# BIOLISTIC-MEDIATED TRANSFORMATION OF *ERAGROSTIS CURVULA* WITH THE HSP 12 GENE

**SANDILE WELCOME NCANANA**

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# Abbreviations

| | |
|---|---|
| BAP | 6-benzylaminopurine |
| bp | base pairs |
| CaMV35S | cauliflower mosaic virus 35 S promoter |
| CTAB | cetyltrimethylammonium bromide |
| $^{0}$C | degrees Celsius |
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| DNA | deoxyribonucleic acid |
| dH$_2$O | distilled water |
| dNTP | deoxynucleoside 5' trisphosphate |
| *E. coli* | *Escherichia coli* |
| *E. curvula* | *Eragrostis curvula* |
| EDTA | ethylenediaminetetraacetate |
| g | gram |
| GFP | green fluorescent protein |
| GTC | guanidine thyocyanate |
| GUS | *β*-glucuronidase |
| HCl | hydrochloric acid |
| h | hour(s) |
| Hsp | heat shock protein |
| IAA | indole 3-acetic acid |
| IPTG | isopropyl β-D thiogalactopyranoside |
| kDa | kilodaltons |
| kPa | kilopascals |
| LEA | Late Embryogenesis Abundant |
| LB | luria broth |
| M | molar |
| MALDI-TOF | matrix assisted laser desorption/ionization-time of flight |
| mg | milligram |
| ml | milliliter |

| | |
|---|---|
| mM | millimolar |
| min | minute(s) |
| mRNA | messenger ribonucleic acid |
| $MgCl_2$ | magnesium chlroride |
| MS medium | Murashige and Skoog medium |
| MW | molecular weight |
| NAA | α-naphthalene-acetic acid |
| NaCl | sodium chloride |
| nm | nanometer |
| NaOAc | sodium acetate |
| OD | optical density |
| PMSF | phenylmethanesulfonylchloride |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| RT-PCR | reverse transcription PCR |
| RNA | ribonucleic acid |
| *S. cerevisiae* | *Saccharomyces cerevisiae* |
| SDS | sodium dodecyl sulphate |
| sec | second(s) |
| TAE | Tris acetate EDTA |
| T-DNA | transfer-DNA |
| TDZ | thidiazuron |
| Ti-plasmid | tumour inducing plasmid |
| TWEEN 20 | polyoxyethylenesorbitan monolaurate |
| Tris-HCl | Tris-(hydroxymethyl) aminomethane-HCl |
| µl | microlitre |
| µm | micrometer |
| µg | microgram |
| V | volts |
| X-Gluc | 1-bromo-2-chloro-3-indolyl-β-glucuronic acid |

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# Abstract

*Eragrostis curvula* is a desiccation sensitive monocotyledonous plant and an economically important forage grass in southern Africa. This species has a potential to be improved for drought and salt resistance among other important agronomic traits. In this study, *E. curvula* was used as a model system to explore the feasibility of producing pasture grasses with increased tolerance to water deficit. To date, no reports have been published on transformation of this species. This study reports the transformation of *E. curvula* with *Saccharomyces cerevisiae* Hsp 12 gene using biolistic-mediated transformation.

Firstly, a tissue culture protocol was established for *E. curvula* that was suitable for transformation studies. Although this species has been previously regenerated *in vitro* using inflorescence tissues, this study established new protocols that utilize leaf and seed as source of material. The aim of which was to find the best regenerable tissue that could-be used for transformation studies. Plant regeneration was achieved from shoot explants cultured on MS medium supplemented with either 0.5 mg/l thidiazuron (TDZ) or 0.5 mg/l 6-benzylaminopurine (BAP) through the process of direct organogenesis. It was found that TDZ was the most effective cytokinin. Plant regeneration was also achieved from callus induced from immature leaves on MS medium supplemented with 2 mg/l 2,4-D, 0.01 mg/l BAP and 6 % (w/v) sucrose. Histological experiments performed gave clear evidence that plant regeneration from callus was through a process of indirect organogenesis. The regeneration protocol was combined with an optimized biolistic-mediated transformation protocol using the PDS-100/He apparatus of which both shoot explants and callus were used as target tissues.

In the molecular aspect of the study, the Hsp 12 gene was ligated in the *Sac* I restriction site of pCAMBIAUbeeQ vector. The successful cloning of the Hsp 12 gene was confirmed by PCR and restriction endonuclease digestion. The resultant vector pCAMBIAUbeeQHsp12 was purified and subsequently used for biolistic transformation of *E. curvula*. The regenerable shoot explants and callus tissue were bombarded with

DNA (pCAMBIAUBeeQHsp12) coated on gold particles. As a comparative study, *Agrobacterium tumefaciens* was transformed with pCAMBIAUBeeQHsp12 vector for subsequent transformation of *Nicotiana tabacum*.

Transient expression of GUS gene in transformed *E. curvula* shoot explants was visualized 72 h after bombardment. Optimized conditions for expression of GUS gene were gold microprojectiles, 7 cm travel distance and helium pressure of 9100 kPa. Transformed tissues were cultured on the regeneration medium without antibiotic selection. Putative transformants were generated and the presence of Hsp 12 gene was verified by PCR and its expression at RNA level was confirmed by RT-PCR. The presence of Hsp 12 protein in the transformed plants was analyzed using SDS-PAGE and MALDI-TOF peptide mass spectrometry.

# Chapter 1

# General introduction

## 1.1 Water deficit

All organisms, including plants, are dependent on water for life. It accounts for 85 % to 95 % of the weight of most plants. Most biochemical reactions in plants occur in water based solutions. Water deficit is the major abiotic factor that depresses plant growth and development and thus affects crop productivity. The restriction in crop productivity does not just affect areas classified as arid or semiarid, but also in areas where the evaporation demand greatly exceeds rainfall during growing season (Day, 1981). Plant water deficit occurs when the rate of transpiration exceeds that of water uptake, and can be caused by low temperature, drought and high salinity (Bonhert *et al.*, 1995). Water deficit can result in a concentration of solutes, changes in cell volume and membrane shape, disruption of water potential gradient, loss of turgor, disruption of membrane integrity and denaturation of proteins (Bray, 1997). The processes such as photosynthesis, respiration and growth are compromised, which may eventually lead to the death of a plant (Gaff, 1989).

## 1.2 Desiccation tolerant plants

Many plants cannot withstand severe, prolonged water stress, but a few are able to survive under severe environmental conditions (Sherwin and Farrant, 1995). The small number of plants called desiccation-tolerant or resurrection plants (Gaff, 1971) can survive drying to an air dry state (Bewley, 1979). This small number of plants can lose more than 90 % of their cellular water and continue full metabolism after rehydration (Gaff, 1971; Vertucci and Farrant, 1995; Bonhert, 2000).

The ability of vegetative tissues to withstand considerable water loss is common to many algae and lichens, and is also found in some liverworts, mosses and ferns. This trait is completely missing from gymnosperms, however, appears again in a few angiosperms (Bewley and Krochko, 1982; Bonhert, 2000). The factor that is common to all these organisms is survival at low water potential. Organisms that successfully deal with lower water potentials can either cope with limited water availability while maintaining high internal water concentration, or tolerate water loss. Thus the organisms that resist water loss are generally considered to be drought tolerant whereas those that survive in spite of the water loss are termed desiccation tolerant (Bewley, 1979; Vertucci and Farrant, 1995; Walters *et al.*, 2002).

