

**GENETIC ENGINEERING OF FINGER MILLET (*Eleusine coracana*) WITH *Aldose reductase* GENE ISOLATED FROM *Xerophyta viscosa* TO ENHANCE DROUGHT AND SALINITY TOLERANCE**

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## **DECLARATION**

I, Njeru Asunta Mukami, declare that the work presented in this thesis is my original work and has not been presented for a degree in any other University or Institute for any other award.

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## **DEDICATION**

I dedicate this work to my dear husband Wamalwa, my daughter Annabel, my parents Moses and Mary and my siblings for their love and support during research period.

## TABLE OF CONTENTS

<b>DECLARATION</b> .....	ii
<b>ACKNOWLEDGEMENT</b> .....	iii
<b>DEDICATION</b> .....	iv
<b>TABLE OF CONTENTS</b> .....	v
<b>LIST OF TABLES</b> .....	vii
<b>LIST OF FIGURES</b> .....	viii
<b>LIST OF APPENDICES</b> .....	ix
<b>ACCRONYMS AND ABBREVIATIONS</b> .....	x
<b>ABSTRACT</b> .....	xi
<b>CHAPTER ONE</b> .....	1
1.0 INTRODUCTION .....	1
1.1 Background of the Study .....	1
1.2 Statement of research problem.....	4
1.3. Objectives .....	4
1.4. Null Hypotheses.....	5
1.5 Justification of the study .....	5
<b>CHAPTER TWO</b> .....	6
2.0 LITERATURE REVIEW .....	6
2.1 Taxonomy and origin of finger millet.....	6
2.2 Agronomic conditions for finger millet cultivation .....	6
2.3 Economic importance of finger millet .....	7
2.4 Constrains of finger millet production.....	7
2.5 Plant drought tolerance mechanism .....	8
2.6 Plant salinity tolerance mechanism.....	10
2.7 Generation of drought and salinity tolerant finger millet.....	11
2.8 Plant tissue culture and <i>Organogenesis</i> .....	12
2.9 <i>Xerophyta viscosa</i> as a model for studying abiotic stress tolerance .....	13
2.10 The <i>XvAld1</i> genes .....	14
2.11 Strategies for expression of foreign genes in finger millet .....	15
<b>CHAPTER THREE</b> .....	18
3.0 MATERIALS AND METHODS.....	18
3.1 Direct Regeneration of finger millet .....	18
3.1.1 Plant material and explants preparation .....	18
3.1.2 Shoot induction, multiplication and elongation. ....	18
3.1.3 Root induction.....	19

3.1.4 Hardening and acclimatization.....	19
3.2 Genetic transformation.....	19
3.2.1 <i>Agrobacterium. tumefaciens</i> strain and expression plasmid .....	19
3.2.2 Finger millet transformation .....	20
3.3 Molecular analysis of putative transgenic finger millet plants .....	21
3.3.1 Detection of positive finger millet transformants .....	21
3.3.2 RNA isolation and reverse transcription-PCR .....	22
3.4 Drought tolerance assay .....	22
3.5 Salt tolerance assay .....	23
3.6 Data analysis .....	23
<b>CHAPTER FOUR.....</b>	<b>24</b>
4.0 RESULTS .....	24
4.1 Effects of MS medium on germination.....	24
4.2 Effect of shoot induction using shoot apical meristems.....	24
4.3. Root induction.....	27
4.4 Hardening and Acclimatization .....	28
4.5 Generation of transgenic finger millet .....	28
4.6 Detection of transgenic events by PCR analysis.....	30
4.7 Reverse transcription-polymerase chain reaction analysis .....	31
4.8 Germination rate during drought stress.....	31
4.9 Effects of drought stress on number of green leaves .....	34
4.10 Effects of drought stress on total chlorophyll content .....	36
4.11 Effects of salinity stress on germination .....	38
4.12 Effects of salinity stress on number of green leaves .....	40
4.13 Effects of salinity stress on total chlorophyll content. ....	42
<b>CHAPTER FIVE .....</b>	<b>45</b>
5.0 DISCUSSION .....	45
<b>CHAPTER SIX .....</b>	<b>53</b>
6.0 CONCLUSION AND RECOMMENDATIONS.....	53
6.1 Conclusions.....	53
6.2 Recommendations.....	53
<b>REFERENCES.....</b>	<b>54</b>
<b>APPENDICES .....</b>	<b>71</b>

## LIST OF TABLES

Table 4. 1 Germination efficiencies of six finger millet varieties .....	24
Table 4. 2 Shoot induction on BAP after 12 days of six finger millet varieties .....	26
Table 4. 3 Shoot multiplication on BAP after 24 days of six finger millet varieties ...	27
Table 4. 4 Root induction on IAA .....	28
Table 4. 5 Transformation and regeneration of XvAld1 finger millet lines .....	30
Table 4. 6 Effects of drought on germination .....	33
Table 4. 7 Effects of drought stress on number of green leaves .....	35
Table 4. 8 Effects of drought stress on total chlorophyll content .....	37
Table 4. 9 Effects of salinity stress on germination.....	39
Table 4. 10 Effects of salinity stress on the number of green leaves.....	41
Table 4. 11 Effects of salinity stress on total chlorophyll content.....	43

## LIST OF FIGURES

Figure 2.1 Appearance of the <i>X. viscosa</i> along the dehydration and rehydration .....	14
Figure 2.2 The polyol pathways.....	15
Figure 3.1 Plasmid vectors used in finger millet transformation.....	20
Figure 4.1 Finger millet plants obtained via direct regeneration.....	26
Figure 4.2 Transgenic finger millet plants obtained by direct regeneration .....	29
Figure 4.3 Amplification of XvAld1 from genomic DNA .....	30
Figure 4.4 RT-PCR analysis confirming expression of XvAld1 .....	31
Figure 4.5 Appearance of finger millet plants before and after drought stress.....	38
Figure 4.6 Appearance of finger millet plants before and after salinity stress .....	44



## LIST OF APPENDICES

Appendix 1 Surface sterilization protocol .....	71
Appendix 2 DNA extraction protocol.....	71
Appendix 3 Explant transformation.....	71
Appendix 4 XvAld1 Gene Primer Sequence (5'- 3').....	72
Appendix 5 Regeneration media.....	72

## ACCRONYMS AND ABBREVIATIONS

2,4-D	2, 4-Dichlorophenoxyacetic acid
AKR	Aldo-Keto Reductase
ANOVA	Analysis of variance
BAP	Benzylaminopurine
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of United Nations
IAA	Indole-3-acetic acid
LB	Luria-Bertani
MS	Murashige and Skoog
LB	Luria-Bertani
NADPH	Nicotinamide adenine dinucleotide phosphate
nosT	Nopaline synthase terminator
<i>nptII</i>	<i>neomycin phosphotransferase gene</i>
OD	Optical density
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse transcription PCR
SAMs	Shoot Apical Meristems
SIM	Shoot Induction Medium
SIS	Shoot Induction and Selection medium
T-DNA	Transfer DNA
v/v	Volume per Volume
w/v	Weight per volume
WT	Wild type

## ABSTRACT

Finger millet is chief food crop for millions of people in the world. It is ranked third in cereal production in semi-arid regions after sorghum and pearl millet. It is generally grown all over the world especially the developing countries with stable yield and strong adaptability to a number of agro-ecological environments. Despite its importance, finger millet increase in production is limited by abiotic stresses especially drought and salinity which affects the plant in the field during seed germination and early phases of seedling development. Unique plants such as *Xerophyta viscosa*, (a resurrection plant) that uses a number of physiological and molecular responses in order to survive under extreme stress conditions, are valuable sources of useful genes for crop improvement. Accordingly, *XvAld1* gene that encodes aldose reductase has previously been isolated from *X. Viscosa* under dehydration stress. The objective of this study was to establish a direct regeneration protocol of Kenyan finger millet varieties and develop transgenic drought and salinity tolerant finger millet plants expressing *XvAld1* gene via *Agrobacterium*- mediated transformation. Six finger millet varieties GBK043137, GBK043128, GBK043124, GBK043122, GBK043094 and GBK043050 were used. As a prerequisite, a rapid and reproducible direct regeneration protocol was established using shoot apical meristems. The study established that the highest shoot induction was obtained in MS media supplemented with 1.75mg/l BAP while highest rooting events was obtained in MS media supplemented with 4.0  $\mu$ M. In order to produce drought and salinity tolerant finger millet plants, *XvAld1* gene controlled by stress-inducible *XvPsap1* promoter was introduced into finger millet via *Agrobacterium*-mediated transformation and the transgenic events regenerated through direct organogenesis. The polymerase chain reaction (PCR) and the reverse transcription PCR (RT-PCR) confirmed the integration and the expression of *XvAld1* gene with 1 positive event recorded in each finger millet line. In order to evaluate the drought and salinity tolerance, the rate of germination, number of green leaves and the total chlorophyll content of the transgenic compared to the wildtype plants was examined under simulated drought and salinity stress using mannitol and sodium chloride respectively. The study established that transgenic plants were more tolerant to drought and salinity stresses than the wildtype plants. The results of this study demonstrate a rapid, adoptable and effective system to transform and regenerate finger millet plant. Genetic enhancement of finger millet will improve yield and ensure food security even during crop failure due to hostile weather conditions associated with climate change.

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Background of the Study

Finger millet, *Eleusine coracana* (L.) Gaertn.) is a valuable crop in Poaceae family and subfamily Chloridoideae (Gimode *et al.*, 2016). It is cultivated worldwide in more than four million ha with a total production of 5 million tons of grains and is a major food crop for millions of people in tropical and sub-tropical areas (Satish *et al.*, 2016). This crop comes third after sorghum and pearl millet in production of cereals in semi-arid areas of the world (Fetene *et al.*, 2011). The crop is grown as food grain both in Africa and South East Asia, mainly India and Nepal (Upadhyay *et al.*, 2006). In addition to its' use as human and animal feed, finger millet is likewise a raw material in the production ethanol (Tekaligne *et al.*, 2015). In comparison, finger millet has outstanding nutritional qualities as it is rich in phosphorus, cysteine, iron, tyrosine, calcium, tryptophan, and methionine than in other cereals (Sharma *et al.*, 2017).). Finger millet is mainly used to make beer, bread, soup, roti (flat bread) and for porridge in Africa. Other latest provisions prepared from this crop that has become common among younger generation include; vermicelli, pasta, noodles, sweet products, snacks among other bakery products.

Despite its many uses, farming of finger millet is limited in many areas of the world by agronomic practices and several unfavourable abiotic and biotic stresses (Saha *et al.*, 2016). Among the abiotic stresses are drought and salinity (Maharajan *et al.*, 2018). These two environmental stresses adversely affect potential yields as seed germination, seedling development and seed setting stages are susceptible to water scarcity and salt stress (Ramakrishnan *et al.*, 2017). With the current climate change perturbations, development of improved drought and salinity tolerant varieties to increase finger millet yields is desirable, particularly for dryland areas where water resources for agronomic use is a major problem.

Drought is among the chief limitations restricting production of crops globally (Shao *et al.*, 2009). Drought disturbs water relations, inhibits normal growth, and decreases water utilization efficiency in plants. The vulnerability of plants to water deficiency is different depending on of extent of stress, other factors accompanying the stress, plant species, and

plant developmental stages (Demirevska *et al.*, 2009). Plants exhibit different mechanisms of withstanding drought (Chaves and Oliveira, 2004). Such mechanisms include; reducing the rate at which plants lose water by increasing resistance to diffusion, increasing absorption with increased deep root systems, and reducing the size of leaves to decrease water loss through transpiration, production of osmolytes, such as proline, glycinebetaine, polyols and organic acids which play a critical role in supporting cell physiology under drought (Rhodes and Samaras, 1994). Plant growth regulators such as cytokinins, auxins, salicylic acid, gibberellins and abscisic acid are also produced in response to drought. Other substance that help plant adjust to drought stress include auxins, salicylic acid, cytokinins, gibberellins, and abscisic acid adjust plant responses toward drought. Citrulline, Polyamines, and several enzymes have antioxidant activity and help reduce unfavourable effects of water scarcity (Farooq *et al.*, 2012).

Though trait enhancement in finger millet is not widely reported, conventional improvement methods such as mutation breeding, mass selection and pure- line selection have previously been applied (Nandini *et al.*, 2010). Mass selection has been used in cultivar purification and development of varieties bred by pure- line or pedigree breeding. In this method, plants are specially chosen from farmers' varieties that are improved and the resultant plants with better characteristics such as resistance to pests and diseases, early maturity as well as improved grain yield are further tested and at different locations and eventually released as farmers' varieties. Pure- line selection has also been extensively employed in improvement of finger millet. In this approach, a single plant is selected from farmers' varieties and different characteristics tested such as grain yield and resistance to pests and diseases (Harinarayana, 1986). Mutation breeding has also been efficiently employed fast maturing varieties, making of polygenic variability and generation of complete/partial male- sterile lines. Gamma irradiations which may be physical, chemical and combinations of mutagens are employed for this purpose (Nayar *et al.*, 1979). Finger millet improvement using the aforementioned approaches has yielded limited success as the processes are slow, time consuming and laborious. To overcome this, genetic engineering provides a timelier and robust response to challenges that face finger millet production (Delmer, 2005).

The trait enhancement of finger millet through genetic engineering technology has been inadequate irrespective of its significance (Lata, 2015). Since Gupta (2001) reported

transformation of finger millet, only a few other reports are available to date on genetic enhancement of this crop (Ceasar and Ignacimuthu, 2009; Sharma *et al.*, 2011; Babu *et al.*, 2012; Jagga-Chugh *et al.*, 2012; Bayer *et al.*, 2014; Hema *et al.*, 2014 and Satish *et al.*, 2017). Two methods frequently employed in delivery of new genes into plants are biolistic and *Agrobacterium*-mediated transformations (Travella *et al.*, 2005). These approaches have been employed in development of transgenic finger millet plants.

*Agrobacterium tumefaciens*-mediated transformation method is frequently used for many plant species due to its efficiency, simplicity, and stability of the introduced gene (Joshi and Joshi, 1991; Hansen and Wright, 1999; Yu *et al.*, 2007). Most of *Agrobacterium*-mediated approaches depend on indirect organogenesis and regeneration of the explants. However, only one study has reported on direct regeneration and *Agrobacterium*-mediated transformation of finger millet crop (Satish *et al.*, 2017). The previous studies reported a cultivar-dependent response along with low transformation and regeneration efficiency. The success of genetic transformation of a crop species requires an effective and dependable transformation and regeneration procedure. *Agrobacterium tumefaciens*-mediated transformation method in combination with direct plant regeneration and with less cultivar-reliance is critically required for development of an efficient genetic engineering system which will ease biotechnological manipulation of this important crop.

Genes which can confer tolerance to abiotic stresses can be gotten from plants acknowledged as the resurrection plants (Griffiths *et al.*, 2014). These plants have developed means which aid them endure adverse humidity scarcity and are distinctive since they can endure drying of their vegetative tissues. These unique plants lose over 90% of their relative water content and endure in the dried state for prolonged periods and then recover when water is available again (Dinakar and Bartels, 2013; Griffiths *et al.*, 2014). A number of genes are expressed due to dehydration in resurrection plants. These genes generally code for proteins which avert stress-related damage of the cells and play a part in antioxidant defense (Farrant, 2007; Dinakar and Bartels, 2013).

