Agrobacterium tumefaciens-mediated transformation of Sudanese maize genotypes using NPK1 gene for enhancing drought stress tolerance

By

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A thesis presented in partial fulfilment of the requirement for the award of the degree of Master of Science in Biotechnology in the School of Pure And Applied Science, Kenyatta University.

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DECLARATION

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

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This work is dedicated to my mother, who has spent a lifetime of personal sacrifice for me to have the best in life. Her kindness and encouragement is my strength.
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ABSTRACT

Drought is one of the most important abiotic factor affecting maize production worldwide. *Agrobacterium*-mediated gene transfer technique has been established as a versatile way of improving important crops for tolerance to biotic and abiotic factors. Through this technique, the drought tolerance gene, *NPK1*, has been used in the transformation of temperate maize after its isolation and characterization from tobacco. Recovered transgenic events were observed to have enhanced tolerance to water stress. The accelerated adoption of the transformation technique in Africa, and indeed in Sudan, will depend on the ease with which transgenes of agronomic importance can be integrated into appropriate germplasms. This study aimed at screening important Sudanese maize inbred lines and open pollinated varieties (OPVs) for transformability via the integration of the *NPK1* gene. Eight inbred lines and three OPVs were evaluated. A188 was used as the standard inbred line check while KAT was used as the local OPV check. Freshly isolated immature embryos of maize were inoculated with *Agrobacterium* strain EHA101 harbouring the plasmid pSHX004 in LS infection media for 5 minutes and then co-cultivated on LS co-cultivation media for 3 days. Embryos were then transferred to selection media supplemented with 250mg/l cefotaxime and 1.5mg/L bialaphos. After two weeks on this media, calli were subcultured on selection media containing 3.0 mg/L bialaphos for 4 weeks. Bialaphos resistant callus events were then transferred to maturation media supplemented 3mg/L bialaphos for 2 weeks before transferring to shooting media. Shoots were then transferred to rooting media. Plantlets with well-formed root system were transferred from the *in vitro* environment to green house for hardening. Hardened plantlets were transplanted to soil in the greenhouse and maintained till they set seeds. To confirm the presence of the transgene, PCR analysis was done on putative transgenic plants using the *Bar* primers. Out of a total of 4401 immature embryos from the 13 genotypes infected, 327 survived selection in bialaphos. Bialaphos resistant calli emerged 3-4 weeks after selection. IL3, IL15, Hudiba-2, IL1, IL38, Hudiba-1, A188 and KAT produced compact calli from their scutella surfaces while IL28, IL42, IL43, Mojtamaa-45 and IL16 established watery nonembryogenic calli. Statistically significant differences (*p*<0.05) were observed between the genotypes with respect to transformation frequency (TF). IL3 was identified as the most amenable to transformation with a TF of 31.7% and proved to be superior to A188, which recorded a TF of 5.82%. Hudiba-2 was identified as the most transformable OPV with a TF of 8.7% compared to that of 7.3% for KAT. IL1 and Mojtamaa-45 proved to be poor responders to transformation with TFs of 2.5% and 1.7%, respectively. Putative transgenics were recovered from IL3, IL15, Hudiba-2, IL1, IL38, Mojtamaa-45, A188 and KAT. The frequency of regeneration of bialaphos resistant shoots varied from 6.9% for IL38 to 100% for Mojtamaa-45. PCR analysis indicated a 540bp fragment in the DNA extracts from transgenic R1 plants. Transformation efficiency (TE) was found to depend on the genotype used. The highest TE was observed for IL3 (3.7%), while the lowest TE of 0.0% was observed in IL42 IL43, IL16 and IL28. Various abnormalities were observed in putative transformants including dwarfism, tussel seed and lack of ear. However, plants grew to maturity and were able to establish seeds in spite of these abnormalities. In conclusion, the inbred line IL3 and the OPV Hudiba-2 proved to be the most amenable Sudanese genotypes to *A. tumefaciens*-mediated transformation. Future research in maize improvement through biotechnologies such as tissue culture and genetic transformation should be focussed on these good responders.
<table>
<thead>
<tr>
<th>CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>iv</td>
</tr>
<tr>
<td>Abstract</td>
<td>v</td>
</tr>
<tr>
<td>List of figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of tables</td>
<td>x</td>
</tr>
<tr>
<td>List of appendices</td>
<td>xi</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER ONE</td>
<td>1</td>
</tr>
</tbody>
</table>

1 INTRODUCTION | 1
1.1 Background to the study | 1
1.1.1 Maize production in Sudan | 1
1.1.2 Maize production constraints in Sudan | 2
1.1.3 Improvement of maize for drought stress tolerance | 2
1.1.4 Transformation of maize for drought stress tolerance | 3
1.1.5 Plant responses to drought stress | 3
1.2 Justification | 4
1.3 Hypothesis | 5
1.4 Research objectives | 5
1.4.1 General objective | 6
1.4.2 Specific objectives | 6
1.5 Expected output | 6
LIST OF FIGURES

Figure 1. Model of MAPK cascade.................................................................16

Figure 2. Vector transformation map of the NPKI gene construct..................19

Figure 3. Schematic representation of summarized protocol used for transformation of maize.................................................................24

Figure 4. Resting of Agrobacterium-infected immature embryos of maize.........27

Figure 5. Selection of stable transformation events in bialaphos......................28

Figure 6. Callus formation in maize..............................................................29

Figure 7. Transformation frequency of maize.................................................32

Figure 8. Regeneration of putative Agrobacterium-transformed maize genotypes...37

Figure 9. Plantlet formation from bialaphos resistant events of OPV maize........38

Figure 10. Plantlet formation from bialaphos resistant events of inbred lines.........39

Figure 11. Rhizogenesis in Sudanese maize...................................................40

Figure 12. Hardening of plantlets regenerated from putative transgenic inbred lines.................................................................43

Figure 13. Hardening of plantlets regenerated from putative OPV transformants...44

Figure 14. Green house growth of hardened inbred line plants.......................45

Figure 15. Green house maintenance of hardened OPV plants.......................46

Figure 16. Abnormalities observed in R₀ transgenic plants...............................47

Figure 17. Tussel seed Abnormality observed in R₀ transgenic plants.................48

Figure 18. Fertile seed of putative OPV transformants..................................50

Figure 19. Fertile seed from putative inbred line transformants......................51

Figure 20. Gel electrophoresis of genomic DNA of putative transformants..........52

Figure 21. Polymerase chain reaction (PCR) screening for the bar gene in R₁ transgenic plants.................................................................53
LIST OF TABLES

Table 1. Putative transformation frequency and efficiency of various maize genotypes ..............................................................31

Table 2. Number of shoots per regenerating putative transformed callus .................36

Table 3. Abnormalities observed in putative R₀ transgenic plants .........................42
LIST OF APPENDICES

Appendix I. Composition of media used for *agrobacterium* culture......................78

Appendix II. Basal media.......................................................................................79

Appendix III. Maize transformation media..............................................................80
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASARECA</td>
<td>Association for Strengthening Agricultural Research in Eastern and Central Africa</td>
</tr>
<tr>
<td>KAT</td>
<td>Katumani composite B</td>
</tr>
<tr>
<td>IL</td>
<td>Inbred line</td>
</tr>
<tr>
<td>ARC</td>
<td>Agricultural Research Cooperation</td>
</tr>
<tr>
<td>DAP</td>
<td>Days after pollination</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetate</td>
</tr>
<tr>
<td>Bar</td>
<td>Bialaphos resistance gene</td>
</tr>
<tr>
<td>NPKI</td>
<td>Nicotiana Protein Kinase 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization</td>
</tr>
<tr>
<td>PMI</td>
<td>Phosphomannose isomerase</td>
</tr>
<tr>
<td>YEP</td>
<td>Yeast extract peptone</td>
</tr>
<tr>
<td>OPV</td>
<td>Open pollinated variety</td>
</tr>
<tr>
<td>LS</td>
<td>Linsmaier and Skoog basal media</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog basal media</td>
</tr>
<tr>
<td>IM</td>
<td>Infection media</td>
</tr>
<tr>
<td>CCM</td>
<td>Co-cultivation media</td>
</tr>
<tr>
<td>RM</td>
<td>Resting media</td>
</tr>
<tr>
<td>SI</td>
<td>Selection media I</td>
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<td>SII</td>
<td>Selection media II</td>
</tr>
<tr>
<td>RI</td>
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<td>RII</td>
<td>Regeneration media II</td>
</tr>
</tbody>
</table>
CHAPTER ONE

1 INTRODUCTION

1.1 Background to the study

Maize is the world’s third most important crop after rice and wheat. About half of maize is grown in the developing countries, where maize is staple food for majority of the population (Ofori and Kyei-Baffour, 2006; Pingali, 2000). In Sub-Saharan Africa, maize provides 50% of the basic calories (Machuka, 2001; CIMMYT, 2001) and it is an important source of carbohydrate, protein, iron, vitamin B, and minerals. Africans consume maize as a starchy base in a wide variety of porridges, pastes, grits, and beer. Green maize (fresh on the cob) is eaten parched, baked, roasted or boiled and plays an important role in filling the hunger gap after the dry season (Ofori and Kyei-Baffour, 2006).

