ENHANCEMENT OF DROUGHT TOLERANCE IN TROPICAL MAIZE THROUGH SILENCING OF POLY (ADP-RIBOSE) POLYMERASE-1 GENE

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APRIL 2014
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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I84/11576/2008

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DEDICATION

To Maximillah and Samuel.
ACKNOWLEDGEMENTS

First I thank God for bringing me this far. I acknowledge that He works in ways that we cannot understand but are for the good of those who love Him.

Prof. Jesse Machuka, your death filled me with regret and sadness. But I thank God that I found favour and confidence before you while you lived. You trusted me to handle the practical sessions for many of your BSc and MSc classes. You also recruited me as your research assistant to manage your lab. You guided, encouraged and counselled me wisely throughout my PhD study. All this exposure made me the expert I am in the field of plant molecular biology. I never got the chance to honestly thank you. I thank you. Till we meet again Prof, fare thee well!

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<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>O2</td>
<td>Superoxide radical</td>
</tr>
<tr>
<td>ABA</td>
<td>Abscicic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Ago</td>
<td>Argonaute protein</td>
</tr>
<tr>
<td>amiRNA</td>
<td>Artificial microRNA</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APX</td>
<td>Ascorbate peroxidise</td>
</tr>
<tr>
<td>AQP</td>
<td>Aquaporins</td>
</tr>
<tr>
<td>ASI</td>
<td>Anthesis silking interval</td>
</tr>
<tr>
<td>BAP</td>
<td>Biotinylated alkaline phosphatase</td>
</tr>
<tr>
<td>Bar</td>
<td>Gene for phosphinothricin acetyltransferase</td>
</tr>
<tr>
<td>BecA</td>
<td>Biosciences East and Central Africa</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>B&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Basta resistant plants</td>
</tr>
<tr>
<td>B&lt;sup&gt;S&lt;/sup&gt;</td>
<td>Basta sensitive plants</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower mosaic virus</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cotransformation frequency</td>
</tr>
<tr>
<td>CIM</td>
<td>Callus induction media</td>
</tr>
<tr>
<td>CIMMYT</td>
<td>International Maize and Wheat Improvement Center</td>
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</table>
CIP  Calf intestinal alkaline phosphatase
Cre  Cre recombinase enzyme of the cre gene
CV   Coefficient of variation
dH2O Distilled water
DHS  Dehydration stress
DNA  Dioxyribonucleic acid
dNTP Deoxy nucleotide triphosphate
DRB  Double right border
dsRNA Double stranded RNA
EB   Elution buffer
ECA  Eastern and Central Africa
EDTA Ethylene diamine tetraacetate
EF1-A Translation elongation factor1-alpha
GAPDH Gene for glyceraldehyde-3-phosphate dehydrogenase
gDNA Genomic DNA
GOI  Gene of interest
GUS  β glucuronidase
H2O  Water
H2O2 Hydrogen peroxide
HCL  hydrochloric acid
HKG  Housekeeping gene
hpRNA Hairpin RNA
HSP  Heat shock protein
ILRI International Livestock Research Institute
KmR  Kanamycin resistant plants
<table>
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<tr>
<td>Km&lt;sup&gt;S&lt;/sup&gt;</td>
<td>Kanamycin sensitive plants</td>
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<tr>
<td>LB</td>
<td>Left T-DNA border</td>
</tr>
<tr>
<td>LEA</td>
<td>Late Embrogenesis Abundant protein</td>
</tr>
<tr>
<td>LMP</td>
<td>Low melting point agarose gel</td>
</tr>
<tr>
<td>LS</td>
<td>Linsmaier and skoog (1965) media formulation</td>
</tr>
<tr>
<td>LSDc</td>
<td>LS-based cocultivation medium</td>
</tr>
<tr>
<td>LS-If</td>
<td>LS-based infection medium</td>
</tr>
<tr>
<td>MABC</td>
<td>Marker-Assisted Back Cross</td>
</tr>
<tr>
<td>MARS</td>
<td>Marker-Assisted Recurrent Selection</td>
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<tr>
<td>MAS</td>
<td>Marker assisted selection</td>
</tr>
<tr>
<td>mcs</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MDAR</td>
<td>Monodehydroascorbate reductase</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>ML</td>
<td>Milliliter</td>
</tr>
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<td>MV</td>
<td>Methyl viologen</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<td>Sodium chloride</td>
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<tr>
<td>NAD</td>
<td>Nicotineamide adenine dinucleotide</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Bio-labs Inc., MA, USA</td>
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<tr>
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<td>Nanogram</td>
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<td>Nos</td>
<td>Nopaline synthetase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NPK1</td>
<td>Nicotiana phosphokinase 1 gene</td>
</tr>
<tr>
<td>NTC</td>
<td>Non-Template Control</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees celsius</td>
</tr>
<tr>
<td>OD</td>
<td>Overdrive sequence</td>
</tr>
<tr>
<td>OH⁻¹</td>
<td>Hydroxyl ion</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMI</td>
<td>Phosphomanose isomerase</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PPT</td>
<td>Phosphinothricin</td>
</tr>
<tr>
<td>PPT&lt;sup&gt;R&lt;/sup&gt;</td>
<td>PPT resistant plants</td>
</tr>
<tr>
<td>PPT&lt;sup&gt;S&lt;/sup&gt;</td>
<td>PPT sensitive plants</td>
</tr>
<tr>
<td>Psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>PTGS</td>
<td>Post transcriptional gene silencing</td>
</tr>
<tr>
<td>PTL</td>
<td>Plant transformation laboratory</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription-PCR</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait locus</td>
</tr>
<tr>
<td>RB</td>
<td>Right T-DNA border</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RM</td>
<td>Rooting medium</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
</tbody>
</table>
RT  Reverse transcriptase
RT-PCR  Reverse transcription-PCR
RWC  Relative water content
SAP  Shrimp alkaline phosphatase
SD  Standard deviation
sdH$_2$O  Sterile distilled water
SDS  Sodium dodecyl sulphate
SE  Standard error
SIM  Shoot induction medium
siRNA  Small interfering RNA
SM  Shooting medium
SMG  Selectable marker gene
SOD  Superoxide dismutase
sRNA  Small RNA
T$_0$  Zero generation transformant
T$_1$  Second generation transformant
T$_2$  Third generation transformant
T-DNA  Transferred DNA
TUA  Gene for $\alpha$-tubulin
Ubi1  Maize ubiquitine 1 promoter
V  Variance
WT  Wild type (non-transformed) plant
WUE  Water use efficiency
x-GlcA  5-Bromo-4-chloro-3-indolyl-$\beta$-D-glucuronic acid
YM  Yeast mannitol medium
ABSTRACT

In most of the production areas under maize, mild drought can significantly reduce yields while severe drought can sometimes completely destroy an entire plantation. Consequently, the livelihood of farmers is affected due to reduction in their incomes leading to poverty. Currently, very few highly transformable maize genotypes such as B73, W22, A188 and CM216 are used in genomic studies and genetic engineering of traits such as drought tolerance. Following trait enhancement through genetic engineering, consumer uptake of the transgenic product may be hindered due to presence of undesirable genetic elements such as selectable marker genes (SMG). Therefore generation of transgenic plants free of SMG is important to make them biosafe. The objective of this study was to generate SMG-free maize plants having an artificial microRNA (amiRNA) targeting the poly (ADP-ribose) polymerase 1 (PARP1) gene and assess transgenic plants for tolerance to severe oxidative and drought stress. In earlier studies using arabidopsis and rapeseed, the PARP1 gene silencing approach was demonstrated to enhance drought tolerance through preservation of cellular energy and reduced damage by reactive oxygen species. In this study, maize PARP1 gene silencing constructs (amiRNA-PARP1) were cloned in the same T-DNA region as the pmi or the bar SMG or placed in a separate T-DNA region for cotransformation of plants. The cotransformation vectors were first validated in tobacco before use in transformation of different maize genotypes. Maize transformation was achieved by co-cultivation of immature embryos with Agrobacterium tumefaciens harboring the amiRNA-PARP1 construct. Transgenic plants were assessed for downregulation of the PARP1 gene using qRT-PCR. The effect of PARP1 gene downregulation on drought tolerance was also assessed. Out of 13 genotypes evaluated, two (TL03B6754A-20 and TL03B6757-68) were found to be highly regenerable and were chosen for recovery of transgenic plants using either the PMI/mannose or bar/PPT selection system. Using the PMI/mannose selection system, the two maize genotypes were found to be highly transformable, averaging transformation frequencies (TF) of 48% and 34.16%, respectively. The TF for the control genotype CML216 was 32.19%. The TF under PPT selection’ for the inbred lines CML216, TL03B 6757-68 and TL03B 6754A-20 was 26.16%, 14.81%, and 21.69% for pMarkfree3.1 and 27.22%, 32.10% and 36.32% for pMarkfree3.2, respectively. A qRT-PCR analysis conducted on six amiRNA-PARP1 transgenic lines revealed that the expression of the PARP1 gene was reduced in plants exposed to methyl viologen-induced oxidative stress. However, the level of PARP1 gene expression was higher in non-transformed plants. Plants with reduced expression of the PARP1 gene were tolerant to oxidative and drought stress indicated by higher chlorophyll content, relative water content, growth and biomass as well as reduced anthocyanin accumulation, compared to non-transformed plants. In conclusion TL03B6754A-20 and TL03B6757-68 genotypes are highly responsive to transformation. Therefore these inbred lines extend the pool of highly transformable genotypes available for genomic and trait improvement studies. In addition the cotransformation binary vectors developed here (pMarkfree3.0, pMarkfree2.1 or pMarkfree2.2) are applicable in any plant to produce SMG-free plants containing one or more transgenes of interest. In the long term, the drought tolerant transgenic maize plants produced in this study can help stabilize maize yields and increase production during water stressed conditions. This will impact targeted farmers and their households by increasing their incomes thereby improving their livelihoods.
CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Drought causes extensive losses in crop productivity and limits the geographical distribution of important crop species (Chaves and Oliveira, 2004). Although grown in diverse environments, maize (Zea mays L.) is drought-sensitive. In most of the production areas under maize, mild drought can significantly reduce yields while severe drought can sometimes completely destroy an entire plantation. Drought affects maize throughout its developmental cycle. However, it is most injurious to the maize plant during the seedling and flowering stages (Boyer and Westgate, 2004).

To solve the problem of injury in maize by drought, research has been conducted to transfer tolerance genes from tolerant plants to sensitive ones. When this has been attempted using conventional breeding, such efforts have proven to be slow and laborious and have rarely delivered varieties capable of tolerance to drastic water stress (Collins et al., 2008). The main challenge to development of true drought tolerant varieties has been the complexity of drought tolerance due to its determination by multiple genes (Fleury et al., 2010).
Remarkable levels of dehydration tolerance have been obtained using new and promising genetic engineering strategies designed to manipulate a variety of processes (Collins et al., 2008). One such approach is the use of genetic engineering in preservation of cellular energy under extreme or persistent abiotic stress conditions. Evidence of the influence of stress on energy balance is now emerging. Stresses cause high energy consumption (De Block et al., 2004; Schulz et al., 2012) and enhance respiration with a linked production of reactive oxygen species (Atkin and Macherel, 2009; Lawlor and Tezara, 2009). Identification of stress-induced activation of poly (ADP-ribose) polymerase (PARP) as the main energy depletion process in animals has significantly enlarged our understanding of how stresses affect energy metabolism in plants and has provided a new means for improving drought tolerance in crops through inhibition of poly (ADP-ribose) polymerase (PARP). Inhibition of PARP genes to enhance tolerance to drought has been demonstrated for some plant species, including *Brassica napus* and *Arabidopsis thaliana* (De Block et al., 2004; Schulz et al., 2012). The aim of this study was to determine the effect of introduction of amiRNA-PARP1 transgene in maize on tolerance to oxidative and drought stress.

**1.2 Problem statement**

The most persistent and devastating problem affecting agriculture today is drought. Maize has been reported to be very sensitive to drought particularly during
flowering (Otegui et al., 1995; Boyer and Westgate, 2004). Yield reduction exceeding 90% has been recorded when drought stress occurs in the period just before silking and during grain filling (NeSmith and Ritchie, 1992). This coupled with lack of suitable varieties that perform well under insufficient and erratic distribution of rain, significantly reduce production and productivity of maize. Consequently, the livelihoods of farmers and their households are negatively impacted due to reduced incomes, leading to poverty. Development and production of drought tolerant maize varieties can help to stabilize yields and food security under drought.

Currently, trait improvement and genomics studies in maize utilize very few genotypes such as B73, W22, A188, H99, HiII and CM216 due to their superior response to genetic transformation and regeneration. Identification of more genotypes amenable to transformation will help expand this pool and offer alternatives to the scientific community. During trait enhancement through genetic engineering, identification of transgenic tissues and plants is accomplished with the help of a selectable marker gene (SMG). However, the continued presence of SMGs in transgenic plants may cause environmental and biosafety problems. Therefore removal of SMG from plants is a desirable objective. For tropical maize transformation, this is a challenging objective because of 1) the unavailability of
marker gene-removal systems and 2) the difficulty and high cost associated with development of new marker removal systems.

1.3 Justification

In this study, maize is targeted for improvement because of its global importance as a food and cash crop. It is also highly susceptible to drought, with zero yield recorded when intense water stress occurs during the flowering and grain filling stages. Several genes have been transferred into maize to confer tolerance to drought. When this has been attempted by traditional breeding, success has been low. However, with genetic engineering approaches, tolerance to severe drought stress has been successfully achieved. Maintenance of energy balance in plants through downregulation of poly (ADP-ribose) polymerase (PARP) gene expression is a gene transfer approach aimed at increased tolerance to drought. This approach has been demonstrated successfully in model plant species such as Arabidopsis thaliana and Brassica napus (De Block et al., 2004; Schulz et al., 2012) and holds great promise for agronomically important plant species such as maize.
1.4 Hypotheses

I) Single and multiple T-DNA binary vectors having an amiRNA transgene against maize \textit{PARP1} gene can be developed using conventional cloning procedures.

II) Selectable marker-free plants can be produced through \textit{Agrobacterium tumefaciens}-mediated transformation with binary vectors having multiple T-DNA regions.

III) Maize regeneration and \textit{Agrobacterium}-mediated genetic transformation is genotype dependent.

IV) Expression of \textit{PARP1} gene in tropical maize can be downregulated by a targeting amiRNA transgene.

V) Downregulation of \textit{PARP1} gene can increase tolerance to oxidative and drought stress in tropical maize.

1.5 Objectives

1.5.1 General objective

To stably transform different tropical maize genotypes with an amiRNA against the \textit{PARP1} gene and assess transgenic plants for tolerance to severe oxidative and drought stress.
1.5.2 Specific objectives

The specific objectives of this study were to:

i) construct vectors bearing amiRNA-PARP1 constructs for maize transformation

ii) assess the functionality of new cotransformation vectors in a model plant species (tobacco) for generation of marker-free plants

iii) assess the regeneration and transformation response of tropical maize genotypes with amiRNA1-PARPI gene constructs

iv) assess the efficiency of cotransformation and selectable marker gene removal from maize when transformed using cotransformation vectors

v) establish the effect of oxidative and drought stress on performance of transgenic maize plants stably transformed with an amiRNA-PARPI transgene
CHAPTER TWO

LITERATURE REVIEW

2.1 Impact of drought on maize production

Maize is the most widely cultivated cereal crop covering about 160 million hectares in the world (FAOSTAT, 2010). It is a staple food crop in East and Central Africa (ECA) grown on more than 5.5 million hectares mostly by small and medium scale farmers. It provides over 50% dietary calories with a per capita consumption of about 100 kg/year. In the ECA region maize yield is often inadequate due to numerous biotic and abiotic stresses. Among the abiotic stresses, drought is a major stress affecting productivity of maize (Otegui et al., 1995; Boyer and Westgate, 2004).

Maize has been reported to be very sensitive to drought particularly during flowering (Otegui et al., 1995; Boyer and Westgate, 2004). Yield reductions exceeding 90% have been recorded when drought stress occurs in the period just before silking and during grain filling (NeSmith and Ritchie, 1992). This coupled with lack of suitable varieties that perform well under insufficient and erratic distribution of rain significantly reduce production and productivity of maize, with actual grain yield of 1.3 tons/ha, compared to the potential of over 10 tons/ha.
Drought injury in most plant tissues result from severe oxidative damage due to accumulation of reactive oxygen species (ROS). These ROS including superoxide ('O₂), hydroxyl (OH⁻¹) and hydrogen peroxide (H₂O₂) accumulate rapidly under drought. These ROS cause oxidative damage to various cellular biomolecules and may lead to cell death. Attack of plant tissues by ROS causes peroxidation of lipids leading to damage of the cell and organelle membranes. This compromises cell functioning due to increased membrane fluidity and permeability. Drought is also associated with oxidative damage to proteins. This in effect causes protein denaturation (Sharma and Dubey, 2005). Reactive oxygen species cause oxidative damage to nuclear, mitochondrial and chloroplastic DNA leading to partial or complete inactivation of encoded proteins (Imlay and Linn, 1988).

Under moderate to severe drought stress, accumulated ROS cause damage to photosystem II resulting in decreased photosynthetic efficiency (Jagtap et al., 1998). This problem is further compounded by high heat stress which is normally associated with drought and has been shown to substantially reduce plant photosynthetic rates and productivity (Jagtap et al., 1998; Yamashita et al., 2008).
2.2 Changes in maize plants associated with drought tolerance

Acquisition of drought tolerance leads to different changes at the biochemical, molecular and morphological levels in maize plants. These include changes in levels of proteins, nucleic acids, carbohydrates and amino acids.

Drought tolerance is associated with differential accumulation of stress protective proteins such as the late embryogenesis abundant (LEA) proteins and aquaporins (AQP). In response to drought, the LEA proteins are highly expressed in plant embryos while aquaporins accumulate in the cell membranes. These proteins play significant roles in protection of membranes and other proteins. Expression of the heat shock proteins (HSP) is also highly related to acquisition of drought tolerance (Zhou et al., 2012). The HSP help in recovery of cell functions after abiotic stress by preventing protein thermal aggregation (Lee et al., 1995). Proteins in the dehydrin family have also been found to accumulate to high levels in response to various stresses including drought (Close, 1997).

Drought tolerance in plants is related to efficient reduction of oxidative stress which is due to accumulation of ROS. Reactive oxygen species, which are mainly produced in the chloroplasts (Mittler, 2002), reduce photosynthetic activity of plants during stress. Tolerance to drought is therefore highly correlated with
increased antioxidant and photosynthetic capacity. Both enzymatic and non-enzymatic factors participate in antioxidant activity during stress.

Enzymatic factors found to accumulate to high levels following drought stress include superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDAR) (Bowler et al., 1992; Willekens et al., 1997). Accumulation of nonenzymatic factors has been found to be highly correlated with drought tolerance in many plants. These include glutathione, alpha tocopherol and citrulline (Akashi et al., 2001; Munne-bosch et al., 2013). Oxidative stress has been reported to trigger synthesis of anthocyanins (Nagata et al., 2003; Vanderauwera et al., 2013), the role of which is to protect plants against ROS. The most abundant antioxidant that is synthesized during oxidative stress is ascorbic acid (Nagata et al., 2003). It accumulates highly in all plant cell components including cell walls. In the chloroplasts, the levels of ascorbate can exceed 20 mM. The main role of ascorbate is to detoxify H$_2$O$_2$ (Smirnoff and Wheeler, 2000).

Drought tolerance is also associated with increase in the content of chlorophyll in plants. Drought related changes in the content of chlorophyll $a$, chlorophyll $b$ and chlorophyll ($a + b$) has been reported in many plant species (Anjum et al., 2003; Rad et al., 2012). The decrease in chlorophyll under drought stress is mainly as a
result of damage to chloroplasts caused by active oxygen species (Smirnoff, 1995). Therefore enhanced chlorophyll capacity plays a role in increasing the photosynthetic ability of plants during drought. Drought resistance in terms of enhanced chlorophyll content has been demonstrated in different plant species (Rad et al., 2012).

Drought tolerance is also linked to synthesis of amino acids. Increased amino acid synthesis under drought stress has been reported for different plants (Yoshiba et al., 1997; Ozturk et al., 2002; Talame et al., 2007). Proline is the most common osmolyte in plants and accumulates highly under drought stress conditions. For example, in barley, the concentration of proline has been found to increase 11.1 folds under drought stress (Talame et al., 2007).

2.3 Changes in expression of genes associated with drought tolerance

Tolerance to water stress is associated with changes in expression of genes that are responsible for many biochemical and physiological processes. The expression of the genes is altered by either drought or dehydration stress (DHS) or both. Among transcripts induced by drought are those involved in jasmonate biosynthesis (allene oxide synthase), proline biosynthesis (D-pyrroline-5-carboxylate synthetase) and other osmoprotectant biosynthesis-related proteins (e.g arginine decarboxylase)
Drought tolerant plants also highly accumulate transcripts that encode sugar transporters which may be involved in osmotic adjustment during drought. Others include transcripts encoding kinases believed to be involved in the signalling cascade from sensing the stress event to the activation of defence and acclimation pathways, have been detected in drought stressed plants (Seki et al., 2002).

Other transcripts upregulated during drought are those involved in protection against effects of dessication such as those encoding LEA proteins, lipid transferases and dehydrins (Seki et al., 2002; Talame et al., 2007). Talame et al. (2007) identified genes strictly induced by dehydration only through the use of a cDNA microarray composed of 1654 cDNA clones. They included transcripts related to photosynthesis and amino acid metabolism. One transcript codes for an extensin and two transcripts are involved in protein transport. Both drought and DHS induce the expression of various genes including genes encoding HSP, metallothionein-like proteins, jasmonate-responsive proteins (Lipoxygenase 2) and LEA proteins (Seki et al., 2002; Talame et al., 2007).
2.4 Morphological changes associated with drought tolerance

Drought affects various aspects of plant morphology such as leaf, stem and root growth which suggests that the features play a role in the development of tolerance to drought. Plants exhibiting increased stem length under drought have been reported (Shao et al., 2008). Increase in stem length results from enhanced cell expansion and intermodal enlargement.

An optimal leaf area is essential for photosynthesis and plant dry matter yield. A moderate drought tolerance in terms of leaf growth has been observed in different plant species (Sacks et al., 1997; Shao et al., 2008). Drought tolerant plants achieve higher leaf growth and subsequently leaf area due to enhanced osmoregulation during water stress. Osmotic regulation can enable the maintenance of cell turgor which in turn enhances cell expansion and cell growth.

A common undesirable effect of water stress is reduction in a plant’s fresh and dry mass production. This suggests that drought plays a role in accumulation of plant mass. A moderate drought tolerance in terms of shoot dry mass has been observed in some plants including different rice cultivars (Lafitte et al., 2007).
2.5 Drought-induced expression of transcription factors

Drought tolerance is associated with accumulation of the phytohormone abscisic acid (ABA). ABA serves as an endogenous messenger in biotic and abiotic stress responses (Raghavendra et al., 2010). Increased ABA levels increase plant stress tolerance through activation of stress protective genes. For example overexpression of the ABA-responsive transcription factors ABF3 (ABA-responsive element-binding factor 3) confers tolerance to drought alone (Oh et al., 2005). ABF3 induces the expression of LEA (rd29B and rab18) and protein phosphatase 2C (ABI1 and ABI2) genes. Other transcription factors such as homeobox transcription factor 7 (AtHB7) and AtMYB96 lead to tolerance to multiple stresses by inducing expression of ABA/stress responsive genes, such as LTI65, ERD4, ERD7, ERD10, KIN1, RD29A and Cor15a (Yanhui et al., 2006).

Drought tolerance has been associated with accumulation of CBF3/DREB1B. These transcription factors bind and activate genes containing the CRT/DRE elements at the promoter regions. Expression of genes including cor15a, rd29A, kin1, cor6.6, and cor47/ rd17 (enhanced by CBF3) has been reported. Other transcription factors that accumulate following drought stress include DREB1A and DREB2A (Sakuma et al., 2006; Suo et al., 2012).
2.6 Attempts to transfer drought tolerance to tropical maize

2.6.1 Conventional breeding

Initially the basis of improvement of maize for drought tolerance was through conventional breeding methods such as intra-population, inter-population improvement scheme and backcross breeding. Through this approach increase in drought tolerance and yield has been achieved in maize populations adapted to the tropics at the International Maize and Wheat Improvement Centre (CIMMYT) (Edmeades et al., 1999). Yield increase is normally associated with improved flowering-related traits during drought stress such as anthesis-silking interval (ASI), time to anthesis and tassel primary branch number in maize populations adapted to the tropics (Edmeades et al., 1999).

From the improved populations, drought tolerant inbred lines such as those with a reduced ASI, senescence and barrenness are extracted for use as parents in breeding programs to generate drought tolerant hybrids. Selection of drought tolerant hybrids under controlled stress conditions is an important practice for success of this method (Bänziger et al., 2004). Other practices that have resulted in improved maize hybrid performance during drought include fertilizer input, high planting densities, tillage, crop rotation and weed control.
2.6.2 Marker assisted breeding approach

In marker assisted breeding (MAB), genetic markers are used as a replacement of phenotypic selection to accelerate breeding and release of new cultivars. The two popular MAB approaches used to improve drought tolerance in maize are the marker-assisted backcross (MABC) and marker-assisted recurrent selection (MARS).

MABC involves transfer of a limited number of loci such as drought tolerance transgene or loci from one genetic background to another. Using the MABC method, Ribaut and Ragot (2007) introgressed five QTL alleles for short ASI from a drought-tolerant donor to an elite, drought-susceptible inbred line. Resultant hybrids yielded at least 50% more than control hybrids under high water stress conditions. However, at lower stress intensity, this yield advantage decreased (Ribaut and Ragot, 2007). MARS involves identification and selection of several genomic regions for relatively complex traits such as disease resistance, drought tolerance and grain yield within a single population. This was successfully applied in maize to identify and introgress four early flowering QTLs from a drought tolerant line (NSE626) to an agronomically important line (NSE331) (Ragot et al., 1999).
Marker-assisted backcross is associated with serious drawbacks that have constrained its application in improvement of plant traits. For example, use of QTL involved in drought tolerance traits has been highly associated with linkage drag, a situation in which deleterious alleles at loci linked to the selection markers are unintentionally co-selected. The other limitation of MAB is the lack of contrasting alleles between a given pair of parental lines at the target loci (Tuberosa et al., 2002).

Since its inception decades ago, backcross breeding has become an important technique in plant trait improvement. Today, it is indispensable in the development of agriculturally important transgenic plants. In particular, the MABC approach is incorporated in the process of development of transgenic plants. For example in gene or trait stacking, MABC facilitates the process of combining more than one transgene in a given inbred line. The technique has also enabled breeders to identify progenies with the recombinant chromosomes and the donor-parent genome in a more direct manner. MABC is also used for elimination of unwanted regions of the donor-parent genome linked to the transgene and selection of unlinked regions of the recurrent-parent genome.
2.6.3 Genetic engineering

Some attempts have been made to enhance drought tolerance in maize through genetic engineering. For example, transgenic maize plants highly expressing the NPK1 gene show improved tolerance to drought (Shou et al., 2004). Engineering betA gene in maize resulted in drought tolerance at the germination, seedling and reproductive stages (Quan et al., 2004). However, this approach is constrained by endogenously low levels of choline which hinder glycine betaine accumulation in transgenic plants. Maize plants transformed with a maize transcription factor ZmNF-YB2 exhibit improved tolerance to drought and yield under severe water stress conditions (Nelson et al., 2007). Under moderate drought conditions, maize plants highly expressing C4 phosphoenolpyruvate carboxylase (C4-PEPC) exhibit an increase in water use efficiency (WUE) and dry weight (Jeanneau et al., 2002).

2.7 Plant poly (ADP-ribose) polymerases

Plant poly (ADP Ribose) polymerases (PARPs) are abundant nuclear enzymes that are activated when DNA is damaged and are involved in several cellular processes (Kim et al., 2005; Luo and Kraus, 2012). Plants contain two genes that code for PARPs: parp1 and parp2 (Babiychuk et al., 1998; Mahajan and Zuo, 1998) that differ in domain organization and function. PARP1 is composed of three functional domains: an N-terminal DNA-binding domain containing two zinc finger motifs, a central automodification domain and a C-terminal catalytic
(NAD\(^+\)-binding) domain. The catalytic domain contains a very conserved block of 50 amino acids, referred to as the PARP signature. PARP2 differs from PARP1 in lacking the zinc fingers at its N-terminal DNA binding domain. Apart from their structural differences, \textit{parp1} and \textit{parp2} genes differ in their type of inducers. While both are induced by DNA breaks, stresses such as dehydration and heavy metals mainly induce \textit{parp2} (Doucet-Chabeaud \textit{et al.}, 2001; De Block \textit{et al.}, 2004).

The exact biological role of plant PARPs is not yet clear. However, experimental evidence indicates that PARPs are involved in DNA repair (Amours \textit{et al.}, 1999). Upon activation by DNA breaks, PARP binds to single strand DNA breaks through its N-terminal Zinc finger and recruits the DNA repair machinery, thereby having a role in base excision repair (BER) (Mahajan and Zuo, 1998).

### 2.8 Maize PARPs

The maize PARPs have been purified, cloned and characterized, and have been found to be similar to those of other eukaryotes in their catalytic and structural properties as well as nucleotide and amino acid sequences (Chen \textit{et al.}, 1994; Babiychuk \textit{et al.}, 1998; Mahajan and Zuo, 1998). There are two PARPs in maize; the classical Zinc finger-containing polymerase (ZAP) and the structurally non-
classical PARP protein (NAP), which lacks the Zinc finger motifs (Babiychuk et al., 1998; Mahajan and Zuo, 1998). In addition to the N-terminal domain, the maize PARPs also contain other conserved regions including a putative nuclear localization signal, the automodification domain and the NAD\(^+\)-binding domain (Mahajan and Zuo, 1998). The highest similarity between the two maize PARPs appears to be at the PARP signature region. Otherwise ZAP and NAP are very distinct structurally and perhaps functionally (Babiychuk et al., 1998).

The purified maize ZAP is a 115 KDa protein encoded by a nuclear localised gene that produces a 4 kb transcript. The maize ZAP protein is similar in sequence to PARPs of other plants and animals (Mahajan and Zuo, 1998). It has all the functional features characterising the PARP molecule including DNA-dependent poly (ADP-ribo) sylation of proteins (Babiychuk et al., 1998).

2.9 Role of PARP in stress-induced energy consumption and cell death

The main cause of energy depletion is from the stress-induced accumulation of PARP1 and to a lesser extent PARP2. This energy depletion is due to the poly (ADP-ribo) sylation activity of PARP. Poly (ADP-ribo) sylation involves the transfer of ADP-ribose moiety from NAD\(^+\) to glutamic acid residues in proteins followed by further transfer of ADP-ribose monomers onto newly formed aduct
Overactivation of PARP, therefore, causes a rapid breakdown of the NAD$^+$ pool. As a consequence, resynthesis of NAD$^+$ is stimulated, whereby between three and five molecules of ATP are used for each molecule of NAD$^+$ synthesized. In this way, cellular ATP is depleted which leads to necrotic cell death (Ha and Snyder, 1999).

**2.10 PARP inhibition and enhanced tolerance to multiple stresses in crop plants**

Various experimental studies have shown that inhibition or downregulation of plant PARP confers tolerance to multiple stresses including drought, heat, high light and salt (De Block *et al.*, 2004; Babiychuk *et al.*, 1998; Schulz *et al.*, 2012). For example the use of chemical inhibitors of PARP in various plants including *Brassica napus* and *Arabidopsis* conferred tolerance to oxidative stress and salt stress (De Block *et al.*, 2004; Schulz *et al.*, 2012).

Inhibition of PARP has been linked to improved energy and cellular redox balance. For example, RNAi down-regulation and chemical inhibition of PARP leads to an increased NAD$^+$ content in stress conditions (De Block *et al.*, 2004; Pellny *et al.*, 2009; Schulz *et al.*, 2012). Improvement in energy homeostasis may therefore explain the observed tolerance to severe drought stress.
More recently, it has been shown that inhibition or knockout of PARP1 and PARP2 reduces stress-induced accumulation of secondary metabolites including anthocyanins and ascorbate in plants (Schulz et al., 2012). However, this reduced antioxidant capacity was found not to have any significant effect on the chlorophyll content and therefore photosynthetic performance of plants under oxidative stress conditions. In a previous study, altered anthocyanin accumulation was found to compromise pathogenic defensive ability of plants (Adams-Phillips et al., 2010). A common undesirable effect of drought stress is negative effects on plant growth and productivity (Kasuga et al., 1999; Suo et al., 2012). However, drought tolerance resulting from inhibition of PARP is not associated with any negative effects on growth and seed production (De Block et al., 2004; Vanderauwera et al., 2007).

Non-specificity of PARP inhibitors as well their inhibition of other enzymes renders the enzyme inhibition approach to stress tolerance unpopular. An alternative approach which is more specific is RNA interference (RNAi). This approach involves transformation of crops with a construct having a single- or double-stranded RNA and testing the resultant transgenics under stress conditions (Helliwell, 2003).
2.11 RNA silencing

RNA silencing is a post transcriptional gene silencing (PTGS) process in which double stranded RNA (dsRNA) molecules trigger the degradation of complementary RNA sequences transcribed from an endogenous or exogenous gene (Vazquez, 2006). RNA silencing is found in both plants and animals and may have arisen as a host defence mechanism against viruses and other intruding nucleic acids (Waterhouse et al., 1998; Waterhouse et al., 2001).

RNA silencing has been described differently in plants as PTGS and animals as RNA interference (Hannon, 2002). However, the various types of PTGS are related due to the presence of small/short RNA (sRNA) of approximately 21-25 bp generated by the action of members of the dicer enzyme family. The sRNA of the RNAi and PTGS pathways are known as small interfering RNAs (siRNAs), and are derived from viral and cellular dsRNA (Hannon, 2002). The micro-(mi) RNAs genes found in the genome of animals and plants are processed to give rise to other sRNA species called miRNA (Ossowski et al., 2008).