Among the resurrection plants is the *Xerophyta viscosa*, a monocotyledonous plant with the ability to tolerate severe abiotic stress conditions (Vicré *et al.*, 2004). Investigations into

extreme drought tolerance in *X. viscosa* has provided understandings to the desiccation tolerance mechanisms utilized by these plants to assist them survive under extremely adverse environmental conditions. Several genes have been isolated from *X. viscosa* that conferred functional adequacy to osmotically stressed *E. coli* (srl: Tn10) (Mundree *et al.*, 2000). Among these genes are the *XvAld1* gene (Maredza, 2007) which encode aldose reductase. This study therefore sought to generate drought and salinity tolerant finger millet plants expressing *XvAld1* transgenes driven by a stress-inducible *XvPsap1* promoter (Oduor, 2009) via *Agrobacterium*-mediated transformation.

## **1.2 Statement of research problem**

Finger millet is an economically important food crop in arid and semi-arid areas of the world where its production is constrained by biotic and abiotic constraints such as drought and salinity. To achieve this, conventional breeding strategies such as pure-line breeding, mutational breeding and hybridization have been employed. However, these methods are time consuming, laborious and yield limited success. As an alternative, genetic engineering which allows direct incorporation of genes of interest into host plant genome offers a rapid improvement strategy of this crop to tolerate production stresses.

## **1.3. Objectives**

### **1.3.1 General objective**

The goal of this study was to generate transgenic finger millet germplasm with enhanced drought and salinity tolerance for better food security, incomes and overall livelihoods.

### 1.3.2 Specific objectives

- i. To optimise a rapid and reproducible direct organogenesis and regeneration protocol for Kenyan farmers preferred finger millet varieties.
- ii. To genetically transform selected Kenyan finger millet varieties with *XvAld1* gene via *A. tumefaciens* - mediated transformation.
- iii. To evaluate drought and salinity tolerance in transgenic finger millet plants expressing *XvAld1* gene.

### 1.4. Null Hypotheses

The null hypotheses for the study were;

- i. Kenyan farmer preferred finger millet varieties cannot be regenerated via direct organogenesis.
- ii. Kenyan farmer preferred finger millet varieties are not transformable with *XvAld1* gene via *Agrobacterium tumefaciens*-mediated transformation.
- iii. The ability of *XvAld1* transgene to confer improved drought and salinity tolerance in transgenic finger millet cannot be evaluated.

### 1.5 Justification of the study

The use of modern crop improvement biotechnology provides a judicious response to the crop production threats such as drought and salinity stress. Genetic enhancement of finger millet varieties ensures improved food security and overall livelihoods in arid and semi-arid areas of the world. There is therefore a need for genetic improvement of finger millet by transferring drought and salinity resistance genes to finger millet plants. The development of transgenic finger millet by transferring genes such as *XvAld1* is one of the best options available to overcome water and salinity stress arising from climate change and increase the yield of finger millet.



## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Taxonomy and origin of finger millet

Finger millet belongs to Poaceae family of monocotyledonae group and subfamily Chloridoideae (Dida *et al.*, 2008). It is an allotetraploid ( $2n = 4 \times = 36$ ) with AABB genome. It is normally recognised that the A genome originator is the wild diploid species *E. indica* ( $2n = 2 \times = 18$ ) or one of its close relatives, including *E. tristachya*, while the B genome originator is unidentified or most likely extinct (Liu *et al.*, 2011). Liable on cytogenetic, morphological, and molecular proofs, it's held that today's finger millet (*E. coracana* sub species coracana) was cultivated from undomesticated finger millet (*E. coracaca* sub species *africana*) populations (Hilu and Johnson, 1992). The *E. coracana* subsp. coracana was grown around 5000 years ago in Ethiopia and western Uganda highlands. Subsequently, finger millet was introduced into Western Ghats of India about 3000 BC. Thus, India became the secondary centre of finger millet diversity.

#### 2.2 Agronomic conditions for finger millet cultivation

*Eleusine coracana* is a monocotyledonous, herbaceous annual grass that grows up to 170 cm high (FAO, 2012). It produces a cluster of flowers recognized as panicles that have uneven number of spikes between 3 and 20 and set in a bird's foot style. The panicles resemble the hand fingers, hence the name "finger millet". Finger millet is a quick-growing plant and ripens in 3 to 6 months and infrequently within 45 days (Dida and Devos, 2006). It is normally grown in altitudes between 1000 and 2000 in Southern and Eastern parts of Africa and, likewise up to 2500-3000 m in the Himalayas (FAO, 2012). It does best temperature of 23°C though it can tolerate cooler and hotter conditions (FAO, 2012), as long as it is well spread across the growing season finger millet grows well in annual rainfall ranging between 500 and 1000mm (Dida and Devos, 2006). Finger millet can acclimatize to varying soil conditions but does better in fertile, sandy to sandy loam soils that are well drained and with a PH between 5 and 7. However, it can grow in black heavy vertisols or lateritic and has some levels of endurance to moderately saline soils and alkaline soils (Dida and Devos, 2006).

### **2.3 Economic importance of finger millet**

Finger millet is an essential crop economically. Its' seeds can be stored for long periods as 10 years thus warranting sustainable food supply even when the harvest fails (Mgonja *et al.*, 2007). It is made into a variety of products, particularly in the rural areas. Among these are porridge, soup, bread, beer and roti (flat bread). Other latest provisions prepared from finger millet that has become common among younger generation include, noodles, vermicelli sweet products, pasta, snacks and different bakery products. It is a vital diet for breastfeeding and pregnant women and kids in addition to the financial system of subsistence farmers. It has exceptionally good nutritional qualities when compared to other cereals since the grain is rich in iron, calcium, phosphorus, cysteine, tryptophan, tyrosine, and methionine (Latha *et al.*, 2005). The plant straw is also useful as animal feed containing up to a sum of 60% useful nutrients. Finger millet helps in activating some physiological functions of human body so as to prevent serious diseases like intestinal cancer, diabetes, constipation, and high cholesterol formation due to its elevated polyphenols which shows anti-oxidant action and elevated fibre which promotes unhurried absorption and blood sugar activity (Devi *et al.*, 2014). Finger millet plant has well been used as conventional solution to different diseases including pneumonia, leprosy, small pox and measles, and (Dida & Devos, 2006). In addition to its use as foodstuff, finger millet is also used in the production of ethanol (Tekaligne *et al.*, 2015).

### **2.4 Constrains of finger millet production**

Despite the many advantages offered by the cultivation of finger millet, production is mostly in developing countries and by resource poor farmers (Lule *et al.*, 2012). The production of finger millet is significantly hampered by such factors as low yielding varieties, high weed infestation pests and diseases, labour intensity, low soil fertility, high weed infestation, lodging, poor attitude to the crop, salinity and drought emanating from climate change (Mgonja *et al.*, 2007).

Among the most important biotic factors of finger millet production worldwide is blast disease. Blast is caused by *Pyriculariagrisea* and causes up to 90% loss in yield (Mgonja *et al.*, 2007; Nagaraja *et al.*, 2007) as it affects the neck and panicles (Takan *et al.*, 2012). Other serious biotic constraints to production of finger millet in Kenya and globally are insect pests mainly shoot fly (*Atherigonaspp*) and stem borers (*Busseolaspp*, *Chilospp* and *Sesamiaspp*) in addition to weeds. The most important weeds associated with the crop are *Striga hermonthica*

and *Eleusine indica* (Owere *et al.*, 2014). Under poor management conditions, these weeds compete with finger millet for light, water and nutrients subsequently depriving finger millet its essential nutrients and the plants become weak and give poor yields.

The abiotic constraints which significantly affect finger millet production include drought and salinity. Although finger millet has been documented to tolerate drought, prolonged and frequent dry spells and erratic rainfall result into substantial yield reduction (Hebbar *et al.*, 1992) since major areas of cultivation are rain-fed. Drought negatively impacts on growth, establishment, survival and yield of crops thus leading to significant threat to sustainable farming (Barnabás, 2008). Despite the fact that finger millet can tolerate acidic soils (Barbeau and Hilu, 1993), its productivity is highly affected by salinity stress which reduces potential yields (Shailaja and Thirumeni, 2007). High salt concentrations decrease osmotic potential of solutions in the thus generating water deficiency in plants and finally cause ion toxicity.

## **2.5 Plant drought tolerance mechanism**

In marginalized areas where millet is the main cereal crop, water scarcity is the main biotic stress influencing production and ecological widespread leading to significant economic losses. Plants react, adjust and endure during water deficiency by initiating a range of biochemical, physiological and morphological responses (Wang *et al.*, 2001). Drought tolerance is the capability to grow, flower and show economic yield under reduced water supply. Drought stress adversely influence the water relations of plants at all levels of growth and development leading to harm or adjustment responses (Beck *et al.*, 2007). In order to survive drought stress, tolerant plants induce protection strategies against water scarcity (Chaves and Oliveira, 2004).

Morphological mechanisms include; escape and avoidance. Escape from drought occurs via a reduced maturity period or growing season, ensuring reproduction to occur prior the environment becomes dry (Kooyers, 2015). Drought avoidance occurs when plants retain water absorption via a widespread and abundant root system (Turner *et al.*, 2001; Kavar *et al.*, 2007). The root attributes like density, depth, biomass and length, are the major drought averting characteristics which contribute to ultimate productivity during adverse conditions

(Turner *et al.*, 2001). Prolific and extensive root system helps in the water extraction from substantial depths (Kavar *et al.*, 2007).

Plant physiological systems include; reduction of water loss by controlling stomatal transpiration, scavenging defence system, antioxidation osmoprotection and osmotic adjustment. Osmotic adjustment enables plant cell to reduce osmotic potential hence increasing the water gradient. This ensures water entry and balancing of turgor. This mechanism helps maintain functional activities during prolonged times of water scarcity (Sharp *et al.*, 1990). The antioxidant protection mechanism in plant cell comprises of enzymatic and non-enzymatic components. Enzymes involved in drought defence include peroxidase, superoxide catalase, dismutase, and ascorbate peroxidase and glutathione reductase. Other substances include reduced glutathione, cystein and ascorbic acid (Gong *et al.*, 2005). There is a number of lipid-soluble and water-soluble scavenging molecules and/or antioxidant enzymes helps eliminate reactive oxygen species in plants (Hasegawa *et al.*, 2000). The antioxidant enzymes are the main effective strategies to overcome stress due ROS (Farooq *et al.*, 2008).

At the molecular level tolerant plants vary the expression of genes with regard to water deficiency. Different genes are initiated in relation to drought at the transcriptional level, and resultant gene products are believed to have a role in endurance to water scarcity (Kavar *et al.*, 2007). Some of the products that have been reported include stress proteins and Aquaporins. Aquaporins exhibit the capability to aid and control exchange of water between cell membranes. They are present in abundance in the vacuolar and cell membranes. Production of stress proteins is a universal reaction in plants for survival in existing adverse environments which includes water scarcity. Majority of these proteins are soluble in water and thus have a role with regard to the stress adaptation aspect by hydrating the cell organelles (Wahid *et al.*, 2007). Proteins such as heat shock proteins functions in maintaining the structure of other proteins. These proteins are synthesized in response to environmental stress, mainly elevated temperature (Wahid *et al.*, 2007).

Late embryogenic rich proteins and membrane-stabilizing proteins are other useful proteins that confer drought resistance. These proteins enhance the water trapping ability through building a shielded environment for other proteins or structures, known as dehydrins. In addition, they as well function in ions sequestration which is then increased during cell dehydration (Gorantla *et al.*, 2006). These proteins function to prevent breakdown of partner protein by proteinases as well as elimination of denatured and broken proteins.

## **2.6 Plant salinity tolerance mechanism**

Based on adaptive evolution, plants are grouped as halophytes (which can endure salinity) and the glycophytes (which cannot endure salinity and finally die). Most cultivated plants are glycophytes and since they cannot endure salinity stress, it becomes chief adverse environmental stresses which affects crop production globally (Munns and Tester, 2008). Soil salinity is identified as inhibitor of plant growth through osmotic stress which leads to ion toxicity (James *et al.*, 2011). Initially due to salinity stress, water intake ability of roots is reduced and water loss in the leaves is increased as there is increased salt buildup in soil and plants. Amongst the main harmful results of salinity stress is the buildup of Na<sup>+</sup> and Cl<sup>-</sup> ions in plant tissues growing in medium containing elevated NaCl concentrations. Influx of both Na<sup>+</sup> and Cl<sup>-</sup> into the cells results to ion disequilibrium and too much uptake can lead to important functional problems. Elevated Na<sup>+</sup> concentration prevents the absorption of K<sup>+</sup> ions that are crucial factor for plant health and this leads to lowered production and likewise plant death (James *et al.*, 2011).

Plants also respond to salinity stress by generating, hydrogen peroxidase (H<sub>2</sub>O<sub>2</sub>), reactive oxygen species (ROS) and hydroxyl radicals (OH) and. Reactive Oxygen Species bring about oxidative damage to numerous cell compositions including membrane lipids, proteins, nucleic acids, and chlorophyll (Mishra *et al.*, 2011). Plants defend themselves from oxidative damage due to ROS by use of both non-enzymatic and enzymatic defense mechanism. Some of ROS scavenging enzymes in plants include superoxide, guaiacol peroxidase, dismutase, catalase, glutathione reductase and ascorbate peroxidase, and the balance and function of these enzymes help in suppressing toxic ROS within cells (Misra and Gupta, 2005).

Non-enzymatic defense mechanism includes; osmotic adjustment, ion-selective absorption and compartmentalization. Plants increase their osmotic potential by accumulating friendly organic solutes such as organic acids, carbohydrates, and quaternary ammonium compounds such as glycine betaine and proline (Ashraf and Foolad, 2007). Carbohydrates act as water replacement molecules (Crowe *et al.*, 1987), facilitate production of intracellular glasses that put a status on metabolism, minimize ROS-associated molecular alterations (Berjak *et al.*, 2007) and ameliorate the concentration effects of salts and ions accumulated in the vacuole (Munns, 2002).

Salt stress results into ion toxicity in plants through an increased inflow of  $\text{Na}^+$  into cells which results into intracellular ion disequilibrium. Ion toxicity adversely affects cytosolic enzyme activities, nutrition, metabolism and photosynthetic function in plants (Flowers, 2004). Plants under  $\text{Na}^+$  toxicity lower intracellular  $\text{K}^+$  thus balancing  $\text{K}^+$  in the cytoplasm in an environment with elevated  $\text{Na}^+$  concentration. The plants also release  $\text{Na}^+$  from the cells or move it to the less active metabolic areas.  $\text{Na}^+/\text{H}^+$  antiporter in the vacuole is responsible for separating of  $\text{Na}^+$  to the vacuole thus decreasing their concentrations in the cytoplasm (Gaxiola *et al.*, 1999). The cell membrane  $\text{Na}^+/\text{H}^+$  antiporter is responsible for moving of excess  $\text{Na}^+$  from the cells to the external environments.

Controlling of the expression of gene in relation to salinity stress involves wide range of strategies that are applied by plants to upregulate or downregulate the synthesis of definite gene products (RNA or protein). Numerous genes and transcription factors are increased due to salinity stress in different plant species and they serve different functions (Chakraborty *et al.*, 2012). Genetic variations in salt endurance exist, and the rate of salt tolerance differs with species and varieties within a species.

## **2.7 Generation of drought and salinity tolerant finger millet**

Water scarcity and salinity stress are the chief environmental factors involved in reduced crop production (Wani and Gosal, 2011). Improvement in the adaptability to salinity and drought in finger millet could increase yield and increase cultivation area in the world. Despite that traditional breeding has considerably helped to trait upgrading in finger millet, integrating attributes from stress tolerant germplasm lines into farmers' varieties by traditional breeding

yield limited success and is time consuming (Jayaprakash *et al.*, 1992). This limited success is due to the complexity of stress tolerant attributes. Alternatively, genetic engineering provides a considerable potential for upgrading the finger millet as specific alterations in the genome can be attained in a short duration and the transgenics can provide the required traits without losing the of genetic integrity (Tang *et al.*, 2000). Currently, only a limited number of reports are available on expression of genes conferring tolerance to drought and salinity stress in finger millet; Mahalakshmi *et al.*, (2006), Hema *et al.*, (2014) and Jayasudha *et al.* (2014).