Desiccation tolerant angiosperms are widely, but unevenly, distributed geographically. They occur in all continents except Antarctica (Alpert and Oliver, 2002). The highest number of known desiccation tolerant angiosperms are in southern Africa, western Australia and eastern South America (Gaff, 1977). Desiccation tolerant lower order plants grow mainly in the interstices and on the margins of the world's vegetation (Alpert and Oliver, 2002) whereas desiccation-tolerant vascular plants (Gaff, 1997) grow mostly on rocky outcrops where there is little shade and the water supply is limited (Porembski and Barthlott, 2000; Sherwin and Farrant, 1995). Desiccation tolerant plants include the poikilochlorophyllous angiosperms such as *Xerophyta viscosa* Baker and *Eragrostis nindensis*, which disassemble chlorophyll on drying, and the homiochlorophyllous angiosperms such as *Craterostigma wilmsii* and *Myrothamnus flabellifolius*, which retain chlorophyll but protect it from light by leaf folding and anthocyanin accumulation (Gaff, 1971; Farrant, 2000; Vander Willigen *et al.*, 2001).

## 1.3 Damage caused by water deficit

As already stated, water deficit is one of the abiotic stresses most affecting plant productivity. Under water deficit conditions, plants are affected both biochemically and physiologically. Although understanding of the biochemistry and physiology of water stress relies heavily on short-term experiments, usually on isolated cells or whole plants growing under conditions far from their natural environments (Yeo, 1998) some stress-coping mechanisms, such as changes in leaf size and stomatal density are usually observed only in long-term response stress (Amthor and McCree, 1990).

The loss of turgor is the first sign of water deficit in plants, and this occurs at water potentials of about –1 to –2 Mpa (Walters *et al.*, 2002). At lower water potentials, cells lose water and shrink. Shrinkage in leaf area is particularly observed in species which have relatively little sclerenchyma, e.g. in the *Scrophulariaccae.* In *Coleochloa* species and grasses the leaves become inrolled to a tight thread (Gaff, 1977). Other damage caused by water stress includes the disruption or loss of macromolecular integrity occurring as a result of the increase in protein and ion concentration during water deficit and/ or due to oxidative damage to plant cells caused by proliferation of reactive oxygen species. These reactive oxygen species can have deleterious effects on membranes, proteins and DNA (Smirnoff, 1993; Foyer *et al.*, 1994; McAinsh *et al.*, 1996).

## 1.4 Mechanisms of desiccation tolerance

### 1.4.1 Regulation of gene expression during dehydration

Water deficit resistance occurs when a plant withstands the imposed stress, and may arise from either tolerance or mechanisms that permit the avoidance of the stress. For the plant to tolerate or avoid stress it must able to recognize it first. The loss of water from the cell is perceived to trigger a cellular signal transduction pathway in which a physical stress is converted into a biochemical response. Changes such as decrease or loss of turgor, change in cell volume or membrane area and in concentration of solute can signal water deficit to the cell (Bray, 1997).

Following cellular perception of water loss, signaling mechanisms are activated to induce specific genes. One of the major signals operating during drought is the plant hormone abscisic acid (ABA). Water deficit and high salinity *inter alia* cause plants to increase production of ABA. Exogenous supply of ABA also induces a number of genes that respond to stress, including dehydration (Thomashow, 1998). Such genes encode proteins that include mitogen-activated protein (MAP) kinases, calcium-dependent protein kinases (CDPK) and enzymes involved in metabolism of phospholipids, e.g. phospholipase D (PLD) and Inositol- (1,4,5) triphosphate ($IP_3$) (Jonaki *et al.*, 1996; Mizoguchi *et al.*, 1997; Shinozaki *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000) and many others. All these proteins are involved in signaling cascades during water deficit.

Some of the genes that are induced during dehydration, however do not respond to exogenous ABA treatment (Shinozaki and Yamaguchi-Shinozaki, 1999; 2000) and both ABA-independent and ABA-dependent signal transduction pathways are reported (Shinozaki and Yamaguchi-Shinozaki, 2000). The transcription factors that control ABA-independent gene expression in response to dehydration and cold stress include cis-acting dehydration-responsive element (DRE) and cis-acting element (C-repeat) (CRT) and its DNA binding protein. All these elements within the promoter region are important in determining the regulation of the gene under different conditions (Yamaguchi-Shinozaki and Shinozaki, 1994; Thomashow, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000).

### 1.4.2 Protectants

Genes induced in plants that are subjected to water deficit are thought to function in protecting cells by producing important metabolic proteins and cellular protectants and also genes that are involved in transducing the stress response signal (Thomashow, 1998; Ingram and Bartels, 1996; Steponkus *et al.*, 1998).

During dehydration, some genes are upregulated and translated into functional proteins which are useful in the period of water deficit. Some of these proteins are involved in various pathways to synthesize compounds like osmolytes. These are compatible solutes that can accumulate to high levels without disrupting metabolism (Bray, 1997). Osmolytes include amino acids, such as proline, quaternary ammonium compounds e.g. glycine betaine, polyols and sugars e.g. fructans, trehalose and mannitol. These compounds raise the cytoplasmic osmotic pressure and thus maintain cellular turgor, and also stabilize proteins and membranes (Bray, 1997). Glycine betaine, for example, is one of the compatible solutes that has osmotic protection function and is known to protect proteins and enzyme activities under water deficits, and even stabilize membranes during freezing (Xing and Rajashekar, 1999). It has also been suggested that glycine betaine is involved in stabilizing protein tertiary structure and preventing or reversing the disruption of the tertiary structure of proteins caused by non-compatible solutes (Bateman *et al.*, 1992). Some solutes such as citrulline have also been proposed to act as radical scavengers in drought-tolerant watermelon plants (Akashi *et al.*, 2001).

Other protective mechanisms particularly reported to occur in angiosperms include: subcellular re-organization, e.g. the folding of the cell walls in the *Craterostigma wilmsii* and replacement of water in the vacuoles of the *Xerophyta* spp., to minimize mechanical damage related to the loss of turgor (Farrant, 2000; Vicré *et al*., 1999); dismantling of the photosynthetic apparatus and loss of chlorophyll in poikilochlorophyllous types, and leaf folding and chlorophyll shading in homoichlorophyllous type, for prevention of light related damage (Farrant and Sherwin, 1998; Farrant, 2000).

Mechanisms by which the cellular machinery is protected have been investigated in many studies. Such mechanisms include the induction of proteins such as osmotin, chaperones or heat shock proteins (Hsp) and Late Embryogenesis Abundant (LEA) proteins. LEA proteins are hydrophilic globular proteins that accumulate during final desiccation stage of seed development. These LEA proteins have also been found in vegetative organs during dehydration (Ingram and Bartels, 1996; Bray, 1997). Molecular chaperones play important roles in prevention of aggregation of unfolded proteins. They stabilize denatured proteins in order to limit damage caused by water loss (Morimoto *et al*., 1994; Boston *et al*., 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998). These proteins are further reviewed in this chapter since one of the ultimate aims of this thesis is to incorporate a gene Hsp 12 into a desiccation sensitive species in an attempt to improve tolerance to water deficit-stress.

