PERFORMANCE AND NUTRITIONAL ASSESSMENT OF TRANSGENIC TROPICAL MAIZE (*Zea mays* L.) HARBOURING ISOPENTENYL TRANSFERASE GENE UNDER DROUGHT STRESS

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DECLARATION

I, Rose Mweru Muruo duly declare that the work presented in this thesis is my original work and has not been presented for a degree or any other award in any other university or institution.

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DEDICATION

This thesis is dedicated to Misiga Onkundi, Rachael Misiga, Efra Misiga and Esther Muruo for their immense support during my studies.

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ABBREVIATIONS AND ACRONYMS

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
AMP	Adenosine monophosphate
Ag^{2+}	Silver ions
APX	Ascorbate peroxidase
ATAF	Arabidopsis Transcription Activator Factor
ASI	Anthesis-Silking Interval
BR	Brassinosteroids
CaMV	Cauliflower Mosaic Virus
CAT	Catalase
CK	Cytokinin
CIMMYT	International Maize and Wheat Improvement Centre
cm	Centimetres
CTAB	Cetyltrimethy Ammonium Bromide
CUC	Cup Shaped Cotyledon
DMAPP	Dimethylallylpyrophosphate
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DREB	Dehydration-responsive element-binding protein
DTMA	Drought Tolerant Maize for Africa
ECA	East and Central Africa
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization
g	grams
GC-MS	Gas Chromatography Mass- Spectroscopy
GLS	Gray Leaf Spot

GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione-to-Glutathione disulfide bridge
h	hours
H_2O_2	Hydrogen peroxide
ha	Hectares
HDAC	Histone deacetylases
iPA	Isopentenyl Adenosine
IPT	Isopentenyl transferase
ISAAA	The International Service for the Acquisition of Agri-biotech Applications
JA	Jasmonic acid
Kg	Kilograms
L	Liters
LEA	Late Embryogenesis Abundant
MARs	Matrix Attachment Regions
MCMV	Maize Chlorotic Mottle Virus
MCP	Methylcyclopropene
MLN	Maize Lethal Necrosis
mRNA	Messenger ribonucleic acid
MSV	Maize Streak Virus
NAC	NAM, ATAF and CUC
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NAM	No Apical Meristem
NPK	Nitrogen, Phosphorus, Potassium
NT	Non-transgenic
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PMI	Phosphomannose Isomerase

PRF2	Profilin-2
PTL	Plant Transformation Lab
QTL	Quantitative Trait Locus
rDNA	Recombinant Deoxyribonucleic acid
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen species
RT-PCR	Reverse Transcription PCR
RWC	Relative Water Content
SA	Salicylic acid
SAGs	Senescence-associated genes
SARK	Senescence-Associated Receptor Kinase
SCMV	Sugarcane Mosaic Virus
SSA	Sub Saharan Africa
TAE	Tris-Acetate-EDTA
TF	Transcription factor
TLB	*Turcicum* leaf blight
UTR	Untranslated region
WFP	World Food Program
YAP	Yes-associated protein

ABSTRACT

Maize productivity is significantly influenced by various abiotic factors key among them drought. Development of crops that are tolerant to drought via genetic engineering is considered the best possible solution to this problem. However, how these genes are inherited as well as whether the traits persist in successive generations of selfing is not yet known. Additionally, the nutritional profile of this transgenic maize has not been characterized. This study sought to use farmer-preferred maize line (CML-144) transgenic maize previously transformed with *isopentenyl transferase* (*IPT*) gene under the regulation of Senescence-Associated Receptor Kinase (SARK) promoter to confer drought tolerance. The objective of the study was to evaluate the agronomic performance and nutritional composition of the pSARK::*IPT* transgenic maize under drought conditions towards possible advancement to contained and confined field trials. The transgenic maize seeds harboring pSARK::IPT and its non-transgenic control were planted in potted soil. Germinated plants (T3) were screened to determine presence of transgene via polymerase chain reaction (PCR). Selfing was done to generate T4 plants, which were also screened for presence of transgene. PCR-positive plants in T4 generation were selected for drought assay. Fifteen transgenic and 15 non-transgenic controls at eight weeks were exposed to drought stress for three weeks by withholding water then re-watered to maturity. Data on growth of stressed and unstressed plants were collected and analyzed using student's t-test at 95% confidence interval in GraphPad Prism statistical software version 8.4.3.683, followed by correlation analysis. The current study reported that pSARK::IPT transformed plants contained the transfer DNA (T-DNA) in T3 and T4 generations. Out of 125 transgenic events screened via PCR, only 45 were positive for the transgene. T4 generation plants exhibited significantly higher relative water content, higher chlorophyll amounts, and elevated antioxidant enzyme activities as well as delayed senescence compared to the non-transformed plants under drought stress. However, there were no significant differences in yield between the transgenic and non-transgenic maize plants under drought stress. Notably, the well-watered transgenic plants had 45% higher more kernels per ear than their non-transgenic counterparts. Moreover, pSARK::IPT maize kernels had 47% and 7.5% higher crude protein and fat content respectively than their non-transgenic control. The study confirms presence of the pSARK::IPT transgene, a key phenomenon for successful transformation. The pSARK::IPT enhances yield even in well-watered conditions was higher. This study recommends further evaluation of these genotypes for their stability over time so that the transgenic plants can be considered for field trials in ecoregions with unreliable rainfall.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Corn (*Zea mays* L.) is a type of grain that belongs to the Poaceae family (Doebley, 2004). It is a fully domesticated form of the wild grass, teosinte, and native to Central America and is a major cereal crop after wheat and rice (Galinat, 1971). In Sub-Saharan Africa (SSA), its dependency as a source of food income and livelihood is over 80% (Sharma and Misra, 2011; Ranum *et al*., 2014; Pardey *et al.*, 2016).

Globally, maize has been estimated to occupy about 168 million hectares, a production of 854 million tones and productivity of about 5,000 kilograms per hectare (Byakod, 2017). Maize plays a crucial role in the SSA and is used by millions of people as food, beverage, animal feeds, cooking fuel and building materials (FAO, 2017). Lesotho is a leading maize consumer in SSA with a yearly per capita of 117 kg and followed closely by East Africa with a consumption rate of around 100 kg/person/year (Awata *et al*., 2019). In Kenya, maize represents at least 51% of all the staple food crops grown. The consumption rate in Kenya is approximately 103 kg/person/year. On the other hand, the per capita consumption is 31 kg, 52kg and 73 kg for Uganda, Ethiopia and Tanzania, respectively (Kirimi *et al*., 2004; Fisher *et al*., 2015). The major maize importers in SSA from 2004 to 2013 were Kenya and Zimbabwe, hence regarded as great maize consumers with production shortages (Daly *et al*., 2017).

Maize productivity has not improved significantly over the years, notwithstanding its critical significance in food security (Ranum *et al*., 2014) . Water is the most limiting resource of agricultural production (FAO, 2008). Maize is third in high water requirement after sugarcane and rice. Frequent water deficit resulting from sporadic rainfall and soils with low water holding capacity causes a substantial drop in maize yield throughout the tropics (Vörösmarty *et al*., 2000). It is anticipated that change in climate will cause notable impacts on water availability for crop production in the coming years. The United Nations predicted that the world population would rise by more than tenfold in 250 years (Roser, 2019). Rapid population growth obstructs efforts to increase income, guard livelihoods and lessen food shortages, predominantly in rural areas where food insecurity is often most startling (Maltsoglou, Irini and Khwaja, 2010). More than 80% of Kenyan land is considered arid and semi-arid (Birch, 2018), thus making the country food insecure.

Crop losses due to drought arise when water scarcity occurs during sensitive phases of crop growth, particularly during flowering and grain filling (Bodner *et al*., 2015; Ihsan *et al*., 2016;Yang *et al*., 2019). Maize is susceptive to drought around the silking period (Byakod, 2017). Maize and wheat are responsible for 75% of drought-related damage to agricultural gross domestic production (Kim *et al*., 2019). Reports on drought-induced crop losses indicate that between 1983 and 2009, maize had the most prominent economic loss of $ 58 billion or about 35% of the global sum compared with rice, wheat and soy (Kim *et al*., 2019). A possible solution to dealing with the drought stress impasse is the development of crops that can utilize minimal water.

Drought tolerance is a plant's ability to exhibit the lowest yield loss in a water-deficient environment compared to the highest yield in an environment with adequate water for crop growth (Basu *et al*., 2016). Providentially, the advancement from open-pollinated hybrids to single cross has progressively corresponded with greater tolerance to stress due to changes in methods of selection. However, crop yield has not improved enough to sustain the existing demand (Tollenaar and Wu, 1999). Crop cultivar development for tolerance to drought has been slow due to low heritability, lack of comprehension of the mechanism of tolerance and efficient techniques for screening germplasm (Mitra, 2001; Keneni *et al*., 2017).

Conventional methods lack precise knowledge of the critical genes involved and are unpredictable. Recombinant DNA technology using *Agrobacterium*-mediated transfer of transgenes has been considered the most competent technique in developing transgenic maize tolerant to water-deficit stress (Miccah *et al*., 2016). Different maize varieties that are drought tolerant have been developed via recombinant DNA technology focusing on various genes that target various biochemical properties in plants. There are various genes involved in drought tolerance that have been extensively studied, including *isopentyl transferase*. This gene encodes *Isopentenyl transferase* enzyme, which catalyzes a fundamental step in the biosynthesis of Cytokinin (CK) (Akiyoshi *et al*., 1984; Barry *et al*., 1984). CKs are important plant hormones that modulate growth and development in plants as well as regulate plant response to water-deficit stress. It has been reported that CK signaling acts as an intercellular communication network that helps mediate plant stress response during drought stress by interacting with other types of phytohormones and their regulating pathways (Hai *et al*., 2020).

Many studies have been done and many are still ongoing on the development of transgenic maize that confers tolerance to drought stress. *Isopentyl transferase* gene driven by different promoters has been used to transform maize (Robson *et al*., 2004; Oneto *et al*., 2016). However, how these traits persist over generations is not yet known. This study focused on transgenic maize generated at the plant transformation laboratory of Kenyatta University expressing *IPT* transgene driven by senescence-associated receptor kinase promoter (SARK). The study sought to ascertain whether transgenes are passed over to subsequent generations, determine whether the transgenic maize shows tolerance to drought as well as determine differences or lack thereof in the nutritional profiles of transgenic and non-transgenic maize.

1.2 Statement of the problem and justification

The primary threat to food security worldwide is climate change and rapid population growth (Lesk *et al*., 2016). The demand for food globally is postulated to double by the year 2050 (Godfray *et al*., 2010; Tilman *et al*., 2011; Doering and Sorensen, 2018). Drought is a key climatic constraint to yield (Mishra and Cherkauer, 2010; Lobell *et al*., 2014; Lesk *et al*., 2016; Zipper *et al*., 2016; Matiu *et al*., 2017). In the past four decades, a combined loss of 1.82 billion tonnes in maize, rice and wheat has been associated with drought stress (Lesk *et al*., 2016). Moreover, in severe drought, the expected yield loss can be over 70% for soybean and maize, while rice and wheat are about 64% and 68%, respectively (Leng and Hall, 2019). Studies published between 1980 and 2015 indicated a global average yield reduction of 21% in wheat (*Triticum aestivum* L.) and 40% in *Zea mays* L as a result of water stress (Daryanto *et al*., 2016).

Cultivars that can tolerate drought would offer a solution to this menace and this can be achieved through genetic engineering or conventional breeding. In cytokinin biosynthesis, the rate-limiting step is catalyzed by Isopentenyl transferase (Zhang *et al*., 2013; Mens *et al*., 2018). Previous work with transgenic maize (Leta *et al*., 2016), cotton (Kuppu *et al*., 2013), tobacco (Rivero *et al*., 2010) and rice (Peleg *et al*., 2011) showed that regulated expression of isopentenyl transferase (IPT) gene under the control of Senescence-Associated Receptor Kinase (SARK) promoter led to improved drought tolerance. SARK is a promoter that was first isolated from *Phaseolus vulgaris* and is induced by senescence and stress (Delatorre *et al*., 2012). The promoter is upregulated during tissue maturation and drought, at the beginning of senescence (Rivero *et al*., 2010).

Tropical maize CML-144 was transformed with the IPT gene, under the regulation of pSARK (Leta *et al*., 2016). The pSARK::IPT maize plants showed delayed senescence and higher yields than the non-transgenic counterparts under drought stress imposed around the flowering period. Southern blot analysis of the transgenic events showed multiple copies of the transgene. Consistent expression of the transgene within the plant genome is a major concern for transgenic crops. For successful transformations, the genes must be inherited in a predictable manner (Schetelig *et al*., 2011).

The effectiveness of the desirable trait ultimately depends on the transgene stability (De Buck *et al*., 2013). Unintended changes in the nutritional profile of transgenic maize are a key concern (Tang *et al*., 2013). This study therefore assessed the inheritance of these transgenes in subsequent generations as well as the nutritional composition of the transgenic maize. This study, therefore, assessed the stability of the transgene in T3 and T4 generations, as well as the persistence of drought tolerance traits in T4. The study also analysed the nutritional profiles of the kernels of T4 generation. This information is important for advancement of germplasms to field trials.

1.4. Hypotheses

i. *Isopentenyl transferase (IPT)* transgene is not present in the subsequent generations of transformed maize.

ii. Transgenic maize harbouring *IPT* transgene is not drought tolerant.

iii. The nutritional profile of *IPT* transgenic maize is not similar to that of the non-transgenic equivalent.

1.5 Objectives of the study

1.5.1 General Objective

To determine the agronomic performance and nutritional profile of transgenic maize harbouring *isopentenyl transferase* gene under drought stress

1.5.2 Specific Objectives

i. To determine the presence of *IPT* transgene in T3 and T4 generations of transformed tropical maize.
ii. To determine the agronomic performance of T4 transgenic maize containing *isopentenyl transferase* gene under drought stress.
iii. To determine the nutritional profile of kernels from transgenic maize harbouring *isopentyl transferase* gene compared to the non-transgenic.

CHAPTER TWO

LITERATURE REVIEW

2.1 Maize production in Sub-Saharan Africa (SSA)

Zea mays L. (maize) belongs to the family Poaceae and is a diploid with 20 chromosomes (n=10). It is purported to have originated from Teosintes (wild grass) nearly 100 centuries ago in Mexico, Guatemala and Honduras (Eichten *et al*., 2011; Schnable, 2015). Maize is adapted to different ecosystems of temperate as well as tropical zones of the globe. Maize production is optimum when soil pH is between 5.5 and 7 and temperatures of 17 to 33 °C (Sánchez *et al*., 2014; Sikalengo, 2019). Its optimum water requirement per season ranges from 450 to 700 mm (Hammad *et al*., 2011; Udom and Kamalu, 2019).

Maize has a global annual production of about 844 million tonnes and an estimated area of 162 million hectares in the world (Production *et al*., 2014). This could be due to its wider adaptability and high production potential (Byakod, 2017). Maize covers an area of about 100 million ha in third world nations and is one of the three predominantly cultivated crops in 60% of those countries (Shiferaw *et al*., 2011). Maize is Kenya's utmost vital crop. It has been reported that at least 2.1 million hectares of land were under maize cultivation between 2011 and 2013. Its yield was about 1622 kg/ha and an average output of about three million tons (DTMA, 2015). However, according to a review on yield increase between the years 1980 and 2013, the average yield in Kenya has seen a slight decrease of roughly 1kg /ha/ year, against the growing numbers of 146 kg, 121 kg, 120kg, 97 kg and 93 kg for South Africa, Mali, Ethiopia, Zambia and Malawi respectively. The average decline for the SSA was estimated at 31 kg/ha/year (DTMA, 2015).

2.2 Economic significance of maize

Globally, maize generates revenue of about $219 billion, making it the third most dominant crop, after rice and wheat. Africa's share in global maize trade was estimated at 20% by revenue and 2 to 4% by volume in 2013 (Daly *et al*., 2017. Maize is mainly used to make animal feed as well as fuel production in first world countries, whereas in emerging nations, it is used as a source of food for domestic consumption. It is an excellent source of dietary fibre, proteins, fats and starch (Bathla *et al*., 2020). More than 80% of the SSA population relies on it for food and revenue (Sharma and Misra, 2011; Ranum *et al*., 2014; Pardey *et al*., 2016; Awata *et al*., 2019). Lesotho and East Africa region are the leading consumers of maize in the entire region of SSA, with a per person average consumption of 117 kg and 100 kg per year, respectively (Awata *et al*., 2019).