## **2.8 Plant tissue culture and *Organogenesis***

Tissue culture is the production of plant cells, tissues or organs on specially prepared nutrient media in septic environment and regulated conditions of light, humidity and temperature (Kumar and Rao, 2012). The capability to grow plant cells and tissues and to regulate their growth forms the centre of many useful applications including genetic engineering (Sharma *et al.*, 2011). However, there is no universal protocol for species. The three main ways of using tissue culture deliver new gene into plants cells and regenerate into plants are through protoplast, somatic embryo and direct organogenesis (Narusaka *et al.*, 2012). The success of protoplast and somatic embryo methods are explant and species dependent.

Organogenesis occur either directly or indirectly. In direct organogenesis, already differentiated cells dedifferentiate and then redifferentiate to produce shoot and root meristems. Cytokinins are used for direct organogenesis while combination of cytokinins and auxins are used for indirect shoot organogenesis (Pati *et al.*, 2004). Direct organogenesis through shoot-tips has been reported to be a successful and cultivar independent method. However, direct organogenesis systems can bring about generation of chimeras (Lou *et al.*, 2006). Regeneration via direct organogenesis has been achieved in many plant species including finger millet (Satish, 2015).

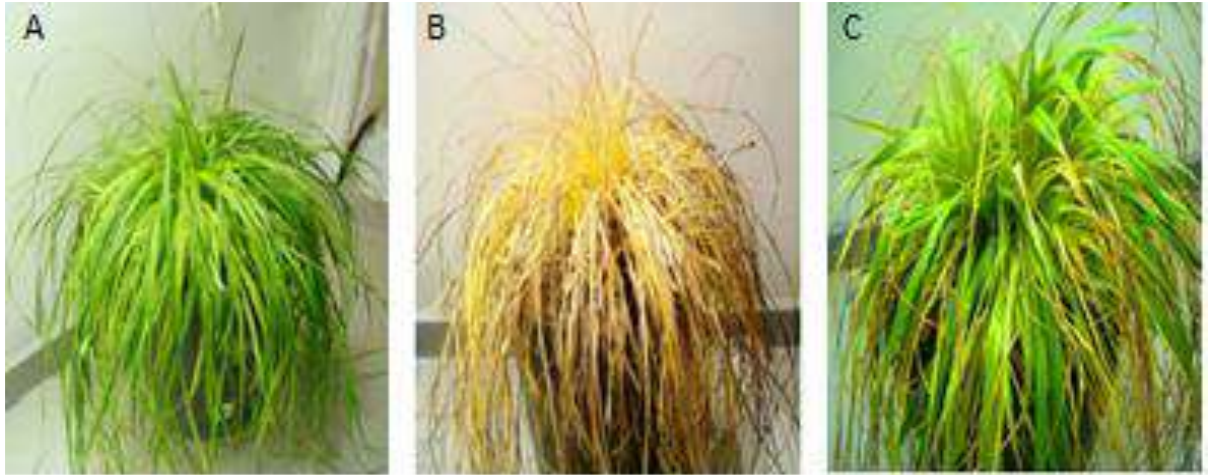
Indirect organogenesis occurs through somatic embryogenesis in which plant is grown from callus or calli. Somatic cell form structures that resemble zygote which eventually form plants (Jiménez, 2005). Somatic embryogenesis can be induced using two systems; directly and indirectly. Directly, tissues are employed as explants whereby plants are genetically

indistinguishable and indirectly by use of undifferentiated cells. Somatic embryogenesis is induced by body cells derived from whichever part of the plant. Usually cells that are highly capable produce somatic embryos are the ones obtained from immature tissues and young zygotic embryos. Moreover, leaves, stem and roots, stem, and can employed too. Somatic embryos are normally initiated by simple manipulations of the *in vitro* culture conditions. One of the chief essentials in the culture medium is the plant growth hormones such as gibberellins, abscisic acid, cytokinins and auxins and among other components. Amongst the aforementioned growth hormones, auxins are the commonly used elements in the induction of the process (Jiménez, and Thomas, 2006).

### **2.9 *Xerophyta viscosa* as a model for studying abiotic stress tolerance**

*Xerophyta viscosa* (Baker) is a monocotyledonous resurrection plant from the family *Vellociaceae* found in summer-rainfall areas of Lesotho, Swaziland and South Africa (Farrant *et al.*, 2015). It occupies rocky lands in bare grasslands where it regularly encounters times of water scarcity. This plant can tolerate dehydration to an air-dry state, but rehydrate between 24 and 80 hours upon rewatering (Mundree *et al.*, 2002) as shown in **Fig 2.1**. This unique trend can therefore be efficiently used to study abiotic stress tolerance. *Xerophyta viscosa* also serves like a fine model scheme to study drought tolerance and for the detection of new genes which can be employed for genetic upgrading of crop varieties (Mundree *et al.*, 2006). *Xerophyta viscosa* has been broadly investigated in order to understand the molecular system of dehydration adaptation (Farrant, 2000; Mundree *et al.*, 2000; Marais *et al.*, 2004; Walford *et al.*, 2004; Mundree *et al.*, 2006; Garwe *et al.*, 2006; Ingle *et al.*, 2007; Iyer *et al.*, 2008). From these studies, several interesting genes have been isolated in *X. viscosa* which are up-regulated due to different abiotic stresses and among them is *XvAld1*. This gene has been transformed into dicots like tobacco and *A. thaliana* they confer abiotic stress endurance to the resulting transgenes under various environmental stress (Garwe *et al.*, 2006; Govender *et al.*, 2016; Mundree *et al.* 2006; Maredza, 2007; Kumar *et al.* 2013b).





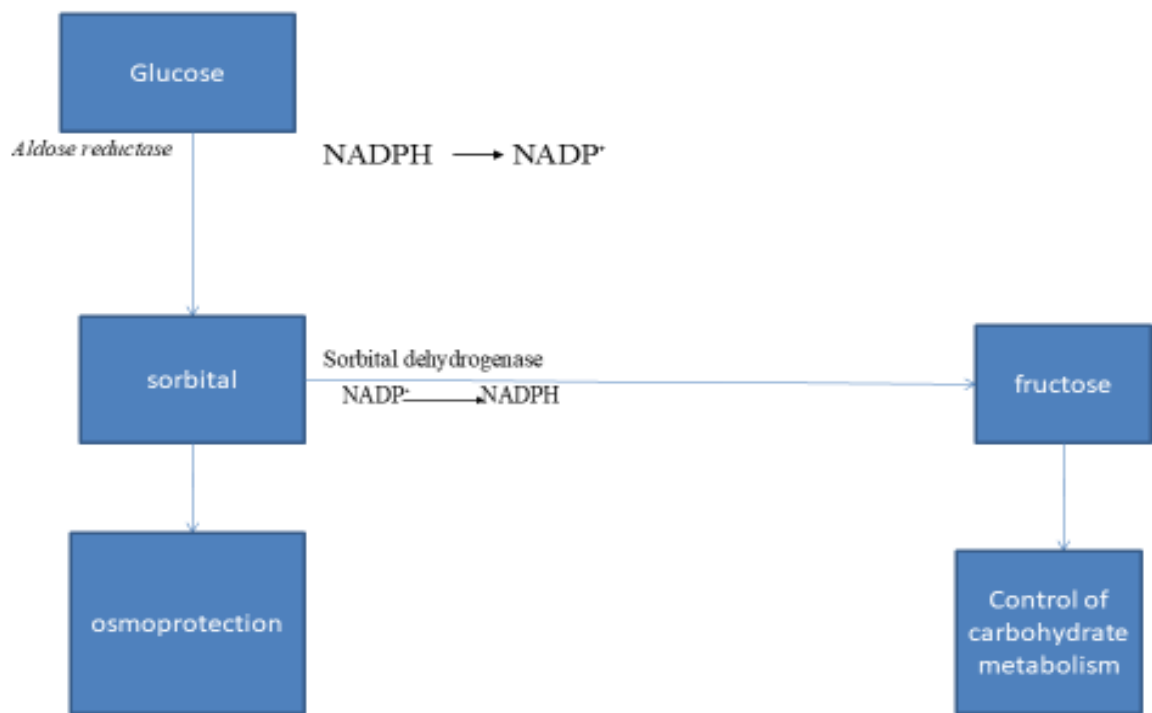
**Figure 2.1 Appearance of the *X. viscosa* along the dehydration and rehydration.** A. Potted fully hydrated (100% RWC) *X. viscosa* plant in a controlled environment. B. The same plant dehydrated at 5% RWC. C. The same plant 80 hours after rewatering (Abdalla, 2009).

### 2.10 The *XvAld1* genes

Plant genes that are homologous to aldose reductase have been isolated from various species including some resurrection plants (Negm, 1986; Bartels *et al.*, 1991; Lee and Chen, 1993; Li and Foley, 1995; Roncarati *et al.*, 1995; Mundree *et al.*, 2000; Gavidia *et al.*, 2002). Aldose reductases may have multiple physiological functions that may interact with defence mechanisms to counter oxidative and osmotic stress. The *X. viscosa* aldose reductase, *XvAld1* (AF133841; Mundree *et al.*, 2000), which is a member of the aldo-keto reductase (AKR) superfamily it's a 36-kDa monomeric protein (Mundree *et al.*, 2000). According to Maredza, (2007), *XvAld1* gene is organised into nine exons and eight introns spanning ~2.9 kb, excluding the 5' and 3' untranslated regions (UTRs). The southern blot analysis also demonstrated the existence of other *XvAld1* related genes in *X. viscosa*.

The physiological roles of many AKR proteins are largely unknown and little is known about how the expression of *XvAld1* is regulated in *X. viscosa*. However, in a study to investigate how *XvAld1* gene is regulated in *X. viscosa* (Maredza,2007), it was found that the expression of *XvAld1* is partly regulated by hormones. Hormones control many characteristics of plant growth and development, in addition to playing a role in abiotic and biotic stress signalling. Expression of *XvAld1* is induced by dehydration and it is expressed in many different tissues (stems, meristems, mesophyll, vascular tissue, guard cells) depending on the prevailing stress

conditions or developmental stages of plants. The *XvAld1* gene product is involved in the catalytic reaction of reducing D-glucose to its corresponding alcohol, sorbitol that is metabolically converted to D-fructose by sorbitol dehydrogenase (**Figure 2.2**). Sorbitol is linked with stabilizing of osmotic equilibrium in the cytoplasm as well as safeguarding of macromolecules in both plants and animal systems, when under desiccation pressure (Singh *et al.*, 2015).



**Figure 2.2 The polyol pathways.** Glucose is converted to sorbitol by aldose reductase using a cofactor NADPH. The sorbitol that accumulates in the plant cell leads to osmoprotection. Sorbitol dehydrogenase catalyses metabolism of sorbitol to fructose which is used as a source of energy or as a second messenger for the control of carbohydrate metabolism (Maredza, 2007).

### 2.11 Strategies for expression of foreign genes in finger millet

Genetic enhancement of finger millet is limited when compared to the efforts made for other major cereals (Ceasar and Ignacimuthu, 2009). Gupta *et al.* (2001) initiated the earliest effort on transformation of finger millet using biolistic method by comparing the efficiency of five gene promoters (cauliflower mosaic virus 35s(CaMV35S), rice

actin gene promoter(ActI), maize ubiquitin(UqI), ribulose-1,5-biophosphite carboxylase small subunit gene promoter(RbcS), Flaveria trinervia  $\beta$ -glucuronidase (*FtuidA*) on the expression of the  $\beta$ -glucuronidase (GUS) reporter gene.

Following this study, a few studies on regeneration and transformation were reported. Latha *et al.*, (2005) transformed finger millet using PIN gene via biolistic method. Porteresia coarctata's serine-rich protein (PcSrp) gene was overexpressed in finger millet under salinity condition (Mahalakshmi *et al.*, 2006). The transgenic finger millet treated with 250Mm NaCl exhibited normal growth, flower and seed rescuing from saline conditions. Ignacimuthu and Ceasar (2012) introduced a rice Chitinase 11 gene (Chi11) into genotype GPU45 of finger millet through *Agrobacterium*- mediated transformation to develop leaf blast resistance. The transgenic plants overexpressing foreign gene exhibited resistance to leaf blast disease compared to non-transformed control plants. Jayasudha *et al.* (2014) produced a transgenic finger millet by introducing Na<sup>+</sup>/H<sup>+</sup> antiporter of *Pennisetum glaucum* (PgNHX1) and *Arabidopsis thaliana* vacuolar H<sup>+</sup>-pyrophosphatase (AVP1) for salinity stress tolerance through *Agrobacterium*-mediated transformation. The transgenic finger millet showed higher level of salinity tolerance compared to wild type plants. Transgenic finger millet expressing a bacterial mannitol-1-phosphate dehydrogenase (mtlD) gene was developed through *Agrobacterium*-mediated transformation (Hema *et al.*, 2014). Transgenic finger millet plants expressing mtlD gene had better growth under drought and salinity stress compared to wild-type. The transgenic plants also showed better osmotic stress tolerance with chlorophyll retention under drought stress compared to the wild-type plants (Hema *et al.*, 2014).

It is evident that only a limited number of reports are available on expression of genes conferring tolerance to drought and salinity stress in finger millet. Currently, only one report (Satish *et al.*, 2017) is available on *Agrobacterium*-mediated transformation of finger millet shoot apical meristems (SAMs) and direct regeneration with no callus mediated phase after *Agrobacterium*-mediated transformation. Such a transformation system, with direct plant regeneration and less genotype dependence, is very much needed for finger millet. The development of an effective genetic transformation method across finger millet cultivars will enable rapid and effective production of

transgenic finger millet plants improved the yield. In the present study, a rapid and reproducible protocol was validated and *XvAld1* was engineered into selected six finger millet varieties to confer resistance to drought and salinity.

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Direct Regeneration of finger millet

##### 3.1.1 Plant material and explants preparation

Six Kenyan farmers preferred finger millet varieties were used: GBK-043122, GBK-043124, GBK-043128, GBK-043137, GBK-043050 and GBK-043094. The seeds of these varieties were procured from Kenya Agricultural and Livestock Research Organization, Gene Bank, Nairobi, Kenya. All seeds were surface sterilized by washing with sterile distilled water followed by incubating them with 70 % (v/v) ethanol for two minutes then transferred to 20% sodium hypochlorite containing a drop of Polysorbate 20 for 23 minutes. Surface sterilized seeds were rinsed thrice with double distilled water and germinated aseptically on Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) supplemented with 30% sucrose. The medium pH was adjusted to 5.8 before adding 3 g/l gelrite, followed by autoclaving at 121°C for 15 minutes under 15 kPa. The cultures were incubated at 25±2°C in the dark for germination for three days. The germination efficiency of the six varieties was calculated after 3 days of culture.

##### 3.1.2 Shoot induction, multiplication and elongation.

Aseptically grown 3-day-old shoot tips, comprising of the apex and a part of mesocotyl, were excised and utilized as explants. 15 shoot tips (4-6mm) per variety were cultured on shoot induction medium (SIM) comprising of MS basal medium supplemented with 30% sucrose and different concentrations of 6-benzylaminopurine (BAP) (1, 1.5, 1.75 and 2mg/l). Shoot tips cultured in MS media devoid of BAP was used as control. The culture bottles were incubated in the growth room with 16/8-hours light/dark at 26±2°C for 12 days. Twelve days after culture, the formed shoots (10 shoots per variety) were transferred to fresh SIM and incubated in the growth room with 16/8-hours light/dark at 26±2°C for a further 12 days to elongate the induced shoots. The percentages of the number of shoots that formed in each shoot clump and the mean number of shoots induced in each explant were calculated following 24 days of culture.