### 1.4.2.1 Heat shock proteins

A severe increase in the temperature of an organism results in the induction of heat shock proteins. Heat shock proteins are found in highly conserved protein families, of which there are constitutive and heat-inducible members (Parsell and Lindquist, 1993). Besides induction by heat, Hsps were also found to be induced in other stressful treatments such high concentration of ethanol, arsenite, or heavy metals and during the normal functioning of the cells (Craig *et al*., 1993; Hendrick and Hartl., 1993; Parsel and Lindquist,1993; Morimoto *et al*., 1994; Netzer and Hartl, 1998). The induction of Hsp synthesis is also triggered by accumulation of foreign proteins, denatured proteins or proteins containing structurally destabilized amino acid substitutions in the cells (Goff and Goldberg, 1985; Ananthan *et al.*, 1986; Parsell and Sauer, 1989).

The ways in which Hsps could help the cells to cope with stress-induced damage to polypeptides include: 1) Hsps could promote degradation of abnormal proteins by their proteolytic activities. 2) Hsps could reactivate stress-damaged proteins (Parsell and Lindquist, 1993). Some Hsps function as molecular chaperones, the proteins that mediate the folding of other polypeptides and in some cases their assembly into oligomeric structures (Hightower, 1991; Ellis, 1990). They can also mediate in holding polypeptides in an unstructured form competent for transport across the membranes; maintaining proteins in a specific conformation required for their proper functioning; preventing aggregation of unfolded proteins and promoting renaturation of aggregated proteins (Gething and Sambrook, 1992; Hendrick and Hartl, 1993; Morimoto *et al.*, 1994; Boston *et al.*, 1996; Johnson and Craig, 1997; Netzer and Hartl, 1998). The most abundant Hsps or molecular chaperones are Hsp 60, Hsp 70 and Hsp 90. Hsp 70 in particular, protects cells against the damaging effects of various stresses. It binds to the damaged proteins and thereby protects the interactive domain and may prevent the proteins from aggregating (Sanchez *et al.*, 1993; Becker and Craig, 1993).

### 1.4.2.2 LEA proteins

As already stated above, LEA proteins are synthesized at the prior to of desiccation in maturing seeds and in vegetative organs exposed to water deficit. They are highly hydrophilic; as a result they have been predicted to play important roles such as in sequestration of ions to reduce the effective ionic strength, binding of water and they may also operate as chaperones (Bray, 1997; Martilla *et al.*, 1996). LEA proteins have been grouped into at least 6 families based on sequence homologies (Ingram and Bartels, 1996; Colmenero-Flores *et al.*, 1997). The feature common to all these groups is their high hydrophilicity and high percentage of the amino acid glycine (Baker *et al.*, 1988). Because they have been identified in a wide range of different species they are considered ubiquitous in plants (Ingram and Bartels, 1996; Close, 1997; Garay-Arroyo *et al.*, 2000). For instance, HVA1, which confers tolerance to water deficit and salt stress in rice, is a group 3 LEA protein from barley and 3 LE25, a group 4 LEA protein, is from tomato (Xu *et al.*, 1996).

Other LEA proteins include dehydrins which are present in a range of photosynthetic organisms, including lower plants, algae and cynobacteria (Close and Lammers, 1993; Close, 1997). Dehydrins, like other LEA proteins, probably function to mitigate the physiochemical problems encountered in the plant cytosol during dehydration by protecting the structural integrity of membranes and proteins (Baker *et al.*, 1988). More recently, a dehydrin gene has been implicated to have a vital role in the desiccation-tolerant *Eragrostis nindensis* (Zekarias Gebremedhin, unpublished).

### 1.4.2.3 The LEA-like protein Hsp 12

On the basis of some limited sequence homology between Hsp 12 and known heat shock proteins, Hsp 12 was identified as a putative heat shock protein (Praekelt and Meacock, 1990). Subsequently, there has been a suggestion that *Saccharomyces cerevisiae* Hsp 12 should rather be classified as a LEA-like protein because of the similarities shared by the two proteins (Mtwisha *et al.*, 1998). The suggested similarities include: 1) Both Hsp 12 and LEA proteins remained soluble at $80^0$ C, the temperature where non-chaperones would be expected to precipitate due to their high hydrophobic amino acid content. 2) Amino acid composition of Hsp 12 and pea group 1LEA p11 protein were showed to be similar (Russouw *et al.*, 1995, 1997). 3) Both have hydropathy plots that are negative (Parsell and Lindquist, 1993; Mtwisha *et al.*, 1998).

These small heat shock proteins or LEA-like Hsp 12 proteins are encoded by the Hsp 12 gene, the expression of which has been associated with stress-responsive elements (STREs) and High-Osmolarity Glycerol 1 (*HOG*1) in *Saccharomyces cerevisiae.* Hsp 12 gene is activated by the High-Osmolarity Glycerol pathway, and CCCCT elements (STREs) are essential for induction of the Hsp 12 gene by salt stress. The protein accumulates in large quantities in yeast cells exposed to heat shock, osmostress, high concentrations of alcohol and in early stationary phase cells (Praekelt and Meacock, 1990; Varela *et al.*, 1995; Sales *et al.*, 2000). The LEA-like protein Hsp 12 is presumed to be located in the plasma membrane of *S. cerevisiae*, and it protects the membrane against desiccation and ethanol-induced stress (Sales *et al.*, 2000).

## 1.5 Desiccation-sensitive grass, *Eragrostis curvula* provides a good model for studying improved desiccation tolerance

*Eragrostis curvula* Schrad. cv. Ermelo, is an important forage grass in southern Africa. It is a warm season $C_4$ grass. Its leaf growth is relatively insensitive to soil drying and it is thought to be drought resistant (Puliga *et al.*, 1996). However, *E. curvula* is desiccation sensitive (Vander Willigen *et al.*, 2001). In the current study *E. curvula* is therefore used as the model for studying the genetic improvement of important pasture grasses for drought *tolerance*. As indicated above, some genes are upregulated and express proteins that directly or indirectly protect the plants in response to water deficit. Some of these genes have been cloned to other species, which improved their desiccation tolerance, e.g. overexpression of HVA1 improved drought and salinity resistance in transgenic rice (Xu *et al.*, 1996). Thus also important in this study is the understanding of plant transformation systems that are utilized to produce new desiccation tolerant plants.

## 1.6 Transformation

Plant transformation or plant genetic engineering entails the isolation, introduction and expression of foreign DNA into the plant genome. The resultant plants are termed transgenics, which simply refers to plants that express foreign genes. The expression of foreign genes was reported for the first time in tobacco (De Block *et al.*, 1984). It was shown that foreign genes can be systematically integrated into the plant genome and that transformed plants are capable of normal growth. Subsequently such research has been performed on many plant species, including grasses. Pivotal to genetic engineering of plants is the establishment of a regeneration system of a plant to be transformed. Tissue culture makes use of totipotency, that is, every cell in a plant has full genetic make up and is capable of producing the entire plant (see Chapter 2). Besides tissue culture techniques which complement classical means of plant breeding (Lichtenstein and Fuller, 1987), development of a vector system to deliver the foreign DNA to the plant is also important (see Chapter 3). The vector systems can be those based on plant virus, tumor-inducing (Ti) plasmid of *Agrobacterium* and/ or direct gene transfer using particle gun technology.

