic DNA of different transgenic maize plants digested with *Eco*R1 (1 and 2 were different events, 4 and 5 were the same events, 6 to 8 were the same events, 10 is independent event and 13 to 18 were the same events), **N:** Negative control (genomic DNA of untransformed CML144 digested with *Eco*R1).

4.3.3.3 Expression assay with reverse transcription -PCR

Reverse transcription-PCR for CML144 and Staha transformed with *XvPrx2* gene construct using internal *XvPrx2* gene specific primers was done to determine the expression of the *XvPrx2* gene in transgenic maize plants. In comparison to *Zm-actin* gene amplification as an internal control, the *XvPrx2* gene expression in transgenic plants was detected in transgenic CML144 (Figure 4.20) and Staha (Figure 4.21).

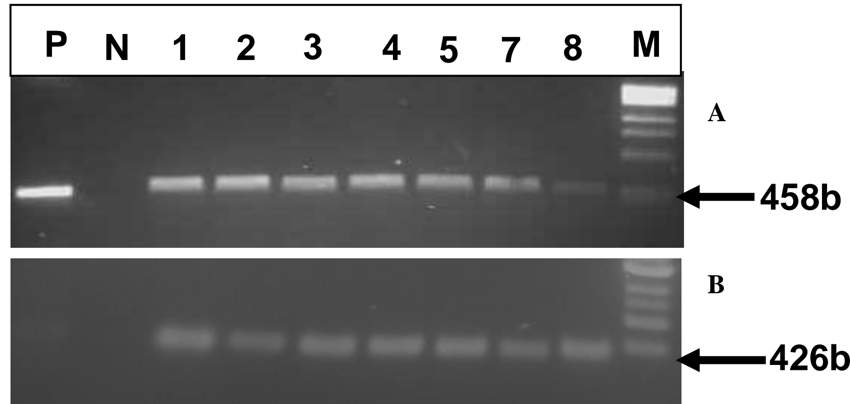

Figure 4.20: RT-PCR on transgenic and non-transgenic CML144 maize.
Panel A: Results obtained using *XvPrx2* specific primers, **Panel B:** The same substrates amplified with maize actin gene primers for loading control. **M:** 1 Kb ladder (New England Biolab, UK); **P:** Positive control (PCR on plasmid 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 non transgenic maize under dehydration. Expected band size for *XvPrx2* and *Zm-actin* gene was 458 bp and 426 bp, respectively.

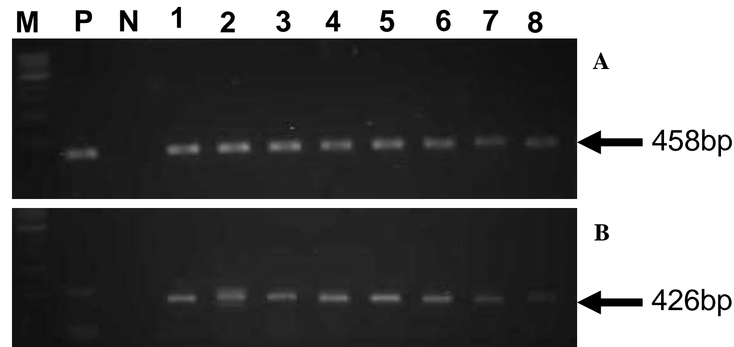

Figure 4.21: RT-PCR on transgenic and non-transgenic Staha maize.
Panel A: Results obtained using *XvPrx2* specific primers, **Panel B:** The same substrates amplified with maize actin gene primers for loading control. **M:** 1 Kb ladder (New England Biolab, UK); **P:** Positive control (PCR on plasmid of the construct used for maize transformation, **N:** Negative control (RNA untreated by first strand superscript III reverse transcriptase), **Lanes 1-2:** RT-PCR product from transgenic maize plants cDNA under dehydration, **Lanes 3-4:** cDNA from transgenic maize under well watered condition, **Lanes 5-6:** RT-PCR on cDNA of untransformed Staha maize under dehydration, **Lanes 7-8**: RT-PCR on cDNA of non transgenic Staha under well watered condition. Expected band size for *XvPrx2* and *Zm-actin* gene was 458 bp and 426 bp, respectively.

Reverse transcription-PCR for CML144 transformed with *XVSAP1* gene construct using XvPsap1 primers was done to determine the expression of the *XVSAP1* gene in transgenic maize plants. In comparison to *Zm-actin* gene amplification as an internal control, the *XVSAP1* expression in transgenic plants was detected (Figure 4.22).

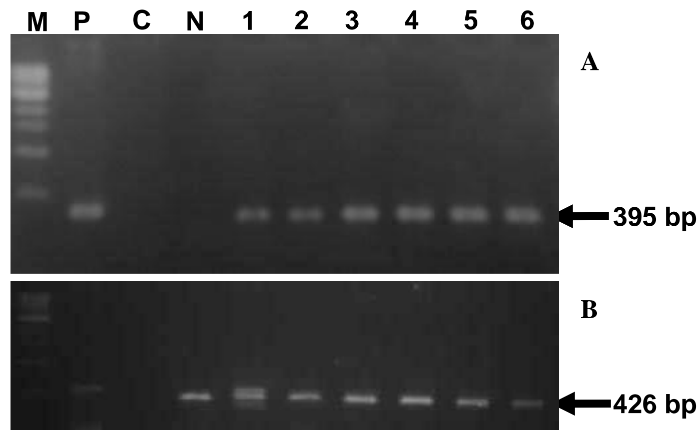

Figure 4.22: RT-PCR on CML144 transformed with *XVSAP1* gene construct. Panel A: Results obtained using XvPsap1 promoter specific primers, **Panel B:** The same substrates amplified with maize actin primers for loading control, **M:** 1Kb ladder (New England Biolab, UK), **P:** Positive controll (Plasmid construct used for maize transformation), **C:** Non template control, **N:** Negative control (Plant cDNA from untransformed CML144 plant), **1-6:** cDNA from 6 transgenic plant events. Expected band size for XvPsap1 and *Zm-actin* gene was 395 bp and 426 bp, respectively.

4.4 DISCUSSION

Genetic engineering has become an integral strategy in crop improvement particularly for polygenic traits like drought stress tolerance. Successful genetic transformation depends much on explant response to *in vitro* culture. Plant response to *in vitro* culture is influenced by endogenous hormonal balance and other chemical composition in the cells of an explant and in the external environment such as media composition. In the present study, immature embryos responded by forming calli originating from their scutellum. This response can be associated with media formulation where by the presence of silver nitrate and 2, 4-D played a role in calli formation particularly in the RM. Silver nitrate has been known to increase callus formation and inhibiting recurrence of *A. tumefaciens* thereby improving transformation efficiency (Kumar *et al*., 2007).

In this study, *XvPrx2* gene was successfully introgressed into CML144 (QPM) and Staha maize genotypes whereas *XVSAP1* gene was only transformed into CML144 maize genotype. Selection of putative transgenic calli events under mannose was an effective system in maize. Following infection, most untransformed embryos as well as their control counterparts turned necrotic and ultimately died whereas those that were transformed increased in size and developed calli. This was because transformed embryos were able to take up mannose and convert it to mannose 6-phosphate by endogenous hexokinase (Reed *et al.,* 2001). Accumulation of mannose 6-phosphate in non-transformed plant cells inhibits

glycolysis thus depriving cells of energy (Reed *et al.,* 2001). In the present study, the stringency of PMI selection was observed with several calli events dying allowing only transgenic cells to thrive.

Transformation frequency (TF), transformation efficiency (TE) and regeneration frequency (RF) are the parameters that are commonly used to determine the success of transformation and regeneration experiments. Transformation frequency (TF) for CML144 transformed with *XvPrx2* gene construct, ranged from 9.8 to 15.3% with an average of 12.9% (Table 4.9) whereas for Staha, the range was from 13.9 to 27.3% with an average of 23.9% (Table 4.10). These TF values are low compared to TF of 30% and 35% on temperate maize and wheat reported by Negrotto *et al.* (2000) and Reed *et al.* (2001), respectively using the same selection system. However, TF values for CML144 transformed with *XVSAP1* gene construct (Table 4.11) were at per with those reported on temperate maize and wheat (Negrotto *et al.,* 2000; Reeds *et al.,* 2001). Although tropical maize genotypes have been reported to be recalcitrant to transformation as compared to temperate maize genotypes, the disparity between these findings may also be attributed to the type of the explant, the quality and age of embryos, media composition and selectable marker genes used. Other factors that may affect TF include *Agrobacterium* concentration and incubation conditions.

The type of selectable marker and the amount of selective agent used is also an important consideration in transformation experiments. Use of sub-optimal amount of selective agent may result in an inefficient selection thereby leading to plant escapes. On the other hand excessive use of selective agent may select against weakly expressing transgenic cells. Hence, subjecting calli cells to optimal levels of PMI selection system, in the present study, facilitated the recovery of transgenic calli and enhanced regeneration of transgenic plants with no escapes as all resulting plants tested positive with PCR.

In this study, regeneration frequency values were very low, which may be attributed to the fact that not all putatively transformed calli were able to regenerate. The failure in sprouting of shoots in some calli may be as a result of roots formation, which suppressed shoot formation. Other calli were affected by some phenol-like chemical compounds exuded by some dead calli cells. The phenol like chemical compounds was suspected to be hamful to other neighbouring health calli (Seth *et al.,* 2012). PCR detection also revealed the presence of transgenes in almost all plants tested implying that mannose selection was an efficient system for maize.

Few somaclonal variants were observed in regenerated transgenic plants (particularly in CML144 transformed with *XVSAP1* gene construct). Somaclonal variations are usually epigenetic in nature. Epigenetic changes appear to be a

direct effect of the tissue culture process, being physiological in origin and perhaps resulting from effects of the growth regulators in the medium as described earlier by Fluminhan *et al.* (1996). Epigenetic changes observed in this study are of no value for crop improvement, as they were not expressed in sexual progeny although they had tremendous negative effect in plant development. Examples of effects were observed as a result of somaclonal variations include impaired fertility and growth of R_0 or T_0 plants observed in three plants in this study. However, all other T_0 plants were fertile and were able to produce anthers and silks. Self pollination of T_0 plants resulted in good yield of T_0 seeds.

PCR results for screening of putative transgenic maize events were positive in all the events tested implying the presence of *XvPrx2* and *XVSAP1* genes in the genome of transgenic maize. Since no sequence amplification was detected by PCR using *VirG* gene specific primers in the DNA of transgenic maize plants, it was therefore arguable that the T_0 transgenic plants obtained were free from plasmid backbone and *Agrobacterium* contamination. This means that only the T-DNA region was integrated into maize plant genome. However, earlier studies have shown that the problem associated with *Agrobacterium* mediated transformation is the possibility for the DNA sequences outside the T-DNA region integrating into the plant genome alongside the T-DNA (Kononov *et al.,* 1997; Shou *et al.,* 2004b). T-DNA vector backbones are often fused with bacterial antibiotic resistance genes that can create biosafety concerns. It is therefore

important to consider the technique that would reduce the transgene copies and minimize or eliminate the presence of T-DNA vector backbone sequences. Recent studies have suggested that backbone integration into plant genome occurs more frequently when using small T-DNA binay vector systems (Oltmanns *et al.,* 2010; Gelvin *et al.,* 2012). However, the observation that large T-DNAs only integrate into plant genome seldomly reveals a possible reason as to why chromosomal integration of T-DNA results in transgenic plants lacking vector backbone sequences.

Southern blot analysis of genomic DNA from transgenic T_1 plants was carried out to ascertain stable integration of the transgenes and determine the copy number in the recovered events. Transgene copy number is defined as the number of exogenous DNA insert(s) in the genome. In this study, stable gene integration was achieved in both maize plants transformed with *XvPrx2* and *XVSAP1* gene constructs with considerably low copy numbers of transgenes. Out of all the transgenic events selected for southern analysis, only one in each case of gene construct used, showed a single copy whereas the rest had 2 - 3 copies. Such observation of multi-gene copy occurring at one or more loci is common in transgenic studies mediated by *Agrobacterium.* Wu *et al.* (2006) described this observation to result from the use of additional *vir* genes to enhance transformation efficiencies in recalcitrant cereal species such as maize. In the present study pNOV2819 vector with an added *VirG* sequence in its backbone was

also used to enhance the transfer of the transgenes to the host plants. While working on wheat using both *Agrobacterium* and particle bombardment, Cheng *et al.* (1997) reported that out of 26 *Agrobacterium*-mediated transformants, more than one-third contained a single T-DNA insert, half contained 2 - 3 copies, and the remainder (about 15%) contained 4–5 copies and no transformants contained more than five T-DNAs. Travella *et al.* (2005) conducted similar experiments in barley and showed that all the *Agrobacterium*-derived lines contained 1–3 copies of the transgene, while 60% of the transgenic lines derived by particle bombardment contained more than eight copies.

Transgene copy number is a key issue for transgenic studies since it is directly relevant to the effectiveness of transgenic event and data interpretation. Transgene copy number is important in the genetic analysis of gene function. Multiple transgene copies can lead to extremely high expression of the gene (Stavolone *et al.,* 2003), and occasionally result in transgene silencing (Tang *et al.,* 2007). For this reason, transgene copy number determination is an essential part of transgenic studies. However, in this study some events could not show signal with Southern blot although they were positive with PCR, which could be as a result of insensitivity of the Southern kit attributed to low gene dosage (concentration) in the DNA samples used for hybridization.

Transgene expression in transgenic T_1 plants was also observed as validated by reverse transcription polymerase chain reaction. The XvPsap1 is drought stress inducible promoter that could only drive downstream gene expression under dehydration stress. A maize *Zm-actin* gene (GenBank accession no: AY107106) was used as an internal control gene. As expected, the transgenic plants of *XvPrx2* construct expressed *XvPrx2* genes whereas little or no expression was observed in the control plants (non-transformed and/or unstressed transgenic plants). Although peroxiredoxin2 is found across species, its expression is highly dependent on controlling elements in different species. The XvPsap1 promoter used in this study, is stress inducible and the differential expression exhibited by peroxiredoxin2 transcripts between the transgenic under stress, unstressed and non-transgenic plants revealed that, XvPsap1 is a strong stress inducible promoter. These findings are in agreement with Oduor *et al.* (2009) who reported on the potential function of full length XvPsap1 promoter (2083 bp) sequence (also used in this study) as compared to other two truncated fragments designated XvPsap2 (1577 bp) and XvPsap3 (1127 bp) promoters.

In this study, stable gene integration in transformed CML144 (QPM) and Staha maize was successfully achieved. However, the transformation successes described in this chapter would not be complete if drought stress assays were not done to evaluate the performance of developed transgenic lines. The next chapter

discusses the performance of these transgenic maize plants evaluated under drought stress in the glasshouse.