### 3.1.3 Root induction

The elongated shoots 5-6cm (10 shoots per variety) were transferred to rooting medium comprising of MS basal medium supplemented with 30% sucrose and various concentrations of indole-3- acetic acid (IAA;1, 2, 3, 4 and 5  $\mu$ M). The plantlets were then incubated in the growth room under light16/8-hours light/dark at  $26\pm 2^{\circ}\text{C}$  to induce rooting. The total number of roots initiated per shoot was calculated after 28 days of culture. Shoots cultured on MS devoid of IAA were used as control.

### 3.1.4 Hardening and acclimatization

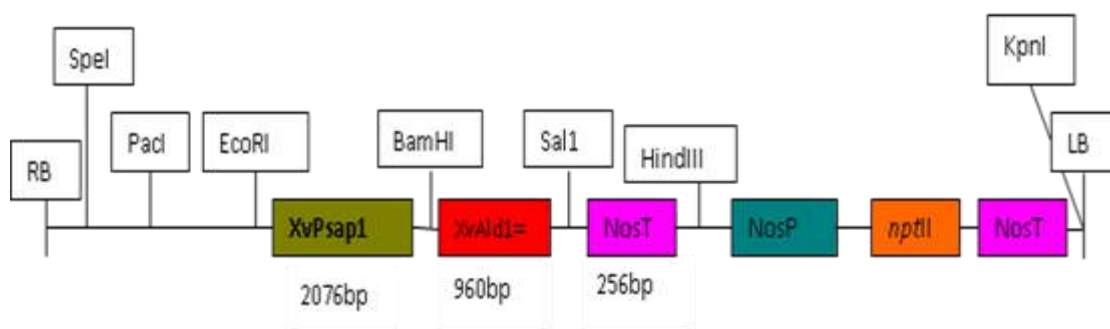
Rooted plants were washed with double distilled water to remove medium on the plantlets. The plants were thereafter transferred into peat moss in plastic cups (11 $\times$ 15 cm) for hardening for 5 days after which the plants were transferred to soil in pots and incubated in greenhouse for acclimatization. The plants were watered regularly and data on survival rate of plants recorded after 4 weeks of culture. Plant survival rate was calculated as:

$$\text{Survival rate} = \frac{\text{surviving plants}}{\text{total plantlets}} \times 100 \text{ (Ko et al., 2008)}$$

## 3.2 Genetic transformation

### 3.2.1 *Agrobacterium tumefaciens* strain and expression plasmid

*Agrobacterium tumefaciens* strain EHA101 bearing the standard binary vector (kindly provided by Dr. Wilton, Pwani University) was used. The pNOV2819 vector (Syngenta Biotech. Laboratory, North Carolina, USA) was used. The study used a stress inducible promoter XvPsap1 to drive the expression of *XvAld1* gene and terminated by nopaline synthase (*nosT*) terminator whereas *nopaline synthase* promoter (*nosP*) was used to control the *neomycin phosphotransferase (nptII)* plant selectable marker gene for selection with kanamycin (**Figure 3.1**). The stress inducible promoter XvPsap1 and the gene of interest XvAld1 (kindly provided by Prof. Jennifer Thomson, Department of Molecular and Cell Biology, University of Cape Town, South Africa) had previously been isolated from *X. viscosa*.



**Figure 3.1 Plasmid vectors used in finger millet transformation.** XvAld11 T-DNA. **RB**, right border of T-DNA; **XvPSap1**, stress inducible promoter from *X. viscosa*; **nptII**, neomycin phosphotransferase gene for plant kanamycin resistance; **NosT**, nopaline synthase terminator; **XvSap1**-gene from *X. viscosa*; **LB**, left border of T-DNA.

### 3.2.2 Finger millet transformation

The transformation procedure used for transformation and regeneration was a modification of a protocol described by Satish *et al.*, (2017). A single colony of the *Agrobacterium* strain was grown overnight at 28 °C in Luria-Bertani (LB) media (pH 7.2) containing 100 mg/l streptomycin and 100 mg/l kanamycin, to late log phase (Optical Density = 1.0–1.5). The bacterial cells were collected by centrifugation, resuspended in infection media comprising of MS basal salts + 30 g/l of sucrose and acetosyringone (100 µM), pH 5.8 and left on the shaker for 4 hours. Aseptically grown 3-day-old shoots tips (15 per variety), consisting of the apex and part of mesocotyl, were excised and placed in the *Agrobacterium* cell suspension for 20 minutes, and then air dried briefly. The *Agrobacterium*-inoculated explants were then blot-dried, and incubated on co-cultivation media comprising of MS basal salts + 100 µM of acetosyringone, 1.75mg/l BAP, 0.9 µM 2, 4-dichlorophenoxyacetic acid (2,4-D), 500 mg/l casein enzymichydrolysate, 750 mg/l proline, 2.0 mg/l glycine, 30 g/l of sucrose, 3% gelrite, pH 5.8 for 5 days in the dark at 25 ± 2°C.

#### 3.2.2.1 Shoot induction

Five days after transformation, the explants were washed with MS basal salts supplemented 150 mg/l carbenicillin and 150 mg/l cefotaxime for 15 minutes to remove excess *Agrobacterium*. The explants were then transferred to shoot induction and selection (SIS) medium comprising of MS basal salts + 1.75mg/l BAP, 0.9 µM 2,4-D, 500 mg/l casein

enzymichydrolysate, 750 mg/l proline, 2 mg/l glycine, 100 mg/l kanamycin, 150 mg/l cefotaxime, 30 g/l of sucrose, and 3% gelrite, pH 5.8 for 12 days in the growth room under (16/8 light/dark). Uninfected shoot tips were incubated in shoot induction and selection media (SIS) and MS media devoid of kanamycin and used as a negative control.

### **3.2.2.2 Shoot elongation and root induction**

After 12 days the formed shoots were incubated onto MS basal salts supplemented with 11.0  $\mu$ M BAP, 0.9  $\mu$ M 2, 4-D, 500 mg/l casein enzymichydrolysate, 750 mg/l proline, 2 mg/l glycine, 100 mg/l kanamycin, 150 mg/l cefotaxime, 30 g/l of sucrose, 3 g/l gelrite, pH 5.8 for 12 days in light to elongate the shoots. To initiate rooting, the elongated shoots (5-6cm) were transferred to the half-strength MS basal salts supplemented with 3  $\mu$ M IAA, 30 mg/l kanamycin, 1.5% (w/v) sucrose and 4.5% (w/v) gelrite, pH 5.8 for 28 days for rooting.

### **3.2.2.3 Hardening and Acclimatization**

Rooted plants were rinsed with sterile water to remove the excess medium. The plants were then transferred into jiffy cups containing peat moss for hardening and acclimatization after which the plants were grown to maturity in the greenhouse.

## **3.3 Molecular analysis of putative transgenic finger millet plants**

### **3.3.1 Detection of positive finger millet transformants**

Genomic DNA was isolated from the putative transformants and the untransformed wild plants using DNA extraction kit (Thermoscientific) following manufacturers' instruction and quantified by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). The XvAld1 primers: Forward: 5'-GGGCACTCAATCCCCGCAGTT-3', Reverse: 5'-TCTCCATCTGGCACACCGAG-3') were used to amplify the XvAld1 gene coding sequences in putative transformants. Thermal-cycling parameters were as follows: after an initial denaturation at 95°C for 2 minutes, samples were subjected to a cycling regime of 30 cycles (denaturation 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 60 seconds). At the end of the final cycle, an additional extension step was carried out for a further 5 minutes at 72°C. The amplified DNA fragments were separated by electrophoresis on 2% (w/v) agarose gel stained with SYBR green for 1 hour. The genomic



DNA from the untransformed control plants and pNOV2819 plasmid containing *nptII* gene were used as negative and positive controls, respectively.

### **3.3.2 RNA isolation and reverse transcription-PCR**

Finger millet plants were grown at 28 °C in soil for four weeks and then exposed to water and salinity stress treatments. Leaf samples were collected on 15<sup>th</sup> day after exposure and then stored at -80 °C. Total RNA was extracted using Spectrum Plant Total RNA Kit (RNeasy Plant Mini Kit (50)) according to the manufacturer's instructions. To remove traces of genomic DNA, the RNA samples were digested with DNase I by using the DNA-free Kit (Ambion, Austin, USA) and quantified by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). The First strand cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's described protocol and primer sequences.

After CDNA synthesis, PCR was carried out. Thermal-cycling parameters were as follows: after an initial denaturation at 95°C for 2 minutes, samples were subjected to a cycling regime of 30 cycles (denaturation 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 60 seconds). At the end of the final cycle, an additional extension step was carried out for a further 5 minutes at 72°C. The PCR products were resolved in 2% (w/v) agarose gel electrophoresis for 1 hour.

### **3.4 Drought tolerance assay**

The procedure used for drought assay was a modification of the procedure described by Singh, (2014). The transgenic finger millet and wildtype finger millet seeds were germinated in pots containing sterile soil. The drought stress was induced by irrigating the seeds with various concentrations of mannitol (0, 200, 400 and 600 mM mannitol) at an interval of 3 days for two weeks. Observations on the rate of germination were scored on the 17<sup>th</sup> day after treatment. This experiment was repeated in which the transgenic and wildtype seedlings were exposed to drought stress and observations made for a period of 21 days. Data on number of green leaves and the total chlorophyll content were scored on the 25<sup>th</sup> day after treatment. Chlorophyll a, b and total

chlorophylls (a + b) were determined according to Arnon (1949). Total chlorophyll content in each sample, expressed in mg/g fresh mass(FM) was calculated using Arnon's 1949 formula:  $TC = 20.2(A645) + 8.02(A663) \times V/1000 \times W$  where V corresponds to the volume of total extract per litre and W is the mass of the fresh material. This experiment was done in triplicates for assessment of the performance of the different finger millet lines during mannitol treatments.

### **3.5 Salt tolerance assay**

The procedure used for salt assay was a modification of the procedure described by Garwe *et al.*, (2006). The transgenic finger millet and wildtype finger millet seeds were germinated in pots containing sterile soil. The salinity stress was induced by irrigating the seeds with various concentrations of NaCl (0, 100, 200 and 300 mM NaCl) at an interval of 3 days for two weeks. Observations on the rate of germination were scored on the 17<sup>th</sup> day of treatment. This experiment was repeated in which the transgenic and wildtype finger millet seedlings were exposed to salinity stress and observations made for a period of 21 days. Data on number of green leaves and the total chlorophyll content were scored on the 25<sup>th</sup> day after treatment. This experiment was done in triplicates for assessment of the performance of the different finger millet lines during salt treatments.

### **3.6 Data analysis**

The data on regeneration was analysed in terms of germination percentage, number of shoots induced, number of shoot clumps induced and number of roots induced. The data on regeneration of putative transformants was analysed in terms of transformation frequency and transformation efficiency. Transformation frequency was calculated as the total number of putative divided by total number of infected explants. The transformation efficiency was calculated as the number of positive events divided by total number of putative transformants obtained. The data on drought and salinity tolerance was analysed in terms of germination percentage, total number of green leaves and total chlorophyll content. The variability in data was expressed as mean  $\pm$  standard error (SE). The data collected were analysed using one-way analysis of variance (ANOVA) with Minitab statistical computer software v.17. Means were separated using the Fisher's protected LSD test at a confidence level of 95% ( $p \leq 0.05$ ).

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 Effects of MS medium on germination

The seeds of the six selected finger millet varieties were germinated on MS basal medium containing with 30 % sucrose and different germination percentage recorded after three days of incubation in dark. GBK-043137 had the highest germination percentage of 84.33% followed by GBK-043128, GBK-043050 and GBK-043124 with seed germination efficiencies of 82.33%, 80%, and 72.70% respectively. Varieties GBK-043122 and GBK-043094 had the least germination percentage of 62.67% (**Table 4.1**). Significantly higher difference in germination was observed for varieties GBK-043137, GBK-043128, GBK-043050, GBK-043124, GBK-043122 and GBK-043094 (**Table 4.1**). However, for varieties GBK-043137, GBK-043128, GBK-043050 there was significantly lower difference in germination percentage (**Table 4.1**).

**Table 4.1 Germination efficiencies of six finger millet varieties**

Variety	Number of seeds	Germination percentage	P value
GBK-043137	100	84.33± 0.048 <sup>a</sup>	0.021
GBK-043128	100	82.33±0.034 <sup>a</sup>	
GBK043050	100	80.00±0.012 <sup>a</sup>	
GBK-043124	100	72.70±0.10 <sup>ab</sup>	
GBK-043122	100	62.67±0.013 <sup>b</sup>	
GBK-043094	100	62.67±0.013 <sup>b</sup>	

Germination percentages were calculated as the percentage of germinated seeds. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

#### 4.2 Effect of shoot induction using shoot apical meristems

When 3-day-old meristemic shoot tips (**Fig.4.1A**), consisting of the apex and part of mesocotyl were cultured on MS basal medium containing various concentrations of BAP, shoot induction was observed within 1 day of incubation. After one day of incubation, the explants induced shoot which were white in color (**Fig. 4.1B**) and formed a single leaf-like

structure which thereafter became green(**Fig.4.1C**) and formed multiple shoots after 24 days (**Fig. 4.1D**). The six finger millet varieties tested exhibited remarkably different regeneration responses depending on the concentration of BAP. The best shoot induction was observed in medium containing 1.75mg/l BAP (**Table 4.2**). Shoot apical meristems explants of all finger millet varieties tested responded well to different concentrations of BAP tested in the shoot induction medium. However, shoot induction response and number of shoots per explant also varied based on the variety and BAP concentration in the shoot induction medium. Statistical analysis of variance indicated significant differences among the varieties in plant regeneration response. Induction medium supplemented with 1.75 mg/l BAP exhibited significantly better response of shoot induction than the other BAP concentration tested, ranging from 3.00 to 1.28 shoots per explant. The variety GBK-043050, showed significantly higher response of shoot induction; GBK-043128, GBK-043124, GBK-043137 and GBK-043122 produced a moderate response; GBK-043094 produced a significantly lowest response of 1.62 shoots per explant in shoot induction medium supplemented with 1.75 mg/l BAP (**Table 4.2**). The lowest shoot induction was observed in 1.0mg/l on GBK-043094 with an average number of shoots of  $1.28 \pm 0.13$  (**Table 4. 2**). The height of the plant varied from 5 to 6cm.

Similarly, shoot clumps developed on all varieties when the shoots were sub-cultured in shoot induction medium containing BAP. The best response was observed in MS basal medium containing 1.75mg/l BAP with 12 total shoots (**Table 4.3**). However, shoot clumps sub-cultured to the same induction medium containing 1.0mg/l BAP and no response of shoot multiplication was observed on MS basal medium lacking plant growth regulators. The shoot multiplication response also varied among the 6 varieties evaluated (**Table 4.3**).



**Figure 4. 1 Finger millet plants obtained via direct regeneration.** (A) Three day-old finger millet seedlings germinated on plant growth-regulators free MS medium; (B) Initiation of shoots from shoot apical meristems (4-6cm) inoculated on MS medium containing BAP; (C) Shoots formed in 12 days; (D) Multiple shoots formed in 24 days; (E) Root development in MS medium containing IAA; (F) Acclimated plantlets in plastic cups containing sterile peat moss; (G) Two weeks after hardening off on peat moss.