1.1.1 Maize production in Sudan

Maize is an important food and forage crop in Sudan. In Northern Sudan, maize was introduced into the existing farming system by the state government in recognition of its high potential as a cash crop (Abdelrahman et al., 2001). Research in maize in Northern Sudan has been going on since the sixties and has been focussed mainly on breeding for increased yield. In northern Sudan, maize is produced through irrigation and is grown during the late summer and early winter periods. In this part of Sudan, maize yield is greatly affected by high temperature (Abdelrahman et al., 2001).
In Southern Sudan, maize is the second most important cereal after sorghum. The annual cereal production in southern Sudan is estimated at 460,000 tonnes, 40% of which is maize. However, the cereal requirements are estimated at 494,000 tonnes from 556000 ha leading to a deficit in cereal production (FAO, 2000).

1.1.2 Maize production constraints in Sudan

Apart from such socio-economic factors as insecurity and lack of agricultural inputs, maize production in Sudan is constrained by biotic and abiotic factors. The most serious biotic factors affecting yield are weeds including *Striga* (*Striga hermonthica*) and insects such as stem borer (FAO, 1998). Abiotic factors include low soil fertility, high temperatures, and drought. Drought is the major abiotic constraint to maize production in Sudan. Drought stress alone can account for a significant percentage of average yield losses and is one of the greatest yield reducing factors in maize production (Ofori and Kyei-Baffour, 2006). It has been estimated that drought causes 17% loss in maize production, but under severe conditions loses as high as 70% have been realized (Edmeades *et al.*, 1994).

1.1.3 Improvement of maize for drought stress tolerance

Conventional breeding methods have been used to introgress desirable traits such as drought tolerance in maize cultivars. However, these methods are laborious and space-, and time-consuming (CIMMYT, 2001). Additionally they are restricted to the narrow genetic variability within the genotypes (Jones and Qualset, 1984). Genetic engineering provides a new breeding tool whereby DNA may be inserted into the maize genome from a wider range of donors than is possible by conventional breeding procedures.
1.1.4 Transformation of maize for drought stress tolerance

The first transgenic maize was produced using biolistic gun as a gene delivery system (Gordon-Kamm et al., 2002). *Agrobacterium tumefaciens*-mediated maize transformation offers a better alternative for delivery of transgenes to maize. This gene delivery system results in greater proportion of stable low copy number transgenic events than does the other systems (Ishida et al., 1996). It also offers the possibility of transferring large DNA segments into recipient cells (Hamilton et al., 1996). Despite the recalcitrance of elite germplasm to tissue culture manipulation and plant regeneration procedures, maize transformation technology has served as an important tool in germplasm development and research (Armstrong, 1999).

1.1.5 Plant responses to drought stress

In order to survive under stress condition, plants respond and adapt to environmental stresses, such as drought, at the molecular, cellular levels, physiological and biochemical levels (Xiong and Zhu, 2001: 2002). A common plant response to different abiotic stresses at the molecular level is the accelerated generation and/or accumulation of reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) (Kaur and Anil, 2005). Hydrogen peroxide is an active signalling molecule and its accumulation (oxidative stress) leads to a variety of cellular responses. Plants respond to oxidative stress by activating a series of protein phosphorylation events involving mitogen activated protein kinases (MAPKs) (Meskiene and Hirt, 2000). The activated MAPK cascade induces the expression of stress-response genes that protect plants from diverse environmental stresses. This includes the production of antioxidant enzymes, such as glutathione- S-transferases (GSTs), as well as the activation of
protective genes encoding heat shock proteins (HSPs) and pathogenesis-related proteins (Kovtun et al., 2000). Accumulation of both proteins can reduce damage, especially to the photosynthesis machinery, caused by drought, heat or chilling and protect plants from environmental stresses (Kovtun et al., 2000), thereby improving the yield potential of the major cereal crop, maize.

When NPKI gene was engineered into maize using A. tumefaciens, transgenic plants maintained significantly higher photosynthesis rates than did the non-transgenic control under drought conditions. Thus, it was suggested that NPKI induced a mechanism that protected photosynthesis machinery from dehydration damage (Shou et al., 2004). It is therefore hoped that the expression of NPKI will increase drought stress tolerance and improve the yield potential of maize grown in drought areas of Sudan.

1.2 Justification

Maize is the third most important food crop in the world. Its consumption is highest in the rural areas of southern Sudan, with an estimated annual consumption of 494,000 tonnes (FAO, 2000). However production levels lag behind consumption demands. Drought is the most important abiotic factor affecting maize productivity in Sudan. Traditional breeding as an approach to manage drought stress tolerance in plants has had limited success due its being limited to the narrow genetic variability available within the germplasm (Jones and Qualset, 1984). Moreover, it’s time- and space-consuming as well as laborious. Through genetic engineering, genes conferring drought tolerance can be directly introduced into the maize genome from a wide range of organisms. However the effectiveness of this technology for genetically
transforming the maize plant has been limited by maize insensitivity to *A. tumefaciens* infection (Carvalho *et al.*, 2004). Furthermore tropical elite germplasm have been known to be rather recalcitrant to tissue culture manipulation and plant regeneration procedures (Bohorova *et al.*, 1995), making the regeneration of transgenic maize plant a challenging objective. In spite of such challenges, *A. tumefaciens*-mediated transformation remains a popular method for improvement of germplasm for agronomic important traits such as drought tolerance. *Agrobacterium tumefaciens* -mediated transformation using the drought tolerance gene (*NPKI*) has been successfully achieved in temperate maize. Evaluation of the transgenic plants under drought conditions revealed a significantly higher photosynthesis rate than the non-transgenic controls (Shou *et al.*, 2004). The accelerated adoption of the transformation technique in Africa, and indeed in Sudan, will depend on the ease with which transgenes of agronomic importance can be integrated into appropriate germplasms. This will go a long way in improving production levels of main food crops, thereby alleviating food insecurity. There is therefore need to engineer drought tolerance in Sudanese elite germplasm and *A. tumefaciens* provides a good gene delivery method for maize.

### 1.3 Hypothesis

It is not possible to transform and regenerate Sudanese maize varieties with the gene conferring drought stress tolerance (*NPKI*) via *Agrobacterium tumefaciens*.

### 1.4 Research objectives
1.4.1 General objective

To genetically transform Sudanese maize eight inbred lines and three open pollinated varieties with \textit{NPKI} gene.

1.4.2 Specific objectives

i) Introduce the \textit{NPKI} gene into Sudanese maize inbred lines and commercial varieties via \textit{Agrobacterium tumefaciens}.

ii) To regenerate transformants and confirm presence of the transgene in the maize genome through molecular analysis.

1.5 Expected output

It is expected that:

a) One or more Sudanese maize varieties responding well to \textit{A. tumefaciens} -mediated transformation will be identified.

b) Sudanese maize transformed with the drought tolerance gene, \textit{NPKI}, will be produced and characterised.
CHAPTER TWO

2 LITERATURE REVIEW

2.1 Direct and indirect gene transfer systems

Genetic transformation has become an important tool for crop improvement. At the same time, gene transfer by *Agrobacterium* is the established method of choice for the genetic transformation of most plant species compared to direct gene transfer methodologies (particle bombardment, electroporation etc). *Agrobacterium* mediated transformation offers several advantages such as the possibility to transfer only one or few copies of DNA fragments carrying the gene of interest at higher efficiencies with lower cost and the transfer of very large DNA fragment with minimal rearrangements (Hansen and Wright, 1999; Hiei *et al.*, 1997; Negrotto *et al.*, 2000). The most important advantage, however, is the possibility of producing transgenic plants, which are free of marker gene (Komari *et al.*, 1996; Matthews *et al.*, 2001).

2.2 Transformation using *Agrobacterium*

Some bacteria such as *A. tumefaciens* and *A. rhizogenes* have evolved the capability of delivering DNA from tumour inducing plasmid (Ti-plasmid) into cells of a wide variety of dicotyledonous and some monocotyledonous plants. Incorporation of the transferred DNA (T-DNA) into the nuclear genome of the plant cell and its resultant expression causes pathogenic response characteristic of the transformed cell. The cellular basis of this transformation has been intensively studied over the past 10 years (Binns and Thomashaw, 1988; Zambryski, 1988). Beside elucidating many of the crucial steps of the transformation process, a variety of studies have led to the
development of Ti-based vectors for use in efficient and widely used strategies to generate transgenic plants. One of the most important finding relating to this practical utilization of the Agrobacterium Ti-plasmid system was that disarmed T-DNA, that is T-DNA lacking functional oncogenes, can be transferred and integrated into plant genome allowing the regeneration of transgenic plants. Any DNA fragment can be transferred from Ti-plasmid as long as it’s between the 25bp direct repeats, called borders that flank wild type Ti-plasmid (Barker et al., 1983). The virulence genes virB, virG, virC are required for T-DNA transfer.

2.3 Agrobacterium tumefaciens T-DNA transfer process

The T DNA region is located on a large plasmid called Ti-plasmid, which also contains other functional parts for virulence (vir), conjugation (con) and the origin of its own replication (ori). Any genes located in the T-DNA region in principle can be transferred, but they themselves are dispensable for this process. Only the 25bp direct repeats at the right and the left borders are necessary (Wei et al., 2000).