CHAPTER FIVE

EVALUATION OF THE PERFORMANCE OF TRANSGENIC MAIZE PLANTS UNDER DROUGHT STRESS

5.1 INTRODUCTION

This chapter describes the performance of transgenic CML144 and Staha maize genotypes in comparison to their non transgenic maize counterparts under drought stress conditions. In this report, only CML144 and Staha transformed with *XvPrx2* gene construct were studied. The *XvPrx2* gene encodes an antioxidant enzyme peroxiredoxin2 responsible for scavenging of excess reactive oxygen species (ROS) generated in plant cells under drought stress condition.

Relative water content (RWC) and photosynthetic pigments were used as indicators for screening tolerance against drought stress in different transgenic plants. RWC is a valuable indicator of plant water status in comparison to other water potential parameters under drought stress (Keles and Oncel, 2004) and high RWC has been associated with drought tolerance in plants. Drought affects photosynthetic components such as enzymes, chlorophylls, and carotenoids. Changes in these photosynthetic components depend on the severity and duration of stress (Ashraf and Harris, 2013) and on plant species (Dubey, 1994). Photosynthetic pigments play an important role in light harvesting and dissipation of excess energy. It has been reported that both chlorophyll *a* and *b* content

changes under drought stress (Farooq *et al.* 2009). Carotenoids participate in energy dissipation thereby protecting chlorophylls against oxidative damage by O_2 thus improving plants' tolerance against drought stress and high irradiance (Aono *et al.,* 1993; Gunes *et al.,* 2008).

In the present study CML144 and Staha maize transformed with *XvPrx2* gene construct were evaluated for tolerance to drought stress against their non transgenic maize counterparts. RWC, amount of chlorophyll *a*, *b*, *a*+*b*, chla/b ratio and carotenoids were determined in both transgenic and non transgenic maize plants. These parameters were used as indicators for drought stress tolerance in transgenic maize plants compared with their non transgenic maize counterparts.

5.2 MATERIALS AND METHODS

5.2.1 Experimental materials and design

Transgenic T_0 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. The experiment was carried out in the glasshouse at a temperature of 43±2ºC and relative humidity of 55 to 60%. Germinated T_1 plants were screened for the presence of the transgene by PCR. Then plants were thinned and positive plants were transplanted into other pots such that the number remained one plant per pot. Eight positive transgenic T_1 maize plants and 8 non-transgenic controls at the age of 6 and 8 weeks for CML144 and Staha, respectively were selected for the experiment. From germination plants were watered with half a litre of water once (every morning) daily before imposing drought stress. From each group (transgenic and non-transgenic) of plants, 4 maize plants were used for drought stress treatment while 4 were watered with half a litre once daily throughout the experiment. The experiment was arranged in 2x4x3 factorial (2 genotpes (transgenic and non transgenic maize) 4 observation points under drought stress (days and recovery) and 3 replications) in a complete radondomized design.

5.2.2 Determination of watering regime for plants during drought stress assays

Plant water requirement for daily irrigation was determined emperically 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 again weighed (W2). The soil was then 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 taken. 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/cm^3.

5.2.3 Procedures for drought stress experiment

Before commencement of drought 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. In 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 non transgenic maize plants. Plant photos were taken to mark as a reference for comparison of maize responses under drought stress. Thereafter, sampling was done from same plants at mid day on day 7, 14 and 21 after withholding water in transgenic and non-tarnsgenic CML144. In transgenic and non-transgenic Staha sampling was done from same plants at mid day on day 5 and 10 after withholding water. Sampling was also done after 24 h following one time re-watering of plants at the end of drought stress 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 non transgenic maize plants

5.2.4 Determination of leaf relative water content

To determine RWC, 3 pieces of leaf samples (3 cm x 4cm) 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. In 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):

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

Where: FW is the fresh weight

SW is the saturation or turgid weight

DW is the dry weight

5.2.5 Determination of total chlorophylls, chlorophyll *a*, *b*, chlorophyll *a/b* ratio and total carotenoids

Photosynthetic pigments were extracted using 2 ml of 100% (v/v) of acetone per sample as descibed by Lichtenthaller 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 in a pestle and motor. 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 cuvets for OD reading at A_{662nm}, A_{645nm} and $A_{470nm,}$. One cuvet with acetone served as a blank. Absorbance for specific pigment was determined using 722N visible spectrophotometer (EVERICH MEDCARE LTD, Nanjing, China). Maximum absorbance for chlorophylls (Chl) *a*, *b* and carotenoids (*Car*) were recorded at A_{662nm}, A_{645nm} and $A_{470nm,}$ respectively. The respective pigments were calculated using the formula by Lichtenthaller and Wellburn (1983):

Chl*a* (µg/gfw) = $11.75A_{662}$-$2.350A_{645}$

Chl*b* (µg/gfw) = $18.61A_{645}$-$3.960A_{662}$

Car (µg/gfw) = $1000A_{470}$-2.270Chl*a* (µg/gfw)-81.4Chl*b* (µg/gfw)/227

Total Chl (µg/gfw) = Chl*a* (µg/gfw) + Chl*b* (µg/gfw)

Chlorohyll ratio was simply obtained by dividing Chl*a* (µg/gfw) by Chl*b* (µg/gfw)

5.2.6 Drought stress data management and analysis

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_{662nm}, A_{645nm} and $A_{470nm,}$ were used to compute the amount/content of chlorophyll *a*, chlorophyll *b* and total carotenoids, respectively. Analysis of

variance (ANOVA) was carried out using GenStat Discovery Edition 4 (VSN International software for biosciences, www.vsni.co.uk/software/genstat/) to test the statistical significance of differences among the genotypes (transgenic and non transgenic maize genotypes) and the days of exposure to drought stress. Pair wise comparison of means (for RWC, chlorphylls *a* content, chlorophylls *b* content, total chlorophylls, chlorophyll *a/b* ratio and total carotenoid content) was carried out using least significance difference (LSD) test at 5% probability level. Figures were prepared using Excel software (Microsoft).

5.3 RESULTS

5.3.1 Effect of drought stress on relative water content

RWC (%) of transgenic and non transgenic maize plants were found to be significantly different from each other at $p<0.05$ (Table 5.1 and 5.2). Pairwise comparison of RWC between transgenic and non transgenic maize also revealed significant difference according to LSD at $p<0.05$ in all sampling points. Generally, there was a decrease in RWC in both non transgenic and transgenic maize plants upon exposure to drought stress. However, the effect was more pronounced in non transgenic maize plants (CML144 and Staha) compared to the transgenic plants (CML144-XvPrx2 and Staha-XvPrx2) (Figure 5.1). RWC decreased from 85.22% (day 0) to 78.23% (day 7), 73.25% (day 14) and 62.06% (day 21) in untransformed CML144, whereas in transgenic (CML144-XvPrx2) the RWC decreased from 85.52% on day 0 to 82.31% on day 7, 78.73% on day 14 and 72.26% on day 21. RWC determined 24 h after re-watering increased from 62.06% to 74.89% in non transgenic CML144 whereas in transgenic plants (CML144-XvPrx2) the increase ranged from 72.26% to 82.43% (Figure 5.1A).

Likewise for Staha, the RWC decreased from 81.25% (day 0) to 58.02% (day 5), 43% (day 10) in non transgenic Staha, whereas in transgenic (Staha-XvPrx2) the RWC decreased from 85.23% (day 0) to 71.28% (day 5), 61.39% (day 10). In non transgenic Staha the RWC increased from 43% to 55.43% 24 h after re-watering while in transgenic Staha, the RWC increased from 61.39% to 95.18% (Figure

5.1B). Furthermore, upon commencement of drought stress, leaf wilting was observed within three day in untransformed maize while in transgenic maize wilting was noted on the fifth day of drought stress. Upon re-watering, recovery in transgenic maize genotypes was noted within 6 to 24 h compared to 4 days in non transgenic maize counterparts (Figure 5.2 and Figure 5.3).

Table 5. 1: Analysis of variance for total chlorophyll, *a*, *b*, Chl*a*/Chl*b*, carotenoids and RWC of transgenic CML144 and non transgenic CML144 maize genotypes as affected by drought stress

Source of variation	**Df**	**Mean Square**					
		Chl*a*	**Chl*b***	**TChl**	**Chl *a/b* ratio**	***Car***	**RWC**
Genotypes (G)	1	5.01982**	15.9036**	38.7933**	0.244247**	0.63704**	228.682**
Days under 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:** Genotype by days under stress interaction, **E:** Residual (Error), **CV (%):** Coefficient of variation, **Chl*a*:** Chlorophyll a, **Chl*b*:** Chlorophyll b, **TChl:** Total chlorophyll, **Chl*a/b*:** The ratio of chlorophyll a to that of b, ***Car*:** Carotenoids, **RWC (%):** Leaf relative water content.

Table 5. 2: Analysis of variance for total chlorophyll, *a*, *b*, Chl*a*/Chl*b*, carotenoids and RWC of transgenic Staha and non transgenic Staha maize genotypes as affected by drought stress

Source of variation	df	Mean squares					
		RWC	**Chl*a***	**Chl*b***	**TChl**	**Chla/Chlb ratio**	***Car***
Genotype (G)	1	2131.48**	10.50588**	196.4512**	297.817**	1.365048**	5.04104**
Days under stress (D)	3	1087.47**	2.72643**	41.2504**	61.184**	0.244550**	1.99792**
GxD	3	344.82**	0.84396**	7.1468**	12.461**	0.064708**	0.05065**
Residual (E)	16	16.74	0.09645	0.8106	1.075	0.003072	0.04433
CV (%)		5.9	1.8	6.4	3.3	4.1	10.6

**Significant differences at 5 % probability level.

Df: Degrees of freedom, **GxD:** Genotype by days under stress interaction, **E:** Residual (Error), **CV (%):** Coefficient of variation, **Chl*a*:** Chlorophyll a, **Chl*b*:** Chlorophyll b, **TChl:** Total chlorophyll, **Chl*a/b*:** The ratio of chlorophyll a to that of b, ***Car*:** Carotenoids, **RWC (%):** Leaf relative water content.

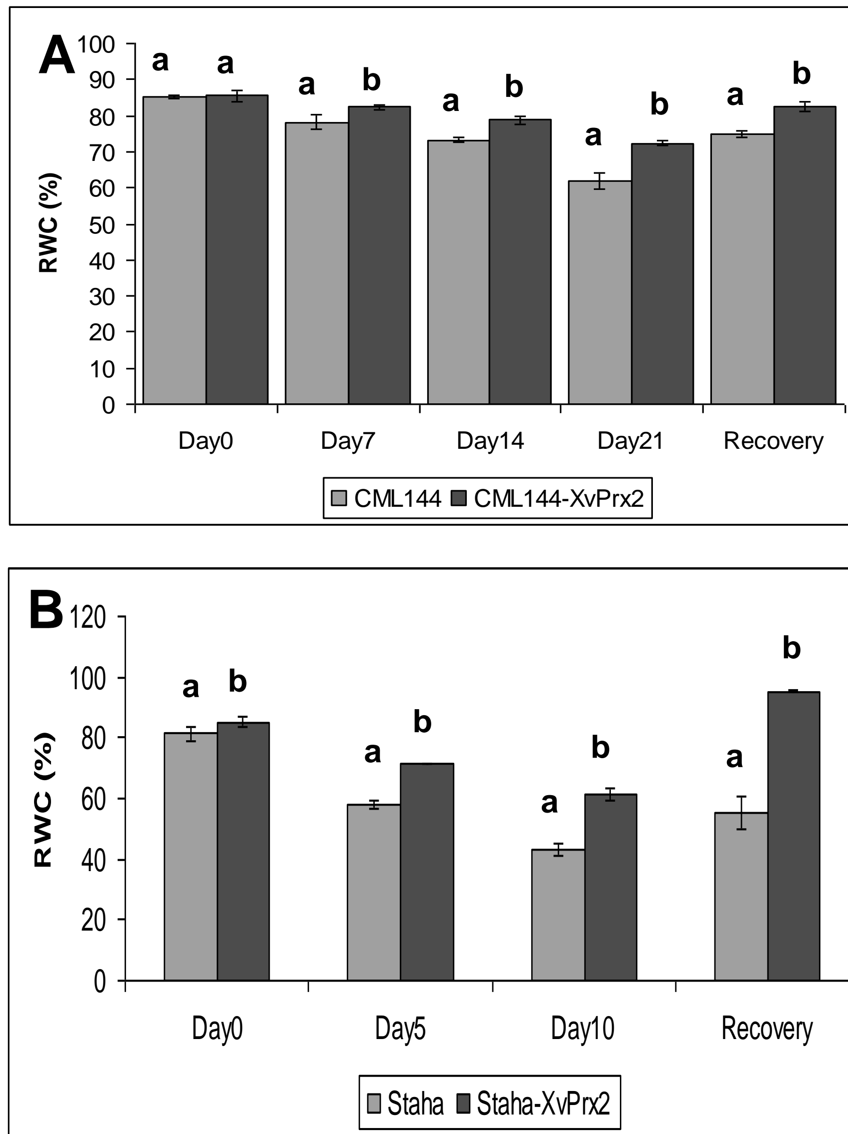

Figure 5.1: Leaf RWC as affected by drought stress and recovery after re-watering in transgenic maize and non transgenic maize plants.
Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at 5 % probability level. **A:** Transgenic (CML144-XvPrx2) and non transgenic CML144, **B:** Transgenic (Staha-XvPrx2) and non transgenic Staha.

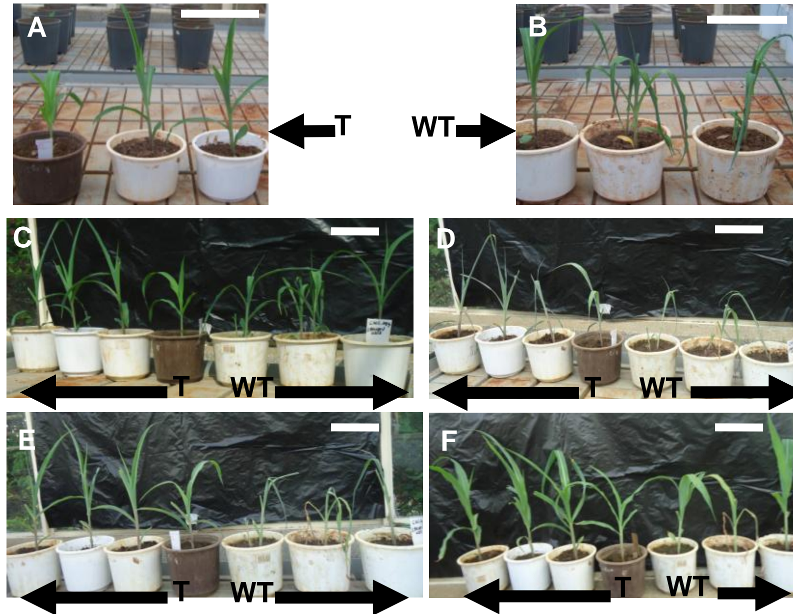

Figure 5.2: Six week old transgenic and non transgenic CML144 maize genotypes under different stages of drought stress.