In the East and Central African region, the highest amount of maize is consumed in Kenya and Tanzania, with annual per capita consumption of more than 120 kg, which is the highest in Africa and among the highest in the world (Odendo *et al*., 2001). Kenya's trade balance for maize was erratic from 1961 to 1990, when it was mainly a net exporter. However, Kenya has been a net importer of corn since the late 1990s (Daly *et al*., 2017). Between 2000 and 2011, the country imported almost 292,000 tons of maize grain at an estimated cost of nearly US$ 76 million per year. Kenya's net imports peaked in 2009 when the country imported over 1.5 million tons of maize grain worth over $435 million (DTMA, 2015). It is estimated that by 2050, the demand for maize will double in developing countries. By 2025, it is expected to become the most productive crop globally (Hellin *et al*., 2012).

However, in Kenya, agricultural production, especially maize production, has either stayed stable or increased at a relatively modest rate over the decades (Birch, 2018). The number of people in need of food assistance in Kenya rose from 0.7 million to 2.6 million in a span of one year (2018-2019), of whom 2.3 million had an acute shortage of food and 300,000 were experiencing emergency levels (Ofda, 2019). Despite the immense agricultural significance of maize and the extensive research being conducted, its productivity in the region is still lower than the average global yield as a result of multifaceted biotic and abiotic limitations (Awata *et al*., 2019).

2.3 Factors affecting maize production in SSA

Even though maize is a critical staple food crop, Africa has the lowest average output per hectare in the world (Jayne and Ameyaw, 2016). Furthermore, the annual yield increase in Africa has failed to keep up with the rising population (FAO, 2017). The constraints of productivity in sub-Saharan Africa (SSA) include pests, little irrigation capacity, farming systems, diseases, climate change and poor policy systems (Jayne and Ameyaw, 2016). This leads to massive yield decreases of 30-100%, resulting in severe food insecurity and millions of dollars in economic loss dollars (FAO, 2017; Natol *et al*., 2018 ; Awata *et al*., 2019; Raheem *et al*., 2021).

2.3.1 Pests and Diseases

Aphids, fall armyworms, weevils, rodents and mites, are all pests that are particularly detrimental to maize productivity (Mulungu, 2017; Kumar *et al*., 2018). The fall armyworm, reported in West Africa in the year 2016, has been considered the most devastating pest to maize and other grain crops in recent years, and it is rapidly spreading throughout sub-Saharan Africa (SSA) (Prasanna *et al*., 2018). In Africa, key maize diseases include maize chlorotic mottle virus (MCMV), common rust, downy mildew, maize streak virus (MSV), sugarcane mosaic virus (SCMV), *Turcicum* leaf blight (TLB), maize lethal necrosis (MLN), gray leaf spot (GLS) and aflatoxin. These diseases result in substantial losses of yield. The tropical climate encourages pests and pathogens to thrive (Sharma and Misra, 2011; Macauley, 2015).

2.3.2 Weeds

Maize weeds can be grouped into grasses, broadleaf and sedges (Rai *et al*., 2018). *Striga hermonthica* is the most important constraint to maize production, causing more than 50% of the yield loss in SSA (Kanampiu *et al*., 2018). It is prevalent in Western Kenya and causes tremendous yield loss (Atera *et al*., 2013). *Cyperus rotundus* is one of the prevailing sedge weeds in maize (Kumar *et al*., 2015; Swetha *et al*., 2018). Additionally, *Dactyloctenium aegyptium*, *Cynodon dactylon*, *Rottboelli aexaltata, Digitaria sanguinalis* and *Echinochloa spp* have been reported as the most common grass weeds in maize experimental fields. In contrast *Commelina benghalensis*, *Euphorbia geniculata*, *Parthenium hysterophorus*, *Amaranthus viridis*, *Trianthema portulacastrum*, and *Digera arvensis* are among the broadleaved weeds (Swetha *et al*., 2015).

Weeds cause substantial yield losses of 18–85% depending on infestation level, types of weeds and time of occurrence (Rai *et al*., 2018). Maize yield loss caused by weeds ranges from 40 % to 60 %, depending on the type of weed and its intensity in the field (Sunitha *et al*., 2012). Besides, Kumar *et al*. (2015) estimated the loss of maize grain yield due to weeds at 50.3%.

2.3.3 Poor soil fertility and land degradation

Most SSA soils have high erodibility, low soil organic matter, nutrient and moisture retention (Jones *et al*., 2013; Tully *et al*., 2015; Middendorf *et al*., 2017). The magnitude of nutrient mining is vast. Nutrient mining across Africa ranges from 9 kg Nitrogen, Phosphorus, Potassium (NPK)/ha per year in Egypt to 88 kg in Somalia. Nitrogen (N) loss is estimated at 4.1 and 52.3 kg/ha yearly in South Africa and Somalia, respectively, while that of phosphorus is 0 and 9.2 kg/ha per year in arid North Africa and Burundi, respectively. On the other hand, Potassium losses vary at about 6.5 kg/ha and 30.4 kg/ha per year in Algeria and Gabon, respectively. In SSA, total nutrient mining is approximately 7.26 million tonnes of NPK per year. Kenya recorded a loss of 68kg NPK/ha per year in the 2002- 2004 cropping season (Henao and Baanante, 2006). According to Detchinli and Sogbedji (2015), land degradation is mainly caused by increased population, deforestation, soil management problems, climate change, uncertain land ownership systems and types of soil in various ecosystems.

2.3.4 Poor policy

Factors such as insufficient credit, small land size, illiteracy, labor, inadequate resources, political instability, poor land ownership systems, poor infrastructure lead to low yield in yield in SSA (Pardey *et al*., 2016; Deininger *et al*., 2017; Bjornlund *et al*., 2020); Santpoort, 2020). Other studies (Onono *et al*., 2013); Mbithi and Van Huylenbroeck, 2000) have associated decline in maize production to policy shifts (liberalization) and prices for inputs and output.

2.3.5 Climate change

Maize is substantially affected by changes in climate such as flooding, heat stress and sporadic rainfall (Hellin *et al*., 2014; Raza *et al*., 2019). Bender *et al*. (2011) postulated that climate change could directly impact maize quality and yield. The potential global maize production has already been reduced by 3.8% due to the impact of climate change during 1980 and 2008 (Lobell *et al*., 2011), countervailing some yield improvements from breeding as well as other scientific developments. Maize yield reductions of 12% and 20% have been projected in SSA as a result of climate change by 2050 and 2080, respectively

(Tesfaye *et al*., 2015). However, it has been stipulated that climate change might improve maize production, especially in areas where low temperatures prevail (Ewert, 2012).

2.3.5.1 Drought as a constraint to maize production

Drought is a chief abiotic factor that alters growth and development in plants by upsetting their water status (Gao *et al*., 2018). This has a significant impact on all physiological functions in a plant, such as photosynthesis, mineral uptake and respiration (Wang *et al*., 2016). Drought alters the function of stomata impedes respiration, consequently causing overproduction of reactive oxygen species (ROS) and build-up of oxidative stress (Kar, 2011). Moreover, drought impedes leaf expansion, cell division, growth of stem leaf surface and root proliferation (Anjum *et al*., 2011).

Maize is the third most vital grain crop requiring higher amounts of water after paddy and sugarcane (Byakod, 2017). The average losses in yield from a particular drought period globally were reported at 8%, 3% and 7% for wheat, rice and soy and maize, respectively (Kim *et al*., 2019). A study based on superposed epoch analysis revealed a loss of approximately 5% cereal yields between the years 1964 to 2007 (Lesk *et al*., 2016). Approximately 40 % of maize cultivated regions in Africa experience sporadic water stress, with losses in yield estimated at 10–25 % (Fisher *et al*., 2015). Over 80% of the total land in Kenya is under arid and semi-arid agro-ecology (Birch, 2018), thus making these countries food insecure.

Drought tolerance is best defined as the capacity to withstand minimal water content in tissues during dehydration stress (Basu *et al*., 2016). They tend to develop adaptive traits that mainly incorporate the preservation of cell membrane turgor via cellular elasticity and osmotic regulation as well as increase protoplasmic resistance (Basu *et al*., 2016). The progress in developing a cultivar tolerant to dehydration stress has been slow as a result of insufficient knowledge on the mechanism of tolerance and its inheritance as a trait, as well as the lack of efficient techniques for screening germplasm (Mitra, 2001; Keneni *et al*., 2017). Developing crops that are tolerant to drought whose yields are equal to those of susceptible crops grown in a well-watered environment is a possible solution to this problem. Luckily, the advancement from open-pollinated to single cross hybrids has progressively led to better tolerance to stress tolerance owing to modifications in selection techniques. Nevertheless, the current demand on grain yield has not been met (Tollenaar and Wu, 1999).

2.5 Plant response to dehydration

The key approaches used by plants to tolerate dehydration stress are drought avoidance, escape and tolerance (Osmolovskaya *et al*., 2018). The escape mechanism involves plants completing their life cycle before injury occurs. The plants can alter their reproductive and vegetative growth based on the water available, fundamentally via mechanisms involving slow growth in the dry period coupled with the production of a limited number of seeds and flowers. However, during the rainy period, they grow indeterminately, producing numerous seeds (Basu *et al*., 2016). The strategy of drought avoidance seeks to increase water uptake while decreasing its loss through increased rooting, hydraulic conductance, reduced transpiration and radiation absorption. On the other hand, drought tolerance relies on mechanisms such as increased cellular elasticity, antioxidant capacity, osmotic adjustment, tolerance to desiccation and protoplasmic resistance (Osmolovskaya *et al*., 2018), as shown in Figure 2.1.

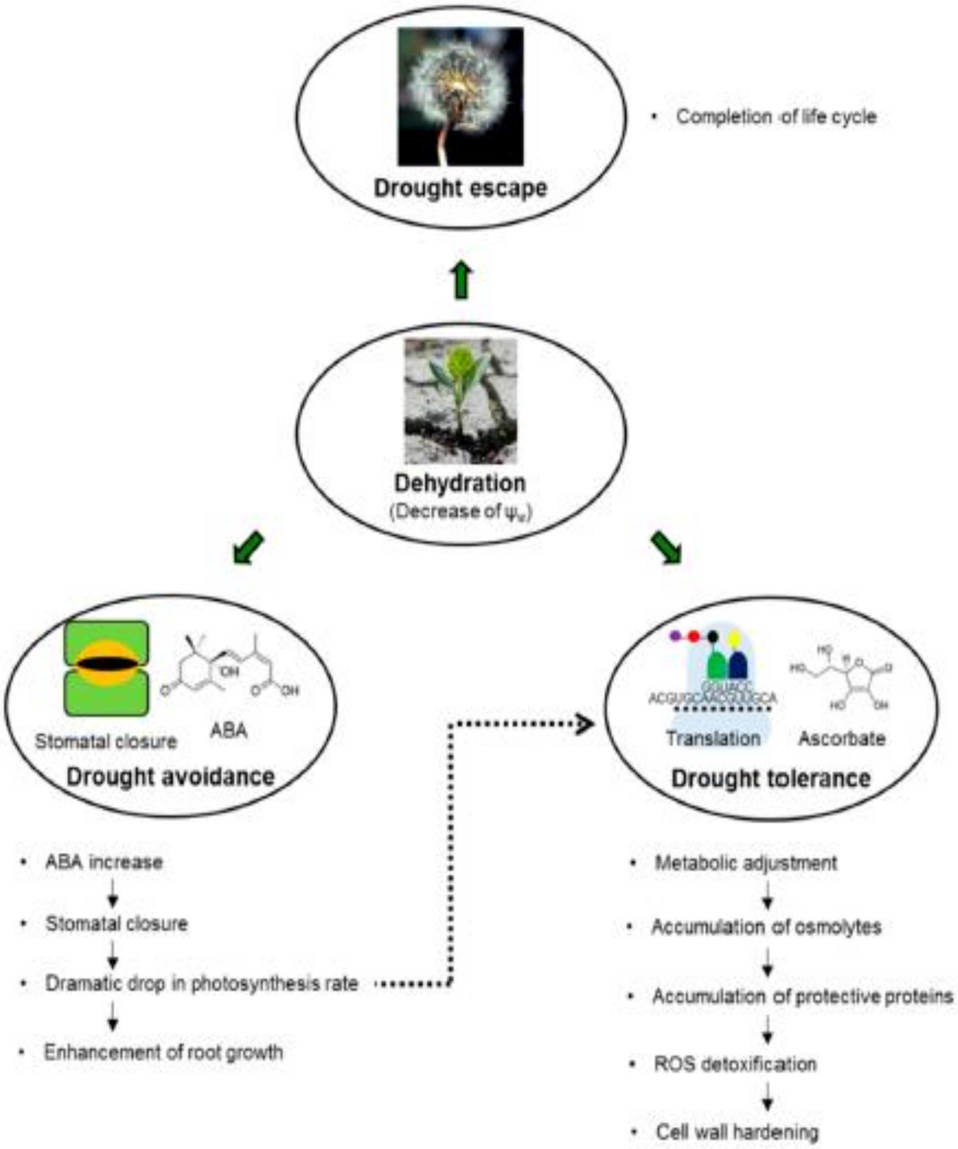

Figure 2.1 Drought resistance strategies in plants

Maize growth stages are classified into seedling growth (VE and V1), vegetative growth (V2, V3... Vn), flowering and fertilization (VT, R0, and R1) and grain filling and maturity (R2 to R6) (Abendroth *et al*., 2011). When water deficit occurs during the vegetative stage, it leads to decreased growth rate along with changes in the distribution of carbohydrates in maize. Short-term drought stress has been reported to cause losses in dry weight of about 30% in the vegetative growth phase and an estimated loss of 66–93% during flowering (Çakir, 2004). During the V9 phase, numerous ear shoots are produced, and kernel rows are set (Abendroth *et al*., 2011). A fast and consistent increase in dry weight and nutrients starts at the V10 stage and progresses into the reproductive phase.

Furthermore, a prolonged water deficit (21 days) that occurs few weeks to flowering has been reported to delay tasselling and silking as well as decrease the average size of certain internodes and leaves. This consequently leads to yield losses of 15 to 25% (Wang *et al*., 2019). The number of kernels per row is determined between the V12 and V15 stage and dehydration stress at this period substantially decreases the kernel number per plant (Aslam *et al*., 2015). When drought stress was imposed for five days around the pollination phase, abnormal embryos were formed and a striking reduction in the number of kernels was evident (Wang *et al*., 2019). Additionally, Setter *et al*. (2001) reported reduced kernel set predominantly in the apical regions of the ear when water deficit was imposed for five days at the phase bracketing pre-pollination and early post-pollination.

Flag leaf in maize has a very substantial impact on biomass accumulation attributable to high photosynthetic rate. According to Liu *et al*. (2015), kernel yield photosynthates are predominantly generated by five to six leaves above and close to the ear. Water deficit leads to a substantial decrease in the rate of photosynthesis, consequently impeding growth by decreasing the size of the "source" in plants (Aslam *et al*., 2015). Leta *et al*. (2001) reported grain yield reduction of 60%, 40% and 15% at grain filling, pollination and vegetative growth stages, respectively, when drought stress was induced at the various stages.

2.6 Markers of drought stress

2.6.1 Water status and photosynthetic parameters

A reduction in leaf relative water content (RWC) is an accurate parameter for evaluating the degree of water loss in a plant (Soltys-Kalina *et al.*, 2016). This can be achieved by calculating the ratio of dry weight to fresh weight (Silva *et al.*, 2011). Notwithstanding its straightforwardness and destructive nature, the method generates data that is reproducible (Tanentzap *et al.*, 2015). A better substitute for this method is an involuntary analysis of short-wave infrared irradiation reflected from the surface of the leaf (Tanentzap *et al.*, 2015).

In addition to the degradation of photosynthetic pigments, drought stress adversely impacts the entire photosynthetic apparatus (López-Jurado *et al.*, 2016). A very reliable indicator of this is reduced activity of photosystem II (PS II) (Chen *et al.*, 2016). The relative content of chlorophyll as well as the efficiency of PS II can be measured with ease using pulse amplitude modulation (PAM) fluorometry (Klughammer and Schreiber, 2008; Rühle *et al.*, 2018). Chlorophyll can also be estimated using spectrophotometry after extraction with different solvents such as acetone (Pérez-Patricio *et al.*, 2018), dimethylsulphoxide (DMSO) (Kumari *et al.*, 2018) and N, N-dimethylformamide (DMF) (Hu *et al.*, 2013).

2.6.2 Phytohormones

Water stress causes cascades of signal transduction to be activated, including those mediated by phytohormones (Daszkowska-Golec and Szarejko, 2013). Stomatal closure is regulated by abscisic acid (ABA) and its signalling pathways (Agurla *et al.*, 2018; Haworth *et al.*, 2018; Bharath *et al.*, 2021). According to a study performed with *Zea mays* L., early ABA build-up as well as activation of related signalling genes correlated with reduced conductivity of stomata as revealed by molecular, physiological and biochemical techniques (Oneto *et al*., 2016).