### 1.6.1 *Agrobacterium*-mediated transformation

*A. tumefaciens* is a Gram negative soil-borne bacterium that is naturally capable of transferring a portion of the Ti plasmid to plant cells. The transferred segment is termed T-DNA. The infection of plants by *A. tumefaciens* is attributed to the presence of phenolic compounds which are believed to function as elicitors for T-DNA transfer (Engström *et al.*, 1987). Ti plasmid is comprised of three genetic components for infection of plant cells- T-DNA, *vir* genes and chromosomal virulence loci (Figure 1.1). The chromosomal virulence loci specify the binding of *A. tumefaciens* to plant cells during infection. The *vir* region includes eight gene operons, *vir* A to *vir* H of which six provide the product required for plant cell recognition and T-DNA transfer (Douglas *et al.*, 1985; Hooykaas and Schisperoort, 1992). The *vir* D genes encode a site-specific (T-DNA) endonuclease enzyme. This enzyme nicks each border repeat 3 bases from the 3' end of the T-DNA (Jasper *et al.*, 1994). It is suggested that this nicking reaction is the beginning of the generation of a single-stranded linear copy of the T-DNA. The T-DNA strand unwinds from the Ti plasmid and is then transferred into the plant cell as a single stranded molecule likely linked to some other *vir* D proteins (Tinland *et al.*, 1994; Lichtenstein and Draper, 1985).

T-DNA is a structural element flanked by 25 bp directly repeated sequences called T-DNA borders. Located within T-DNA borders are three genes that code for enzymes that are involved in the synthesis of growth regulatory substances whose expression in plant cells causes tumor or crown gall growth (Schröder *et al.*, 1984). These T-DNA genes can be deleted to form new derivatives of Ti plasmids, termed disarmed Ti plasmids (Hood *et al.*, 1993; Torisky *et al.*, 1997).

*Agrobacterium* based vectors have a broad host-range. *Agrobacteium* infects mostly dicotyledonous plants (An *et al.*, 1988). The *Agrobacterium*-mediated transformation method has also been used to transform a few monocotyledonous plants (e.g. rice, Hiei *et* *al.*, 1994). Monocotyledonous plants are thought not to produce appropriate phenolic eliciting compounds (Stachel *et al.*, 1985), making transformation using *Agrobacterium* difficult. Various types of Ti vectors that can be used to transform both dicotyledonous and monocotyledonous plants are now available commercially. They can be classified into 2 types of vectors, cointegrate and binary vectors. The cointegrate vectors are disarmed Ti plasmids in which the border sequences flank a copy of the *E. coli* plasmid vector pBR322. Foreign DNA cloned into a pBR322-like vector is cointegrated into disarmed Ti plasmids by homologous recombination. This vector therefore requires an intermediate plasmid that can be used to clone the gene of interest (Rogers *et al.*, 1988) (Figure 1.1).

Binary vector systems use the *trans* acting function of the *vir* region on a Ti plasmid to transfer modified T-DNA region to plant cells. This vector is able to replicate in both *E. coli* and *A. tumefaciens* and contains T-DNA border sequences flanking multiple cloning sites and selectable marker genes. This plasmid is transferred from *E. coli* to *Agrobacterium* strain containing a second helper plasmid which confers virulence functions, thus allowing the infection of a plant (Hoekema *et al.*, 1985; An *et al.*, 1988).

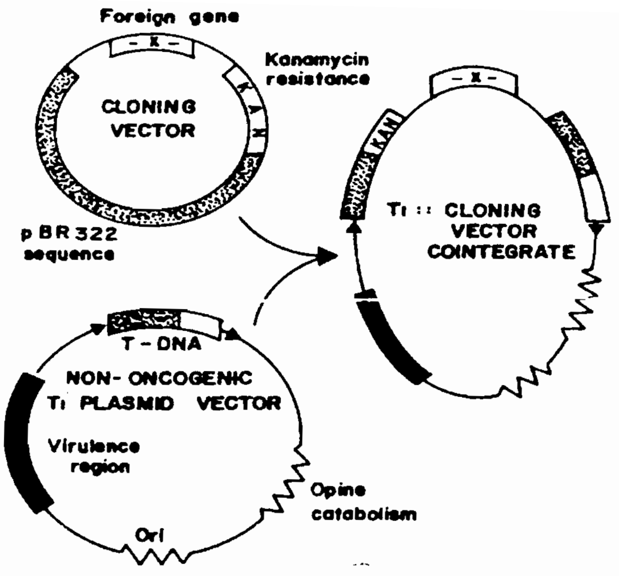


Figure 1.1. Cointegrate vector system based on *Agrobacterium* Ti plasmid vector.

### 1.6.2 Direct gene transfer

Direct gene transfer techniques can be used in most plant species and have alleviated the problems encountered in crops recalcitrant to genetic manipulation. With direct gene transfer method, the foreign DNA is transferred directly into the cells or protoplast without the use of a plasmid vector (except biolistic transformation which requires plasmid vector). The protocol includes: 1) Protoplast transformation (Paszkowski *et al.*, 1985), a method that involves the treatment of protoplasts with gene of interest and *in vitro* culture of protoplasts to regenerate plants. The uptake of DNA by protoplasts can be mediated by treatment of the cultures with polyethylene glycol (PEG), calcium ions, or magnesium (Krens *et al.*, 1982; Paszkowski *et al.*, 1985); 2) Electroporation, a method that uses electrical pulses (either short or long) to induce pore formation in the membrane through which genes of interest are inserted in the plant genome (Shillito *et al.*, 1985; Lurquin, 1997); 3). Particle gun transformation, first described by Klein *et al.*, 1987, in which DNA is delivered into plant cells using high velocity microprojectiles. This is the most preferred method for genetically transforming plants.

Particle gun transformation or gene gun or biolistics, is a relatively new gene transfer technique which employs microprojectiles. Microprojectiles, inert particles e.g. gold or tungsten which are coated with DNA to be transferred, are accelerated into plant cells, penetrating walls and membranes. In the original experiments microprojectiles were used to carry RNA or DNA into epidermal cells of onion tissues and these genes were subsequently expressed genetically. The method was also used in transformation of important cereal species such as rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.). Tungsten particles carrying plasmid DNA were bombarded into rice and wheat callus using the method of Klein *et al.*, (1987).

Microprojectiles are delivered using gas shock. The gas shock can be derived by use of a chemical explosion (gunpowder), an electric discharge or helium shock (Sanford *et al.*, 1987; Christou *et al.*, 1988). Apparatus driven by helium is preferred as it is clean, safe and can be controlled. Helium is a light gas and expands much faster than air, therefore it imparts higher velocities to the microprojectiles (Sanford *et al.*, 1993). Also important with particle bombardment devices are parameters that affect velocity. These parameters include pressure, distance from the power source to microprojectiles and target distance. Higher pressure, shorter gap distances and long microprojectile flight distances give higher launch velocities.