These various sRNAs are loaded into RNA-induced silencing complex (RISC) where they bind to argonaut (Ago) protein family members. Through sequence complementarity with the bound small RNA, RISC binds and degrades (siRNAs)
and/or represses (miRNA) target RNAs thereby causing gene silencing. Small interfering RNAs can also direct gene silencing at the transcriptional level through the methylation of DNA or formation of repressive heterochromatin or both (Hannon, 2002). This type of silencing is a form of genome protection afforded through the suppression of the action of repeated sequences such as transposons and retro-elements (Waterhouse et al., 2001).

The RNA silencing phenomenon has been exploited to silence any cellular gene of interest that is endogenous or exogenous in occurrence (Waterhouse et al., 2001). This has been through introduction of either a hairpin RNA (hpRNA) or pre-amiRNA transgene against the gene of interest into cells (Helliwell, 2003).

2.11.1 Gene silencing using a hpRNA transgene

The hpRNA transgene consists of a plant promoter, and inversely repeated sequence of the target gene interrupted by a spacer fragment and terminator (Helliwell, 2003). The most common plant promoter used in hpRNA transgene construction has been the constitutive cauliflower mosaic virus (CaMV) 35S promoter. However, tissue-specific promoters, such as the napin and lectin promoters active in seeds (Smith et al., 2000; Stoutjesdijk et al., 2002) and the A-type and B-type MADS-box gene promoters active in flowers (Byzova et al.,
2004) have also been used effectively. The sequence of the target gene on the hpRNA transgene is normally a 300 and 800 bp fragment of the coding region of an mRNA. The 5’ or 3’ untranslated regions have also been used as targets for gene silencing (Wesley et al., 2001; Brummell et al., 2003). After transcription, the RNA from the hpRNA transgene hybridises with itself to form a hairpin structure. The hairpin comprises a dsRNA structure (the stem) encoded by the inverted repeats and a single stranded loop encoded by the spacer fragment. The stem is used as a substrate by dicer for the generation of siRNA, while the loop is important for the stability of the transgene construct. HpRNA transgenes have been used to effectively silence a wide variety of endogenous genes including those encoding transcription factors for biosynthetic enzymes and viral sequences (Waterhouse et al., 2001).

2.11.2 Plant gene regulation by miRNA

Micro RNA (MiRNA) is a class of sRNA (21 nucleotides long) molecules that are expressed from endogenous genes to regulate the expression of the same or other endogenous genes (Jones-Rhoades et al., 2006). Biogenesis of miRNA begins when RNA polymerase II enzyme transcribes miRNA genes into long transcripts called the pri-miRNA. In plants, pri-miRNAs are normally more than 1000 bp in length (Jones-Rhoades et al., 2006). These pri-miRNA transcripts have the ability to fold back onto themselves to produce imperfectly double-stranded stem loop
precursor structures called pre-miRNA (Jones-Rhoades et al., 2006). The pre-
mRNA (about 70 nucleotide long) transcripts is bound and cleaved by an RNase
III like enzyme called dicer 1 to release a double stranded transcript called the
miRNA/miRNA* duplex (Kurihara and Watanabe, 2004). The duplex is then
transported out of the nucleus by exportin. In the cytoplasm the miRNA duplex
dissociates and the antisense strand gets incorporated into the RISC where it acts
as a probe to guide degradation of the target mRNA through complementary base
pairing (Hammond et al., 2000).

Many miRNA identified in plants are involved in a wide range of roles such as
organ development and regulation of small RNA metabolism (Nath et al., 2003;
Allen et al., 2005). Some research findings have shown that miRNA may be
involved in reponses to environmental stresses including abiotic (Sunkar and Zhu,
2004; Zhang et al., 2005; Luo and Krau, 2012), biotic (Zang et al., 2005)
oxidative (Sunkar et al., 2006) and nutrient stress (Fujii et al., 2005) as well as
mechanical stress in trees (Lu et al., 2005).

2.11.3 Genetic engineering using amiRNA

Synthetic or artificial microRNA (amiRNA) are small RNA that are generated by
exploiting endogenous miRNA precursors. Micro RNA precursors produce one
sRNA duplex, the miRNA-miRNA* duplex. When both sequences within the miRNA precursor genes are exchanged while maintaining the pattern of matches and mismatches in the foldback, this often leads to high level accumulation of an miRNA of desired sequence (Khraiwesh et al., 2008; Ossowski et al., 2008). Expression of amiRNA is effective with either constitutive or tissue-specific promoters and results in specific and effective interference with reporter and endogenous gene expression (Khraiwesh et al., 2008; Ossowski et al., 2008).

To date there exists a few reports detailing the application of amiRNA in efficient gene silencing in plants. Khraiwesh et al. (2008) established that silencing of Physcometrella PpFtsZ2-1 and PpGNT1 genes using amiRNA was just as effective as silencing due to targeted disruption of the genes. AmiRNA have also been used to overcome viral attack in Arabidopsis (Niu et al., 2006; Qu et al., 2007; Duan et al., 2008) and achieve silencing of various genes (Alvarez et al., 2006; Schwab et al., 2013). However, the use of amiRNA for silencing of tropical maize genes and the subsequent analysis of transgenic lines for tolerance to biotic or abiotic stress has not been reported.

The use of the amiRNA technique in gene regulation offers several advantages over the dsRNA approach. The dsRNA technique results in amplification of siRNA in plant cells leading to accumulation of too much small RNAs. This
results in nonspecific events due to activation of innate immune responses. However, expression of amiRNA results in comparatively lower concentrations of resulting small RNA which reduces the nonspecific effects. Gene silencing using dsRNA is associated with incomplete complementarity with the target gene. This may lead to inadvertent downregulation of genes having incomplete complementarity with the siRNA leading to problems with toxicity (Xu et al., 2006; Birmingham et al., 2006). In addition, with amiRNA-mediated gene silencing it is easier to express multiple amiRNAs (Sun et al., 2006; Qu et al., 2007; Zhu et al., 2007; Hu et al., 2009) and achieve tissue-specific gene silencing.

2.12 Agrobacterium-mediated transformation of maize

Agrobacterium tumefaciens-mediated transformation is a popular method of transferring genes of interest to plants. This is because it is cheaper and more accessible compared to direct gene transfer approaches such as particle bombardment. In addition, this technique results in integration of few copies of the transgene into cells as well as minimizing rearrangement of the transgene. Maize transformation using Agrobacterium has achieved low transformation frequencies mainly due to the resistance of maize to infection by the bacterium. In addition, other transformation requirements including the right Agrobacterium-vector combination (Li et al., 2000), the use of bactericides (e.g. cefotaxime) (Zhao et al., 2002; Zhang et al., 2003) and negative plant selection chemicals (e.g.
Phosphinothricin) in transformation media further reduce transformation frequency. Nevertheless, some tropical maize genotypes have been transformed successfully (Ombori et al., 2013; Rasha et al., 2013) with frequencies as high as 30% with the pmi gene (Mgutu, 2011). PMI is a positive selectable marker for selection of transformants on the sugar mannose and may be a better selection system than the negative selection systems not only due to its stimulatory effect on transformation efficiency (Negrotto et al., 2000) but also because it may not elicit concerns about food and environmental safety (Jaiwal et al., 2002). In addition it may not lead to severe physiological disturbance associated with high expression of negative selection marker genes (Bregitzer et al., 2007; Brown et al., 2013).

2.13 Approaches for removal of selectable marker genes

2.13.1 Recombination based methods

Different recombination strategies have been devised for elimination of selectable marker genes from plants. Among them the Cre/Lox recombination system is the most popular. The system makes use of the Cre recombinase enzyme (a 38-kDa product of the cre gene) to catalyse the excision of a selectable marker gene cloned between two 34 base pair loxP sequences derived from bacteriophage P1. This system has been used successfully to produce SMG-free plants of different species including maize (Zhang et al., 2003) and tobacco (Kovalchuk et al., 2003). A similar strategy based on Saccharomyces cerevisiae FLP recombinase and the
compatible FRT recombination sites was demonstrated in mammalian and insect cells (Chou and Perrimon, 1992; Kanegael et al., 1995) but was not effective in maize (Lyznik et al., 1993).

There are several disadvantages that limit the application of the recombination based systems. The systems require either retransformation of a transgenic plant containing a gene of interest with the recombinase enzyme or crossing of the transgenic plant with another expressing the recombinase enzyme (Zhang et al., 2003). The systems are therefore not suitable for vegetatively propagated plants. Continued presence of recombination sequences in the transgenic plants, as well as prolonged expression of the recombinase enzyme may cause unwanted changes to the genome.

2.13.2 Transposon based methods

Use of transposable elements for marker gene removal involves several steps. First, the marker gene is inserted into a transposon, a segment of DNA that jumps around in the plant’s genome. Second, is the co-transformation with gene of interest and finally segregation of the marker gene (Goldsbrough et al., 1993). The most common transposable element used in these sytems is the Ac transposable element. The Ac element transcribes its own transposase and so it conveniently
excises itself and the SMG cloned between it. This makes it unnecessary to perform sexual reproduction for marker gene removal (Ebinuma, 1997). Marker-free maize (Bregitzer and Brown, 2013), rice (Cotsaftis et al., 2002; Jin et al., 2003) and tomato (Goldsbrough et al., 1993) plants have been generated using this system. However, the main drawback of using a transposable element-based system for marker gene removal is the low efficiency of marker-free transgenic plant generation because of the tendency of transposable elements to randomly reinsert elsewhere in the genome.

2.13.3 Agrobacterium-mediated cotransformation

Cotransformation involves introduction of a gene of interest (GOI) and SMG, harboured between separate T-DNA regions, into the plant cells. If the two transgenes integrate in unlinked genomic loci, they can be separated from each other in subsequent generation of the cotransformants through genetic segregation (Figure 2.1). Four different approaches have been used successfully to transform plants with multiple T-DNA and recover SMG-free transgenic plants. The most popular approach is transformation of plants with multiple T-DNA regions harboured on one binary vector (Komari et al., 1996; Hong-Yan et al., 2003). Marker removal using the system involves (i) insertion of two separate T-DNAs, one bearing the SMG and the other bearing the GOI, onto one binary vector (ii)
co-transformation of plants with the two T-DNA regions using Agrobacterium (iii) segregating the GOI and the SMG for subsequent marker removal (Figure 2.1).

Plants may also be cotransformed with single T-DNA plasmids harboured either in one (Daley et al., 1998) or different (Hong-Yan et al., 2003; Park et al., 2004) Agrobacterium cells. A single T-DNA vector in which the GOI and the SMG are separated by an additional T-DNA right border sequence is the most recent approach. The general design of this double right border (DRB) vector is LB-GOI-RB2-SMG-RB1. This implies that two distinct inserts may be independently transferred and integrated into the plant genome, starting either from RB1 to LB, or RB2 to LB. There exists a high possibility of a RB1 to RB2 insertion, which may significantly reduce or prevent generation of marker-free plants. Once integrated into the plant genome at unlinked locations, the second insert (RB2 to LB) which carries the GOI only can be selected for in progenies of cotransformants, while plants having the RB1 to RB2 insertion are eliminated. This DRB vector system has been demonstrated to result in high cotransformation, coexpression and segregation of two transgenes in tobacco and rice plants (Lu et al., 2001; Li et al., 2006; Xu et al., 2008).
2.13.3.1 Factors affecting *Agrobacterium*-mediated cotransformation

There are two main parameters used to assess the efficacy of an *Agrobacterium* cotransformation technique: (1) the cotransformation frequency (CF) and (2) the frequency of unlinked transfer of multiple T-DNA regions. These attributes must be sufficiently high for an effective cotransformation system. Several factors may influence these attributes:

2.13.3.1.1 The cotransformation method used

Cotransformation systems differ significantly in their ability to deliver multiple T-DNA into plants. The two T-DNA system has proved to be superior to the other
cotransformation techniques in CF. Using a two T-DNA system, Miller et al. (2002) obtained a CF of 93% for maize. This contrasted remarkably with a CF of 11.7% for the mixed strain method (Miller et al., 2002). For rice, the two T-DNA and the mixed strain method were reported to yield CF of 47% and 14%, respectively (Komari et al., 1996).

2.13.3.1.2 Strain of *Agrobacterium* used

The frequency of unlinked transfer of multiple T-DNAs is highly dependent on the strain of *Agrobacterium* used. Currently, octopine derived strains are the most commonly used strains because they favour integration at unlinked loci at high rate enabling generation of marker-free plants. LBA4404 is the most extensively used octopine strain, and has been used to derive marker free barley (Matthews et al., 2001; Kapusi et al., 2013), maize (Miller et al., 2002; Ishida et al., 2004), rice (Komari et al., 1996) and tobacco (Komari et al., 1996; Daley et al., 1998; McCormac et al., 2001; Hong-Yan et al., 2003; Park et al., 2004) plants. Nopaline derived strains were initially found to favour linked transfer of multiple T-DNA (De Block and Debrouwer, 1991; Poirier et al., 2000; Radchuk et al., 2005). Recently, there are reports detailing successful use of the nopaline strains EHA101 and its derivative EHA105 to deliver multiple T-DNA regions in unlinked genomic locations and generation of marker-free plants (Xing et al., 2000; Breitler et al., 2004).
2.13.3.1.3 Size and location of T-DNA regions on the binary vector

The size of the cotransforming T-DNA has been altered to positively influence cotransformation frequency and unlinked transfer of multiple T-DNA. Cotransformation frequencies higher than 90% were achieved in tobacco when the selectable marker T-DNA was two times larger than the T-DNA containing GOI (McCormac et al., 2001). Apart from the size, genetic distance between cotransformed T-DNA may be an important factor influencing cotransformation. The two T-DNA regions were placed opposite each other in a super binary vector when the two T-DNA system was first proposed (Komari et al., 1996). Using an octopine strain, this vector design lead to cotransformation frequency of 50% and at unlinked genomic locations in tobacco. In maize, a similar vector constructed by Ishida et al. (2004) yielded cotransformants at 50.6% and more than 42% of the primary cotransformants produced marker-free plants. Similarly, cotransformed maize tissues were generated at frequency of over 93% and marker-free plants at 64.4% (Miller et al., 2002). Though very effective, the super binary vectors were extremely large requiring homologous recombination to assemble. Smaller ordinary binary vectors in which the two T-DNA regions are placed close together without intervening sequences have been developed using conventional cloning techniques (Hong-Yan et al., 2003; Sun et al., 2008). By cloning a small ‘right-border/left-border’ fragment into the mcs of an ordinary binary vector, Mathews et al. (2001) demonstrated that the approach was a convenient and practical means of generating marker-free barley plants.
2.13.3.1.4 Type of cotransformation vector backbone used

The replication origin of a plasmid determines the number of copies of that plasmid in an *Agrobacterium* cell (RamanaRao and Veluthambi, 2010). Consequently, the number of copies of a binary vector in an *Agrobacterium* cell influences the CF by that vector. RK2 vectors existing in high copy numbers have been demonstrated to produce higher CF than those that existed in low copy numbers. Sripriya *et al.* (2011) were able to increase cotransformation when using a vector with a PVS replication origin from around 30% to 86% when a vector with the RK2 replication origin was used. The source of T-DNA border sequences also affects CF. Vectors with T-DNA border sequences derived from octopine strains promote better transformation when in an octopine derived *Agrobacterium* strain. In addition, the presence of additional sequences at the T-DNA RB called overdrive (OD) has been shown to enhance transformation (Toro *et al.*, 1988). In this study, all cotransformation vectors are RK2 vectors bearing border sequences derived from octopine strains. Other factors determining the efficiency of cotransformation and marker gene removal include the plant species to be transformed and the transformation procedure used.
CHAPTER THREE

CONSTRUCTION OF AMIRNA-PARPI BINARY VECTORS FOR USE IN AGROBACTERIUM-MEDIATED TRANSFORMATION OF PLANTS

3.1 INTRODUCTION

Many \textit{in vitro} gene manipulations and cloning are performed on the binary vector (Lee and Gelvin, 2008). The binary vector consists of the T-DNA region which is defined and delimited by the left (LB) and right (RB) border repeat sequences. For successful \textit{Agrobacterium}-mediated gene transfer, the foreign DNA must be present between the T-DNA borders of the binary vector (Joos \textit{et al.}, 1983).

Within the T-DNA borders, a selectable marker gene expression cassette is cloned for selection of transformed plant cells. The neomycin phosphotransferase II gene (\textit{nptII}) is a commonly used selectable marker gene (SMG) that confers plant resistance to kanamycin, paromomycin and geneticin. However, the bialaphos resistance (\textit{bar}) gene, that confers resistance to the herbicide phosphinothricin, is a preferred selectable marker gene for transformation of monocot plants including rice, wheat and maize. The \textit{pmi} gene is also a popular SMG for transformation of monocot plants (Negrotto \textit{et al.}, 2000).
A reporter gene expression cassette may be contained in the T-DNA of the binary vectors. In gene transfer experiments, reporter genes are useful in quick detection of transformed events. The gus gene encoding β-glucuronidase (Jefferson et al., 1987) is an example of a reporter gene commonly used in plant transformation.

Development of binary vectors bearing either a single T-DNA or two T-DNA for maize transformation is reported in this chapter. Single T-DNA vectors were constructed to contain the amiRNA1-PARP1 transgene on the pNOV2819 (SYGENTA Biotech Laboratories, North Carolina, USA) backbone while the multiple T-DNA vectors had an amiRNA3-PARP1 construct on the pSCV backbone. The vectors were developed using standard cloning procedures described by Sambrook et al. (1989).

3.2 MATERIALS AND METHODS

3.2.1 DNA manipulation and cloning

DNA manipulation and cloning was achieved using techniques described by Sambrook et al. (1989). Enzymes such as restriction endonucleases, alkaline phosphatase, DNA polymerase were purchased exclusively from New England Bio-labs Inc., MA, USA. DNA ligases were procured from Invitrogen Corp. Carlsbad CA, USA.
3.2.2 Oligo synthesis and DNA sequencing

All oligonucleotides for PCR and sequencing were designed using the RealTimeDesign™ software (Biosearch Technologies) and synthesised in Bioneer Corporation (Korea) through Biosciences Eastern and Central Africa (BecA) Hub (ILRI, Nairobi, Kenya). Oligonucleotides for qRT-PCR were designed using D-LUX™ Designer and ordered from Invitrogen Corp. Carlsbad CA, USA. DNA sequencing services were provided by the SEGOLI division of the BecA Hub.

3.2.3 Preparation of plasmid DNA (minipreps protocol)

3.2.3.1 Preparation of crude plasmid DNA

Plasmid DNA was isolated from bacteria using the alkaline denaturation procedure described in Sambrook et al. (1989) with some modifications. Bacteria harboring plasmid DNA was streaked on LB agar supplemented with the appropriate antibiotics and grown by incubating at 37 °C overnight. A loopful of the bacteria was inoculated into 3 ml LB broth amended with the appropriate antibiotics and incubated at 37 °C with vigorous shaking for 12-16 hours.

One milliliter of the culture was transferred into 1.5 ml tube and the bacterial cells centrifuged at 12000 rpm for 1 minute. The supernatant was removed and the pellet resuspended in 300 µl of resuspension buffer. Lysis buffer (300 µl) was
added, mixed gently and incubated on ice for 5 min. Lysis buffer contained SDS for lysis of bacteria thereby releasing cellular contents including DNA. It also contains NaOH for alkaline denaturation of double stranded chromosomal DNA. The supercoiled plasmid DNA remains intact. Three hundred microliters of neutralization buffer was added, mixed gently and tubes incubated on ice for 5 min. The neutralization buffer has a lower pH than the lysis buffer. This causes the aggregation of single stranded DNA into a mass. Centrifuging at 14000 rpm for 10 min caused the chromosomal DNA and the cellular debris to collect at the bottom of the tube. The supernatant was transferred into a clean 1.5 ml tube and DNA precipitated using 560 µl ice cold isopropanol. Precipitated DNA was collected by centrifugation at 12000 rpm for 30 min. The supernatant was removed and the DNA pellet washed by adding 1 ml of 70% (v/v) ethanol followed by centrifugation at 12000 rpm for 5 min. The supernatant was removed carefully and the DNA pellet air dried for 5 min. DNA was dissolved in 50 µl of elution buffer (buffer EB).

3.2.3.2 Preparation of pure plasmid DNA

Plasmid DNA was isolated from *E. coli* (strain DH5α) according to the user manual of QIAGEN plasmid mini kit (Qiagen, Maryland, USA). A single colony of DH5α cells containing the plasmid DNA of interest was inoculated in 2 to 5 ml LB medium containing the appropriate selective antibiotic. This starter culture was
incubated for about 8 hours at a temperature of 37 °C with vigorous shaking (about 300 rpm). The starter culture was diluted 1/1000 into 3 ml of antibiotic-containing LB medium and grown at 37 °C for 12 to 16 hours with vigorous shaking. The cells were harvested by centrifugation at 13000 rpm for 1 min at 4 °C. The bacterial pellet was resuspended in 300 μl of buffer P1. Buffer P2 was added and mixed thoroughly by vigorously shaking the mixture 4 to 6 times. The mixture was incubated at room temperature for 5 minutes. Finally, 300 μl of chilled buffer P3 was added and mixed immediately and thoroughly by shaking the mixture 4 to 6 times. The tubes were centrifuged at 14000 rpm for 10 minutes. The supernatant was transferred to an equilibrated QIAGEN-tip 20 column and allowed to enter the resin by gravity flow. The QIAGEN-tip 20 was washed twice with 2 ml of buffer QC by allowing buffer QC to move through the QIAGEN-tip 20 by gravity flow. The QIAGEN-tip was placed in a 1.5 ml eppendorf tube, and DNA eluted with 800 μl Buffer QF. To the eluate, 540 μl isopropanol was added and mixed well. Precipitated DNA was pelleted by centrifuging the tubes at 10,000 rpm for 30 minutes. The samples were carefully taken out of the centrifuge and the supernatants discarded. The DNA was washed by adding 1 ml of 70% (v/v) ethanol followed by centrifugation at 13000 rpm for 10 minutes. After centrifuging the ethanol was removed and the tubes left open until the ethanol had evaporated. DNA was dissolved in 50 μl buffer EB and stored at 4 °C.
3.2.4 Agarose gel electrophoresis

An appropriate amount of agarose was weighed and placed into a conical flask containing 100 ml of 1× TAE electrophoresis buffer. The flask was covered with a cling foil at the mouth and a small hole of about 0.5 cm punctured. The flask was placed in a microwave and heated till the agarose melted completely. This took about 5 min. The Gel was allowed to cool to about 50 °C before casting into a gel tray with a 14 well comb and left to solidify. Solidification was signified by a gel colour change from clear to white. The gel was placed in the electrophoresis tank containing sufficient amount of 1× TAE buffer and the comb removed. The appropriate volume of the sample was loaded in each well after staining with 1 μl of 10000× SYBR green. The stained sample was also mixed with 6× DNA loading buffer to a final concentration of ×1. Electrophoresis was done at 120 V until the dye front had migrated 2/3 of the gel. The gel was placed in a UV transilluminator box and documented using a digital camera.

3.2.5 DNA purification

3.2.5.1 Purification from agarose gel

DNA fragments were separated by electrophoresis in agarose gel in 1× TAE buffer. DNA was purified from agarose gel according to the protocol described in the user manual of QIAquick gel extraction kit (Qiagen, Maryland, USA). Briefly, The DNA fragment was excised from the gel with a sterile sharp scalpel. The
excised DNA was weighed and 3 volumes of buffer QG added to 1 volume of the gel. The mixture was incubated in a 50 °C water bath for 10 min to completely dissolve the gel. One gel volume of isopropanol was added. DNA was bound by placing 800 µl of the sample in a QIAquick column followed by centrifugation at 13000 rpm for 60 seconds. Bound DNA was washed by adding 0.75 ml of buffer PE to the column followed by centrifugation at 13000 rpm for 60 seconds. The flow-through was discarded before briefly centrifuging the tubes to get rid of any remaining ethanol. The DNA was eluted with 30 to 50 µl of buffer EB, then analysed for quality and quantity on a 1.2% (w/v) agarose gel.

3.2.5.2 Purification from other sources

DNA from other applications including PCR amplification, restriction enzyme digestion, DNA ligation, fill-in reactions, CIP treatment reactions and crude preparations was purified using the Qiaquick PCR purification kit (Qiagen, Maryland, USA). Five volumes of Buffer PBI were added to 1 volume of the sample and mixed. The mixture was applied to a QIAquick column and centrifuged at 13000 rpm for 60 seconds. The flow-through was discarded and the column returned into the same collection tube. The bound DNA was washed by adding 0.75 ml of buffer PE and centrifuging at 13000 rpm for 60 seconds. The flow-through was centrifuged and columns centrifuged again to get rid of residual
ethanol. To eluate the bound DNA, the column was placed in a sterile 1.5 ml tube 30 to 50 ul Buffer EB added.

3.2.6 DNA quantitation

The amount of DNA in a preparation was routinely estimated by assessing its concentration relative to the concentration of DNA of known molecular weight. To achieve this between 1 to 3 µl of the pure DNA was initially separated by electrophoresis on agarose gel. Five microliters (0.5 µg) of 1 kb DNA ladder (New England Bio-labs inc., Ma, USA) was ran alongside the purified DNA in a separate sample well. Sample concentration was then determined by comparing with concentration of reference ladder bands of known molecular weight. The 1 kb DNA ladder (0.5 µg/µl) was diluted with 2× DNA loading buffer to a final concentration of 0.1 µg/µl. Five microliters of the diluted ladder was used for DNA quantification.

3.2.7 Restriction enzyme digestion of DNA

About 0.5 to 1 µg of DNA was digested using the restriction enzyme digestion mixture whose content is shown in Table 3.1. The digestion mixture was mixed gently and centrifuged briefly. This was followed by incubation of the mixture at the appropriate temperature for 1 to 2 hours. Enzymes were inactivated by heating
the restriction mixture at 70 °C for 15 minutes. Alternatively, restriction endonucleases were removed by purifying the restricted DNA using the column purification method described in section 3.2.5.2.

**Table 3.1:** Reagents used for preparation of restriction enzyme digestion reaction mixture

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume for 1 reaction (30 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 to 1 µg of DNA</td>
<td>10 µl</td>
</tr>
<tr>
<td>Water, nuclease free</td>
<td>14.7 µl</td>
</tr>
<tr>
<td>10× recommended buffer</td>
<td>3 µl</td>
</tr>
<tr>
<td>Restriction enzyme 1</td>
<td>1 µl</td>
</tr>
<tr>
<td>Restriction enzyme 2</td>
<td>1 µl</td>
</tr>
<tr>
<td>BSA (optional for some enzymes)</td>
<td>0.3 µl</td>
</tr>
</tbody>
</table>

3.2.8 **Creation of blunt ends on digested DNA**

Blunt ends were produced on restriction enzyme-digested DNA using DNA polymerase I, large (klenow) fragment (New England Bio-labs Inc., MA, USA). DNA polymerase I, large (klenow) fragment has a 5’→3’ polymerization activity as well as the 3’→5’ exonuclease activity. To blunt double-stranded DNA molecules with 5’ protruding termini restriction enzymes in a digestion mixture were first removed by column purification. Alternatively, the enzymes were inactivated by heating the mixture at a temperature of 75 °C for 20 min. One microgram of restriction enzyme-digested DNA was placed in a 200 µl tube. NEB
buffer 2 New England Bio-labs Inc., MA, USA) was added to a final concentration of 1×. Each dNTP was added to a final concentration of 33 μM and mixed thoroughly. DNA polymerase I, large (klenow) fragment was added to the mixture to a final concentration of 1 U/μg of DNA. The mixture was toped-up to 50 μl with sterile distilled water. The reaction mix was incubated at 25 °C for 15 minutes before stopping the reaction by addition of 10 mM EDTA. The Klenow enzyme was inactivated by heating the reaction mixture at a temperature of 75 °C for 20 minutes. Finally, DNA in the mixture was purified by the column purification method described in section 3.2.5.2.

### 3.2.9 Dephosphorylation of vector DNA

Alkaline phosphatase is used to prevent self-ligation of linear DNA fragments thereby reducing vector background in cloning strategies. Calf intestinal alkaline phosphatase (CIP) catalyses the removal of 5’ and 3’ phosphate groups from nucleic acids. Unlike shrimp alkaline phosphatase (SAP), CIP cannot be completely inactivated by heating and therefore it has to be removed by spin-column purification. Dephosphorylation was performed by first placing blunted vector DNA (1 μg) into a 200 μl tube. NEB buffer 2 (New England Bio-labs Inc., MA, USA) was added to a final concentration of 1×. The volume of the mixture was brought up to 50 μl with sterile distilled water. One microliter of CIP (New England Bio-labs Inc., MA, USA) was added, mixed well and the mixture
incubated at 37 °C for 60 minutes. DNA was purified by spin-column purification and eluted in 50 μl of EB buffer.

3.2.10 Ligation of DNA

3.2.10.1 Routine DNA ligation

The concentration of DNA fragments (insert and vector DNA) was initially estimated as outlined in section 3.2.6 before ligation. Ligation was initiated by mixing insert and vector DNA to a final volume of 10 μl in a 3:1 molar ratio. To attain this ratio, the mass of vector DNA in the 20 μl ligation reaction was always kept constant at 50 ng. However, the appropriate mass of insert DNA for use in ligation was determined empirically using the following formula:

\[
\text{Insert mass in ng} = 3 \times (\text{length of insert in bp}) \times \text{mass of vector in ng} / (\text{length of vector in bp})
\]

To the insert/vector mixture, 10 μl of 2× ligation buffer and 1 μl (0.3 U) of T4 DNA ligase were added. The ligation reaction was mixed thoroughly and centrifuged briefly. This was followed by incubating the mixture at 16 °C for 12 to 16 hours.
3.2.10.2 Rapid DNA ligation

For quick ligation experiments, the NEB quick ligation kit (New England Bio-labs Inc., MA, USA) was used. The kit is suitable for ligation of cohesive end or blunt end DNA fragments in 5 min at 25 °C. DNA ligation was performed by first combining 50 ng of vector with a 3-fold molar excess of insert. The volume was adjusted to 10 μl with sterile distilled water. Ten microliters of 2× Quick ligation buffer was added and mixed. To this mixture, 1 μl Quick T4 DNA Ligase was added and mixed thoroughly. The ligation mixture was incubated at 25 °C for 5 to 10 minutes before being used for transformation.

3.2.10.3 Ligation of DNA embedded in a gel

Ligation of insert and vector DNA embedded in low melting point (LMP) agarose gel was accomplished using the NEB quick ligation kit (New England Bio-labs Inc., MA, USA). The insert and vector DNA were first combined in a 6:1 ratio. The DNA fragments were added into a 37 °C prewarmed tube to 10 µl or less. The insert/vector DNA mixture was incubated for 10 min at 37 °C. Ten microliters of ice-cold T4 DNA ligase mixture was added and mixed well to dissolve the agarose. However, the ligation mixture was observed to solidify after the experiment, warranting melting of the agarose. Such melting is not recommended when using the quick ligase kit due to the resultant reduction in transformation efficiency. The problem was resolved by adding 20 μl (instead of 10 μl) of ligation
mixture to 10 µl of DNA. The ligation mixture was incubated at between 20 to 25 °C for 16 hours.

### 3.2.11 Transformation of *E. coli*

All plasmids were introduced into the *E. coli* strain DH5α for maintenance and propagation. Plasmid transfer into cells was by the heat shock method. Before transformation, agarose gel-embedded DNA was first melted at 70 °C and equilibrated at 37 °C. Occasionally, DNA was purified before transformation. Five to ten microliters of the ligation mixture was chilled in a 1.5 ml tube. The ligation mixture was added to 200 µl competent cells and the contents mixed quickly but gently by inversion of the tubes. The DNA/cell mixture was incubated in ice bath for 30 minutes. The mixture was heat-shocked at 42 °C for 90 seconds, then chilled on ice for 5 minutes. Room temperature SOC medium (800 µl) was added and incubated at 37 °C for 60 minutes. The cells were centrifuged and 800 µl of the supernatant removed immediately. The cells were resuspended in the remaining medium using a pipetter and spread on SOB medium containing the appropriate antibiotics. The plates were incubated at 37 °C for development of colonies.
3.2.12 Transformation of *Agrobacterium tumefaciens*

Competent LBA4404 cells were transformed using the freeze thaw method (Raviraja and Sridhar, 2007) by first mixing with 1 μg or more of pure plasmid DNA. This cell/DNA mix was frozen in liquid nitrogen for 10 minutes. The frozen mixture was thawed in 37 °C water bath for 5 min followed by addition of antibiotic-free YM medium (YM). This mixture was incubated at 28 to 30 °C with shaking for 2 to 4 hours. The cells were centrifuged at 12000 rpm for 1 min and resuspended in 200 μl YM. Resuspended cells were spread on warm (37 °C) YM medium containing 1 mg/l rifampicin for selection of LBA4404 strain. The medium also contained 50 mg/l kanamycin or 100 mg/l spectinomycin for selection of the plasmid. Plates were incubated at 28 to 30 °C for 2 to 3 days for colony establishment.

3.2.13 Polymerase chain reaction (PCR)

3.2.13.1 PCR on plasmid and genomic DNA

Standard PCR conditions were used for detection of genes in plasmids and plant genome. PCR reagents were mixed in a 200 μl tube to a final reaction volume of 25 μl as outlined in Table 3.2. PCRs were done using the Eppendorf Mastercycler Pro (Eppendorf AG, Hamburg) programmed as shown in Table 3.3. Difficult PCRs were carried out by optimization of reaction conditions, especially the
annealing temperature, using the cycler’s gradient PCR feature. All PCR reagents used were procured from New England Biolabs Inc., MA, USA.