Ethylene, Auxins and CKs function to impede the closure of stomata triggered by ABA, whereas salicylic acid, brassinosteroids as well as jasmonic acid, work hand in hand with ABA to induce stomata closure (Wilkinson and Davis, 2010). Jasmonic acid, alongside its derivatives, acts synergistically with ABA to initiate ABA signalling (Wang *et al*., 2020). In contrast, ethylene triggers the opening of stomata by impeding nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the leaves, whose role is to activate ROS-dependent pathways (Arraes *et al*., 2015; Salazar *et al*., 2015). Besides, ethylene triggers senescence. Conclusively, the effect of phytohormones on drought is evidentially substantial. Nevertheless, they have a complicated pattern of actions which limit their use as markers for water stress (Daszkowska-Golec and Szarejko, 2013).

2.6.3 Metabolites

Under drought stress, several solutes that are metabolically neutral and osmotically active such as organic acids, various sugars, polyamines, amino acids (mainly glycine and proline) as well as betaine, accumulate in plants (Paudel *et al*., 2016). Stomata closure precedes metabolic adjustment as plants respond to dehydration. Metabolic adjustment functions to maintain physiological functions of a plant as well as its water status during short durations of drought (Krasensky and Jonak, 2012; Bhargava and Sawant, 2013).

The comprehensive profiles of the primary metabolites may be evaluated comprehensively using gas chromatography-mass spectrometry (GC-MS) method, providing relative (Tarakhovskaya *et al*., 2017; Birkemeyer *et al*., 2019) and absolute (Milkovska-Stamenova *et al*., 2015) amounts of individual analytes. Glycine betaine, an osmo-protectant, is specific to plant species like sugar beet (*Beta vulgaris*), sunflower (*Helianthus annuus*), spinach (Spinacia oleracea), wheat (*Triticum* aestivum), pepper (*Capsicum annuum*) and barley (*Hordeum vulgare*) (Yang *et al*., 2021) while increased proline content is the most conserved and characteristic for a wide range of plants (Giri, 2011).

2.6.4 Protective Proteins

During water deficit, synthesis of a wide range of protective proteins, mainly late embryogenesis abundant (LEA) proteins, chaperones and antioxidant enzymes, are upregulated. Chaperones form and maintain the structure of native proteins (Park and Seo, 2015) and are mostly represented by heat shock proteins and polypeptides involved many adaptation responses to stress (Banerjee and Roychoudhury, 2018). Xiang *et al*. (2018) reported that heat shock protein Osnsp50.2 overexpression in rice decreased water loss and improved tolerance of plants to osmotic stress.

LEA proteins represent a class of polypeptides that protect chloroplasts, mitochondria as well other cellular structures from mechanical damage by creating a membrane-protecting shield, hence preventing membrane lipids peroxidation (Chen *et al*., 2019; Ali *et al*., 2020). It has been reported that a greater number of LEA genes are overexpressed in drought-tolerant cultivars of *Gossypium tomentosum* (Hawaiian cotton) than the ones that are drought-sensitive (Magwanga *et al*., 2018).

2.6.5 Oxidative Stress

Destabilizing the balance between detoxification and generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS) elicits oxidative stress. Increased ROS generation in water-limiting environments is well studied and widely reviewed (Ahanger *et al*., 2017; Hasanuzzaman *et al*., 2020). ROS are very reactive, enormously toxic and can mar biomolecules. When the oxidative stress persists, the damage becomes irreversible, resulting in cell death (Sharma *et al*., 2012). Oxidative phosphorylation, which results from reduced nucleotide forms, causes excessive production of ROS in plant cells during dehydration (Sharma *et al*., 2012).

The rate of production of ROS depends on how severe the drought is (Mattos and Moretti, 2016). Consequentially, the compounds that are produced during oxidative stress can act as biochemical markers of drought. ROS production generates lipid hydro-peroxides by breaking the double bonds in polyunsaturated fatty acids (Ayala *et al*., 2014). Consequently, compounds such as malondialdehyde, are produced which become a reliable indicator of lipid peroxidation (Farmer and Mueller, 2013; Huang *et al*., 2019). Moreover, hydrogen peroxide (H_2O_2) concentration in plant tissue can be used to estimate the severity of drought as it is very stable and can be measured with relative ease (Habib *et al*., 2020).

Specific antioxidant enzymes effectively cushion plants against damage by ROS (Osmolovskaya *et al*., 2018). The ascorbate–glutathione pathway consists of antioxidant enzymes that detoxify H_2O_2 when plants suffer dehydration stress (Foyer and Noctor, 2011; Hasanuzzaman *et al*., 2020). Ascorbate peroxidase, the principal antioxidant enzyme, scavenges H_2O_2 in plant cells and utilizes ascorbic acid as a source of electrons (Akram *et al*., 2017). The dehydro-ascorbate produced can be regenerated by NADPH reaction catalyzed by mono-dehydroascorbate reductase (Hasanuzzaman *et al*., 2020). The resulting monodehydroascorbate is toxic and is instantly reduced by dehydroascorbate reductase to ascorbic acid, equivalent to glutathione-to-glutathione disulfide bridge (GSSG) oxidations. Glutathione reductase catalyzes the consequential production of glutathione (GSH) that helps plants survive in a stressed environment (Anjum *et al*., 2010; Hasanuzzaman *et al*., 2020). The proportion of GSH to oxidized ascorbate maintains a favourable redox status of cells, a significant indicator of adaptability of plants to stress (Xiao *et al*., 2021).

2.6.6 Leaf senescence

Leaves provide a photosynthetic environment for the fixation of carbon dioxide for carbohydrate synthesis. Senescence is closely linked to variations in expression profiles of different genes with concomitant deterioration of cells and nutrient recycling (Guo and Gan, 2014). As the plant organs senesce, biomolecules are degraded and the nutrients recycled to actively growing regions (Sade *et al*., 2018). Leaf senescence and abscission during dehydration stress enable plants to reduce the size of their canopy. Perennial plants use this mechanism to survive as it permits them to finish their life cycle in a water-limiting environment. Nonetheless, this decreases the yields causing financial loss to farmers (Gregersen *et al*., 2013; Joshi *et al*., 2019).

It has been reported that when senescence occurs after harvesting vegetable crops as well as ornamental plants, there is degradation of nutrients like vitamin C, which consequently reduces the yield quality (Guo and Gan, 2014). Various biotic and biotic factors such as heat, drought, darkness, nutrient stress as well infections by pathogens can all trigger senescence (Guo and Gan, 2012; Ma *et al*., 2020). Internal factors such as growth regulators/hormones, reproductive growth and aging are also closely linked to senescence (Jibran *et al*., 2013). All these factors can be used to manipulate the progression of senescence.

The surgical removal of inflorescences is an artificial way of delaying senescence. Low temperature is used to increase the shelf life of most fruits and greens by lowering the rate of metabolism (Pott *et al*., 2020). Previous reports denote that senescence was delayed when Nitrogen and Phosphorus were applied to plants (Bieker and Zentgraf, 2013). The plant hormones known to induce senescence include ethylene, jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), auxin and brassinosteroids (BR), whereas polyamines and cytokinins impede senescence (Jibran *et al*., 2013; Wang *et al*., 2020). Ethylene inhibitors such as 1-Methylcyclopropene (1-MCP) and Silver ions (Ag^{2+}) have been used to delay senescence in vegetables, fruits and flowers (Ziv and Fallik, 2021). Genes associated with senescence are control leaf senescence at the molecular level (Wang *et al*., 2019).

2.7 Generation of drought-tolerant maize

Development of climate-resilient germplasm through coupling conventional, molecular and transgenic approaches to breeding is a key requirement of the present day. Molecular breeding approach and phenotyping can be potential and highly efficient techniques for generating germplasm that is well-adapted to future climates (Xu *et al*., 2017). Drought tolerant donors assimilated into the breeding program and unique alleles linked to drought tolerance can yield promising germplasm together with the establishment of maize germplasm that is well adapted to the prevailing climate (Singh *et al*., 2016).

Understanding tolerance to drought at the molecular level in plants is key for a researcher to establish superior genotypes through conventional breeding or biotechnological approaches (Kirui, 2012). Various genes have exhibited a positive response to water-deficit stress in multiple species (Osakabe *et al*., 2014). These genes protect plants from dehydration by secreting vital protein metabolites as well as regulating signal transduction genes against water-deficit stress (Merewitz *et al*., 2016).

2.7.1 Conventional breeding

The International Maize and Wheat Improvement Centre (CIMMYT) introduced tropical maize that is tolerant to drought through genetic improvement in the 1970s (Edmeades *et al*., 2017). Complex biochemical responses to dehydration stress and limited genetic diversity are significant barriers to conventional breeding in improving drought tolerance (Miccah *et al*., 2016). The insertion of Quantitative Trait Loci (QTLs) genomic portions involved in drought tolerance is associated with undesirable characteristics from the donor parents (Bhatnagar-Mathur *et al*., 2008).

2.7.2 Genetic engineering

Tolerance to insect and herbicide in maize were the first major traits addressed through genetic engineering (Raman, 2017). Increasing the level of glycine betaine in maize plants via genetic engineering using *betA* gene isolated from *E. coli* reportedly improved tolerance of maize to drought stress at the seedling phase, consequently improving yields by 10-23% (Leta *et al*., 2016). Maize transformed with mitogen-activated protein kinase (Nicotiana *PK1)* isolated from tobacco showed better tolerance to drought and increased grain weight than non-transgenic plants (Rasha *et al*., 2013).

ZmNF-YB16, an essential member of the NF-YB superfamily as well as that of transcription factor complex, was overexpressed in maize. The transgenic maize increased photosynthetic rate and grain yield under well-watered and water-stressed conditions (Wang *et al*., 2019). Miccah *et al*. (2016) transformed maize using *XvPrx2* and *Xvsap1* genes. *XvPrx2* gene codes for type II peroxiredoxin, which is important in scavenging the reactive oxygen species produced under dehydration stress. On the other hand, the *Xvsap1* gene encodes an integral membrane protein that stabilizes the membrane integrity during dehydration stress. The two genes were extracted from *Xerophyta viscosa*, a plant that can survive in extreme drought conditions. Transgenic plants tolerated drought stress better than their wild counterparts.

The isopentenyl transferase (*IPT*) gene is a bacterial gene isolated from *Agrobacterium tumefaciens*. This gene codes for the *Isopentenyl transferase* enzyme that catalyzes a major step in biosynthesis of cytokinins. It promotes tolerance to drought by boosting cytokinin, which delays leaf senescence induced by drought in transgenic crops. A study to transform tropical maize genotypes with *IPT* gene through *A. tumefaciens*-mediated genetic transformation was designed by Leta *et al*. (2016) to develop tolerance to water-deficit stress. Transformed maize exhibited tolerance to drought by delaying senescence in leaves.

2.7.3. Development of an auto-regulatory cytokinin production system

Cytokinins are phytohormones that regulate most of the stages of plant growth, including germination, flowering and senescence (Hwang *et al*., 2012; Akhtar *et al*., 2020). Kinetin has been shown to delay senescence when applied exogenously on Alfafa (Zwack and Rashotte, 2013). Cytokinins have also been reported to delay senescence in plants transformed using the Isopentenyl transferase gene (*IPT*) (Kant *et al*., 2015; Shan *et al*., 2019).

IPT gene encodes the IPT enzyme that catalyses the condensation of 5′-Adenosine monophosphate (AMP) and dimethylallylpyrophosphate (DMAPP) to isopentenyl adenosine (iPA) 5′-phosphate, a key stage in cytokinin synthesis (Guo and Gan, 2014). *IPT* gene isolated from bacteria has been reported to boost cytokinin concentration in transgenic canola (Kant *et al*., 2015), cauliflower (Nguyen *et al*., 2021), tomato (Merewitz *et al*., 2010), peach (Ricci *et al*., 2020), tobacco (Rivero *et al*., 2010), cucumber (Shivhare *et al*., 2020), *Arabidopsis* (Kuppu *et al*., 2013) and lettuce (Guo and Gan, 2014). However, some undesirable phenotypes such as stunted growth, small leaves, reduced internodes, under-developed root systems, wider stem diameter as well as reduced apical dominance were reported when expression of *IPT* gene was regulated via constitutive promoters, for instance, the *35S* promoter of the cauliflower mosaic virus (CaMV) (Thirukkumaran *et al*. 2009) and the native *IPT* promoter (Guo *et al*. 2010 ; Ma and Liu, 2009). Inducible promoters, as well as those that are tissue-specific, were used to generate transgenic plants with improved productivity and minimal growth defects (Guo and Gan, 2014).

Senescence-associated gene 12 (*SAG12)* encodes a vacuolar cysteine proteinase and was first identified in *Arabidopsis* (James *et al*., 2018; James *et al*., 2019). The promoter was linked to *IPT* to create an auto-regulatory machinery to impede senescence (Wu *et al*., 2021). The promoter triggers *IPT* expression at the beginning of senescence, consequentially increasing cytokinin levels which ultimately delays leaf senescence. (Peleg *et al*., 2011)) reported that transgenic rice carrying pSARK::IPT construct produced higher yields than the non-transgenic when the plants were grown in water-limiting conditions. Furthermore, Qin *et al*. (2011) conducted multi-year field studies and reported a 58% increase in peanut yield when pSARK-*IPT* peanut plants were imposed to drought stress. Delay of leaf senescence using this promoter has been documented in maize (Oneto *et al*., 2016; Leta *et al*., 2016).

2.8 Molecular genetic approaches to senescence.

2.8.1 Transcription factor biology-based technology

Senescence-associated genes (*SAG*s) are activated by binding of transcription factors to specific regulatory sequences, which consequently trigger transcription by RNA polymerase II. Expression of a considerable gene count may be altered if the expression of a major transcription factor gene occurs within a plant during senescence (Guo, 2013). This shows the potential to manipulate transcription factor genes that are senescence-specific to alter the progression of leaf senescence. *AtNAP* is a NAC (No Apical Meristem (NAM), Arabidopsis Transcription Activator Factor (ATAF) and Cut Shaped Cotyledon (CUC) family gene whose expression is senescence specific. The Knockout mutant of the gene delayed senescence in *Arabidopsis* leaves for 10 days (Li *et al*., 2018).

2.8.2 Translation initiation factor biology-based technology

Regulation of plant senescence occurs during translational as well as during post-translational proteins modification. Inhibitors of translation have been shown to impede senescence (Guo and Gan, 2014). Studies on translation initiation factors reveal that *eIF-5A* is implicated in programmed cell death that typifies leaf senescence and softening of fruits after harvesting (Xu *et al*., 2011; Wang *et al*., 2012). It is postulated that the *eIF-5A* initiation factor transports mRNAs associated with senescence for translation in the cytoplasm (Thompson *et al*., 2004).

2.8.3 Hormone biology-based technology

Suppression of hormones that trigger senescence, for instance, ethylene or overproduction of those that inhibit senescence such as cytokinins, are some of the approaches that can be used to delay plant senescence (Jibran *et al*., 2013; Khan *et al*., 2013; Wojciechowska *et* *al*., 2018). Previous studies show that repressing genes that code for enzymes involved in ethylene biosynthesis, 1-Aminocyclopropane-1-carboxylic acid (ACC) oxidase and ACC synthase increases ethylene prolonging the shelf life of transgenic tomato (Liu *et al*., 2015). The senescence of flowers in torenia was also delayed (Shibuya, 2018). Moreover, flower senescence has been delayed in horticultural crops by altering genes involved in ABA biosynthesis (Chang *et al*., 2014) and gibberellins (Lü *et al*., 2014) has also been reported.

2.9 Stability of transgenes

Successful genetic engineering necessitates that transgenes exhibit stable expression, which must be passed on to subsequent generations in a predictable pattern (Ahuja and Fladung, 2014). Transgene inheritance and expression levels are highly dependent on various factors, such as transformation methodologies, promoter strength, insertion sites and copy numbers of the transgene (Bandopadhyay *et al*., 2010). Analysis of transgene expression has been conducted on various staple crops like barley (*Hoedeum vulgare* L.) (Yi *et al*., 2020), maize (*Zea mays* L.) (Erasmus *et al*., 2019), wheat (*Triticum aestivum* L.) (Liu *et al*., 2020) and rice (*Oryza sativa* L.) (Zhao *et al*., 2011). In majority of the studies, silencing or loss of the transgene was reported. Additionally, 10% of transgenic lines had transgene expression in progeny silenced (Dolgova and Dolgov, 2019).

Inheritance of transgenes in soybean (*Glycine max*) transformed for herbicide resistance has been reported. Of the four transformed lines, three were inherited in Mendelian pattern up to the fifth generation (T5). Nonetheless, one transgenic line revealed that the T1 generation did not express transgenes, yet it was inherited in the plants. Moreover, when the plants were inoculated with the *Soybean mosaic virus* (SMV), silencing of transgene was not repressed, an indication that the silencing was not post-transcriptional (Kavas *et al*., 2016; Yi *et al*., 2017).