A: Transgenic maize after 7days of drought stress, *bar*=150 mm, **B:** Non transgenic maize after 7days 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:** Non-transgenic CML144.

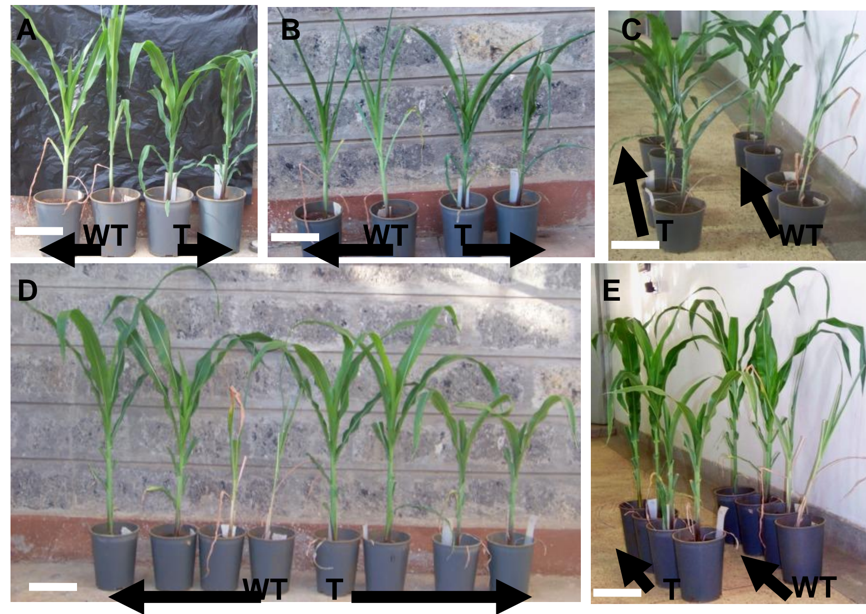

Figure 5.3: Eight week old transgenic and non transgenic Staha maize genotypes under different stages of drought stress.

A: Transgenic and non transgenic Staha maize just before commencement of drought stress, *bar*=170 mm, **B:** Transgenic (T) and non transgenic (WT) 5 days after drought stress, *bar*=170 mm, **C:** Transgenic and non transgenic Staha 10 days after stress just before re-watering, *bar*=170 mm, **D:** Recovery irrigation of transgenic and non transgenic Staha 3 h after re-watering, *bar*=170 mm, **E:** Recovery irrigation of transgenic and non transgenic Staha after 4 days of watering re-watering (Behind are unstressed transgenic and non transgenic (Staha) controls in their respective rows), *bar*=170 mm. **T:** Transgenic (Staha-XvPrx2), **WT:** Non-transgenic Staha.

5.3.2 Effect of drought stress on total chlorophyll content

According to analysis of variance (Table 5.1 and 5.2), total chlorophyll (TChl) content was significantly different between the transgenic and non-transgenic maize genotypes 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 non transgenic maize was significantly different according to LSD at $p<0.05$ in all sampling points. The TChl content for non transgenic CML144 decreased from 39.23 µg/gfw (day 0) to 35.77 µg/gfw (day 7), 28.85 µg/gfw (day 14) and 22.05 µg/gfw (day 21). In transgenic CML144-XvPrx2, the TChl content increased from 35.88 µg/gfw (day 0) to 37.93 µg/gfw (day 7) and thereafter it decreased to 35.65 µg/gfw and 28.89 µg/gfw on day 14 and day 21, respectively (Figure 5.4A). Twenty four hours after re-watering, the total chlorophyll content increased from 22.05 µg/gfw (day 21) to 28.37 µg/gfw in non transgenic CML144 whereas in transgenic CML144, the TChl content decreased from 28.89 (day 21) to 27.29 µg/gfw (Figure 5.4A). For non transgenic Staha, TChl content decreased from 34.16 µg/gfw (day 0) to 25.76 µg/gfw (day 10) whereas in transgenic (Staha-XvPrx2) the TChl decreased from 38.35 µg/gfw (day 0) to 35.87 µg/gfw (day 5) to 33.70 µg/gfw (day 10) (Figure 5.4B). Similarly, TChl content assessed after 24 h of plant recovery in non transgenic Staha increased from 25.76 µg/gfw (day 10 of drought stress) to 26.83 µg/gfw whereas in transgenic (Staha-XvPrx2), the TChl decreased from 33.70 µg/gfw (day 10) to 32.21 µg/gfw (Figure 5.4B).

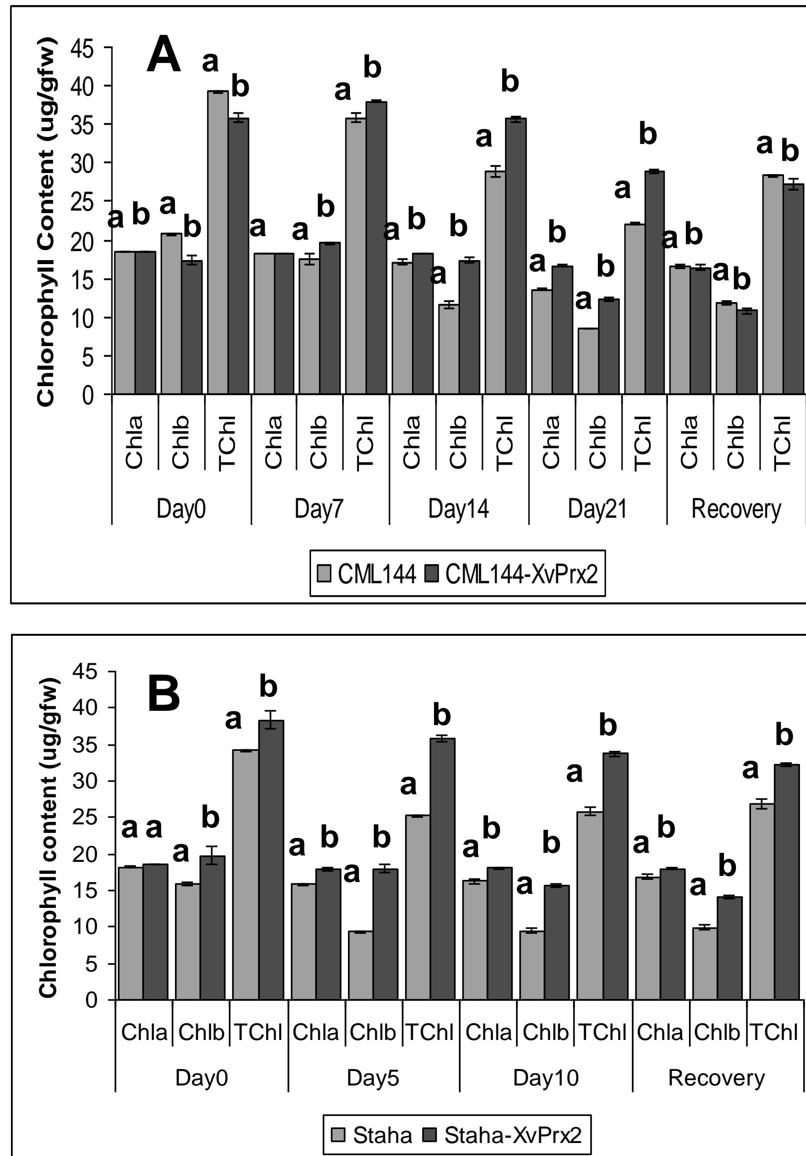

Figure 5.4: Effect of drought stress and recovery re-watering on chlorophyll content.

Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p<0.05$. **A:** Chlorophyll contents in transgenic and non-transgenic CML144, **B:** Chlorophyll contents in transgenic and non transgenic Staha.

5.3.3 Effect of drought stress on chlorophyll a (Chl*a*) content

The Chl*a* 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 5.1 and 5.2). Pairwise comparison of Chl*a* between transgenic and non-transgenic maize also revealed significant difference according to LSD at $p<0.05$ in all sampling points. Under drought stress, Chl*a* content in non transgenic CML144 decreased from 18.49 μg/gfw to 18.26 μg/gfw, 17.15 μg/gfw and 13.57 μg/gfw on day 0, 7, 14 and 21, respectively. In CML144-XvPrx2 plants, Chl*a* content decreased from 18.49 μg/gfw (day 0) to 18.32 μg/gfw, 18.26 μg/gfw and 16.65 μg/gfw on day 7, 14, and 21, respectively (Figure 5.4A). During recovery watering, Chl*a* content increased from 13.57 μg/gfw (day21) to 16.59 μg/gfw after 24 h in non transgenic CML144 whereas in transgenic CML144-XvPrx2 plants, Chl*a* content slightly decreased further from 16.65 μg/gfw (day 21) to 16.45 μg/gfw (Figure 5.4A). In non transgenic Staha, Chl*a* content decreased from 18.23 μg/gfw (day 0) to 15.83 μg/gfw (day 5) then rose to 16.29 μg/gfw (day 21) under drought stress. In transgenic Staha under drought stress, Chl*a* content changed from 18.60 μg/gfw (day 0) to 17.89 μg/gfw and 18.03 μg/gfw on day 5 and day 10 respectively (Figure 5.4B). On recovery watering, Chl*a* levels in non transgenic Staha rose from 16.29 μg/gfw (day 10) to 16.89 μg/gfw whereas in transgenic (Staha-XvPrx2), Chl*a* levels decreased further from 18.03 μg/gfw (day10 of drought stress) to 18.01 μg/gfw in 24 h (Figure 5.4B).

5.3.4 Effect of drought stress on chlorophyll *b* content

Pairwise comparison of chlorophyll *b* (Chl*b*) between transgenic and non transgenic maize also revealed significant difference according to LSD at $p<0.05$ in all sampling points. Chlorophyll *b* content in non transgenic CML144 decreased from 20.74 µg/gfw (day 0) to 17.51 µg/gfw (day 7), 11.70 µg/gfw (day 14) and 8.48 µg/gfw (day 21). For transgenic CML144-XvPrx2 plants, the content of Chl*b* increased from 17.39 µg/gfw (day 0) to 19.61 µg/gfw (day 7), followed by a steady decrease to17.39 and 12.25 µg/gfw on day 14 and 21, respectively (Figure 5.4A). After re-watering, the content of Chl*b* in non transgenic CML144 increased from 8.48 µg/gfw (day 21) to 11.77 µg/gfw in 24 h, whereas in transgenic counterpart, Chl*b* content slightly decreased from 12.25 µg/gfw (day 21) to 10.84 µg/gfw (Figure 5.4A). Similar trend was observed in Staha whereby in non transgenic, Chl*b* levels decreased from 15.93 µg/gfw (day 0) to 9.37 µg/gfw and 9.47 µg/gfw on day 5 and 10, respectively. In transgenic (Staha-XvPrx2), Chl*b* levels decreased from 19.75 µg/gfw (day 0) to 17.98 µg/gfw (day 5) and 15.67 µg/gfw (day 10) (Figure 5.4B). After re-watering, the content of Chl*b* in non transgenic Staha increased from 9.47 µg/gfw (day 10) to 9.93 µg/gfw in 24 h of recovery, whereas in transgenic counterpart, Chl*b* content slightly decreased from 15.67 µg/gfw (day 10) to 14.19 µg/gfw (Figure 5.4B).

5.3.5 Effect of drought stress on chlorophyll (Chl *a/b*) ratio

Chlorophyll (Chl *a/b*) ratios were significantly different ($p<0.001$) between the transgenic and non transgenic maize genotypes and days under drought stress. There was also a significant interaction ($p<0.001$) between genotypes and days under exposure to drought stress (Table 5.1 and 5.2). Pairwise comparison of Chl *a/b* ratio between transgenic and non transgenic maize also revealed significant difference according to LSD at $p<0.05$ in all sampling points. The Chl *a/b* ratios were steadily increasing with prolonged drought stress across the days for non transgenic CML144 and Staha. The transgenic CML144-XvPrx2 and Staha-XvPrx2 maize genotypes also showed relative increase in the Chl *a/b* ratios compared to their non transformed counterparts (Figure 5.5). Upon re-watering, the ratio decreased for the non transgenic CML144 and Staha whereas a slight increase was observed in the transgenic CML144-XvPrx2 and Staha-XvPrx2 plants (Figure 5.5).

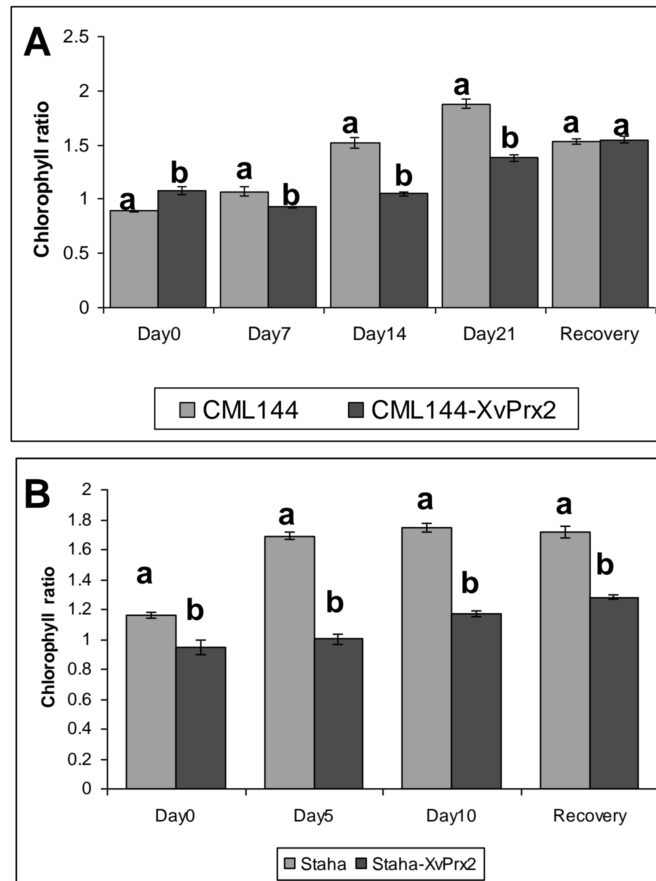

Figure 5.5: Effect of drought stress to chlorophyll (Chl *a/b*) ratio.
Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p<0.05$. **A:** Chlorophyll ratio in transgenic (CML144-XvPrx2) and non transgenic CML144, **B:** Chlorophyll ratio in transgenic (Staha-XvPrx2) and non transgenic Staha.