Gene transfer from Agrobacterium to plant cell occurs essentially through five steps: bacterial colonization, induction of bacterial virulence genes, integration of T DNA into plant cell and integration of T DNA into plant genome. Bacterial colonization involves the attachment of the Agrobacterium to the surface of the plant cell following injury of the plant. The injured plant cells release some small compounds such as the phenolic acetosyringone (AS) and certain class of monosaccharides which act synergistically to activate VirA by autophosphorylation (Wei et al., 2000). Activated VirA in turn phosphorylates and activates VirG which subsequently binds to and induces the expression of other vir genes called vir box.
The activated *vir* genes mediate the generation of the single-stranded copy of the T-DNA. The proteins VirD1 and VirD2 play a key role in this step, recognizing the T-DNA border sequences and nicking the bottom strand at each border. The generated T-strand is then transferred into plant cells as a VirD2/T-strand complex together with another ssDNA binding protein named VirE2. Once into the plant cell, many VirE2 molecules bind cooperatively to the T-strand, forming T-complex which is then targeted into the nucleus by the nuclear target signals (NLSs) of its associated VirD2 and VirE2, where the T-DNA randomly integrates into the plant genome as single or multiple copies. The integration is shown to preferentially occur in the transcription active and/or repetitive regions of the genome through a process of illegitimate recombination (Wei *et al.*, 2000).

**2.4 Explant material**

An ideal system of *Agrobacterium*-mediated transformation must have a high rate of T-DNA transformation into the plant cells (Wei *et al.*, 2000). This means that the target cells must have a high division activity. Initially the best explants for *Agrobacterium*-mediated transformation was reported to be embryogenic calli derived from mature seeds (Hiei *et al.*, 1997; Hiei *et al.*, 1994) due to their active cell division (Cheng *et al.*, 2004). These were however, very genotype dependent and freshly isolated immature embryos were later found to be the best explants for many plants including rice (Aldemita and Hodges, 1996) and barley (Tingay *et al.*, 1997).

The only maize explants reported so far to be highly competent for *Agrobacterium* infection are immature embryos (Cheng *et al.*, 2004). However these maize immature embryos show variations in their competence for *Agrobacterium* infection depending
on their developmental stage. Very young embryos (at 10 DAP or less), which consist of undifferentiated apical shoot meristem, have been shown to be incompetent for \textit{Agrobacterium} infection (Schlappi and Hohn, 1992). This lack of competence may be due to production of factors by the early embryos that inhibit \textit{vir} gene induction of \textit{Agrobacterium} (Schlappi and Hohn, 1992). Using temperate maize, Schlappi and Hohn (1992) found that 14-16 DAP was the age at which immature embryos peaked in susceptibility to \textit{Agrobacterium}. Immature embryos of between 1.2 and 2.0mm have been the explants of choice in many \textit{Agrobacterium}-mediated transformations experiments of maize (Frame \textit{et al.}, 2002; Ishida \textit{et al.}, 1996; Negrotto \textit{et al.}, 2000; Zhao \textit{et al.}, 2001).

For successful transformation by \textit{Agrobacterium}, the explant must have a strong regeneration potential (Wei \textit{et al.}, 2000). Maize cultures have been initiated from virtually all plant parts but these have been found to be largely non regenerable. Immature embryos have been the most widely used explants for initiation of regenerable cultures (Armstrong and Green, 1985; Phillips \textit{et al.}, 1988). Immature embryos can initiate two types of callus cultures from their scutella surface: Type I and Type II callus. Type I is compact and organogenic and easily obtained from immature embryos. On the other hand, Type II is friable and embryogenic and is initiated at a lower frequency than Type I (Carvalho \textit{et al.}, 1997; Phillips \textit{et al.}, 1988). Only a few tropical genotypes have been shown to be capable of initiating Type II callus (Carvalho \textit{et al.}, 1997). Type II callus are more desirable for transformation than Type I because they have been found to be more regenerable (Armstrong and Green, 1985).
2.5 Plant regeneration through somatic embryogenesis

Maize plant regeneration can take place through two avenues, that is, organogenesis or somatic embryogenesis. Organogenesis involves the formation of organs (shoots and roots) directly from the explant, or indirectly from a callus. On the other hand, somatic embryogenesis involves the formation of scutella-like structures from the explant or callus (Slater et al., 2004). Somatic embryogenesis is the most common avenue of plant regeneration (Oduor et al., 2006; Phillips et al., 1988).

2.6 Agrobacterium strains and T-DNA vectors

Although there are many Agrobacterium strains, successful transformation of maize has been achieved using a few strains. These include the disarmed supervirulent strain EHA101 and its derivative EHA105 and the regular strain LBA4404 (Cheng et al., 2004). Transformation frequency has been shown to be dependent on the choice of the Agrobacterium strain. Mburu (2007) showed that EHA101 yielded a significantly higher transformation frequency than that of LBA4404 when used to transform tropical (Kenyan) maize.

To date, two vector systems have been used for Agrobacterium-mediated transformation of maize; the super binary vector that carries extra copies of virB, virC and virG and the standard binary vector (Cheng et al., 2004; Komari et al., 1996). While the former kind was previously used intensely to infect immature zygotic embryos (Ishida et al., 1996; Negrotto et al., 2000), the later kind has had successful utility in Agrobacterium-mediated transformation of maize (Ishida et al., 2003; Negrotto et al., 2000).
There are indications that the strain-vector combination may influence the efficiency of transformation of some plant species. For example efficient transformation systems were established for maize only with super binary vectors in LBA4404, while a standard binary vector (pTF102) in a super virulent train (EHA101) showed low transformation frequency (5.5%) even with improved co-culture conditions (Frame et al., 2002).

2.7 *Agrobacterium* inoculation and co-culture

Increased transformation efficiency in maize via *Agrobacterium* demands the inclusion of chemicals e.g. acetosyringone for vir gene induction (Ishida et al., 1996; Zhao et al., 2001). Other chemicals including antioxidants e.g. cystine and anti-ethylene e.g. silver nitrate in the co-culture medium are recommended in most monocot *Agrobacterium*-mediated transformation protocol (Frame et al., 2002; Zhao et al., 2001). The addition of silver nitrate is also important as it suppresses the growth of *Agrobacterium* on the target explants thereby facilitating plant cell recovery resulting in increased efficiency of transformation (Cheng et al., 2004).

After co-culture, antibiotics such as cefotaxime and carbenicillin are regularly included in *Agrobacterium*-mediated transformation media to suppress or eliminate *Agrobacterium* (Cheng et al., 2004). However, cefotaxime at concentrations of 250mg/L has been shown to have a detrimental effect on callus induction, making carbenicillin the bactericide of choice for maize transformation (Zhang et al., 2003; Zhao et al., 2001).
2.8 Selectable marker genes

A critical step in the regeneration of transgenic plants is the ability to distinguish between transformed plant cells with an integrated transgene and the bulk of non-transformed cells. The traditional way to achieve this goal is to use marker genes within the transgene and to select for their expression (Shrawat and Lörz, 2006).

The conventionally used selectable markers in monocot transformation are the genes encoding for hygromycin phosphotransferase (hpt) phosphinothricin acetyltransferase (pat or bar) and neomycin phosphotransferase (nptI1). These markers work well with the 35s promoter from cauliflower mosaic virus or the ubiquitin promoter from maize (Cheng et al., 2004). To improve transformation efficiency, introns are inserted into the markers gene’s coding region (Wang et al., 1997), as a strategy to not only to enhance transgene expression and to reduce copy numbers of the marker gene but also to enable better Agrobacterium growth during the transformation process (Shrawat and Lörz, 2006).

In this study, the bar gene marker under the control of the 35S promoter (Figure 2) was used in the selection of stable transformants using bialaphos. Bialaphos (phosphinothricylalanylalanine sodium) is a tripeptide antibiotic consisting of phosphinothricin (PPT) and two alanine residues. Removal of these residues by peptidases leaves PPT, a potent inhibitor of glutamine synthase. Phosphinothricin inhibits glutamine synthase, causing a rapid accumulation of ammonia that leads to plant cell death.

The bialaphos resistance gene (bar) codes for a phosphinothricin acetyl-transferase, that acetylates phosphinothricin at the free NH₂ group and acetylated phosphinothricin
is no longer inhibitory to glutamine synthase. A chimeric bar gene has been used to confer resistance to transformed tobacco, potato, and tomato plants as well as maize (De Block et al., 1987).

An alternative to the conventional marker system, which involves detoxification of the selective agent, is the so-called positive selectable markers, in which the selective agent is converted into a metabolizable product that promotes the growth of transgenic plants (Joersbo et al., 1998). These include the Phosphomannose Isomerase (PMI) and xylose isomerase (xyIA) systems (Negrotto et al., 2000). The PMI system has been adapted to Kenyan open pollinated varieties and hybrids where transgenic events were recovered at frequencies ranging from 30% to 40% (Mburu, 2007).

2.9 Drought tolerance genes

A number of genes have been described that respond to drought stress. For example, Peroxiredoxine (XvPer1), which protects nucleic acids against oxidative stress-induced injury, and XvSap1, which encodes membrane-binding protein, from drought stress tolerant plant Xerophyta viscosa (Mowla et al., 2002; Seki et al., 2001). These genes are induced by dehydration, osmotic stress, high temperature, heat, abscisic acid and high light intensity (Machuka et al., 1999).