## 1.7 Foreign gene expression in plants

The ultimate aim for bioengineering plants is to obtain transgenic plants that show the characteristics of foreign genes. Therefore transformation is considered successful if the target cells survive the transformation process, and the newly transferred gene is transcribed into messenger RNA (mRNA) and is subsequently translated into protein such that the plant changes its natural metabolism to include the new introduced trait. All these processes are determined by transcriptional control signals and promoters. Numerous studies have been done to unravel novel promoters that would effect the high-level of expression in all plant species. A promoter that has been widely used in chimeric gene constructs in plant is 35 S RNA from the cauliflower mosaic virus (CaMV) (Ow *et al.*, 1987). Besides 35 S RNA promoter, the ubiquitin promoter from maize is also one of the promoters that results in high gene expression more particularly in monocotyledonous plants (Christensen and Quail, 1996).

The monitoring of transgene expression is done by using reporter genes which form part of the vector construct. The expression of a reporter gene in plant cells means the gene of interest was successfully transferred into plant cells (see Chapter 4). Reporter genes are chosen on the basis that they are not present in plants, e.g. *β*-glucuronidase (GUS) gene, the predominantly used reporter gene from *E. coli* (Jefferson *et al.*, 1987), and that they are also easily detected. Other reporter genes that are often used include *E. coli β*-galactosidase (Helmer *et al.*, 1984); luciferace (Ow *et al.*, 1986) and combination of GUS and green fluorescent protein (GFP) (Quaedvlieg *et al.*, 1998).

## 1.8 Transgenic analysis

The measure of success for any transformation system is determined by molecular and phenotypic analysis of putative transformants. Since the regeneration of plants on selection medium, or the transient expression of a reporter gene, do not give conclusive evidence for successful stable transformation, molecular and physiological analysis are required to determine if the gene is present, transcribed and translated in the plant genome.

The presence of the foreign gene in a transformed plant is determined by Southern blotting (Southern, 1975; Sambrook *et al.*, 1989) and Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987; Higgins *et al.*, 2000). In the Southern blot method, genomic DNA is digested with restriction enzymes and separated by gel electrophoresis, transferred and linked to a membrane and hybridized with DNA strands complementary to the transgene, which is subsequently visualized radioactively. This method can be used to estimate the number of insertion events and copies of transferred DNA (Schaff, 1991). Though this method is still widely used, the PCR method has been a standard procedure to screen putative transformants at any early developmental stage where plant material would be limiting for other assays. In this method plant genomic DNA is used as template, then amplified using transgene specific primers and visualized after DNA separation by gel electrophoresis. Considering high demands (time and resources) associated with plant transformation systems as well as regeneration of transformed plants, PCR method is often the preferred method.

The transcription of a transgene can be evaluated at the RNA level using Northern blot analysis. The method differs from Southern blotting basically due to the fact that RNA secondary structure has to be denatured for size fractionation during gel electrophoresis. The presence of transgenic RNA demonstrates that the transgene is actively transcribed. Alternatively, reverse transcription PCR (RT-PCR) can also verify that RNA is transcribed from the transgene. Both techniques provide information on whether transcription is constitutive, tissue specific or does not occur at all (Schaff, 1991). RT-PCR is the preferred technique for analyzing mRNA in extremely low abundance (Pfaffl and Hageleit, 2001). RT-PCR needs primers specific to the transgene of interest, and reverse transcriptase polymerase enzyme, to synthesize a double-stranded template from the target RNA that is transcribed from the transgene, which is subsequently amplified by PCR.

Translation of a transgene into encoded protein can be demonstrated with SDS-PAGE, Western blotting, enzyme immunosorbent assay (ELISA) or Matrix Assisted Laser Desorption/Ionization Time Of Flight (MALDI-TOF) mass spectrometry to obtain peptide mass fingerprint. The Western blotting or ELISA requires antibodies directed against the transgene protein to verify that it is synthesized. Once the molecular analysis verifies that the plant is indeed transgenic, the stability of the transgene in consecutive meiotic generations, phenotypic change and expression of the transferred trait under field conditions are all evaluated (Schaff, 1991).

## 1.9 Aims of this research

To date, no reports have been published on methods for *Eragrostis curvula* transformation. The aim of this research was to establish a transformation protocol for *Eragrostis curvula* cultivar Ermelo with an ultimate aim of transforming the species with Hsp 12 gene in order to develop plants more tolerant of water-deficit stress. The research conducted here is to be used as a model system for genetically improving important crops for drought and desiccation tolerance in the long term. To this end, studies were conducted to find tissues that were suitable explants for regeneration protocols such that gene transfer can be effected. Plant regeneration in *E. curvula* was achieved through immature leaf and shoot culture. The Hsp 12 gene from *Saccharomyces cerevisiae* was cloned into the pCAMBIAUbeeQ vector, bombardment conditions optimized and regenerated plants were tested for transformation efficiency. As a comparative study, a new construct pCAMBIAUbeeQHsp12 was also introduced into *Nicotiana tabacum* cv *Xanthi* via leaf disc co-cultivation with *A. tumefaciens,* the aim of which was to express Hsp 12 protein in dicotyledonous plant. Putative transformants were generated and the presence of the Hsp 12 gene was verified by PCR and its expression at RNA level was confirmed by RT-PCR. Analysis of Hsp 12 gene expression at protein level was performed using SDS-PAGE and MALDI-TOF peptide mass spectrometry.

# Chapter 2

## Establishment of tissue culture protocol(s) for transformation of *Eragrostis curvula*

### 2.1 Introduction

The prerequisite for achieving optimal transformation is the establishment of an efficient plant regeneration system or tissue culture protocol for the plant species to be transformed. Since transformation frequency can be low as 1-5 x $10^{-2}$ (Shillito *et al.*, 1985) large quantities of regenerable target tissue become important in order to achieve optimal transformation. The most important point about tissue culture is that it provides large quantities of uniform material or single cells that can be regenerated into entire plants. In addition to quantity of the material, tissue culture also allows the use of different types of tissue that can be transformed ranging from callus, embryos to organogenic shoots.

Tissue culture has played an increasingly vital role in genetic transformation of many plant species in recent years. For instance, production of transgenic maize has been achieved using friable embryogenic callus or suspension cultures (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990). To date, no reports have been published on transformation of *E. curvula*. This plant species has been regenerated *in vitro* using inflorescence tissues (Echenique *et al.*, 1996), which have been recognized as important source of totipotent cells (Tisserat, 1985). However, since time required for development of inflorescence on the parent is long, it would be useful to have protocols that utilize leaf and seed as source material. Thus aim of this section of the work was to develop a tissue culture protocol from leaves and seeds *of E. curvula*.

To accomplish this task, several routes were attempted viz: indirect and direct embryogenesis and indirect and direct organogenesis. For indirect embryogenesis, callus tissue that consists of aggregates of parenchyma cells, interspersed amongst which are regions of active division, is induced from leaf and seeds cells (Dodds and Robert, 1982).