(Tizaoui and Kchouk, 2012) studied the pattern of segregation of three subsequent generations of transgenic tobacco. Five of the transformation events confirmed Mendelian inheritance, whereas six of the transformation events produced progenies that were non-segregating, a finding that could not be explained at the time. Moreover, Pinheiro *et al*. (2009) reported that the rep-trap-ren genes were inherited in the transgenic bean line consistently in Mendelian fashion.

2.9.1 Scaffold attachment regions (SARs)/ Matrix attachment regions (MARs) effect on transgene expression

The chromosomal position effect is a key problem associated with transgenic plants as it causes transgene silencing or mixed expression. This effect is linked to the fact that the transgene is integrating within the euchromatin. The structure of the chromatin at the integration site ultimately defines the level of expression by acting as a gene silencer or enhancer (Bandopadhyay *et al*., 2010; Dong *et al*., 2021). This impact of chromosomal position can essentially be avoided if elements of the DNA capable of binding to the nuclear matrix (MARs/ SARs) flank the transgene (Dolgova and Dolgov, 2019; Abrahamian *et al*., 2021). It is postulated that the MARs location within regulatory elements of transcription could help move these sequences of DNA closer to the scaffold, thus boosting promoter and enhancer function through enabling association with transcription factors (Roychowdhury and Chattopadhyay, 2020).

2.9.2 Effect of DNA Methylation and Acetylation on transgene expression

As reported by Bandopadhyay *et al*. (2010), DNA acetylation decreases the affinity of histones to DNA by neutralizing the lysine residues on their amino terminal. *Arabidopsis* reportedly has 18 histone deacetylase (HDAC) members (Yang *et al*., 2018). Some are involved in silencing transgenes, such as AtHDA6 (Su *et al*., 2015) while others, such as AtHD1, have been reported to regulate transcription during *Arabidopsis* growth (Li *et al*., 2017). Moreover, studies have shown that DNA methylation plays a fundamental role in the silencing of plant genes (He *et al*., 2011). Methyl CpG-binding protein (MeCP2) through a corepressor has been reported to recruit HDAC to methylated DNA. Additionally, hemi-methylation has been reported to inhibit transient expression of genes, whereas non-methylated genes are expressed normally (He *et al*., 2011; Sharif and Koseki, 2018).

2.9.3 Transcriptional factors effect on transgene expression

Transcription factors are proteins that bind DNA that are sequence-specific. The transcription factors work together with the promoter to control the rate at which RNA polymerase II initiates the synthesis of mRNA (Sanders and Mason, 2016; Caramori *et al*., 2020). Overexpression and antisense technology are commonly used to evaluate the function of transcription factors with regard to transgene expression. When the transcription factor in a transformed plant has a relatively high expression, it favours its binding to sites with low affinity, causing gene expression to be activated from promoters that are non-cognate (Bandopadhyay *et al*., 2010).

It has been reported that transgenic rice with dehydration-responsive element-binding protein/C-repeat binding factors (DREB 1s/CBF) or OsDREB 1A/1B transcription factor interacted specifically with DRE/CRT or OsDRE cis-acting elements, regulating the expression of various genes induced by stress (Kumari *et al*., 2021; Yang *et al*., 2021). Additionally, studies show that the expression of yeast yes-associated protein 1 (YAP1) gene caused greater tolerance to salt in transgenic Arabidopsis (Mansour, 2013). Nuclear YAP1 governs the expression of multiple genes linked to oxidative stress that results from high salinity (Zhao *et al*., 2009; Rodrigues-Pousada *et al*., 2019).

2.9.4 Effect of introns on transgene expression

Introns are defined as non-coding DNA regions that are located between exons. For expression of some genes, an intron must be present in addition to an efficient promoter (Barrett *et al*., 2012). Expression of a profilin-2 (PRF2) promoter and the β-glucuronidase in Arabidopsis requires PRF2 intron. Additionally, conversion of PFR5:GUS fusion to a vegetative pattern from a reproductive one is facilitated by PRF2 (Zhong *et al*., 2020). Introns can boost the level of expression of a gene via enhancer element, which is an alternative activity of promoter, or independently through intron-mediated enhancement (IME) (Rose, 2019). The most significant aspect is the intron position within the gene. Previous studies indicate that the introns in the 5’ untranslated region (UTR) of *rubi3* gene enhanced expression in rice (Shi *et al*., 2020; Liu *et al*., 2021). Currently, there are several introns that can significantly impact the actual size as well as the amount of expression of a gene (Bandopadhyay *et al*., 2010; Back and Walther, 2021).

2.9.5 Promoter effect on transgene expression

The 5’ upstream regions of a gene provide regions for initiation factors to attach. These factors are involved in transcription. The initiation factors attach to short regions of a specific DNA sequence (promoter) (Ponomarenko *et al*., 2013). TATA box, a key promoter element, is about 25bp long and provides a binding site for RNA polymerase II (Ray and Fry, 2015).

Enhancers are consensus DNA sequence motifs associated with place, level, and timing of expression, in response to biotic or abiotic (internal or external) factors. They can be found downstream, upstream, within gene coding regions, downstream or in the intron sequences (Pennacchio *et al*., 2013). The activation of enhancer elements controls the expression of genes at transcription level. Silencers, which are cis-elements, can silence or repress gene expression (Biłas *et al*., 2016).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Establishment of maize plants

CML 144 maize variety transformed with *isopentenyl transferase* (*IPT*) gene under regulation of senescence associated receptor kinase promoter (pSARK) was used (Leta *et al*., 2016). First, seeds were surface - sterilized using 10% sodium hypochlorite for about 10 minutes and thereafter rinsed thoroughly thrice using distilled water. Five seeds per pot were pre-germinated in sterilized vermiculite. Two weeks old seedlings were transferred into 20L- capacity plastic pots containing 15kg of soil mixed with manure at a ratio of 1:1 and maintained in the glasshouse at a temperature of 40/26 ±2 ºC (day/night), relative humidity of 60±5% and 16 h /8 h (light/dark) photoperiod. Untransformed CML 144 was used as the non-transgenic control throughout the experiment.

3.2 DNA extraction

Leaves from three weeks old pSARK::IPT plantlets were used to extract total genomic DNA using the CTAB method (Tamari *et al*., 2013). Briefly, 250 mg of maize leaf tissue was collected and crushed in a mortar containing liquid nitrogen. Preheated extraction buffer (Cetyl trimethylammonium bromide, sodium chloride, 0.5 M ethylenediaminetetraacetic acid, 1 M Tris, polyvinylpyrrolidone and water) was added to the leaf tissue and incubated in a water bath at 65 ºC for 30 minutes and mixed by mechanical inversion every 10 minutes. The supernatant was then pipetted into a new micro centrifuge tube and an equal volume of Chloroform: Isoamyl alcohol at a ratio of 24:1 added, mixed by inversion and incubated at room temperature (25 ºC) for three minutes prior to centrifuging (10,000 rpm for 5 minutes). The aqueous phase was pipetted-out into a new micro centrifuge tube and two third the volume of ice-cold Isopropanol added, mixed and incubated at -20 ^oC for 4 h. This was later centrifuged for 10 minutes (10,000 rpm) and the pellet washed in 70% ethanol. Upon spinning at 13200 rpm for five minutes, the supernatant was discarded and pellet dissolved in 40 µl of nuclease-free water and stored at -20 ^oC.

3.3 PCR amplification for detection of IPT transgene

To confirm presence of the transgenes, PCR was done using primers specific to the *IPT* and *PMI* genes, the selectable marker gene. IPT-forward 5'-CCAACTTGCACAGGAAAGACGACG-3' and *IPT*-reverse 5'-TCCAGA TGAAGACAGGTGCGAC-3’ primers were used to amplify 690 bp of the IPT gene transcripts while PMI-forward 5’-ACAGCCACTCTCCATTCA-3' and *PMI*-reverse 5' GTTTGCCATCACTTCCAG-3' were used to amplify the 0.5 kb PMI transcripts (Leta *et al.*, 2016).

The PCR amplification was conducted as per the procedure described by (Lorenz, 2012). In summary, synthesized primers were used to amplify genes using Bioline My Taq PCR master mix that included buffer, magnesium chloride and deoxy-nucleotide triphosphate primers and DNA template adjusted to a total volume of 25 µl. The PCR machine (Eppedorf AG 22331, Hamburg, Germany) was programmed as follows; initial denaturation temperature at 95 ºC for five minutes followed by 35 cycles each of 95 ºC for 15 seconds, annealing at 55 ºC for 30 seconds, initial elongation at 72 ^oC for 1 minute, final extension at 72 ºC for 7 minutes and held at 4 ºC to infinite. The same procedure was used for PMI primers except that annealing was done at 53 ^oC. The amplicons were resolved on

1.5% (w/v) TAE agarose gel at 80 V for 45 minutes. PCR-positive plants were selected for drought stress assays.

3.4 Drought Stress Assays

3.4.1 Determination of plant performance under drought stress

Soil (15kg) was mixed with compost at a ratio of 1:1 and placed in 20 L plastic pots. At least 35 seeds of each of the two genotypes (pSARK::IPT transformed and non-transgenic maize) were sown individually with an average of three seeds per pot. They were then thinned to two plants per pot. The drought stress assay analyzed five transgenic events which were replicated three times totalling to 15 plants per treatment. The well-watered controls for each of the genotypes were included in the setup. The experiment was arranged in the glasshouse in a completely randomized design.

The optimum amount of water for each plant was estimated following the procedure described by (Rathod *et al*., 2017). Briefly, 5 L of water was added slowly and uniformly on top of the soil in each of the three pots until the soil was completely saturated. The pots holding the saturated soil were covered with a black polythene bag and allowed to stand on a water-collecting tray for 48 h to collect dripping water until all downward movement of excess water ceased completely. After 48 h, the volume of the water collected in the tray was measured using a measuring cylinder and the same amount deducted from the five litres of water used to saturate the soil. This observation was repeated three times and the average of 2L was taken as the volume of water that should be applied to this soil daily to keep the soil moisture at a level available for optimum plant growth.

The plants were watered daily with tap water and maintained in the glasshouse. Sixty days post sowing, a cycle of drought conditions was induced by withholding water for 21 days. A control set was maintained by continuously watering the plants daily. After 21 days of water deficit, the plants were watered for three days for recovery. Sampling was done for relative water content and chlorophyll determination on days zero, seven, fourteen and twenty-one of stress as well as three days after re-watering. Phenotypic parameters such as height of plants, leaf number per plant, number of leaves showing senescence per plant, number of rolled leaves per plant, days to silking days to anthesis, seed number per plant, ear length and seed weight were measured and recorded at maturity.

Shoot fresh weight of each plant was measured after cutting at the base of the stem with a sharp scalpel. The weight of fresh roots was determined after washing roots with running tap water to minimize damage then air-dried to remove excess water. The dry weight of each root and shoot was taken after collecting each root and shoot individually in khaki bags and oven drying at 80 ^{o}C for 48 h. In all sampling days (days 0, 7, 14 and 21), plants were photographed for comparison of how the two groups responded morphologically to drought stress.

3.4.2 Determination of chlorophyll content of plants under drought stress

The photosynthetic impact of dehydration stress on the pSARK::IPT and non-transgenic maize was determined by extracting the total chlorophyll content under drought stress and well-watered conditions. Chlorophyll was extracted from leaf 5 of each plant in triplicate using dimethyl sulfoxide (DMSO) non-destructive method according to (Kumari *et al.*, 2018) with some modification.

Concisely, 3 ml of preheated DMSO was added to a 15 ml centrifuge tube containing 40 mg of fresh leaf tissue. The tubes were incubated for 1 hr at 65 ^{o}C in a water bath in the dark, then removed from incubation and topped up to 4ml with DMSO. The optical density (OD) of the extract was measured at 649 and 665 nm (722N Visible Spectrophotometer, EVERICH MEDCARE LTD, Nanjing, China), calibrating to zero with pure DMSO. Measurements and calculations were performed according to (Wellburn, 1994).

$$Chla = 12.19A665 - 3.45A649$$

$$Chlb = 21.99A649 - 5.32A665$$

3.4.3 Determination of leaf relative water content

The relative water content in leaves was assessed based on the method by Miccah *et al*. (2016). Three samples of leaves (3cm x 4cm) were cut from each experimental plant and leaf fresh weight (FW) recorded. The leaves were then incubated overnight at 4 ^{o}C in sterile distilled water and thereafter blotted with paper towels and saturation weight (SW) recorded. Subsequently, the leaves were oven-dried (Combi-H12, FINEPCR, Korea) for 24 h at 80 ^{o}C and dry weight of the leaf (DW) recorded. RWC was computed in accordance with the formula by (Soltys-Kalina *et al*., 2016):

$$RWC = \frac{(FW - DW)}{SW - DW} X\,100$$

Where: FW is the fresh weight, SW is the saturation weight and DW is the dry weight.

3.4.4 Determination of antioxidant enzyme activity

3.4.4.1 Preparation of crude leaf extract

The crude leaf extracts were prepared according to the method by (Elavarthi and Martin, 2010). Fresh leaf tissues were collected from both genotypes under stressed and well-watered. About 200 mg of leaf tissue was weighed and ground into a fine powder using a pre-chilled mortar and pestle containing liquid nitrogen. The powder was completely homogenized in 1.2 ml of 0.2 M potassium phosphate buffer (pH 7.8). Thereafter, the samples were spun at 15,000×g for 20 minutes at 4 ^{o}C. The supernatant was removed, the pellet re-suspended in 0.8 ml of the same buffer, and the suspension centrifuged for another 15 minutes at 15,000 ×g. The supernatants were combined and refrigerated, ready to be used to determine the activities of antioxidant enzymes.

3.4.4.2 Determination of Catalase (CAT) activity

The activity of Catalase was measured as per the procedure described by (Elavarthi and Martin, 2010). Decomposition of hydrogen peroxide was followed as a drop in absorbance at 240 nm in a UV/Vis spectrophotometer (Specord 200 analytik Jena). The 3 ml assay mixture comprised 2ml leaf extract (diluted 200 times in 50 mM potassium phosphate buffer, PH 7.0) and 10 mM hydrogen peroxide. The extinction coefficient of hydrogen peroxide (40 mM^{-1} cm^{-1} at 240 nm) was used to compute the enzyme activity, which was expressed in milli-moles of hydrogen peroxide per minute per gram of fresh weight as follows;

$$Enzyme\ activity\ (U/\ mg/\ min) = \frac{\Delta Abs \times Total\ assay\ volume}{\Delta t \times l \times \varepsilon \times enzyme\ sample\ volume}$$

Where;

ΔAbs is change in absorbance
Δt is time of incubation
ε is extinction coefficient of the enzyme in mM^{-1}cm^{-1}
l is cuvette diameter (1 cm)

3.4.4.3 Determination of Ascorbate Peroxidase (APX) activity

The activity of Ascorbate peroxidase was evaluated by modifying the standard technique of (Senthilkumar *et al*., 2021). The enzyme activity was assessed from the drop in absorbance (Specord 200 analytik Jena) at 290 nm resulting from oxidation of ascorbate in the reaction. The 1 mL assay mixture comprised of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.5 mM H_2O_2 (hydrogen peroxide), and 10 µl of crude leaf extract. H_2O_2 was finally added to start off the reaction and the drop in absorbance recorded for three minutes. An extinction coefficient of 2.8 mM−1 cm−1 for reduced ascorbate was used to compute the activity of the enzyme which, was expressed in terms of milli-mole of ascorbate per minute per gram of fresh weight.

$$Enzyme\ activity\ (U/\ mg/\ min) = \frac{\Delta Abs \times Total\ assay\ volume}{\Delta t \times l \times \varepsilon \times enzyme\ sample\ volume}$$

Where;

ΔAbs is change in absorbance
Δt is time of incubation
ε is extinction coefficient of the enzyme in $mM^{-1}cm^{-1}$
l is cuvette diameter (1 cm)

3.5 Determination of selected agronomic parameters

3.5.1 Induction of leaf senescence in the dark

Five leaves at the same developmental stage (leaf five) were excised from the plant and photos taken. This was done in triplicate. They were thereafter placed on damp filter paper and incubated in the dark at room temperature (25 ^{o}C). Photographs were taken at three and seven-days post-incubation.

3.5.2 Number of leaves showing senescence

The number of senesced leaves per plant was counted at the 7th, 14th and 21st of the drought period. Leaves showing at least 10% yellowing of the leaf blade were considered senesced.

3.5.3 Number of rolled-up leaves

The number of rolled-up leaves and the severity of rolling were scored per plant and recorded at 3 pm (hottest part of the day) at day 0 and day 3 of stress. A leaf rolling scale with scores of 1-5 was used to assess severity where 1 was considered normal (no rolling) while 5 was considered severely rolled up.

3.5.4 Plant height

The height of individual plants was measured from the soil level to the tip of the uppermost fully expanded leaf at the end of the stress period.

3.5.5 Leaf number per plant

The total number of leaves was counted manually in each plant at the end of the stress period and the average was recorded as the number of leaves per plant.