3.2.13.2 PCR using bacteria cells as a source of template DNA

Colony-PCR was performed to determine existence of plasmid in *E. coli* or *Agrobacterium*. To achieve this, distinct colonies growing on antibiotic-containing medium were picked using the tip of a sterile toothpick and swirled in 30 µl of sD2O. The mixture was boiled for 3 minutes and centrifuged at 14000 rpm for 2 minutes to pellet cell debris. The debris was discarded while 20 µl of the supernatant was transferred into a clean tube. Of these, 2 µl were used in PCR to amplify a desired gene in the colony-derived plasmid. PCR was carried out as described for plasmid or genomic DNA in section 3.2.13.1. The remaining portion of the colony was inoculated in 50 ml LB broth containing antibiotics for plasmid selection and incubated overnight at 37 °C. Three millilitres of the overnight cultures were used for plasmid minipreps. Plasmid extracts were subjected to PCR analysis to confirm the presence of the gene of interest in the plasmid.
Table 3.2: Composition of master mix for PCR amplification

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
<th>Final volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (× 10)</td>
<td>× 1</td>
<td>5</td>
</tr>
<tr>
<td>dNTPs(10 mM)</td>
<td>0.5 mM</td>
<td>2.5</td>
</tr>
<tr>
<td>MgCl₂(50 mM)</td>
<td>2.5 mM</td>
<td>2.5</td>
</tr>
<tr>
<td>Primer1(2 µM)</td>
<td>0.25 µM</td>
<td>6.25</td>
</tr>
<tr>
<td>Primer2(2 µM)</td>
<td>0.25 µM</td>
<td>6.25</td>
</tr>
<tr>
<td>Taq(5 U/µl)</td>
<td>1 U/rxn</td>
<td>0.5</td>
</tr>
<tr>
<td>Template (10 ng/µl)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>To 25 µl</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Conditions for PCR amplification

<table>
<thead>
<tr>
<th>Step</th>
<th>No. of cycles</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Temperature (°C)</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>94</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>94</td>
</tr>
<tr>
<td>Annealing</td>
<td>25</td>
<td>65</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Final extension</td>
<td>1</td>
<td>72</td>
</tr>
</tbody>
</table>
3.2.14 Preparation of chemically competent cells

3.2.14.1 Preparation of chemically competent *Agrobacterium* cells

The protocol for preparation of *A. tumefaciens* strain LBA4404 was modified from Xu and Li (2008). LBA4404 cells were made competent by first inoculating them on fresh solid YM medium containing 1 mg/l rifampicin (YM+RIF-1) and incubating at 30 °C for 24 hours. One colony was inoculated in 25 ml of liquid YM+Rif-1 and incubated at 30 °C for 24 hours. The cells were centrifuged at 6000 rpm for 5 min. The pellet was resuspended in 4 ml YM+RIF-1 and aliquoted into four 1 ml tubes in volumes of 1 ml. The cells were incubated in ice bath for 10 min. This was followed by centrifuged the cells at 12000 rpm for 1 minute in a chilled bench top centrifuge. The microfuge centrifuge was chilled by keeping at 4 °C for 1 to 2 hours. Competent cells were resuspended in 200 μl of 20 mM CaCl₂ and kept briefly in an ice bath awaiting transformation.

3.2.14.2 Preparation of chemically competent *E. coli* cells

The need to frequently prepare chemically competent *E. coli* cells for cloning purposes necessitated using an easy and convenient procedure that achieves high transformation efficiency. This meant altering the protocol by Tu *et al.* (2005). Tips were held at -20 °C before use, while CaCl₂ solution and tubes were kept in an ice bath during the preparation period. A single DH5α colony was inoculated
into 5 ml of LB medium and grown by incubating at 37 °C overnight with shaking at 240 rpm. One millilitre of the overnight culture was added to 25 ml of LB and incubated at 37 °C for 90 minutes. The cells were incubated at -20 °C until they almost froze. The cells were aliquoted into chilled 2 ml tubes in volumes of 2 ml and centrifuged at 12000 rpm for 60 seconds in a chilled bench top microcentrige. The cell pellet was resuspended in 1 ml of chilled 100 mM CaCl$_2$ and incubated in ice bath for 40 to 60 minutes. The competent cells were centrifuged as before and resuspended in 0.2 ml of chilled 100 mM CaCl$_2$.

3.2.15 Construction of pNOV2819-amiRNA binary vectors

The amiRNA-PARP1 constructs used in this study were originally from the pExamiRNAL2 plasmid (Figure 3.1) provided by Prof. Mieke Van Lijsebettens (Ghent University, Belgium). The pNOV2819-amiRNA1 and pNOV2819-amiRNA3 vectors were constructed through several steps as outlined in section 3.2.15.1 to 3.2.15.3.

3.2.15.1 Subeloning of amiRNA1-PARP1 into Ubi/NC1300

The complete pre-amiRNA1-PARP1 and pre-amiRNA3-PARP1 regions (414 bp) were amplified from pExamiRNAL2 vectors using the amiRNA forward primer (5’ tatcacacccgggtccccaaacacacgctcg 3’) having the Smal restriction site and the
amiRNA reverse primer (5’ atacagagctcctccccatggcgatgccttaa 3’) having the SacI restriction site. All reactions were performed in volumes of 100 µl. PCR reaction mixture preparation and amplification was performed as described in section 3.2.13. About 5 µl of the PCR products were separated on a 1% (w/v) agarose gel to check for the presence of appropriate bands. The remaining PCR products were purified using the spin-column purification method described in section 3.2.5.2. Pure PCR products were eluted with EB buffer in volumes of 30 µl. One microliter of the pure PCR products was used for DNA quantification. The plasmid Ubi/NC1300 (Figure 3.1) provided by Dr. Steven Runo (Kenyatta University, Nairobi, Kenya) was introduced into *E. coli* and confirmed by restriction enzyme digestion before use. The plasmid was purified from *E. coli* cells and eluted using 30 µl of EB buffer. Before use, the purified Ubi/NC1300 was quantified as described in section 3.2.6.

AmiRNA-PARP1 PCR products and the Ubi/NC1300 plasmid were first diluted with 1× restriction buffer (or sdH2O) to a final DNA concentration of 0.5 µg/µl. The PCR products and the Ubi/NC1300 plasmid were digested separately with *SmaI* and *SacI* using the protocol outlined in section 3.2.7. The digestion mixture was agitated gently and centrifuged briefly. The mixtures were incubated at a temperature of 25 °C for 12 hours to enable *SmaI* to cut, then at a temperature of
Figure 3.1: Map of pExamiRNAL2 (A) and Ubi/NC1300 (B) vector
37 °C for optimum \textit{SacI} activity. Restriction enzymes were inactivated by heating the digestion mixture at 70 °C for 15 min. Digested AmiRNA-PARP1 PCR products and Ubi/NC1300 vector were separated in a 1\% (w/v) LMP agarose gel. The bands were quickly cut out from the gel, under UV illumination (360 nm) using a sterile scalpel blade and placed in a sterile 1.5 ml tube. Before ligation, the gel slices were melted at 70 °C and equilibrated at 37 °C. Ligation was performed using the protocol for ligation of DNA embedded in gel described in section 3.2.10.3. Five to ten microliters of the ligation mixture were immediately added to 200 µl competent \textit{E. coli} cells and the contents mixed quickly but gently by inversion of the tube. \textit{E. coli} cells were transformed as described earlier 3.2.11. Transformed \textit{E. coli} cells were spread on LB agar medium containing 50 mg/l kanamycin and incubated at 37 °C for 12 hours.

Colony-PCR was carried out on possibly transformed \textit{E. coli} colonies to determine true transformants. To achieve this, four distinct colonies were picked using the tip of a sterile toothpick and subjected to colony PCR as described under section 3.2.13.2. The primers amiRNA forward and the amiRNA reverse were used in the colony PCR to amplify the amiRNA-PARP1 construct. The remaining portion of the colony was inoculated in 30 ml LB broth containing 50 mg/l kanamycin and incubated at 37 °C overnight. Three millilitres of overnight cultures were used for
plasmid mini prep as described in section 3.2.3.1. Plasmid extracts were then subjected to PCR and restriction digestion for confirmation of positive colonies.

Plasmid DNA from three confirmed clones for each of the amiRNA-PARP1 construct was sent for sequencing with the amiRNA forward and amiRNA reverse primers. The obtained sequence was analysed for any PCR-induced errors and the presence of restriction sites at both ends of the pre-amiRNA1-PARP1 and pre-amiRNA3-PARP1 insert. The online software, Clustal W, was used for multiple sequence alignment. The constructs with the error-free pre-amiRNA-PARP1 sequence was named Ubi/NC1300-amiRNA1 and Ubi/NC1300-amiRNA3.

3.2.15.2 Cloning of Tnos into Ubi/NC1300-amiRNA1 and Ubi/NC1300-amiRNA3 vectors

The full nos terminator sequence (288 bp) was amplified by PCR using the Tnos forward primer (5’ tatcagagctcgtcaacatttgca 3’) having SacI restriction site and Tnos reverse primer (5’ atacactagtgaatcccgatctagtaacatag 3’) having SpeI restriction site at their 5’ ends. PRESQ100 plasmid (Provided by Dr. Allan Mgutu, Kenyatta University, Nairobi, Kenya) was used as the template for the PCR reaction. PCR was performed using the PCR conditions outlined in section 3.2.13.1 but with 60 °C as the annealing temperature.
PCR amplicons of *Tnos* were purified using the spin-column purification method and subsequently the concentration of the amplicons determined. Ubi/NC1300-amiRNA1 and Ubi/NC1300-amiRNA3 vector DNA were purified from *E. coli* cells using the spin-column method and their concentration determined.

Five microliters of the *Tnos* PCR product and 1 μg of the Ubi/NC1300-amiRNA vectors were digested by SacI and SpeI restriction enzymes. Restriction digestion mixture was incubated at 37 °C overnight. Enzymes were heat inactivated at 65 °C for 20 minutes before ligation. Digested *Tnos* was ligated into the cut Ubi/NC1300-amiRNA vectors using the procedure described for insertion of pre-amiRNA-PARP1 PCR products into Ubi/NC1300.

Competent *E. coli* cells were transformed with the ligation mixture followed by culturing on LB agar containing 50 mg/l kanamycin. Two colonies were selected for each recombinant molecule (either Ubi/NC1300::amiRNA1::Tnos or Ubi/NC1300::amiRNA3::Tnos) to determine the presence of *Tnos* insert in the vectors using PCR. PCR was performed as per the previously outlined protocol (section 3.2.13.1). However, the amiRNA forward and the *Tnos* reverse primers were used to identify the clones. Amplification using the primers was expected to produce a 726 bp band on DNA from the true transformants. A control PCR reaction comprising the Ubi/NC1300-amiRNA1 was included.
Restriction digestion was performed on plasmid DNA from positive colonies to confirm success of *Tnos* insertion into Ubi/NC1300-amiRNA vectors. DNA was isolated from PCR positive clones and digested with *HindIII* and *SpeI* enzymes. The control plasmid DNA (Ubi/NC1300-amiRNA1) was also digested with the same enzymes.

Verified Ubi/NC1300-amiRNA1::Tnos and Ubi/NC1300-amiRNA3::Tnos recombinant plasmids were purified and quantified before sending for sequencing. Sequencing was carried out with Tnos Forward and Tnos reverse primers to verify the sequence of the cloned *Tnos* gene. The *Tnos* sequence from the clones was compared to that from the NCBI database using Clustal W multiple sequence alignment program. Constructs with the correct *Tnos* sequence were named Ubi/NC1300-amiRNA1Tnos and Ubi/NC1300-amiRNA3Tnos.

3.2.15.3 Subeloning of amiRNA-PARP1 expression cassette into pNOV2819

PNOV2819 (obtained from SYGENTA Biotech Laboratories, North Carolina, USA) was extracted from *A. tumefaciens* strain EHA101 cells using the alkaline denaturation method described in section 3.2.3.1. The extracted plasmid was then inserted into DH5α cells using the heat shock method described in section 3.2.11. PCR was performed on 5 ng of plasmid DNA from selected *E. coli* colonies using
PMI forward (5’ acagccactctcattca 3’) and PMI reverse (5’ gttgccccatctcagcag 3’) primers at an annealing temperature of 55 °C. Clones were confirmed by digesting plasmid DNA with KpnI and SpeI restriction enzymes. These enzymes were expected to released a 1982 bp fragment, comprising the pmi cassette and the multiple cloning site from the plasmid.

Ubi/NC1300-amiRNATnos and pNOV2819 plasmids (Figure 3.2) were digested by HindIII and SpeI restriction endonucleases for 2 hours. Digestion with these enzymes was expected to release a 2618 bp DNA fragment from Ubi/NC 1300::amiRNA::Tnos vectors. However, only one visible linear DNA fragment was expected upon digestion of pNOV2819. Products of digestion were analysed in a 1.2% (w/v) LMP agarose gel. Bands of expected mass were purified from the gel. Subsequently, ligation of the amiRNA-PARP1 transgene cassettes (Ubi1::amiRNA1::Tnos and Ubi1::amiRNA3::Tnos ) to pNOV2819 (vector) was carried out by T4 DNA ligase using the quick ligation protocol described in section 3.2.10.2. Following ligation, 10 µl of the ligation mixture was mixed with 200 µl competent *E. coli* cells and transformed as described in section 3.2.11.

To identify colonies that had received recombinant pNOV2819::Ubi1::ZmPARP1-amiRNA1::Tnos or pNOV2819::Ubi1::ZmPARP1-amiRNA3::Tnos plasmid, PCR was performed on selected colonies using primers for pre-amiRNA-PARP1. The
PCR products were resolved on a 0.8% (w/v) agarose gel. To confirm presence of the transgene cassette in pNOV2819, restriction enzyme digestion was done on PCR-positive DNA. Digestion was accomplished using *HindIII* and *SpeI* restriction enzymes.

Figure 3.2: Map of pNOV2819 vector
3.2.16 Construction of cotransformation vectors

3.2.16.1 Preparation of transgene T-DNA vector

The vector pSCV1.6 (Vaughan et al., 2006) was chosen for assembly of the transgene and selectable maker T-DNAs. The pSCV1.6 contains, within its T-DNA region, the β-glucuronidase (GUS) and nptII genes both driven by the P35S promoter. The plasmid DNA was first confirmed by PCR using primers for nptII (NPTII forward: 5’ ggattgcacgcaggttctc 3’, NPTII reverse: 5’ ctcttcagcaatatcacgggt 3’) and gus (Gus forward: 5’ acgtcctgtagaaaccccaa 3’, Gus reverse: 5’ cccgcttcaaaacccaa 3’) gene. The authenticated pSCV1.6 was digested with HindIII to delete the gus and nptII gene cassettes from the T-DNA region. The resultant vector (pSCV∆NPTIIGUS) was gel-purified and self-ligated.

To obtain the amiRNA3-PARP1 expression cassette, the plasmid pExamIRNAL2 (Figure 3.1) was restricted by SacI and HindIII to release a 1803 bp fragment (P35S::amiRNA3::T35S). The cassette was gel-purified and ligated into the SacI/HindIII site of Ubi/NC1300 (Figure 3.1). Ligation products were transformed into competent E. coli DH5α cells and colonies carrying the pCAMBIA1300::P35S::amiRNA3::T35S recombinant vector (designated pCAMBIA1300-amiRNA3) screened by digesting using SacI and HindIII.
The P35S::amiRNA3::T35S fragment (1810 bp) was excised from the vector pCAMBIA1300-amiRNA3 with XbaI and HindIII and purified from gel. This insert was subcloned into the XbaI and HindIII site of pSCVΔNPTIIGUS. This new intermediate vector containing the amiRNA3-PARP1 gene cassette only was named pSCVamiRNA3.

3.2.16.2 Preparation of selectable marker T-DNA vectors

3.2.16.2.1 Preparation of pSCVBar vector

PTF101.1 (Paz et al., 2004) was used as the source of the transformation marker gene, bar. The plasmid was inserted in E. coli and purified as described in section 3.2.3.2. Pure pTF101.1 was digested using BglII and HindIII to excise P35SBar fragment. The fragment was ligated with pSCVΔNPTIIGUS vector pre-digested with BglII and HindIII to form the new recombinant construct pSCV::P35SBar. Recombinant clones were identified by colony PCR using BorderB-OD2 reverse (5’ atacaatcagtcggggegcctcaatacttagcagctgatcacttaca 3’) and BarORF-multiplex reverse (5’ ggtcaacttcgtccattcggg 3’) primers.

A T35S PCR gene sequence was cloned upstream the bar gene in pSCV::P35SBar recombinant vector. This was achieved by first designing T35SspeI-fwd (5’ tatcacaactagtagtcgtgcagttttgttttaggaattag 3’) and T35SspeI-rev (5’
atacaactagtccgcatgtcagactgcgtcggcaaaatccacca3’) primers having SpeI restriction site at their 5’ end. They were designed based on the T35S sequence of the vector pCambia1300-amiRNA3. The primers were used to amplify the T35S sequence on pCambia1300-amiRNA3 by PCR. However, PCR was performed using a mixture of the ordinary Taq DNA polymerase (New England Bio-Labs Inc., MA, USA) and the Phusion high fidelity DNA polymerase (New England Bio-Labs Inc., MA, USA) in a ratio of 9:1 Units. The PCR products were purified and their concentration estimated as described in section 3.2.5.2 and 3.2.6, respectively.

PSCV::P35SBar vector (1 µg) was digested by BglII, and purified using the Qiaquick PCR purification kit. Purified DNA was eluted in 60 µl of 1× NEB buffer 2. The vector was blunted by DNA polymerase I, large (klenow) fragment as described in section 3.2.8. Blunted DNA was purified and eluted in 60 µl of 1× NEB buffer 3. The DNA was dephosphorylated using Alkaline phosphatase, calf intestinal (CIP) as outlined in section 3.2.9. The DNA was purified by spin-column purification and analysed for quantity on an agarose spi (Duchefa, Haarlem Netherlands) gel.

The typical protocol for DNA ligation outlined in section 3.2.10.1 was followed in ligation of the T35S PCR amplicon into the blunt-ended pSCV::P35SBar vector. However, T4 DNA ligase (Invitrogen Corp. Carlsbad CA, USA) was allowed to
ligate the DNA fragments at 14 °C for 12 to 16 hours. Ligation mixture was used to transform competent *E. coli* cells. DNA was extracted from selected colonies, and subjected to PCR using BorderB-OD2-rev (5’ atacaatcgatctcgagggcgcccaaacaaacatacacagcgacttttcaca 3’) and T35SspeI-rev primers to identify recombinant clones in which T35S inserted into pSCV::P35SBar vector in the correct orientation. The vector with the correct T35S insertion was named pSCVBar.

3.2.16.2.2 Preparation of marker T-DNA in pBluescript(SK-)

A left border primer (BorderA-fwd: 5’ tataactcgagggcgcccaaacaaacatacacagcgacttttcataaatgtgtaaat 3’) harboring a *NotI* restriction site at its 5’ end and a right border primer (BorderB-OD2-rev: 5’ atacaatcgatctcgagggcgcccaaacaaacatacacagcgacttttcaca 3’) bearing a *ClaI* site at the 5’ end were designed based on sequence information of the *A. tumefaciens* octopine plasmid pTiach5 (GenBank accession number K00549.1). The primers were used in a PCR reaction to amplify the T-DNA region of the plasmid pSCVΔ*NPTIIGUS*. The PCR products were purified and their concentration estimated as described in section 3.2.5.2 and 3.2.6, respectively.
Purified PCR product was digested using *NotI* and *ClaI* and ligated into pBluescript(SK-) (pBlu2SK) (Stratagene, Cambridge, UK) pre-digested with the same restriction enzymes. Ligation products were used to transform competent *E. coli* cells. DNA was extracted from colonies that had possibly received the new recombinant vector (pBlu2SK::EmptyTDNA). The vector was analysed by PCR using BorderA-fwd and BorderB-OD2-rev primers. PCR positive vector was confirmed by digestion with the enzyme *BglII*.

P35SBar insert was subcloned into pBlu2SK::EmptyTDNA vector DNA in the same way it was subcloned into pSCVaNPTIIGUS. Analysis of transformed colonies was performed using BorderB-OD2 reverse and BarORF-multiplex reverse. Subcloning of the terminator, T35S, upstream of the *bar* gene in pBlu2SK::TDNA::P35SBar vector was achieved in a similar manner as insertion of T35S into pSCV::P35SBar to produce the recombinant plasmid pBlu2SK::TDNA::P35Sbar::T35S. Insertion of T35S in the right orientation was confirmed by PCR using T35SspeI-fwd and BarORF-multiplex-rev primers. The resultant new vector was named pBluTDNABar.
3.2.16.3 Construction of dual T-DNA pMarkfree2.1 and pMarkfree2.2 vectors for cloning gene of interest

The restriction sites on pBluTDNABar vector was removed by digesting with *HindIII* and *Sacl*. The resultant vector (pBluTDNABarΔMCS) was filled in by DNA polymerase large (klenow) fragment and self-ligated. The marker T-DNA element in pBluTDNABarΔMCS was excised by digesting with *Ascl* and *NcoI* and purified from a LMP agarose gel. Pure marker T-DNA fragment was blunted using DNA polymerase large (klenow) fragment as described in section 3.2.8. The vector pSCVΔNPTIIGUS was digested with *XhoI* and treated with klenow DNA polymerase and CIP as described in section 3.2.8 and 3.2.9, respectively. Blunted marker T-DNA insert and dephosphorylated linear pSCVΔNPTIIGUS vector DNA were ligated by T4 DNA ligase and subsequently transformed into competent *E. coli* cells. Recombinant clones were identified by PCR using primers specific to the *bar* gene (Bar-orfmultiplex fwd: 5’ gatctcggtgacggcagga 3’ and Bar-orfmultiplex rev: 5’ ggtcaacttccgtaccgagc 3’). Clones that amplified the correct *bar* gene fragment size were investigated further to establish the orientation of the inserted marker T-DNA. This was achieved by digesting plasmid DNA extracts from the clones with *EcoRI*. 
3.2.16.4 Construction of the pMarkfree4.0

To construct the double right border vector pMarkfree4.0, the binary vector pSCVamiRNA3 was used as the backbone. It was digested with XhoI to make it linear. It was filled-in using DNA polymerase large (klenow) fragment before dephosphorylating with CIP. The DNA was subsequently purified using spin-column purification method and quantified.

A bar T-DNA element was removed from pBlu2SKTDNABar as a HindIII-Ascl fragment. This fragment was gel-purified, blunted and ligated into the cut pSCVamiRNA3. Products of ligation were transformed into competent E. coli cells. Resulting colonies possibly bearing the new construct were identified by colony PCR using bar gene specific primers. The positive colonies were investigated further to establish the orientation of the inserted bar T-DNA element. This was achieved by performing PCR on DNA isolated from the clones using BarORF-multiplex fwd and T35SspeI reverse. A clone with the expected 1805 bp amplicon was identified as having the correct construct. This correct binary vector was named pMarkfree4.0.
3.2.16.5 Construction of the cis control binary vector, pSCVamiRNA3Bar

A good positive control binary vector is needed for monitoring transformation efficacy of maize with double T-DNA and double right border vectors. The binary vector pSCV-Bar was used as the backbone for construction of the cis control vector. It was cut open with *HindIII* and *EcoRI*, purified and its concentration estimated. The amiRNA-PARP1 transgene cassette was removed from pSCV-amiRNA3 as a *HindIII*-*EcoRI* fragment and ligated with the cut pSCV-Bar backbone. Ligation products were transformed into competent *E. coli* cells and transformants identified by PCR using T35Sspei reverse and BarORF-multiplex forward primers. The new constructs were verified by digestion with *HindIII* and *EcoRI*. The verified binary vector was named pSCVamiRNA3Bar.

3.2.16.6 Construction of the double right border pMarkfree3.0 vector for cloning a gene of interest

PSCVΔ*NPTII*GUS vector, digested with *XhoI*, was treated with DNA polymerase I large (Klenow) fragment before dephosphorylating with CIP. The CIP’d DNA was purified using a PCR purification kit. The *bar* T-DNA flanked by a copy of the right border sequence was removed from pBluBarTDNA as an *Ascl/HindIII* fragment. The fragment was blunted by DNA polymerase I large (klenow) fragment and purified. The pure DNA fragment was ligated into the CIP’d and blunted pSCVΔ*NPTII*GUS vector. Ligation products were transformed into
competent *E. coli* cells. DNA from emerging colonies were screened by digesting with *KpnI* to determine the orientation of the bar TDNA fragment in the pSCVΔNPTIIGUS vector. The correct vector was named pMarkerfree3.0 and sequenced to confirm the orientation of the TDNA border sequences.

### 3.2.17 Transformation of *A. tumefaciens* with new constructs

Binary vectors pSCVBar, pSCVamiRNA3, p2TDNA-amiRNA3.1 and p2TDNA-amiRNA3.2 pDRB-amiRNA3, pSCVamiRNA3Bar, pDRB-scv1.6BarTDNA and pNOV2819-UBIamiRNA1 were mobilized into LBA4404 (pAL4404, pSB1) using the freeze thaw technique. Colonies of *A. tumefaciens* possibly transformed with each construct were selected to determine if they had taken up their respective vectors. To achieve this, colony PCR was performed on selected colonies. Amplification products were resolved in 1.2% (w/v) agarose gels. One confirmed clone for each construct was inoculated in liquid and solid YM media containing 1 mg/l rifampicin and 50 mg/l kanamycin or 100 mg/l spectinomycin and grown by incubating at 28 °C overnight. Glycerol stocks were made from liquid cultures and preserved in a -80 °C freezer. Cultures on solid medium were used in plant transformation experiments.
3.3 RESULTS

3.3.1 Analysis of clones for presence of Ubi/NC1300::amiRNA plasmids

PCR performed using amiRNA-PARP1-specific primers on colonies likely transformed with the Ubi/NC1300::amiRNA recombinant vectors amplified the expected 438 bp band. The band was similar in size to the one observed on the control plasmid (Figure 3.3). Therefore the clones may be positive colonies carrying the desired recombinant vectors.

PCR on plasmid DNA extracted from the PCR positive colonies produced amplification similar to that produced using colony PCR analysis. The clones were confirmed further by digesting the extracted DNA with \textit{XbaI}. \textit{XbaI} released a 450 bp fragment from the recombinant plasmids. However, the band was absent in the control plasmid (Figure 3.3). These results confirm cloning of amiRNA-PARP1 constructs in Ubi/NC1300 vector.

Figure 3.4 show the output for Clustal W multiple sequence alignment between cloned and the reference pre-amiRNA1-PARP1 and pre-amiRNA3-PARP1 sequences. From the analysis, it was observed that the sequence of the cloned pre-amiRNA1-PARP1 and pre-amiRNA3-PARP1 were identical to the respective reference sequences.
Figure 3.3: Construction of Ubi/NC1300::amiRNA vectors. (A) Colony PCR confirmation of the presence of pre-amiRNA-PARP1 in Ubi/NC1300 using pre-amiRNA-PARP1 sequence-specific primers. Expected fragment size is indicated. Lane M, 1 Kb DNA ladder; Lane 1, Amplification of control (pEXamiRNAL2 plasmid.) plasmid; Lane 2 to 5, Amplification on colonies putatively transformed with Ubi/NC1300::amiRNA1; Lane 6 to 9, PCR amplification on Ubi/NC1300::amiRNA3 putative transformants. (B) Analysis by XbaI restriction digestion of DNA isolated from PCR positive clones. Lane 1, control DNA (Ubi/NC1300); Lane 2 and 3, digestion of Ubi/NC1300::amiRNA1 recombinant vectors; Lane 4 and 5, digestion of Ubi/NC1300::amiRNA3 recombinant vectors; Lane 6, 2-log DNA marker. (C) Map of the newly developed Ubi/NC1300-AmiRNA1 vector.
**Figure 3.4:** Clustal W alignment of the nucleotide sequence of the cloned pre-amiRNA1-PARP1 with a known sequence of pre-amiRNA1-PARP1.
3.3. 2 Analysis of clones for presence of Ubi/NC1300::amiRNA::Tnos vectors

Tnos cloned into the recombinant Ubi/NC1300::amiRNA vectors was detected by amplification of both the Tnos and amiRNA-PARP1 sequences using the vector-specific amiRNA forward primer and an insert-specific Tnos reverse primer. Amplification products (726 bp) were observed only for the recombinant clones. However, PCR reactions for water and Ubi/NC1300-amiRNA vector used as negative controls had no observable amplifications (Figure 3.5).

Digestion of DNA with HindIII and SpeI resulted in release of a 2554 bp band from the recombinant clones. However, this fragment was absent from the control vector (Ubi/NC1300-amiRNA) which lacks a cloned Tnos gene. Instead the control vector released a band which was slightly smaller than the one released by the recombinant clones (Figure 3.5). The above analysis confirms that a Tnos fragment had been cloned in the desired restriction sites of the Ubi/NC1300-amiRNA vector. One colony carrying a verified Ubi/NC1300-amiRNA vector (Figure 3.5) was stored in a -80°C freezer as glycerol stock.

Sequences of the Tnos PCR product cloned into Ubi/NC1300-amiRNA were compared with that of a Tnos gene from database using Clustal W multiple sequence alignment software. The analysis revealed that the sequence of the Tnos
Figure 3.5: Construction of Ubi/NC1300-AmiRNA1 vector. (A) Confirmation of insertion of Tnos into Ubi/NC1300::amiRNA vector by PCR using amiRNA reverse and Tnos forward primers. The expected fragment size is shown. Lane 3 and 4, PCR of DNA from two Ubi/NC1300::amiRNA1::Tnos putative transformants; Lane 5 and 6, PCR of DNA from two Ubi/NC1300::amiRNA3::Tnos putative transformants; Lane 2, PCR of plasmid Ubi/NC1300-amiRNA; Lane 1, PCR on non-template control; Lane 1, 1 Kb DNA ladder. (B): Analysis of recombinant Ubi/NC1300::amiRNA::Tnos plasmid DNA extracted from selected PCR-positive clones by digestion using HindIII and SpeI restriction enzymes. The Tnos-containing vectors (lane 3 and 4) released a 2554 bp band which was absent in the control Ubi/NC1300 vector (lane 2) containing the amiRNA-PARP1 alone. Lane 1 contained a 1 Kb DNA ladder. (C) Map of the newly developed Ubi/NC1300-AmiRNA1Tnos vector.
from clone f2 was identical with that of the *Tnos* from the NCBI nucleotide database (Figure 3.6).

**Figure 3.6:** Clustal W alignment of the nucleotide sequence of two cloned *Tnos* genes with that of the *Tnos* gene from the database.
3.3.3 Analysis of clones having pNOV2819::Ubi1::amiRNA::Tnos vectors

Insertion of the amiRNA-PARP1 expression cassette into pNOV2819 proved to be difficult. Even after altering different ligation parameters such as total DNA concentration, ligase concentration and ligation temperature (from 16 to 25) no success was achieved. Results were achieved when gel purification for pNOV2819 vector after restriction enzyme digestion was excluded to prevent subsequent possible inhibition of ligase by agarose. Though effective, this approach was found to result in establishment of colonies having vectors that did not have the insert. Digestion of the ligation mixture with SalI before transformation into competent E. coli resulted in less background.

Results of PCR performed on DNA extracted from three colonies for each amiRNA-PARP1 recombinant vector (pNOV2819::amiRNA1::Tnos and pNOV2819::amiRNA3::Tnos) revealed that one clone per recombinant vector amplified a band (438 bp) that was similar in size to that observed on the control plasmid (Figure 3.7).

Digestion of DNA from the PCR positive recombinant clones using HindIII and SpeI enzymes resulted in the release of the expected 2554 bp fragment from the recombinant plasmids and the control plasmid (Ubi/NC1300::amiRNA1::Tnos).
Figure 3.7: Construction of the recombinant pNOV2819:: Ubi1::amiRNA::Tnos plasmids. (A) Colony PCR Confirmation of insertion of Ubi1::amiRNA::Tnos cassette into pNOV2819 by PCR. Lane 1: 1 kb DNA ladder. Lane 2, PCR on positive control (Ubi/NC1300::Ubi1::amiRNA::Tnos) DNA; Lane 3, PCR of a non-template negative control; Lane 4 to 10, PCR on selected PNOV2819::Ubi1::amiRNA1::Tnos (lane 4 to 6) and PNOV2819::Ubi::amiRNA3::Tnos colonies (lane 7 to 10). (B) Confirmation of insertion of Ubi1::amiRNA::Tnos cassette into the PNOV2819 by digestion of DNA derived from PCR-positive clones with *HindIII* and *SpeI*. Lane 1, 1 kb DNA ladder; Lane 2, Digestion of control (Ubi/NC1300::amiRNA::Tnos) DNA; Lanes 3, Digestion of PNOV2819::Ubi1::amiRNA1::Tnos; Lanes 4, Digestion of PNOV2819::Ubi1::amiRNA3::Tnos recombinant plasmid. (C) Map of the final pNOV2819-UbiamiRNA vector.
Also as expected, the vector fragment from the recombinant plasmids was smaller (7599 bp) than that from the control (8958 bp) (Figure 3.7) vector. This final verification indicates that the amiRNA-PARP1 expression cassette was subcloned in pNOV2819 successfully.

3.3.4 Construction of marker-free vectors

3.3.4.1 Validation of pSCV1.6 vector
Recombinant clones carrying pSCV1.6 (Figure 3.8) were analysed by PCR for the presence of the nptII and gus genes. PCR results indicated the presence of a 1098 bp from the colonies when gus gene primers were used. The same band size was observed for the positive control vector pTF102 (Figure 3.8). NPTII gene-specific primers amplified a 678 bp fragment on clones having pSCV1.6 plasmid as well as on the positive control vector pBin19 (Figure 3.8).