The BetA gene from Escherichia coli, is a key enzyme in the biosynthesis of glycine betaine from choline. Glycine betaine plays an important role in some plants, including maize, in conditions of abiotic drought stress (Chen and Murata, 2002). The PARP (Poly ADP-Ribose Polymerase) genes are activated by DNA damage caused by reactive oxygen species (ROS). Stresses such as drought, high light and heat activate
PARP causing NAD$^+$ breakdown and ATP consumption. When the PARP activity is reduced by means of chemical inhibitors or by gene silencing, cell death is inhibited and the plant becomes tolerant to a broad range of abiotic stresses such as high light, drought and heat (De Block et al., 2005).

Drought and other environmental stresses activate MAPKs (Agrawal et al., 2003). The MAPK signal transduction cascade consists of three functionally interlinked protein kinases: MAPKKK, MAPKK, and MAPK. The activated MAPK genes then encode polypeptides whose sequence and function are highly conserved among eukaryotes (Zhu, 2002). In this phosphorylation module, a MAPKKK is phosphorylated directly downstream of the stimulus. The activated MAPKKK then phosphorylates and activates a particular MAPKK, which in turn phosphorylates and activates a MAPK. Activated MAPK is imported into the nucleus, where it phosphorylates and activates specific downstream signalling components, such as transcription factors to induce cellular responses (Triesmann, 1996) (Figure 1).

The activated MAPK cascade induces the expression of stress-response genes that protect plants from diverse environmental stresses. This includes the production of antioxidant enzymes, including glutathione- $S$-transferases (GSTs), peroxidases, superoxide dismutases, and catalases, as well as the activation of protective genes encoding heat shock proteins (HSPs) and pathogenesis-related proteins (Mizoguchi et al., 2000).
Figure 1. Model of MAPK cascade depicting how MAPK phosphorylation system serves as a link between upstream receptors and downstream signalling components such as transcription factors to induce cellular response. (Figure modified from Kaur and Anil, (2005).

When NPK1 gene, a MAPK from tobacco, was engineered into maize using A. tumefaciens, transgenic plants maintained significantly higher photosynthesis rates than did the non-transgenic control under drought condition, suggesting that NPK1 induced a mechanism that protected photosynthesis machinery from dehydration damage (Shou et al., 2004).
In this study, the *NPK1* gene will be engineered into Sudanese maize using *A. tumefaciens* harbouring the gene construct shown in Figure 2. It’s hoped that stable transgene-containing plants will be recovered and will show improved ability to withstand drought stress.
CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Plant materials
Transformability was evaluated in eight Sudanese maize inbred lines (IL1, IL3, IL15, IL16, IL28, IL38, IL42 and IL43) and three commercial maize varieties (Hudiba-1, Hudiba-2 and Mojtamaa-45) obtained from Sudan Agriculture Research Corporation. KAT was used as a local open pollinated variety check, while A188 was used as the inbred line check. Seeds were planted in pots in the research field of Plant Transformation Laboratory at Kenyatta University. On maturing, plants were self-pollinated and immature zygotic embryos (IZEs), 15-16 days after pollination, excised and used as explants.

3.2 Agrobacterium strain and plasmid
*A. tumefaciens* strain EHA101 containing the standard binary vector pSHX004 (Shou *et al.*, 2003) was used to infect the explants materials (Figure 2). pSHX004 contains a broad host range origin of replication and a spectinomycin-resistant marker gene (*aadA*) for bacterial selection; the cauliflower mosaic virus (CaMV) 35S promoter (P35S) to drive both the *bar* selectable marker and the *NPK1* gene; a tobacco etch virus (TEV) translational enhancer in the 5’3 end of the *bar* gene; the soybean vegetative storage protein terminator in its codon region to prevent *NPK1* activity in *Agrobacterium* cells.
3.3 Embryo dissection

Ears 15-16 DAP were harvested and either used immediately or refrigerated for 1-2 days at 4°C while still in the husk. Dehusked ears were surface sterilized for 3 minutes in 70% alcohol followed by soaking in 2.5% sodium hypochlorite containing one drop of wetting agent (Tween 20) for 20 minutes. They were then rinsed 3 times with sterile distilled water under aseptic conditions. Immature embryos of length 1.0-1.5mm were targeted for excision. A long forceps was used to spear the end of the ear on the silk scar side of the kernel to act as a handle while isolating the embryo. The
top half of kernel was cut off with a sterile scalpel while the kernels were still attached on the cob. The embryos were excised aseptically with a spatula.

3.4 Transformation of maize

The generalised protocol for transformation of Sudanese maize via Agrobacterium tumefaciens and the subsequent molecular analysis to verify stable transformation was done as summarised in Figure 3. The detailed protocol is described below.

3.4.1 Agrobacterium preparation

The vector system, pSHX004 in EHA101, was maintained on yeast extract peptone (YEP) medium containing 100mg/L spectinomycin (for pSHX004) and 50mg/L kanamycin (for EHA101). Bacterial cultures for weekly experiments was initiated from stock plates stored for up to two weeks at 4°C.

3.4.2 Agrobacterium infection

Agrobacterium cultures were grown for three days at 28°C on YEP solid medium amended with 100mg/L spectinomycin, 50mg/L kanamycin and 25mg/l chloramphenicol. One full loop (3 mm) of bacterial culture was scraped from the three-day old plate and was suspended in 5 ml of liquid infection medium (IM) supplemented with 100 µM acetosyringone (IM+AS) in a 50 ml centrifuge tube. The tube was fixed horizontally to a bench-top shaker and shaken on speed (~200rpm) for one hour at room temperature. This pre-induction step was carried out for all experiments. For infection, immature zygotic embryos from one ear were dissected to bacteria-free IM+ AS medium (2 ml) in 150x15mm sterile petri-plate (20 to 100 embryos). This was followed by the addition of 20ml of Agrobacterium suspension
(OD$_{550} = 0.3$ to $0.4$) to the embryos and left to stand in the dark for five minutes with occasional swirling.

### 3.4.3 Co-cultivation

After infection, embryos were transferred to the surface of solid co-cultivation medium (CCM) and excess *Agrobacterium* suspension pipetted off the medium surface. Embryos were oriented with the embryo-axis side in contact with the medium (scutellum side up). Plates were then wrapped with aluminium foil and incubating in the dark at 20°C for three days.

### 3.4.4 Resting

After three days of co-cultivation, all embryos were transferred to resting medium (RM) and plates wrapped with parafilm before incubating at 28°C in dark for 14 days.

### 3.4.5 Selection for stable transformation events

After 14 days on RM, embryos responding or not responding to callus induction were transferred to selection medium containing 1.5 mg/L bialaphos (SI) for 2 weeks. They were sub-cultured for two more 2-week passages on 3 mg/L bialaphos (SII) and wrapped with parafilm throughout selection. After the six weeks selection period, embryos surviving bialaphos selection were counted for calculation of Transformation frequency (TF). Transformation frequency was computed according to Frame *et al.* (2006) as the percentage of the number of calli surviving bialaphos selection compared to the number of embryos infected.
3.4.6 Regeneration of transgenic plants and acclimatization

Acclimatization of regenerated plants was accomplished in soil in the glasshouse as described by Oduor et al. (2006). The number of regenerated plants was counted in order to compute the regeneration frequency (RF). RF was computed as the percentage of the number of regenerated shoots compared to the number of calli regenerating at least one shoot. Plantlets were maintained in the glasshouse till they matured and set fertile seeds.

3.5 Transgenic plant analysis

To conform the presence, R₁ seedlings were screened for the presence of the Bar gene by Polymerase Chain Reaction (PCR). Transformation efficiency was computed on PCR positive events according to Frame et al. (2002) as the percentage of the number of PCR positive events per 100 Agrobacterium-infected embryos.

3.5.1 PCR analysis

Leaf genomic DNA was prepared according the CTAB extraction method of Shanghai Maroof et al. (1984). The primers used to amplify the bar gene were forward primer 5'-GTCTGCACCATCGTCAACC-3' and reverse primer 5'-GAAGTCCAGCTGCCAGAAAC-3' described in Liu et al. (1998). The PCRs were carried out in a 25μL solution comprising 10 ng of genomic DNA, 50 mM KCl, 10 mM Tris–HCl buffer (pH 8.8), 3 mM MgCl₂, 0.1% (w/v) Triton X-100, 0.24 mM each dNTP, 1 U Taq DNA polymerase, and 0.16 μmol of each primer. Denaturation was performed at 94 °C for 1 min followed by 30 amplification cycles (94 °C for 30 s, 64 °C for 2 min, and 72 °C for 2 min) were separated in 0.8% (w/v) agarose gels.
3.5.2 Agarose gel electrophoresis

The PCR amplification products (5 μL) were mixed with loading dye (0.4% bromophenol blue, 50% glycerol) and DNA marker (1kb ladder) loaded as a standard into individual well in 0.8 % agarose gel containing ethidium bromide (0.5μg/ml). The electrophoresis gel tanks were buffered by 1 x TAE [0.1m Tris/acetic acid, 20mM EDTA]. Electrophoresis was carried out at 100 volts for 1 hour. PCR product visualization and photography was achieved using the Genesnap image acquisition software from Syngene (Synoptics LTD).
Figure 3. Schematic representation of summarized protocol for transformation of maize.
### 3.6 Data management and analysis

All experiments were set up as completely randomised block design and replicated six times per genotype. Analysis of variance (ANOVA) was used to test the differences in responses to transformation frequency and efficiency among the genotypes using the Genstat for Windows (Discovery edition) statistical software. Means were compared using least significant difference (LSD) at the 95% confidence level. All percentage data were square root transformed before analysis.
CHAPTER FOUR

4 RESULTS

4.1 Selection of stable transformants

After a period of co-culture and resting (Figure 4), infected embryos responding or not responding to callus induction were transferred and maintained on selection media, containing bialaphos, for six weeks to select for stable transformation (Figure 5). All 13 genotypes used in this study were observed to initiate calli that survived and grew in bialaphos-containing media (Figure 5). However, the genotypes were different in the type of callus they initiated. Calli of IL3, Hudiba-2 and IL15 were observed to be white in colour, dry and compact. Moreover, friable embryos were clearly observable on the surface of these callus cultures (Figure 6A, B and C). IL15 calli were observed to comprise of compact and friable sections and watery sections on their surface (Figure 6C).