**Table 4.2 Shoot induction on BAP after 12 days of six finger millet varieties**

Variety	BAP (mg/l)			
	1.0	1.5	1.75	2.0
GBK-043137	1.70±0.23 <sup>b</sup>	1.98±0.11 <sup>ab</sup>	2.02±0.11 <sup>b</sup>	1.88±0.28 <sup>b</sup>
GBK-043128	1.55±0.08 <sup>b</sup>	2.22±0.11 <sup>ab</sup>	2.40±0.31 <sup>ab</sup>	1.60±0.21 <sup>b</sup>
GBK-043124	1.67±0.32 <sup>b</sup>	1.96±0.15 <sup>ab</sup>	2.01±0.15 <sup>b</sup>	2.02±0.16 <sup>ab</sup>
GBK-043122	1.84±0.29 <sup>ab</sup>	1.75±0.17 <sup>b</sup>	2.07±0.41 <sup>ab</sup>	1.72±0.07 <sup>b</sup>
GBK-043094	1.28±0.13 <sup>b</sup>	1.89±0.29 <sup>ab</sup>	1.62±0.29 <sup>b</sup>	1.74±0.13 <sup>b</sup>
GBK-043050	2.38±0.26 <sup>a</sup>	2.71±0.65 <sup>a</sup>	3.00±0.39 <sup>a</sup>	2.61±0.39 <sup>a</sup>
Control	0.00±0.00 <sup>c</sup>	0.00±0.00 <sup>c</sup>	0.00±0.00 <sup>c</sup>	0.00±0.00 <sup>c</sup>

The induction efficiency was calculated in terms of the number of shoots induced per explant. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD

**Table 4.3 Shoot multiplication on BAP after 24 days of six finger millet varieties**

Variety	BAP (mg/l)			
	1	1.5	1.75	2.0
GBK-043137	3.60±0.15 <sup>a</sup>	4.13±0.19 <sup>b</sup>	4.43±1.44 <sup>cd</sup>	3.77±0.23 <sup>c</sup>
GBK-043128	3.30±0.32 <sup>a</sup>	3.63±0.09 <sup>bc</sup>	10.33±0.88 <sup>ab</sup>	6.00±0.58 <sup>bc</sup>
GBK-043124	2.00±0.00 <sup>b</sup>	3.00±0.00 <sup>c</sup>	8.00±1.53 <sup>bc</sup>	7.33±0.33 <sup>ab</sup>
GBK-043122	3.83±0.32 <sup>a</sup>	3.43±0.55 <sup>bc</sup>	4.13±0.63 <sup>cd</sup>	4.00±0.57 <sup>bc</sup>
GBK-043094	3.00±0.58 <sup>a</sup>	3.47±0.29 <sup>bc</sup>	4.00±1.08 <sup>d</sup>	3.70±0.59 <sup>c</sup>
GBK-043050	3.67±0.29 <sup>a</sup>	5.00±0.00 <sup>a</sup>	12.00±2.31 <sup>a</sup>	10.00±2.89 <sup>a</sup>
Control	0.00±0.00 <sup>c</sup>	0.00±0.00 <sup>d</sup>	0.00±0.00 <sup>e</sup>	0.00±0.00 <sup>d</sup>

Shoots were cultured on shoot multiplication and elongation media. The shoot multiplication response was calculated as the number of shoot clumps formed per shoot. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

### 4.3. Root induction

Rooting occurred after two weeks after culturing elongated shoots on MS basal medium containing different concentrations of IAA (**Fig 4.1E**). In all the medium concentrations, proper root system was achieved in four weeks of culture. The highest root growth responses were observed in MS medium supplemented with 4  $\mu$ M IAA which produced 10.28 roots (**Table 4.4**). Shoots cultured on rooting medium produced varied responses of rooting based on the variety and concentration of IAA. Majority of the varieties produced optimal root induction response on MS supplemented with 3  $\mu$ M IAA. The variety GBK 043124 showed significantly higher response of root induction (8.89 roots); GBK043137, GBK043128, GBK043122 and GBK 043094 produced moderate response; GBK 043050 produced the significantly low response of 4.25 roots. However, root induction in varieties GBK-043124 and GBK-043050 was better achieved in 4  $\mu$ M and 2  $\mu$ M with 10.28 and 5.70 roots respectively. The least root induction responses were observed in MS medium supplemented with 1  $\mu$ M and 5  $\mu$ M across the six varieties (**Table 4.4**).

**Table 4.4 Root induction on IAA**

Variety	IAA( $\mu$ M)				
	1	2	3	4	5
GBK-043137	4.33 $\pm$ 0.17 <sup>ab</sup>	7.13 $\pm$ 0.35 <sup>a</sup>	8.03 $\pm$ 0.42 <sup>ab</sup>	4.50 $\pm$ 0.25 <sup>b</sup>	3.39 $\pm$ 0.59 <sup>ab</sup>
GBK-043128	3.70 $\pm$ 0.29 <sup>bc</sup>	6.50 $\pm$ 0.12 <sup>ab</sup>	7.86 $\pm$ 0.29 <sup>ab</sup>	4.65 $\pm$ 0.80 <sup>b</sup>	2.63 $\pm$ 0.18 <sup>bcd</sup>
GBK-043124	4.54 $\pm$ 0.27 <sup>a</sup>	6.74 $\pm$ 0.54 <sup>ab</sup>	8.89 $\pm$ 0.11 <sup>a</sup>	10.28 $\pm$ 0.20 <sup>a</sup>	4.0 $\pm$ 0.00 <sup>a</sup>
GBK-043122	3.88 $\pm$ 0.07 <sup>abc</sup>	4.41 $\pm$ 0.51 <sup>c</sup>	7.60 $\pm$ 0.48 <sup>b</sup>	4.81 $\pm$ 0.51 <sup>b</sup>	3.23 $\pm$ 0.44 <sup>abc</sup>
GBK-043094	2.51 $\pm$ 0.39 <sup>d</sup>	4.44 $\pm$ 0.50 <sup>c</sup>	6.25 $\pm$ 0.25 <sup>c</sup>	5.25 $\pm$ 0.30 <sup>b</sup>	2.47 $\pm$ 0.15 <sup>cd</sup>
GBK-043050	3.48 $\pm$ 0.42 <sup>c</sup>	5.70 $\pm$ 0.49 <sup>b</sup>	4.25 $\pm$ 0.53 <sup>d</sup>	3.15 $\pm$ 0.52 <sup>c</sup>	2.01 $\pm$ 0.13 <sup>d</sup>
Control					

Elongated shoots were cultured on root induction media and the response calculated as the number of roots per shoot. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

#### 4.4 Hardening and Acclimatization

The rooted plants were transferred to peat moss and maintained in growth room for 5 days (**Fig. 4.1F**) Following the 5 days' culture in growth room, the plants were transferred to soil in plastic pots with 100% survival rate and incubated in the greenhouse where the plants were watered regularly. The plants exhibited phenotypic homogeneity and had no observable abnormalities when compared to field-grown finger millet plants derived from seeds. (**Fig. 4.1G**).

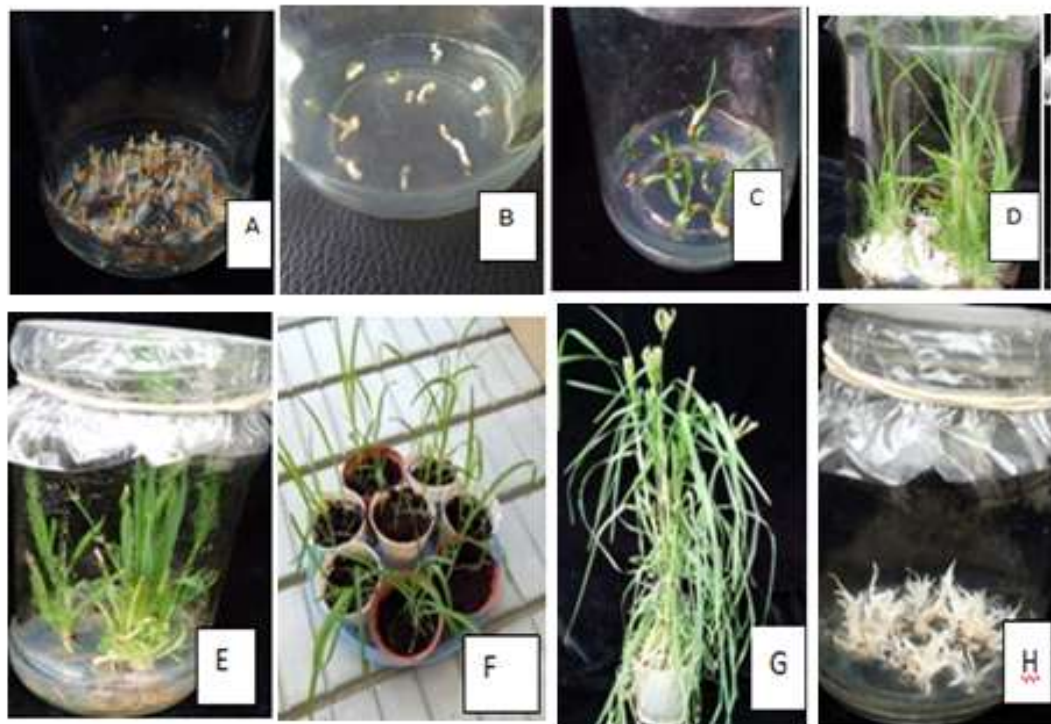
#### 4.5 Generation of transgenic finger millet

Transgenic finger millet plants were generated using *A. tumefaciens* strains EHA101 carrying the binary plasmids pNOV2819-XvAld1 (**Fig. 3.1**). The putative transgenic finger millet lines were established and propagated in the green house. All transgenic lines were phenotypically indistinguishable from the wildtype plants.

Thirty days after infecting finger millet shoot apical meristems with *Agrobacterium tumefaciens*, many putative transgenic shoots developed and chloritized and were separated in every subculture (**Fig.4.2 D**). Kanamycin resistance of the shoots indicated that shoots regenerated were possibly transgenic. The shoots that survived in shoot induction and selection medium were rooted in root induction and selection medium (**Fig.4.2 E**). After 28 days, shoots with well-developed root system were hardened and acclimatized in peat moss(**Fig.4.2F**) for 5 days after which they were transferred to the soil and grown to maturity in the greenhouse (**Fig. 4.2G**). Uninfected SAM cultured on SIS and MSIS medium were used

as negative control and uninfected SAMs inoculated on shoot induction medium and multiple shoot induction medium lacking kanamycin was used as a positive control. All the uninfected SAMs in SIS produced shoot clumps that necrotized and died (**Fig. 4.2H**).

The transformation of the finger millet varieties was variety dependent (**Table 4.5**). Overall, GBK043122 showed higher response in terms of transformation frequency (39%), followed by GBK043124 and GBK043128 and GBK043094 (**Table 4.5**). Although the statistical analysis of variance indicated that there was no significance difference in the transformation efficiency, transgenic GBK043128 recorded superior response with 10.83% followed by GBK043124 and GBK043094 with 10.00% and 4.77% respectively.



**Figure 4.2 Transgenic finger millet plants obtained by direct regeneration.**(A)Three day-old finger millet seedlings germinated on plant growth-regulators free MS medium; (B) Infected shoot apical meristems on after 5 days in cocultivation media; (C)Shoots initiated on shoot induction and selection media; (D)Shoot clumps formed in multiple shoot induction and selection media; (E) Root development in root induction and selection media; (F)Acclimated plantlets in plastic cups containing sterile peat moss; (G) Young transgenic finger millet plantlets growing in the soil in the green house; (H)shoot clumps produced by uninfected shoot apical meristems in shoot induction/multiplication and selection media.



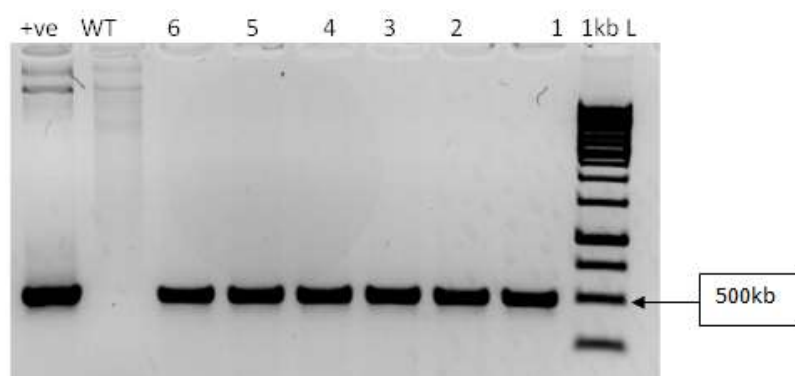
**Table 4.5 Transformation and regeneration of XvAld1 finger millet lines**

Variety	No. infected Explants	No. of putative shoots	+ transgenic shoots	TF (%)	TE (%)
GBK043137	13.33±3.33 <sup>b</sup>	0.33±0.33 <sup>b</sup>	0.33±0.33 <sup>a</sup>	1.67±0.02 <sup>d</sup>	3.33±0.33 <sup>a</sup>
GBK043128	23.67±6.33 <sup>ab</sup>	6.00±1.00 <sup>a</sup>	0.67±0.33 <sup>a</sup>	26.67±0.03 <sup>abc</sup>	10.83±0.06 <sup>a</sup>
GBK043124	37.67±7.22 <sup>a</sup>	8.33±1.67 <sup>a</sup>	0.67±0.33 <sup>a</sup>	32.00±0.09 <sup>ab</sup>	10.00±0.06 <sup>a</sup>
GBK043122	19.33±5.21 <sup>b</sup>	7.33±1.67 <sup>a</sup>	0.33±0.33 <sup>a</sup>	39.00±0.03 <sup>a</sup>	3.70±0.04 <sup>a</sup>
GBK043094	15.00±2.89 <sup>b</sup>	1.33±0.67 <sup>b</sup>	0.33±0.33 <sup>a</sup>	1.90±0.10 <sup>bcd</sup>	4.77±0.05 <sup>a</sup>
GBK043050	24.00±4.00 <sup>ab</sup>	4.67±2.33 <sup>ab</sup>	0.33±0.33 <sup>a</sup>	1.00±0.06 <sup>cd</sup>	1.67±0.17 <sup>a</sup>

TF (%), Transformation frequency= total number of putative transgenic plantlets over total number of explants infected. TE (%), Transformation efficiency=total number of positive plantlets over total number of putative transgenic plantlets Mean± standard error. Means are from three replicates. Means (± SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

#### 4.6 Detection of transgenic events by PCR analysis

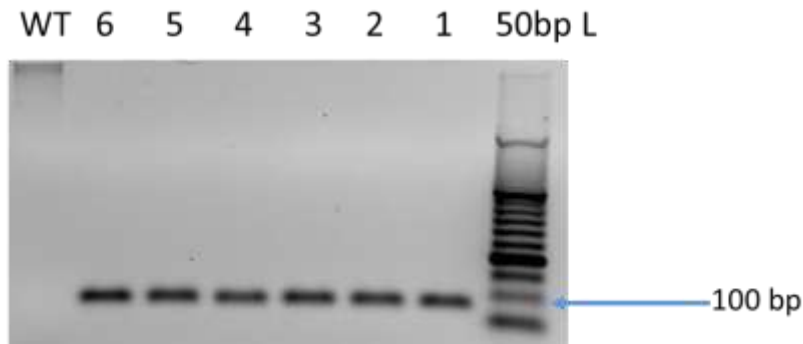
Putative transgenic finger millet plants regenerated were identified by PCR amplification of *XvAld1* gene. The transformants showed an amplification product of 511bp which was consistent with the expected size of amplified products while no amplification of the target gene were observed in wildtype control (**Figure 4.3**). For the 84 putative transgenic finger millet plants analysed, 6 were positive, 1 each for GBK043122, GBK043124, GBK043128, GBK043137, GBK043050 and GBK043094.



**Figure 4.3 Amplification of XvAld1 from genomic DNA.** The numbers(1-6) represents the transgenic plant lines(GBK043122, GBK043124, GBK043128, GBK043137, GBK043050 and GBK043094) , WT is the non-transformed plants for negative control, +ve is the positive control and L, is 1-kb ladder.