5.3.6 Effect of drought stress on total carotenoid content.

Carotenoid (*Car*) content was significantly influenced ($p<0.001$) by the interaction between the genotype and the level of imposed drought stress (Table 5.1 and 5.2). Pairwise comparison of total carotenoid content between transgenic and non transgenic maize also revealed significant difference according to LSD at $p<0.05$

in all sampling points. Results indicated that total carotenoids content increased in both transgenic and non transgenic maize genotypes as drought stress was prolonged with non transgenic plants indicating higher total carotenoid content compared to their transgenic counterparts (Figure 5.6). After 24 h following re-watering, total carotenoid content decreased in non transgenic maize plants whereas in transgenic CML144-XvPrx2 and Staha-XvPrx2 maize, the increase was slightly below the non transgenic counterparts (Figure 5.6).

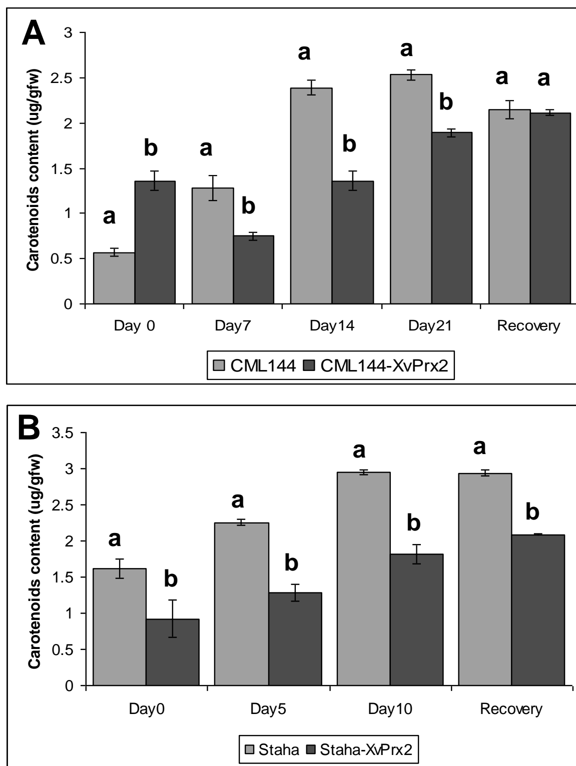

Figure 5.6: Effect of drought stress and recovery after re-watering on carotenoid content.

Bar graphs followed by different letters indicate that their means are statistically different from each other according to LSD at $p<0.05$. **A:** Carotenoid content in transgenic (CML144-XvPrx2) maize and non transgenic CML144, **B:** Carotenoids content in transgenic (Staha-XvPrx2) maize and wnon transgenic Staha.

5.4 DISCUSSION

The effect of drought on maize plant development and productivity is probably the most devastating of all abiotic stresses. One of the effects of drought stress in maize plants is the turnover of reactive oxygen species (ROS). The physiological, molecular and biochemical knowledge of plant responses to drought offers some insight towards successful crop improvement to mitigate the effect of climate changes such as drought and erratic rainfall (Reddy *et al*., 2004). The present study revealed the protective role of peroxiredoxin2 enzyme in transgenic (CML144-XvPrx2 and Staha-XvPrx2) maize compared to non transgenic (CML144 and Staha) maize genotypes under the effect of drought. In this study, some physiological parameters that were affected by drought stress studied to explain the performance of transgenic and non transgenic maize genotype response to drought. The parameters studied were leaf relative water contents, total chlorophylls content, contents of chlorophylls *a* and *b*, chlorophyll *a/b* ratios and total carotenoids contents. These parameters were used as indicators to compare the level of drought stress tolerance in transgenic maize against non-transgenic couterparts.

Drought stress significantly reduced RWC of both transgenic and non-transgenic maize genotypes. However, transgenic CML144-XvPrx2 and Staha-XvPrx2 maize genotypes had higher RWC compared to their non transgenic counterparts at all sampling points. While both genotypes indicated a decrease in RWC under

drought stress, the effect was more pronounced in non transgenic maize plants than in the transgenic plants. High RWC observed in transgenic maize plants under drought stress might have been contributed by rapid build up of peroxiredoxin2 an antioxidant enzyme as a product of *XvPrx2* gene expression. Peroxiredoxin2 might have been involved in ROS scavenging thereby maintaining integrity of the cell membrane by preventing shrinkage of the plasma membrane away from the cell wall and subsequence cytorhesis. The *XvPrx2* gene product may have suppressed oxidative stress caused by the production of reactive oxygen species (ROS) and damage to macromolecules such as DNA and proteins thereby allowing cellular osmotic balance. Similar findings were reported by other researchers in which drought tolerant genotypes exhibiting higher RWC compared to the drought sensitive genotypes in different species such as horsegram (Bhadwaj and Yadav, 2012), *Phaseolus vulgaris* (Turkan *et al.,* 2005), barley (Kocheva and Georgiev, 2003), wheat (Sairam and Srivastava, 2001) and *Vicia faba* (El-Tyeb, 2006). This implied that transgenic CML144-XvPrx2 and Staha-XvPrx2 genotypes in this study were relatively tolerant to drought stress compared to their non-transgenic counterparts.

The percent water loss by non transgenic CML144 and Staha maize after 21 and 10 days of drought stress, respectively, was almost twice the amount lost by transgenic (CML144-XvPrx2 and Staha-XvPrx2) maize genotypes. Transgenic maize maintained high RWC under drought stress for long compared to non

transgenic plants. Reduced RWC in drought stressed plants has previously been reported by other researchers (Fu and Huang, 2001; Shaw *et al.,* 2002) implying that physiological changes observed in the present study could be the result of harmful effect of water deficit on important metabolic processes as well as a defence mechanisms by the plant in responses to drought stress (Talebi *et al.,* 2013).

The fact that transgenic plants absorbed less water than the non transgenic maize plants during 24 h of recovery after one time re-watering gave an indication that the cells of the transgenic plants could stay functional even at lower water content. In addition, transgenic maize plants had quick vegetative recovery compared to the non transformed genotypes. Higher RWC in transgenic maize also delayed the onset of wilting compared to non transgenic maize plants.

Under drought stress stomatal conductance is reduced to allow more water conservation in C4 plants such as maize. This result in reduced CO_2 fixation and consequently the rate of photosynthesis is reduced as noted by Flexas *et al.* (2004). Reduced photosynthesis translates to poor assimilate, plant development and productivity. Chlorophylls are important photosynthetic pigments responsible for trapping solar energy and convert it to chemical energy in higher plants and photosynthetic bacteria. However, under drought stress condition the amount and function of plant chlorophylls are reduced or completelty obstructed.

Inflicting drought stress during vegetative growth substantially decreased the content of TChl, Chl*a* and *b* in both transgenic and non-transgenic plants. This reduction in photosynthetic pigments was brought by oxidative damage caused by RWC reduction in stressed plants. These findings are similar to report by Terzi and Kadioglu, (2006) who noted reduction in photosynthetic pigments while studying drought stress tolerance and antioxidant enzyme system in *Ctenanthe setosa*. However, the reduction of TChl, Chl*a* and Chl*b* was less in transgenic maize compared to non-transgenic maize genotypes. Transgenic maize plants maintained a relatively higher content of chlorophylls compared to the non-transgenic maize plants. The different in chlorophyll turnover between transgenic and no transgenic plants might have been attributed by the reduced degradation or sustained synthesis in transgenic plants. These results complement the reports by Pastori and Trippi (1992) and Zaeifyzadeh and Goliov (2009) who showed that tolerant genotypes of wheat and corn had higher chlorophyll content than sensitive genotypes under drought induced oxidative stress.

Comparing the transgenic and non-transgenic plants in this study, it was clear that the presence of *XvPrx2* gene in transgenic maize had an effect in plant respose to the effect of drought stress. Maintance of high RWC and chlorophyll levels in transgenics compared to non-transgenic plants suggests that *XvPrx2* could have a role in protecting or sustaining synthesis of chlorophylls and enhancement of osmotic adjustment in transgenic plants. While working with *A. thaliana*,

Lamkemeyer *et al.* (2006) reported that the absence or presence of *Prx Q* gene (also belongs to peroxiredoxin gene family), in transgenic plants had an effect on chlorophyll *a* fluorescence parameter suggesting a role in protecting photosynthesis.

Excessive energy absorption in the photosynthetic apparatus act as the main cause of uncoupling of electron transfer leading to excessive generation of ROS and plants tend to avoid this through degradation of chlorophylls (Herbinger *et al.,* 2002). The high chlorophyll content observed in transgenic maize plants under the present study might have been attributed to the presence of peroxiredoxin2 which protects chlorophylls by quenching off ROS and sustaining pigment synthesis.

The increase in Chl *a/b* ratio with increase in drought severity observed in this study suggests that Chl*a* was relatively stable under drought stress than Chl*b*. However, high Chl *a/b* ratios were observed in non transgenic maize compared to transgenic maize. These results are in agreement with Ashraf *et al.* (1994) who reported that drought stress reduces the concentration of chlorophyll *b* more than chlorophyll *a* in wheat plants. Chlorophylls lost during drought stress can be attributed to loss of pigment in the light-harvesting Chl *a/b* protein, which may result in elevated Chl *a/b* ratios and reduced light harvesting and ultimate low rate of photosynthesis (Anjum *et al.,* 2003; Farooq *et al.,* 2009). Higher Chl *b* levels allow light interception in wider wave length bands. Therefore, the transfer of a

large amount of energy to reaction centres is expected. This means that the Chl *a/b* ratio may be used as indicator of plant response to environmental stress such as drought. Therefore, lower Chl *a/b* ratio observed in transgenic maize plants in the present study may indicate better adaptation to drought stress. This is contrary to report by Mafakheri *et al.* (2010) who worked on chickpea a C3 plant and observed no effect on the chlorophyll *a/b* ratio, thereby implying that Chl*b* was not more sensitive to drought than Chl*a*. Often, decrease or unchanged chlorophyll contents may happen as a result of duration of drought stress.

The progressive increase in carotenoids observed in the present study as drought stress intensified may be associated with its role (Talebi *et al.,* 2013). In the present study, carotenoids were higher in non transgenic maize plants (CML144 and Staha) than in transgenic maize (CML144-XvPrx2 and Staha-XvPrx2) under drought stress. Carotenoid forms part of pigments of photosynthesis process by playing a central role of absorbing excessive light energy that chlorophyll cannot absorb and mobilize it to PSII. Carotenoids have also been implicated in protecting cells by scavenging oxygen radicals (Develin and Withman, 2002). Increase in carotenoids under drought stress may imply protection of cell membrane and its components such as lipoprotein and glycoproteins from degradation of chlorophylls. ROS produced under drought stress cause lipid peroxidation which affects cell membrane thereby destroying the osmotic balance of the cell (Sairam *et al.,* 1998). Therefore, high carotenoids content in non transgenic CML144 and

Staha maize under drought stress observed in this study may be associated with its role of protecting cell membrane by quenching off ROS and thus in transgenic plants, the role of carotenoids could have been taken over by the antioxidant enzyme peroxiredoxin2 as a product of introduced *XvPrx2* gene.

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 GENERAL DISCUSSION

Since the first transgenic crop was developed in the early 1983 in United States of America by four groups lead by Mary-Dell Clinton (Washington University group), Jeff Schell and Marc Van Montagu (Ghent, Belgium), Robert Fraley, Stephen Rogers and Robert Horsch (at Monsanto), and John Kemp and Timothy Hall (Wisconsin group), several agronomically important genes have been identified, characterized, cloned and introduced into crops (Shinozaki and Yamaguchi-Shinozaki, 2003; Yamaguchi and Blumwald 2005). Some of these genes are those responsible for abiotic (drought, heat, cold and salinity), biotic (herbicides, pathogenic and pests) resistance, value addition such as nutrients and yield components and these have been used to improve crop plants through genetic transformation. The predicted changes in climate, environmental degradation that worsen drought, increased soil salinity and elevated temperatures remain the current and future challenges in food production needed to support the ever increasing population.

The current and past contribution of conventional plant breeding can not be underestimated. However, the method is arguably slow, time consuming, labour

intensive, expensive and only allows access of genes that are from close and compatible plant species. With the current biotechnology tools such as tissue culture, marker-assisted selection (MAS) or molecular breeding and more recently, genetic engineering or genetic transformation, have enabled the incorporation of various genes into crop plants which could not have been possible through conventional breeding.

The main objective in this study was to assess the regeneration ability of selected tropical maize genotypes and genetically engineer the most regenerable ones to enhance drought stress adaptability. Open pollinated maize varieties namely Situka M-1, Staha and Kito were found to be the most responsive varieties, whereas for inbred lines, CML395 was the most regenerable genotype under all 2,4-D concentration levels tested. This was closely followed by CML443 at lower concentration of 1 mg/l 2,4-D. Although Kito was the most responsive genotype, a large number of somatic embryos from this genotype aborted in maturation medium. However, somatic embryo abortion in Kito could partly be avoided by bypassing the maturation stage. In this study Staha and CML144 maize genotypes were selected and used in the transformation experiments to enhance drought stress tolerance. Development of transgenic Staha and CML144 maize (Staha-XvPrx2, CML144-XvPrx2 and CML144-XVSAP1) in the present study was successfully achieved. These transgenic plants were subjected to preliminary indicative drought stress studies under containment in the glasshouse to compare

their performance with the non transgenic maize counterparts. The results were promissing.

In nature, plants are affected by multiple stresses at the same time. In the present study transgenic maize plants were stressed at temperatures of 43±2ºC and relative humidity of 55 - 60% by denying water. This temperature is very high compared to normal temperatures in sub-Saharan Africa. The transgenic maize were tolerant to such stresses and were able to remain green and physiologically functional for longer periods of drought stress compared to the non transgenic maize under similar conditions. The RWC in transgenic maize plants remained higher than those for non transgenic maize counterparts in all sampling stages. Chlorophyll levels and their ratio under drought stress were higher in transgenic maize than in non-transgenic maize plants. This suggests that chlorophylls were not hugely affected in transgenic maize plants compared to those for non-transgenic maize. Higher carotenoids contents observed under drought stress in non-transgenic CML144 and Staha maize compared to transgenic maize plants implies that carotenoid contents were elevated to protect the cell membrane and its components to counteract the effect of drought stress. On the other hand, the role of carotenoids in protecting cell membrane by scavenging ROS in transgenic plants, could have been taken over by the peroxiredoxin2 an antioxidant enzyme expressed by the introduced *XvPrx2* gene in transgenic maize plants, hence the low turnover of carotenoids observed. The recovery processes was also observed to be rapid in

transgenic maize whereas in non transgenic maize plants, the recovery was slow with occasional death of plants. Confined field trials (CFT) coupled with other detailed analysis is therefore required to further validate these findings.