The inbred lines IL42, IL28 and IL43 were observed to induce a callus type that was watery and cream-colored in appearance (Figure 6D, E and F). These calli had a soft and spongy texture with no distinct embryo like structures observable on their surface. This callus type has been designated Type I (Phillips et al., 1988).

Rhizogenesis was also observed in some maize genotypes. Hudiba-1 and IL28 were particularly observed to germinate red, green and some times white pigmented roots during selection.
Figure 4. Resting of *Agrobacterium*-infected immature embryos of IL1, IL15, IL3 and Hudiba-1. A: Immature embryos of IL1 after 5 days of culture on resting medium (RM). B: Immature embryos of IL15 after 10 days on RM. C: Immature embryos of IL3 after 10 days on RM. D: Immature embryos of Hudiba-1 after 10 days on RM. All immature embryos are on resting media with 250mg/L cefotaxime. (Bars=1cm).
Figure 5. Selection of stable transformants in selection II media (SII). A: IL28 calli after 1 week culture on selection II media (SII). B: IL3 calli after 1 week culture on SII. C: KAT calli after 4 weeks culture on SII. D: IL15 calli after 4 weeks culture on SII. Calli were transferred from selection I media (SI) containing 1.5mg/l bialaphos to SII with 3.0 mg/l of bialaphos. Red arrows point to dead calli, while black arrows point to growing calli. Bars =1cm.
Figure 6. Callus formation in maturation media with bialaphos. A: Embryogenic callus of IL3 after culture on regeneration 1 media (R1), showing (a) somatic embryos. B: Embryogenic callus of Hudiba-2 after culture on R1. Arrow (a) points to somatic embryo. C: IL15 callus showing (b) embryogenic section and (c) watery section. D: Calli of IL1 showing the absence of somatic embryos. E: Calli of IL42 that is watery and lacks somatic embryos. F: Calli of IL16 that lacks somatic embryos.
4.2 Effect of genotype on transformation frequency

Transformation frequency (TF) was computed for all the 13 genotypes on which transformability was evaluated. The average transformation frequency for all the 13 genotypes was 8.24% (Table 1). The Open pollinated variety, KAT, and the inbred line, A188 used as checks gave average transformation frequencies of 7.27% and 5.82% respectively.

The highest average TF of 31.70% with a range of 14.54 and 43.48% was observed in IL3 (Figure 7). The next highest TF was obtained in IL43, which ranged from 4.76 to 15.00% with an average of 10.09%. Hudiba-2 had a TF of 8.65% with a range of between 6.06 and 9.67%. The lowest TF ranged from 0.00 to 4.24%, with 1.69% as the average in Mojtamaa-45. The average TF obtained for IL1 was among the lowest obtained in this study (Figure 7). IL1 gave TF of between 0.00 and 6.06%, with an average of 2.47%. This was followed, in ascending order, by mean TFs of 5.19%, 5.99% and 6.34% produced by IL42, IL16 and IL15, respectively (Figure 7). The highest TF observed for IL42 was 5.56% while the lowest was 2.67%. IL16 had TF of 8.33% as the highest and 2.08% as the lowest, while the TF of IL15 ranged from 0.00% to 13.00%.

Comparable average transformation frequencies were obtained for the genotypes IL28 IL38 and Hudiba-1 (Table 1). The Average TF recorded by IL28 was 7.13% with a range of 3.64% to 10.00%. IL38 had a TF of 7.24% with a range of between 2.70 and 12.12%. The TF of Hudiba-1 ranged from 3.43% to 11.54 % with 7.56 % as the average.
Table 1. Putative transformation frequency and efficiency of various maize genotypes.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Experiments</th>
<th>Number of embryos infected</th>
<th>Number of bialaphos resistant calli</th>
<th>Putative transformation frequency (%)</th>
<th>Putative transformation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAT</td>
<td>6</td>
<td>442</td>
<td>30</td>
<td>7.273±1.222</td>
<td>0.555± 0.555</td>
</tr>
<tr>
<td>Mojtamaa-45</td>
<td>6</td>
<td>295</td>
<td>5</td>
<td>1.686±0.842</td>
<td>0.355±0.355</td>
</tr>
<tr>
<td>Hudiba-1</td>
<td>6</td>
<td>549</td>
<td>37</td>
<td>7.553±1.213</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>Hudiba-2</td>
<td>6</td>
<td>263</td>
<td>23</td>
<td>8.650±0.705</td>
<td>1.232±0.789</td>
</tr>
<tr>
<td>A188</td>
<td>6</td>
<td>433</td>
<td>24</td>
<td>5.818±1.024</td>
<td>0.976±0.500</td>
</tr>
<tr>
<td>IL3</td>
<td>6</td>
<td>253</td>
<td>76</td>
<td>31.700±2.108</td>
<td>3.780±0.856</td>
</tr>
<tr>
<td>IL16</td>
<td>6</td>
<td>257</td>
<td>14</td>
<td>5.996±1.083</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>IL28</td>
<td>6</td>
<td>334</td>
<td>23</td>
<td>7.186±0.858</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>IL42</td>
<td>6</td>
<td>374</td>
<td>19</td>
<td>5.192±0.824</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>IL38</td>
<td>6</td>
<td>419</td>
<td>29</td>
<td>7.240±1.380</td>
<td>0.177±0.177</td>
</tr>
<tr>
<td>IL15</td>
<td>6</td>
<td>292</td>
<td>16</td>
<td>6.341±1.962</td>
<td>0.222±0.222</td>
</tr>
<tr>
<td>IL43</td>
<td>6</td>
<td>232</td>
<td>23</td>
<td>10.093±1.839</td>
<td>0.000±0.000</td>
</tr>
<tr>
<td>IL1</td>
<td>6</td>
<td>258</td>
<td>8</td>
<td>2.468±0.951</td>
<td>0.253±0.253</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4401</strong></td>
<td><strong>327</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| P value  | <0.0001                | <0.0001                    |
| LSD      | 4.997                   | 1.149                      |

Values are the means of six replications ±S.E.
Figure 7. Putative transformation frequency of various maize genotypes. Values are means of six replications and vertical bars are standard errors.
Analysis of variance (ANOVA) showed that there were significant differences among the genotypes used in this study in transformation frequency \( (p<0.0001) \) (Table 1). Mojtamaa-45 gave the lowest average transformation frequency, which was significantly lower \( (p<0.05) \) than that of all the other genotypes. IL1 was inferior to other genotypes as its TF was observed to be the lowest \( (p<0.05) \). Differences were observed in TF between IL3 and the other genotypes, in that the TF produced by IL3 (31.70%) was found to be significantly higher \( (p<0.0001) \).

Significant differences were observed among the OPVs used in this study. The local check OPV (KAT) was observed to produce a significantly higher TF \( (p<0.005) \) than that of Mojtamaa-45, a Sudanese OPV. KAT, Hudiba-1 and Hudiba-2 produced almost comparable TFs. No significant differences \( (p>0.05) \) in TF were found among them. Variations were found among Sudanese OPVs with respect to transformation frequency. Mojtamma gave a significantly lower TF \( (p<0.005) \) compared to other Sudanese OPVs (Table1).

ANOVA also showed significant differences between Sudanese inbred lines and the A188 genotype used as the check inbred line. The Putative TF of 31.70% produced by IL3 was found to be significantly higher \( (p<0.0001) \) than that of A188 (5.82%) as well as the other Sudanese inbred lines \( (p<0.0001) \). However, no significant differences \( (p>0.05) \) were found between A188 and the rest of the Sudanese inbred lines.
IL1 gave the lowest average of TF. A significant difference \( (p < 0.0149) \) was observed between IL1 and A188 with respect to TF. Moreover, significant differences in TF \( (p < 0.005) \) were found between IL1 and all other Sudanese inbred lines.

### 4.3 Effect of genotype on putative transformation efficiency

Putative transformation Efficiency (TE) was calculated for all the maize genotypes on which transformation was attempted. Results indicate that most of the Sudanese varieties were particularly incompetent in establishment of mature and fertile plants. This is reflected by the low putative TE values they recorded, that are as low as 0.00% (Table 1). Only eight genotypes produced mature and fertile seed-setting plants and so relatively high TE values were obtained from them. The standard control inbred line (A188) and the local check OPV (KAT) recorded TE of 0.98 and 0.56% respectively. The highest TEs were observed in IL3 and Hudiba-2. IL3 averaged 3.78% in TE, with a range of 2.70% - 5.46%. Hudiba-2 was observed to yield an average TE of 1.23% with a range of between 0.00% and 4.17%. IL15 and IL1 recorded TEs that were lower than those of the control genotypes (Table 1).