#### 4.7 Reverse transcription-polymerase chain reaction analysis

The RT-PCR analysis detected the expression of mRNA in all six transgenic finger millet plants but none in the WT line (Figure 4.4) showing the expression of *XvAld1* gene in those transgenic plants. The transformants showed transcripts amplification products of 80bp and this was consistent with the expected size of amplified products while no PCR products were observed in WT control (Figure 4.4)



**Figure 4.4 RT-PCR analysis confirming expression of *XvAld1*.** Numbers 1-6 are transgenic plant lines, WT, non-transformed plants for negative control and L is 50bp ladder.

#### 4.8 Germination rate during drought stress

When transgenic and wildtype finger millet seeds were germinated on soil and irrigated with various concentrations of mannitol, germination was first observed in the pots without mannitol treatment. Germination of the seeds under drought stress induced by mannitol was delayed. However transgenic seeds under drought stress germinated earlier than the wildtype under the same stress levels. Even after the elapse of the two weeks of treatment, the wildtype seeds under stress levels of 600 mM mannitol recorded zero germination (Table 4.6). Generally, there was a decrease in germination rate in both transgenic and the corresponding wildtype with increasing drought stress. However, both transgenic and wildtype lines recorded high responses under no drought stress. While the wildtype lines recorded 0% germination at 600mM drought stress, their corresponding transgenic lines recorded relatively higher germination percentages even under such drought stress. Plants under drought stress to 200mM recorded relative significance differences with the transgenic GBK043137 and wildtype GBK043137 recording the highest response of 62.50% and 55% respectively. Under the drought stress of 400 mM transgenic GBK043122 recorded superior response of 16.25% germination followed by transgenic GBK043128. Under the same stress, wildtype

GBK043122 recorded the highest response with 16.25% while the rest of the wildtype lines recorded moderate responses. The statistical analysis of variance indicated that there was no significance difference in the germination percentages of plants under drought stress of 600mM mannitol (**Table 4.6**).

**Table 4.6 Effects of drought on germination**

Variety	Mannitol (mM)							
	0		200		400		600	
	T	WT	T	WT	T	WT	T	WT
GBK-043137	82.50±4.79 <sup>a</sup>	83.75±4.73 <sup>a</sup>	62.50±4.79 <sup>a</sup>	55.00±6.45 <sup>a</sup>	5.00±2.04 <sup>b</sup>	0.00±0.00 <sup>b</sup>	2.50±1.44 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043128	56.30±10.7 <sup>b</sup>	65.00±14.0 <sup>ab</sup>	10.00±3.54 <sup>b</sup>	3.75±2.39 <sup>b</sup>	6.25±8.26 <sup>ab</sup>	1.25±1.25 <sup>b</sup>	1.25±1.25 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043124	71.25±4.27 <sup>ab</sup>	65.00±7.36 <sup>ab</sup>	16.25±6.57 <sup>b</sup>	3.75±2.39 <sup>b</sup>	5.00±2.04 <sup>b</sup>	0.00±0.00 <sup>b</sup>	1.25±1.25 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043122	70.00±9.13 <sup>ab</sup>	72.50±4.33 <sup>ab</sup>	15.00±4.56 <sup>b</sup>	16.25±3.75 <sup>b</sup>	16.25±8.26 <sup>a</sup>	16.25±8.26 <sup>a</sup>	1.25±1.25 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043094	55.00±4.56 <sup>b</sup>	51.25±5.91 <sup>b</sup>	12.50±9.24 <sup>b</sup>	8.75±7.18 <sup>b</sup>	5.00±0.00 <sup>b</sup>	0.00±0.00 <sup>b</sup>	1.25±1.25 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043050	61.30±10.1 <sup>ab</sup>	66.25±9.66 <sup>ab</sup>	10.00±2.04 <sup>b</sup>	6.25±3.15 <sup>b</sup>	2.50±1.44 <sup>b</sup>	0.00±0.00 <sup>b</sup>	1.25±1.25 <sup>a</sup>	0.00±0.00 <sup>a</sup>

Transgenic and wildtype finger millet seeds were irrigated with mannitol and the germination percentages per treatment per line calculated. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

#### **4.9 Effects of drought stress on number of green leaves**

After 21 days of treatment, both transgenic and wildtype finger millet seedlings had decreased the number of green leaves. While the wildtype lines had wilted and completely dried leaves, this was not observed in the transgenic lines (**Figure 4.5**). Generally, there was a decrease in the number of green leaves with increasing drought stress. However transgenic lines recorded relatively higher response than wildtype lines ranging from 3.20 to 0.20 respectively. Under the drought stress of 200mM there was no significance difference in both transgenic and wildtype lines. However, transgenic GBK043122 and GBK043094 and wildtype GBK043137 recorded relatively higher response with 3.20 and 2.00 leaves respectively. Under the drought stress of 400mM there was no significance difference recorded in the six transgenic lines with transgenic GBK043128 and GBK043122 recording high number of leaves 2.60 leaves (**Table 4.7**). Under the same stress levels, the wildtype GBK043128 recorded high numbers of leaves (1.80 leaves) which was not statistically significant different from that of GBK043137, GBK043124, GBK043122, GBK043094 and GBK043060. At this stress level, GBK043124 recorded the least number of leaves with 0.20 leaves. Under the drought stress of 600mM both transgenic and wildtype plant didn't record significance differences.

**Table 4.7 Effects of drought stress on number of green leaves**

Variety	Mannitol (mM)							
	0		200		400		600	
	T	WT	T	WT	T	WT	T	WT
GBK-043137	3.00±0.55 <sup>a</sup>	3.20±0.49 <sup>a</sup>	2.20±0.49 <sup>a</sup>	2.00±0.32 <sup>a</sup>	1.40±0.40 <sup>a</sup>	1.20±0.20 <sup>ab</sup>	1.00±0.45 <sup>a</sup>	0.40±0.25 <sup>a</sup>
GBK-043128	3.20±0.58 <sup>a</sup>	3.40±0.51 <sup>a</sup>	2.40±1.03 <sup>a</sup>	1.40±0.60 <sup>a</sup>	2.60±0.75 <sup>a</sup>	1.80±0.74 <sup>a</sup>	1.20±0.37 <sup>a</sup>	0.80±0.49 <sup>a</sup>
GBK-043124	4.00±0.55 <sup>a</sup>	4.00±0.45 <sup>a</sup>	3.00±0.78 <sup>a</sup>	1.60±0.51 <sup>a</sup>	2.20±0.66 <sup>a</sup>	0.20±0.20 <sup>b</sup>	1.20±0.37 <sup>a</sup>	1.20±0.58 <sup>a</sup>
GBK-043122	3.40±0.40 <sup>a</sup>	3.80±0.20 <sup>a</sup>	3.20±0.97 <sup>a</sup>	1.60±0.68 <sup>a</sup>	2.60±0.84 <sup>a</sup>	1.40±0.60 <sup>ab</sup>	1.60±0.40 <sup>a</sup>	0.40±0.40 <sup>a</sup>
GBK-043094	4.00±0.63 <sup>a</sup>	3.80±0.58 <sup>a</sup>	3.20±0.86 <sup>a</sup>	1.20±0.58 <sup>a</sup>	2.00±0.84 <sup>a</sup>	1.00±0.63 <sup>ab</sup>	1.20±0.49 <sup>a</sup>	1.40±0.60 <sup>a</sup>
GBK-043050	4.00±0.32 <sup>a</sup>	3.40±0.60 <sup>a</sup>	1.60±0.25 <sup>a</sup>	1.00±0.63 <sup>a</sup>	1.60±0.25 <sup>a</sup>	0.40±0.40 <sup>ab</sup>	0.40±0.40 <sup>a</sup>	1.20±0.49 <sup>a</sup>

Transgenic and wildtype finger millet seedlings were irrigated with mannitol and the number of green leaves per treatment per line calculated. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

#### **4.10 Effects of drought stress on total chlorophyll content**

The varieties recorded different responses in different treatments. However, the transgenic lines had relatively higher amounts of total chlorophyll content compared to their wildtype lines (**Table 4.8**). Generally, at drought stress of 200mM mannitol, three transgenic and wildtype lines i.e. GBK043137, GBK043124 and GBK043050 recorded a significant increase in total chlorophyll content while line GBK043128, GBK043122 and GBK043094 recorded a decrease in total chlorophyll content. At drought stress of 400mM, four transgenic lines i.e. GBK043122, GBK043050, GBK043094 and GBK043124 had a significant increase in chlorophyll content. Similarly, under the same stress levels, the wildtype GBK043122, GBK043094 and GBK043050 recorded an increase in chlorophyll content. At 600 mM drought stress, while other lines recorded a decrease in total chlorophyll content, the line transgenic GBK043128 and wildtype GBK043128 and GBK043137 recorded an increase in total chlorophyll content (**Table 4.8**).

**Table 4.8 Effects of drought stress on total chlorophyll content**

Variety	Mannitol (mM)							
	0		200		400		600	
	T	WT	T	WT	T	WT	T	WT
GBK-043137	15.04±0.01 <sup>c</sup>	14.69±0.03 <sup>d</sup>	19.17±0.06 <sup>b</sup>	18.85±0.12 <sup>b</sup>	18.39±0.70 <sup>ab</sup>	9.25±0.04 <sup>bc</sup>	14.13±0.37 <sup>a</sup>	13.49±0.06 <sup>a</sup>
GBK-043128	21.19±0.69 <sup>a</sup>	23.08±0.03 <sup>a</sup>	14.22±0.18 <sup>d</sup>	14.19±0.15 <sup>d</sup>	11.97±3.54 <sup>cd</sup>	6.51±1.41 <sup>c</sup>	13.89±0.57 <sup>a</sup>	12.81±0.41 <sup>a</sup>
GBK-043124	12.79±0.67 <sup>d</sup>	7.67±0.04 <sup>f</sup>	17.04±0.71 <sup>c</sup>	16.38±0.25 <sup>c</sup>	17.19±0.41 <sup>bc</sup>	13.42±4.12 <sup>bc</sup>	7.93±3.38 <sup>b</sup>	6.57±2.98 <sup>b</sup>
GBK-043122	16.36±0.05 <sup>b</sup>	15.89±0.04 <sup>c</sup>	8.69±0.31 <sup>e</sup>	6.89±0.19 <sup>e</sup>	10.24±0.55 <sup>d</sup>	12.41±1.90 <sup>bc</sup>	7.15±1.46 <sup>b</sup>	5.99±1.13 <sup>b</sup>
GBK-043094	11.98±0.24 <sup>d</sup>	10.58±0.04 <sup>e</sup>	6.79±0.17 <sup>f</sup>	4.77±0.17 <sup>f</sup>	14.43±2.70 <sup>bcd</sup>	14.43±2.70 <sup>b</sup>	13.43±2.21 <sup>a</sup>	12.65±2.93 <sup>a</sup>
GBK-043050	16.06±0.01 <sup>bc</sup>	16.79±0.42 <sup>b</sup>	23.67±0.59 <sup>a</sup>	23.29±0.42 <sup>a</sup>	23.67±1.10 <sup>a</sup>	23.62±1.12 <sup>a</sup>	15.98±0.48 <sup>a</sup>	15.85±0.37 <sup>a</sup>

Transgenic and wildtype finger millet seedlings were irrigated with mannitol and the total chlorophyll per treatment per line calculated. Total chlorophyll=20.2(A645) +(8.02(A663) \*0.0015\*0.2. Means (± SE) followed by different alphabets in each column are significantly different (P ≤0.05) using Fishers LSD.





**Figure 4.5 Appearance of finger millet plants before and after drought stress. (A, C) Transgenic finger millet plants; (B,D) Wildtype finger millet plants.**

#### **4.11 Effects of salinity stress on germination**

When transgenic and wildtype finger millet seeds were cultured on soil and irrigated with various concentrations of NaCl, germination was first observed in pots without NaCl treatment after 10 days. Seeds under salinity stress of 300mM recorded the latest seed germination emergence after 13 days of sowing (**Table 4.9**). Generally, there was a decrease in the germination percentage with the increasing salinity stress (**Table 4.9**). Both wildtype and transgenic lines recorded remarkable high responses at 0mM NaCl with transgenic and wildtype GBK043137 recording 73.57% and 90.00% respectively. Under salinity stress of 100 mM transgenic GBK043124 recorded high response with 51.25% while GBK043137 recorded the least with 7.50%. Under the same salinity stress, the wildtype GBK043124 recorded superior responses followed by GBK043122 with 46.25 and 45.00% respectively. At 200mM transgenic GBK043124 recorded relatively higher response with 6.25% and while at 300Mm, transgenic GBK043128 recorded higher with 3.75% though statistical analysis of variance indicated there was no significant difference recorded at these levels. Transgenic lines exhibited tolerance to severe salinity stress and therefore recorded germination at 200mM and 300mM NaCl. However, the wildtype lines could not tolerate severe salinity stress and thus recorded 0% germination under 200mM and 300mM (**Table 4.9**).

**Table 4.9 Effects of salinity stress on germination**

Variety	NaCl (mM)							
	0		100		200		300	
	T	WT	T	WT	T	WT	T	WT
GBK-043137	78.75±2.39 <sup>ab</sup>	90.00±3.54 <sup>a</sup>	7.50±3.23 <sup>c</sup>	3.75±3.75 <sup>c</sup>	5.00±2.04 <sup>a</sup>	0.00±0.00 <sup>a</sup>	2.50±1.44 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043128	65.00±2.04 <sup>ab</sup>	65.00±3.54 <sup>bc</sup>	31.3±13.0 <sup>abc</sup>	20.00±3.54 <sup>c</sup>	5.00±3.54 <sup>a</sup>	0.00±0.00 <sup>a</sup>	3.75±1.25 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043124	65.00±12.1 <sup>ab</sup>	80.00±8.42 <sup>a</sup>	51.25±8.51 <sup>a</sup>	46.25±9.66 <sup>a</sup>	6.25±3.75 <sup>a</sup>	0.00±0.00 <sup>a</sup>	2.50±1.44 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043122	62.50±3.23 <sup>b</sup>	56.25±5.15 <sup>c</sup>	45.00±7.36 <sup>ab</sup>	45.00±8.90 <sup>ab</sup>	2.50±1.44 <sup>a</sup>	0.00±0.00 <sup>a</sup>	2.50±1.44 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043094	73.75±2.39 <sup>a</sup>	63.75±1.25 <sup>bc</sup>	28.75±4.27 <sup>abc</sup>	22.5±11.6 <sup>bc</sup>	5.00±2.04 <sup>a</sup>	0.00±0.00 <sup>a</sup>	2.50±1.44 <sup>a</sup>	0.00±0.00 <sup>a</sup>
GBK-043050	63.75±2.39 <sup>ab</sup>	76.25±3.75 <sup>ab</sup>	21.3±10.9 <sup>bc</sup>	18.75±5.54 <sup>c</sup>	5.00±2.04 <sup>a</sup>	0.00±0.00 <sup>a</sup>	1.25±1.25 <sup>a</sup>	0.00±0.00 <sup>a</sup>

Transgenic and wildtype finger millet seeds were irrigated with NaCl and the germination percentages per treatment per line calculated. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

#### **4.12 Effects of salinity stress on number of green leaves**

After 10 days of treatment with NaCl, the effect of treatment on seedlings was observed. Wildtype lines lost their firmness while transgenic seedlings remained firm. Both transgenic and wildtype lines had decreased the number of green leaves after 21 days of treatment (Figure 4.6). There was a concomitant decrease in the number of green leaves in both transgenic and wildtype lines with increase salinity stress. However, transgenic lines recorded remarkable higher responses compared to their wildtype lines at all stress levels. At 100mM transgenic GBK043124 and wildtype GBK043050 recorded relatively higher number of leaves with 3.00 and 1.60 leaves respectively though there was no significant difference recorded at this stress level in both transgenic and wildtype lines. At 200 mM NaCl, transgenic GBK043122 recorded high number of leaves with 2.40 leaves while transgenic GBK043137 recorded least with 1.60 leaves. The other transgenic lines under this treatment recorded moderate response. Under the same salinity stress, the wildtype lines recorded relatively lower responses with GBK043050 recording the least number of leaves with 0.60. At 300 mM both transgenic and wildtype lines recorded the least number of leaves though transgenic and wildtype GBK043050 recorded relatively higher responses with 1.80 and 1.00 leaves respectively (**Table 4.10**).