3.3.4.2 Verification of putative pCAMBIA1300-amiRNA3 recombinant clones
Digestion of DNA was extracted from two likely pCAMBIA1300-amiRNA3 transformants using SacI and HindIII resulted in the release of the expected 1800 bp fragment (Figure 3.9), indicating that the amiRNA transgene cassette had been successfully subcloned into Ubi/NC1300 vector. The map of the developed vector is shown in Figure 3.9.
3.3.4.3 Analysis of possible pSCV::P35S::amiRNA3::T35S transformed clones

PCR of colonies possibly transformed with the pSCV::P35S::amiRNA3::T35S recombinant vector revealed presence of the desired amplicon (438 bp) in two out of ten selected colonies. The band was similar in size to that from the positive control vector (Figure 3.10). The other colonies amplified bands of higher mass than that of the positive control. XbaI and HindIII was used to digest DNA isolated from three clones whose PCR amplicons were similar in mass to that of the positive control. Results indicated that two of the colonies released a fragment (1815 bp) of the expected size (Figure 3.10). The DNA fragment was similar in size to the one released from the positive control vector. Release of this insert from the vector indicated its successful subcloning into the desired restriction sites of the pSCVΔNPTIIGUS vector. Figure 3.10 shows the map of the final plant expression vector pSCV-amiRNA3.
Figure 3.8: Analysis of pSCV1.6. (A) Map of pSCV1.6. (B) PCR confirmation of pSCV1.6 vector using *gus* gene-specific primers. Lane L, 1Kb ladder; Lane +, PCR on positive control (pTF102) plasmid; Lane 2, PCR on non-template control; Lane 1 and 2, PCR on two colonies having pSCV1.6 plasmid. (B) PCR Confirmation of pSCV1.6 vector using *nptII* gene-specific primers. Lane L, 1Kb DNA ladder; Lane +, PCR on positive control (pBin19) plasmid; Lane 2, PCR on non-template control; Lane 1 and 2, PCR on two colonies having pSCV1.6 plasmid.
Figure 3.9: Construction of pCAMBIA1300-amiRNA3 vector. (A) Confirmation of insertion of P35S::amiRNA3::T35S into the pCAMBIA1300 by digestion of colony-derived plasmid DNA with SacI and HindIII. Lane M, 1 Kb DNA ladder; Lane 1 and 2, E. coli colonies carrying the recombinant vector pCAMBIA1300::P35SamirNA3Tnos.
Figure 3.10: Construction of the recombinant vector pSCV::P35S::amiRNA3::T35S. (A) Confirmation of insertion of P35S::amiRNA3::T35S into the pSCVΔNPTII GUS by colony PCR using amiRNA-PARP1 construct-specific primers. Lane M, LMW DNA ladder; Lane 1, PCR on positive control vector (p1300amiRNA3); Lane 1 to 10, PCR on 10 colonies likely transformed with pSCV::P35S::amiRNA3::T35S recombinant vector. (B) Analysis, by digestion with XbaI and HindIII, of DNA extracted from three PCR positive colonies. Lane +, digestion of the positive control vector, p1300amiRNA3; Lane M, 1 kb DNA ladder; Lane 1, 2 and 3, digestion of plasmid DNA from PCR positive colony 3, 5 and 6, respectively. (C) Map of the T-DNA region of the plant expression vector pSCV-amiRNA3.
3.3.4.4 Confirmation of clones having pSCV::P35SBar recombinant vector.

Three colonies were identified by colony PCR using bar gene-specific primers to have possibly received the PSCV::P35Sbar recombinant vector. PCR, repeated on DNA isolated from the three clones using a vector-specific and a bar gene-specific primer revealed the expected fragment size (Figure 3.11). Plasmid DNA that was confirmed to have the P35SBar insert was named PSCV::P35SBar.

3.3.4.5 Confirmation of clones having pSCV::P35SBar::T35S recombinant vector.

PCR results revealed that the correct fragment size (330 bp) was generated using T35S gene-specific (T35SspeI-rev) and vector-specific (BorderB-OD2_rev) primers on DNA extracted from three out of six tested clones (Figure 3.11). This indicated that the three colonies carried the vector pSCV::P35Sbar with T35S inserted correctly. The results also indicated that Phusion high fidelity DNA polymerase had produced a blunt ended PCR product, which could be ligated to a blunted vector without requiring further modifications. The correct final vector was designated pSCVBar (Figure 3.11).
Figure 3.11: Construction of the recombinant vector PSCV::P35S::Bar and PSCV::P35Sbar::T35S. (A) Colony PCR confirmation of insertion of P35S::Bar into the pSCVΔNPTII GUS vector. Lane M: LMW DNA ladder. Lane 1 to 3, PCR on plasmid DNA from three colonies. (B) PCR analysis of plasmid DNA from clones possibly transformed with the recombinant plasmid PSCV::P35Sbar::T35S. Lane +, PCR on positive control (PSCVamiRNA3 plasmid DNA); Lane M; LMW DNA ladder. Lane -, PCR on non-template control; Lane 1-6, PCR on plasmid DNA isolated from selected clones. (C) Map of the T-DNA region of the plant expression vector pSCV-Bar.
3.3.4.6 Analysis of clones having pBlu2SK::EmptyTDNA vector

Colony PCR was performed on 8 clones using BorderA-fwd and BorderB-OD2 reverse primers to detect the presence of the T-DNA insert in pBlu2SK. Results indicated that two of the clones had possibly taken up the pBlu2SK::EmptyTDNA recombinant vector judging by the presence of the expected fragment size in their PCR profiles (Figure 3.12). Digestion of the plasmid DNA from the two PCR-positive colonies using BglII produced a linear DNA band of 3100 bp (Figure 3.12). Since the BglII site is within the T-DNA fragment introduced into pBlu2SK vector, the results confirmed successful cloning of the ‘empty T-DNA’ fragment. However, no digestion was observed for the pBlu2SK vector without the insert indicating the absence of the BglII site and consequently, the insert.

3.3.4.7 Analysis of colonies carrying pBlu2SK::TDNA::P35SBar

PCR was performed on DNA isolated from 10 colonies using the insert-specific (Bar-ORF multiplex rev) and vector-specific (BorderB-OD2 rev) primers to confirm the recombinant vector pBlu2SK::TDNA::P35SBar. Amplification of the correct fragment size (450 bp) was observed in the PCR profile of all 10 colonies assessed (Figure 3.13). These results confirm the presence of P35SBar insert in pBlu2SK::emptyTDNA. Vector map of the final pBluTDNAP35SBar vector is shown in Figure 3.13.
3.3.4.8 Confirmation of clones for the presence of the recombinant vector

pBluTDNABar

Putative recombinant clones were analysed via PCR using vector-specific (BarORF-multiplex-rev) and an insert-specific (T35SspeI-fwd) primers. Amplification of the desired fragment size (600 bp) was observed for three out of the seven clones analysed (Figure 3.14). This indicates that the three clones had the T35S in PBluTDNAP35SBar vector inserted in the desired restriction sites in the right orientation. One of the clones having the confirmed pBluTDNABar vector (Figure 3.14) was selected for subsequent experiments.
Figure 3.12: Construction of pBluEmptyTDNA vector. (A) Colony PCR confirmation of the presence of T-DNA insert in pBluscriptIIISK using BorderA-forward and BorderB-OD2 reverse primers. Lane +, PCR on positive control pSCVΔNPTIIGUS plasmid; Lane M, LMW DNA ladder; Lane 3-9, PCR on colony 1 to 6. (B) Confirmation by BglII restriction enzyme digestion of PCR-positive clones 3 and 4 likely harboring pBlu::EmptyTDNA recombinant vector. Lane 1, Uncut Pblu2SK; Lane 2 and 3, digestion of plasmid DNA isolated from the two clones; Lane 4, 1 Kb DNA ladder. (C) Map of pBluEmptyTDNA.
**Figure 3.13:** Construction of PBluTDNAP35SBar. (A) PCR confirmation of the presence of P35SBar fragment within PBlu::EmptyTDNA vector extracted from selected clones. The expected fragment size is indicated. Lane M, LMW ladder; Lane 1 to 10, PCR profile of plasmid DNA isolated from 10 colonies. (B) Map of the final pBluTDNAP35SBar vector.
Figure 3.14: Construction of the recombinant vector pBlu2SK::TDNA::P35SBar ::T35S. (A) Colony PCR confirmation of insertion of T35S fragment into pBlu2SK::TDNA::P35SBar using T35SspeI-fwd and BarORF-multiplex-rev primers. The expected amplicon size shown. Lane M, LMW DNA ladder; Lane 1 to 7, PCR profile of plasmid DNA isolated from seven clones. (B) Map of pBluTDNABar.
3.3.5 Analysis of clones carrying pMarkfree2.1 and pMarkfree2.2 vectors

A total of eight recombinant clones were screened with *bar* gene-specific primers to establish insertion of the *bar* T-DNA in the pSCVΔNPTII GUS vector. PCR amplification of the correct fragment size using BarORF-multiplex forward and BarORF-multiplex reverse primers was achieved for six out of eight colonies (Figure 3.15). Two different profiles were observed for plasmid DNA from four of the PCR positive clones following digestion with *EcoRI*. Approximately 1800 bp fragment was released from plasmid DNA derived from colony 4 and 6 (Figure 3.15). Plasmid DNA from the two colonies was designated pMarkfree2.1. PMarkfree2.2 was used to designate plasmid DNA from colony 5.

3.3.6 Confirmation of clones having the pMarkfree4.0 vector

Clones carrying the pmarkfree4.0 vector were verified by PCR using vector-specific (T35Sspe1 reverse) and insert-specific (Bar-ORF multiplex forward) primers. A fragment of about 1805 bp was observed for three colonies (colonies 3, 5 and 8). This fragment was almost similar in size to that amplified on the positive control vector, pMarkfree3.2 (1856 bp) (Figure 3.16). Clones producing the 1805 bp PCR band had the cloned fragment inserted in the desired orientation. Colony 3 was cultured further for preparation of glycerol stock for cryopreservation as well as for plasmid minipreps for subsequent plant transformation work. No amplification was observed on colony 12. The remaining colonies (13, 14, 16...
and 17) amplified smaller bands signifying cloning of the insert in the reverse orientation.

**Figure 3.15:** Construction of the pMarkfree2.1 and pMarkfree2.2. (A) Colony PCR confirmation of insertion of the bar T-DNA fragment into pSCVΔNPTIIGUS using bar gene-specific primers. Lane M: LMW DNA ladder. Lane +, pMarkfree3.0 used as positive control; Lane 1 to 8, PCR profile of plasmid DNA isolated from eight clones. (B) *EcoRI* digestion of plasmid DNA from different PCR positive clones to identify those bearing pMarkfree2.1 and pMarkfree2.2. Lane 1-5, digestion profile of plasmid DNA isolated from colonies 1, 3, 4, 5 and 6, respectively.
Figure 3.16: Construction of the double right border vector, pMarkfree4.0. (A) Analysis, by PCR, of plasmid DNA isolated from possible pMarkfree4.0 transformants. Lane +, PCR on the positive control (pMarkfree3.2) vector; Lane M, 1 kb DNA ladder; Lane 1 to 8, PCR on clones 3, 5, 8, 12, 13, 14, 16 and 17, respectively. (B) Map of the T-DNA regions of pMarkfree4.0.
3.3.7 Analysis of clones carrying the cis control vector

Primers T35Sspei reverse and BarORF-multiplex forward were used in PCR to screen 6 clones to identify true transformants. The expected band size was amplified on three colonies as well as from the control DNA (Figure 3.17). DNA from the three PCR positive colonies was digested by HindIII and EcoRI. The expected 1851 bp fragment was released from plasmid DNA of the three colonies (Figure 3.17) thereby confirming subcloning of the amiRNA3-PARP1 expression cassette in pSCV-Bar vector.

3.3.8 Verification of pmarkfree3 vector

Out of five colonies screened for the presence of pMarkfree3 by digestion with KpnI, one possessed a plasmid that released a band similar to the one released by the positive control (pBlubar) vector (Figure 19). In addition, the plasmid had the same vector profile as the pSCV∆NPTIIGUS vector without an insert. To verify it further, pMarkfree3 (Figure 3.18) was sequenced beginning from the left border region to the first right border (RB1) of the vector. The results confirmed that the bar T-DNA was oriented correctly in the pMarkfree3 vector (Figure 3.18).
Figure 3.17: Construction of the cis control vector, pSCVamiRNA3Bar. (A) PCR confirmation of insertion of amiRNA3-PARP1 expression cassette into pSCV-Bar. PCR was done with T35sspei reverse and BarORF-multiplex reverse primers. Lane +, PCR profile for the positive control plasmid pMarkfree4.0; Lane 3, 1 kb DNA ladder; Lane 1 to 6, PCR profile of plasmid DNA from clones B, C, 4, 5, 7, and 10, respectively. (B) Analysis of plasmid DNA from PCR-positive clones by digestion with HindIII and EcoRI. Lane M, 1 kb DNA ladder; Lane 1 to 3, Digestion profile of clones B, C and 5, respectively. (C) T-DNA region of the final pSCVamiRNA3Bar plant expression vector.
Figure 3.18: Construction of the double right border vector, pMarkfree3. (A) Verification of clones possibly carrying pMarkfree3 by digestion with \textit{KpnI}. Digestion identified one vector (clone 1) to be the correctly oriented vector because its digestion released a fragment that was similar in size to the one released by the positive control vector (lane C1). In addition, the digestion profile of the vector’s backbone was similar to that of the pSCV\textit{∆NPT\textsc{II}}\textsc{GUS} vector without an insert (lane C2). (B) Sequence analysis of the T-DNA region of pMarkfree3.0. Underlined sequence in small case indicates a filled-in \textit{XhoI} restriction site. (C) The T-DNA region of the constructed plant expression vector pmarkfree3.0.
A

B

C

98
3.3.9 Analysis of LBA4404 for uptake of prepared vectors

Following transformation, LBA4404 colonies emerged 2-3 days later and were screened to establish uptake of the developed plant expression vectors. PCR using \textit{pmi} gene-specific primers on control (pNOV2819) DNA revealed amplification of a 532 bp fragment that was also present in the profile of two colonies carrying the pNOV2819::Ubi1::amiRNA1::Tnos and pNOV2819::Ubi1::amiRNA3::Tnos (Figure 3.19). No visible amplification was observed for the negative control.

The presence of the two T-DNA vectors pMarkfree3.1 and pMarkfree3.2 was investigated by colony PCR targeting the \textit{bar} gene. Results indicate amplification of the desired fragment size (400 bp) on two colonies haboring pMarkfree3.1 (Figure 3.19) when primers specific to the \textit{bar} gene were used in PCR. The results also reveal the presence of the pMarkfree3.2 vector in two colonies due to the presence of the expected 400 bp band in their PCR profile (Figure 3.19). These analyses confirm successful transformation of LBA4404 with the chosen vectors.
Figure 3.19: Confirmation of transformation of *A. tumefaciens* with constructed vectors. (A) Colony PCR, using bar gene-specific primers, on five colonies carrying pSCV-Bar construct. Lane 1 to 5: PCR on five selected colonies. Lane M, LMW DNA ladder; Lane +, PCR on positive control plasmid, PBlu2SKTDNABar. (B) PCR analysis, using bar gene-specific primers, of LBA4404 transformed with the double T-DNA vectors. Lane 1 and 2, PCR on colonies haboring pDTDNA-amiRNA3.1 trasformants; Lane 3 and 4, PCR profile of colonies having pDTDNA-amiRNA3.2 transformants; Lane 5, PCR of positive control vector (PBlu2SKTDNABar); Lane M; LMW DNA ladder. (C) Analysis of LBA4404 colonies harboring pNOV2819-amiRNA1 by PCR using amiRNA1-PARP1 gene construct-specific primers. Lane M, LMW DNA ladder; Lane +, PCR of positive control (pNOV2819-amiRNA1) vector; Lane -, PCR on non-template control; Lane 1 to 3, Colony PCR profile of three colonies.
3.4 DISCUSSION

One of the primary objectives of the study was to develop vectors that could enable generation of plants that are free of selectable marker genes. The first vectors developed were the pNOV2819-UbiamiRNA vectors bearing the *pmi* gene as the plant selectable marker. PNOV2819-UbiamiRNA1 (Figure 3.7) and pNOV2819-UbiamiRNA3 consist of the maize Ubi1 promoter, the PARP1-amiRNA and the Tnos ligated into the vector pNOV2819 as the Ubi1::amiRNA::Tnos expression unit. The expression cassette is oriented with the Tnos proximal to the T-DNA left border of pNOV2819.

One DRB vector (pMarfree3.0) and two dual T-DNA vectors (pMarkfree2.1 and pmarkfree2.2) that can be used for cloning one or more genes of interest have been developed. These vectors may facilitate removal of the plant selectable marker gene *bar* from plants by cotransformation and segregation. The DRB binary vector pMarkfree3.0 has the structure LB-*mcs*-RB2-*bar*-RB1. A multiple cloning site (mcs) comprising the *EcoRI, BamHI, SmaI, XbaI* and *HindIII* restriction sites is located between RB2 and LB. These restriction sites are unique to the pmarkfree3.0 vector and most other vectors that carry a gene of interest. This makes it possible to generate unique sticky ends on the DRB vector to facilitate easy cloning of any GOI. The pMarkfree2.1 and pmarkfree2.2 each contains two distinct T-DNA regions, one with a *bar* gene as the transformation marker gene
and the other containing a MCS (similar to that of pMarkfree3.0) for cloning the desired gene expression cassette(s).

PMarkfree4.0 is a binary vector comprising two T-DNA right border sequences flanking the bar expression cassette, followed by the amiRNA3-PARP1 expression unit then the T-DNA left border sequence. The vector was constructed by moving the bar expression unit including the right border sequence from pSCV-Bar to a site just outside the Right border sequence pSCV-amiRNA3. The bar expression unit is oriented with CAMV 35S promoter proximal to the right border of the construct pSCV-amiRNA3.

Two intermediate vectors pSCV-amiRNA3 and pSCV-Bar were constructed to facilitate construction of the cotransformation vectors. PSCV-amiRNA3 is a binary vector with amiRNA3-PARP1 under the control of CaMV 35S promoter and CaMV 35S terminator. It was prepared such that the CaMV 35S terminator is proximal to the T-DNA right border of the vector pSCVΔNPTIIGUS. PSCV-amiRNA3 was prepared for fitting the bar T-DNA element to produce the double right border vector (pMarkfree4.0). PSCV-Bar is a vector which can express bar gene in plants. It has the bar gene driven by a long the CaMV 35S promoter. The vector’s bar expression cassette is oriented with the CaMV 35S terminator in close proximity to the RB of the pSCVΔNPTIIGUS vector. The vector was prepared for
fitting the amiRNA3-PARP1 T-DNA element in constructions of the cis control vector pSCVamiRNA3Bar. When introduced into the same or different Agrobacterium cells, pSCV-amiRNA3 and pSCV-Bar can be used in cotransformation of plants.

The developed cotransformation vectors may be useful in generation of transgenic plants that are free of SMGs. This may be achieved by simply inserting any gene(s) of interest into the developed cotransformation vectors pMarfree3.0, pMarkfree2.1 and pMarkfree2.2. The resultant vectors can then be used in A. tumefaciens-mediated transformation to produce the desired cotransformed plants. Marker-free segregating plants can then be selected from among the progeny derived from selfed cotransformants.
CHAPTER FOUR

PRODUCTION OF MARKER-FREE TRANSGENIC TOBACCO PLANTS USING A NEW DOUBLE RIGHT BORDER BINARY VECTOR

4.1 INTRODUCTION

Removal of selectable marker gene (SMG) from transgenic plants is increasingly becoming an important objective for the plant biotechnology research community and is viewed as a good laboratory practice. Elimination of SMGs from transgenic plants can be beneficial for the following reasons: (1) it enables reuse of the selectable marker for identification of transformants during retransformation of a transgenic plant with a gene for the same or a different trait (2) It allows greater probability of acceptance of transgenic plants by consumers (3) It obviates the need to assess the SMG in the transgenic plant for environmental or toxicological safety in compliance with regulatory requirements (Miki and McHugh, 2004).

Different techniques for elimination of selectable marker genes have been developed including some based on site-specific recombination (Gleave et al., 1999) and transposition (Cotsaftis et al., 2002). However, cotransformation stands out as a conceptually simple and cheap system to develop. Cotransformation involves introduction of a gene of interest (GOI) and SMG, harboured between separate T-DNA regions, into the plant cells. If the two transgenes integrate in
unlinked genomic loci, they can be separated from each other in subsequent generation of the cotransformants through genetic segregation. Cotransformation of plants with a GOI and SMG has been achieved successfully by particle bombardment or by *Agrobacterium tumefaciens*. With particle bombardment mediated cotransformation, the transgenes are integrated in the genome in a complex manner and rarely segregate (Wakita *et al.*, 1998). However, cotransformation mediated by *Agrobacterium tumefaciens* has the advantage of being capable of efficiently segregating transgenes due to the ability to integrate transgenes in a simple pattern and in few copies.

Over the past few years, different techniques for removal of SMGs have been developed. However, many remain inaccessible because they are protected by patents (Komari *et al.*, 1998; Baszczynski *et al.*, 2002; Richael, 2011; Ye *et al.*, 2012). In addition, development of marker-removal techniques is cost prohibitive and difficult due to the large size and complexity of the vector systems. On the other hand, cotransformation vectors are, in concept, simple to develop. Most require adapting available binary vectors to the desired cotransformation vector design. In this chapter, the development of a new pilot DRB vector pMarkfree5.0 is reported. The vector contains a selectable maker gene between RB1 and RB2. The *nptII* and *gus* gene were placed between RB2 and the LB and can be replaced with any gene(s) of interest. This binary vector was introduced into *Agrobacterium*
*tumefaciens* strain LBA4404 which was in turn co-cultivated with tobacco leaf discs. In addition, efficient cotransfer and coexpression of transgenes contained in the different T-DNA regions in primary transformants is reported. Finally, it is reported here the identification of plants free of the T-DNA region containing the selectable marker gene.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of pMarkerfree5.0 vector and *Agrobacterium* strain

PMarkerfree5.0 was constructed from pMarkerfree3.0. Preparation of pMarkerfree3.0 vector for cloning a gene of interest is described in section 3.2.16.6. The *SalI/BglII* fragment haboring the *gus* and *nptII* gene cassettes was removed from pSCV1.6 and end-filled. The fragment was subcloned into pMarkerfree3.0 predigested with *HindIII* to produce pMarkerfree5.0. PMarkerfree5.0 was mobilized into the *A. tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) using the freeze thaw technique (Raviraja and Sridhar, 2007). The new *Agrobacterium* strain was cultured on yeast extract-mannitol (YM) medium (Lin, 1994) containing rifampicin (1 mg/L) and kanamycin (50 mg/L). Fifteen inoculating loopfuls of the LBA4404 were transferred directly from the plates to a 50 ml sterile tube containing 10 ml of tobacco shoot induction (SM) media. SM media was based on MS basal salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg *et al.*, 1968). Composition of the SM medium is shown in Table 4.1.
Table 4.1: Composition of media used for tobacco shoot induction

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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<tbody>
<tr>
<td>MS basal salts including B5 vitamins (Duchefa)</td>
<td>4.4 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 g</td>
</tr>
<tr>
<td>NAA</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>BAP</td>
<td>1 mg</td>
</tr>
<tr>
<td>Agar</td>
<td>8 g</td>
</tr>
<tr>
<td>Water to</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH to 5.9 with 1M KOH</td>
<td></td>
</tr>
</tbody>
</table>

4.2.2 Transformation of tobacco with pMarkfree5.0

Leaf discs of *Nicotiana tabacum* were transformed using the co-cultivation method (Horsch *et al.*, 1985). Healthy and green fully expanded leaves were excised from 3-5 week old tissue culture grown tobacco plants and cut into 0.8 to 1.0 cm squares. The leaf pieces were transferred into sterile 50 ml tubes containing 2 ml of SM liquid media. LBA4404 was suspended in SM liquid media was transferred to the 50 ml tube with explants. Leaf explants were incubated with the *Agrobacterium tumefaciens* at 28 ºC with gentle shaking at 100 rpm for 30 min. The bacterium suspension was poured off and the explants transferred to solid SM media covered with sterile filter paper. Ten to 15 explants were placed on each plate with the lower epidermis uppermost. Plates were sealed with parafilm and incubated under dim light for 24 hours, and then for 3 days at 28 ºC, 16h/8h
day/night photoperiod in bright light (cool white fluorescent, 56 micromoles/m/s). Cultures were monitored daily for signs of *Agrobacterium tumefaciens* overgrowth.

### 4.2.3 Shoot regeneration

After co-cultivation, leaf pieces were washed by immersing in 30 ml liquid SM medium supplemented with 1000 mg/l timentin and stirring vigorously for 3 minutes. The explants were rinsed with liquid SM twice (3 minutes per time). Washed explants were then blot dried on a sterile filter paper. Explants were placed firmly (lower epidermis lowermost) on SM with selection antibiotics (100 mg/l kanamycin and 300 mg/l timentin) and incubated in a 28 ºC growth room under 16h/8h light/dark photoperiod for 2 weeks. Leaf explants were transferred to new SM plates with fresh selection antibiotics and incubated for an additional 10 days to 2 weeks during which the first or additional shoots appeared.

### 4.2.4 Rooting of kanamycin resistant regenerants

Shoots (1 to 3 cm in height) were transferred by cutting cleanly away from any callus and the original leaf piece at a 45 degree angle. The shoots were placed approximately 5 mm deep into RM (Table 4.2) containing 300 mg/l kanamycin and 300 mg/l timentin. Leaf pieces from which each shoot was obtained were discarded to eliminate the possibility of selecting non-independent transformants.
Table 4.2: Composition of media used for tobacco root induction

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS basal salts</td>
<td>4.7 g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30 g</td>
</tr>
<tr>
<td>Agar</td>
<td>8 g</td>
</tr>
<tr>
<td>Water</td>
<td>to 1000 ml</td>
</tr>
<tr>
<td>pH to 5.9 with KOH</td>
<td></td>
</tr>
</tbody>
</table>

4.2.5 Preparation of regenerants for transfer to glasshouse

After substantial root growth (at least 3 to 5 cm in length and 3 to 5 main roots), the shoots were hardened prior to transfer to glasshouse. Rooted shoots were removed from RM and excess agar washed off in a sink using clean (unsterile) water. The plants were placed in jam jars containing 50 ml of sterile 1/10 MS medium and let to sit on lab bench with closed lid for 24 hours, then with lid ajar for an additional day. Plants were transferred to autoclaved, prewetted mixture of peat moss and soil (1:1) in 7 cm diameter plastic pots. Plants were watered again with tap water to settle soil around roots. Plants were not watered again until after five days in the glasshouse. The plants were placed in a 20 cm deep basin and two layers of cheese cloth placed on top and secured in place with a tape.
4.2.6 Growth in glasshouse: water stress induced early flowering

After two days under glasshouse conditions, one layer of cheese cloth was removed. The other was removed after two more days. Once well established, plants were water stressed by allowing them to dry just to the wilting point between watering. Root development was restricted by maintaining the plants in the same 7 cm pots (half filled with soil) until maturity. Nitrogen phosphorus potassium (NPK) Fertilizer was applied at one and three weeks after initial transfer to the glasshouse. Subsequent watering was with tap water only.

4.2.7 Controlled pollination and seed harvesting

Self-pollination of putative transformants was conducted by placing pollination bags over inflorescences beginning when corolla of first flower showed color prior to opening. Seed capsules were harvested when they first begun to turn brown (approximately 15 to 20 days after pollination). Alternatively the capsules were harvested when, upon dissection, brown seeds could be seen. The pods were stored in sealed coin envelops. A 60W incandescent light bulb placed on a shelf was used to aid drying of seed capsules for about 2 to 3 days.

4.2.8 Screening of tobacco cotransformants by multiplex PCR

Motars and pestles used for grinding of plant material were cleaned by soaking in 0.25 M HCl for atleast 20 minutes. The acid-washed motar and pestles were rinsed
by wiping using a piece of cotton wool soaked in sterile distilled water. A fresh piece of tobacco leaf (100 to 200 mg) was harvested from plants growing in the glasshouse and placed in the cleaned motar. The piece of leaf was ground to a very fine paste using 100 mg sterile acid-washed sand wetted in 200 µl of 0.1 M Tris-HCl (pH 8.0). The acid-washed sand was prepared by soaking cleaned sand in 0.25 M HCl for 30 minutes followed by rinsing five times in water before sterilizing by autoclaving. To the fine paste, 3 ml of urea DNA extraction buffer was added and ground gently to ensure thorough mixing. Three milliliters of the mixture was transferred into a 10 ml tube and incubated at 65 ºC for 2 hours with occasional mixing. The mixture was extracted by adding 3 ml of Phenol:chloroform:isoamylalcohol (prepared as outlined in appendix I) and mixed thoroughly. The mixture was centrifuged at 6000 rpm for 20 minutes to separate the phases. The top aqueous phase (2.5 ml) was removed and transferred to a clean 10 ml tube. RNaseA (20 µg/ml) was added to the mixture and incubated at 37 ºC for 30 minutes to digest RNA. RNaseA treated mixture was extracted with 2.5 ml phenol:chloroform:isoamylalcohol mixture (25:24:1) as indicated above. The mixture was centrifuged at 6000 rpm for 20 minutes before transferring 2 ml of the aqueous layer into a clean 10 ml tube. A final extraction with an equal volume of chloroform was performed and phases separated by centrifuging at 6000 rpm for 20 minutes. The upper phase (about 1.5 ml) was transferred into a clean tube and DNA was precipitated by adding 1/10 volume of 0.3 M sodium acetate (pH 8.0) and 6/10 volume of isopropanol. DNA was precipitated overnight at -20 ºC.
Precipitated DNA was collected by centrifugation at 6000 rpm for 1 hour. The pellet was washed by adding 3 ml of 70% (v/v) ethanol and centrifuging for 20 minutes at 6000 rpm. Ethanol was discarded and the tube centrifuged again for 2 minutes to collect the remaining ethanol to the bottom of the tube. This was aspirated off with a P1000 pippeter. DNA was completely solubilised in 0.8 mM NaOH and stored at -20 ºC. Quality and quantity of the DNA was determined by agarose gel electrophoresis.

Tobacco genomic DNA (20 to 250 ng) was used in PCR to detect the presence of *bar* and *nptII* genes. Each 50 µl reaction contained 1× PCR reaction buffer containing 3 mM MgSO₄, 0.5 µM each primer, 1 mM of dNTP mixture and 2U Taq DNA polymerase. The primers used for amplification of the *bar* gene were Bar-fwd: 5’ gatctcggtgacgggac 3’ and Bar-rev: 5’ ggtcaaccttcgatcgg 3’. Primers for amplification of *nptII* gene were NPTII-forward: 5’ ggattgcacgcaggttctc 3’, NPTII-reverse: 5’ ctcttcagcaatatcaggttctc 3’. The PCR profile involved initial denaturation at 94 ºC for 10 minutes, followed by 35 cycles of denaturation at 94 ºC for 3 minute, annealing at 63 ºC for 1 minute and extension at 72 ºC for 1 minute with a final extension at 72 ºC for 8 min. Amplicons were separated in a 1.5% (w/v) agarose gel by electrophoresis and results documented using a digital camera.
4.2.9 Basta leaf paint assay

To assess functionality of the bar gene, Basta® leaf paint assay was applied on cotransformed plants. The plants were swabbed with Basta (0.02% (v/v) applied using a piece of cotton wool to a small section of a tobacco leaf. The section of leaf to be painted with Basta was first marked using a permanent marker pen. After seven days, putative transgenic tobacco plants were scored for response to the applied herbicide. Plants that showed no leaf damage were classified as Basta resistant (B$^R$) while those that were damaged were classified as Basta sensitive (B$^S$).

4.2.10 Leaf bleach and histochemical assays

The leaf bleach assay was performed on plants growing in soil to identify those containing a functional nptII gene. The assay was performed by applying assay solution containing paromomycin (Duchefa Biochemie B.V., Haarlem, The Netherlands) and kanamycin (Phytotechnology) each at 1,000 mg/l and 0.06% (v/v) of Silwet L-77 (Lehle Seeds, Texas, USA) on a small section of a leaf using a piece of cotton wool. Results were recorded 7 days post application. Plants that showed no bleaching were categorized as kanamycin resistant (Km$^R$) while those that bleached were categorized as kanamycin sensitive (Km$^S$). Histochemical assay for β-glucuronidase (GUS) activity were performed on leaf tissues as described previously (Jefferson et al., 1987). Briefly, tobacco tissues were
immersed in 0.5 ml of buffer P in a microfuge tube and incubated at 37 ºC for 1 hour. The buffer was removed and 0.3 ml of buffer X added and incubated for 18 hours at 37 ºC. Green tissues were cleared off chlorophyll by incubating in 70% ethanol for at least 12 hours. The tissues were immersed in 50% (v/v) glycerol for 1 hour before finally mounting in 100% glycerol. The tissues were examined for change in color under stereomicroscope.

4.2.11 Phenotypic and molecular assays of T₁ seedlings

To identify cotransformed lines that were segregating the bar gene, T₁ seedlings were screened for resistance to PPT. Seeds (T₀) derived from self-pollinated cotransformed plants were placed in a 2ml tube and sterilised by soaking in 1ml of 70% ethanol for 30 seconds. The ethanol was replaced with 1 ml of 2.5% (v/v) sodium hypochlorite containing 0.1% (v/v) tween-20 and incubated for 20 minutes with occasional vortexing. The seeds, now sterile, were rinsed three times with sterile distilled water. After rinsing, the water was removed and 1 ml of absolute ethanol was added to the tubes to resuspend the seeds. The resuspended seeds were quickly dispensed onto sterile filter paper in a sterile petri dish. Upon evaporation of the ethanol, the paper was tapped gently to allow about 50 to 100 seeds to spread onto MS medium containing 10 mg/l PPT. Plates were sealed with parafilm and incubated at 27 ºC in 16h/8h light/dark photoperiod. Fourteen days later, survival of tobacco seedlings to PPT was examined. Plants that were green and growing vigorously were categorized as PPT-resistant (PPT<sup>R</sup>) while those that
were small and bleached were classified as PPT-sensitive (PPT\textsuperscript{S}). Ratio of resistant-to-sensitive plants on PPT (PPT\textsuperscript{R/S}) was used to calculate the efficiency of cotransformation-mediated marker removal.

To identify plants free of the *bar* gene, T\textsubscript{0} events with single-copy of *bar* gene were selected for T\textsubscript{1} segregation analysis. Seedlings (T\textsubscript{1}) were germinated on MS medium and transplanted to soil. Once established, the plants were assayed for resistance to Basta (0.02\% (v/v) as described previously. The leaf bleach assay was performed on T\textsubscript{1} seedlings previously assayed for Basta resistance to identify plants expressing the *nptII* gene. The marker-free plants identified based on phenotypic assays (Basta and kanamycin resistance) were advanced for confirmation through PCR as described previously.