ANOVA shows that there was an influence by the genotype on the observed T.E. response (Table 1). The T.E of IL3 was significantly higher \( (p < 0.05) \) than those of all the other genotypes, including A188. The best performing OPV with regard to T.E. was Hudiba-2, whose efficiency of transformation was significantly higher \( (p < 0.005) \) than that of other OPVs.
4.4 Regeneration of putative transformants

Bialaphos surviving calli from all the genotypes were placed on maturation (Regeneration 1) media and cultured for 14 days in the dark (Figure 8). During this period, somatic embryos of A188, IL3, IL15, IL1, IL38, Mojtamaa-45, Hudiba-2 and KAT calli matured, that is they formed a scutellum (that later turned white), a coleoptile-like structure (that turned green) and a rootlet (Figure 8E). Calli of the other genotypes had no observable response to maturation.

Mature calli transferred to shoot induction media (Regeneration) started greening 2-5 days after transfer to light. From majority of the calli, shoots and roots regenerated on R1 (Figure 9 and 10) and transfer to rooting medium was therefore unnecessary. The calli of Hudiba-1 and IL28 cultured on R1 responded to maturation by forming roots, which were mostly red pigmented (Figure 11). Calli of Mojtamaa-45, IL1, IL43 and IL28 had no other observable response to regeneration except slight greening.

Since all the genotypes produced bialaphos resistant calli, regeneration was attempted for all the genotypes. However, not all genotypes could regenerate plants. Regeneration was achieved only in IL3, IL15, and IL1, IL38, Hudiba-2, Mojtamaa-45, A188 and KAT. These genotypes regenerated 19, 4, 3, 2, 10, 5, 7 and 3 plants, respectively (Table 2). Mojtamaa-45 had the highest regeneration frequency (Table 2). However regenerated shoots were weak and delicate in growth resulting in the death of some of them. Moderately high regeneration frequency of 43%, 37% and 29% were observed for Hudiba-1, IL1 and A188, respectively. The lowest regeneration frequency was recorded by IL38 (Table 2).
Table 2. The number of shoots per regenerating putative transformed callus.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of bialaphos resistant calli to regeneration</th>
<th>Number of regenerating calli</th>
<th>Number of shoots</th>
<th>Fertile R₀ plants</th>
<th>Regeneration Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL3</td>
<td>76</td>
<td>10</td>
<td>19</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>IL15</td>
<td>16</td>
<td>1</td>
<td>4</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>IL1</td>
<td>8</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>37.5</td>
</tr>
<tr>
<td>Hudiba-2</td>
<td>23</td>
<td>3</td>
<td>10</td>
<td>6</td>
<td>43.48</td>
</tr>
<tr>
<td>Mojtamaa-45</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>KAT</td>
<td>30</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>IL38</td>
<td>29</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>A188</td>
<td>24</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>29.17</td>
</tr>
<tr>
<td>Total</td>
<td>211</td>
<td>23</td>
<td>53</td>
<td>28</td>
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</tr>
</tbody>
</table>
Figure 8. Regeneration of putative Agrobacterium-transformed maize genotypes. 
A: Hudiba-2 calli after 2 weeks on regeneration I media (RI) with 3 mg/l bialaphos. 
B: IL 43 calli after 2 weeks on RI with 3 mg/l bialaphos C: A188 calli after 2 weeks 
on RI with 3 mg/l bialaphos. Bars =1cm. D: IL3 mature putative transformed calli on RI. Red arrows show root regeneration and black arrow shows shoot regeneration. Bar =1.5cm. 
E: Mojtamaa-45 mature putative transformed calli on RI. Red arrow shows root regeneration and black arrow shows shoot regeneration. Bar = 0.5cm.
Figure 9. Plantlet formation from bialaphos resistant calli of OPV maize. A: Shoot and root regeneration from a bialaphos resistant callus of Hudiba-2 after 2 weeks on regeneration II medium (RII). B: Mojtamaa-45 callus forming plantlets after 2 weeks on RII. C: Katumani calli regenerating plantlets on RII. Bars=2cm. Calli were transferred from bialaphos-containing regeneration I and maintained on regeneration II media without bialaphos.
Figure 10. Plantlet formation from bialaphos resistant calli of some inbred lines. 
A: A188 callus with multiple shoots formation after 2 weeks on RII. B: IL3 callus regenerating a shoot after 2 weeks on RII. C: IL15 callus with an emerging shoot 3 days after culture on RII. D: IL1 callus regenerating plantlets after 1 week RII. Bars =2cm. Calli were transferred from bialaphos-containing regeneration I and maintained on regeneration II media without bialaphos.
Figure 11. Rhizogenesis in Sudanese maize. A: Hudiba-1 calli showing profuse regeneration of red-pigmented roots in regeneration 1 media B: IL28 calli showing root geminating in regeneration media (Bars =1cm).
4.5 Acclimatization and growth of regenerants in glass house

After about 2-3 weeks of culture in shooting or rooting media, plantlets with a well developed root system were transplanted to peat moss for glass house conditioning (Figure 12 and 13). Most of the plantlets survived hardening and were transferred to the soil. A total of 53 regenerants were obtained from 8 regenerating genotypes. Plants were maintained to maturity in the glass house (Figure 14 and 15).

Although regenerated plants appeared fertile, some abnormal phenotypes were observed in regenerated plants (Figure 16 and 17). The most commonly observed abnormalities in R0 regenerants were dwarfism, lack of tussel and tassel ears, which occurred at frequencies of 78%, 58% and 33% respectively (Table 3). Plants with dwarf, tasteless and earless phenotypes failed to set seeds. However other plants (28 in total) produced viable seeds despite the presence of abnormalities (Table 2). These seeds were mostly established from tassels of mature plants (Figure 18 and 19).
Table 3. Abnormalities observed in putative R₀ transgenic plants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Abnormality</th>
<th>Silk and tassel from same place</th>
<th>Lack of tassel</th>
<th>Dwarfism</th>
<th>Silk growth from apex</th>
<th>Long silk</th>
<th>Multiple cobs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL3</td>
<td></td>
<td>2/6 (33%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL15</td>
<td></td>
<td>1/2 (50%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1/2 (50%)</td>
<td>-</td>
</tr>
<tr>
<td>Hudiba-2</td>
<td></td>
<td>1/6 (17%)</td>
<td>3/6 (33%)</td>
<td>4/6 (67%)</td>
<td>-</td>
<td>-</td>
<td>1/6 (17%)</td>
</tr>
<tr>
<td>A188</td>
<td></td>
<td>-</td>
<td>2/3 (75%)</td>
<td>3/3 (100%)</td>
<td>1/3 (33%)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>KAT</td>
<td></td>
<td>-</td>
<td>1/2 (50)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IL1</td>
<td></td>
<td>-</td>
<td>1/1 (100)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mojtamaa-45</td>
<td></td>
<td>1/1 (100%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>5/15 (33%)</td>
<td>7/12 (58%)</td>
<td>7/9 (78%)</td>
<td>1/3 (33%)</td>
<td>1/2 (50%)</td>
<td>1/6 (17%)</td>
</tr>
</tbody>
</table>

Values are the number of plants with the observed abnormality compared to the total number of regenerated plants. Values in parenthesis are percentages of plants with the abnormality over the total number of regenerated plants.
Figure 12. Hardening of plantlets regenerated from putative transgenic inbred lines. A: Hardening of A188 regenerants. B: Hardening of IL15 plantlets C: Hardening of IL3 regenerants. D: Hardening of IL1 plants. Bars= 5cm. All plants are growing in small pots containing peat mose and covered with a polythene bag.
Figure 13. Hardening of plantlets regenerated from putative OPV transformants. A: Hardening of Hudiba-2 regenerants. B: Hardening of Mojtamaa-45 regenerants. C: Hardening of Katumani plantlets. Bars = 4cm. All plants growing in small pots containing peat mose and covered with a polythene bag.
Figure 14. Glass house growth of hardened inbred line plants. A: Maturing IL3 plants. B: Maturing IL15 plants. C: A188 plants 3 weeks after hardening. D: IL1 plants 3 weeks after hardening. All plants growing in 20 liter pots containing loam soil in the glass house.
Figure 15. Glass house maintenance of hardened OPV plants. A: Silking Katumani plant. B: Mojtamaa-45 plant forming silks. C: Silking Hudiba-2 plant. All plants growing in 20 liter pots containing loam soil in the glass house.
Figure 16. Abnormalities observed in $R_0$ transgenic plants A: multiple ears sprouting in Hudiba-2. B: dwarfism in A188 C: dwarf and tussel seed variants of Hudiba-2 D: lack of leaf spread in an IL3 plant.
Figure 17. Tussel seed abnormality observed in R₀ transgenic plants. Formation of tussel seeds in IL3 (A), IL15 (B), IL1 (C) and IL3 (D) regenerants.
4.6 Screening for stable transformation in R₁ plants using PCR