3.5.6 Shoot and root fresh weight

At physiological maturity, plants were cut at the soil level using a sharp knife. The shoots were weighed after removing the comb while the roots were washed thoroughly in running tap water, allowed to drip off excess water, blotted with paper towels and weighed. The weight was recorded individually in grams per plant.

3.5.7 Shoot and root dry weight

The shoots and roots for each maize were oven-dried separately at 70 ^{o}C for 48 h after harvesting the seed-bearing cob. After removing the seeds, the cob was dried separately and dry weight recorded.

3.5.8 Days to anthesis, days to silking and anthesis-silking interval

Days to anthesis were reported as the number of days taken by the plant from germination to the day at least a pollen was shed by the tassel. Days to silking were determined as the number of days a plant had taken from germination to the day at least a single silk had emerged at least 2 cm from the ear sheath. Anthesis-silking interval (ASI) was determined as the difference between the number of days to silking and the number of days to anthesis.

3.5.9 Ear length

The length of the ear was measured from the base to the tip and the value was recorded as ear length in centimetres.

3.5.10 Seed yield and seed weight per plant

The number of the seed per plant was counted manually and recorded as seed yield per plant.Weight of 10 seeds was computed on sensitive balance (Citizon scales HZT-A+200) and the value recorded as seed weight in grams.

3.6 Proximate analysis

3.6.1 Determination of moisture content

The method described by (Bradley, 2010) was used to estimate moisture content in the ground sample. Concisely, empty sample pans were dried in the oven (Combi-H12, FINEPCR, Korea) at 105 ^{o}C for 1 h and cooled in the desiccator (Pyrex 3120-200) prior to the experiment. The sample pan with the cover was weighed and weight recorded (W_0) and tared. Five grams of finely ground sample was put in the sample pans in triplicate and the weight recorded (W1). The pans with the cover were thereafter oven-dried at 105 ^{o}C for 3 h then cooled in a desiccator. The weight was measured (W2) and the moisture content of the sample calculated as follows:

$$Moisture\ content\ (\%) = \frac{(100\ x\ W1) - (W2 - W0)}{W1}$$

3.6.2 Determination of crude ash content

Crude ash content was assessed following the procedure by (Marshall, 2010) with some modifications. In a nutshell, weight of the ashing dishes was determined by heating in a muffle furnace (Eisklo LN 120) at 550 ^{o}C for one hour. They were thereafter cooled to room temperature in a desiccator (Pyrex 3120-200) and weighed accurately (W_0). The maize kernels were ground in a blender finely and 2 g put in the ashing crucible and weighed (W_1). This was then incinerated at 550 ^{o}C in a muffle furnace overnight. Light grey to white ash was deposited in the ashing dish. The dishes were cooled in a desiccator to room temperature. The dish with the lid and ash was weighed accurately (W_2). The crude ash content was calculated as follows:

$$\%\ crude\ ash = \frac{(100\ x\ Weight\ after\ ashing\ W2) - (Weight\ of\ crucible\ W0)}{(Weight\ before\ ashing\ W1) - (Weight\ of\ crucible\ W0)}$$

3.6.3 Determination of crude fat content

Crude fat content was estimated by the soxhlet extraction method ((Min and Ellefson, 2010). In brief, the weight of the empty thimble was recorded, and 2 g of sample was transferred into the thimble. Weight of the sample plus thimble was then recorded. Empty volumetric flask weight was recorded, and 250 ml of petroleum ether added. This was then boiled for 3 h at 60 ^{o}C. The thimble was then removed and placed in the oven at 105 ^{o}C for 25 minutes and final weight of the thimble recorded.

$$Crude\ fat\ (\%) = \frac{W3 - W4}{W2 - W1} x100$$

Where,
W_1 = Weight of empty thimble (g)
W_2 = Weight of thimble + sample (g)
W_3 = Weight of thimble + sample before extraction (g)
W_4 = Weight of thimble + sample after extraction (g)

3.6.4 Determination of crude fibre content

Crude fibre was evaluated by sequential acid and alkali hydrolysis technique according to (Möller, 2014). Succinctly, 3g of defatted dry sample was put in a flask and 100 ml of 1.25% sulphuric acid solution added, boiled for 30 minutes and weighed (weight C). Lining the Buchner funnel with filter paper, cotton wool and boiling water was done. After boiling, the flask was removed, allowed to rest for a minute and the contents filtered carefully using suction. Thereafter, the filter paper was cleaned with boiling water. The residue was then transferred to the flask, 100 ml of 1.25 % sodium hydroxide added and boiled for 30 minutes.

The residue was thereafter washed with 5 ml hot water, followed by 5 ml ethanol, 5 ml petroleum ether and 5 ml diethyl ether. The crucible was then placed in an oven at 105 ^{o}C for 30 minutes then into a desiccator for cooling. The crucible with the residue was weighed immediately (weight A) then placed in a furnace set at 550 ^{o}C for 3 h It was thereafter left to cool in a desiccator and weighed again (weight B). Crude fibre was computed using the equation as follows;

$$Crude\ fibre\ (\%) = \frac{A-B}{C} X100$$

Where,
A = Weight of crucible with dry residue
B = Weight of crucible with ash (g)

C = Weight of sample (g)

3.6.5 Determination of crude protein content

The method described by (Nielsen, 2017) with little modification was used to estimate crude protein content in the sample. Succinctly, 0.3 g of finely ground sample was added in 10 ml of digestion mixture (sulphuric acid and catalyst) into a Kjeldahl flask. This was digested at 110 ^{o}C for 1 h, followed by 330 ^{o}C for another hour and finally at 360 ^{o}C in a digester (Kjeldahl BD50 digestion block) till the sample was clear. It was then filtered in a volumetric flask and topped to volume (50 ml). Twenty millilitres of 4% boric was pipetted into a 250 ml conical flask and mixed indicator (methylene blue and methyl red) added. In a distillation flask, 10 ml of sample was diluted with 20 ml of distilled water, then 10 ml of 40 % sodium hydroxide added to neutralize the sulphuric acid. This was distilled into the boric acid containing mixed indicator. About 40 ml of aliquot was distilled to the boric acid-containing flask.

Upon completion of distillation, boric acid was titrated against 0.005 N sulphuric acid and the digestion mixture (without sample) was also titrated against 0.005 N sulphuric acid as a blank. Titration was repeated three times. The volume of the titre was determined and calculated as below;

Moles of H_2SO_4 = moles of NH_3 = moles of N in the sample

$$\%N = NH2SO4\ x \frac{Corrected\ acid\ volume}{g\ of\ sample}\ x \frac{14gN}{mol}\ x\ 100$$

where: N H_2SO_4 = normality of H_2SO_4 in mol/1000 ml; Corrected acid vol. = (ml std. acid for sample) – (ml std. acid for blank) and 14 = atomic weight of nitrogen

$$\%\ N\ x\ 6.25 = \%\ protein$$

3.7 Statistical analysis

Plant growth parameters including number of leaves per plant, number of senesced leaves, number of rolled-up leaves, plant height, shoot and root fresh weight, shoot dry and root dry weight, ear length, anthesis-silking interval, number of seeds per plant, seed weight, chlorophyll and relative water content of plants under stressed and watered conditions was subjected to descriptive statistics which were expressed as mean ± SEM. Pearsons normality test was used to test for normality of data. Student’s t-test at 95 % confidence interval was used to compare the two genotypes followed by correlation analysis using GraphPad Prism statistical software version 8.4.3.683. All data were presented in tables and graphs.

CHAPTER FOUR

RESULTS

4.1 Stability of pSARK::IPT transgene in T3 and T4 generations of *Zea mays* L.

Agarose gel electrophoresis at 100v for 30 minutes revealed that the isolated DNA had no contamination and was not fragmented (Plate 4.1A). Screening through PCR was conducted on T3 and T4 generations of *Zea mays* L. Fragments of 500bp and 690pb were amplified with PMI (Plates 4.1B and C) and IPT (Plates 4.1D and E) primers, respectively. In T3 generation, three out of seven plants had the T-DNA, while in T4, one out of seven plants had T-DNA.

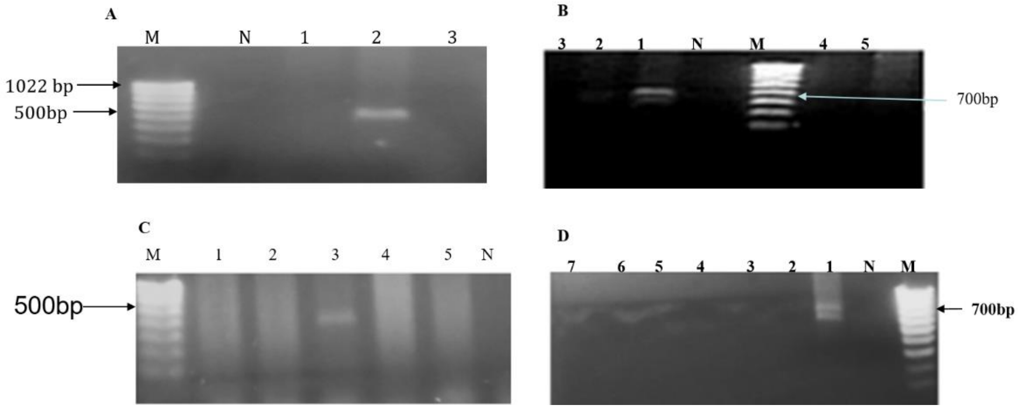

Plate 4.1: PCR products of transformed maize amplified using gene-specific primers. **A**: PCR products (lanes 1-3) for detection of *PMI* gene in T4 generation of maize; **B**: PCR products (lanes 1-5) for detection of I*PT* gene in T4 generation of maize; **C**: PCR products for detection of *PMI* gene (lanes 1-5) in T3 generation of maize; **D**: PCR products (lanes 1-7) for detection of *PMI* gene in T3 generation of maize; **M**: 100bp ladder (HyperLadder™ - Bioline), N: negative control (no template)

4.2. Effect of drought on leaf relative water content

Leaf relative water content (RWC), which was assessed at different times points (0, 7, 14, 21 days) in leaf seven, revealed a decrease in RWC in both non-transgenic and pSARK::IPT maize plants upon exposure to drought stress. Unpaired Student’s t-test analysis showed that the pSARK::IPT maize had significantly ($p<0.05$) higher RWC than the non-transgenic at all sampling points except at day zero because drought stress was not yet induced (Figure 4.1). The RWC decreased from 79.10 % to 8.01% in non-transgenic maize after 21 days of stress, whereas in pSARK::IPT maize, the decrease was from 71.20% to 43.64%. Upon re-watering for three days, there was a 91% recovery in pSARK::IPT, while the non-transgenic had 10% of the RWC before dehydration stress (Figure 4.1).

The RWC did not decrease significantly ($p>0.05$) in pSARK::IPT maize after seven days of stress, while tremendous loss was recorded with the non-transgenic plants within seven days of stress (Figure 4.1). Decreases from 79.10 % (day zero) to 63.20 % (day seven), 46.38 % (day 14) and 8.01% (day 21) was recorded in non-transgenic plants, whereas in pSARK::IPT plants, the RWC decreased from 77.83% on day 0 to 71.20% on day seven, 57.28% on day 14 and 43.64% on day 21. After re-watering the plants for three days, the RWC increased from 8.06% to 8.48% in non-transgenic plants, while in pSARK::IPT transformed plants, the increase was from 43.64% to 70.53% (Figure 4.1).

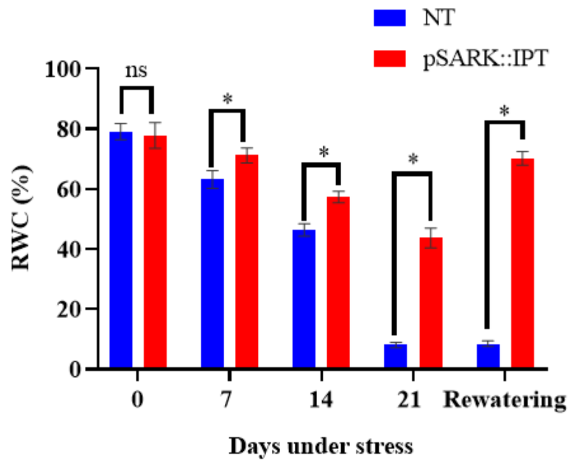

Figure 4.1: Leaf relative water content measured in 60-days old pSARK::IPT and non-transgenic maize. at different time points. The results (n=15) are expressed as the mean ±SE. Bars marked with asterisk (*) indicate that their means are statistically different from each other according to Students t-test at 95% confidence interval.

Additionally, leaf rolling was observed during the hottest part of the day (3 pm) before and during the stress period (Figures 4.2 and plate 4.2). Severe leaf rolling was observed in non-transgenic maize within three days of dehydration stress, while in pSARK::IPT maize, leaf rolling was mild (Plate 4.2 and Figure 4.2). The number of rolled-up leaves at day 0 and day 3 was statistically significant ($p<0.05$) between the two groups (Figure 4.2B). About 80 % and 12.22% of leaves per plant were rolled up in non-transgenic and pSARK::IPT plants respectively at day 0, while after 3 days of stress, rolled leaves were 83.82% in non-transgenic and 27.6% in pSARK::IPT maize. After seven days of stress, both groups exhibited leaf rolling.

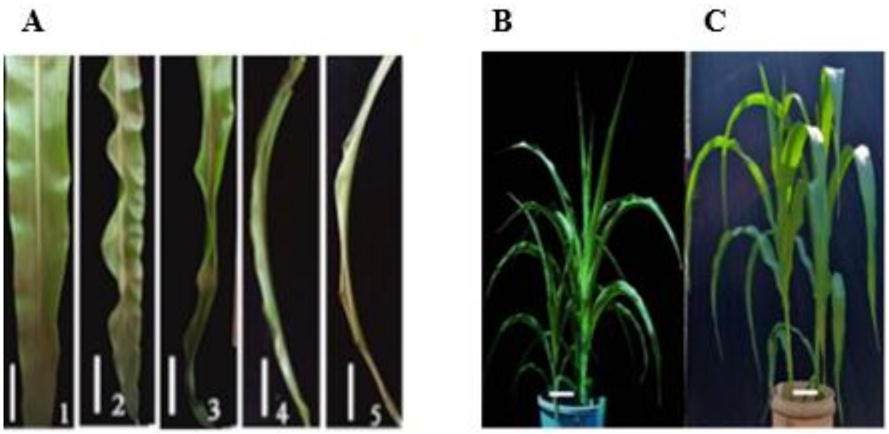

Plate 4. 2 Leaf rolling in non-transgenic and pSARK::IPT plants under drought stress. **A**: Leaf rolling scale with 1 representing normal leaf and 5 showing severe rolling. **B and C**: Leaf rolling in non-transgenic and pSARK::IPT plants respectively after 3 days of dehydration stress.

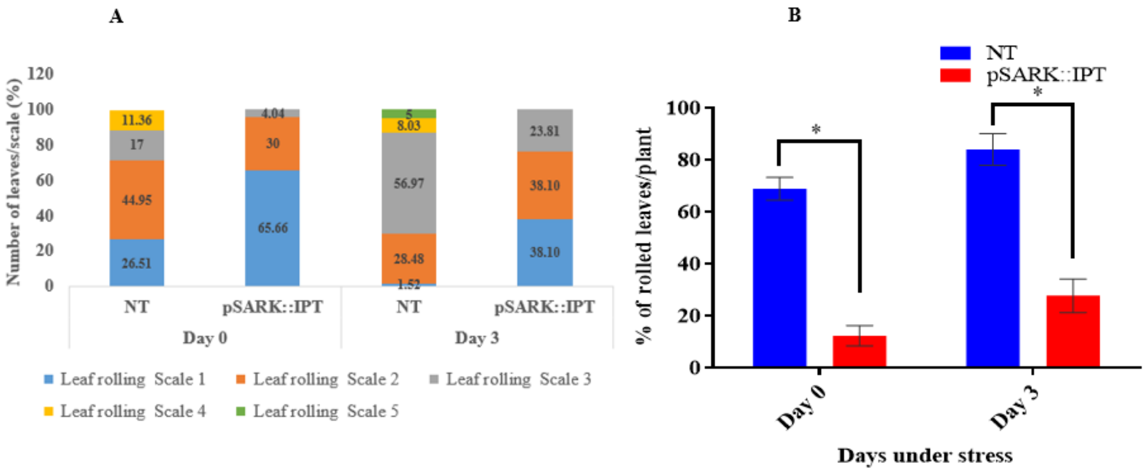

Figure 4.2: The number and severity of leaf rolling in pSARK::IPT and non-transgenic maize after three days of dehydration stress. **A**: Number of rolled leaves per scale. **B**: Number of rolled leaves per plant. The results (n=15) are expressed as the mean ±SE. Bars marked with asterisk (*) indicate that their means are statistically different from each other according to Student's t-test at 95% confidence interval.