**Table 4.10 Effects of salinity stress on the number of green leaves**

Variety	NaCl (mM)							
	0		100		200		300	
	T	WT	T	WT	T	WT	T	WT
GBK-043137	2.60±0.40 <sup>ab</sup>	2.20±0.58 <sup>a</sup>	2.20±0.20 <sup>a</sup>	1.40±.25 <sup>a</sup>	1.60±0.25 <sup>b</sup>	1.00±0.00 <sup>a</sup>	0.80±0.37 <sup>abc</sup>	0.80±0.37 <sup>a</sup>
GBK-043128	2.80±0.37 <sup>ab</sup>	2.00±0.32 <sup>a</sup>	3.00±0.00 <sup>a</sup>	1.00±0.00 <sup>a</sup>	2.00±0.00 <sup>ab</sup>	1.00±0.00 <sup>a</sup>	1.20±0.49 <sup>ab</sup>	0.80±0.37 <sup>a</sup>
GBK-043124	2.00±0.32 <sup>b</sup>	2.20±0.49 <sup>a</sup>	3.00±0.78 <sup>a</sup>	1.40±0.40 <sup>a</sup>	2.00±0.00 <sup>ab</sup>	1.00±0.00 <sup>a</sup>	0.40±0.25 <sup>bc</sup>	0.40±0.25 <sup>a</sup>
GBK-043122	2.00±0.32 <sup>b</sup>	2.20±0.20 <sup>a</sup>	2.40±0.25 <sup>a</sup>	1.00±0.00 <sup>a</sup>	2.40±0.40 <sup>a</sup>	1.20±0.20 <sup>a</sup>	1.40±0.40 <sup>ab</sup>	0.80±0.20 <sup>a</sup>
GBK-043094	2.60±0.40 <sup>ab</sup>	2.40±0.40 <sup>a</sup>	2.40±0.25 <sup>a</sup>	1.40±0.25 <sup>a</sup>	2.00±0.00 <sup>ab</sup>	1.00±0.00 <sup>a</sup>	0.00±0.00 <sup>c</sup>	0.40±0.40 <sup>a</sup>
GBK-043050	3.20±0.58 <sup>a</sup>	2.00±0.00 <sup>a</sup>	2.80±0.58 <sup>a</sup>	1.60±0.25 <sup>a</sup>	1.80±0.37 <sup>ab</sup>	0.60±0.25 <sup>b</sup>	1.80±0.37 <sup>a</sup>	1.00±0.32 <sup>a</sup>

Transgenic and wildtype finger millet seedlings were irrigated with NaCl and the number of green leaves per treatment per line calculated. Means ( $\pm$  SE) followed by different alphabets in each column are significantly different ( $P \leq 0.05$ ) using Fishers LSD.

#### **4.13 Effects of salinity stress on total chlorophyll content.**

Both the transgenic and wildtype line recorded different responses at different salinity stress regimes. Generally, plants recorded relatively high amount of chlorophyll when they were not exposed to salinity stress with transgenic GBK043122 and GBK043050 recording high with 10.04 and 10.10 respectively while GBK043124 recorded the least response with 5.85mg/g (**Table 4.11**). At 0mM, the wildtype GBK043122 recorded high amount of chlorophyll with 10.36mg/g and GBK043124 recorded the least with 5.87mg/g. At 100mM NaCl four transgenic lines (GBK043128, GBK043122, GBK043094 and GBK043050) recorded a decrease in total chlorophyll content compared to transgenic GBK043124 and GBK043137 which recorded an increase. Under the same stress level, the while the other wildtype lines recorded a decrease, wildtype GBK043124 recorded an increase. Similarly, under salinity stress of 200mM both transgenic and wildtype recorded a decrease in total chlorophyll content in exception of transgenic GBK043124 and wildtype GBK043124 and GBK043122 which recorded an increase. Under salinity stress of 300mM transgenic lines recorded a decrease in total chlorophyll with transgenic GBK043122 recording the highest with 7.38mg/g and transgenic GBK043137 recording the least with 4.48mg/g. at the same treatment regime, the wildtype lines recorded a decrease in total chlorophyll in exception of GBK043050 which recorded an increase (**Table 4.11**).

**Table 4.11 Effects of salinity stress on total chlorophyll content**

Variety	NaCl (mM)							
	0		100		200		300	
	T	WT	T	WT	T	WT	T	WT
GBK-043137	8.28±0.36 <sup>b</sup>	8.42±0.38 <sup>b</sup>	9.68±1.41 <sup>a</sup>	8.09±1.84 <sup>a</sup>	6.29±2.03 <sup>a</sup>	5.02±0.38 <sup>c</sup>	4.48±0.55 <sup>c</sup>	4.36±0.58 <sup>b</sup>
GBK-043128	9.02±0.88 <sup>ab</sup>	9.08±0.99 <sup>ab</sup>	7.65±1.44 <sup>a</sup>	7.47±1.48 <sup>a</sup>	6.80±0.62 <sup>a</sup>	6.35±0.53 <sup>abc</sup>	5.80±0.09 <sup>bc</sup>	5.49±0.08 <sup>ab</sup>
GBK-043124	5.85±0.23 <sup>c</sup>	5.87±0.06 <sup>c</sup>	7.35±0.25 <sup>a</sup>	6.84±0.13 <sup>a</sup>	7.53±0.24 <sup>a</sup>	7.28±0.21 <sup>ab</sup>	6.03±0.29 <sup>abc</sup>	5.94±0.13 <sup>a</sup>
GBK-043122	10.04±0.08 <sup>a</sup>	10.36±0.24 <sup>a</sup>	7.84±1.18 <sup>a</sup>	7.45±0.34 <sup>a</sup>	7.89±0.38 <sup>a</sup>	7.64±0.33 <sup>a</sup>	7.38±0.93 <sup>a</sup>	6.61±0.66 <sup>a</sup>
GBK-043094	8.82±0.67 <sup>ab</sup>	9.06±0.64 <sup>ab</sup>	7.72±0.09 <sup>a</sup>	7.41±0.07 <sup>a</sup>	7.58±0.14 <sup>a</sup>	7.19±0.13 <sup>ab</sup>	6.42±0.34 <sup>ab</sup>	6.42±0.13 <sup>a</sup>
GBK-043050	10.10±0.72 <sup>a</sup>	10.23±0.77 <sup>ab</sup>	7.78±1.18 <sup>a</sup>	6.82±0.51 <sup>b</sup>	6.87±0.14 <sup>a</sup>	5.67±1.26 <sup>bc</sup>	5.94±0.12 <sup>abc</sup>	5.73±0.13 <sup>a</sup>

Transgenic and wildtype finger millet seedlings were irrigated with NaCl and the total chlorophyll per treatment per line calculated. Total chlorophyll=20.2(A645) +(8.02(A663) \*0.0015\*0.2. Means (± SE) followed by different alphabets in each column are significantly different (P ≤0.05) using Fishers LSD.



**Figure 4.6** Appearance of finger millet plants before and after salinity stress (A, C) Transgenic finger millet plants; (B,D) Wildtype finger millet plants.

## CHAPTER FIVE

### 5.0 DISCUSSION

Genetic engineering techniques are increasingly becoming important tools in achieving rapid improvements of finger millet cultivars. To successfully achieve this objective and efficiently produce bioengineered crops, improvements to the existing laborious and time-consuming protocols for *in vitro* regeneration need to be established. In this study, procedures for rapid and effective shoot-regeneration of six Kenyan farmer preferred finger millet varieties that have potential for application in genetic engineering experiments were optimized. In order to establish rapid and efficient plant regeneration procedures that could be used in genetic engineering experiments, hormone regimes which have previously been reported in successful finger millet transformation were tested. The optimized regimes promoted shoot regeneration of 2-12 shoots per explants within 7 weeks from initiation of shoot induction treatment. These results are comparable to the previous studies that reported regeneration of finger millet within 20 weeks (Anju *et al.*, 2016), 18 weeks (Kashyap, *et al.*, 2018), and 8 weeks (Pande *et al.*, 2015) via indirect organogenesis. The choice of shoot organogenesis over somatic embryogenesis was because it is fast and also circumvents prolonged callus stages, therefore minimising chances of somaclonal variation (Karp, 1991). Moreover, shoot apical meristems are also easily handled compared to other explants and can be induced to produce multiple shoots (Arockiasamy and Ignacimuthu, 2007).

Shoot apical meristems derived from mature seeds as an initial explant for efficient and reproducible direct regeneration protocol for finger millet were used. The successful use of shoot apical meristems explants in regeneration and transformation of finger millet confirms that they are a better choice for plant regeneration in cereals when compared to other explants. This present study therefore confirms previous reports by Satish *et al.* (2017) and Ceasar and Ignacimuthu (2010) who found shoot apex as a suitable explant for regeneration and agrobacterium mediated transformation finger millet. Mature seeds derived explants are better source material for tissue culture research than others because of the availability of seeds, easy of storage of seeds, and homogeneity quality of the explants (Yang *et al.*, 2013). Cytokinins play an important role in shoot growth. 6-Benzylaminopurine is Cytokinin, commonly used for *in vitro* regeneration of cereals and other monocot plants (Ramakrishnan



*et al.*, 2013). The genotype dependent variations in shoot induction among finger millet varieties observed in our study were also noted in previous studies for monocot cereal plants (Pazuki and Sohani, 2013).

Shoot clumps obtained from the shoot induction medium were subcultured to shoot elongation medium with various concentrations of BAP. The medium supplemented with 1.75mg/l BAP produced more number of shoots with 10.33 shoots per explant. To the best of my knowledge, this is the highest number ever reported in finger millet. Pande *et al.* (2015) reported optimal multiple shoot induction response was recorded on MS basal media supplemented with 3.0 mg/l of BAP while Satish *et al.* (2015) reported 8.3 shoots per explants of finger millet variety 'CO(Ra)-14' in MS basal medium containing 17.6  $\mu$ M 6 BAP, 0.9  $\mu$ M, 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 750 mg/l proline, 500 mg/l casein enzymatic hydrolysate and 2 mg/l glycine. When compared to other monocots, 30.33 shoots per explant of Sakon Nakhon in MS medium containing 50  $\mu$ M 6-BAP in rice (Pilahome *et al.* (2014) and 5.7 shoots from inbred lines of Kenyan maize KAT and TLO8 from shoot apices on MS basal medium containing 26.64  $\mu$ M BAP, 296  $\mu$ M adenine and 9  $\mu$ M 2,4-D (Muoma *et al.* (2008) have been reported. Earlier research work on maize genotypes have stated 4.3 shoots (CM300) and 1–3 shoots (LM5) in MS medium supplemented with 4.4  $\mu$ M BA and 2.8  $\mu$ M IAA from 14-day-old immature embryos (Rakshit *et al.* 2010; Manivannan *et al.* 2010). Similarly, 9 shoots from mature embryo in maize genotype HQPM-1 on MS basal medium containing 8.8  $\mu$ M BA, 4.6  $\mu$ M Kinetin and 2.6  $\mu$ M 1-naphthaleneaceticacid (NAA) were reported (Pathi *et al.*, 2013)

The direct regeneration system reported in here is rapid, effective and proficient and offers mass multiplication of finger millet within 7 weeks. Results from various work on other plants including *Zea mays* (Ramakrishnan *et al.*, 2014), *Curcuma attenuata* (Kou *et al.*, 2013), *Hippophae rhamnoides* (Sriskandarajah and Lundquist, 2009), *Metabriggsia ovalifolia* (Ma *et al.*, 2010), *Primulina tabacum* (Yang *et al.*, 2012), and *Pulsatilla koreana* (Lin *et al.*, 2011) using the two-stage tissue culture system has also proven it to be efficient in other plants.

In order to achieve the *in vitro* rooting of regenerated shoots, indole-3- acetic acid at different concentrations was used. The study found 3  $\mu$ M to be the optimal concentration for four finger

millet varieties; GBK-043137, GBK-043128, GBK-043122, GBK-043094. Varieties GBK-043124 and GBK-043050 however, showed best root induction at 4  $\mu$ M and 2  $\mu$ M respectively. Interestingly poor rooting was observed in 1  $\mu$ M and 5  $\mu$ M across all the varieties. These results indicate that *in vitro* rooting in finger millet can be induced with IAA at concentrations between 2  $\mu$ M and 4  $\mu$ M. Peat moss was used for acclimatization and hardening of the rooted plants because of its high water retention capacity recorded in the previous study (Ng'etich *et al.*, 2018). Consequently, all the plants were successfully acclimatized and hardened with 100% survival rate. This high survival rates could also be attributed to the well-developed root system and greenhouse conditions. Plants were regenerated within 52 days.

Genetic engineering has been considered as the most promising and the best method for improving crop plants since specific changes in the genome can be achieved within a short period of time and the transgenic plants could provide the desired traits without the loss of genetic integrity (Tang *et al.*, 2000). Genetic engineering of finger millet for improved abiotic stress tolerance is hampered by lack of an efficient method for genetic transformation. Biolistic genetic transformation has been one of the methods used for transforming finger millet (Mahalakshmi *et al.*, 2006; Latha *et al.*, 2005). However, this method is expensive and usually results in multiple insertions of the transgene in the genome. Multiple insertions negatively impact both laboratory research and commercial release of transgenic plants (Oltmanns *et al.*, 2010). *Agrobacterium* mediated plant transformation is one of the main strategy for gene delivery into plant genome. Contrary to particle gun-mediated method, this method is simple, permits large scale experiments with less cost and has higher reproducibility (Yu *et al.*, 2007)

Regeneration and transformation of six finger millet varieties via *Agrobacterium* mediated plant transformation of six finger millet varieties (GBK043137, GBK043128, GBK043124, GBK043122, GBK043094 and GBK043050) was successfully established in this study. Naturally, monocots are recalcitrant to *Agrobacterium* infection but successful attempts have been made in the past years by incorporating phenolics and modifying genetic and environmental aspects during transformation process. Several parameters have been

previously investigated and reported to affect *Agrobacterium*-mediated transformation of monocot cereals (Cheng *et al.*, 2004; Shrawat and Lo 'rz, 2006).

Shoot apical meristems (SAMS) were considered as the suitable explants for *Agrobacterium*-mediated transformation in this study. The results of the present study indicate shoot apical meristems (SAMs) are the ideal explants for transformation of finger millet as they are readily available as explants and have simple transformation process as well as rapid regeneration. Shoot apex explants have been used in millets for both regeneration (Arockiasamy *et al.*, 2001; Ceasar and Ignacimuthu 2008, 2010) and transformation (Latha *et al.*, 2005, 2006) studies. SAMs have also been used effectively to develop regeneration systems across the other cereals and the used as starting material to recover stably transformed wheat (Hamada *et al.*, 2017), rice (Arockiasamy and Ignacimuthu, 2007), oat (Cho *et al.*, 2003), and millet (Satish *et al.*, 2017). Leaf sheath segments (Gupta *et al.*, 2001), shoot tips (Latha *et al.*, 2005), shoot apex (Ceasar and Ignacimuthu, 2011; 2015; Ignacimuthu and Ceasar, 2012) and embryogenic seed (Sharma *et al.*, 2011; Babu *et al.*, 2012; Jagga-Chugh *et al.*, 2012; Bayer *et al.*, 2014; Hema *et al.*, 2014) were used as the explants in earlier transformation studies of finger millet.