Previous works on confined filed trials of many transgenic lines have persuasively indicated that there are no yield penalties or any negative consequences of the transgene integration and expression (Vasil, 2007). Furthermore, apart from the useful traits acquired, the transgenic lines have shown no difference from their non transgenic counterparts (Vasil, 2007). Therefore it is important that the developed transgenic versions of Staha and CML144 be tested for their stability and other agronomic characteristics under field conditions.

Going forward on the commercialization of the developed transgenic drought tolerant Staha and CML144 maize, it is arguably important for the regulatory authorities in the ECA regions to approval the confined field testing of these products. Although Kenya is ahead in the region regarding the eshtablishment of biosafety bill, the current ongoing discussions in Uganda, Ethiopia and Tanzania are certainly in the right direction. As soon as these biosafety frame works are in place, it is anticipated that the approvals for confined field trials and subsequent incorporation of these germplasms in the breeding programs will be rapid thus leading to rapid commercialization..

6.2 CONCLUSIONS

Efficient *in vitro* procedures for calli initiation, maintenance and regeneration of four commercially important Tanzanian open pollinated varieties of maize and CIMMYT inbred lines were developed. For open pollinated maize varieties, Situka M-1, Staha and Kito were found to be the most responsive varieties, whereas for inbred lines, CML395 was the most regenerable genotype under all 2,4-D concentration levels tested followed by CML443 at lower concentration of 1 mg/l 2,4-D. Although Kito was the most responsive genotype, a large number of somatic embryos from this genotype were aborted in maturation medium. However, somatic embryo abortion in Kito could partly be avoided by bypassing the maturation stage. TMV-1 was the least responsive in terms of calli induction and embryogenic calli formation among the OPVs although it demonstrated a relatively higher regeneration frequency than Kito and Situka M-1. This work represents the first report of *in vitro* regeneration of Tanzanian maize varieties and thus will act as a basis for future *in vitro* improvement of Tanzanian open pollinated maize varieties through genetic transformation, recovery of somaclonal variants, haploidisation, somatic hybridization and micropropagation.

Stable gene integration in transformed CML144 (QPM) and Staha maize was successful achieved. The use of PMI selection system has currently become an attractive opportunity for recovery of safe commercially important transgenic

crops. This has given a road map towards crop improvement of complex traits like drought tolerance through genetic engineering approach.

Over-expression of *XvPrx2* gene under drought stress has been shown to confer relative tolerance in transgenic CML144 and Staha maize compared to the non-transformed (CML144 and Staha) maize under similar drought stress conditions. Higher RWC, Chl *a*, *b*, *a/b* ratios observed in transgenic maize compared to the non-transgenic CML144 and Staha maize plants are as a results of the presence of *XvPrx2* gene transcripts that manages the ROS under drought stress. *X. viscosa* peroxiredoxin2 gene together with its native promoter has shown to work efficiently in maize. These results show that the potential of genetic engineering may offer a powerful tool in crop improvement for recalcitrant traits like drought stress tolerance. This represents the first report on fertile transgenic drought stress tolerant tropical maize ever developed in Eastern and Central Africa by local scientists of the region using locally adapted germplasms.

6.3 RECOMMENDATIONS AND SUGGESTIONS FOR FURTHER STUDIES

This study resulted in the following recommendations and opportunity for future research:

- Controlled glasshouse drought stress assays should be done to verify the physiological, morphological and agronomic performance of maize transformed with *XVSAP1* gene against the non transgenic maize genotype.
- For successful commercialization of the developed transgenic CML144 and Staha maize events, a confined field trial is required to validate the performance of transgenic under drought condition against their non transgenic maize counterparts.
- Agronomic data should be generated and compared between the transgenic maize events and non-transgenic maize under confined field condition.
- The results of this study are largely accessible to scholars in peer reviewed journals and abstracts yet the ultimate consumer of the resulting transgenics remain oblivious of these progress. As such, a deliberate interaction with all stakeholders should be conducted to enhance acceptability.

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APPENDICES

Appendix I

Media composition and supplements for tissue culture and regeneration

Macro-and micro-nutrients used to prepare the transformation media

LS Macronutrients	mg/l	10X stock	Working solution	
			1L	500ML
NH_4NO_3	1650	16.5 g	100 ml	50 ml
KNO_3	1900	19.0 g	100 ml	50 ml
$CaCl_2$. $2H_2O$	332.02	3.32 g	100 ml	50 ml
$MgSO_4.7H_2O$	180.54	1.8 g	100 ml	50 ml
KH_2PO_4	170	1.7 g	100 ml	50 ml
MS Micronutrients	**mg/l**	**1000X stock**		
$CoCl_2.6H_2O$	0.025	0.025 g	1 ml	0.5 ml
$CuSO_4.5H_2O$	0.025	0.025 g	1 ml	0.5 ml
FeNaEDTA*	**36.7**	**100X**	**10 ml**	**5 ml**
H_3BO_3	6.2	6.200 g	1 ml	0.5 ml
KI	0.83	0.830 g	1 ml	0.5 ml
$MnSO_4.H_2O$	16.9	16.900 g	1 ml	0.5 ml
$Na_2MoO_4.2H_2O$	0.25	0.250 g	1 ml	0.5 ml
$ZnSO_4.7H_2O$	8.6	8.600 g	1 ml	0.5 ml

*FeNaEDTA was prepared separately

Modified LS vitamins

LS vitamins*	mg/l	X1000	1000 ml Medium	500 ml Medium
Nicotinic acid	0.5	0.500 g	1 ml	0.5 ml
B6(Pyrodoxine)	0.5	0.500 g	1 ml	0.5 ml
B1(Thiamine)	1	1.000 g	1 ml	0.5 ml
Myo-inositol	100	100.00 g	1 ml	0.5 ml

*Vitamins were filter sterilize and store at -20 ºC until needed

Phytohormones

Hormone	Stock	W. Conc	Volume required used per volume of medium				
	(mg/ml)	(mg/l)	100 ml	200ml	250ml	500ml	1000ml
2, 4-D	2	2	100 µl	200 µl	250 µl	500 µl	1000 µl
NAA	1	1	100 µl	200 µl	250 µl	500 µl	1000 µl
Kinetin	0.5	0.5	100 µl	200 µl	250 µl	500 µl	1000 µl

Antibiotics

Antibiotics	Stock	W.Conc	Volume required used per volume of medium							
	(mg/ml)	(mg/l)	10 ml	20 ml	50 ml	100 ml	200 ml	250 ml	500 ml	1000 ml
Spectinomycin	100	100	10 µl	20 µl	50 µl	100 µl	200 µl	250 µl	500 µl	1000 µl
Streptomycin	100	100	10 µl	20 µl	50 µl	100 µl	200 µl	250 µl	500 µl	1000 µl
Kanamycin	50	50	10 µl	20 µl	50 µl	100 µl	200 µl	250 µl	500 µl	1000 µl
Chloranmphenical	30	30	10 µl	20 µl	50 µl	100 µl	200 µl	250 µl	500 µl	1000 µl
Ampicillin	100	100	10 µl	20 µl	50 µl	100 µl	200 µl	250 µl	500 µl	1000 µl
Carbenicilin	250	250	10 µl	20 µl	50 µl	100 µl	200 µl	250 µl	500 µl	1000 µl

All antibiotics and hormones were prepared using appropriate solvents (1N NaOH for NAA; 2,4-D and kinetin, absolute ethanol for chloramphenicol, and water for the rest of antibiotics) and made to volume using sterile distilled water (sdH2O) and then filter sterilised using 0.22 micropore filters under sterile laminar flow cabinet then stored at -20ºC

1N Sodium hydroxide (NaOH)

A stock solution of 1 N NaOH was prepared by dissolving 4.0 g of NaOH in 100 ml of sterile distilled water.

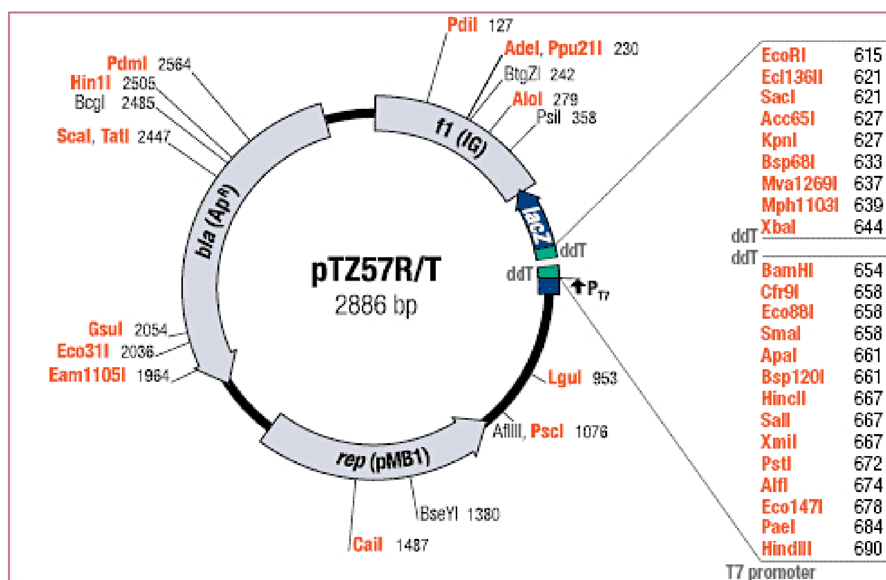
Media for callus induction, maintenance and regeneration

Media	Composition*
CIM	4.43 g/l MS premix, 3 % sucrose, 2.9 g/l proline, 0.1 g/l casein hydrolysate, 10 mg/l $AgNO_3$, 0.8 % agar, supplemented with 1, 1.5, 2 or 2.5 mg/l 2, 4-D, pH 5.8
CMM	4.43 g/l MS premix, 3 % sucrose, 2.9 g/l proline, 0.1 g/l casein hydrolysate, 0.8 % agar, supplemented with either 1, 1.5, 2 or 2.5 mg/l 2, 4-D, pH 5.8
EMM	4.43 g/l MS premix, 6 % sucrose, 1 mg/l NAA, and 0.8 % agar, pH 5.8
SM	4.43 g/l MS premix, 3 % sucrose, and 0.8 % agar, pH 5.8
RM	2.2 g/l MS premix, 1.5 % sucrose, and 0.8 % agar, pH 5.8

*All components were mixed and the pH adjusted accordingly before autoclaving for 20 min at 121 ºC and 15 psi. Vitamins were added just before dispensing the media at a temperature of 40-50 ºC.

Appendix II

Vector maps and sequences



M13/pUC sequencing primer (-20), 17-mer (#S0100)
615 EcoRI Ecl136II SacI Acc65I KpnI Bsp68I Mva1269I Mph1103I XbaI Eco32I

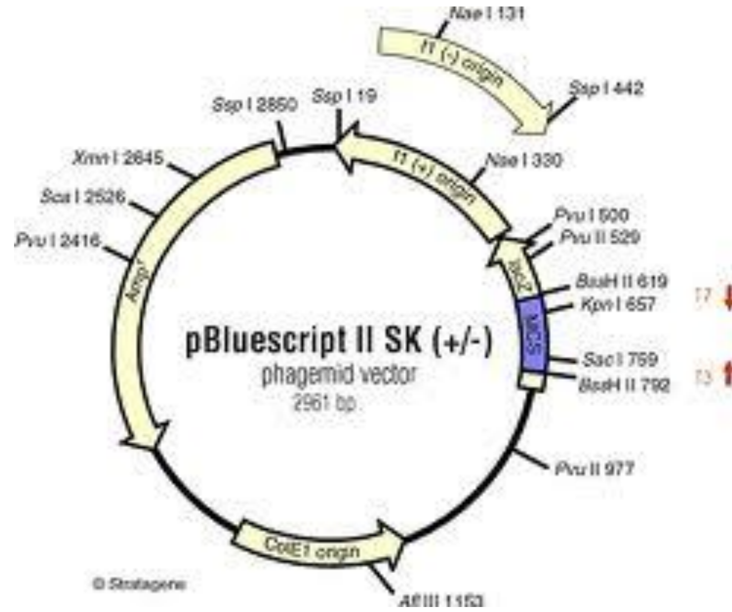
5′ G TAA AAC GAC GGC CAG TGA ATT CGA GCT CGG TAC CTC GCG AAT GCA TCT AGA TAT C
3′ C ATT TTG CTG CCG GTC ACT TAA GCT CGA GCC ATG GAG CGC TTA CGT AGA TCT ATA G
LacZ ← Val Val Ala Leu Ser Asn Ser Ser Pro Val Glu Arg Ile Cys Arg Ser Ile

BamHI Cfr9I Eco88I SmaI ApaI Bsp120I HincII SalI XmiI PstI AlfI Eco147I PaeI HindIII 695

GG ATC CCG GGC CCG TCG ACT GCA GAG GCC TGC ATG CAA GCT TT
CC TAG GGC CCG GGC AGC TGA CGT CTC CGG ACG TAC GTT CGA AA
Pro Asp Arg Ala Arg Arg Ser Cys Leu Gly Ala His Leu Ser Glu