4.3 Effect of drought on leaf senescence

Leaves were considered senesced when at least 10% of the leaf blade had started yellowing. The average number of senesced leaves per plant was scored at day zero and weekly thereafter, following commencement of drought stress. At any given time point, the number of senesced leaves was significantly higher ($p<0.05$) in non-transgenic than in pSARK::IPT plants (Figure 4.3). After seven days of stress, 16.89 % of the leaves in pSARK::IPT plants were senesced in contrast to 72.92 % in non-transgenic plants. By the 14th day of stress, about 92.6 % of non-transgenic leaves had senesced compared to 47.6 % in pSARK::IPT. Towards the close of the stress period, almost all leaves in non-transgenic had senesced (98.4 %), unlike in pSARK::IPT plants where only 58.4% showed senescence (Figure 4.3 and Plate 4.3A). Leaf five of all plants in both groups was closely monitored and the image analysis showed slower progress of senescence in pSARK::IPT plants compared to non-transgenic under water deficit (Plate 4.3 B). Further, induction of senescence in the dark showed delay in pSARK::IPT plants compared to non-transgenic (Plate 4.3C).

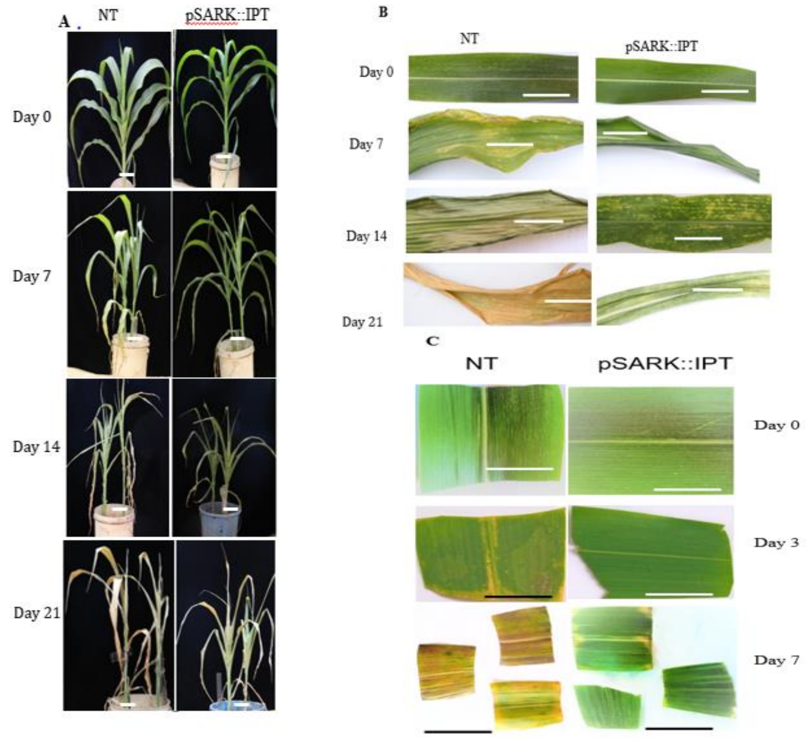

Plate 4.3: Progression of senescence in non-transgenic and pSARK::IPT plants at different time points. **A**: Progression of senescence in non-transgenic and pSARK::IPT plants **B**: Progression of senescence in leaf 5. **C**: Induction of senescence in the dark in leaf 7. Scale bar = 1 cm.

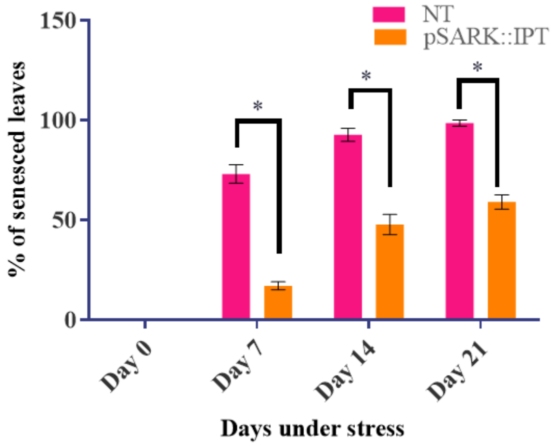

Figure 4.3: Number of senesced leaves in non-transgenic and pSARK::IPT maize at different time points.
The results (n=15) are expressed as the mean ±SE. Bars marked with asterisk (*) indicate that their means are statistically different from each other according to student's t-test at 95% confidence interval. Scale bar = 1 cm

4.4 Effect of drought on chlorophyll content

Chlorophyll, which was extracted using the non-destructive DMSO method before commencement of drought stress (day zero) and weekly thereafter till the end of the stress period, was used as a marker of senescence.Chlorophyll a, the primary pigment for photosynthesis, was non-significantly higher ($p<0.05$) in pSARK::IPT maize than in the non-transgenic up to the 14th day of the stress period (Figure 4.4). However, significant variations ($p<0.05$) between the two genotypes were recorded after 21 days of stress (2.23mg/gfw in pSARK::IPT and 1.73 mg/gfw in non-transgenic).

Significant differences ($p<0.05$) were also reported after rewatering the plants for three days as pSARK::IPT plants had 1.73 mg/gfw while non-transgenic had 0.45 mg/gfw of chlorophyll a. Comparison within individual genotypes showed a significant ($p<0.05$) decrease from 3.00 mg/gfw to 2.22 mg/gfw in pSARK::IPT maize and 3.21mg/gfw to 2.04 mg/gfw in non-transgenic maize after seven days of stress (Figure 4.4). The amounts of chlorophyll a continued to decrease in both groups, albeit non-significantly ($p<0.05$), up to 21 days of stress. However, upon rewatering for 3 days, the decrease in chlorophyll a was significant ($p<0.05$) but more severe in non-transgenic (1.7 mg/gfw to 0.45mg/gfw) than in pSARK::IPT maize (2.23mg/gfw-1.73 mg/gfw) as shown in Figure 4.4.

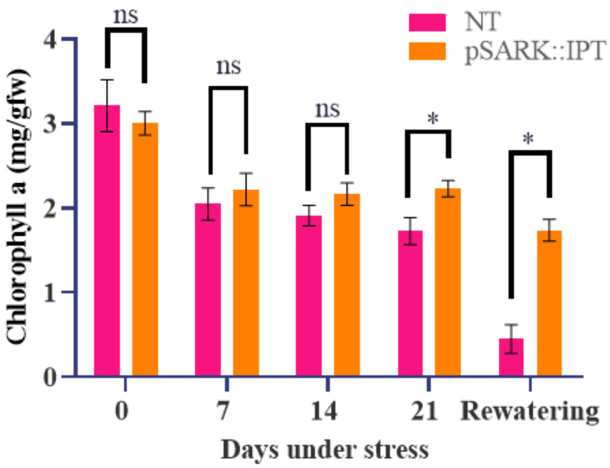

Figure 4.4: Chlorophyll a from leaves of non-transgenic and pSARK::IPT plants at different time points. A: Comparison of chlorophyll a between the two groups. **B**: Comparison of chlorophyll a within individual group. The results (n=15) are expressed as the mean ±SE. Bars marked with asterisk (*) indicate that their means are statistically different from each other according to student's t-test at 95% confidence interval.

On the other hand, chlorophyll b (the accessory pigment) was non-significantly ($p>0.05$) higher in pSARK::IPT maize than in non-transgenic up to 14th day of the stress period (Figure 4.5). However, significant ($p<0.05$) differences were reported after 21 days of stress (3.80mg/gfw in pSARK::IPT vis a vis 2.47mg/gfw in non-transgenic plants). Moreover, chlorophyll b was significantly ($p<0.05$) three times higher in pSARK::IPT than in non-transgenic plants after rewatering for three days. Comparison within individual genotypes indicated that no significant decrease ($p>0.05$) in the pSARK::IPT plants throughout the stress period but showed a significant reduction ($p<0.05$) in the wild-type from 14 days of stress to 3 days rewatering (Figure 4.5).

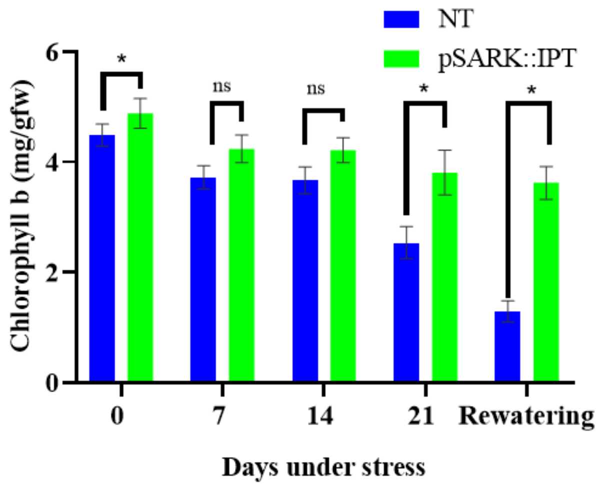

Figure 4.5: Effect of drought stress on chlorophyll b at different time points in non-transgenic and pSARK::IPT plants under drought stress. Bars marked with asterisk indicate that their means are statistically different from each other according to student's t-test $p<0.05$.

Total chlorophyll content of 6.14 mg/gfw in pSARK::IPT was significantly higher ($p<0.05$) than 4.43mg/gfw in non-transgenic plants after 21 days of stress. Moreover, total chlorophyll continued to decrease significantly ($p<0.05$) between the two groups, with that of pSARK::IPT plants being three times higher than non-transgenic after rewatering for three days. Total chlorophyll decreased significantly ($p<0.05$) in the non-transgenic plants after 14 days (from 5.92mg/gfw to 4.25mg/gfw after 21 days of stress) and continued to drop significantly ($p<0.05$) to 1.57mg/gfw upon rewatering for three days.

Conversely, the pSARK::IPT transformed plants had no significant decrease in total chlorophyll after seven days of stress, as shown in Figure 4.6 A. There was no significant association ($R^2 = 0.0927$; p-value = 0.6184) between total chlorophyll and relative water content in pSARK::IPT maize (Figure 4.6 C) while a strong positive correlation (R^2 =

0.7945; p-value = 0.0423) between the above parameters was observed in non-transgenic (Figure 4.6 D) according to Pearson correlation at $p<0.05$.

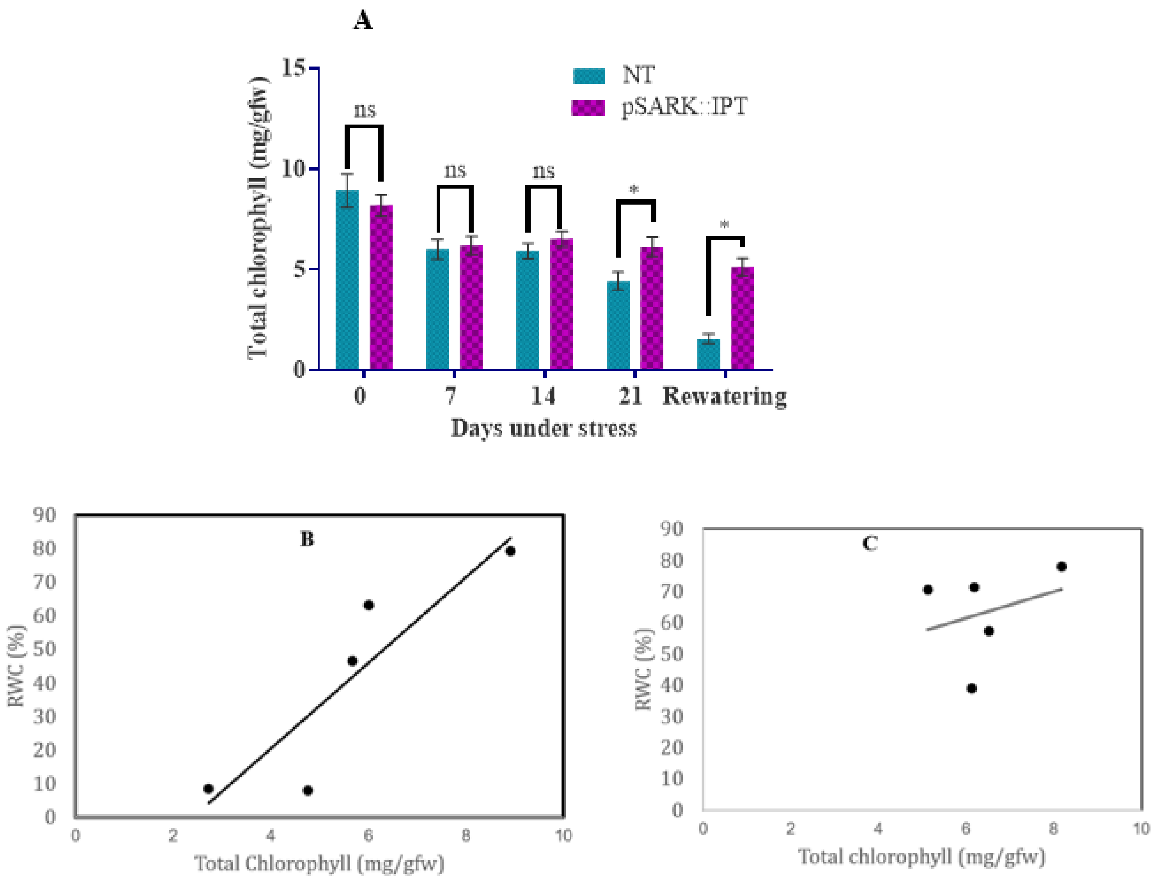

Figure 4.6: Effect of drought stress on total chlorophyll. **A:** Comparison of total chlorophyll (mg/gfw) between pSARK::IPT and non-transgenic plants. **B and C:** Correlation between total chlorophyll and relative water content in non-transgenic and pSARK::IPT plants respectively. Bars marked with asterisk indicate that their means are statistically different from each other at $p \leq 0.05$.

Regarding the ratio of chlorophyll a to chlorophyll b, there was no significant ($p<0.05$) variation between the two genotypes throughout the stress period. Notably, the ratio of chlorophyll a to chlorophyll b increased after 21 days of stress in both groups from 0.4825 to 0.6509 in non-transgenic and from 0.4977 to 0.6565 in pSARK::IPT (Figure 4.7).

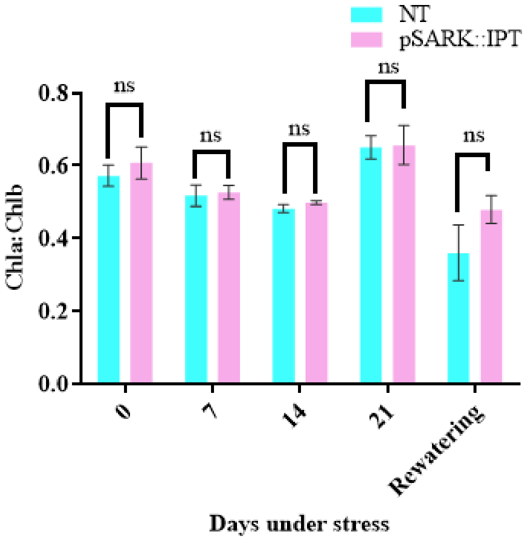

Figure 4.7: Comparison of chlorophyll a to chlorophyll b ratios between pSARK::IPT and non-transgenic plants under drought stress.

4.5 Effect of drought on antioxidant enzymes activity

Leaf samples collected in both stressed and well-watered plants of pSARK::IPT after three days of stress, revealed that catalase (CAT) activity was three times (0.013 U mg-1 min-1) significantly higher ($p<0.05$) in pSARK::IPT plants under drought stress than in well-watered conditions (0.04 U mg-1 min-1). On the other hand, CAT activity did not differ significantly ($p>0.05$) between non-transgenic plants grown in well-watered (0.003 U mg-1 min-1) and stressed conditions (0.003 U mg-1 min-1). Moreover, CAT activity was four times (0.013 U mg-1 min-1) significantly higher ($p<0.05$) in pSARK::IPT plants than in non-transgenic plants (0.003 U mg-1 min-1) under drought stress (Figure 4.8A).