4.2.12 Data analysis

Chi-square goodness-of-fit tests were performed on data from the T\textsubscript{1} populations derived from self-pollinated cotransformed tobacco plants to determine if the observed segregation ratios of PPT, Basta or NPTII resistant plants to PPT, Basta or NPTII sensitive plants fit the expected Mendelian 3:1 or 1:1 phenotypic ratios, respectively.
4.3 RESULTS

4.3.1 Analysis of clones having pMarkfree5.0 vector

PMarkfree5.0 transformants were identified by PCR using BorderB-OD forward and BarORF-multiplex-fwd primers. Out of the 28 colonies analysed, the desired band size was observed in three clones only. When the PCR analysis was repeated on DNA extracted from the three clones a fragment size of 1412 bp was observed on the three clones (Figure 4.1). The positive control vector pmarkfree3.2 produced a slightly bigger fragment due to an extra 108 bp that included the left border sequence. PMarkfree3.1 and pCis-amiRNA3 plasmids used as negative controls produced no observable amplification. The T-DNA region of the newly constructed pMarkfree5.0 vector is shown in Figure 4.1.

4.3.2 Production of kanamycin resistant tobacco plants using the pMarkfree5.0 binary vector

The transformation frequency of pMarkfree5.0 was tested by assessing resistance of transformed shoot-producing leaf discs to kanamycin selection. All explants that were transformed with pMarkfree5.0 construct and cultured on SM without antibiotics survived and shooted (Figure 4.2). However, majority of the explants that were transformed with pMarkfree5.0 construct and placed on SM containing 100 mg/l kanamycin died (Figure 4.2). Surviving explants produced shoots after 8 days but the explants were smaller and had fewer shoots compared to explants on
Figure 4.1: Construction of the DRB vector pMarkfree5.0. (A) Confirmation of clones harbouring pMarkfree5.0 through PCR. Lane 1, PCR on pMarkfree3.1 as negative control; Lane 2, PCR for pCis-amiRNA3 vector used as negative control; Lane 3, PCR profile of the positive control vector pMarkfree3.2; lane 4, PCR on positive control (pMarkfree4) vector; Lane M, 1 Kb DNA ladder; Lane 5 to 7, PCR on colonies having pMarkfree5.0. (B) Schematic map of the T-DNA region of the binary vector pMarkfree3.0 and insertion of the gus and nptII expression cassettes to create pMarkfree5.0. P35S, cauliflower mosaic virus 35S promoter; T35S, cauliflower mosaic virus 35S terminator; int-gus, β-glucuronidase gene with catalase intron; nos 3’, nopaline synthase gene polyadenylation signal; LB, left T-DNA border; RB, right T-DNA border; OD, overdrive sequence.
non-selective SM medium (Figure 4.2). More than 55% of explants transformed with pMarkfree5.0 survived and produced at least one shoot on kanamycin selection (Table 4.3). A total of 78 independent kanamycin-resistant plants were obtained from the explants surviving kanamycin selection (Table 4.3). These T₀ plants were maintained in a glasshouse.

Table 4.3: Frequency of regeneration of plants from tobacco leaf discs putatively transformed with pMarkfree5.0 and cultured on selective or non-selective medium

<table>
<thead>
<tr>
<th>Expt</th>
<th>Rep</th>
<th>Total explants</th>
<th>Regenerating explants</th>
<th>Expt</th>
<th>Rep</th>
<th>Total explants</th>
<th>Regenerating explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>13</td>
<td>5</td>
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</tr>
<tr>
<td>Total</td>
<td></td>
<td>140</td>
<td>78</td>
<td>96</td>
<td>96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Regeneration frequency on selective medium was 55.7%, expressed as total number of regenerating explants per 100 explants infected with LBA4404 (pMarkfree5.0). Expt, Experiment; Rep, Replication.
Figure 4.2: Regeneration and establishment of putatively transformed tobacco plants. Proliferation of shoots from putatively transformed tobacco leaf tissues after 21 days on non-selective (A) and selective (B) shoot induction medium. (C) Putative transgenic tobacco plants establishing in the glasshouse. (D) Histochemical staining of leaf discs to detect gus activity in different putatively transformed T₀ tobacco plants. Codes T1 to TA2 refers to independently transformed T₀ plants.
4.3.3 Stable integration of the nptII and bar transgenes into tobacco genome

Putative transgenic tobacco plants produced using the cotransformation vector pMarkfree5.0 were analysed for the presence of the nptII and/or bar T-DNA by multiplex PCR. A representative gel image showing results of a multiplex PCR analysis for 22 putatively transformed tobacco plants is shown in Figure 4.3. An nptII PCR fragment of approximately 700 bp was obtained from genomic DNA of 60 out of the 73 T₀ plants. Plants (66.67%) positive for the nptII gene were found to possess a 300 bp fragment of the bar gene. Therefore 33.33% (20/60) of the analysed plants contained the nptII gene only and were consequently excluded from further analyses. Similarly plants that possessed the bar gene only were discarded.

4.3.4 Expression of co-inserted nptII/gus and bar transgenes

Transient expression of gus gene was assessed in leaf tissues obtained from plants that were establishing in the glasshouse (Figure 4.2) to help in rapid identification of transgenic plants. A total of 73 Kanamycin-resistant plants were screened using the histochemical assay. Figure 4.2 shows the gus staining results for a few of the regenerated T₀ plants. The blue stain was observed in 47.95% (35/73) of the tested kanamycin resistant T₀ plants.
Figure 4.3: Multiplex PCR assay for *nptII* and *bar* transgenes in transgenic T₀ plants transformed using the cotransformation vector pMarkfree5.0. Lane TD33 to TD3, gDNA of independently cotransformed plants; Lane Wild type, gDNA from a non-transgenic plant; Lane pMarkfree5, pMarkfree5.0 used as positive control.
Stable expression of the introduced *bar* and *nptII* transgenes was confirmed by performing the basta and leaf bleach assays on leaves of cotransformed plants. The basta leaf paint assay on some of the cotransformed plants is shown in Figure 4.4. Over 89% of the regenerated T₀ plants were resistant to the application of 0.3% Basta herbicide (Table 4.4). Among the basta sensitive plants were two plants confirmed to be cotransformed (event TAY3 and TAC1) by PCR analysis. These plants showed severe leaf damage even after application of relatively lower (0.02%) concentration of Basta®.

**Table 4.4:** Functional analysis of *bar* and *nptII* genes in transformed T₀ plants

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Number of plants assayed</th>
<th>Number of tolerant plants</th>
<th>Transformation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basta leaf paint</td>
<td>78</td>
<td>70</td>
<td>89.73%</td>
</tr>
<tr>
<td>Kanamycin/paromomycin Leaf bleach</td>
<td>34</td>
<td>32</td>
<td>94.12%</td>
</tr>
</tbody>
</table>

Using the kanamycin leaf bleach assay, over 94% of the primary regenerants showed no bleached spots on their leaves and were therefore kanamycin resistant (Table 4.4). Resistance to kanamycin is an indication of the presence of an active *nptII* gene. Four primary transformed lines (TV2 and TAA1) had bleached spots on their leaves indicating kanamycin sensitivity and implying absence of an active *nptII* gene.
Figure 4.4: Evaluation of expression of the *bar* and *nptII* gene in T₀ and T₁ transgenic plants using the Basta and leaf bleach assay. A, Effect of Basta on T₀ plants; B and C, response of wildtype plants to leaf bleach and Basta, respectively; D, Effect of Basta on T₁ progeny plants derived from the cotransformed line TD81; E and F, response of basta resistant T₁ plants derived from the cotransformed line TD81 to leaf bleach.
4.3.5 Independent segregation of the \textit{nptII} and \textit{bar} transgenes

A PPT resistance assay was conducted to identify cotransformed events segregating the \textit{bar} gene for advancement to segregation analysis. PPT-resistant progeny plants were clearly distinguishable from the sensitive ones 14 days after plating of T\textsubscript{1} seeds on medium containing 10 mg/l PPT. The PPT-resistant plants were green and grew vigorously while sensitive were small and completely bleached. Sensitivity to 10 mg/l PPT was not detected in T\textsubscript{1} progenies derived from 50% (3/6) of the cotransformed lines (Table 4.5).

\begin{table}
\centering
\begin{tabular}{lcccc}
\hline
T\textsubscript{0} event & Number of T\textsubscript{1} plants evaluated & Number of T\textsubscript{1} plants & Test ratio & $\chi^2$
\hline
TD52 & 49 & 21 & 28 & 1:1 & 0.7451 (NS)
TD48 & 50 & 50 & 0 & - & -
TD63 & 73 & 73 & 0 & - & -
TD 81 & 30 & 22 & 8 & 3:1 & 0.0444 (NS)
TD56 & 18 & 14 & 5 & - & -
TD46 & 35 & 35 & 0 & - & -
\hline
\end{tabular}
\caption{Segregation analysis on phosphinothricin (PPT) of T\textsubscript{1} seeds, collected from T\textsubscript{0} plants cotransformed with the \textit{bar} and \textit{nptII} genes}
\end{table}

NS, Not significant
Segregation of PPT resistance was observed in 50% (3/6) of the tested cotransformed lines (Table 4.5). Segregation of PPT resistance in line TD81 and TD81 was observed to occur in the expected Mendelian ratio of 3:1, while a ratio of 1:1 was observed for line TD52.

Seeds from cotransformed plants that produced PPT sensitive (marker-free) progeny plants were grown in soil for confirmation of out-segregation of the *bar* gene using the basta leaf paint assay. After screening with basta, all seedlings were evaluated using paromomycin/kanamycin leaf bleach assay to identify kanamycin resistant plants. Herbicide sensitive tobacco plants could be distinguished from the resistant ones three days after painting with 0.02% (v/v) Basta. Seven days after application, clear effects of the herbicide were observed (Figure 4.4). Application of the leaf kanamycin/paromomycin leaf bleach solution lead to damage effects on leaves of tobacco seedlings that were similar to those of Basta (Figure 4.4). Leaf damage resulting from basta application was observed on 9 out of 19 T1 plants derived from the cotransformed line TD52. Subsequent leaf bleach test revealed leaf damage in 10 of the 19 T1 progeny plants assayed. These phenotypic assay results confirmed that the *bar* and *nptII* transgenes were segregating in the T1 plants. Leaf damage developed on 4 out of 19 T1 seedlings derived from the cotransformed line TD81 while the rest were completely resistant to the damaging effect of Basta (Table 4.6). The same cotransformed line TD81 failed to segregate
kanamycin resistance to its progenies. This is because all of the T<sub>1</sub> progeny plants derived from cotransformed line TD81 contained a functional \textit{nptII} gene (Table 4.6).

\begin{table}[h]
\centering
\caption{Segregation of Basta and kanamycin resistance in T<sub>1</sub> plants derived from cotransformed plants}
\begin{tabular}{llllllll}
\hline
& \text{\textit{T<sub>0</sub> event}} & \text{T<sub>1</sub> plants assessed by Basta leaf painting} & \text{T<sub>1</sub> plants analysed by kanamycin leaf bleach assay} & \text{Number of T<sub>1</sub> plants} & \text{Frequency of generation of marker-free plants} \\
\hline
& \text{B<sup>R</sup>} & \text{B<sup>S</sup>} & \text{Km<sup>R</sup>} & \text{Km<sup>S</sup>} & \text{B<sup>R</sup>Km<sup>R</sup>} & \text{B<sup>R</sup>Km<sup>S</sup>} & \text{B<sup>S</sup>Km<sup>R</sup>} & \text{B<sup>S</sup>Km<sup>S</sup>} \\
TD56 & 15 & 8 & 15 & 8 & 15 & 0 & 0 & 8 & 0.00\% \\
TD81 & 15 & 4 & 19 & 0 & 15 & 0 & 4 & 0 & 21.05\% \\
TD52 & 8 & 11 & 9 & 10 & 6 & 4 & 3 & 6 & 15.79\% \\
\hline
\end{tabular}
\end{table}

\begin{itemize}
\item B<sup>R</sup>, Basta resistant; B<sup>S</sup>, Basta sensitive; Km<sup>R</sup>, kanamycin resistant; Km<sup>S</sup>, kanamycin sensitive.
\end{itemize}

\textbf{4.3.6 PCR confirmation of genetic separation of \textit{bar} and \textit{nptII} transgenes}

Removal of the \textit{bar} gene from progeny plants derived from cotransformants was confirmed by molecular analyses. T<sub>1</sub> progeny plants derived from the cotransformed line TD52 exhibited the 700 bp fragment of the \textit{nptII} gene in 9 plants. Of these, 6 revealed the presence of a 300 bp \textit{bar} gene fragment. Therefore in 3 of the progeny plants, the \textit{bar} gene was absent (Figure 4.5). PCR conducted on 19 progeny plants of TD81 revealed the presence of a 300 bp fragment of the
bar gene in 12 of the plants. However, the 700 bp band diagnostic of the nptII gene was present in all the 19 progenies assayed. PCR analyses also revealed that all T1 progeny plants derived from line TD56 were either negative or positive for both the bar and nptII T-DNA insertions (Figure 4.6).

**Figure 4.5:** Multiplex PCR analyses for the presence of bar and nptII in 19 T1 progeny plants derived from cotransformed line TD52. Lane +, genomic DNA from TD52 line used as positive control; Lane -, non-template control; Lane M, HindIII/lambda DNA; Lane 1 to 18, T1 plants derived from the cotransformed line TD52.
Figure 4.6: PCR analyses of T1 progeny plants derived from cotransformed line TD56 for the presence of the bar and nptII T-DNA insertions. Lane TD56, gDNA from the cotransformed line TD56; Lane NTC, non-template control; Lane WT, gDNA from a non-transgenic plant; Lane 56/1 to 56/15, gDNA of different T1 progeny plants derived from the cotransformed line TD56.
4.4 DISCUSSION

A double right border (DRB) binary vector pMarkfree5.0 having the structure LB-
\textit{nptII::gus}-RB2-\textit{bar}-RB1 was developed for a quick validation of the DRB vector
system in model plants. This could be achieved through assessment of segregation
of the \textit{nptII::gus} and \textit{bar} T-DNA regions by gus staining or basta leaf painting. The
\textit{gus} and \textit{nptII} genes are under the control of CaMV35S promoter and nos
terminator. The \textit{bar} expression unit was composed of a long CaMV35S promoter, the \textit{bar} gene and the CaMV35S terminator.

The first indication of a functional cotransformation system is its ability to produce
the expected coinsertions. In tobacco, cotransformation frequencies (CF) have
varied depending on the cotransformation approach. For example CF has ranged
from 59.25 (Hong-Yan \textit{et al.}, 2003) to 50\% (Komari \textit{et al.}, 1996) for the two T-
DNA system. With mixtures of two strains of \textit{A. tumefaciens}, CF of 20.0-47.7\%
(Hong-Yan \textit{et al.}, 2003) and 54\% (Park \textit{et al.}, 2004) have been reported. Daley \textit{et al.}
(1998) obtained a CF of 52\% using two vectors in one strain of \textit{A. tumefaciens}.
Evidently, about half of the primary transformants will possess multiple
transgenes. Results of this study fall within this expectation.
Although *nptII/gus* transgene was detected in 60 primary transformants by PCR, in only 47.95% of the T₀ plants was the *gus* gene activity detectable. This phenomenon whereby a big proportion of transgenic plants possessing the *gus* transgene don’t show any *gus* activity by histochemical staining has been reported elsewhere (Parkhi *et al.*, 2005). Truncation of *Agrobacterium* T-DNA region during integration in the plant genome has been used to explain this observation. In this study, the *gus* gene expression cassette was positioned closest to the T-DNA LB of pMarkfree5.0. This may have exposed it to truncation which occurs mostly at the LB (Rossi *et al.*, 1996).

Two primary transformed lines though surviving high kanamycin selection were devoid of an active *nptII* gene. These two lines were confirmed negative for the presence of the *nptII* gene by the multiplex PCR analysis. These plants were possibly escapes, transgenic-chimeras or could not express enough NPTII protein in their leaves to confer resistance. On the basis of the leaf bleach assay the frequency of transformation of tobacco with the *nptII* gene using the DRB pMarkfree5.0 vector was found to be 94.12%. Similar frequencies have been reported for transformation of tobacco using vectors that contain only one T-DNA region. For example a TF of 85% was reported for vector pGA482 (Komari *et al.*, 1996). This therefore, indicated that the developed DRB vector does not compromise the ability to deliver transgenes into the plant genome. The *bar* gene
failed to segregate in the progenies because it was integrated in multiple copies in the genome of the parent plants. A PPT resistance segregation ratio of 3:1 was observed in line TD81 and TD52, implying that these cotransformants were hemizygous transgenic lines harboring a single copy of the bar transgene.

The observed 1:1 ratio of segregation of PPT resistance for line TD52 may be attributed to the failure of passing the bar transgene to the next generation due to pollen lethality or due to a mutation affecting the female gametes (Limanton-Grevet and Jullien, 2001). Consequently, transmission of the bar transgene will occur either through the male or female gamete only. Progenies that died in PPT selection were devoid of the bar gene, indicating that the bar gene had been out-segregated in those progeny plants.

From the present study, the frequency of removal of the bar gene from transgenic T₁ plants was between 15.79 and 21.05%. Similarly, Hong-Yan et al. (2003), using a DRB system, observed that 19.5% of T₁ plants derived from tobacco plants cotransformed with nptII and bar transgenes were free of the nptII gene. Therefore recovery of SMF T₁ plants from T₀ plants cotransformed using pmarkfree5.0 is highly efficient.
Three T₁ plants derived from a segregating T₀ line TD52 were confirmed to possess the GOI but not the selectable marker-gene (bar). This indicates that the bar gene was not inherited in these three progenies and were therefore marker-free. The presence of the nptII gene in all the T₁ plants derived from line TD81 suggests that it did not segregate. This is because of existence of more than once copy of the nptII gene in the T₀ event TD81. However, the bar gene segregated in the T₁ plants, indicating its existence as a single copy in the parental plant TD81.

All T₁ plants derived from the cotransformed line TD56 were either negative or positive for both the bar and nptII transgenes. This indicates absence of genetic separation between the bar and nptII T-DNAs regions. This means that the integration of the two T-DNA regions was in the same genomic location in the cotransformants making the two inserts to segregate together. Therefore no marker-free progeny plants were produced by the parental line TD56.

Successful genetic separation of two T-DNA is dependent on various factors. One of the most important factor seems to be the strain of A. tumefaciens used to deliver the multiple T-DNAs. Nopaline-derived A. tumefaciens strains may favour insertion of multiple T-DNA in linked genomic loci. Initially, when two distinct T-DNAs were separately inserted in two nopaline-derived Agrobacterium, marker-free Arabidopsis thaliana or Brassica napus were produced at a very low
frequency (De Block and Debrouwer, 1991; Poirier et al., 2000; Radchuk et al., 2005). Recently, the frequency of generation of maker-free plants increased when the same strain (EHA101) or its derivative (EHA105) was used in combination with a two T-DNA vector (Xing et al., 2000; Breitler et al., 2004). This implies that differences in the plant species and strain/vector used could alter genetic linkage relationship.

Currently, cotransformation systems are mainly based on octopine-derived *A. tumefaciens* strains possibly because they favour unlinked transfer of independent T-DNAs (McCormac et al., 2001). Among the octopine-derived *A. tumefaciens* strains, LBA4404 is the most popular. Using this strain high efficiencies of marker-free plants have been obtained from different species including barley (Matthews et al., 2001; Kapusi et al., 2013), maize (Miller et al., 2002; Ishida et al., 2004), rice (Komari et al., 1996) and tobacco (Komari et al., 1996; Daley et al., 1998; McCormac et al., 2001; Hong-Yan et al., 2003; Park et al., 2004). Apart from the *A. tumefaciens* strain used, the type of the T-DNA border regions on the cotransformation vector may affect unlinked transfer of multiple T-DNAs.
CHAPTER FIVE

USE OF PHOSPHOMANOSE ISOMERASE TO RECOVER AMIRNA1-PARP1 TRANSGENIC TROPICAL MAIZE PLANTS

5.1 INTRODUCTION

For the genetic engineering systems, the SMG is of immense importance in the identification of transgenic tissues. However, the continued presence of antibiotic and herbicide resistance genes in the final transgenic plant may cause serious environmental and biosafety concerns. The use of marker systems with proven environmental and human safety status has been proposed as an alternative to these controversial marker systems. The phosphomannose isomerase (pmi) is a plant SMG that has undergone careful risk assessment (Privalle et al., 1998) and has emerged as a useful alternative to antibiotic and herbicide resistance genes. The pmi gene is a useful SMG that helps in identification of plants containing the transgene of interest (Negrotto et al., 2000).

Different genetic engineering approaches to improving drought tolerance in plants have been explored. Maintenance of energy homeostasis during severe stresses is a new approach that has been successfully demonstrated in the model plant Arabidopsis thaliana and Brassica napus (De Block et al., 2004; Schulz et al., 2012). This was through reduction of activity of PARP. Plants with reduced PARP
activity were resistant to multiple stresses including high light, heat and water stress (De Block et al., 2004; Schulz et al., 2012). Silencing of the PARP gene was previously achieved using hpRNAi (De Block et al., 2004). The use of amiRNA to cause specific and effective gene silencing in different crops including rice (Warthmann et al., 2008; Chen et al., 2012) has also been reported.

In this study, the identification of maize genotypes with high embryogenic callus induction and plant regeneration potential is reported. Transformation of maize immature embryos, derived from different genotypes, by co-cultivation with A. tumefaciens is also reported. Finally, the use of the PMI/mannose system to recover plants stably transformed with the amiRNA-PARP1 construct is demonstrated.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

Seeds of thirteen tropical maize genotypes (Table 5.1) obtained from International Maize and Wheat Improvement Center (CIMMYT) were planted outdoors at the research field of Kenyatta University Plant Transformation Laboratory (PTL). Maize inbred line IL3, CML216 and A188 were included in the study as control
genotypes. Growing plants were maintained by water and fertilizer application. Pesticides were also applied to prevent destruction by plants insects and pests.

**Table 5.1:** Pedigree of different maize genotypes used in this study

<table>
<thead>
<tr>
<th>Genotype code</th>
<th>Pedigree</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL03B 6755-9</td>
<td>1760A G1 Am x Comp.-B-3-3-B-B-B-B</td>
</tr>
<tr>
<td>TL03B 6753A-29</td>
<td>CML 329/ MBR C3 Am F103-1-1-1-B</td>
</tr>
<tr>
<td>TL03B 6753A-21</td>
<td>CML 329/MBR C3 Am F48-2-1-1-B</td>
</tr>
<tr>
<td>TL03B 6753A-30</td>
<td>CML 329/MBR C3 Am F103-1-1-2-B</td>
</tr>
<tr>
<td>TL03B 6757-99</td>
<td>MBR C5 Bc F60-2-1-2-B-B-B x CML 384-B-1-1-B</td>
</tr>
<tr>
<td>TL03B 6757-5</td>
<td>[[TuxpSeq]C1F2/P49-SR]F2-45-7-5-1-BBBB x MBR C5 Bc F4-4-1-2-1-B-B-B-B-1-1-B</td>
</tr>
<tr>
<td>TL03B 6757-68</td>
<td>MBR-ET(W) C1 F139-2-1-B-2-B-B-B-B-B-B x MBR C5 Bc F13-3-3-2-1-B-B-B-B-1-2-B</td>
</tr>
<tr>
<td>TL03B 6754A-2</td>
<td>CML 311/ MBR C3 Bc F3-1-1-2-B</td>
</tr>
<tr>
<td>TL03B 6754A-37</td>
<td>CML 311/ MBR C3 Bc F63-1-2-1-B</td>
</tr>
<tr>
<td>TL03B 6752B-23</td>
<td>MBR C6 Bc F325-1-B-#-1-2-B-B-B</td>
</tr>
<tr>
<td>TL03B 6757-7</td>
<td>MBR C5 Bc F114-3-1-1-B-B-B x P43C9-1-1-1-1-1-BBBB-B-1-1-1-B</td>
</tr>
<tr>
<td>TL03B 6754A-20</td>
<td>CML 311/MBR C3 Bc F35-2-2-1-B</td>
</tr>
<tr>
<td>AF03B 5751B-61-B</td>
<td>MIRTC5 Bco F19-1-1-2-1-1-1-B</td>
</tr>
</tbody>
</table>

5.2.2 **Callus induction from immature embryo explants**

Ears were harvested 14 to 15 days after pollination, dehusked and sterilized before excision of embryos. Sterilization was achieved by soaking the dehusked ears in 70% (v/v) ethanol for 30 seconds. The surface sterilized ears were transferred into a clean laminar flow hood. Ethanol was removed and the ears soaked in 2.5% (v/v) sodium hypochlorite for 20 minutes. Sterile ears were rinsed three times with sdH2O. Immature embryos (1.5 to 2 mm in length) were excised onto callus induction medium (CIM) using a sterile spatula following removal of the top half
of the kernels. The CIM for induction of calli from the embryos was prepared by adding 2 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D), 0.7 g/l proline and 1.6 mg/l silver nitrate to the LSDc medium described by Negrotto et al. (2000). However, dicamba was omitted from the LSDc media. The plated embryos were kept in the dark at 28 °C. To avoid depletion of nutrients and auxin, embryos were transferred to fresh CIM every 14 days. Four weeks after embryo culture, the type of callus induced was determined. Callus induction frequency was determined by comparing the number of callusing embryos per 100 cultured embryos. Frequency of embryogenic callus induction was also determined as the percentage of the number of embryogenic callus per the total number of callus produced.

5.2.3 Regeneration of shoots from embryogenic callus of TL03B 6757-68 and TL03B 6754A-20

To determine regenerability, callus pieces (about 2 mm in diameter) of the most embryogenic genotypes were cultured on shoot induction medium (SIM) and kept at a photoperiod of 16 hour light, 8 hour dark. SIM comprised MS medium (Murashige and Skoog, 1962) and 30 g/l sucrose. The number of shoots measuring at least 2 cm in height were recorded for every callus 14 days after culture. The inbred line IL3 was used as a control.
5.2.4 Transformation of immature embryos of TL03B 6757-68, TL03B 6754A-20 and CML 216 genotypes

The two inbred lines identified as being highly regenerable (TL03B 6757-68 and TL03B 6754A-20) were used for amiRNA-PARP1 transgene insertion using *A. tumefaciens*. Their response to *A. tumefaciens*-mediated genetic transformation was compared with that of inbred line CML 216. Transformation of maize was achieved using the *A. tumefaciens* strains LBA4404 harboring pNOV-UbiamiRNA1. PNOV-UbiamiRNA1 vector was constructed as outlined in section 3.2.15. PNOV-UbiamiRNA1 is a binary vector containing one T-DNA region comprising a *pmi* gene under the control of CMPS promoter and nos terminator. Within the T-DNA region is also the amiRNA-PARPI under the control of maize ubiquitine promoter and the nos terminator. The vector was introduced into LBA4404 (pAL4404, pSB1) by the freeze thaw method (Raviraja and Kandikere, 2007).

Maize immature embryos (1.5 to 2 mm) were excised aseptically as described in section 5.2.2 and transformed using a modified protocol of Negrotto *et al.* (2000). Modifications of the protocol were at the inoculation, co-cultivation, and resting steps. During inoculation, embryos were collected in a 2 ml eppendorf tube containing 1.5 ml of the reported LS-if + 100 µM acetosyringone and vortexed for 5 seconds. The infection medium was aspirated off after vortexing. Two loopfuls
of *A. tumefaciens* (instead of the use of 10 small colonies reported in Negrotto *et al.* (2000) were placed in a 2 ml tube containing 1 ml infection medium, resuspended by vortexing briefly and added directly into the tube containing embryos. The tube was vortexed for 30 seconds and allowed to stand at room temperature for 5 min for infection to occur. Infected embryos were co-cultivated as described by Negrotto *et al.* (2000) but incubated at 20 °C (instead 28 °C as reported by Negrotto *et al.* (2000) for at most 4 days (instead of the reported 10 days in Negrotto *et al.* (2000). A resting step was introduced, whereby cocultured embryos were transferred to LSDc medium lacking acetylsyringone but having timentin to suppress the growth of *A. tumefaciens*. The embryos were cultured on this medium at 28 °C in the dark for 7 to 10 days for completion of embryogenic callus development. Selection of transformed tissues was achieved essentially as described by Negrotto *et al.* (2000).

### 5.2.5 Regeneration of plants from putative amiRNA-PARP1 transgenic tissues

Callus surviving mannose selection was transferred to SIM and plantlet regeneration performed as described in section 5.2.3. Plantlets with an established root system were transferred to sterile peat moss and hardened as described in Ombori *et al.* (2013). Hardened regenerants were transferred into soil and maintained until seed establishment.
5.2.6 DNA extraction and analysis by PCR

Motars and pestles used for grinding of plant material were cleaned by soaking in 0.25 M HCl for at least 20 minutes. The acid-washed motar and pestles were rinsed by wiping using a piece of cotton wool soaked in sterile water. A fresh piece of maize leaf (100 to 200 mg) was harvested from plants growing in the glasshouse and placed in the cleaned motar. The piece of leaf was ground to a very fine paste using 100 mg sterile acid-washed sand wetted in 200 µl of 0.1 M Tris-HCl (pH 8.0). The acid-washed sand was prepared by soaking cleaned sand in 0.25 M HCl for 30 minutes followed by rinsing five times in water before sterilizing by autoclaving. To the fine paste, 3 ml of urea DNA extraction buffer was added and ground gently to ensure thorough mixing. Three millilitres of the mixture was transferred into a 10 ml tube and incubated at 65 ºC for 2 hours with occasional mixing. The mixture was extracted by adding 3 ml of Phenol:chloroform:isoamylalcohol (prepared as outlined in appendix 1) and mixed thoroughly. The mixture was centrifuged at 6000 rpm for 20 minutes to separate the phases. The top aqueous phase (2.5 ml) was removed and transferred to a clean 10 ml tube. RNaseA (20 µg/ml) was added to the mixture and incubated at 37 ºC for 30 minutes to digest RNA. RNaseA treated mixture was extracted with 2.5 ml phenol:chloroform:isoamylalcohol mixture (25:24:1) as indicated above. The mixture was centrifuged at 6000 rpm for 20 minutes before transferring 2 ml of the aqueous layer into a clean 10 ml tube. A final extraction with an equal volume of chloroform was performed and phases separated by centrifuging at 6000 rpm for
20 minutes. The upper phase (1.5 ml) was transferred into a clean tube and DNA was precipitated by adding 1/10 volume of 0.3 M sodium acetate (pH 8.0) and 6/10 volume of isopropanol. DNA was precipitated overnight at -20 °C. Precipitated DNA was collected by centrifuging at 6000 for 1 hour. The pellet was washed by adding 3 ml of 70% (v/v) ethanol and centrifuging for 20 minutes. Ethanol was discarded and the tube centrifuged again for 2 minutes to collect the remaining ethanol to the bottom of the tube. This was aspirated off with a P1000 pipetter. DNA was completely solubilised in 0.8 mM NaOH and stored at in a -20 °C freezer.

The DNA was assessed by agarose gel electrophoresis to determine its quantity. However, to determine its suitability as a PCR template, the extracted DNA was used in PCR to detect the maize α-Tubulin (TUA) gene. A 50 µl reaction to amplify the TUA gene comprised 1× thermopol reaction buffer, 1 mM dNTP mix, 0.5 µM of reverse and forward primer, 2.5 mM MgSO4, 100 ng of template DNA and 1U of Taq DNA polymerase. The primers Zmaystubfwd (5’ ggatccacttcagcttctg 3’) and Zmaystubrev (5’ gggaaccacctaccacgggtacag 3’) were used in the PCR. PCR conditions used were initial denaturation at 94 ºC for 10 min followed by 35 cycles of denaturation at 94 ºC for 3 min, annealing at 63 ºC for 1 min, extension at 72 ºC for 1 min and a final extension at 72 ºC for 10
min. Amplification products were resolved by electrophoresis in a 1.5% agarose gel.

High quality DNA was used to determine integration of the \textit{pmi} gene or amiRNA1-PARP1 gene in the genome of putative transformants by PCR. PCR was performed as described for \textit{TUA} gene but using the PMIF (5’ctcgctgcagaccttagtg3’) and PMIR (5’ttgtaaacacgctaaacg3’) and amiRNA1Fwd2 (5’caaacacagctaggacgcatattac3’) amiRNA1-rev2 (5’tacaaaagagagacgcttgcttgactac3’) for amplification of the \textit{pmi} gene and amiRNA-PARP1 construct, respectively. The annealing temperature was 55 and 63 °C for amplification of the \textit{pmi} and amiRNA1-PARP1 transgene, respectively.

\textbf{5.2.7 Southern blot hybridization analysis of transgenic plants}

Southern hybridization of plant genomic DNA was performed based on the protocol described by Southern (Southern, 1975) but using the biotin labelling and detection kits (New England Bio-labs Inc., MA, USA). Zeta-Probe blotting membranes (BIO-RAD laboratories, 2000 Alfred Nobel Drive, Hercules, CA 94547) were used for DNA transfer.
5.2.7.1 Restriction enzyme digestion of DNA

Genomic DNA was extracted from transgenic and non-transformed plants as described in section 5.2.8. The DNA was measured using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific Inc., Delaware, USA) to determine its concentration. Plant genomic DNA (30 µg) was digested with HindIII restriction enzyme. The digestion mixture is outlined in Table 5.2.

Table 5.2: Composition of restriction enzyme digestion mixture

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genomic DNA (100 ng/ µl)</td>
<td>30</td>
</tr>
<tr>
<td>sdH₂O</td>
<td>77</td>
</tr>
<tr>
<td>Buffer (10×)</td>
<td>12</td>
</tr>
<tr>
<td>Enzyme (10 U/ µl)</td>
<td>1</td>
</tr>
</tbody>
</table>

The digestion mixture was centrifuged briefly and incubated at 37 ºC for 12 to 16 hours. DNA in the mixture was concentrated using ethanol precipitation method. This involved adding 1/10 volume of 3 M sodium acetate, pH 5.2 and two volumes of absolute (99.5%) ethanol. The mixture was incubated at -20 ºC for 12 to 24 hours. Precipitated DNA was collected by centrifuging at 12000 rpm for 30 min. DNA pellet was washed by adding 70% (v/v) ethanol and centrifuging at 12000 rpm for 5 minutes. The DNA was dissolved in 25 µl of sdH₂O.
5.2.7.2 End labeling of DNA ladders with biotin

The 1 Kb DNA ladder and the HindIII/lambda DNA were used to estimate the size of fractionated genomic DNA. To achieve this, the ladders were first labelled with biotin using DNA polymerase large (klenow) fragment (New England Biolabs) and biotinylated dNTPs. The composition of the labeling mixture is shown in Table 5.3.