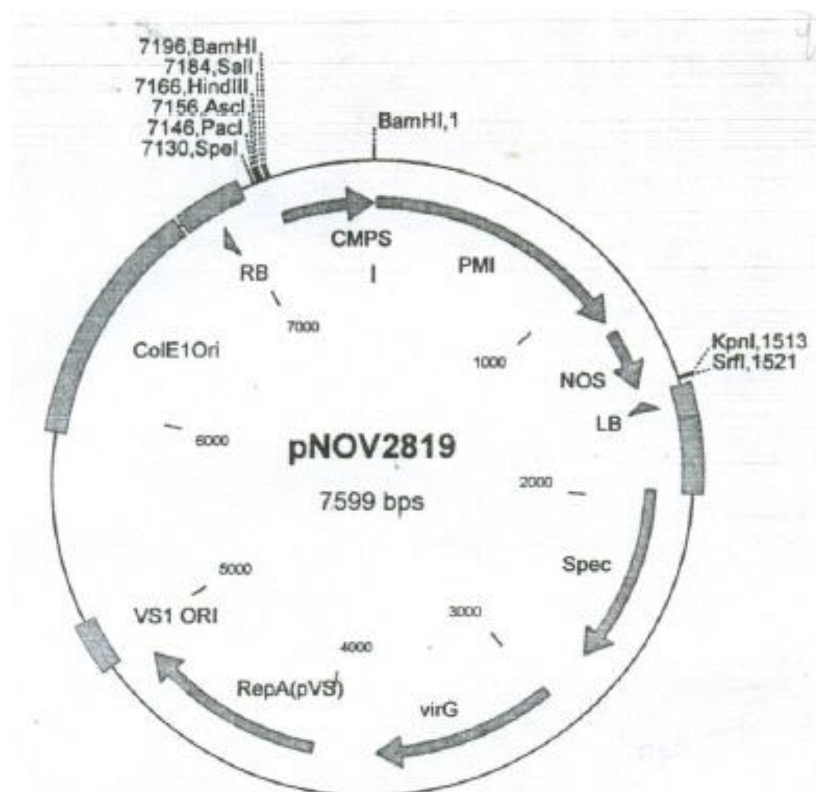
C CCT ATA GTG AGT CGT ATT AGA GCT TGG CGT AAT CAT GGT CAT AGC TGT TTC CTG 3′
G GGA TAT CAC TCA GCA TAA TCT CGA ACC GCA TTA GTA CCA GTA TCG ACA AAG GAC 5′
T7 transcription start T7 promoter M13/pUC reverse sequencing primer (-26), 17-mer (#S0101)
Arg Tyr His Thr Thr Asn Ser Ser Pro Thr Ile Met Thr Met

pTZ57R/T PCR Cloning Vector map and multiple cloning site sequence

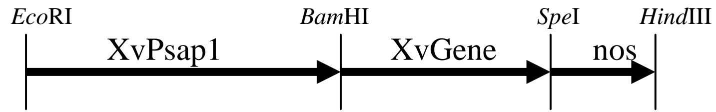


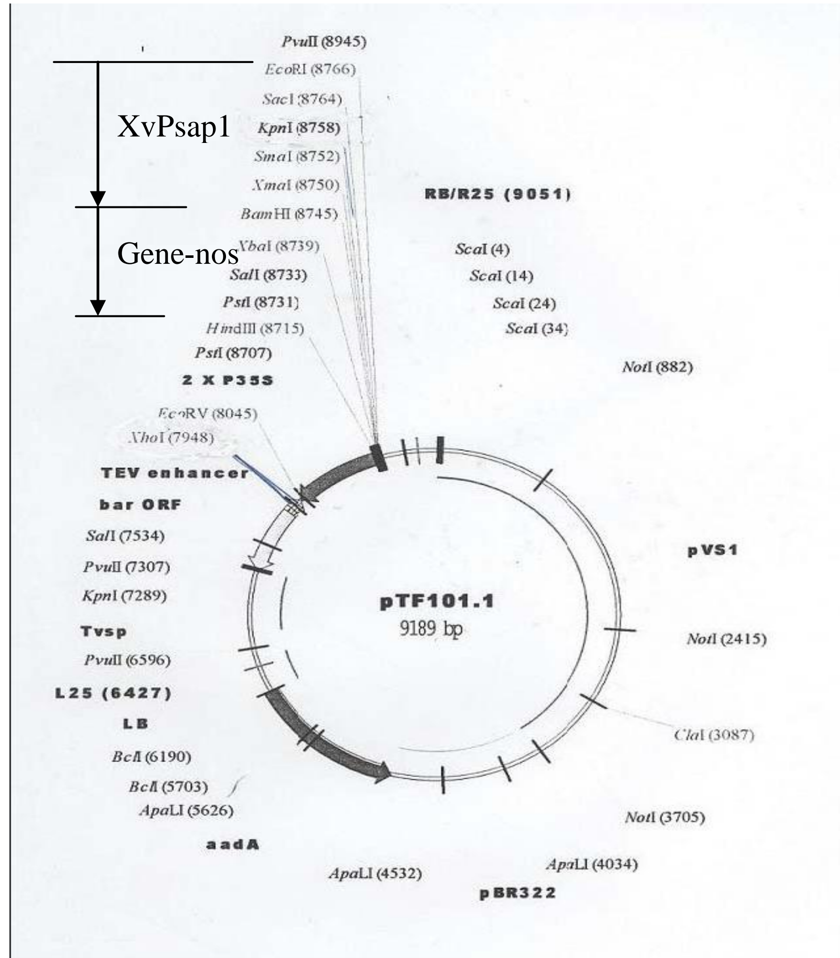

```
M13/pUC sequencing primer (-20), 17-mer (#S0100)      T7 promoter               T7 transcription start
5' G TAA AAC GAC GGC CAG TGA GCG CGC GTA ATA CGA CTC ACT ATA GGG CGA ATT
3' C ATT TTG CTG CCG GTC ACT CGC GCG CAT TAT GCT GAG TGA TAT CCC GCT TAA
LacZ ←   Val Val Ala Leu Ser Ala Arg Tyr Tyr Ser Glu Ser Tyr Pro Ser Asn

        BtgI
        Cfr42I       Eco52I                                   Cfr9I
653 Ecl136II BstXI                                            
    SacI     OliI    NotI      XbaI   BcuI   BamHI   SmaI    PstI   EcoRI
GGA GCT CCA CCG CGG TGG CGG CCG CTC TAG AAC TAG TGG ATC CCC CGG GCT GCA GGA ATT C
CCT CGA GGT GGC GCC ACC GCC GGC GAG ATC TTG ATC ACC TAG GGG GCC CGA CGT CCT TAA G
Ser Ser Trp Arg Pro Pro Pro Arg Glu Leu Val Leu Pro Asp Gly Pro Ser Cys Ser Asn

                            HincII   PspXI   Eco0109I
                            SalI             ApaI      Acc65I
Eco32I  HindIII  Bsu15I     XmiI     XhoI    Bsp120I   KpnI  760
GA TAT CAA GCT TAT CGA TAC CGT CGA CCT CGA GGG GGG CCC GGT ACC CA
CT ATA GTT CGA ATA GCT ATG GCA GCT GGA GCT CCC CCC CGG GCC ATG GGT
Ser Ile Leu Ser Ile Ser Val Thr Ser Arg Ser Pro Pro Gly Pro Val Trp

GCT TTT GTT CCC TTT AGT GAG GGT TAA TTG CGC GCT TGG CGT AAT CAT GGT CAT AGC TGT TTC CTG 3'
CGA AAA CAA GGG AAA TCA CTC CCA ATT AAC GCG CGA ACC GCA TTA GTA CCA GTA TCG ACA AAG GAC 5'
     ←T3 transcription start        T3 promoter                 ←M13/pUC reverse sequencing primer (-26), 17-mer
Ser Lys Asn Gly Lys Thr Leu Thr Leu Glu Ser Ser Pro Thr Ile Met Thr Met
```

pBluescript II SK (+/-) phagemid Cloning Vector map and multiple cloning site sequence

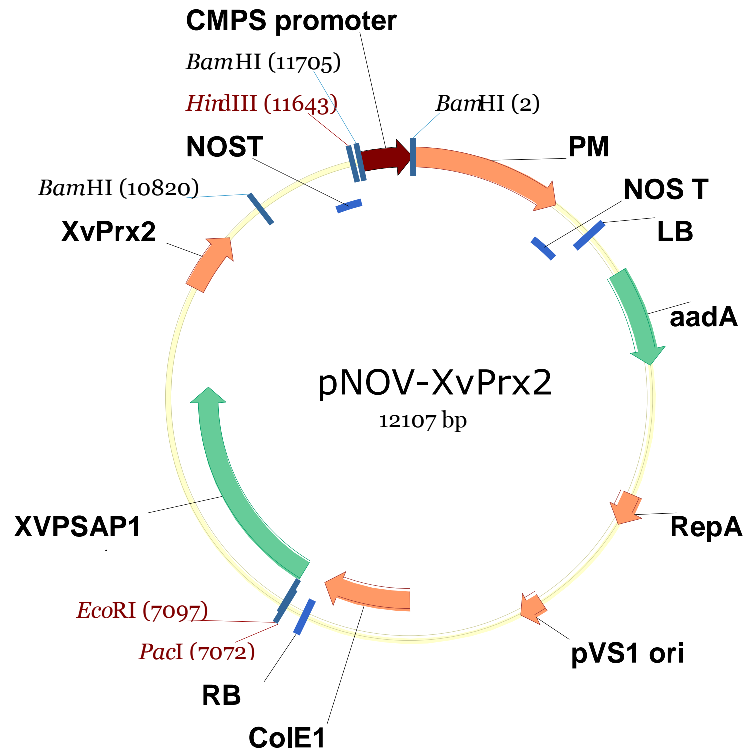


Map of pNOV2819 plant expression vector to which genes were cloned

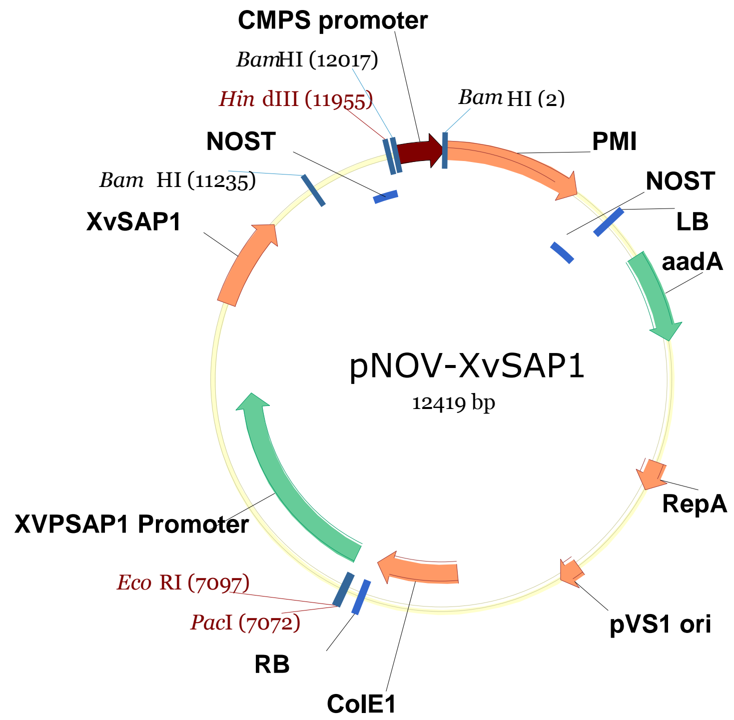




Map of pTF101.1 vector originally carrying the gene cassettes



Final construct harbouring *XvPrx2* gene used for maize transformation



Final construct harbouring *XVSAP1* gene used for maize transformation

Appendix III

Luria-Bertani medium for bacterial growth and maintenance

All components were mixed as in Table and the pH adjusted to 7.0 and autoclaved for 20 min at 121 ºC and 15 psi. The appropriate antibiotic was filter sterilised and added prior to use. For blue-white screening for positive clones, X-gal and IPTG were added to the LB Agar.

LB Media

Components	LB Broth (1L)	LB Agar (1L)
Tryptone	10 g/l	10 g/l
Yeast extract	5 g/l	5 g/l
NaCl	5 g/l	5 g/l
Bacto-Agar	–	15 g
dH_2O	Add up to1L	Add up to 1L

Appendix IV

Media for transformation, selection and regeneration of maize

Medium	Composition
LS-Inf	LS macro- and micro-salts (Linsmaier and Skoog, 1965), modified LS-vitamins, 1.5mg/L 2, 4-D, 1g/L casein hydrolysate, 34.25 g/L Sucrose, 18 g/L glucose, 100µM Acetosyringone (As), pH 5.2
LSAc	LS macro- and micro-salts, modified LS-vitamins, 1.5 mg/L 2, 4-D, 700 mg/L proline, 500 mg/L MES, 100µM As, 30 g/L Sucrose, 10g/L glucose, 8 g/L agar, pH 5.8
RM	LS macro- and micro-salts, modified LS vitamins, 2 mg/L 2, 4-D, 700 mg/L praline, 500mg/L MES, 30g/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, 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, 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, 20 g/L sucrose, 5 g/L mannose, 8 g/L agar, 250 mg/L carbenicillin, pH 5.8

Media supplement preparations

Silver nitrate ($AgNO_3$)

A stock solution of silver nitrate was prepared as 1000 times in volume of 50 ml (10 mg/ml) by dissolving 0.5 g in 50 ml of distilled water. The stock was filter sterilized and kept at 4 ^{o}C in dark. Silver nitrate was used at 10 mg/l in callus induction in regeneration whereas a concentration of 1.6 mg/l was used in resting medium in transformation experiments

100 µM Acetosyringone

A stock of acetosyringone was prepared as 1000 times concentration by dissolving 19.62 g in 1000 ml DMSO. This was filter sterilized using 0.22 micro-pore filters and stored at -20 ºC. Acetosyringone was used at a final concentration of 100 µM per 1000 ml of medium (i.e. 1 ml)

Appendix V

General reagents and standard solutions for molecular analysis

Plant genomic DNA extraction buffers, solutions and reagents

Supplies: 1.4 M NaCL, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% β-mercaptoethanol (Optional), Absolute Isopropanol, Absolute Ethanol, 70% Ethanol, TE Buffer (1mM Tris, 0.1mM, EDTA, and 10mM Tris, 1 mM EDTA pH 8.0 and 3 M Sodium acetate (NaOAc) pH 5.2

Table A.8 DNA extraction buffer[1] (2% CTAB buffer)

Stock	Conc	10 ml	50 ml	100 ml	200 ml	1000 ml
sdH_2O		7.3 ml	36.5 ml	73 ml	146 ml	730 ml
Tris-7.5	1.0 M	1.0 ml	5.0 ml	10.0 ml	20.0 ml	100 ml
NaCl	5.0 M	1.4 ml	7.0 ml	14.0 ml	28.0 ml	140 ml
NaEDTA-8.0	0.5 M	0.2 ml	1.0 ml	2.0 ml	4.0 ml	20 ml
β-ME (μl)[2]		0.1	0.5	1.0	2.0	10
CTAB[3]		0.2 g	1.0 g	2.0 g	4.0 g	20 g

[1]Use freshly made
Add β-ME (β-Mercaptoethanol) to warmed buffer (60-65ºC), just prior to use
CTAB-Cetyltrimethyl-ammoium bromide

0.5 M Di Sodium EDTA (Na_2EDTA) pH 8.0

Dissolve 186.12 g Na2EDTA.2H_2O (Mwt 372.24) (Disodium tetra acetic acid) in approx. 750 ml of dH_2O. Add Sodium hydroxide (NaOH) pellets to bring pH to 8.0. After EDTA is in solution, bring to1000 ml with dH_2O. Autoclave.