Seedlings of putative transformants were assessed for the presence of the bar gene. Gel electrophoresis of genomic DNA extract from the leaves of the seedlings revealed minimal degradation during extraction (Figure 18). The presence of bar gene in the putative transformed R₁ plants was detected by PCR amplification of the 540 bp fragment in the DNA extracts from transgenic plants. This fragment represents the bar gene element on the pSHX004 plant expressible cassette. DNA from a non-transformed Sudanese maize genotypes was used as the negative control whereas pSHX004 plasmid DNA was used as the positive control. There was amplification in the sample containing the plasmid DNA and no amplification occurred in the negative controls (Figure 21).
Figure 18. Seed of putative OPV transformants. A and B: Hudiba-2 R₀ seeds established on the tussel C: Katumani R₀ ear seeds
Figure 19. Fertile seed from putative inbred line transformants. A and B: IL3 $R_0$ seeds from the tassel and ear. C: IL15 $R_0$ seeds established on the tassel.
D: 188 $R_0$ ear seeds.
Figure 20. Gel electrophoresis of genomic DNA of putative transformants. Lane M: 1Kb DNA Marker, Lane 1-4: IL3, Lanes 4-10: Hudiba-2, Lanes 11-12: IL15.
Figure 21. Polymerase chain reaction (PCR) screening for the bar gene in R₁ transgenic plants. M: 1kb DNA marker lanes 1-4: non-transformed (negative control) lane 5: pSH004 plasmid DNA (positive control) lane 6: IL15, lanes 7-8: A188, lanes 9-10: Hudiba-2, lanes 11-12: IL3, lane 13: KAT.
CHAPTER FIVE

DISCUSSION

Necrosis and growth arrest was observed in embryos after 7-14 days of selection in bialaphos. Bialaphos selection has been observed to cause the browning and eventual death of untransformed maize embryos by a number of investigators working with tropical maize (Binnot et al., 2005; Mburu 2007). Non-transformed cells exposed to bialaphos die as a result interruption of protein synthesis by phosphinothricin (the active component of bialaphos). Phosphinothricin (PPT) is a potent inhibitor of glutamine synthase. Inhibition of glutamine synthase leads to a rapid accumulation of ammonia in the cells leading to cell death (De block et al., 1987).

Despite the presence of bialaphos, a small percentage of infected embryos were observed to grow and initiate the formation of callus. Survival of these embryos because they harbored the bar gene which codes for the phosphinothricin acetyltransferase (PAT) enzyme that acetylates PPT. Acetylated PPT is no longer inhibitory to glutamine synthase (De block et al., 1987).

Necrosis and cell death was also observed in some genotypes to start at the co-cultivation stage. IL1, IL16 and Mojtamaa-45 embryos were especially observed to respond to Agrobacterium co-cultivation with necrosis and arrested growth. It has been shown that Agrobacterium infection causes tissue necrosis and mortality. Plant cells respond to invading pathogens by activating signal transduction leading to hypersensitive reaction characterized by a rapid localized cell death around the infection area (). Agrobacterium induced necrosis has been reported in maize under
co-cultivation conditions (Hansen, 2000). However, tissue necrosis and mortality have been reported to occur rapidly in *Agrobacterium*-free medium (Das et al., 2002; Perl et al., 1996). Factors that influence the degree of necrosis include explant age, preculture period, bacterial density and infection duration. Kumaria et al. (2001) observed that a high bacterial density and prolonged infection time adversely affected the growth and regeneration of indica rice during *Agrobacterium*-mediated transformation.

Necrosis and tissue death induced by *Agrobacterium*, especially as observed in recalcitrant species, has been proposed to be caused by the over-production of ROS (oxidative burst) at the site of *Agrobacterium* infection (Kuta and Tripathi, 2005). The generated oxygen radicals may lead not only to plant cell death and necrosis, but also to bacterial cell death and induction of pathogenesis-related genes, followed by the production of antimicrobial substances (phytoalexins, etc) and oxidation of sugar and base moieties of DNA (Mehdy, 1994). Plant tissue necrosis and death induced by *Agrobacterium* mediated T-DNA transfer may be arrested by reprogramming a resistant plant cell into a susceptible one. This may be achieved through two main approaches. Induction of cellular dedifferentiation and rejuvenation through preculture of the explant before *Agrobacterium* infection (Sangwan et al., 1992), and addition of acetosyringone (Wu et al., 2003). Additionally, compounds with potential to quench oxidation burst can be used to arrest *Agrobacterium*-induced oxidative burst in host tissues, thereby improving transformation efficiency especially in ‘recalcitrant’ species (Cheng et al., 2004).
Immature embryos of genotypes used for transformation in this study initiated two types of embryogenic callus types under selection conditions, Type I and Type II callus. Maize immature embryos have been shown to initiate the two types of calli (Armstrong and Green, 1985). Type I callus is dirty white, compact and organogenic while type II is white friable and embryogenic. IL3, Hudiba-2, Mojtabaaa-45, KAT, A188, IL15, IL38 and IL28 formed Type II callus while IL16, IL43 and IL42 initiated Type I callus. The type of callus induced has been shown to be dependent on the genotype (Carvalho et al., 1997). Two genes have been implicated in the inheritance of callus induction and plant regeneration. These genes find expression in the middle and basal portion of the scutellum of immature embryo. In case of responsive genotypes, these regions proliferate to form embryogenic callus while in non-embryogenic genotypes, they don't (Bronsema, 1997).

Although some genotypes such as IL28 initiated Type II callus, the callus failed to regenerate into plants but rather continued forming roots on maturation media. Carvalho et al. (1997) reported that not all tropical genotypes that initiated embryogenic calli could regenerate plants and some genotypes classified as nonembryogenic produced plants. They concluded that such a classification does not accurately predict the regenerative ability of a callus from a given genotype (Carvalho et al., 1997). This implies that plant regeneration is achievable for both embryogenic and non-embryogenic genotypes under appropriate tissue culture condition.

The two types of callus were also observed on the same embryo for some genotypes such as IL15. This observation is similar to that of Bronsema et al. (1997) who
reported formation of compact embryogenic callus at the middle part of the scutellum and friable embryogenic callus at the basal side of the scutellum.

The genotypes Hudiba-1 and IL28 showed formation of compact callus in selection media. It was also observed that from this compact callus, roots emerged from all directions. This type of callus (rhizogenic callus) has been observed in both embryogenic and nonembryogenic maize varieties and inbred lines (Green and Phillips, 1975; Tomes, 1985). These results are similar to those Rao et al. (2006) who reported that such a response could be dependent on the genotype used. It has been shown for some maize genotypes that the transition from embryogenesis to rhizogenesis can be prevented by addition of sugars such as mannitol in the callus proliferation media (Emons and Kieft, 1995). Plant regeneration from rhizogenic calli could not be achieved under these culture conditions. It is possible that differences in responses to regeneration of a rhizogenic callus depends on the presence of other factors in the culture media. For example the addition of dicamba in the culture media resulted in regeneration of a rhizogenic callus of the inbred line A632 (Duncan et al., 1985).

Nine inbred lines and four OPVs were studied in relation to their response to Agrobacterium-mediated transformation. It was observed that all the inbred lines and the OPVs are transformable since some callus events survived selection in bialaphos. Similar results have been reported by other workers with tropical maize (Binnot et al., 2005; Ombori et al., 2006). However, not all bialaphos resistant events were regenerable. Although IL16, IL42, IL28, IL43 and Hudiba-1 had some callus surviving bialaphos selection, no shoots could regenerate from them. These genotypes
recorded TE of zero. Regenerability has been found to be genotype-dependent (Armstrong 1985). Furthermore, regeneration of transformants may be influenced negatively by prolonged exposure to bialaphos and 2,4-D (MAFF, 2004).

Results indicate that the putative transformation frequency response varied with the genotype used. Similar findings were reported by Ombori et al. (2006) who observed genotype specific responses in TF when Agrobacterium was used to transform tropical maize. A188, a temperate inbred line, has been proven to be an effective Agrobacterium host and performs well in culture. Therefore, it is used widely to extend the range of maize genotypes susceptible to Agrobacterium (Ishida et al., 1996; Zhao et al., 2001). Results indicate that A188, used a standard check inbred line, produced a putative TF of 5.8%. A TF of 5.5% was obtained for A188 by Frame et al., (2002) utilising the Agrobacterium strain EHA101 harbouring pTF102 binary vector construct. However, TFs of between 5% and 30% have been realised in A188 using Agrobacterium harbouring a superbinary vector (Hiei et al., 1994). The average TF obtained for the Sudanese Inbred lines was 8% and ranged from 2.5% for the IL1 to 31.7% for IL3. A high TF was obtained for IL3 despite the recalcitrance of tropical maize, especially inbred lines, to Agrobacterium-mediated transformation (Carvalho et al., 1997). In fact, the putative TF of IL3 was observed to be higher than that of the standard check, A188. Consequently, IL3 can be said to be better than A188 in transformability under the particular culture conditions used in this study. IL1 on the other hand is inferior to A188, and indeed all other genotypes evaluated in TF.

KAT is an OPV that is widely grown across the Eastern and Southern African region due to its fast maturity and drought resistance (Muasya and Diallo, 2001; Mugo et al.,
Recent research in KAT has shown that it responds well to tissue culture (Matheka, 2007; Oduor et al., 2006; Ombori et al., 2006) and transformation (Mburu, 2007). A transformation frequency of 16.6% was obtained for KAT by Mburu (2007), using the PMI system in the *A. tumefaciens* strain EHA101. The TF obtained for KAT in this study was relatively lower. These differences may largely be attributed to the different selective agents used. While the negative selection agents such as bialaphos may have a detrimental effect on the growth of calli (MAFF, 2006), positive selective agents such as mannose promote the growth of transformed cells (Joersbo et al., 1998). The Sudanese OPV that was most amenable to transformation was Hudiba-2 as it showed no significant differences in TF compared to KAT.