In an appropriate medium this callus is induced to produce embryos which closely parallels that of zygotic embryos, showing all stages of development: globular, heart and torpedo shaped stages. The induced embryos are germinated in an appropriate medium, producing the entire plant. This process of indirect somatic embryogenesis was reported by Bekele *et al.* (1995) from leaf and root explants of *Eragrostis tef.* Direct embryogenic processes occur in the absence of undifferentiated callus, but embryos are formed and developed as described for indirect embryogenesis (Hicks, 1980).

In organogenic processes, plant production can be achieved through one of the 2 modes (Tisserat, 1985):

A. Production of adventitious organs from callus derived from the explant and
B. Emergence of adventitious organs directly from the explant without intervening callus phase.

This process has been reported by a number of authors including Echenique *et al.* (1996) for *E. curvula* and more recently by Ramgareeb *et al.* (2001) for the pasture grass *Cynodon dactylon.*

In the current study, the influence of various concentrations of the plant growth regulators BAP, TDZ, 2,4-D, and sucrose, in the callus induction and plant regeneration media was studied. By simply manipulating the concentrations of growth regulators and carbon source and environmental conditions (light/dark conditions), indirect and direct organogenesis processes are reported here for *E. curvula.*

## 2.2 Methods and materials

### 2.2.1 Source of seed material

The *Eragrostis curvula* (cultivar ermelo) seed material was obtained from Agricultural Research council, Grassland Research, Unit Roodepoort, South Africa.

### 2.2.2 Sterilisation of seeds

Seeds (approximately 100) were sterilised in 1 ml of 70 % (v/v) ethyl alcohol for 5 min. Ethyl alcohol was removed and replaced with 0.35 % (v/v) sodium hypochlorite, containing 2 drops of Tween 20, for 10 min. Seeds were rinsed by 5 washes with shaking in sterile distilled water.

### 2.2.3 Callus induction from seeds

Induction of callus was performed using Murashige and Skoog (MS) salts (1962), 2 % (w/v) sucrose, 0.8 % (w/v) agar (Purified agar from Sigma). In the first method, the MS callus induction medium was supplemented with either 1 mg/l, 5 mg/l, 10 mg/l, or 30 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and either 1 mg/l or 2 mg/l benzylaminopurine (BAP). In the second method, supplementation was with 2,4-D only (1 mg/l, 5 mg/l, 10 mg/l, and 30 mg/l). In the third method, supplementation was with 10 mg/l of 2,4-D and napthaleneacetic acid (NAA) at (0.1 mg/l, 0.2 mg/l, 2 mg/l and 3 mg/l) and in the fourth method, supplementation was with 10 mg/l 2,4-D and 0.5 mg/l thidiazuron (TDZ). The summary of the methods is shown in Table 2.1. The pH of the media of all treatments was adjusted to 5.8 with 1 M KOH prior to autoclaving and the medium was sterilised by autoclaving at 121 $^0$C for 20 min. 20 ml aliquots of the autoclaved medium were dispersed into 9 cm diameter Petri dishes then allowed to set overnight in a laminar flow hood. *E. curvula* seeds, 10 in each Petri dish, were cultured on the assorted media. Cultures were either kept at 22 $^0$C in dark or in a continuous light (45 $\mu$mol/m$^2$/sec) conditions.

Table 2.1. Showing different composition of auxin and cytokinin used in the callus induction media for seeds. The basal medium consisted of MS salts and 0.8 % (w/v) agar.

| **Method** | **Auxin** mg/l | **Cytokinin** mg/l | **Sucrose** % (w/v) |
|---|---|---|---|
| 1 | **2,4-D** | **BAP** | |
| | 1 | 1 | 2 |
| | 5 | 1 | 2 |
| | 10 | 2 | 2 |
| | 30 | 2 | 2 |
| 2 | **2,4-D** | | |
| | 1 | | 2 |
| | 5 | | 2 |
| | 10 | | 2 |
| | 30 | | 2 |
| 3 | **NAA** | **BAP** | |
| | 1 | 0.1 | 2 |
| | 2 | 0.2 | 2 |
| | 3 | 2 | 2 |
| | 10 | 3 | 2 |
| 4 | **2,4-D** | **TDZ** | |
| | 1 | 0.5 | 2 |
| | 5 | 0.5 | 2 |
| | 10 | 0.5 | 2 |
| | 30 | 0.5 | 2 |

### 2.2.4 Aseptic germination of seeds and callus induction from immature leaves.

The sterilised seeds (10) were cultured in 100 ml tissue culture bottles on MS medium containing 0.8 % (w/v) agar and 3 % (w/v) sucrose. The sterilised seeds were germinated for 10 days in the dark at 22 $^0$C. After 10 days, immature leaf explants (approximately 2 cm) were excised and used for callus induction. In addition to the methods shown in Table 2.1, the MS basal medium was also supplemented with 2 mg/l 2,4-D, either 0.01

mg/l or 1 mg/l BAP and 6 % (w/v) sucrose as summarised in Table 2.2. Eight explants of an immature leaf (2 cm segments) were cultured into each Petri dish, cultures were maintained at 22 $^{0}$C in the dark or in continuous light (45 µmol/m$^{2}$/sec).Ten replicates were made for each treatment.

Table 2.2. Showing different composition of auxin and cytokinin used in the callus induction media for leaves. The basal medium consisted of MS (1962) salts and 0.8 % (w/v) agar.

| **Method** | **Auxin** mg/l | **Cytokinin** mg/l | **Sucrose** % (w/v) |
|---|---|---|---|
| 1 | **2,4-D** | **BAP** | |
| | 1 | 1 | 2 |
| | 5 | 1 | 2 |
| | 10 | 2 | 2 |
| | 30 | 2 | 2 |
| 2 | **2,4-D** | | |
| | 1 | | 2 |
| | 5 | | 2 |
| | 10 | | 2 |
| | 30 | | 2 |
| 3 | **NAA** | **BAP** | |
| | 1 | 0.1 | 2 |
| | 2 | 0.2 | 2 |
| | 3 | 2 | 2 |
| | 10 | 3 | 2 |
| 4 | **2,4-D** | **TDZ** | |
| | 1 | 0.5 | 2 |
| | 5 | 0.5 | 2 |
| | 10 | 0.5 | 2 |
| | 30 | 0.5 | 2 |
| 5 | **2,4-D** | **BAP** | |
| | 2 | 0.01 | 6 |
| | 2 | 1 | 6 |

### 2.2.5 Regeneration from callus

Regeneration from callus was performed using 3 methods summarised in Table 2.3.

*Method A*: calli obtained after 8 weeks on callus induction medium (Table 2.1 and Table 2.2, methods 1, 2, 4 and 5) were transferred onto regeneration medium. Regeneration was optimized using MS salts with various treatments with BAP, 2,4-D and kinetin (either 0.4 mg/l 2,4-D, 0.5 mg/l BAP or 0.5 mg/l kinetin and 2 % (w/v) sucrose).

*Method B*: callus (same as in Method A) was cultured on an MS medium supplemented with 0.4 mg/l 2,4-D, 0.5 mg/l BAP, 0.5 mg/l kinetin, 1 mg/l ABA and 2 % (w/v) sucrose.