The Ascorbate peroxidase (APX) activity was significantly ($p<0.05$) six time higher (0.155 U mg-1 min-1) in pSARK::IPT plants grown in well-watered conditions than under drought stress and significantly ($p<0.05$) three times higher (0.061 U mg-1 min-1) in non-transgenic plants under drought stress than under well-watered conditions and non-transgenic plants, respectively. Besides, the enzyme activity was significantly ($p<0.05$) three times higher (0.155 U mg-1 min-1) in pSARK::IPT than in non-transgenic plants (0.061 U mg-1 min-1) under drought stress (Figure 4.8 B)

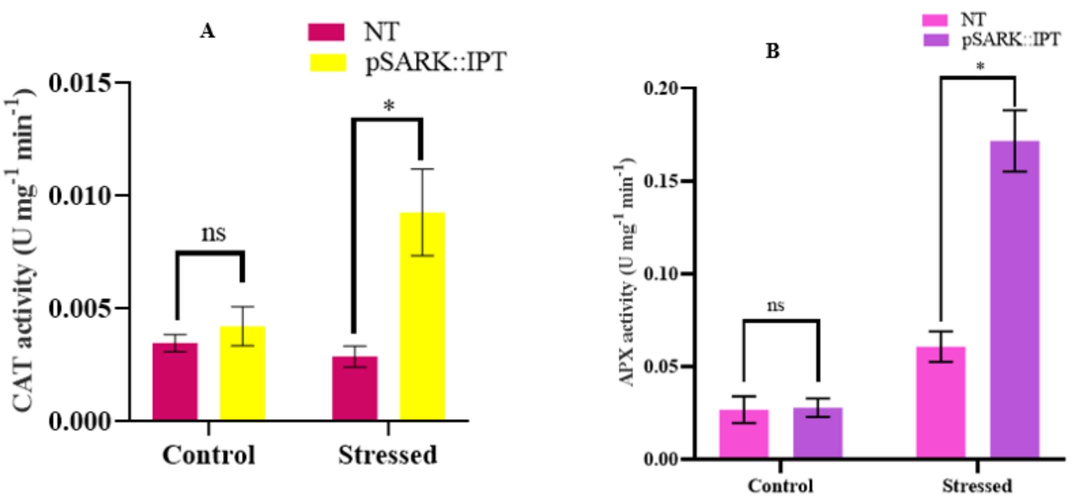

Figure 4.8: Antioxidant enzyme activities under well-watered and stressed conditions. **A**: CAT enzyme activity. **B**: APX enzyme activity. The results (n=10) are expressed as the mean ±SE. Asterisks (*) indicates a statistical significance at $p<0.05$.

4.6 Effect of drought on plant height and leaf number

Height of plants as well as the total number of leaves per plant recorded at the end of the stress period revealed that the pSARK::IPT plants were significantly taller (87.7 cm) than the non-transgenic (70.25cm) under drought. However, no significant ($p>0.05$) variation in height was observed between the two genotypes under well-watered conditions (112.94 cm in WT and 107.61 cm in pSARK::IPT as indicated in Figure 4.9 A. On average, stressed plants were significantly ($p<0.05$) shorter (78.98 cm) than well-watered controls (110.28 cm).

Under drought stress, number of leaves per plant did not differ significantly (p>0.05) between non-transgenic and pSARK::IPT plants (eight leaves per plant) under drought stress and even in well-watered conditions where the non-transgenic plants had fewer leaves per plant (8) and pSARK::IPT had 10 leaves per plant (Figure 4.9 B).

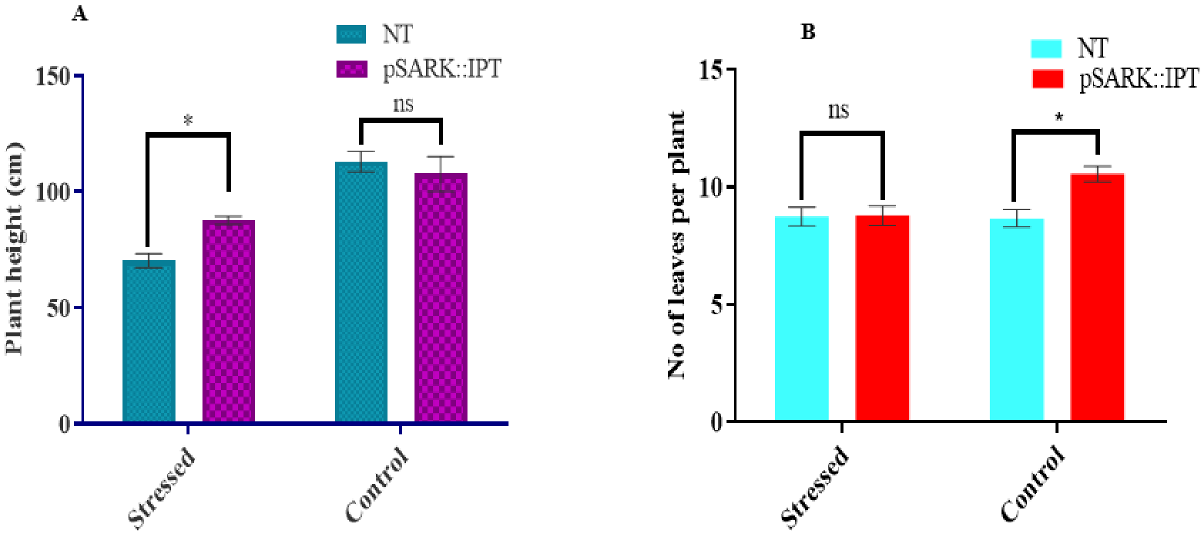

Figure 4.9: Effect on drought on plant height and leaf number.

A: Comparison of plant height between non-transgenic and pSARK::IPT plants under drought stress and well-watered conditions. **B**: Comparison of number of leaves per plant between pSARK::IPT and non-transgenic plants under drought stress and well- watered conditions. The results (n=15) are expressed as the mean ±SE. Bars marked with asterisk indicate that their means are statistically different from each other at p<0.05.

4.7 Effect of drought on Anthesis-Silking Interval (ASI)

Plants under drought stress tasselled two weeks after the well-watered plants. None of the non-transgenic plants silked under drought stress. Most plants under drought stress produced anthers with few or no pollen. The ASI was 13 days on average in pSARK::IPT under drought stress which was significantly (p<0.05) lower than that of the non-transgenic plants (90 days) (Figure 4.10). On the other hand, ASI was five days in both non-transgenic and pSARK::IPT conditions under well-watered conditions.

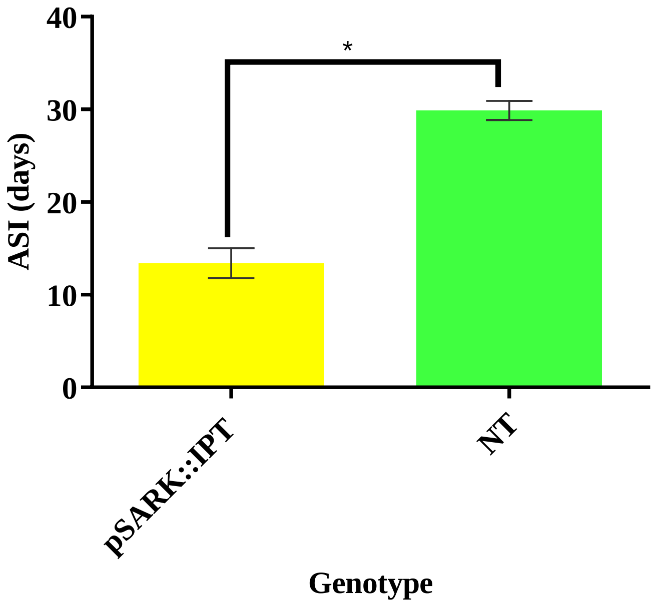

Figure 4.10: Comparison of ASI between pSARK::IPT and non-transgenic plants under drought stress. The results (n=15) are expressed as mean ± SEM. Bar marked with asterisk indicates that their means are statistically different from each other at $p<0.05$.

4.8 Effect of drought on maize yield and yield components

Pollination was severely affected under drought stress. The tassels had few pollen. In most cases, pollen mortality was evident. The non-transgenic plants had no ears since they didn't silk, while pSARK::IPT plants had ears with no kernels (Figure 4.11 and Plate 4.4). Moreover, under well-watered conditions, the average seed weight (weight of 10 seeds) was significantly ($p<0.05$) higher in pSARK::IPT plants than in non-transgenic plants (Table 4.1). Additionally, pSARK::IPT plants had significantly ($p<0.05$) more seeds per comb (139) than the non-transgenic plants (95) (Table 4.1).

Table 4.1 Comparison of yield and yield components between pSARK::IPT and non-transgenic plants under well-watered conditions.

Yield component	Wild-type	pSARK::IPT	P-value
Seed Number	95.75 ± 15.92	139.3 ± 9.801*	0.0239
Seed weight	2.00 ± 0.1317	2.582 ± 0.1414*	0.0075
Ear length	11.89 ± 0.4771	12.87 ± 0.4023	0.1305

The results (n=15) are expressed as mean ± SE. Asterisks (*) indicate that their means are statistically different from each other within a row at $p<0.05$.

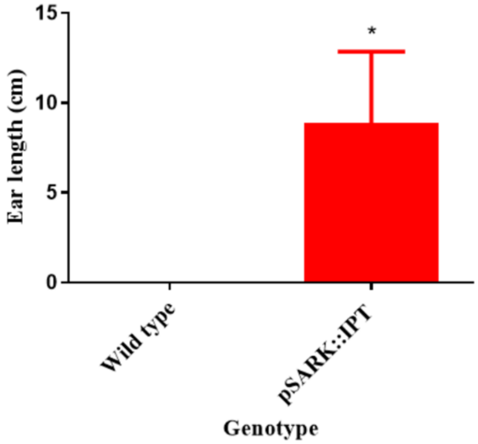

Figure 4.11: Ear length in wild-type and pSARK::IPT plants. Bar marked with Asterisk indicate that their means are statistically different from each other at $p<0.05$.

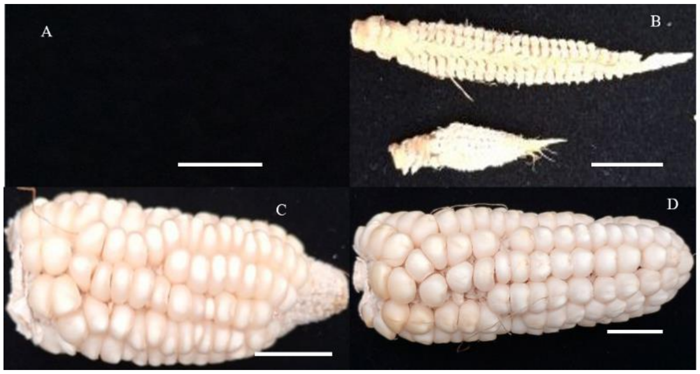

Plate 4.4: Comparison of yield and yield components between pSARK::IPT and non-transgenic plants under drought stress and well-watered conditions. **A and B**: Non-transgenic and pSARK::IPT ears under drought stress respectively. **C** and **D:** Ears of non-transgenic and pSARK::IPT ears under well-watered conditions respectively. Scale bar = 1 cm

4.9 Effect of drought on roots and shoots

Upon cutting the plant from the base of the soil and carefully uprooting and cleaning roots with running tap water, the primary roots under drought stress were found to be significantly ($p<0.05$) shorter in non-transgenic plants (26.91 cm) than that of maize having *IPT* transgene (31.10 cm). Moreover, the number of seminal roots was 1.4 times significantly ($p<0.05$) higher in pSARK::IPT maize than in non-transgenic maize, as shown in Figure 4.12 and Plate 4.5. Under well-watered conditions, seminal roots were significantly more ($p<0.05$) in pSARK::IPT plants (40) than in non-transgenic plants (30). Additionally, the primary roots were non- significantly ($p>0.05$) longer in pSARK::IPT than in non-transgenic maize (Figure 4.12 and Plate 4.5).

Root and shoot fresh weights were significantly ($p<0.05$) 10 and 8 times higher under well-watered conditions than under drought stress respectively. Furthermore, shoot fresh weight in pSARK::IPT was non-significantly ($p>0.05$) 1.3 times higher than non-transgenic under well-watered conditions. Generally, under both conditions, no significant ($p>0.05$) difference was recorded in shoot and root weights between pSARK::IPT and non-transgenic plants (Figures 4.12 C and D).

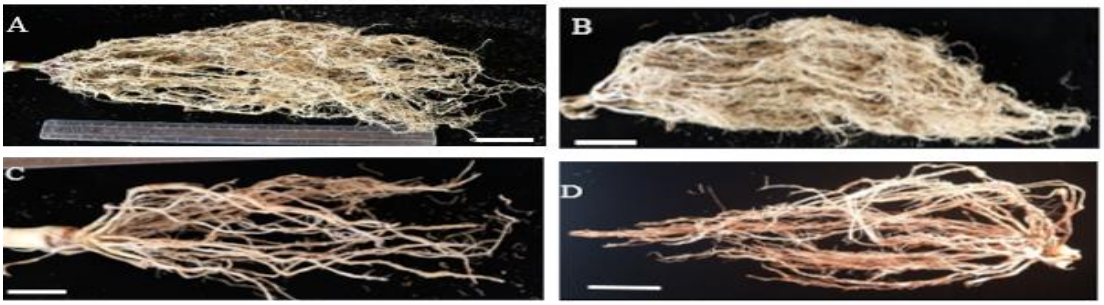

Plate 4.5: Effect of drought on roots of non-transgenic and pSARK::IPT plants. A and B: Roots of non-transgenic and pSARK::IPT plants respectively under well-watered conditions. C and D: Roots of non-transgenic plants and pSARK::IPT plants respectively under drought stress. Scale bar = 1 cm.

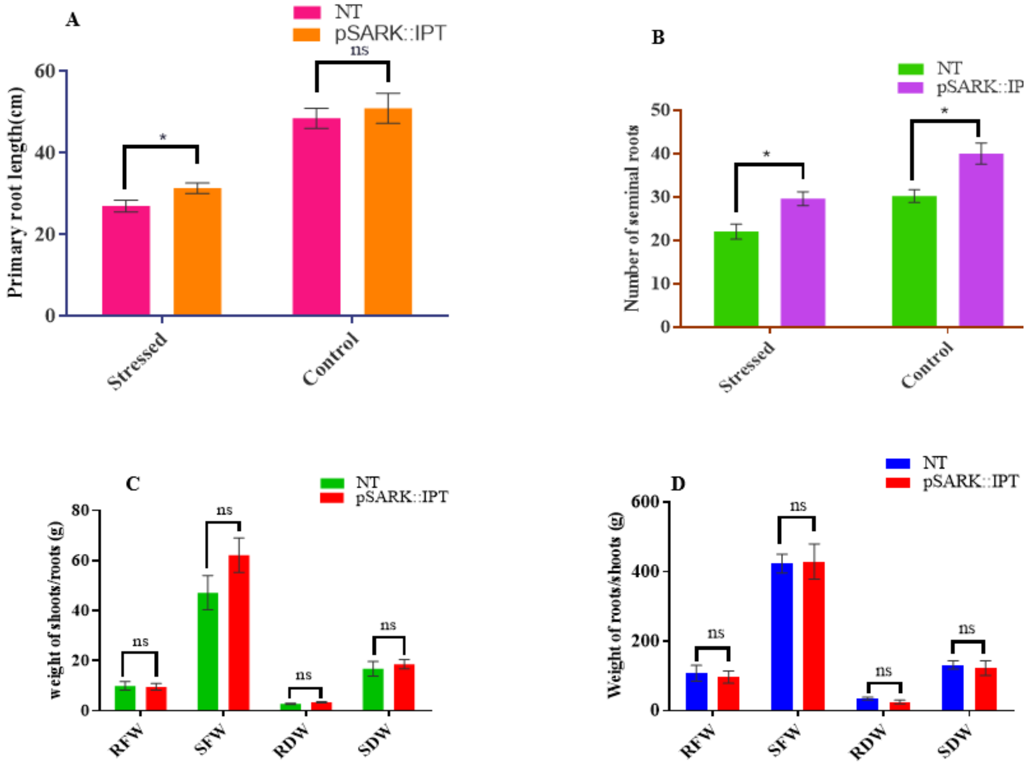

Figure 4.12: Effect of drought on roots and shoots

A: Primary root length in pSARK::IPT maize and non-transgenic under drought and well-watered conditions. **B**: Number of seminal roots in pSARK::IPT maize and non-transgenic under drought and well-watered conditions. **C:** Root and shoot fresh and dry weight of non-transgenic and pSARK::IPT maize under drought stress. **D**: Root and shoot fresh and dry weight of non-transgenic and pSARK::IPT maize under well-watered conditions. The results (n=15) are expressed as the mean ±SE. Asterisks (*) indicates a statistical significance ($p<0.05$). Scale bar = 1 cm. **RFW**: root fresh weight; **RDW**: root dry weight; **SFW**: shoot fresh weight; **SDW**: shoot dry weight

4.10 Proximate analysis of mature maize kernels

Upon harvesting the maize and quantifying yield, food component analysis of the mature kernels grown in well-watered conditions showed that crude fat was significantly higher ($p<0.05$) in pSARK::IPT (13.82%) than in non-transgenic (5.59%). Moreover, the crude protein content was significantly lower ($p<0.05$) in non-transgenic (3.18%) than in pSARK::IPT (5.69%). However, no significant differences were observed in moisture content, ash content as well a crude fibre content between the two groups as shown in Figure 4.13.