Transformed calli were observed to regenerate between 1-3 shoots per callus, indicating a relatively high capacity of bialaphos resistant calli to regenerate shoots. Regeneration frequencies of bialaphos resistant shoots varied from 100% for Hudiba-2 to 6% for A188. Tissue culture response and plant regenerability has been shown to be under complex genetic control. Separate genes have been shown to govern these processes (Carvalho, 1997; Peng and Hodges, 1989; Phillips et al., 1988).

Most of the regenerated plantlets survived the hardening process but some died. The transition from the *in vitro* environment to the glasshouse constitutes a stressful stage (Lutts et al., 1999), and this may have brought about the death of some plantlets. Moreover, poor root formation and lack of proper root penetration in the peat moss may have further reduced plantlet survival.
As the plants grew to maturity in the glasshouse, they were observed to be aberrant in phenotypes like height, tassel and ear formation. These aberrations are typical of tissue-cultured cells, plants derived from such cells, and progenies of such plants. They have been termed as somaclonal variations and have been described either as epigenetic or genetic in occurrence (Larkin and Scowcroft, 1981). Epigenetic changes are physiological and so cannot be passed on from one generation to another. On the other hand, genetic changes are heritable and arise as a result of changes in the chromosome structure and number. Several factors have been proposed to lead to such changes in the cultured cells including tissue culture components (e.g. 2,4-D), tissue culture conditions (e.g. high temperature) and in vitro manipulations (e.g. time in culture). Apart from the tissue culture-related factors, cellular stresses imposed by the transformation process increases the chances of recovering plants with increased somaclonal variations (Choi et al., 2000). Such transformation process as loss of cell turgor during infection, selection of transformed tissues, and the necessity of transformed tissue to grow in presence of dying or dead tissue, causes cellular stress, thereby causing an impact on the integrity of the chromosome (Choi et al., 2000).

In conclusion, this study demonstrates that transformation using Agrobacterium tumefaciens is possible for a number of Sudanese maize genotypes. Successful transformation of Sudanese inbred lines (IL3, IL1, IL15 and IL38) and OPVs (Hudiba-2 and Mojtamaa-45) using the NPK1 gene as revealed by PCR analysis was achieved. A total of 28 putative transgenic plants were obtained from these genotypes. The information provided here can therefore be used to yield reproducible results in other Sudanese maize varieties.
CONCLUSION

This study aimed at assessing the transformability of Sudanese maize genotypes. All the Sudanese genotypes evaluated were successfully selected in bialaphos-containing media, indicating the successful transformation of these genotypes. The different genotypes used in this study produced varying transformation frequencies, indicating a genotype dependent response.

Not all bialaphos resistant callus events were regenerable. Only six Sudanese maize genotypes, one standard inbred line check (A188) and a local OPV check (KAT) regenerated shoots, an indication that regeneration is genotype specific. Frequencies of transformation were also found to vary with the genotype used.

The high Transformation frequency and efficiency obtained for IL3 indicate that the quest to identify Sudan maize inbred line that responds well to transformation and regeneration has been attained. This inbred line can therefore be used to extend the range of other local, and indeed international, germplasm for Agrobacterium-mediated transformation through crossing. This implies that the introgression of useful agronomic traits in local varieties will be more effective and faster than is achievable using conventional breeding.

Two commercial varieties (Mojtamaa-45 and Hudiba-2) were successfully transformed indicating the possibility of indirect genetic engineering of OPV which eliminates the necessity of breeding.
To my Knowledge, this is the first study aimed at transforming any Sudanese crop. Therefore, it will have relevance to corresponding research in other Sudanese crops such as sorghum, cotton and wheat. Moreover, it’s the first report on the genetic engineering of maize genotypes with a gene for drought tolerance using *A. tumefaciens*. This study will therefore form a reference point in the improvement of other maize genotypes.
RECOMMENDATIONS

1) Optimisations of *Agrobacterium* mediated transformation conditions to improve the efficiency of maize transformation for both poor and good responders should be done.

2) Southern hybridization analysis to establish stable integration of the *NPKI* gene into the maize plant genome should be done on transgenic plants.

3) The agronomic performance of the transgenics under water stress conditions should be evaluated.
REFERENCES


APPENDIX I. Composition of media used for *Agrobacterium* culture

**YEP medium**

- **Yeast extract**: 5g/l
- **Peptone**: 10g/l
- **NaCl**: 5g/l
- **Bactoagar**: 15g/l

Adjust pH to 6.8 with 1N NaCl. Autoclave and let cool to 60°C.

Then add:

- **Chloramphenicol**: 25mg/l
- **Kanamycin**: 50mg/l
- **Spectomycin**: 100mg/l
## APPENDIX II. Basal Media

<table>
<thead>
<tr>
<th>Medium name</th>
<th>Macronutrients</th>
<th>Micronutrients</th>
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</thead>
<tbody>
<tr>
<td><strong>Murashige and</strong></td>
<td>NH$_4$NO$_3$ (1650 mg/l)</td>
<td>H$_3$BO$_4$ (6.2 mg/l)</td>
</tr>
<tr>
<td><strong>Skoog (MS)</strong></td>
<td>CaCl$_2$.2H$_2$O (332.02 mg/l)</td>
<td>COCl$_2$.6H$_2$O (0.025 mg/l)</td>
</tr>
<tr>
<td><strong>basal media</strong></td>
<td>MgSO$_4$.7H$_2$O (180.54 mg/l)</td>
<td>CuSO$_4$.5H$_2$O (0.025 mg/l)</td>
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<tr>
<td></td>
<td>KNO$_3$ (1900 mg/l)</td>
<td>FeNaEDTA (36.7 mg/l)</td>
</tr>
<tr>
<td></td>
<td>KH$_2$PO$_4$ (170 mg/l)</td>
<td>FeSO$_4$.7H$_2$O (27.8 mg/l)</td>
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<td></td>
<td></td>
<td>MnSO$_4$.H$_2$O (16.9 mg/l)</td>
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<tr>
<td></td>
<td></td>
<td>KI (0.83 mg/l)</td>
</tr>
<tr>
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<td></td>
<td>Na$_2$MoO$_4$.2H$_2$O (0.25 mg/l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnSO$_4$.H$_2$O (8.6 mg/l)</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td>Myoinositol (100 mg/l),</td>
<td></td>
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<tr>
<td></td>
<td>Glycine (2.0 mg/l),</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nicotinic acid (0.5 mg/l),</td>
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<tr>
<td></td>
<td>Pyridoxine HCL (0.5 mg/l),</td>
<td></td>
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<tr>
<td></td>
<td>Thiamine HCL (0.1 mg/l).</td>
<td></td>
</tr>
<tr>
<td><strong>Linsmaier and</strong></td>
<td>Same as MS</td>
<td>Same as MS</td>
</tr>
<tr>
<td><strong>Skoog (LS)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>basal media</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td>Myoinositol (100 mg/l),</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiamine HCL (0.4 mg/l).</td>
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APPENDIX III. Maize Transformation Media (Adapted From Frame et al., 2002)

<table>
<thead>
<tr>
<th>Medium name</th>
<th>Composition</th>
</tr>
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<tr>
<td></td>
<td><strong>Pre-autoclaving</strong></td>
</tr>
<tr>
<td><strong>LS Infection</strong></td>
<td>LS macro and micro-salts, LS vitamins, 1.5 mg/l, 2,4-D 0.7 g/l L-proline, 0.5g/l MES, 68.4 g/l sucrose, 36g/l glucose (pH 5.2). Filter</td>
</tr>
<tr>
<td>(IM)</td>
<td>sterilise then add filter-sterilized acetosyringone (AS, 100mM)</td>
</tr>
<tr>
<td><strong>LS cocultivation</strong></td>
<td>LS salts, 1.5 mg/l 2,4-D, 0.7 g/l L-proline, 0.5g MES, 30 g/l sucrose, 10g/l glucose, 8 g /l Agar (pH 5.8)</td>
</tr>
<tr>
<td>(CCM)</td>
<td></td>
</tr>
<tr>
<td><strong>LS resting</strong></td>
<td>Same as LS CC</td>
</tr>
<tr>
<td>(RM)</td>
<td></td>
</tr>
<tr>
<td><strong>LS selection I</strong></td>
<td>Same as LSR</td>
</tr>
<tr>
<td>(SI)</td>
<td></td>
</tr>
<tr>
<td><strong>LS selection II</strong></td>
<td>Same as LSSI</td>
</tr>
<tr>
<td>(SII)</td>
<td></td>
</tr>
<tr>
<td><strong>Regeneration I</strong></td>
<td>MS salts, 60 g /l sucrose, 3 g/l gelrite (pH 5.8).</td>
</tr>
<tr>
<td>(RI)</td>
<td></td>
</tr>
<tr>
<td><strong>Regeneration II</strong></td>
<td>MS Salts, 30 g/l sucrose, 3g/l gelrite, (pH 5.8).</td>
</tr>
<tr>
<td>(RII)</td>
<td></td>
</tr>
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</table>