*Agrobacterium tumefaciens* strain EHA101 harbouring the binary vector pNOV2819 modified to contain neomycin phosphotransferase *nptII* gene to enable selection of transgenic plants using kanamycin was used in the present study for finger millet transformation. The vector contained *XvAld1* gene driven by a stress-inducible *XvPsap1* promoter. Other strains of *Agrobacterium* such LBA4404 (pSB1) (Ignacimuthu and Ceasar, 2012) and EHA105 strain (Bayer *et al.*, 2014; Satish *et al.*, 2017) have been used in genetic engineering of finger millet. While the previous studies used herbicide resistant gene for trifluralin (Bayer *et al.*, 2014) and antibiotic resistant gene for hygromycin (Bayer *et al.*, 2014; Satish *et al.*, 2017), the present study used *nptII* plant selectable marker gene for selection with kanamycin to recover kanamycin resistant transgenic finger millet transformants. Kanamycin has been commonly used in plants genetic engineering because it inhibits the growth of plant cells by binding to the 30S ribosomal subunit, thereby inhibiting initiation of plastid translation (Wilmink Dons, 1993). Plant cells transformed with the *nptII* gene can detoxify the antibiotics in the selection medium (Kapaun and Cheng, 1999). Correspondingly, the putative transformants obtained in this study maintained their chlorophyll and had no observable abnormalities when

compared to uninfected control explants in medium devoid of kanamycin. However, the untransformed explants lost their chlorophyll after 1 week of inoculation in the selection medium. The latter also had retarded growth and later died. This kind of selection enabled rapid separation of the transformed plantlets from untransformed. It also provided the initial screening of transformants before the molecular analysis. Although high number of putative transgenic plants were obtained, the number of positive transgenic plants was low. This could be attributed to high levels of resistance to kanamycin by the plantlets. Therefore, higher concentrations of kanamycin could assure the selection efficiency. It has been reported that the primary transformants produced from *Agrobacterium* infected SAMs can be chimeric. However, the multiplication of *Agrobacterium* transformed SAMs could be turned into the developmental stage under *in vitro* conditions, and the plant growth regulator-based manipulation of transformed SAMs induces multiple shoot regeneration and produce more stable transformants (Zhong *et al.*, 1996; Yookongkaew *et al.*, 2007). Accordingly, *Agrobacterium* infected SAMs were grown under *in vitro* conditions using various plant growth regulators and subjected to kanamycin selection, thereby decreasing the chance of getting chimeras.

Although the significant difference in transformation frequency was recorded in GBK043122 and GBK043137, GBK043050 and GBK043094, the transformation efficiencies of the six finger millet varieties were significantly not different statistically suggesting that the transformation efficiencies are variety- independent. SAM based plant regeneration and genetic transformation have been reported to be genotype-independent as it gives a possible target for genetic transformation through *Agrobacterium* and direct T-DNA delivery method (Sticken and Oraby, 2005). The transformation method used in this study resulted in relatively high transformation efficiency of 10.83% with shorter tissue culture period (60days). After *Agrobacterium*- mediated infection, plant defense mechanism result in hypersensitive response causing tissue browning, necrosis and cell death. These are common factors leading to low transformation efficiencies in many crops. The elimination or control of an overgrowth of *Agrobacterium* is frequently accomplished by the addition of one or more antibiotics, individually or in combination, to the culture medium. Most of the procedures use one or two antibiotics in the selection and regeneration media to control the overgrowth of *Agrobacterium* and to recover putative transgenic plantlets. In this study, two antibiotics i.e.

carbenicillin and cefotaxime (150mg/l each) were used and this could have improved the regeneration and transformation frequency in all the six finger millet varieties. Similar results were observed in rice (Tran and Sanan-Mishra, 2015) and finger millet (Satish, 2017) transformation by adding a combination of carbenicillin and cefotaxime antibiotics in the regeneration and selection media. The high transformation frequency recorded could be attributed to the correct choice of explant, transformation and regeneration system. The results of PCR and RT-PCR of putative transgenic and positive transgenic lines of six finger millet lines indicated that *XvAld1* transgene was stably integrated into the finger millet genome.

Plants have developed various physiological and biochemical strategies to tolerate stress condition (Joshi *et al.*, 2016), however, it is necessary to increase the stress tolerance level of crops by engineering stress tolerant genes so as to survive under severe stress condition. To date many studies have demonstrated that genes can be transformed into crops to enhance tolerance to abiotic stresses such as drought and salinity. Earlier reports on finger millet (Babu *et al.*, 2012; Hema *et al.*, 2014; Jayasudha *et al.*, 2014), rice (Datta *et al.*, 2012; Jeong *et al.*, 2013) and maize (Omer *et al.*, 2013) have demonstrated that development of transgenic plants effectively enhanced protection against drought and salinity stress. However, only a few scholars have focused on drought mechanism of finger millet, identification methods and indicators of drought tolerance (Ramados, 2014; Kumar *et al.*, 2017 and Bartwal *et al.*, 2016). Drought stress is induced by withholding water. Polyethylene glycol and mannitol have also been used to induce drought stress. Mannitol is an osmotic regulation macromolecule that cause minimal toxicity to cells and act as an ideal material to simulate the soil drought. In the present study, mannitol was used to induce osmotic stress to the transgenic and wildtype lines. The present study found used mannitol simulated drought stress to investigate the difference of drought resistance on germination, number of green leaves and total chlorophyll content in order to determine the tolerance of transgenic and wildtype lines as well as provide theoretical guidance for drought resistance mechanism in finger millet. This study found that the growth traits including germination percentages and the number of green leaves decreased with the increase in stress level in both transgenic and wildtype lines. However, transgenic lines recorded relatively superior responses compared to the wildtype lines. This suggested that transgenic lines were more tolerant to drought stress compared to wildtype lines.

Salinity is also one of the major abiotic stresses affecting finger millet growth, development and productivity across the world. However, only a few scholars have addressed the aspect of salinity tolerance in finger millet (Ediga *et al.*, 2013 and Satish *et al.*, 2016). Satish *et al.*, (2016) reported a decrease in leaves and shoot growth at 150mM NaCl, increased root growth, levels of electrolyte leakage, proline content, hydrogen peroxide and caspase-activity (at 0-200mM), decrease in chlorophyll content and relative water content with increasing salinity. The present study used NaCl to induce osmotic stress in transgenic finger millet plants and wildtype finger millet plants. The transgenic plants marginally performed better compared to the corresponding wild-type plants under salinity stress. For example, *XvAld1* gene expressing transgenic plants recorded higher germination efficiencies and higher number of green leaves under salinity stress when compared to the corresponding wildtype varieties. It is possible that *XvAld1* was expressed in transgenic finger millet plants which lead to accumulation of sorbitol that acted as an osmoprotectants and protected the cells from radicals in addition to inducing several other stress tolerance mechanisms and hence leading to increase in their tolerance compared to wildtype plants.

Chlorophyll is an important photosynthetic pigment. Under osmotic stress, membranes become damaged and chloroplasts become degraded (Alberte *et al.*, 1977). Plants can respond to drought by increasing the accumulation of chlorophyll and carotenoids which protect the plants by getting rid of excessive energy by thermal dissipation (Reddy *et al.*, 2004). Chlorophyll content was investigated in order to further compare the physiological mechanisms of drought and salinity tolerance between transgenic and wildtype finger millet plants. Under drought stress, a variety of responses were recorded in both transgenic and wildtype lines. While there was no notable trend recorded in other lines, transgenic GBK043124 and GBK043050 and the wildtype GBK043050 recorded a significant accumulation of total chlorophyll content with increase in drought stress. The highest response was recorded under stress levels of 200mM and the least was recorded under stress levels of 600mM. Similarly, under salinity stress, plants produced remarkable different responses at various stress levels. However, transgenic and wildtype GBK043124 plants recorded a significant accumulation of total chlorophyll content with the increase in salinity stress. The results are consistent with findings of (Jayasudha, 2014) who reported that the transgenic finger millet lines co-expressing PgNHX1 and AVP1 showed higher chlorophyll

stability index than wild type treated plants. It has also been reported that that accumulation of total chlorophyll in IE 4757, IE 5091 and IE 6537 indicates drought tolerance in these three finger millet accessions (Ramados, 2014).

Abiotic stress is a multi-genetic trait mediated by a number of biochemical and physiological processes (Zhu, 2000). It has been suggested that meaningful tolerance to abiotic stresses, such as salt and drought, can only be achieved by pyramiding several stress-responsive genes in a single genotype (Cushman *et al.*, 2000). However, the expression of single genes has been shown to improve tolerance to various stresses. The expression of an isopentenyltransferase gene in transgenic sweet potatoes has been shown to result in increased drought tolerance (Nawiri *et al.*, 2018). It has also been shown that also showed that the expression of a superoxide dismutase gene from *Nicotiana plumbaginifolia* in transgenic alfalfa resulted in increased drought tolerance under field conditions (McKersie *et al.*, 1996) Although a number of genes have been shown to be up-regulated by abiotic stresses in resurrection plants (Oliver, *et al.*, 1998; Mundree *et al.*, 2000; Mowla *et al.*, 2002; Bockel *et al.*, 1998 and Neale *et al.*, 2000), very little has been reported on the expression of these stress-associated genes in plant system. The present study demonstrates that the expression of *XvAld1* gene results in increased tolerance to drought and salt stress. Transgenic finger millet plants exhibited greater germination efficiencies, high number of green leaves and greater accumulation of total chlorophyll content under osmotic and water- deficit stress conditions. However, there was no morphological difference between transgenic finger millet plants and the control plants when the plants were grown in stress free media than when the plants were subjected to stress treatment. This could be argued that, *XvAld1* actually conferred tolerance than promoting growth.

## CHAPTER SIX

### 6.0 CONCLUSION AND RECOMMENDATIONS

#### 6.1 Conclusions

A rapid, efficient and reproducible regeneration protocol for Kenyan farmers preferred finger millet varieties GBK043137, GBK043128, GBK043124, GBK043122, GBK043094 and GBK043050 via direct organogenesis from the cultures of shoot apical meristems was optimized for finger millet. The regeneration system optimized in this study can be excellently employed in genetic transformation for improvement of finger millet varieties as the results of this study demonstrated the ability of transformation and regeneration of finger millet through *Agrobacterium*-mediated strategy using shoot apical meristem as explants. This study also validated the ability of expression of *XvAld1* gene in transgenic finger millet in enhancing drought and salinity tolerance.

#### 6.2 Recommendations

The regeneration system developed in this study should be replicated using other explants such as leaves or root and or other crops.

The transgenic finger millet should be screened for other physiological and biochemical characters in order to determine the effect of expression of *XvAld1* gene.

The stability of the gene over several generations should be determined.



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## **APPENDICES**

### **Appendix 1 Surface sterilization protocol**

- i. Wash the finger millet seeds in running tap
- ii. Transfer the seeds to sterile culture bottles under sterile environment
- iii. Wash the seeds with 70% ethanol for two minutes
- iv. Rinse thrice with double distilled water.
- v. wash further with 20% sodium hypochlorite with tween20 for 23minutes
- vi. Rinse 3-4 times with double distilled water.

### **Appendix 2 DNA extraction protocol**

- i. Wash the leaf in normal saline in an Eppendorf tube
- ii. Add 200µl of lysis buffer(CTAB)
- iii. Grind leaves in buffer in Eppendorf tube
- iv. Vortex for 15 seconds. Add 200µl of CTAB lysis buffer.
- v. Add 50 µl of mercaptoethanol and vortex for 30seconds
- vi. Incubate in water bath (65<sup>0</sup>c) for 1.5hours with intermittent inversions
- vii. Centrifuge and pick 450 µl of liquid phase. Transfer to fresh Eppendorf tube,
- viii. Add 450 µl chloroform: isoamyl (24:1) or phenol: chloroform: isoamyl (24:24:1)
- ix. Mix gently and incubate in a freezer for 1hour.
- x. Centrifuge at 13000rpm for 10minutes
- xi. Transfer the supernatant to fresh tube and precipitate with absolute ethanol (450 µl)
- xii. Incubate for 1 hour (or overnight) in a freezer
- xiii. Centrifuge at 13000rpm for 5-8 minutes
- xiv. Wash the pellet with 70% ethanol (450 µl)
- xv. Centrifuge for 2 minutes at 13000rpm
- xvi. Dry the pellet by inverting on sterile filter paper.
- xvii. Add 40 µl of elution buffer (TE buffer)

### **Appendix 3 Explant transformation**

- i. Streak actively growing bacteria (possibly in the evening).

- ii. On the following day morning, scoop (using a sterile tip or loop) the bacteria and suspend in the infection media with acetosyringone ( $OD_{600}=0.5$ ) and incubate in a shaker for 4 hours.
- iii. Excise the explants, place in sterile petriplates and suspend in infection media without bacteria
- iv. Remove the infection media with pipette then pour the bacteria solution
- v. Cover the plates and place the aluminium foil to allow darkness for 10 minutes
- vi. Remove the bacteria solution with pipette and place the explants using forceps on co-cultivation media. Seal the plate using parafilm and incubate in dark at  $21^{\circ}C$  for 5 days
- vii. Wash the explants using antibiotics and transfer to selection media then incubate in growth room at  $25^{\circ}C$  -  $28^{\circ}C$ .

#### Appendix 4 XvAld1 Gene Primer Sequence (5'- 3')

Primer	Name	Sequence
Forward	Ald-F	GGGCACTCAATCCCCGCAGTT
Reverse	Ald-R1	TCTCCATCTGGCACACCGAG

#### Appendix 5 Regeneration media

Medium	Medium composition
Initiation medium	MS basal salts, 30% sucrose, 3g/l gelrite, pH5.8.
Shoot induction medium	MS basal salts, 30% sucrose, 3g/l gelrite, BAP (1, 1.5, 1.75, 2.0mg/l), pH5.8.
Rooting medium	MS basal salts, 30% sucrose, 3g/l gelrite, IAA (1,2,3,4,5 $\mu$ M), pH5.8.
Co-cultivation medium	MS basal salts + 100 $\mu$ M of acetosyringone, 17.6 $\mu$ M BAP, 0.9 $\mu$ M 2,4-D, 500 mg/l casein enzymichydrolysate, 750 mg/l proline, 2.0 mg/l glycine, 30 g/l of sucrose, 3% gelrite, pH 5.8
Infection medium	MS basal salts + 30 g/l of sucrose and acetosyringone (100 $\mu$ M), pH 5.8
Shoot induction and selection medium	MS basal salts + 17.6 $\mu$ M BAP, 0.9 $\mu$ M 2, 4-D, 500 mg/l casein enzymichydrolysate, 750 mg/l proline, 2 mg/l glycine, 100 mg/l kanamycin, 150 mg/l, 150 mg/l cefotaxime, 30 g/l of sucrose, and 3% gelrite, pH 5.8
Shoot multiplication and selection medium	MS basal salts supplemented with 11.0 $\mu$ M BAP, 0.9 $\mu$ M 2, 4-D, 500 mg/l casein enzymichydrolysate, 750 mg/l proline, 2 mg/l glycine, 100 mg/l kanamycin, 150 mg/l cefotaxime, 30 g/l of sucrose, 3 g/l gelrite, pH 5.8
Rooting and selection medium	Half strength MS basal salts supplemented with 2.8 $\mu$ M IAA, 30 mg/l kanamycin, 1.5% (w/v) sucrose and 4.5% (w/v) gelrite, pH 5.8