5.0 M Sodium Chloride (NaCl)

Dissolve 292.2 g NaCl (Mwt 58.44) in dH2O to a final volume of 1000 ml. Autocalve.

3.0 M Sodium acetate pH 5.2

Dissolve 408.24 g of Sodium acetate in 600 ml dH_2O and adjust pH to 5.2 with glacial acetic acid. Make volume to one litre. Autoclave.

1.0 M Tris-Base 7.5, 8.0

Dissolve 121.2 g Tris-Base in approx. 750 ml dH_2O. Add conc. HCl until desired pH is reached (75 ml HCl-pH 7.5, 49 ml HCl-pH 8.0). Bring solution to 1000 ml with dH_2O.

Preparation of 1.0M Tris Buffer

Tris	Water	HCl to be added (ml)		
		pH 7.5	**pH 8.0**	**Final***
121.2	800	60	42	1000
60.55	300	30	21	500
30.28	150	15	10.5	250
12.11	75	6	4.2	100

*Final volume to be made up with dH_2O, check and adjust the pH before making up the final volume. Sterilize by Autoclaving at 15 psi for 30 min and store at a temperature of 4 ºC.

Chloroform Iso-Amylalcohol 24:1

Mix 240 ml Chloroform with 10 ml iso-amylalcohol and store in refrigerator

10 mg/ml RNAse A

Dissolve 100 mg of pancreatic RNAse A (RNAse A) in 10 mM Tris 7.5, 15 mM NaCl. Heat in boiling waterfor 15 min and allow cooling slowly to room temperature. Dispense in to aliquots and store at -20ºC. Working stocks may be stored at 4 ºC.

Tris EDTA, (TE buffer)

TE buffer for long storage of DNA was prepared as indicated in Table below

Preparation of Tris EDTA (TE buffer)

Stock	Conc.	50 ml	100 ml	500 ml	1000 ml
Tris-8.0*	1.0 M	0.5 ml	1.0 ml	5.0 ml	10.0 ml
Na_2EDTA-8.0	0.5 M	0.1 ml	0.2 ml	1.0 ml	2.0 ml

Adjust the volume using dH_2O. Sterilize by autoclaving and store at 4 ºC
*For preparation of TE buffer of pH 7.5, 8.0 use 1 M Tris solution of pH7.5, 8.0 respectively.

Appendix VI

In vitro regeneration data analysis

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Analysis of variance on the effect of 2,4-D levels on calli induction frequencies of selected tropical maize genotypes

Two-way design- Anova table:

Variate: CIF_%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	6	80931.4	13488.6	89.96	<.001
%2_4_D_Level	3	197.4	65.8	0.44	0.726
Variety.%2_4_D_Level	18	3098.7	172.2	1.15	0.334
Residual	56	8396.8	149.9		
Total	83	92624.3			

Tables of means

Variate: CIF_%

Grand mean 62.7

Variety	CML395	CML443	CML488	KITO	SITUKA M-1	STAHA
	78.8	51.2	0.0	84.4	92.7	88.7

Variety	TMV-1
	43.3

%2_4_D_Level	1.0	1.5	2.0	2.5
	61.9	62.5	65.3	61.3

Variety	%2_4_D_Level	1.0	1.5	2.0	2.5

CML395	93.7	74.7	81.3	65.3
CML443	40.7	60.0	52.0	52.0
CML488	0.0	0.0	0.0	0.0
KITO	73.3	90.7	87.6	86.2
SITUKA M-1	96.0	94.8	89.3	90.7
STAHA	89.3	84.0	92.0	89.3
TMV-1	40.0	33.3	54.7	45.3

Standard errors of means

Table	Variety	%2_4_D_Level	Variety %2_4_D_Level
rep.	12	21	3
d.f.	56	56	56
e.s.e.	3.53	2.67	7.07

Standard errors of differences of means

Table	Variety	%2_4_D_Level	Variety %2_4_D_Level
rep.	12	21	3
d.f.	56	56	56
s.e.d.	5.00	3.78	10.00

Least significant differences of means (5% level)

Table	Variety	%2_4_D_Level	Variety %2_4_D_Level
rep.	12	21	3
d.f.	56	56	56
l.s.d.	10.01	7.57	20.03

Stratum standard errors and coefficients of variation

Variate: CIF_%

d.f.	s.e.	cv%
56	12.25	19.5

Pairwise comparison of means

All pairwise comparisons are tested.

Variance = 149.9428 with 56 degrees of freedom

Tukey's 95 % confidence intervals

Mean	- Mean	Lower	Difference	Upper	significant
SITUKA M-1	STAHA	-11.2502	4.0370	19.3243	No
SITUKA M-1	KITO	-7.0280	8.2593	23.5465	No
SITUKA M-1	CML395	-1.3335	13.9537	29.2409	No
SITUKA M-1	CML443	26.2498	41.5370	56.8243	Yes
SITUKA M-1	TMV-1	34.0831	49.3704	64.6576	Yes
SITUKA M-1	CML488	77.4165	92.7037	107.9909	Yes
STAHA	KITO	-11.0650	4.2222	19.5095	No
STAHA	CML395	-5.3706	9.9167	25.2039	No
STAHA	CML443	22.2128	37.5000	52.7872	Yes
STAHA	TMV-1	30.0461	45.3333	60.6206	Yes
STAHA	CML488	73.3794	88.6667	103.9539	Yes
KITO	CML395	-9.5928	5.6944	20.9817	No
KITO	CML443	17.9905	33.2778	48.5650	Yes
KITO	TMV-1	25.8239	41.1111	56.3983	Yes
KITO	CML488	69.1572	84.4444	99.7317	Yes
CML395	CML443	12.2961	27.5833	42.8706	Yes
CML395	TMV-1	20.1294	35.4167	50.7039	Yes
CML395	CML488	63.4628	78.7500	94.0372	Yes
CML443	TMV-1	-7.4539	7.8333	23.1206	No
CML443	CML488	35.8794	51.1667	66.4539	Yes
TMV-1	CML488	28.0461	43.3333	58.6206	Yes

Identifier	Mean		
SITUKA M-1	92.70	|	
STAHA	88.67	|	
KITO	84.44	|	
CML395	78.75	|	
CML443	51.17		|
TMV-1	43.33		|
CML488	0.00		

Analysis of variance on the effect of 2,4-D levels on embryogenic calli formation frequencies of selected tropical maize genotypes

Two-way design- Anova table:

Variate: ECF_%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Variety	6	36853.5	6142.2	29.12	<.001
%2_4_D_Level	3	1855.1	618.4	2.93	0.041
Variety.%2_4_D_Level	18	11801.7	655.6	3.11	<.001
Residual	56	11812.5	210.9		
Total	83	62322.8			

Tables of means

Variate: ECF_%

Grand mean 33.7

Variety	CML395	CML443	CML488	KITO	SITUKA M-1	STAHA
	38.7	27.2	0.0	69.5	49.8	35.4

Variety	TMV-1
	15.7

%2_4_D_Level	1.0	1.5	2.0	2.5
	39.2	37.6	29.1	29.0

Variety	%2_4_D_Level	1.0	1.5	2.0	2.5
CML395		34.3	41.3	52.4	26.7
CML443		30.0	34.7	25.3	18.7
CML488		0.0	0.0	0.0	0.0
KITO		61.3	64.0	75.3	77.3
SITUKA M-1		85.9	64.9	8.3	40.0
STAHA		52.0	36.0	29.3	24.4
TMV-1		10.7	22.7	13.3	16.0

Standard errors of means

Table	Variety	%2_4_D_Level	Variety %2_4_D_Level
rep.	12	21	3
d.f.	56	56	56
e.s.e.	4.19	3.17	8.39

Standard errors of differences of means

Table	Variety	%2_4_D_Level	Variety %2_4_D_Level
rep.	12	21	3
d.f.	56	56	56
s.e.d.	5.93	4.48	11.86

Least significant differences of means (5% level)

Table	Variety	%2_4_D_Level	Variety %2_4_D_Level
rep.	12	21	3
d.f.	56	56	56
l.s.d.	11.88	8.98	23.76

Stratum standard errors and coefficients of variation

Variate: ECF_%

d.f.	s.e.	cv%
56	14.52	43.0

Pairwise comparison of means

All pairwise comparisons are tested.

Variance = 210.9383 with 56 degrees of freedom

Tukey's 95 % confidence intervals

Mean	- Mean	Lower	Difference	Upper	significant
KITO	SITUKA M-1	1.5895	19.7214	37.8533	Yes
KITO	CML395	12.6827	30.8147	48.9466	Yes
KITO	STAHA	15.9327	34.0647	52.1966	Yes
KITO	CML443	24.1975	42.3294	60.4613	Yes
KITO	TMV-1	35.6975	53.8294	71.9613	Yes
KITO	CML488	51.3641	69.4960	87.6279	Yes
SITUKA M-1	CML395	-7.0387	11.0932	29.2252	No
SITUKA M-1	STAHA	-3.7887	14.3432	32.4752	No
SITUKA M-1	CML443	4.4760	22.6079	40.7399	Yes
SITUKA M-1	TMV-1	15.9760	34.1079	52.2399	Yes
SITUKA M-1	CML488	31.6427	49.7746	67.9065	Yes
CML395	STAHA	-14.8819	3.2500	21.3819	No
CML395	CML443	-6.6172	11.5147	29.6466	No
CML395	TMV-1	4.8828	23.0147	41.1466	Yes
CML395	CML488	20.5495	38.6814	56.8133	Yes

STAHA	CML443	-9.8672	8.2647	26.3966	No
STAHA	TMV-1	1.6328	19.7647	37.8966	Yes
STAHA	CML488	17.2995	35.4314	53.5633	Yes
CML443	TMV-1	-6.6319	11.5000	29.6319	No
CML443	CML488	9.0348	27.1667	45.2986	Yes
TMV-1	CML488	-2.4652	15.6667	33.7986	No

Identifier	Mean				
KITO	69.50				
SITUKA M-1	49.77	|			
CML395	38.68	|	|		
STAHA	35.43	|	|		
CML443	27.17		|	|	
TMV-1	15.67			|	|
CML488	0.00				|

Analysis of variance on *in vitro* regeneration frequencies of selected tropical maize genotypes

Two-way design- Anova table:

Variate: Rf_%

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Variety ignoring %2_4_D_Level	6	149328	24888	1.80	0.115
Variety eliminating %2_4_D_Level	6	149295	24882	1.80	0.115
%2_4_D_Level ignoring Variety	3	81226	27075	1.96	0.130
%2_4_D_Level eliminating Variety	3	81192	27064	1.96	0.130
Variety.%2_4_D_Level	18	264998	14722	1.07	0.407
Residual	55	758672	13794		
Total	82	1254191	15295		

Information summary

Design unbalanced, analysed by GenStat regression

Predictions from regression model

Response variate: RF_%

	Prediction
Variety	
CML395	130.04
CML443	7.83
CML488	0.00
KITO	3.37
SITUKA M-1	12.01

STAHA	35.52
TMV-1	18.73

Approximate effective standard errors

Variety	
CML395	33.91
CML443	33.91
CML488	33.91
KITO	33.91
SITUKA M-1	33.91
STAHA	35.83
TMV-1	33.91

Discrepancy between sed and value calculated from ese's

Maximum discrepancy	0
Maximum % discrepancy	0.00

Minimum standard error of difference	47.96
Average standard error of difference	48.35
Maximum standard error of difference	49.33
Minimum least significant difference	96.11
Average least significant difference	96.90
Maximum least significant difference	98.86

Predictions from regression model

Response variate: RF_%

%2_4_D_Level	Prediction
1.0	81.35
1.5	26.89
2.0	7.09
2.5	2.84

Approximate effective standard errors

%2_4_D_Level	
1.0	25.64
1.5	26.42
2.0	25.64
2.5	25.64

Discrepancy between sed and value calculated from ese's

Maximum discrepancy 0
Maximum % discrepancy 0.00

Minimum standard error of difference 36.26
Average standard error of difference 36.54
Maximum standard error of difference 36.81
Minimum least significant difference 72.67
Average least significant difference 73.22
Maximum least significant difference 73.78

Predictions from regression model

Response variate: RF_%

%2_4_D_Level	Prediction 1.0	1.5	2.0	2.5
Variety				
CML395	413.89	75.00	28.65	0.00
CML443	30.95	0.00	0.00	0.00
CML488	0.00	0.00	0.00	0.00
KITO	11.67	1.75	0.00	0.00
SITUKA M-1	7.78	41.67	0.00	0.00
STAHA	63.69	34.72	22.22	21.43
TMV-1	40.00	35.71	0.00	0.00

Approximate effective standard errors

%2_4_D_Level	1.0	1.5	2.0	2.5
Variety				
CML395	67.81	67.81	67.81	67.81
CML443	67.81	67.81	67.81	67.81
CML488	67.81	67.81	67.81	67.81
KITO	67.81	67.81	67.81	67.81
SITUKA M-1	67.81	67.81	67.81	67.81
STAHA	67.81	83.05	67.81	67.81
TMV-1	67.81	67.81	67.81	67.81

Discrepancy between sed and value calculated from ese's

Maximum discrepancy 0
Maximum % discrepancy 0.00

Minimum standard error of difference 95.90
Average standard error of difference 96.70
Maximum standard error of difference 107.21
Minimum least significant difference 192.2

Average least significant difference 193.8
Maximum least significant difference 214.9

Coefficient of variation and standard error of a single unit

%cv	se
397.45	117.4

Appendix VII

Analysis of data from drought stress experiments

GenStat Release 10.3DE (PC/Windows XP) 15 June 2013 23:07:28
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GenStat Procedure Library Release PL18.2

Data imported from Excel file: C:\Documents and Settings\Miccah\Desktop\CML144-Prx2Chl-RWC data sheet.xls
on: 15-Jun-2013 23:07:43
taken from sheet ""Sheet3"", cells A2:AD31

Analysis of variance on the effect of drought on chlorophyll a content in transgenic and non-transgenic CML144 maize

Two-way design- Anova table:

Variate: Chla_g_gfw

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	5.01982	5.01982	65.90	<.001
Day	4	47.60538	11.90134	156.25	<.001
Genotype.Day	4	11.05085	2.76271	36.27	<.001
Residual	20	1.52338	0.07617		
Total	29	65.19943			

Tables of means

Variate: Chla_g_gfw

Grand mean 17.223

Genotype	CML144	CML144-Prx2
	16.814	17.632

Day	D0	D7	D14	D21	24R
	18.488	18.290	17.704	15.108	16.523

Genotype	Day	D0	D7	D14	D21	24R
CML144		18.491	18.262	17.151	13.570	16.594
CML144-Prx2		18.485	18.319	18.256	16.647	16.451