*Method C*: callus (same as in Method A) was cultured on the medium that included either 2 mg/l 2,4-D and 0.01 mg/l BAP or 2 mg/l 2,4-D and 1 mg/l BAP and 6 % (w/v) sucrose.

Each treatment contained 10 replicates of 10 pieces of callus. Cultures were incubated in the dark for 3 weeks at 22 $^0$C/ 18$^0$C (day/night) then transferred to 16 h photoperiod conditions, light intensity 7.4 µmol/m$^2$/sec. Calli initiated on MS medium with either 2 mg/l 2,4-D and 0.01 mg/l BAP or 2 mg/l 2,4-D and 1 mg/l BAP were either subjected to low light intensity (7.4 µmol/m$^2$/sec) 16 h photoperiod conditions, or continuous light, 45 µmol/m$^2$/sec for 2 weeks (in Petri dishes), thereafter maintained in 16 h photoperiod conditions in Petri dishes. The cultures were transferred to fresh medium in 100 ml bottles every 4 weeks and shoots that developed were transferred to rooting medium.

Table 2.3. Callus induced from either leaf or seed material cultured on various regeneration media utilized. The basal medium consisted of MS salts, either 2 % (w/v) or 6 % (w/v) sucrose and 0.8 % (w/v) agar.

| **Method** | **Source of callus** | **Plant growth regulators** |
|---|---|---|
| A | leaves and seeds | either 0.4 mg/l 2,4-D, 0.5 mg/l BAP or 0.5 mg/l kinetin |
| B | leaves and seeds | 0.4 mg/l 2,4-D,<br>0.5 mg/l BAP<br>0.5 mg/l kinetin and<br>1 mg/l ABA |
| C | leaves | either 2 mg/l 2,4-D and 0.01mg/l BAP<br>or<br>2 mg/l 2,4-D and 1 mg/ l BAP |

## 2.2.6. Regeneration from shoot and leaf explants

Regeneration was performed using 2 methods summarised in Table 2.4. In the first method, shoot explants (2 cm segments) were cut from callus-shoot aggregates (Figure 2.4A) obtained from seeds cultured on MS medium containing either 10 mg/l 2,4-D and 0.5 mg/l TDZ or 10 mg/l 2,4-D and 0.5 mg/l BAP and 2 % (w/v) sucrose in Petri dishes, kept in the dark for 4 weeks. These shoot explants were placed on MS medium supplemented with either 0.5 mg/l TDZ or 0.5 mg/l BAP in Petri dishes. Other parameters involved culturing the whole callus-shoot aggregate on the same medium. Cultures were maintained at 22 $^0$C / 18$^0$C (day/night) in the dark for 3 weeks. In the fourth week, cultures were transferred to continuous light (light intensity 45 µmol/m$^2$/sec) and were kept for 2 weeks. Thereafter maintained at 22 $^0$C / 18$^0$C (day/night), 16 h photoperiod conditions, light intensity 7.4 µmol/m$^2$/sec. The developed shoots (approximately 4 cm long) were maintained in 100 ml bottles.

In a second method, sterile immature leaf explants were obtained as reported in section 2.2.4 and plated on an MS medium supplemented with 2 mg/l 2,4-D and either 0.01 mg/l BAP 0.1 mg/l BAP or 1 mg/l BAP and 2 % (w/v) sucrose. The Petri dishes with cultures were kept at 22 $^{0}$C / 18$^{0}$C (day/night) in the dark for 30 days. The cultures were then transferred to continuous light for 2 weeks and then to 16 h photoperiod conditions. The cultures were transferred to fresh medium every 4 weeks and shoots that developed were transferred to rooting medium in 100 ml bottles.

Table 2.4. Leaf and shoot explants on various regeneration media utilized. The basal medium consisted of MS salts, 2 % (w/v) sucrose and 0.8 % (w/v) agar.

| **Method** | **Source of explant** | **Plant growth regulators** |
|---|---|---|
| 1 | Shoot explant* | either 0.5 mg/l TDZ or 0.5 mg/l BAP |
| 2 | Leaf explant | either 2 mg/l 2,4-D and 0.01 mg/l BAP, 2 mg/l 2,4-D and 0.1 mg/l BAP or 2 mg/l 2,4-D and 1 mg/l BAP |

* Shoot explants excised from callus-shoot aggregates which were obtained from seeds cultured for 30 days in the dark on an MS medium containing either 10 mg/l 2,4-D and 0.5 mg/l TDZ or 10 mg/l 2,4-D and 0.5 mg/l BAP.

### 2.2.7 Rooting and hardening-off

Shoots (about 6cm long) developed from callus, leaf and shoot explants were all rooted on an MS medium with 3 % (w/v) sucrose but devoid of hormones in 100 ml bottles. Shoots formed via callus were also rooted on an MS medium supplemented with 2 mg/l 2,4-D and 0.01 mg/l BAP (same as medium used to induce callus and shoots). All media contained either 0.25 % (w/v) gelrite or 0.8 % (w/v) agar and the pH was 5.8 prior to autoclaving. Rooting occurred at 16 h photoperiod, light intensity 7.4 μmol/m$^2$/sec at 22 $^0$C for 6 weeks, with subculturing on the same medium after 2 weeks. Plants with well developed root system were planted in an autoclaved mixture of vermiculite, sand and soil (2:1:1) or sand and soil (2:3) in plastic pots, enclosed in plastic bags and transferred to plant growth rooms (22 $^0$C / 18$^0$C (day/night) 16 h photoperiod). Plants were watered 3 times a week with sterilised water and plastics bags covering them were removed permanently after 2 weeks.

### 2.2.8 Histology experiments

Regeneration was studied on shoot explants and callus tissue that had been induced from immature leaf explants. The method for fixation and embedding used was based on the one described by Sherwin and Farrant (1996) for resurrection plants. Samples were fixed overnight in 2.5 % buffered (0.1 M phosphate pH 7.4) glutaraldehyde and 0.5 % caffeine, then washed (3 times for 5 min) with 0.1 M phosphate buffer pH 7.4 and fixed for another 1 h in one part 2 % osmium tetraoxide in phosphate buffer. After fixation, samples were dehydrated using graded ethanol series, infiltrated with epoxy resin for 3 days and polymerised for 16 h at 60 $^0$C. Sections (95 nm thick) were cut using a Rechart Ultracut-S (Leica, Vienna, Austria), mounted on the copper grids and stained with uranyl acetate and lead citrate (Reynoulds, 1963) for 10 min each. Sections were viewed with transmission electron microscopy (Zeiss 109 TEM). For light microscopy, sections were mounted on the glass slide and stained with 1 % toluidine blue.