Table 5.3: Composition of mixture for biotin labelling of a ladder

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA ladder</td>
<td>1 μg</td>
</tr>
<tr>
<td>10× NEB Buffer 2</td>
<td>5 μl</td>
</tr>
<tr>
<td>Biotinylated dNTP mixture (4 mM)</td>
<td>1.65 μl</td>
</tr>
<tr>
<td>Klenow Fragment (1 U/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Water</td>
<td>To 50 μl</td>
</tr>
</tbody>
</table>

The mixture was incubated at 37 °C for at least one hour. The reaction was stopped by adding 10 mM EDTA and subsequently the DNA polymerase large (klenow) fragment inactivated by heating at 75 °C for 20 minutes. DNA was optionally purified with the PCR purification kit.

5.2.7.3 DNA gel electrophoresis and blotting

Digested DNA (20 to 30 μl) was mixed with 5 μl of 10× loading dye and loaded carefully into a 1.5 mm wide sample loading well. A labelled DNA ladder was
also loaded into a sample well to help in sizing of genomic fragments as well as monitor the success of DNA transfer process.

Blotting of DNA fragments from the gel onto the Zeta-probe blotting membrane was achieved in 20× SCC buffer solution (3 M NaCl, 0.3 M sodium citrate, pH 7.0) using the upward capillary transfer method described by Sambrook et al. (1989). After electrophoresis, the gel was soaked in a 0.25 M HCL solution for 15 minutes or until the bromophenol blue completely turned yellow. As depurination of DNA progressed, a small glass dish was placed in a shallow but wide glass dish to serve as a platform for preparation of the transfer setup. A long whatman paper was placed on the platform to act as a wick. Three whatman filter papers of the same size as the gel were soaked in 20× SCC and placed on the wick. Bubbles were removed.

After acid treatment, the gel was rinsed three times by soaking and shaking gently in sdH₂O. DNA in the gel was denatured by soaking the gel in a solution of 1.5 M NaCl and 0.5 M NaOH for upto 30 minutes. The gel was soaked for 30 minutes in 500 ml of 1 M Tris-HCl, 1.5 M NaCl, pH 7.5 to neutralize the gel. The gel was trimmed to ensure it was smaller than the membrane and placed on top of the filter papers and bubbles removed by rolling a boiling tube on top of the gel. The set up was covered with a plastic cling film to ensure its stability. However, a window
was cut out to expose the DNA section of the gel. A filter paper that was slightly bigger than the gel was wetted in 20× SCC and placed over the gel to cover it fully. After removing bubbles, a plastic cling film was placed over the setup and a window cut open as described previously. Five whatman filter papers slightly smaller than the membrane were placed on top of this pile. The bottom-most filter paper had been wetted in 20× SCC. A 3 cm thick blotting paper towels, of the same size as the whatman filter papers were prepared, stacked together and placed on top of the pile. The set up was allowed to stand for 2 to 4 hour to complete the DNA transfer process.

After DNA transfer, filter papers were removed carefully and the gel peeled off the membrane. The membrane was washed by soaking in 2× SCC before air drying. The DNA was crosslinked to the membrane by baking at 80 ºC for 2 hours. The membrane was kept at room temperature between two whatman filter papers awaiting hybridization.

5.2.7.4 Preparation of biotin-labelled probe by PCR

Probe labelled with biotin was prepared using the PCR procedure described in section 3.2.13.1. The PCR reaction mixture was prepared as outlined in Table 5.4.
Table 5.4: Composition of mixture for biotin labelling of a DNA probe

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10× PCR buffer</td>
<td>5</td>
</tr>
<tr>
<td>Biotinylated dNTP mixture</td>
<td>2.5</td>
</tr>
<tr>
<td>Primer 1</td>
<td>5</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>10</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>1</td>
</tr>
<tr>
<td>sdH₂O</td>
<td>To 50</td>
</tr>
</tbody>
</table>

Biotin labelled PCR fragments were purified using the PCR purification kit (Qiagen, Maryland, USA) and their quantity estimated.

5.2.7.5 Preparation of biotin-labelled probe by random primed labelling

5.2.7.5.1 Preparation of template DNA for random primed labelling

A fragment of the amiRNA-PARP1 construct was generated from pNOV2819-UbiamiRNA1 plasmid DNA by PCR using amiRNA-PARP1 specific primers (Table 5.5).
### Table 5.5: Primers used for preparation of PCR DNA fragments for biotin labelling

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Expected size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>amiRNA1-fwd2</td>
<td>caaacacagctcggacgcatattac</td>
<td>272</td>
</tr>
<tr>
<td>amiRNA1-rev2</td>
<td>tacaaaagagagaccgccgaact</td>
<td></td>
</tr>
<tr>
<td>amiRNA3-fwd3</td>
<td>gctcggacgcatattacacatgttc</td>
<td>269</td>
</tr>
<tr>
<td>amiRNA3-rev3</td>
<td>ttggaataaaagagagaggtgccg</td>
<td></td>
</tr>
<tr>
<td>PMIF</td>
<td>ctcgctgcactaccttagtgc</td>
<td>198</td>
</tr>
<tr>
<td>PMIR</td>
<td>ttgtaaacacgcgctaaacg</td>
<td></td>
</tr>
</tbody>
</table>

The PCR products were purified using the column purification procedure described in section 3.2.5.2. To determine the size and quality of the PCR products, between 1 to 2 µl of the purified PCR product was analysed on a 1.5% agarose gel.

### 5.2.7.5.2 Random primed biotin labelling of a DNA fragment

One to two microliters of PCR product was diluted to 17 µl with nuclease free water. The diluted DNA was boiled at 100 °C for 5 minutes before quickly chilling on ice for 5 minutes. To the denatured DNA template, 5 µl of labelling mixture (containing biotin labelled random octamers), 2.5 µl of dNTP mix (containing biotinylated dNTPs) and 0.5 µl of Klenow Fragment (3’→5’exo–). The mixture was incubated at 37 °C for 21 hours. The biotin labeled probe was purified using
PCR purification kit (New England Bio-Labs Inc., MA, USA) and eluted in 50 µl of buffer EB before use.

**5.2.7.6 Hybridization**

Biotinylated probe was hybridized to blotted genomic DNA as described in the user manual of Neblot phototope kit. Briefly, a membrane was first made wet by soaking in 5× SCC, and transferred to a hybridization tube. To the tube, 0.05 ml of pre-hybridization buffer per cm² of membrane was added and incubated at 68 ºC for 1 hour in the hybridization oven. To the tube, a denatured probe was added. The probe was denatured by first boiling it in 100 µl of 0.2 M NaOH at 100 ºC for 5 minutes followed by rapidly cooling in an ice bath. The probe was allowed to hybridize to the target DNA for 4 to 12 hours depending on amount of probe added.

After hybridization, unbound probe was removed by washing the membrane twice with 100 ml of a mixture of 2× SCC and 0.1% SDS at room temperature. The membrane underwent a second stringent washing in 100 ml of a mixture of 0.1× SCC and 0.1% SDS at 68 ºC for 15 minutes. This washing step was repeated. All hybridizations were performed in a hybridization tube in the hybridization oven.
5.2.7.7 Chemiluminiscent detection

The genomic DNA/probe hybrid was detected using the Phototope star detection kit (New England Bio-Labs Inc., MA, USA). In this procedure, streptavidin and biotinylated alkaline are successively bound to biotinylated probe. This is followed by incubation with a chemiluminiscent substrate and the emitted light captured on an X-ray film.

To detect DNA, the membrane was placed in a hybridization tube and 0.05 ml of blocking solution per cm$^2$ of membrane added and incubated for 5 minutes with gentle shaking. The membrane was reacted with 7.5 μl streptavidin in 7.5 ml blocking solution for 5 minutes with gentle shaking. Unbound streptavidin was removed by washing the membrane twice with 0.5 ml of wash solution I per cm$^2$ of membrane. The membrane was reacted with 7.5 μl biotynylated alkaline phosphatase (BAP) in 7.5 ml blocking solution and incubated for 5 minutes with gentle rocking. Excess BAP was removed by washing the membrane with 75 ml of bloking solution for 5 minutes. Blocking solution was replaced with 75 ml wash solution II and the membrane washed twice by incubating for 5 minutes at room temperature with gentle rocking.
The membrane was transferred to a plastic bag and 3.7 ml CDP star added. After 5 minutes of incubation, excess CDP star was drained off and the membrane placed in a photo cassette. A film was placed on the membrane and exposed for 1 to 5 minutes. The film was processed and documented with a Sony cybershot digital camera (14 MP).

5.2.8 Data analysis

Differences in transformation frequency among genotypes were subjected to ANOVA. Means were separated using the LSD at a confidence level of 0.05%. All data analysis was performed using Statview statistical program (SAS, 1998). All data were square-root transformed before analysis.

5.3 RESULTS

5.3.1 Embryogenic and regeneration response of different tropical maize inbred lines

Most of the genotypes tested, including inbred TL03B 6754A-2 (14), induced a callus type that was soft and watery and were therefore categorized as non-embryogenic (Figure 5.1). However, maize inbred lines A188, TL03B 6753A-21 (5), TL03B 6757-5 (12), TL03B 6757-68 (13), TL03B 6754A-20 (18) and CML216 formed numerous ridges on their scutellum surfaces after 7 to 10 days of
culture of their immature embryos on CIM (Figure 5.1). These embryos eventually formed clearly visible globular white, dry and compact embryos 14 days after culture on CIM (Figure 5.1) and are therefore embryogenic genotypes. Among the test inbred lines, TL03B 6757-68 and TL03B 6754A-20 had the highest capacity to induce embryogenic callus (Table 5.6). The highly embryogenic TL03B6754A-20 and TL03B6757-68 and IL3 inbred lines were cultured on SIM to investigate if they were capable of regenerating plants in vitro. Two days after transfer onto SIM, callus of the three genotypes begun to green. Conspicuous shoots were observed 7 to 10 days after culture on shoot induction medium (SIM) (Figure 5.2). The regeneration frequency was highest for the control (IL3) which averaged more than 5 shoots per callus, while TL03B 6754A-20 produced the least number of shoots per callus (Figure 5.2).
Table 5.6: Response of different tropical maize genotypes to embryogenic callus induction

<table>
<thead>
<tr>
<th>Experimental code</th>
<th>Genotype</th>
<th>Number of embryos</th>
<th>Callus induction (%)</th>
<th>Embryogenic callus induction (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL12</td>
<td>TL03B 6757-5</td>
<td>101</td>
<td>96.69</td>
<td>53.92 ± 0.84</td>
</tr>
<tr>
<td>IL15</td>
<td>TL03B 6754A-37</td>
<td>272</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>IL14</td>
<td>TL03B 6754A-2</td>
<td>180</td>
<td>92.08</td>
<td>0</td>
</tr>
<tr>
<td>IL11</td>
<td>TL03B 6757-99</td>
<td>66</td>
<td>100</td>
<td>5.95 ± 2.54</td>
</tr>
<tr>
<td>IL5</td>
<td>TL03B 6753A-21</td>
<td>117</td>
<td>100</td>
<td>17.14 ± 10.51</td>
</tr>
<tr>
<td>188</td>
<td>A188*</td>
<td>315</td>
<td>99.31</td>
<td>68.34 ± 9.92</td>
</tr>
<tr>
<td>IL17</td>
<td>TL03B 6757-7</td>
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<td>99.51</td>
<td>0</td>
</tr>
<tr>
<td>IL7</td>
<td>TL03B 6753A-30</td>
<td>35</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>IL18</td>
<td>TL03B 6754A-20</td>
<td>79</td>
<td>44.44</td>
<td>75.75 ± 7.20</td>
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<td>IL3.0</td>
<td>TL03B 6753A-29</td>
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<td>216</td>
<td>CML 216*</td>
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<td>100</td>
<td>0</td>
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<td>TL03B 6752B-23</td>
<td>107</td>
<td>84.72</td>
<td>0</td>
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<tr>
<td>IL13</td>
<td>TL03B 6757-68</td>
<td>92</td>
<td>100</td>
<td>58.23 ± 0.62</td>
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</tbody>
</table>

*A188 and CML216 were included as check inbred lines. *<sup>a</sup> Values are the means of three replicates ± Standard error.
<table>
<thead>
<tr>
<th>Code</th>
<th>Genotype</th>
<th>7 days on CIM</th>
<th>14 days on CIM</th>
</tr>
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<td>A188</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
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<tr>
<td>GT14</td>
<td>TL03B 6754A-2</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 5.1:** Callus types formed from immature embryos of different maize inbreds lines. All the shown genotypes produced embryogenic callus except TL03B 6754A-2.
Figure 5.2: Regeneration of plants from different genotypes via somatic embryogenesis. (A) Development of shoots from embryogenic calli derived from inbred lines TL03B6754A-20 and TL03B6757-68 14 days after culture. (B) Regeneration response of different tropical maize genotypes.
Purple pigmentation characterized stems of shoots regenerated from TL03B6757-68 (Figure 5.2). However, shoots from TL03B 6754A-20 (18) showed an overall green coloration (Figure 5.2)

5.3.2 Transformation response for the TL03B6754A-20 and TL03B6757-68 genotypes

Putative amiRNA-PARP transgenic tissues were recovered on mannose-containing media following transformation of TL03B6754A-20, TL03B6757-68 and CML216 inbred lines with pNOV2819-amiRNA1 vector. Transgenic TL03B6757-68 callus grew well on mannose and were clearly distinguishable from non-transgenic ones after 14 to 21 days on selection (Figure 5.3). Fourteen days post selection on mannose, non-transgenic tissues were much smaller in size compared to transgenic ones. All non-transgenic tissues turned brown and died within 21 days of culture on mannose-containing media (Figure 5.3). Continued culture, beyond 21 days, caused majority of the surviving callus to regenerate shoots and roots on selection medium (Figure 5.3). Majority of the shoots produced had intense purple pigmentation on their stems.

An average of 34.16% of all callus tissues derived from genotype TL03B6757-68 (GT13) survived mannose selection. This represented a higher transformation
frequency (TF) than that of the control genotype CML216 (Figure 5.4). However, this difference was not statistically significant (Appendix 2).

**Figure 5.3**: Transformation of TL03B6757-68 and regeneration of putative transgenic plants via somatic embryogenesis. (A) amiRNA1-PARP1 transgenic callus tissues cultured 0.5% mannose-containing medium for 21 days. (B) Shooting and rooting on amiRNA1-PARP1 transgenic callus tissues cultured on 0.5% mannose-containing medium for 25 days. (C) Plantlet regeneration from putative transgenic callus tissues. (D) Mature putative T₀ plants showing seed establishment on tassels.
Transgenic callus tissues derived from TL03B6754A-20 could be identified on mannose selection as early as 6 days after transformation. This is because the transgenic tissues grew larger than the non-transgenic ones. Somatic embryos at different stages of development were clearly visible on majority of the mannose resistant calli (Figure 5.5). Necrosis was observed on some of the callus tissues under selection (Figure 5.5). Majority of the tissues failed to necrose and die on mannose selection. However, mannose suppressed their growth completely such that the tissues were not different in size before and after selection (Figure 5.5). These tissues were considered non-transformed and were therefore discarded. The average rate of recovery of transgenic tissues using mannose as selection agent was highest for TL03B6754A-20 (Figure 5.4). However, the mean transformation frequency (TF) was not significantly higher than that of CML216 or TL03B6757-68 (Appendix 2).

Figure 5.4: Transformation frequency for different genotypes transformed with pNOV2819-UbiamRNA1. Values are means of three replicates and horizontal bars show standard errors.
Majority of callus tissues obtained from CML216 became brown and completely dead after 21 days of culture on mannose. However, a few tissues remained whitish and continued to increase in size despite exposure to mannose (Figure 5.6). These surviving tissues were the likely amiRNA1-PARP1 transformants. The rate of recovery of these transgenic CML216 tissues on mannose was between 10.53 to 72.92% with an average of 32.16% (Figure 5.4).

5.3.3 Regeneration of plants from transgenic tissues

Callus derived from TL03B6757-68 (GT13) shoted and rooted while on selection medium (Figure 5.3). These shooting and rooting callus were transferred to SIM directly where the plantlets continued establishing (Figure 5.3). However, putative transgenic callus tissues of TL03B6754A-20 and CML216 underwent selection without shooting or rooting. Callus tissues from these genotypes were first matured before transfer to SIM. Shooting and rooting was well synchronised in SIM for the genotype TL03B6754A-20 (Figure 5.5). However, rooting on SIM was delayed for the lines CML216 and TL03B6757-68. Well established plantlets with a strong rooting system on SIM were transferred to sterile peat moss for hardening (Figure 5.6). Plants establishing in the glasshouse were phenotypically normal except for presence of some somaclonal variations such as dwarfism and tassel seed formation (Figure 5.3). In addition, plants regenerated from the
Figure 5.5: Process of recovery of *pmi* transgenic callus tissues for the inbred line TL03B6754A-20. (A) Appearance of an immature embryo explant three days after infection with LBA4404 harboring pNOV-UbiamiRNA1. (B) Immature embryo tissue after 7 days on resting medium, having an embryogenic (red arrow) and a nonresponsive (black arrow) section. (C) Non-transgenic callus tissue that is dying under selection on mannose. (D) Callus tissue that is surviving on mannose selection, having transgenic (red, yellow and blue arrows) and non-transgenic (black arrow) sectors. The transgenic sectors comprise elongated embryos at different developmental stages including globular (red arrow), torpedo (yellow arrow) and heart (blue arrow) shapes. (E) Regeneration of a shoot and roots from a putative transgenic callus.
Figure 5.6: Regeneration of plants from transgenic CML216 callus tissues. A: recovery of transgenic callus tissues on mannose-containing media. B: Regeneration of plantlets from transgenic callus tissues cultured on shoot induction medium. C: Acclimatization of plantlets 5 days after transfer from shoot induction media. D: Establishment of plants in the glasshouse.
TL03B6757-68 genotype were characterised by purple pigmentation on stems and leaves (Figure 5.3) throughout their growth and development.

5.3.4 Integration of the amiRNA-PARP1 and pmI transgenes into plant genome

DNA extracted from 15 primary transformed CML216 plants were found to be amplifiable due to the presence of a 200 bp fragment of the TUA gene in all the DNA extracts. However, the non-template control had no detectable amplification (Figure 5.7). PCR performed on these DNA extracts using pmI gene-specific primers produced a 300 bp band in 13 plants. Figure 5.8 shows the PCR profile for some of the analysed plants. The PCR profile of the putative transgenic plants was similar to that of the positive control (pNOV2819-amiRNA1). However, no PCR amplicon was observed on the non-template control (NTC) (Figure 5.7). Out of the pmI positive plants analyzed, 11 (PNO2, PNO2, PNO3, PNO4, PNO5, PNO6, PNO7, PNO10, PNO15, PNO16, PNO20) had seeds (Table 5.7) and were selected for further PCR analyses. PCR using amiRNA1-PARPI gene-specific primers revealed the presence of a 200 bp product in 5 of the plants that were pmI positive (Figure 5.8).
**Table 5.7**: Number of seeds produced by different primary transformed maize lines

<table>
<thead>
<tr>
<th>Event</th>
<th>Number of $T_0$ seeds produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNO2</td>
<td>144</td>
</tr>
<tr>
<td>PNO3</td>
<td>57</td>
</tr>
<tr>
<td>PNO4</td>
<td>60</td>
</tr>
<tr>
<td>PNO5</td>
<td>266</td>
</tr>
<tr>
<td>PNO6</td>
<td>256</td>
</tr>
<tr>
<td>PNO7</td>
<td>144</td>
</tr>
<tr>
<td>PNO10</td>
<td>36</td>
</tr>
<tr>
<td>PNO15</td>
<td>130</td>
</tr>
<tr>
<td>PNO16</td>
<td>109</td>
</tr>
<tr>
<td>PNO17</td>
<td>2</td>
</tr>
<tr>
<td>PNO20</td>
<td>168</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1372</strong></td>
</tr>
</tbody>
</table>
Figure 5.7: PCR analysis of different T₀ CML216 plants transgenic for amiRNA1-PARP1. Amplification of the maize *TUA* gene (A) and the amiRNA1-PARP1 transgene (B) on gDNA extracted from independent T₀ amiRNA1-PARP1 transgenic CML216 plants. Lane NTC, Non-template control; Lane WT, gDNA from a non-transformed plant; Lane PNO2 to PNO26, gDNA from independent T₀ transgenic plants.
Figure 5.8: PCR analysis of different T₀ amiRNA-PARP1 transgenic events for the presence of the *pni* gene. Lane NTC, Non-template control; Lane WT, gDNA from a non-transformed plant; Lane PNO2 to PNO20, gDNA from independent T₀ transgenic plants.
5.3.5 Stable integration of amiRNA1-PARP1 transgene into maize genome

Analysis on NanoDrop 2000/2000c spectrophotometer revealed the gDNA for southern blot hybridization was of high quality and quantity. The concentration of the gDNA ranged from 394.1 to 1663.1 ng/µl (Appendix 27). A total of 30 µg of gDNA from every plant was used for southern blot hybridization. The pmi biotin-labelled probe was prepared using random primed labelling. Analysis on 1.5% agarose gel revealed that the PCR DNA template for probe preparation was of the expected size and free from unspecific amplification (Figure 5.9). Additionally, it was of sufficient quantity for random primed labelling. About 500 ng of template was labelled by random primed labelling for 21 hours. This generated approximately 1800 ng of biotin labelled probe. One microliter (about 40 ng) of purified biotin labelled probe and control (HindIII/λ) template were visible as a smear when run on a 1% agarose gel (Figure 5.10). The probe was found to be labelled upto $10^{-4}$ dilution (Figure 5.9). This level of probe biotinylation is considered insufficient. According to the manufacturers of the NEBLOT phototope labelling and detection kit, the biotinylated lambda control and probe template should be visible atleast to the $10^{-5}$ level to be considered sufficiently biotinylated. Consequently, longer hybridization times and/or more probe was used in the hybridization step of southern blot hybridization procedure to compensate for insufficient biotinylation of the probe.
The suitability of the NEBLOT phototope labelling and detection system (New England Bio-Labs Inc., MA, USA) was evaluated using different DNA fragments of varying molecular weights. Plasmid DNA, pmi PCR template, biotin labelled probe and lambda phage DNA were successfully detected using the system (Figure 5.10). This established that the entire southern blot hybridization system was functional and therefore suitable for analysis of transgenic plants. When six independently transformed transgenic plants were assessed for stable integration of the amiRNA-PARP1 by southern blot hybridization, a high molecular weight amiRNA1-PARP1 hybridizing signal was produced in gDNA of the transgenic plants but not in the non-transformed plants (Figure 5.11). These results strongly indicate stable integration of the amiRNA-PARP1 transgene in the transformed plants.
Figure 5.9: Preparation of biotin-labelled probes using random primed labelling method. (A) Analysis of purified *pmi* DNA fragment generated by PCR. The expected fragment size (599 bp) is shown. (B) Analysis of random primed biotin-labelled control (lane 1) and *pmi* (Lane 2) DNA templates following purification. (C) Determination of level of biotinylation of *pmi* probe and control DNA.
**Figure 5.10:** Southern blot hybridization analysis of different template DNA for detection of the *pmi* gene.
Figure 5.11: Southern blot hybridization analysis of genomic DNA isolated from different independently transformed maize T₁ plants. The blot was hybridized with a biotin labelled amiRNA1 probe on undigested gDNA. The hybridization signal appears as high molecular weight smears. Lane M, Biotin labelled 1 kb DNA ladder; WT, gDNA from a wild type plant; Lane PNO4B3 to PNO15E5, gDNA from independent T₁ transgenic plants.
5.4 DISCUSSION

Fifteen inbred lines (including the control lines A188 and CML 216) were tested for their response to embryogenic callus formation. With the exception of A188 and CML 216, four genotypes (TL03B 6757-68, TL03B 6754A-20, TL03B 6753A-21 and TL03B 6757-5) were observed to be competent for somatic embryogenesis. The genotypes TL03B6754A-20 and TL03B6757-68 however stood out as having the greatest capacity to induce embryogenic calli using the adopted protocol. This implies that somatic embryogenesis is dependent on the genotype under evaluation. So far very few tropical genotypes have been reported as being somatic embryogenesis competent including CML216 (Ombori et al., 2008; Muoma et al., 2011) and IL3 (Rasha et al., 2008). Embryogenic callus induction from immature embryos has been shown to be determined by some genomic regions (Krakowsky et al., 2006). The observed QTLs can be used to improve embryogenic response for agronomically useful inbred lines through marker assisted selection (MAS).

Results on shoot regeneration experiments indicated that the genotypes with high embryogenic capacity are also highly regenerable. TL03B6754A-20 and TL03B6757-68 produced an average of 3 and 4 shoots per callus, respectively. The control genotype IL3 was more regenerable than the tested genotypes, averaging 5 shoots per callus. IL3 has been shown to produce between 5 to 7
shoots per callus under similar media and culture conditions (Rasha et al., 2008). Results from this study suggest that the different genotypes vary in their ability to produce plants from a callus tissue. This genotype-dependent regeneration is also exhibited by other tropical maize genotypes (Ombori et al., 2008; Rasha et al., 2008).

The average rate of recovery of transgenic tissues using PMI/mannose system was 48.28, 34.16 and 32.19% for TL03B6754A-20, TL03B6757-68 CML 216, respectively. The PMI/mannose system has also proved to be useful in recovering transgenic tissues from different maize genotypes. Negrotto et al. (2000) recovered transgenic tissues of A188 at an average rate of 32% using *A. tumefaciens* mediated transformation while Wright et al. (2001) reported a TF of 45% for CG00526 via particle bombardment.

Transgenic tissues were recovered from TL03B6754A-20, TL03B6757-68 and CML 261 at comparable rates. This therefore means that TL03B6754A-20, TL03B6757-68 can be transformed as effectively as the control inbred line CML216. CML216 is a popular inbred line and has been reported to be reliably transformable. It has achieved between 3 and 47 % transformation frequency using the PMI/mannose system (Mgutu, 2011) and between 1.4 and 8.7% using the *bar* gene (Ombori et al., 2013). Because of its high transformability, CML216 has
been used in various single cross breeding with lines that are agronomically desirable but with low transformability (Anami et al., 2010). TL03B6754A-20 and TL03B6757-68 can be added to the pool of the few tropical genotypes that are highly transformable and therefore good candidate genotypes for use in enhancement of genetic transformation of agronomically important maize genotypes.

Callus tissues of the TL03B6757-68 genotype were pigmented purple in culture. Plantlets derived from the callus had purple pigmentation on stems and leaves. A TL03B6757-68 plant is characterized by extensive purple pigmentation and hairiness on stems and leaves throughout its growth period. This high level anthocyanin production and hairiness is an important trait to help protect against drought and pest attack. Silking and tasselling in this genotype was well synchronised under optimum growing conditions. Additionally, plants of the genotype matured earlier than CML216 and TL03B6754A-20.

Fifteen independent events from one transformation experiment were analysed for stable transgene integration by PCR and southern blot hybridization. The amiRNA1-PARP1 transgene was stably integrated in 6 out of the 15 regenerants that were analysed by PCR. This represented a transformation efficiency of
18.18% (6/33). Stable integration was confirmed in three $T_0$ events (PNO2, PNO4, AND PNO15) via southern blot hybridization analysis.

In this chapter, two inbred lines with high embryogenic and regeneration capacity have been identified. In addition, the use of the $pmi$ gene to produce plants bearing the amiRNA3-PARP1transgene is reported. It has also been reported that amiRNA1 transgene targeting maize PARP1 gene was stably integrated in the genome of maize plants.
CHAPTER SIX

GENERATION OF MARKER-FREE AMIRNA3-PARP1 TRANSGENIC MAIZE BY AGROBACTERIUM-MEDIATED COTRANSFORMATION WITH BINARY VECTORS HAVING MULTIPLE T-DNA

6.1 INTRODUCTION

Combining genes of agronomic importance with marker elimination strategies is an effective way to produce marker-free transgenic plants. Presence of SMG in transgenic plant raises some ecological and toxicological concerns. Some of the environmental concerns are that the SMG might convert the transgenic crop into an uncontrollable weed, or that it may spread from the crop to wild relatives or other organisms, which consequently become unmanageable. Biosafety issues include the SMG or its product rendering the plant unsuitable for consumption. This is in addition to occurrence of unexpected pleiotropic effects associated with continuous expression of the SMG. Removal of SMG such as the bar gene after transformation is beneficial because it precludes the aforementioned ecological and biosafety issues. It also useful for practical reasons including possibility of reusing the same SMG for stacking of genes into the transgenic plant.

The objective of this chapter was to use A. tumefaciens two T-DNA systems to generate marker-free maize plants containing amiRNA-PARP1. Development of
pMarkfree3.1 and pMarkfree3.2 vectors each bearing the bar and amiRNA-PARP1 transgenes in separate T-DNA regions is reported here. Immature embryos derived from three inbred lines were transformed using these vectors with the help of the A. tumefaciens strain LBA4404. Cotransformation of maize plants with the bar and amiRNA3-PARP1 transgenes and separation of the transgenes in progenies of the cotransformants is also reported. Reported also is the successful generation of maize plants free of selectable marker genes.

6.2 MATERIALS AND METHODS

6.2.1 Development of cotransformation vectors and plant transformation

Cotransformation binary vectors expressing the amiRNA3-PARP1 transgene were utilised in transformation to help produce SMG-free maize. The dual T-DNA vectors pMarkfree3.1 and pMarkfree3.2 were constructed by inserting amiRNA3-PARP1 gene constructs into pMarkfree2.1 and pMarkfree2.2 vectors, respectively. Construction of pMarkfree2.1 and pMarkfree2.2 vectors has been described in section 3.3.4. The amiRNA3-PARP1 gene construct was removed from pCAMBIA1300-amiRNA3 as a HindIII/XbaI fragment and subcloned into the vectors pMarkfree2.1 and pMarkfree2.2 pre-digested with HindIII and XbaI. Ligation products were transformed into competent E. coli cells. Recombinant clones were identified by PCR using primers specific to the amiRNA3-PARP1 gene construct. Plasmid DNA from clones that revealed the expected amplicon
size was digested with EcoRI to confirm the presence of the insert. Verified constructs were transformed into the *A. tumefaciens* strain LBA4404 by the freeze-thaw method (Raviraja and Sridhar, 2007) prior to plant transformation.

Transformation of maize was achieved essentially as described by Ishida *et al.*, (2007). Briefly, *Agrobacterium tumefaciens* harbouring the two T-DNA vectors (pMarkfree3.1 and pMarkfree3.2) and the DRB vector pMarkfree4.0 was scooped from an LB agar plate and two loopfuls resuspended in 1 ml of infection medium: LS (Linsmaier and Skoog, 1965) medium (pH 5.2) containing 2% sucrose, 6% glucose 0.7 g/l proline, 0.5 g/l MES, and 100 µM acetylsyringone. This *A. tumefaciens* suspension was added to a tube containing immature embryo explants excised from maize inbred lines TL03B 6757-68, TL03B 6754A-20 and CML216. The tubes were vortexed at 3000 rpm for 30 seconds and left at room temperature for 5 min. Infected embryos were co-cultivated on LS medium (pH 5.8) containing 2% (w/v) sucrose, glucose 0.7 g/l proline, 0.5 g/l MES, 100 µM acetylsyringone, 1.6 mg/l AgNO₃ and 2 mg/l 2, 4-D for 3-4 days at 20 to 22 °C in the dark. The explants were transferred to resting medium: LS medium (pH 5.8) with 2% (w/v) sucrose, 1% (w/v) glucose, 0.7 g/l proline, 0.5 g/l MES, 1.6 mg/l AgNO₃ and 2 mg/l 2,4-D, 150 mg/l timentin and 100 mg/l cefotaxime for 10 to 14 days at 28 °C in the dark. Explants were transferred to selection I medium: LS medium (pH 5.8) with 2% (w/v) sucrose, 1% (w/v) glucose 0.7 g/l proline, 0.5 g/l MES, 1.6 mg/l AgNO₃ and 2 mg/l 2,4-D, 150 mg/l timentin and 100 mg/l cefotaxime for 10 to 14 days at 28 °C in the dark.
AgNO₃ and 2 mg/l 2,4-D, 150 mg/l timentin, 100 mg/l cefotaxime and 3 mg/l PTT for 42 days at 28 °C in the dark. Explants were transferred to fresh selection I medium every 21 days and surviving tissues on hormone-free MS medium (pH 5.8) with 150 mg/l timentin and 100 mg/l cefotaxime. Shoots were maintained on this medium until they rooted. Plantlets were hardened by transferring onto sterile peat moss kept in the glasshouse for 7 to 14 days. Plantlets were transferred into soil and maintained in the glasshouse until they set seeds.

6.2.2 Analysis of primary transformants for co-insertion of the bar and amiRNA3-PARPi transgenes

Primary transformants were screened for the presence of an active bar gene using the Trait LL lateral flow test strip kit (Strategic Diagnostics Inc.). A plant tissue was placed on a mortar containing 1 ml of buffer PBST and ground gently using a pestle. About 0.5 ml of the mixture was placed in a 1.5 ml tube and a test strip placed in the tube. The setup was allowed to stand for 10 min before interpretation of the results. Plant samples that caused appearance of two bands on the strips were classified as positive while those that produced one band were classified as negative.
T₀ plants were also screened to identify those cotransformed with bar and amiRNA3-PARP1 genes. This was accomplished with PCR using amiRNA3-Fwd3 (5’gctcggacgcatattacacatgttc 3’) and amiRNA3-Rev3 (5’ ttggaatacaaaagagaggtgcg 3’) for the amiRNA3-PARP1 gene and PTF101.1-F and PTF101.1-R primers for the bar gene expression cassette. Only the cotransformed plants were advanced for segregation analysis.