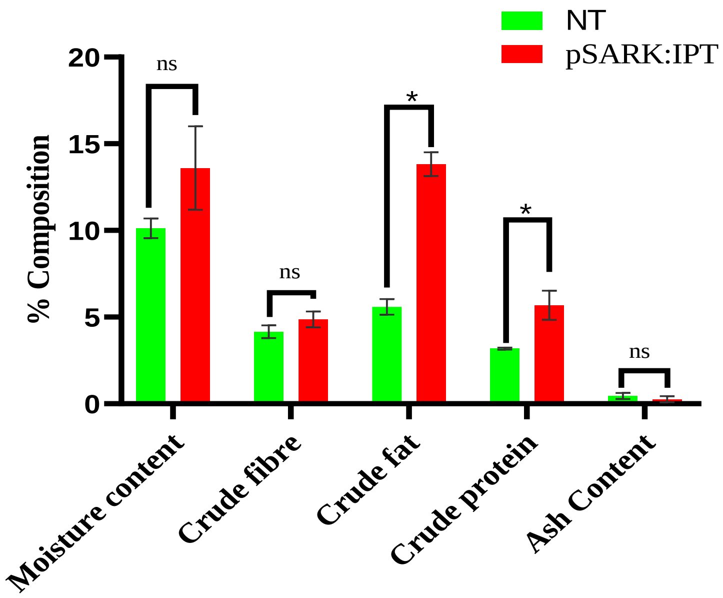

Figure 4.13: Proximate analysis of kernels of non-transgenic (NT) and pSARK::IPT maize under well-watered conditions. The results (n=3) are expressed as the mean ±SE. Asterisks (*) indicates a statistical difference at $p<0.05$.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

This study used transformed tropical maize CML-144 with the *IPT* gene, which was driven by pSARK (Leta *et al*., 2016). The pSARK::IPT maize plants showed delayed senescence and higher yields than the non-transgenic counterparts under drought stress imposed around the flowering period. However, they did not assess the stability of transgenes in subsequent generations as well as the nutritional composition of the transgenic maize for safety and health concerns. This information is key for advancement of these germplasms to field trials.

This study established that pSARK::IPT gene persisted in T3 and T4 generations of maize, an indicator of stable transformation (Plate 4.1). Consistent expression of the transgene within the plant genome is a major concern for transgenic crops. The effectiveness of the desirable trait ultimately depends on the transgene stability (De Buck *et al*., 2013). Moreover, only a portion of the complete genome is expressed at a particular time in a given cell (Bandopadhyay *et al*., 2010). Different nucleic acids and proteins regulate gene expression. These biomolecules determine whether the gene will be transcribed and if the transcription will yield a protein that develops into a phenotype. Likewise, the insert design determines gene expression in transgenic crops (Bandopadhyay *et al*., 2010). The findings of this study corroborate with those of (Oneto *et al*., 2016) but differ in the fact that they did backcrossing (not selfing) up to T2 generation of *Zea mays* L. and used the particle bombardment method of gene delivery.

Relative water content is a suitable parameter for assessing plant water status. This parameter was addressed gravimetrically by computing ratios of dry weight to fresh weight (Silva *et al*., 2011). The relative water content was significantly five times higher ($p<0.05$) in maize carrying pSARK::IPT T-DNA than in non-transgenic maize at the end of the drought period (Figure 4.1). A drop in leaf relative water content is a reliable indicator of the extent of water loss in a plant (Soltys-Kalina *et al*., 2016).

The balance between water supply to tissues in the leaf and its loss through transpiration and evaporation is well reflected by relative water content (Lugojan and Ciulca, 2011). These results validate the findings of (Leta *et al*., 2016), who reported 20% water loss in T1 generation transgenic plants against 40% in non-transgenic after 21 days of water stress. However, the findings differ with those of (Peleg *et al*., 2011) who did not report any difference between T3 generation of the non-transgenic and that of pSARK::IPT transformed rice exposed to water stress for 10 days.

The current study observed that senescence was delayed significantly in pSARK::IPT plants under water deficit stress compared to the control/non-transgenic plants (Figure 4.3 and Plate 4.3). This study further established that total chlorophyll, chlorophyll a and chlorophyll b were significantly higher in pSARK::IPT than in non-transgenic plants at the close of the drought period (21 days). Chlorophyll degradation is a good indicator of senescence. The high chlorophyll content resulted from delay in leaf senescence, a key function of cytokinins. Visible color change is a key characteristic of senescing leaves, which is closely linked to chlorophyll degradation and concomitant synthesis of anthocyanins and carotenoids (Zhao *et al*., 2020). Moreover, senescence associated genes

(SAGs) are upregulated to trigger nutrient recycling from the senescing leaf to the sink (Schippers *et al*., 2015).

These results concurred with the functions of cytokinin (CK) reported in wheat (Joshi *et al*., 2019), maize (Leta *et al*., 2016; Oneto *et al*., 2016 ;), *Arabidopsis* (Talla *et al*., 2016), cotton (Kuppu *et al*., 2013), alfalfa (Zwack and Rashotte, 2013) and creeping bentgrass (Merewitz *et al*., 2012). Water stress reduces cytokinin levels in the xylem by limiting its biosynthesis (Pérez-Alfocea *et al*., 2011). Peleg *et al*. (2011) demonstrated that low levels of CKs induced senescence, inhibited growth and reduced source/sink relationships.

This study further observed that Ascorbate peroxidase (APX) and Catalase (CAT) activities were significantly ($p<0.05$) higher in pSARK::IPT transformed plants than in non-transgenic (Figure 4.8). These results show that the susceptibility to drought was higher in non-transgenic plants than in pSARK::IPT maize plants. Drought stress causes an imbalance in the production of reactive oxygen species (ROS) and its scavenging through antioxidant defence system (Choudhury *et al*., 2017; Verma *et al*., 2019; Wei *et al*., 2019). Enzymes of the antioxidant system have the greatest efficiency as ROS scavengers (Saruhan *et al*., 2012; Osmolovskaya *et al*., 2018). Several studies have confirmed numerous associations between the activity of antioxidant enzymes and CKs (Wu *et al*., 2012; de Moura *et al*., 2017; Hai *et al*., 2020). CKs may influence ROS responses during abiotic stress by altering transcription in plants (Zwack and Rashotte, 2013). Glutathione peroxidase (GPX), APX and CAT are the primary ROS scavengers in plants (Laxa *et al*., 2019).

The findings of this study corroborate with those reported in creeping bentgrass (Xu and Huang, 2017) and eggplant (Xiao *et al*., 2017) transformed with IPT, though gene expression was regulated by SAG12 promoter. They reported that plants exposed to dehydration stress had significantly higher antioxidant enzymatic activities than their non-transgenic counterparts. However, H_2O_2 concentration reportedly increased with elevated CK levels when tobacco was transformed with IPT linked to a dexamethasone-inducible promoter which resulted into elevated lipid peroxidation (Novák *et al*., 2013). When APX from poplar was overexpressed in transgenic tobacco, increased plant performance in a water-limiting environment was reported (Li *et al*., 2009; Cao *et al*., 2017). The principal scavengers, APX and CAT, are strongly activated in species that are tolerant, whereas GPX is strongly activated in species that are susceptible to (Laxa *et al*., 2019).

This study was set up in such a way that the stress period coincided with the pre-anthesis stage and persisted for 21 days, which is considered a long-term drought (Leta *et al*., 2016). This study established that the water deficit treatment affected flowering, grain yield and kernel set of the pSARK::IPT and non-transgenic maize plants. Flowering was delayed and embryo aborted in pSARK::IPT maize, whereas the non-transgenic maize did not produce silk at all. Anthesis-silking interval was longer in stressed plants compared to well-watered ones. The pSARK::IPT plants had an anthesis-silking interval that was significantly shorter ($p<0.05$) than that of non-transgenic plants under drought stress (Figure 4.10).

Drought stress severely affects maize yield when it occurs at the flowering stage (Meseka *et al*., 2018; Jain *et al*., 2019; Sah *et al*., 2020). Gametogenesis, fertilization and embryogenesis are severely affected by drought, limiting the development of seed, consequently reducing yields (Farooq *et al*., 2009; Farooq *et al*., 2014; Dong *et al*., 2017) reported pollen sterility as a common symptom during drought stress. It reduces pollen germination as well as inhibit growth of the pollen tube, consequently impairing fertilization (Fang *et al*., 2010; Gusmao *et al*., 2012). Silk is more susceptible to drought stress than tassel, causing delay in silk emergence (Sehgal *et al*., 2018).

Drought inhibits photosynthesis by decreasing the capacity for regeneration of Ribulose-1,5-bisphosphate as well as its carboxylation efficiency. Whole-plant senescence severely affects seed filling (Sehgal *et al*., 2018). Water deficit in the course of seed development decreases the number of amyloplasts and endosperm cells, consequently reducing kernel/seed sink strength (Ndlovu *et al*., 2021). This negatively impacts the rate and duration at which endosperm gathers starch which ultimately reduces grain weight (Fábián *et al*., 2011).

Ovary abortion occurs due to the increase in non-reducing sugars as well as the inability to accumulate starch under water deficit consequently decreasing grain yield (Jiang *et al*., 2021; Khodaeiaminjan and Bergougnoux, 2021). Moreover, when water stress occurs in the seed filling stage, it triggers early senescence as well as decreases the duration of seed-filling, thus boosting translocation of assimilates from the source to sink (Sehgal *et al*., 2018).

Cytokinins (CKs) stimulate the endosperm cells to divide rapidly, which enhances grain filling (Kong *et al*., 2015). Increased levels of CKs may promote sink strength via up-regulating genes involved in cell division. This can be through sugar signalling, which encompasses increased unloading of phloem as well as import of sugar to the cells of the endospermic via enzyme invertase which is an enzyme associated with the cell wall (Rijavec *et al*., 2009; Sehgal *et al*., 2018).

The findings from this study substantiate those of Oneto *et al*. (2016), who reported that ASI increased in stressed plants. However, non-transgenic maize did not vary significantly from the transgenic plants under stressed conditions with regard to ASI. Furthermore, (Qin *et al*., 2011) reported that peanut plants carrying pSARK::IPT construct produced yields that were significantly higher than in wild-type plants in the field under water-limiting conditions.

Leta *et al*. (2016) also reported higher yield in T1 generation of maize containing pSARK::IPT transgene than in non-transgenic counterpart under drought stress. Oneto *et al*. (2016) discovered that maize plants carrying pSARK::IPT construct had higher yields than non-transgenic under water stress imposed for 20 days intermittently and similar yields with those under well-watered conditions, unlike in our study where plants under drought stress had lesser yield than those that were well watered.

Root and shoot fresh weights were significantly ($p<0.05$) higher in plants grown in well-watered conditions than under dehydration stress (Figure 4.12). This could be due to a reduction in cell turgor associated with a decrease in cell growth and development in the plant’s aerial portion (Blum, 2017). There existed no significant variation ($p>0.05$) in the weight of roots and shoots of the pSARK::IPT and non-transgenic plants (Figures 4.12), possibly because of the severity and duration of stress. Additionally, the number of seminal roots and the primary root length were higher in pSARK::IPT maize than in non-transgenic under drought stress (Figure 4.12). This could be due to tolerance in pSARK::IPT that enabled it to cope with drought stress via accumulation and elongation of root mass.

Roots are the first plant organs to be exposed to the drying soil hence the primary sensors/detectors of drought stress (Aslam *et al*., 2015). The structural root traits affected by drought include volume, density, length and number and these ultimately limits the functioning of the entire plant (Comas *et al*., 2013; Aslam *et al*., 2015). The tolerance in transgenic plants is due to elevated cytokinin levels, which enhance translocation of metabolites to the sinks (roots) via upregulation of cell wall invertase (Hwang *et al*., 2012). These findings uphold those of Qin *et al*. (2011), who established that the roots of the peanut plants transformed with pSARK::IPT were bigger than those of non-transgenic plants. Leta *et al*. (2016) also discovered that the roots of transgenic maize plants were fibrous, while taproots with few seminal roots were evident in the non-transgenic plants.

Interestingly, pSARK::IPT plants performed better than non-transgenic under well-watered conditions. More seminal roots were recorded in pSARK::IPT maize than in non-transgenic maize plants. Furthermore, fresh weight of shoots in pSARK::IPT plants was non-significantly ($p>0.05$) higher compared to that of non-transgenic under well-watered conditions. No significant variations ($p>0.05$) were recorded on dry weight between the studied genotypes. This could be due to water evaporation from the plant during drying reducing variations observed in fresh weights.

Oneto *et al*. (2016) reported that maize transformed by particle bombardment with pSARK::IPT construct had similar biomass under both stressed and well-watered conditions. Moreover, pSARK::IPT plants showed 45 % and 29 % higher seed number and seed weight respectively, than their non-transgenic counterparts under well-watered conditions (Table 4.1). The pSARK::IPT gene has not been reported to improve the yield of maize under well-watered conditions. However, Kant *et al*. (2015) reported improved yields in canola plants when *IPT* was driven by a developmentally-induced promoter AtMYB32xs.

Moreover, this study established that under well-watered conditions, crude fat was significantly higher ($p<0.05$) in pSARK::IPT maize than in non-transgenic maize. Likewise, crude protein content was significantly ($p<0.05$) lower in non-transgenic maize than in pSARK::IPT maize (Figure 4.13). Conversely, it was reported that oil from peanuts transformed with pSARK::IPT had the same quality as that of non-transgenic under an environment that was well-watered (Qin *et al*., 2011). However, transgenic (pSARK::IPT) rice plants had a 15 % increase in grain starch when they were maintained in a well-watered environment (Peleg *et al*., 2011).

It has been reported that genes which code for enzymes of the starch degradation pathway were downregulated in pSARK::IPT transformed rice while those that encode protein kinases Osk24 (Os08g37800) and Osk1 (Os05g45420) were upregulated, when plants were maintained under well-watered environment (Peleg *et al.,* 2011). The role of Osk1 is to unload sucrose through the sucrose synthase pathway, while Osk24 plays a fundamental role in the conversion of sucrose to starch (Raffan *et al*., 2020).

Sugars are the substrates for respiratory system that is involved in the production of energy as well as metabolites required to synthesize macromolecules. Proteins and lipids must bind to sugars for their proper functioning (Morkunas *et al*., 2012). Transcriptomic study of suspension cells of rice that were deprived of sucrose reported a decline in transcripts expression associated with synthesis of fatty acid vis-a-vis an increase in those that are linked to degradation of fatty acids such as fatty acid multifunctional proteins, acyl-CoA oxidase and 3-ketoacyl-CoA thiolase (Wang *et al*., 2007).

The ultimate size and quantity of endospermic cells determine the quantity of proteins and starch that accumulates in the grains, which are influenced by the duration and rate of the grain filling process (Peleg *et al*., 2011). CKs are associated with strengthening of the sink/source relationship in pSARK::IPT plants, which consequently leads to higher yields (Wang *et al*., 2016).

This study hypothesized that the better performance of pSARK::IPT under well-watered conditions could be due to downregulation of enzymes encoding the starch degradation pathway as well as upregulation of SnRK1-type of protein kinase genes. Besides, the activation of cell wall invertase enzyme that enhances translocation of plant nutrients from the source to the sink and increased cell division in the endosperm, which are associated with increased cytokinin, could result in enhanced performance in the transgenic maize.

5.2 Conclusions

The findings from this study can be summarized as follows:

i) The study confirms the presence pSARK::IPT transgene in T3 and T4 generations of selfing, a key phenomenon for successful transformation.

ii) The pSARK::IPT enhances yield even in well-watered conditions. It is presumed that the promoter is not only induced by drought but also by maturation which elevates the levels of cytokinin in well-watered conditions.

iii) Maize transformed with pSARK::IPT has better nutritional value than non-transgenic which gives it an upper hand for consideration for field trials.

iv) The study therefore rejects null hypothesis 1 and 2 because the IPT transgene presence was confirmed, and drought tolerant traits persisted in subsequent generations. However, this study fails to reject the null hypothesis 3 because the nutritional composition of the pSARK::IPT transgenic maize does is not similar to that of the non-transgenic.

5.3 Recommendations

5.3.1 Recommendations from the study

This study recommends that the pSARK::IPT transformed maize (CML144) should be further assessed to validate the severity of drought stress that it can tolerate with a minimal yield penalty. It can thereafter proceed to confined field trials prior to commercialization of the genotype.

5.3.2 Recommendations for further research.

i) Differential gene expression of pSARK::IPT maize under well-watered conditions should be done to confirm whether genes such as those encoding starch degrading enzymes and protein kinases are upregulated/downregulated.

ii) Gene expression analysis of pSARK::IPT at different generational times of transgenic maize under drought stress

iii) Characterization of discrete fatty acids and amino acids in kernels of pSARK::IPT maize under both stressed and well-watered conditions should be done.

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APPENDIX: Research Permit

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