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
e.s.e.	0.0713	0.1127	0.1593

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
s.e.d.	0.1008	0.1593	0.2253

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
l.s.d.	0.2102	0.3324	0.4701

Stratum standard errors and coefficients of variation

Variate: Chla_g_gfw

d.f.	s.e.	cv%
20	0.2760	1.6

Analysis of variance on the effect of drought on chlorophyll b content in transgenic and non-transgenic CML144 maize

Two-way design- Anova table:

Variate: Chlb_g_gfw

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	15.9036	15.9036	40.54	<.001
Day	4	385.9566	96.4891	245.95	<.001
Genotype.Day	4	78.7787	19.6947	50.20	<.001
Residual	20	7.8463	0.3923		
Total	29	488.4852			

Tables of means

Variate: Chlb_g_gfw

Grand mean 14.77

Genotype	CML144	CML144-Prx2
	14.04	15.50

Day	D0	D7	D14	D21	24R
	19.07	18.56	14.55	10.36	11.31

Genotype	Day	D0	D7	D14	D21	24R
CML144		20.74	17.51	11.70	8.48	11.77
CML144-Prx2		17.39	19.61	17.39	12.25	10.84

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
e.s.e.	0.162	0.256	0.362

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
s.e.d.	0.229	0.362	0.511

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
l.s.d.	0.477	0.754	1.067

Stratum standard errors and coefficients of variation

Variate: Chlb_g_gfw

d.f.	s.e.	cv%
20	0.626	4.2

Analysis of variance on the effect of drought on total chlorophyll content in transgenic and non-transgenic CML144 maize

Two-way design- Anova table:

Variate: TChl_g_gfw

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	38.7933	38.7933	67.35	<.001
Day	4	687.0692	171.7673	298.20	<.001
Genotype.Day	4	126.4697	31.6174	54.89	<.001
Residual	20	11.5203	0.5760		
Total	29	863.8525			

Tables of means

Variate: TChl_g_gfw

Grand mean 31.99

Genotype	CML144	CML144-Prx2
	30.85	33.13

Day	D0	D7	D14	D21	24R
	37.55	36.85	32.25	25.47	27.83

Genotype	Day	24R	D0	D14	D21	D7
CML144		28.37	39.23	28.85	22.05	35.77
CML144-Prx2		27.29	35.88	35.65	28.89	37.93

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
e.s.e.	0.196	0.310	0.438

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
s.e.d.	0.277	0.438	0.620

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
l.s.d.	0.578	0.914	1.293

Stratum standard errors and coefficients of variation

Variate: TChl_g_gfw

d.f.	s.e.	cv%
20	0.759	2.4

Analysis of variance on the effect of drought on chlorophyll a/b ratio in transgenic and non-transgenic CML144 maize

Two-way design- Anova table:

Variate: Chla_Chlb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	0.244247	0.244247	90.85	<.001
Day	4	2.113943	0.528486	196.57	<.001
Genotype.Day	4	0.543751	0.135938	50.56	<.001
Residual	20	0.053771	0.002689		
Total	29	2.955712			

Tables of means

Variate: Chla_Chlb

Grand mean 1.2885

Genotype	CML144	CML144-Prx2
	1.3788	1.1983

Day	D0	D7	D14	D21	24R
	0.9861	1.0006	1.2882	1.6257	1.5420

Genotype	Day	D0	D7	D14	D21	24R
CML144		0.8929	1.0667	1.5237	1.8762	1.5343
CML144-Prx2		1.0794	0.9345	1.0527	1.3753	1.5497

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
e.s.e.	0.01339	0.02117	0.02994

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
s.e.d.	0.01893	0.02994	0.04234

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
l.s.d.	0.03949	0.06245	0.08831

Stratum standard errors and coefficients of variation

Variate: Chla_Chlb

d.f.	s.e.	cv%
20	0.05185	4.0

Analysis of variance on the effect of drought on carotenoid content in transgenic and non-transgenic CML144 maize

Two-way design- Anova table:

Variate: Car_g_gfw

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	0.63704	0.63704	32.22	<.001
Day	4	8.82117	2.20529	111.56	<.001
Genotype.Day	4	2.94028	0.73507	37.18	<.001
Residual	20	0.39537	0.01977		
Total	29	12.79386			

Tables of means

Variate: Car_g_gfw

Grand mean 1.639

Genotype	CML144	CML144-Prx2
	1.785	1.494

Day	D0	D7	D14	D21	24R
	0.962	1.017	1.878	2.211	2.130

Genotype	Day	D0	D7	D14	D21	24R
CML144		0.568	1.283	2.396	2.529	2.151
CML144-Prx2		1.356	0.752	1.360	1.892	2.109

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
e.s.e.	0.0363	0.0574	0.0812

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
s.e.d.	0.0513	0.0812	0.1148

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
l.s.d.	0.1071	0.1693	0.2395

Stratum standard errors and coefficients of variation

Variate: Car_g_gfw

d.f.	s.e.	cv%
20	0.1406	8.6

Analysis of variance on the effect of drought on RWC in transgenic and non-transgenic CML144 maize

Two-way design- Anova table:

Variate: RWC_%

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	228.682	228.682	43.56	<.001
Day	4	1080.787	270.197	51.47	<.001
Genotype.Day	4	83.063	20.766	3.96	0.016
Residual	20	104.995	5.250		
Total	29	1497.527			

Tables of means

Variate: RWC_%

Grand mean 77.49

Genotype	CML144	CML144-Prx2
	74.73	80.25

Day	D0	D7	D14	D21	24R
	85.37	80.27	75.99	67.16	78.66

Genotype	Day	D0	D7	D14	D21	24R
CML144		85.22	78.23	73.25	62.06	74.89
CML144-Prx2		85.52	82.31	78.73	72.26	82.43

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
e.s.e.	0.592	0.935	1.323

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
s.e.d.	0.837	1.323	1.871

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	15	6	3
d.f.	20	20	20
l.s.d.	1.745	2.759	3.902

Stratum standard errors and coefficients of variation

Variate: RWC_%

d.f.	s.e.	cv%
20	2.291	3.0

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Data imported from Excel file: C:\Documents and Settings\Miccah\Desktop\Staha-Prx2 Stress repeated Data for analysis.xls
on: 16-Jun-2013 0:37:19
taken from sheet ""Sheet5"", cells A2:I25

Analysis of variance on the effect of drought on chlorophyll a content in transgenic and non-transgenic Staha maize

Two-way design- Anova table:

Variate: Chla_g_ml

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	10.50588	10.50588	108.92	<.001
Day	3	8.17928	2.72643	28.27	<.001
Genotype.Day	3	2.53189	0.84396	8.75	0.001
Residual	16	1.54328	0.09645		
Total	23	22.76032			

Tables of means

Variate: Chla_g_ml

Grand mean 17.472

Genotype	Staha	Staha-XvPrx2
	16.811	18.134

Day	D0	D5	D10	24hRecovery
	18.416	16.860	17.159	17.455

GenotypeDay	D0	D5	D10	24hR	
Staha		18.231	15.826	16.287	16.899
Staha-XvPrx2		18.600	17.894	18.032	18.011

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
e.s.e.	0.0897	0.1268	0.1793

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
s.e.d.	0.1268	0.1793	0.2536

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
l.s.d.	0.2688	0.3801	0.5376

Stratum standard errors and coefficients of variation

Variate: Chla_g_ml

d.f.	s.e.	cv%
16	0.3106	1.8

Analysis of variance on the effect of drought on chlorophyll b content in transgenic and non-transgenic Staha maize

Two-way design- Anova table:

Variate: Chlb_g_ml

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	196.4512	196.4512	242.36	<.001
Day	3	123.7511	41.2504	50.89	<.001
Genotype.Day	3	21.4404	7.1468	8.82	0.001
Residual	16	12.9691	0.8106		
Total	23	354.6118			

Tables of means

Variate: Chlb_g_ml

Grand mean 14.04

Genotype	Staha	Staha-XvPrx2
	11.17	16.90

Day	D0	D5	D10	24hRecovery
	17.84	13.67	12.57	12.06

Genotype	Day	D0	D5	D10	24hRecovery
Staha		15.93	9.37	9.47	9.93
Staha-XvPrx2		19.75	17.98	15.67	14.19

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
e.s.e.	0.260	0.368	0.520

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
s.e.d.	0.368	0.520	0.735

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
l.s.d.	0.779	1.102	1.558

Stratum standard errors and coefficients of variation

Variate: Chlb_g_ml

d.f.	s.e.	cv%
16	0.900	6.4

Analysis of variance on the effect of drought on total chlorophyll content in transgenic and non-transgenic Staha maize

Two-way design- Anova table:

Variate: TChl

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	297.817	297.817	277.11	<.001
Day	3	183.552	61.184	56.93	<.001
Genotype.Day	3	37.383	12.461	11.59	<.001
Residual	16	17.195	1.075		
Total	23	535.948			

Tables of means

Variate: TChl

Grand mean 31.51

Genotype	Staha	Staha-XvPrx2
	27.99	35.03

Day	D0	D5	D10	24hRecovery
	36.25	30.53	29.73	29.52

Genotype	Day	D0	D5	D10	24hRecovery
Staha		34.16	25.20	25.76	26.83
Staha-XvPrx2		38.35	35.87	33.70	32.21

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
e.s.e.	0.299	0.423	0.599

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
s.e.d.	0.423	0.599	0.846

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
l.s.d.	0.897	1.269	1.794

Stratum standard errors and coefficients of variation

Variate: TChl

d.f.	s.e.	cv%
16	1.037	3.3

Analysis of variance on the effect of drought on chlorophyll a/b ratio in transgenic and non-transgenic Staha maize

Two-way design- Anova table:

Variate: Chla_Chlb

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	1.365048	1.365048	444.35	<.001
Day	3	0.733650	0.244550	79.61	<.001
Genotype.Day	3	0.194124	0.064708	21.06	<.001
Residual	16	0.049153	0.003072		
Total	23	2.341975			

Tables of means

Variate: Chla_Chlb

Grand mean 1.340

Genotype	Staha	Staha-XvPrx2
	1.578	1.101

Day	D0	D5	D10	24hRecovery
	1.053	1.347	1.460	1.500

Genotype	Day	D0	D5	D10	24hRecovery
Staha		1.157	1.692	1.746	1.718
Staha-XvPrx2		0.949	1.001	1.173	1.282

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
e.s.e.	0.0160	0.0226	0.0320

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
s.e.d.	0.0226	0.0320	0.0453

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
l.s.d.	0.0480	0.0678	0.0959

Stratum standard errors and coefficients of variation

Variate: Chla_Chlb

d.f.	s.e.	cv%
16	0.0554	4.1

Analysis of variance on the effect of drought on carotenoid content in transgenic and non-transgenic Staha maize

Two-way design- Anova table:

Variate: Car_g_ml

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	5.04104	5.04104	113.71	<.001
Day	3	5.99376	1.99792	45.07	<.001
Genotype.Day	3	0.15194	0.05065	1.14	0.362
Residual	16	0.70933	0.04433		
Total	23	11.89607			

Tables of means

Variate: Car_g_ml

Grand mean 1.984

Genotype	Staha	Staha-XvPrx2
	2.442	1.526

Day	D0	D5	D10	24hRecovery
	1.269	1.768	2.384	2.514

Genotype	Day	D0	D5	D10	24hRecovery
Staha		1.619	2.258	2.950	2.941
Staha-XvPrx2		0.919	1.279	1.818	2.087

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
e.s.e.	0.0608	0.0860	0.1216

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
s.e.d.	0.0860	0.1216	0.1719

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
l.s.d.	0.1822	0.2577	0.3644

Stratum standard errors and coefficients of variation

Variate: Car_g_ml

d.f.	s.e.	cv%
16	0.2106	10.6

Analysis of variance on the effect of drought on RWC of Transgenic and non transgenic Staha maize

Two-way design- Anova table:

Variate: RWC

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Genotype	1	2131.48	2131.48	127.36	<.001
Day	3	3262.40	1087.47	64.98	<.001
Genotype.Day	3	1034.45	344.82	20.60	<.001
Residual	16	267.76	16.74		
Total	23	6696.10			

Tables of means

Variate: RWC

Grand mean 68.85

Genotype	Staha	Staha-XvPrx2
	59.42	78.27

Day	D0	D5	D10	24hRecovery
	83.24	64.65	52.20	75.31

Genotype	Day	D0	D5	D10	24hRecovery
Staha		81.25	58.02	43.00	55.43
Staha-XvPrx2		85.23	71.28	61.39	95.18

Standard errors of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
e.s.e.	1.181	1.670	2.362

Standard errors of differences of means

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
s.e.d.	1.670	2.362	3.340

Least significant differences of means (5% level)

Table	Genotype	Day	Genotype Day
rep.	12	6	3
d.f.	16	16	16
l.s.d.	**3.540**	5.007	7.081

Stratum standard errors and coefficients of variation

Variate: RWC

d.f.	s.e.	cv%
16	4.091	**5.9**

LIST OF PUBLICATIONS ACCRUING FROM THIS THESIS

Bedada LT, **Seth MS**, Runo SM, Tefera W, Mugoya C, Massiga CW, Oduor RO, Blumewald E and Machuka J (2013). Genetic Transformation with IPT Gene Enhances Drought Tolerance and Improves Grain Productivity in Tropical Maize (*Zea Mays* L.) Under Water Limited Environment. Paper presented at the second ASARECA General Assembly and Scientific Conference. 9-13 December 2013, Burundi, Bujumbura.

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Seth MS, Bedada LT, Mneney EE, Oduor RO and Machuka J (2012). *In vitro* regeneration of selected commercial Tanzanian open pollinated maize varieties. *African Journal of Biotechnology* **11(22):** 6043-6049. DOI: 10.5897/AJB11.2420.

Bedada LT, **Seth MS,** Runo SM, Tefera W, and Machuka J (2012). Regenerability of elite tropical maize (*Zea mays* L.) inbred lines using immature embryo explants. *African Journal of Biotechnology* **11(3):** 598-605. DOI: 10.5897/AJB11.812.

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10(6): 506-513 ISSN1682-296X/DOI: 10.3923/biotech.2011.

Oduor RO, **Seth MS**, Bedada L, Iyer R, Thomson JA, Maina R and Machuka J (2011). Plant Genetic Engineering is no longer a “Western Technology” in the sub-Saharan Africa. Keystone symposium on Plant abiotic stress tolerance, water and global agriculture held in Colorado, USA (17th - 22nd Jan).