6.2.3 Analysis of T₁ plants for presence of amiRNA3-PARP1 and absence of bar gene

Progenies derived from cotransformed T₀ plants were assessed for segregation of expression of the bar gene using the basta leaf paint assay. This was achieved by marking a small section of a maize leaf with a permanent marker pen followed by challenging the marked area with 0.02% (v/v) basta using a piece of cotton wool. Seven days after application, plants were assessed for reaction to the applied basta. Plants whose leaves were damaged were classified as basta sensitive (B⁵), while the undamaged plants were categorised as basta resistant (B⁸).

Phosphinothricin acetyl transferase (PAT) negative plants were evaluated further to verify the absence of the bar gene and presence of amiRNA-PARP1. This was accomplished using the PCR. Plants that were bar negative or positive but having
amiRNA3-PARP1 were confirmed by southern blot hybridization. Southern blot hybridization was performed essentially as described in section 5.2.8. However, a bar probe was first labelled using the ALKPHOS direct labelling and detection kit (GE Healthcare Ltd, Buckinghamshire, UK). The probes were hybridized to gDNA digested with *EcoRI*.

6.3 RESULTS

6.3.1 Construction and confirmation of the cotransformation vectors

pMarkfree3.1 and pMarkfree3.2

Two *E. coli* clones that revealed the presence of amiRNA3-PARP1 insert in pMarkfree2.1 and pMarkfree2.2 vector by PCR were analysed further by restriction enzyme digestion. Digestion using *EcoRI* released different DNA fragments sizes from the recombinant clones (Figure 6.1) confirming different positioning of the bar cassette in pMarkfree2.1 and pMarkfree2.2. A 1.8 kb fragment was released from plasmid DNA extracted from colony 12. Plasmid DNA from this clone was named pMarkfree3.2. However, a 3.6 kb fragment was released from plasmid DNA isolated from colony 15. This plasmid was named pMarkfree3.1.
6.3.2 Effect of cotransformation vectors on transformation response of the CML 216 genotype

Three cotransformation vectors (pMarkfree3.1, pMarkfree3.2 and pMarkfree4.0) were compared with an ordinary single T-DNA vector (pSCV-amiRNA3Bar) to investigate their effectiveness in transformation of the inbred line CML216. Recovery of transgenic tissues for the inbred line CML216 is shown in Figure 6.2.

The average transformation frequency for recovery of bar transgenic maize using the pSCV-amiRNA3Bar cotransformation vector was 23.16% (Figure 6.3), while some experiments gave frequencies as high as 38%. However, with pMarkfree3.1, transformation frequencies of between 11.60 and 40.63%, with an average of 26.16%, were exhibited.

Using pMarkfree3.2, transgenic tissues were obtained at frequencies ranging from 16.67 to 40.00% (average 27.22%). On the other hand, using the cotransformation vector pMarkfree4.0, an average transformation frequency of 23.45% (Figure 6.3), with 10.53% as the lowest and 36.36% as the highest was obtained.
Figure 6.1: Construction of pMarkfree3.1 and pmarkfree3.2 vectors. (A) Analysis of clones possibly harbouring pMarkfree3.1 and pMarkfree3.2 vectors by digestion of the extracted plasmid DNA with *EcoRI*. Lane M, 1 Kb DNA ladder; Lane 1 and 3, Analysis of colony 12 and 16, respectively for presence of pMarkfree3.1; Lane 2 and 4, Analysis of colony 15 and 19, respectively for presence of pMarkfree3.2. (B) Map of T-DNA region of the vector pMarkfree3.2 derived from colony 15. (C) Map of T-DNA region of the vector pMarkfree3.1 derived from colony 12.
**Figure 6.2:** Recovery of transgenic tissues for the CML216 genotype. (A) An immature embryo explant infected with *A. tumefaciens* strain LBA4404 bearing the cotransformation vector pMarkfree3.2. (B) Establishment of an embryogenic callus tissue on an immature embryo 7 days after culture on resting medium. (C) Identification of putative transformed callus tissues on PPT-containing medium.
The three cotransformation vectors were not different from each other in transformation frequency by ANOVA (Appendix 3). ANOVA analysis also revealed that transformation frequency for recovery of *bar* transgenic maize tissues for any of the three cotransformation vectors was not different from that of the control vector, pSCV-amiRNA3bar (Appendix 3).

**Figure 6.3:** Transformation frequency for recovery of transgenic callus tissues of CML216 with different vectors. Values are means of three replicates and horizontal bars show standard errors.
6.3.3 Effect of cotransformation vectors on transformation response of different maize genotypes

Genotype TL03B 6757-68, TL03B 6754A-20 and CML216 were transformed with the three marker free vectors (pMarkfree3.1, pMarkfree3.2 and pmarkfree4.0) and transgenic tissues selected on 3 mg/l PPT-containing media for 30 days. Surviving calli of the TL03B 6757-68 genotype were characterized by increase in size and were white to cream in appearance under PPT selection. However, sensitive calli were either arrested in growth or turned brown (Figure 6.4) under selection. A total of 89 independent transgenic callus events of the genotype TL03B 6757-68 were generated using the three cotransformation vectors. Specifically, pMarkfree3.1, pMarkfree3.2 and pMarkfree4.0 generated calli at TF ranging from 4.00 to 22.77% (average 14.81%), 4.00 to 71.74% (average 32.10%), and 10.14 to 21.05% (average 14.84%) respectively, for inbred line TL03B 6757-68 (Figure 6.5). However, the average TF observed for the vectors were not different statistically (Appendix 4).

Possible bar transgenic callus tissues of the TL03B 6754A-20 genotype developed a popcorn-like appearance after 5 to 7 days on SIM (Figure 6.6). Additionally, shoots and roots emerged from callus simultaneously after 7 to 14 days on SIM. TF for TL03B 6754A-20 ranged from 20.00 to 23.38% (average 21.69%), 20.00 to 52.63% (average 36.32%) and 6.63 to 19.23% (average 12.93%) for vectors
Figure 6.4: Transformation of immature embryos of the TL03B 6757-68 genotype and regeneration of plants via somatic embryogenesis. (A) Development of callus on an *Agrobacterium*-infected immature embryo cultured on resting medium for 10 days. Embryogenic (white arrows) and non embryogenic (black arrow) tissues are indicated. (B) Identification of PPT resistant calli by exposure of transformed calli to 3 mg/l phosphinothricin for 21 days. (C) Development of shoots and roots on an embryogenic PPT resistant callus cultured on shoot induction medium for 7 days. (D) Regeneration of multiple plantlets from PPT resistant callus cultured on SIM for 21 days. (E) Establishment of putative transgenic plants in soil at the glasshouse.
pMarkfree3.1, pMarkfree3.2 and pmarkfree4.0, respectively (Figure 6.5). These vectors did not differ from each other in transformation of the inbred line TL03B 6754A-20 (Appendix 4). Transformation frequency for the recovery of transgenic CML216 callus tissues using pMarkfree3.1, pMarkfree3.2 and pmarkfree4.0 ranged from 11.60 to 40.63% (average 26.16%), 16.49 to 40.00% (average 27.22%) and

**Figure 6.5:** Effect of cotransformation vectors on transformation frequency of different genotypes.
10.53 to 36.36% (average 23.44%), respectively (Figure 6.4). However ANOVA revealed that the three cotransformation vectors did not differ significantly in TF ($p>0.05$) for CML216 (Appendix 4).

An ANOVA for the effect of the three cotransformation vectors on transformation frequency of the three genotypes revealed that the effect of the cotransformation vector and genotype on TF was not significant ($p>0.05$). The absence of statistically significant interaction between vector and genotype was also noted (Appendix 4).

6.3.4 Integration of the bar and amiRNA3-PARP1 transgenes in the maize genome

Primary regenerants were screened for the presence of an active bar gene using the lateral test strip test. All the tested plants produced two bands on the test strips. However, the bands were strong for some plants and weak for others (Figure 6.7). Only the transgenic plants that yielded seeds were analysed for co-insertion of the bar and amiRNA3-PARP1 transgenes. Of the 19 independent transgenic plant events transformed using pMarkfree3.1, 15 exhibited the expected 300 bp PCR fragment of the bar gene (Figure 6.7). The 15 bar transgenic plants were
investigated for the presence of the amiRNA3-PARP1. Seven plants were found to contain the amiRNA3-PARP1 transgene (Figure 6.7).

6.3.5 Production of marker-free T1 plants

Basta sensitive T1 maize seedlings from cotransformed parental lines were recovered. Ten days after application of 0.001% basta, resistant T1 maize seedlings were clearly distinguishable from the sensitive ones (Figure 6.8). Sixty percent of the assayed cotransformed lines produced basta sensitive T1 seedlings (Table 6.1). For event 216/3.2/A, 6 out of 20 T1 progeny plants tested (Figure 6.9) showed sensitivity to basta.

6.3.6 Southern blot hybridization confirmation of T1 plants for absence of bar transgene

Southern blot hybridization analysis was performed on genomic DNA from T1 plants that were identified using phenotypic analysis as basta sensitive or tolerant. A bar hybridizing signal was produced on genomic DNA from three T1 plants derived from the cotransformed line 216/1/3.2/B6. However, the signal was absent from genomic DNA of four T1 progeny plants (Figure 6.10). Absence of the bar transgene is indicative of removal of the transgene from transformed plants.
Figure 6.6: Transformation of inbred line TL03B 6754A-20 using pMarkfree3.1 and plant regeneration via somatic embryogenesis. (A) Isolation of transgenic callus tissues on PPT. (B) Appearance of putative transgenic callus of 18 prior to transfer to shooting medium (C) Appearance of a regenerating callus five days after transfer to shoot induction medium. (D) Regeneration of a plantlet from a putatively transformed callus.
Figure 6.7: Analysis of genomic DNA of cotransformed plants for presence of the amiRNA1-PARP1 and bar transgene. PCR analysis to detect the bar (A) and amiRNA3-PARP1 (B) transgenes in transgenic T₀ plants. C: Lateral test strip assay for an active bar gene in different cotransformed plants. Lane P: pMarkfree3.2 plasmid. Lane NTC, non-template control; Lane 1 KB, 1 KB DNA ladder; Lane WT, non-transformed DNA used as negative control; Lane 216/3.1/B1 to 216/3.1/12, independently cotransformed plants.
Table 6.1: Basta resistance in T₁ progeny derived from different cotransformed plants

<table>
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<tr>
<th>T₀ Line</th>
<th>Number of T₁ plants assayed</th>
<th>Number of tolerant T₁ plants</th>
<th>Number of sensitive T₁ plants</th>
<th>Basta R/S</th>
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<tr>
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</tr>
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<td>4</td>
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<td>5</td>
<td>3:1</td>
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<td>10</td>
<td>7</td>
<td>1.43:1</td>
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<tr>
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<td>3</td>
<td>5.67:1</td>
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<td>16</td>
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<td>4:1</td>
</tr>
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Figure 6.8: Application of basta to assess expression of the bar gene in transgenic T₁ maize plants derived from the cotransformed event 216/3.2/O. (A) Normal and albino phenotypes of T₁ plants ready for application of Basta. Image B and C shows reaction to application of basta, respectively, by normal and albino T₁ progeny plants derived from the T₀ event 216/3.2/O. Images were recorded 14 days post application.
Figure 6.9: Response by different T1 plants derived from the cotransformed event 216/3.2/A to application of 0.001% Basta. Images were recorded 21 days after painting. All plants except A and B were sensitive to basta.
Figure 6.10: Southern blot hybridization to detect the bar transgene in different T₁ plants derived from cotransformed event 216/1/3.2/B6. The bar hybridization signal was produced on gDNA from progeny 1, 5, and 6 but not in 2, 3 and 4. The signal is indicated with white arrows. Lane m, 1 KB DNA ladder; wt, gDNA from non-transgenic plant.
6.4 DISCUSSION

The objective of this chapter was to transform different inbred lines (CML216, TL03B 6757-68 and TL03B 6754A-20) with the transgene amiRNA-PARP1 using three cotransformation vectors (pMarkfree4.0, pMarkfree3.1 and pMarkfree3.2). The proportion of resistant callus tissues recovered on PPT for the inbred lines CML216, TL03B 6757-68 and TL03B 6754A-20 was 26.16%, 14.81%, and 21.69% for pmarkfree3.1 and 27.22%, 32.10% and 36.32% for pmarkfree3.2, respectively. This means that the cells had integrated the bar gene and were therefore transgenic. Resistance of the transgenic tissues to PPT is conferred by the PAT enzyme which is encoded by a stably integrated bar gene. The enzyme detoxifies PPT. However, plants cells lacking the enzyme are killed due to PPT-induced accumulation of ammonia and inhibition of photosynthesis.

High frequencies of bar transgenic tissues were exhibited by the developed cotransformation vectors. With the dual T-DNA system pMarkfree3.2 and pMarkfree3.1, TF of 36.32% and 26.16%, respectively were achieved for maize. In an earlier study, bar resistant callus tissues were recovered at a frequency of between 34 and 46.7% (average 42.6) when LB4404 was used to cotransform maize using a two T-DNA vector (Ishida et al., 2004). However, in the study a superbinary vector was used to transform the temperate genotype A188.
The presence of multiple T-DNAs in a binary vector must not compromise the vectors ability to recover primary transformants. Miller et al. (2002) established that a cis control vector produced a slightly higher TF (56.7%) compared to that for the newly developed two T-DNA vector (52.2%). However, No statistical differences were found between these vectors in TF (Miller et al., 2002). In the present study, the efficacy of the cotransformation vectors was also compared to that of a control vector. Using ANOVA no statistically significant difference ($P>0.05$) between the cis control vector and the three cotransformation vectors (pMarkfree3.1, pMarkfree3.2 and pmarkfree4.0) in TF of CML216 was observed. This implies that the developed vector system is capable of transforming this inbred line as efficiently as a single T-DNA vector. A lack of influence of TF by genotype was observed. This suggests that the newly identified genotypes (TL03B 6757-68 and TL03B 6754A-20) are transformable as efficiently as the control genotype CML216. CML216 is one of the very few tropical inbred lines with a high embryogenic and regenerable potential (Ombori et al., 2008; Muoma et al., 2011) and has proved to be highly transformable (Anami et al., 2010; Ombori et al., 2013). However, because CML216 performs poorly agronomically, it and has been used to produce novel lines with increased regeneration and transformability by interbreeding with agronomically desirable but less transformable lines (Anami et al., 2010; Muoma et al., 2011).
The double right border system was developed by Lu and coworkers in 2001 (Lu et al., 2001). The system was reported to transform rice plants at high frequency. Bar transgenic maize tissues were recovered at a frequency of 23.44% with the DRB vector pMarkfree4.0. When applied in tobacco, similar systems have been reported to recover bar transgenic plants at a higher proportions such as at 59.2% (Hong-Yan et al., 2003).

Recovery of basta sensitive T₁ maize seedlings derived from basta resistant parental plants was a good indication of removal of the bar gene from the plants through sexual segregation. Using southern blot hybridization analysis, the basta sensitive plants were confirmed to be devoid of the bar gene. However, basta resistant plants had the bar gene still integrated in their genome.

In this study the bar gene was found to be a useful marker for identification of transgenic tissues from two inbred lines that have not been evaluated before (TL03B6754A-20 and TL03B6757-68). A. tumefaciens-mediated co-introduction of the bar and amiRNA1 transgenes that were harboured in two separate T-DNA regions of new binary vectors (pMarkfree3.1 and pmarkfree3.2) allowed recovery of cotransformed maize plants. Selfing of the cotransformed plants produced amiRNA-PARP1 segregants that were free of the bar SMG.
CHAPTER SEVEN

DOWNREGULATION OF PARP1 GENE TO ENHANCE TOLERANCE TO OXIDATIVE AND DROUGHT STRESS IN TROPICAL MAIZE

7.1 INTRODUCTION

Downregulation of poly (ADP-ribose) polymerase 1 (PARP1) gene is an effective strategy for making plants tolerant to multiple stresses including heat, light and drought. Drought tolerance due to inhibition of PARP is associated with various changes in the transgenic plant. Reduced PARP activity is associated with enhanced energy use efficiency (De Block et al., 2004) and a maintained redox potential during drought (De Block et al., 2004; Schulz et al., 2012).

High tolerance to oxidative stress is also associated with silencing of the PARP1 gene (De Block et al., 2004; Schulz et al., 2012). Tolerance to oxidative stress is associated with maintenance of photosynthesis rates due to protection of the photosynthetic apparatus from damage. Maintenance of high chlorophyll and photosynthetic capacity during oxidative stress is observed in Arabidopsis following inhibition of PARP1 (Schulz et al., 2012). Reduced antioxidant capacity under oxidative stress is also associated with silencing of PARP1 gene in plants. Specifically, the plants have reduced anthocyanin and ascorbate content (Schulz et al., 2012).
Although acquisition of drought tolerance due to overexpression of transgenes is highly associated with negative effects in plant growth, hpPARP1 transgenic plants grow normally without any growth or yield penalty. This has been demonstrated for Arabidopsis and oil seed rape plants (De Block et al., 2004; Vanderauwera et al., 2013). This enhanced accumulation of biomass under stress is due to a combination of factors including maintenance of high levels of energy and photosynthesis and reduction in anthocyanin and ascorbate (De Block et al., 2004; Schulz et al., 2012).

The aim of this chapter was to show that maize plants carrying an amiRNA transgene against PARP1 gene have reduced PARP1 activity than wild type plants. The performance of plants with downregulated PARP1 expression and non-transgenic plants under severe oxidative and drought stress was compared and is reported here.

7.2 MATERIALS AND METHODS

7.2.1 Determination of gene expression

All experiments for measuring the expression of maize endogenous genes were conducted using the Mastercycler ep realplex real-time cycler (Eppendorf Hamburg, Germany) using SYBR green I dye. The 2× QuantiFast SYBR Green
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PCR kit or the 2× QuantiFast SYBR Green QPCR kit (Qiagen, Maryland, USA) was used in preparations of all PCR reaction mixtures.

**7.2.2 Quantitative RT-PCR analysis**

Total RNA was extracted from maize leaf tissues using the RNeasy Plant mini kit (Qiagen, Maryland, USA). Contaminating DNA in the RNA preparations was removed by digesting with RNA-free amplification grade DNase I (Invitrogen Corp. Carlsbad CA, USA), and was confirmed by PCR amplification of glyceraldehyde phosphate dehydrogenase (GAPDH) using GAPDH intron (int-GAPDH) primers (Table 7.1). DNA-free RNA was quantified using NanoDrop 2000/2000c spectrophotometer.

Total RNA (200 ng) was used as a template for synthesis of cDNA using superscript III reverse transcriptase kit (Invitrogen Corp. Carlsbad CA, USA). The resultant cDNA was diluted to 50 ng/µl and then 1.0 µL used as a template in a 20 µL PCR reaction that also consisted of 10 µl QuantiFast SYBR Green PCR Master Mix, 5 µL of RNase-free water and 2 µL of each primer. Primers for qRT-PCR (Table 7.1) were first diluted to a working concentration of 10 µM. The concentration of the primers was confirmed by measuring on a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific Inc., Delaware, USA).
two-step PCR was performed with 40 cycles of denaturation at 95 °C for 15 sec and a combined annealing and extension at 60 °C for 30 sec. An initial activation step at 95 °C for 5 min was performed.

7.2.2.1 Test for specificity of the qRT-PCR reaction

Primers used for amplification of housekeeping and PARP1 genes are shown in Table 7.1. The primers were first tested in silico for specificity with the FastPCR program using the indicated DNA sequences from the NCBI database as template. They were verified routinely by analyzing melting/dissociation curves obtained by heating amplicons from 60 °C to 95 °C. Specificity of PCR products was also checked by agarose gel (2%) electrophoresis.

7.2.2.2 Determining stability of expression of housekeeping genes

Housekeeping genes (HKGs) for use to compare the level of expression of PARP1 gene under oxidative stress were chosen based on their stability. The genes assessed for stability of expression under oxidative stress treatment were EL1-A, TUA and GAPDH.
### Table 7.1: Primers used in qRT-PCR analysis.

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<th>Primer name</th>
<th>Sequence (GenBank accession number)</th>
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<th>Tm (°C)</th>
<th>Expected Amplicon size (bp)</th>
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<td></td>
<td>313 Mgutu, (2011)</td>
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</table>
7.2.3 Methyl viologen (MV) treatment of plants

Methyl viologen treatment was performed on two T<sub>2</sub> plants derived from independent T<sub>0</sub> events. The T<sub>0</sub> plants used were stably transformed CML216 plants. Two Leaf discs (each 7 mm in diameter and 0.003 g in fresh weight) were excised from the fifth leaf of maize plants and floated in 1 ml of different concentrations of MV (0, 5, 30 µM). The leaf discs were kept at room temperature in the dark for one hour. They were illuminated continuously at 22 °C for 24 hours.

7.2.4 Determination of chlorophyll and anthocyanin content

Chlorophyll was extracted from MV treated leaf discs (each 7 mm in diameter and 0.003 g in fresh weight) as described by Robson et al. (2004). Chlorophyll was extracted from leaf discs by floating in 1 ml of chilled methanol in 2 ml tubes. All extractions were performed for 24 hours in the dark at 4 °C. Extracted chlorophyll was measured at 665 nm and 652 nm on a 722N visible spectrophotometer. Values were corrected by subtracting the absorption at 750 nm and the chlorophyll content was calculated according to Wellburn, (1994). Total anthocyanins were extracted from MV treated leaf discs as described by Gould et al. (2000). Leaf discs were excised into 1 ml of chilled acid treated methanol (99:1 (v/v) methanol: HCl). Extractions were carried out for 24 hours in the dark at 4 °C. Extracted
anthocyanin was measured at 530 nm and 657 nm and relative anthocyanin content calculated as described by Sheng et al. (2005).

7.2.5 Measurement of relative water content (RWC)

Transgenic and non-transgenic plants at 8th leaf stage were sprayed with 5 µM MV. After 5 days, RWC was determined. Three pieces of the second last fully expanded leaf was weighed to determine their fresh weight (FW). The leaf pieces were allowed to rehydrate inside a refrigerator overnight. The leaf samples were quickly but carefully blotted dry and weighed to determine their turgid weight (TW). Dry weight (DW) was then determined after oven-drying the samples for 72 hours at 75 ºC. RWC was calculated using the following equation:

\[ \text{RWC} = \left( \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \right) \times 100 \]

7.2.6 Drought tolerance assay of T1 plants

Primary transgenic plants that were confirmed to contain amiRNA3-PARP1 using the southern blot hybridization assay were maintained in the glasshouse till they established seeds. The T0 seeds were planted in pots containing 7000 g of oven-dried loam soil. Pots were well watered and transferred into a growth chamber with temperatures of 29/21 ºC day/night, a 12 hour day length and 65% relative humidity. Plants were maintained by adequate watering and application of a
nutrient solution until they attained the fourth leaf stage. Before application of water stress, plants were screened by PCR using amiRNA-PARP1 specific primers. Plants were marked as positive or negative. For drought stress, water was withheld until the non-transgenic plants showed complete wilting. The pots were rotated every day to avoid any position effects. Measurement of plant height and leaf width was performed every seven days after withholding water. At the end of the 28 day drought period, plants were watered and recovery was recorded 2 days later. After the drought stress period, plant biomass was measured for 10 transgenic and non-transgenic plants.

7.2.7 Data analysis

For Chlorophyll and anthocyanin measurement experiments, two leaf discs (replicated four times) from transgenic and non-transgenic plants were used for each MV level. Absorptions were recorded at different wavelengths for use in calculation of the average anthocyanin and chlorophyll content of transgenic plants. Differences in average chlorophyll and anthocyanin content among MV levels and transgenic and non-transgenic plants were analysed for statistical significance by ANOVA using statview statistical package (SAS, 2003). ANOVA was also used to analyze differences in means of the transgenic and the non-transgenic seedlings.
7.3 RESULTS

7.3.1 Determination of quality and quantity of RNA and cDNA

The concentration of total RNA extracted from transgenic and non-transgenic plants ranged from 10.50 to 34.70 ng/µl (Appendix 24). PCR using GAPDH-int primers (Table 7.1) was used to detect the presence of contaminating DNA in total RNA preparations. The analysis revealed the presence of a band of about 1000 bp in the RNA extracts (Figure 7.1). Therefore all RNA samples were treated with DNase I amplification grade (Invitrogen Corp. Carlsbad CA, USA) before use as a

Figure 7.1: Analysis of RNA and cDNA for contaminating genomic DNA. A: Amplification of GAPDH on total RNA extracted from different plants. B: Amplification of GAPDH on cDNA prepared from DNase I-treated total RNA extracted from different plants. Lane NTC, Non-templated control; Lane WT, non-transgenic sample used as negative control; Lane PNO4A to PNO6J2, RNA and cDNA samples from independent T2 plants.
template for cDNA synthesis. Exactly 200 ng of the RNA was used for cDNA synthesis. When measured using the NanoDrop 2000/2000c spectrophotometer, the concentration of the cDNA varied within a narrow margin of between 536.4 and 551.5 ng/µl (Appendix 25). This suggests a high level of accuracy in the amount of total RNA used as template for cDNA preparation. The 260/280 Absorbance ratio for the cDNA ranged from 1.73 to 1.79 for all samples except PNO6J1 (Appendix 25). This indicates that the quality of the cDNA was very high. However, PCR analysis using GAPDH-int primers on the cDNA samples revealed absence of detectable contaminating gDNA for all samples except for PNO6J1 and PNO6J2 (Figure 7.1). Fresh cDNA for event PNO6J1 and PNO6J2 that was prepared for qRT-PCR was found to be free of contaminating gDNA. CDNA from each sample was diluted to 50 ng/µl for subsequent qRT-PCR analysis. PCR was performed using 1 µl (50 ng) of the diluted cDNA as the template.

7.3.2 Specificity of primers for PARP1 and reference genes

Primers for use in qRT-PCR analysis were first quantified with a NanoDrop 2000/2000c. Quantification results revealed that the working concentration of the primers was slightly higher or lower than 10 µM (Appendix 26). Therefore the primers were accurately diluted to 5 µM before use.
Primers for the PARP1 gene amplified only one PCR product on agarose gel suggesting that they were highly specific. The PCR products were of the expected size when both gDNA and cDNA were used as template (Figure 7.2). In addition, dissociation curves for the PARP1 PCR product revealed a single peak after 40 cycles of amplification (Figure 7.3). No amplification was detected on the agarose gel or by the QPCR machine for the negative control for the three primer pairs.

The three primer pairs used to amplify the reference genes generated single amplicons of expected size from cDNA used as template as shown by the presence of single bands in agarose gel electrophoresis (Figure 7.2) and by single-peak melting curves of the PCR products obtained after 40 amplification cycles. Figure 7.3 shows the dissociation curves for PCR products generated using primers specific to the EL1-A.
Figure 7.2: Specificity of qRT-PCR reactions: amplification of the PARP1 (A) and different reference (B) genes. Lane M, 1 KB DNA ladder; WT, cDNA from non-transgenic plant.
**Figure 7.3:** Specificity of qRT-PCR amplification: Melting curves of *PARP1* gene and the EL1-A HKG showing single peaks (each including five replicates).
7.3.3 Determination of expression levels of *PARP1* gene in T₂ progenies transformed with amiRNA1

Quantitative reverse transcription PCR (qRT-PCR) was used to analyse the level of expression of maize *PARP1* gene in T₂ plants stably transformed with amiRNA1 transgene. The qRT-PCR run data was exported as a CSV file format from the Mastercycler ep realplex real-time cycler (Eppendorf Hamburg, Germany) into the Biogazelle qbase+ software version 2.5.1 (Biogazelle) for analysis. Ct values were first log transformed before analysis. Three reference genes (EL1-A, TUA and GAPDH) were used to normalize *PARP1* gene expression data. The most stably expressed genes were found to be EL1A and GAPDH with a CV value of 0.663 and 0.710, respectively. However, the TUA gene was the most unstably expressed reference gene with a CV value of 1.161. It was therefore excluded from the analysis. Analysis revealed that the expression of *PARP1* gene was significantly reduced (*p* <0.05) in amiRNA1 transgenic plants compared to non-transformed plants (Figure 7.4).
7.3.4 Effect of MV-induced oxidative stress on chlorophyll content

Transgenic and wild type plants were exposed to 5 µM and 30 µM MV for induction of moderate and severe oxidative stress, respectively. After 24 hours of oxidative stress treatment, leaf discs were examined visually for any detectable differences. Increasing levels of MV lead to a progressive decrease in green coloration of leaf tissues for both the transgenic and non-transgenic plants (Figure 7.5).
Figure 7.5: Effect of different concentrations of methyl viologen on chlorophyll content of transgenic and wild type plants.

Increasing concentration of MV-induced oxidative stress lead to decrease in chlorophyll content for the transgenic events PNO4B5, PNO2C2, PNO15E5 and their related control plants. In contrast, the chlorophyll content of the transgenic events PNO4B3 and PNO15E4 and their related wild type plants increased (Figure 7.6 and 7.7). Interestingly, under increasing MV-induced oxidative stress, the chlorophyll content in the transgenic event PNO2C6 increased but decreased in the related non-transgenic plants (Figure 7.6 and 7.7).

Under control conditions (0 µM MV) the content of chlorophyll a and b was higher for the non-transgenic plants than for the transgenic events, with exception of PNO4B5 and PNO15E4 (Figure 7.6 and 7.7). Under mild and severe oxidative stress the transgenic line PNO15E4, PNO4B5 and PNO2C6 maintained a higher
chlorophyll a and b content than the wild type plants. However, the transgenic line PNO15E5 revealed a higher concentration of chlorophyll a and b than the non-transgenic type plants only when exposed to severe MV-induced oxidative stress (Figure 7.6 and 7.7).
Figure 7.6: Effect of MV on the content of chlorophyll a in amiRNA1-PARP1 transgenic and non-transformed plants.
Figure 7.6 (CONTINUED): Effect of MV on the content of chlorophyll a in amiRNA1-PARP1 transgenic and non-transformed plants.
Figure 7.7: Effect of MV on the content of chlorophyll b in amiRNA1-PARP1 transgenic and non-transformed plants.
Figure 7.7 (CONTINUED): Effect of MV on the content of chlorophyll b in amiRNA1-PARP1 transgenic and non-transformed plants.
7.3.5 Effect of MV-induced oxidative stress on anthocyanin content in transgenic plants

The effect of the different treatments of MV (0, 5 and 30 µM) on the anthocyanin contents of transgenic and non-transgenic plants was examined. Under control conditions (0 µM of MV), the amount of anthocyanin was higher in all transgenic plants (except event PNO2C6) than their related non-transformed plants. Results also indicate that for the non-transformed plants the anthocyanin content increased in the presence of 5 µM of MV but decreased upon exposure to 30 µM of MV. However, with the exception of the line PNO2C6, the anthocyanin content in the transgenic plants decreased slightly when treated with 5 µM of MV but increased at 30 µM of MV (Figure 7.8).

Under mild oxidative stress (5 µM of MV), the anthocyanin level for the transgenic events PNO4B3, PNO4B5, PNO2C2 and PNO2C6 was reduced relative to that of their respective control lines (Figure 7.8). It is also noteworthy that under extreme oxidative stress (30 µM of MV), the content of anthocyanin for the transgenic plants PNO4B5 and PNO2C6 was lower than for their respective control plants. The other two events (PNO4B3 and PNO2C2) had relatively higher anthocyanin content than the controls.
Figure 7.8: Effect of MV on the content of anthocyanin in amiRNA1-PARP1 transgenic and non-transformed plants.
Figure 7.8 (CONTINUED): Effect of MV on the content of anthocyanin in amiRNA1-PARP1 transgenic and non-transformed plants.
7.3.6 Effect of MV-induced oxidative stress on relative water content (RWC)

RWC was determined for different transgenic events as well as for a non-transformed plant. The transgenic line PNO15E4 had the highest RWC ranging from 71.3 to 135.1%, with an average of 102%. The transgenic event with the lowest RWC was PNO2C6. Its RWC ranged between 90.6 and 100.9%, with an average of 97.3%. However, the wild type plant had an average RWC of 98.5% (Figure 7.9). Analysis by ANOVA revealed non-significant differences ($p>0.05$) among the transgenic plants and the non-transgenic plant in RWC (Appendix 23).

**Figure 7.9:** Relative water content for different stably transformed $T_1$ transgenic and non-transgenic plants. Values are means of three replicates and horizontal bars show standard errors.
7.3.7 Effect of drought on growth and biomass of transgenic plants

Twenty eight days post water stress (DPWS), non-transgenic plants showed complete wilting. However, plants derived from the transgenic CML216 event PNO2 and PNO4 showed mild wilting. Table 7.2 shows plant height and leaf width for transgenic and non-transgenic plants. ANOVA (Appendix 28) results

<table>
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<tr>
<th>Parameter (cm)</th>
<th>Genotype</th>
<th>Days after withholding water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td><strong>Plant height</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>6.94±0.18a</td>
<td>7.38±0.25b</td>
</tr>
<tr>
<td>PNO2</td>
<td>7.01±0.15a</td>
<td>7.53±0.24b</td>
</tr>
<tr>
<td>PNO4</td>
<td>7.44±0.32a</td>
<td>8.65±0.33bc</td>
</tr>
<tr>
<td><strong>Leaf width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>1.44±0.04a</td>
<td>1.52±0.04b</td>
</tr>
<tr>
<td>PNO2</td>
<td>1.32±0.03a</td>
<td>1.50±0.03b</td>
</tr>
<tr>
<td>PNO4</td>
<td>1.37±0.03a</td>
<td>1.46±0.02b</td>
</tr>
</tbody>
</table>

Values are means ± standard error. For each parameter, values followed by the same letter are not significantly different at $p=0.05$. WT, non-transgenic plants; PNO2 and PNO4, T1 seedlings derived from the transgenic CML216 event PNO2 and PNO4, respectively.
indicate that transgenic plants derived from event PNO4 had a significantly higher plant height ($p<0.05$) than that for non-transgenic plants after 21 and 28 DPWS. ANOVA (Appendix 29) also revealed that the T1 transgenic plants from event PNO2 had significantly higher ($p<0.05$) plant height than non-transgenic seedlings when exposed to water stress.

Leaves of both transgenic and non-transgenic plants were rolled 21-28 DPWS application. However, leaves of non-transgenic plants were rolled more than those of transgenic plants. ANOVA (Appendix 30) revealed that leaf width for transgenic plants derived from PNO2 was non-significantly higher ($p>0.05$) than that for non-transgenic plants. However, a significantly higher leaf width was observed for PNO4 derived plants than for the related non-transgenic plants (Table 7.2).

After watering, most of the stressed transgenic plants recovered fully. However, non-transgenic plants were incapable of recovery, remaining wilted. The mass of plants, 2 days after watering, is shown in Figure 7.10. Analysis of variance (Appendix 32) revealed lack of significant difference ($p>0.05$) in plant biomass between transgenic events PNO4 and PNO2. However, the two transgenic plants had a significantly higher biomass than the non-transgenic plants.
**Figure 7.10:** Biomass of water-stressed transgenic and non-transgenic plants at the fourth leaf stage of growth. Data are means of 10 plants and horizontal bars show standard errors.
7.4 DISCUSSION

*PARP1* silencing was successfully demonstrated in plants stably transformed with the amiRNA1 transgene. Under normal (unstressed) conditions, transgenic plants appeared normal physiologically. This is because differences in chlorophyll, anthocyanin content and RWC observed between the transgenic and non-transgenic maize plants under unstressed conditions were not statistically significant. Similarly, inhibition of the *PARP* genes in *A. thaliana* and *B. napus* plants is not associated with any physiological alteration (De Block *et al.*, 2004; Schulz *et al.*, 2012). However, under moderate oxidative stress conditions transgenic plants had higher RWC than non-transgenic. In addition, the transgenic plants revealed significantly higher content of chlorophyll *a* and *b* when exposed to moderate MV induced oxidative stress. The anthocyanin content of transgenic plants was also higher than that of non-transgenic plants under moderate oxidative stress. Severe oxidative stress caused a decrease in the chlorophyll content of both the transgenic and non-transgenic plants. However, three T₁ transgenic events PNO4B3, PNO2C6 and PNO15E4 maintained significantly higher (*p* < 0.05) levels of chlorophyll than their related non-transformed plants under severe oxidative stress conditions. Significantly lower anthocyanin content (*p* < 0.05) was recorded in event PNO4B3 and PNO2C6 compared to their related control plants under moderate and high oxidative stress.
Plants ability to stay green and enhance photosynthesis under stress can be indirectly determined by measuring the chlorophyll content. This means that plants with enhanced chlorophyll content maintain higher photosynthetic rates. This therefore applies to events which were found to maintain higher chlorophyll content than control plants under moderate and severe oxidative stress. Inhibition of PARP genes in *A. thaliana* and *B. napus* shows a high association with reduced accumulation of anthocyanins. In addition to reduced levels of anthocyanins, inhibition of PARP results in reduced ascorbate production (Schulz *et al*., 2012).

In this study, transgenic plants derived from the T₀ event PNO2 and PNO4 performed better under drought stress compared to non-transgenic plants. This is due to increased growth and biomass accumulation in the transgenic plants relative to the non-transgenic plants. These results agree well with reports that plants with reduced PARP1 expression have increased growth (De Block *et al*., 2004) and biomass (Schulz *et al*., 2012) under drought.
CHAPTER EIGHT

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

8.1 General discussion

So far, very few tropical maize genotypes have been identified as readily regenerable, including CML216, IL3, CML144, CML442 and CML395 (Ombori et al., 2008; Rasha et al., 2008; Mgutu, 2011; Muoma et al., 2011; Bedada et al., 2014). In this study, two tropical maize genotypes (TL03B6754A-20 and TL03B6757-68) were found to be highly responsive to induction of embryogenic callus. Highly embryogenic callus tissues are useful in different biotechnology applications. They are highly desirable in particle bombardment-mediated gene transfer. They are also the tissues of choice in crop improvement through somaclonal variation or for long-term maintenance of plant tissue cultures. In addition, they are useful in development of maize protoplasts. The TL03B6754A-20 and TL03B6757-68 genotypes are also highly regenerable. This therefore adds to the number of genotypes already reported as possessing superior embryogenic and regeneration characteristics. A genotype with a high embryogenic callus induction and plant regeneration ability makes it very attractive for gene transfer experiments and genomic studies.
From this study, two single T-DNA binary vectors containing the amiRNA-PARP1 transgene were developed. In these vectors, the *pmi* gene is present as the plant selectable marker gene. The *pmi* is an effective (Negrotto *et al.*, 2000) and safe (Jaiwal *et al.*, 2002) alternative to the antibiotic and herbicide resistance genes commonly used in gene transfer experiments. Six binary vectors having multiple T-DNA regions were also developed. Three out of the six vectors (that is pMarkfree2.1, pMarkfree2.2 and pMarkfree3.0) had an empty T-DNA region containing a multiple cloning site in which any gene of interest can be inserted. However, these vectors had the *bar* gene which is highly preferred in transformation of monocot plants. They also lack genes for quick identification of transgenic tissues and plants. The amiRNA3-PARP1 transgene was inserted into the empty T-DNA regions of the three vectors (pMarkfree2.1, pMarkfree2.2 and pMarkfree3.0) to produce the cotransformation vectors pMarkfree3.1, pMarkfree3.2 and pMarkfree4.0. All the vectors were developed using conventional cloning procedures (Sambrook *et al.*, 1989) which are laborious and time consuming.

The main challenge to realization of high transformation potential for tropical maize is due to their resistance to gene transfer methods, especially those mediated by *A. tumefaciens*. In this study the transformation response of the TL03B6754A-20 and TL03B6757-68 genotypes was evaluated. They were found to be very
responsive to transformation with the *pmi* and *bar* genes. Using these SMGs, transgenic tissues were recovered from the genotypes at the same rate as the highly transformable genotype CML216. This means that the genotypes can be considered for any trait improvement through *A. tumefaciens*-mediated gene transfer. There is still need for identification of genotypes with very low variation in genetic transformation and regeneration under diverse media and growth conditions. Since these traits are controlled at the gene level, QTL and gene transfer studies may help unlock the potential of tropical maize to transformation and regeneration.

In this study, “clean gene” plants were produced using two approaches: 1) SMG replacement approach, whereby the *bar* gene was replaced with the *pmi* gene in plant transformation vectors. 2) Cotransformation of plants with multiple T-DNAs and segregating SMG-free plants. To achieve this three cotransformation vectors (pMarkfree3.1, pMarkfree3.2 and pMarkfree4.0) each bearing two T-DNA regions were developed. One T-DNA region harboured the GOI (amiRNA-PARP1) while the SMG (*bar*) was on a separate T-DNA region. Transformation of maize with the vectors yielded transgenic tissues at a frequency similar to that for a single T-DNA vector. SMG-free T₁ plants were recovered from selfed cotransformed parental plants. This is the first study that explored the potential of production of amiRNA-PARP1 transgenic marker-free tropical maize plants using
cotransformation vectors. The study therefore opens doors for production of trait-improved transgenic crops that are free of SMG. Production of SMG-free plants has implications on biosafety and environmental safety. SMG-free plants are considered safer and therefore more acceptable than plants having SMGs.

From the study, three independent T₁ CML216 plants having a downregulated PARP1 gene expression were produced. This implies that an amiRNA1 transgene that was stably integrated in the genome of the plants had downregulated the expression of maize PARP1 gene. In the nucleus, amiRNA1 transgene undergoes processing by dicer to produce the amiRNA double stranded transcript (Kurihara and Watanabe, 2004). The duplex is then transported out of the nucleus into the cytoplasm where it dissociates and the antisense strand gets incorporated into the RISC. In the RISC, the amiRNA acts as a probe to guide degradation of the target mRNA through complementary base pairing (Hammond et al., 2000).

Drought and oxidative stress tolerance was expressed in two CML216 plants that had a silenced PARP1 gene. Under oxidative and drought stress, transgenic maize plants accumulated more chlorophyll and biomass than non-transgenic plants. In addition, the plants had a higher RWC and plant growth but reduced anthocyanin content. Similar findings have been reported for A. thaliana and B. napus plants (De Block et al., 2004; Schulz et al., 2012). These results imply that silencing of
the PARP1 gene is an effective approach for enhancement of drought tolerance in maize. This approach can be extended to any crop since all plants contain the PARP genes that can be targeted for silencing using amiRNA.

8.2 Conclusions

The TL03B6754A-20 and TL03B6757-68 in bred lines have very high potential for embryogenic callus formation and plant regeneration. In addition the genotypes are highly transformable. As hypothesized, it is concluded from this study that the response to regeneration and transformation is dependent on the genotype used.

New cotransformation binary vectors (pMarkfree3.0, pMarkfree2.1 or pMarkfree2.2) have been developed in this study. The developed vectors are as effective as ordinary single T-DNA binary vectors in plant transformation. The new cotransformation vectors are capable of generating marker-free plants efficiently. Therefore the vectors can be very useful in recovery of marker-free plants containing one or more transgenes of interest. The transgene (s) of interest can easily be subcloned into the multiple cloning site of either the pMarkfree3.0, pMarkfree2.1 or pMarkfree2.2 vector and used in plant transformation. Marker-free plants can then be produced in T1 progenies after self-pollination of the parental plants.
Transgenic plants having an amiRNA1-PARP1 transgene stably integrated in their genomes have reduced expression of the PARP1 gene. Reduction in expression of tropical maize PARP1 gene results in tolerance to oxidative stress and drought stress at the seedling level. In the long term, the drought tolerant amiRNA-PARP1 transgenic maize plants produced in this study can help stabilize maize yields and increase production under water stressed conditions. This will impact targeted farmers and their households by increasing their incomes thereby improving their livelihoods.

8.3 Recommendations

The protocol for callus induction described in this study is highly suitable, and therefore recommended, for induction of highly embryogenic callus tissues from the genotypes TL03B6754A-20 and TL03B6757-68. It will be interesting to see how different types and combinations of hormones and media will influence the embryogenic and regeneration capacity for the other non-responsive maize inbred lines used in this study.

Inbred line TL03B6754A-20 and TL03B6757-68 are highly responsive to transformation when the bar and pmi genes are used as selectable markers.
Therefore the inbred lines can be considered as alternatives to the commonly used plant models for tropical maize transformation such as CML216.

In this study, three different cotransformation vectors were developed. The vectors (pMarkfree3.0, pMarkfree2.1 and pMarkfree2.2) are useful for cloning genes of interest because they have a multiple cloning site comprising recognition sites for common restriction enzymes. However, the vectors can be made more user friendly by making them gateway compatible. In addition introduction of a reporter gene such as \textit{Egfp} or \textit{gus} into the \textit{bar} T-DNA will facilitate quicker identification of transformed tissues and plants. Replacement of the \textit{bar} gene with the \textit{nptII} and \textit{hpt} gene will extend the application of the marker-free vectors to both monocots and dicots. The cotransformation vectors were demonstrated as an effective way of removing a SMG from tobacco and maize. The system can be applied in other agronomically important species to help produce ‘clean’ plants containing desirable transgene(s).

Transgenic plants produced in this study were tolerant to high oxidative and drought stress under controlled growth chamber conditions. Further studies under glasshouse and field conditions will need to be performed to establish if tolerance at the seedling level translates to tolerance at the reproductive stage. Other transgenic plants were produced in this study and were not evaluated. There is
therefore need to evaluate them for stable transgene integration, expression and tolerance to drought. Finally, the remaining phases of transgenic product development and release must be realized for the developed products to have the desired impact on farmers’ livelihood and food security situation of the East African community.
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APPENDICES

Appendix 1: Preparation of phenol:chloform:isoamylalcohol (25:24:1)

One hundred grams of crystalline phenol (MW 94.14) was placed in a jar and an equal volume of sdH2O added. The jar was capped and placed in 68 °C water baths until the crystals melted. The cap was tightened and the aqueous and organic phases mixed until they formed a fine emulsion. The jar was placed at 4 °C overnight or until phases completely separated. The aqueous layer was aspirated off and replaced with 0.5M Tris-HCL (pH 8.0). A clean stir bar was placed in the jar and the contents mixed well for about 1 hour. The aqueous layer was aspirated off and replaced with 0.1M Tris (pH 8.0) and stirring continued. This step was repeated four times or until pH 8 of the aqueous phase was achieved. The pH of the aqueous layer was checked with pH paper. Twenty milliliter aliquots of equilibrated phenol were overlaid with 10 ml of 0.1M Tris (pH 8.0) and stored at in a freezer at -20 °C. Cloroform and 4ml isoamylalcohol (196 ml) were mixed together in a glass measuring cylinder. Ten millilitres of this mixture was measured accurately and added into a falcon tube containing 10 ml of equilibrated phenol. The mixture was overlaid with 0.1M Tris (pH 8.0) and stored upright on a tube rack in a -20 °C freezer.

Appendix 2: ANOVA for the effect of genotype on frequency of transformation using pNOV2819-UbiamRNAl

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<th>P-Value</th>
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Appendix 3: ANOVA for the effect of binary vector on the frequency of recovery of transgenic tissues from the inbred line CML216

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**Appendix 4:** ANOVA for the effect of cotransformation binary vector on the frequency of recovery of transgenic tissues from different tropical maize genotypes

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**Appendix 5:** ANOVA for the effect of MV treatment on chlorophyll a content of transgenic (PNO4B3) and non-transgenic plants.

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**Appendix 6:** ANOVA for the effect of MV treatment on chlorophyll a content of transgenic (PNO4B5) and non-transgenic plants.

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**Appendix 7:** ANOVA for the effect of MV treatment on Chlorophyll a content of transgenic (PNO2C2) and non-transgenic plants.

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**Appendix 8:** ANOVA for the effect of MV treatment on Chlorophyll a content of transgenic (PNO2C6) and non-transgenic plants.

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**Appendix 9:** ANOVA for the effect of MV treatment on Chlorophyll a content of transgenic (PNO15E4) and non-transgenic plants.

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Appendix 10: ANOVA for the effect of MV treatment on Chlorophyll a content of transgenic (PNO15E5) and non-transgenic plants.

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Appendix 11: ANOVA for the effect of MV treatment on Chlorophyll b content of transgenic (PNO4B3) and non-transgenic plants.

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Appendix 12: ANOVA for the effect of MV treatment on Chlorophyll b content of transgenic (PNO4B5) and non-transgenic plants.

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Appendix 13: ANOVA for the effect of MV treatment on Chlorophyll b content of transgenic (PNO2C2) and non-transgenic plants.

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Appendix 14: ANOVA for the effect of MV treatment on Chlorophyll b content of transgenic (PNO2C6) and non-transgenic plants.

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Appendix 15: ANOVA for the effect of MV treatment on Chlorophyll b content of transgenic (PNO15E4) and non-transgenic plants.

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**Appendix 16:** ANOVA for the effect of MV treatment on Chlorophyll b content of transgenic (PNO15E5) and non-transgenic plants.

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**Appendix 17:** ANOVA for the effect of MV treatment on anthocyanin content of transgenic (PNO4B3) and non-transgenic plants.

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**Appendix 18:** ANOVA for the effect of MV treatment on anthocyanin content of transgenic (PNO4B5) and non-transgenic plants.

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### Appendix 19: ANOVA for the effect of MV treatment on anthocyanin content of transgenic (PNO2C2) and non-transgenic plants.

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### Appendix 20: ANOVA for the effect of MV treatment on anthocyanin content of transgenic (PNO2C6) and non-transgenic plants.

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### Appendix 21: ANOVA for the effect of MV treatment on anthocyanin content of transgenic (PNO15E4) and non-transgenic plants.

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Appendix 22: ANOVA for the effect of MV treatment on anthocyanin content of transgenic (PNO15E5) and non-transgenic plants.

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Appendix 23: ANOVA for the effect of MV treatment on relative water content of transgenic and non-transgenic plants.

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Appendix 24: Concentration of total RNA extracted from different plants and determined using NanoDrop 2000/2000c spectrophotometer

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<th>A280</th>
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<th>Factor</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>A1</td>
<td>10.5</td>
<td>ng/μl</td>
<td>0.263</td>
<td>0.150</td>
<td>1.75</td>
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<td>RNA</td>
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<td>ng/μl</td>
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<td>0.474</td>
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</tr>
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<td>B3</td>
<td>34.7</td>
<td>ng/μl</td>
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<td>4</td>
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<td>ng/μl</td>
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<td>6</td>
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<td>ng/μl</td>
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<td>E5</td>
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<td>ng/μl</td>
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<td>0.351</td>
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<tr>
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<td>0.09</td>
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<tr>
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<td>ng/μl</td>
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<td>0.224</td>
<td>1.93</td>
<td>0.25</td>
<td>RNA</td>
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</table>
Appendix 25: Concentration of cDNA prepared from different total RNA prepared from different plants and determined using NanoDrop 2000/2000c spectrophotometer

<table>
<thead>
<tr>
<th>#</th>
<th>Sample ID</th>
<th>Nucleic Acid Conc.</th>
<th>Unit</th>
<th>A260</th>
<th>A280</th>
<th>260/280</th>
<th>260/230</th>
<th>Sample Type</th>
<th>Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WT</td>
<td>551.5</td>
<td>ng/µl</td>
<td>11.030</td>
<td>6.179</td>
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<td>1.21</td>
<td>DNA</td>
<td>50.00</td>
</tr>
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<td>2</td>
<td>A1</td>
<td>550.0</td>
<td>ng/µl</td>
<td>10.999</td>
<td>6.302</td>
<td>1.75</td>
<td>1.21</td>
<td>DNA</td>
<td>50.00</td>
</tr>
<tr>
<td>3</td>
<td>A2</td>
<td>547.6</td>
<td>ng/µl</td>
<td>10.953</td>
<td>6.261</td>
<td>1.75</td>
<td>1.13</td>
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</tr>
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<td>4</td>
<td>B3</td>
<td>543.2</td>
<td>ng/µl</td>
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<td>6.136</td>
<td>1.77</td>
<td>1.17</td>
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<td>1.17</td>
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<td>6.111</td>
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<td>DNA</td>
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<td>E4</td>
<td>549.1</td>
<td>ng/µl</td>
<td>10.981</td>
<td>6.142</td>
<td>1.79</td>
<td>1.08</td>
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<td>J1</td>
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<td>1.21</td>
<td>DNA</td>
<td>50.00</td>
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<td>10</td>
<td>J2</td>
<td>543.6</td>
<td>ng/µl</td>
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<td>6.267</td>
<td>1.73</td>
<td>1.11</td>
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</table>

Appendix 26: Concentration of primers for qRTPCR as determined using NanoDrop 2000/2000c spectrophotometer.

<table>
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<th>#</th>
<th>Sample ID</th>
<th>Nucleic Acid Conc.</th>
<th>Unit</th>
<th>A260</th>
<th>A280</th>
<th>260/280</th>
<th>260/230</th>
<th>Sample Type</th>
<th>Factor</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>PARP1-F</td>
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<td>ng/µl</td>
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<td>1.119</td>
<td>1.44</td>
<td>1.93</td>
<td>ssDNA</td>
<td>33.00</td>
</tr>
<tr>
<td>2</td>
<td>PARP1-R</td>
<td>66.3</td>
<td>ng/µl</td>
<td>2.010</td>
<td>1.159</td>
<td>1.73</td>
<td>2.15</td>
<td>ssDNA</td>
<td>33.00</td>
</tr>
<tr>
<td>3</td>
<td>EL1-A_F</td>
<td>55.1</td>
<td>ng/µl</td>
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<td>0.976</td>
<td>1.71</td>
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<td>4</td>
<td>EL1-A_R</td>
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<td>ng/µl</td>
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<td>1.293</td>
<td>1.43</td>
<td>1.90</td>
<td>ssDNA</td>
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<td>zmTUA-F</td>
<td>59.4</td>
<td>ng/µl</td>
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<td>1.47</td>
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<td>zmTUA-R</td>
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<td>2.059</td>
<td>1.654</td>
<td>1.95</td>
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<td>7</td>
<td>GAPDH-F</td>
<td>56.2</td>
<td>ng/µl</td>
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<td>8</td>
<td>GAPDH-R</td>
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<td>ng/µl</td>
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<td>0.980</td>
<td>1.86</td>
<td>2.14</td>
<td>ssDNA</td>
<td>33.00</td>
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</table>


<table>
<thead>
<tr>
<th>#</th>
<th>Sample ID</th>
<th>Nucleic Acid Conc.</th>
<th>Unit</th>
<th>A260</th>
<th>A280</th>
<th>260/280</th>
<th>260/230</th>
<th>Sample Type</th>
<th>Factor</th>
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<td>1.81</td>
<td>1.43</td>
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<td>4</td>
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<td>ng/µl</td>
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**Appendix 28:** ANOVA for the effect of drought stress on height of T₁ seedlings derived from the transgenic CML216 event PNO4.

<table>
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<th>Sum of Squares</th>
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<th>P-Value</th>
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<tbody>
<tr>
<td>Days after withholding</td>
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<td>6.59</td>
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<td>water</td>
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<td>39.76</td>
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<td>water * Genotype</td>
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</table>

**Appendix 29:** ANOVA for the effect of drought stress on height of T₁ seedlings derived from the transgenic CML216 event PNO2.

<table>
<thead>
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<th>Sum of Squares</th>
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<td>0.34</td>
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**Appendix 30:** ANOVA for the effect of drought stress on leaf width of $T_1$ seedlings derived from the transgenic CML216 event PNO4.

<table>
<thead>
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<th>Sum of Squares</th>
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<th>P-Value</th>
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<tbody>
<tr>
<td>Days after withholding water</td>
<td>3</td>
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<td>0.01</td>
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<tr>
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</table>

**Appendix 31:** ANOVA for the effect of drought stress on leaf width of $T_1$ seedlings derived from the transgenic CML216 event PNO2.

<table>
<thead>
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<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-Value</th>
<th>P-Value</th>
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</thead>
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<tr>
<td>Residual</td>
<td>72</td>
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</table>
Appendix 32: ANOVA for the effect of drought stress on biomass of T₁ seedlings derived from the transgenic event PNO2 and PNO4 and non-transgenic plants.

<table>
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<tr>
<th></th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
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<td>&lt;0.0001</td>
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<td>Residual</td>
<td>27</td>
<td>0.141</td>
<td>0.005</td>
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</table>

Appendix 33: Preparation of buffers for use in plasmid extraction

Resuspension buffer

Resuspension buffer is composed of 50 mM Tris-HCl, pH8.0, 10 mM EDTA and 100 μg/ml RNaseA. It was prepared by placing 6.06 g of Tris base in a graduated 1L reagent bottle and dissolved in 800 ml distilled water. EDTA-2H₂O (3.72 g) was added and the pH adjusted to 8.0 with 1M HCl. The volume was adjusted to 1000 ml and the solution sterilised by autoclaving at 121 °C, 15 psi for 15 minutes. Upon cooling to room temperature, 100 mg RNaseA was added to the buffer and the buffer stored at a temperature of 4 °C.

Lysis buffer

Lysis buffer comprised 200 mM NaOH and 1% (w/v) SDS. To prepare it, 8.09 g of NaOH pellets were dissolved in 950 ml dH₂O. This was followed by addition of 50 ml of 20% (v/v) SDS solution. The lysis buffer was stored at room temperature.

Neutralization buffer

Neutralization buffer consisted of 3.0 M potassium acetate, pH 5.5. Potassium acetate (294.5 g) was dissolved in 500 ml dH₂O. The pH was adjusted to 5.5 with about 110 ml glacial acetic acid. The volume was adjusted to 1 litter with dH₂O. Neutralization buffer was kept at 4 °C after sterilizing by autoclaving.

Tris-HCl (2M), pH7.6

To prepare 500 ml of 2M Tris-HCl, 157.64 g Tris-HCl (MW: 157.64) was placed in a reagent bottle containing 450 ml dH₂O and a stir bar. The pH was adjusted the pH to 7.6 using 1M HCl. The volume was brought to 500 ml with dH₂O before sterilizing by autoclaving. The buffer was stored at room temperature.
Elution buffer (buffer EB)

Elution buffer is a solution of 10 mM Tris-HCl, pH 7.6. Elution buffer was prepared by measuring 5 ml of a 2 M Tris-HCl stock solution into 950 ml dH₂O. The pH of the solution was adjusted to 7.6 using 1N HCl. The solution was sterilised by autoclaving and stored at room temperature.

Appendix 34: Preparation of buffers and chemicals for use in agarose gel electrophoresis

Na₂EDTA.2H₂O (0.5M) stock

About 300 ml of dH₂O was placed into a reagent bottle and 93.05 g of Na₂EDTA.2H₂O (MW 372.24) added. Ten grams of NaOH pellets was added gradually and the mixture stirred vigorously using a magnetic stirrer. NaOH was added until the EDTA dissolved. The volume was adjusted to 480 ml with dH₂O before adjusting pH to 8.0. The final volume was brought up to 500 ml. The solution was sterilised by autoclaving and stored at room temperature.

TAE (50×) stock

About 600 ml dH₂O was added to a 1 liter reagent bottle and 242 g trizma base, 100 ml of 0.5 M EDTA pH 8.0, and 57.2 ml glacial acetic acid were added and mixed. The final volume was brought to 1000 ml with dH₂O. To make 1× TAE, 20 ml of 50× TAE stock was added to 980 ml dH₂O and mixed vigorously.

DNA loading buffer (6×) stock

DNA loading buffer (6×) is composed of 10 mM Tris-HCl pH 7.6, 0.03 % (w/v) bromophenol blue, 60 mM EDTA and 60% (v/v) glycerol. One hundred millilitre of the buffer was prepared by first mixing 60 ml of sterile glycerol with 34 ml of sdH₂O in a sterile beaker. This was followed by addition of 500 μl of 2M Tris-HCl, pH7.6 and 6 ml of 1 M EDTA. Finally, 0.03 g bromophenol blue dye was added and mixed vigorously until it dissolved completely.

Appendix 35: Preparation of media and stock solutions for use in E. coli transformation

SOB medium

SOB medium comprises 2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 20 mM MgSO₄. One liter of SOB medium was prepared by dissolving 20 g tryptone, 5 g yeast extract, 0.584 g NaCl and
0.186 g KCl in 1000 ml dH₂O. The pH was adjusted to 7.0 and autoclaved. Upon cooling to about 50 °C, 10 ml MgSO₄ was added from a 2 M MgSO₄ stock solution.

Glucose stock (2 M)

Ten millilitres of 2 M glucose solution was prepared by dissolving 3.604 g glucose in a 10 ml dH₂O. The solution was sterilized by filter sterilization.

SOC medium

SOC medium is composed of 2% (w/v) Tryptone, 0.5% (w/v) Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM MgSO₄ and 20 mM glucose. SOC is same as SOB except that it contains 20 mM glucose. It was prepared by adding 1 ml of 2 M glucose stock to 99 ml SOB medium.

Appendix 36: Preparation of media and stock solutions for use in transformation of Agrobacterium tumefaciens

YM medium

YM medium for culturing LBAA4404 is composed of 0.04% (w/v) yeast extract, 1.0% (w/v) manittol, 1.7 mM NaCl, 0.8 mM MgSO₄, 7H₂O, and 2.2 mM K₂HPO₄·3H₂O. One liter of YM medium was prepared by dissolving 0.4 g yeast extract, 10 g manittol, 0.1 g NaCl, 0.2 g MgSO₄·7H₂O and 0.5 g K₂HPO₄·3H₂O in 900 ml dH₂O and the pH adjusted to 7.0. The volume was brought up to 1000 ml with dH₂O and the medium autoclaved at 121 °C, 15 psi for 15 minutes. It was cooled to room temperature before use.

Rifampicin stock (25 mg/ml)

About 1.25 g of rifampicin (Phytotechnology laboratories, shawnee mission, KS, USA) was placed in a sterile 50 ml tube and 50 ml of DMSO added. The mixture was mixed vigorously until all rifampicin crystals dissolved. The solution was aliquoted into sterile 2 ml tubes in volumes of 1 ml and stored at -20 °C. To obtain a final rifampicin concentration of 1 mg/l, 40 μl of the 25 mg/ml rifampicin stock was added to 1 liter of cooled YM medium.

Kanamycin stock (50 mg/ml)

Two and half grams of kanamycin monosulfate salt (Duchefa, Haarlem Netherlands) was dissolved in 10 ml of 1N NaOH before toping up to 50 ml with sdH₂O. The solution was aliquoted into 2 ml tubes in volumes of 2 ml and stored at -20 °C. One milliliter of 50 mg/ml Kanamycin stock was added to 1 liter YM to obtain a final kanamycin concentration of 50 mg/l in the medium.
Spectinomycin stock (100 mg/ml)

Five grams spectinomycin (Phytotechnology laboratories, shawnee mission, KS, USA) was dissolved in 50 ml of sdH₂O. It was aliquoted into 2 ml tubes and kept at -20 °C. One milliliter was obtained from the 100 mg/ml spectinomycin stock and added to 1 liter YM to make the final concentration of spectinomycin in the YM, 100 mg/l.

Appendix 37: Preparation of media and reagents for use in preparation of competent bacteria

LB broth and agar

To prepare 1 liter of LB broth, 10 g trypton, 5 g yeast extracts and 5 g NaCl were placed in a reagent bottle. Distilled water was added to the 1 liter mark and pH adjusted to 6.0. The medium was autoclaved at 121 °C, 15 psi for 15 minutes and allowed to cool to room temperature before use. Preparation of LB agar is similar to that for LB broth except for the addition of 15 g/l of bactoagar in LB agar.

CaCl₂·2H₂O stock (1M)

Exactly 73.5 g of CaCl₂·2H₂O (MW: 147.0) was placed in a reagent bottle and the volume brought up to 500 ml with dH₂O. The solution was autoclaved and cooled to room temperature before use. A concentration of 100 mM CaCl₂ was prepared by mixing 2 ml of 1M CaCl₂·2H₂O with 18 ml of sdH₂O in a sterile 50 ml tube.

Appendix 38: Preparation of hormones for use in preparation of tobacco SM media

BAP (1 mg/ml) stock solution:

Potassium hydroxide (1N) was added to 0.1 g BAP until it dissolved. The solution was topped up to 100 ml with sdH₂O and stored at 4 °C. A final BAP concentration of 1 mg/l was obtained by adding 1 ml of the prepared BAP stock solution to 1L of SM medium.

NAA (1 mg/ml) stock solution:

Potassium hydroxide solution (1N) was added to 0.1 g NAA until it dissolved. The volume of the solution was made up to 100 ml with sdH₂O and stored at 4 °C. One hundred microliters of the stock NAA solution was added to 1L of SM to give a final NAA concentration of 0.1 mg/l.
Appendix 39: Preparation of antibiotics for preparation of selective SM

**Timentin stock solution (300 mg/ml)**

Three grams timentin (Duchefa, Haarlem Netherlands) was weighed and placed in a sterile 25 ml universal bottle and 10 ml of sdH₂O added. After mixing, the solution was aliquoted into 2 ml tubes and stored at -20 °C. One milliliters of the 300 mg/ml Timentin stock solution were added to 1 liter SM medium to give a final timentin concentration of 300 mg/l.

**Kanamycin stock solution (300 mg/ml)**

Ten millilitres of sdH₂O was added to 3 g of kanamycin monosulfate (Phytotechnology Laboratories, Shawnee Mission, KS, USA) and mixed well. The solution was dispensed in 2 ml tubes then kept at -20 °C. A final kanamycin concentration of 300 mg/l was achieved by adding 1 ml of the prepared 300 mg/ml kanamycin stock solution to 1 liter of SM medium.

Appendix 40: Preparation of chemicals and buffers for southern hybridization

**20× SCC and 0.25 M HCL**

One hundred seventy five grams sodium chloride (MW 58.44) and 88 g trisodium citrate (MW 294.10) were mixed in about 800 ml dH₂O using a stirring bar. The volume was adjusted to 1000 ml and the pH adjusted to 7.0 using either 1 N NaOH or 0.1 M HCL. The solution was sterilized by autoclaving at 121 ºC for 15 min and stored at room. Concentrated HCl (12 M) was diluted to 0.25 M.

**SDS (20%)**

About 200 ml of dH₂O was placed in a conical flask containing a stir bar and 50 g sodium dodecyl sulfate (MW 288.4) added gradually and mixed. The volume was adjusted to 250 ml and the solution kept at room temperature.

**Na₂HPO₄, pH 7.2 (1M)**

1M Na₂HPO₄·7H₂O (68.4 ml) was placed in a reagent bottle containing a stir bar and 1M NaH₂PO₄ added until the pH reached 7.2. The volume was adjusted to 100 ml with distilled water and the buffer autoclaved and stored at room temperature.

**Denhardts solution (50×)**

Thirty five millilitres of sdH₂O was placed in a sterile 50 ml tube and 0.5 g ficoll (MW 400,000), 0.5 g polyvinylpyrrolidone (PVP40) and 0.5 g bovine serum
albumin fraction V (Sigma-Aldrich, St. Louis, MO, USA) added and mixed vigorously. The volume was adjusted to 50 ml and stored at 4 ºC.

**Urea hybridization solution**

Sixty millilitres of dH₂O was placed in a beaker and 2 ml of 1M Na₂HPO₄ (pH 7.2) buffer, 10 ml of 50× denhardt solution, 30 ml of 20× SCC added, 12 g of urea and 1 ml of denature salmon sperm DNA (10 mg/ml) added and mixed. The volume was adjusted to 100 ml and the pH adjusted to 7.2. The solution was sterilized by passing through a 0.2 µM filter and stored at 4 ºC.

**Blocking solution**

This solution was prepared by dissolving 7.3 g NaCl, 2.4 g Na₂HPO₄ (dibasic), 1.0 g NaH₂PO₄ (monobasic), and 50 g SDS in 800 ml water. The pH of the solution was adjusted to 7.2 before topping up to 1000 ml with sdH₂O. The solution was autoclaved and stored at room temperature.

**Wash solution I and II**

Wash solution I was prepared by diluting the blocking solution 1 to 10 in water. To prepare 1000 ml of 10× wash solution II, 12 g Tris-HCl base, 5.8 g NaCl and 2 g MgCl₂ was dissolved in about 800 ml water and the pH of the solution adjusted to 9.5 with HCl. The volume of the solution was adjusted to 1000 ml with water. This solution was diluted to 1× before use.

**Appendix 41: Preparation of MV for induction of oxidative stress**

A stock of 50 mM MV (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving 12.86 g MV in 1000 ml sdH₂O. Working MV concentrations of 5 and 30 µM were prepared by diluting from the 50 mM stock solution.