## 2.3 Results

### 2.3.1 Callus initiation

A white, soft and watery callus was visible after 2 weeks on callus induction media from both immature leaf and mature seed explants of *E. curvula* (Figure 2.1). Callus formed from over 96 % of all explants tested. Methods 1, 4 and 5 (Table 2.5) resulted in a relatively large callus weight production as compared to method 2 and 3 (results not shown). The proliferation of callus was observed at all hormone combinations tested except for the combination involving BAP and NAA. For leaf explants cultured using Method 4, combinations of 2,4-D and TDZ did not result in production of callus. However, in seeds, 2,4-D 10 mg/l and 0.5 mg/l TDZ produced large fresh callus weight especially in the dark (Table 2.5). The combination of 2,4-D and BAP also produced large callus weight for both leaf and seeds explants. The most suitable concentration of 2,4-D for callus initiation from leaves was 2 mg/l combined with 0.01 mg/l BAP (Table 2.5, method 5) in the dark (Figure 2.1A and 1B). Generally, large fresh callus weight was obtained from immature leaf explants and from cultures incubated in the dark. Cultures remained healthy as the amount of callus continued to increase for at least 3 months from initiation. But required subculturing after 4 weeks.

Table 2.5. Fresh weight of callus produced from seeds and leaf explants after 4 weeks incubation in Petri dishes containing MS media supplemented as described. * represents cultures kept in the continuous dark, otherwise kept in continuous light.

| **Method** | **Explant** | **Plant growth regulators** | **Average callus fresh weight (g) (n = 20)** |
|---|---|---|---|
| 1* | seeds | 10 mg/l 2,4-D and 1 mg/l BAP | 0.1 ± 0.01 |
| 1 | seeds | | 0.04 ± 0.01 |
| 1* | leaves | 10 mg/l 2,4-D and 1 mg/l BAP | 0.17 ± 0.01 |
| 1 | leaves | | 0.05 ± 0.01 |
| 4* | seeds | 10 mg/l 2,4-D and 0.5 mg/l TDZ | 0.3 ± 0.01 |
| 4 | seeds | | 0.1 ± 0.01 |
| 5* | leaves | 2 mg/l 2,4-D and 0.01 mg/l BAP | 0.25 ± 0.01 |
| 5 | leaves | | 0.06 ± 0.01 |

### 2.3.2 Regeneration from callus

Callus (8 weeks old) was transferred to regeneration medium as described in Table 2.3. Examples of the frequency of regeneration obtained from callus initiated on MS medium with 2 mg/l 2,4-D, 0.01 mg/l BAP and 6 % (w/v) sucrose (Table 2.3, Method C) are presented in Figure 2.2B. Out of 10 pieces of callus on a plate, 2 to 4 regenerated. This callus was regenerated on the same medium as callus induction medium except that cultures were subjected to light regime as described previously. After 3 weeks in the regeneration medium, callus turned light green due to chlorophyll pigment (Figure 2.2B and 2C). After a further 2 weeks, 4-5 plantlets were regenerated per piece of callus (Figure 2.2D and 2E). Ultimately, approximately 50 shoots were produced per piece of callus. Low BAP concentration (0.01 mg/l BAP) resulted in many shoots as compared to 1 mg/l BAP which resulted in shoot regeneration inhibition (Figure 2.3). Exposing the cultures into high light intensity enhanced the production of shoots (Figure 2.3). Method A and B (Table 2.3) did not result in regeneration from callus.

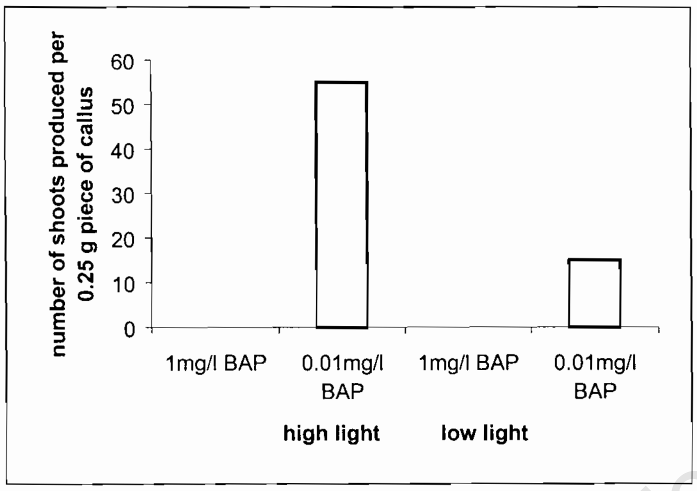


Figure 2.3. Effects of BAP concentrations and light intensity on shoot regeneration from a piece of callus (Histogram) after 4 months of immature leaf culture. High light-45 μmol/m$^2$/sec, low light-7.4 μmol/m$^2$/sec.

### 2.3.3 Regeneration from leaf and shoot explants

*Method* 1: Using an MS medium supplemented with either 10 mg/l 2,4-D and 0.5 mg/l TDZ or 10 mg/l 2,4-D and 0.5 mg/l BAP (Table 2.4, Method 1), seeds germinated into little shoots which was then followed by callus production (Figure 2.4A). When transferred onto an MS medium devoid of auxin 2,4-D, these shoot explants produced axillary shoots (Figure 2.4B). These shoot explants differentiated directly into axillary shoots without an intermediate callus phase (Figure 2.4C and 4D). TDZ was the most effective cytokinin used, with an average of 30 shoots produced per explant. Out of 10 shoot explants on a plate, an average of 8 regenerated.

*Method 2*: Direct development of shoots from leaf explants is shown in Figure 2.5A. Shoots regenerated within 2 to 4 weeks from over 30 % of the explants cultured in low concentrations of 0.01 mg/l BAP and 2 mg/l 2,4-D. Shoot formation was subsequently followed by the proliferation of callus because of the high concentration of 2,4-D (Figure 2.5B). High level (0.1 mg/l) of BAP in the medium resulted in fewer direct regenerants with 1mg/l BAP resulting in an inhibition of shoot regeneration (Figure 2.6). In comparison to Method 1 which resulted in about 30 shoots per explant, Method 2 resulted in approximately 4 shoots per explant.

Acrobat Document

SE = 1
n = 10

Figure 2.6. Influence of BAP concentrations on direct shoot regeneration per plate (plate with 10 immature leaf explants) after 4 weeks culture in the dark. (n = number of samples; SE = standard error).

### 2.3.4 Rooting and hardening-off

Phenotypically normal shoots regenerated from callus, shoot and leaf explants were rooted in MS medium. Roots were formed after 2 weeks in culture without hormones (Figure 2.7A). Plants with well-developed root system were planted in the soil and transferred to growth room, all of the transferred plants survived after acclimatization and developed normally. No phenotypic was variation observed (Compare Figure 2.7B and 2.7C).

### 2.3.5 Histological studies

There were no stages of embryogenic development observed. Instead meristemoids (Figure 2.8A) were observed in callus tissue that had proliferated from immature leaf explants, and were subsequently cultured on regeneration medium (Table 2.3 Method C). These cells when observed using transmission electron microscopy were small with dense cytoplasm, minimal vacuolation and thin cell walls; typical features of meristemoids (Figure 2.8B(2). There was evidence of cell division (Figure 2.8A). The activity of these cells (meristemoids), presumably resulted in the formation of epidermis (Figure 2.8A). Some of the cells observed were large, vacuolated and contained starch (Figure 2.8B(1) and 2.8C). The cells containing chloroplasts (Figure 2.8D) presumably originated from the bigger cells containing starch. Shoot explants cultured on regeneration medium contained shoot primordia and shoot buds which had either developed from parenchyma or meristematic cells (Figure 2.9).

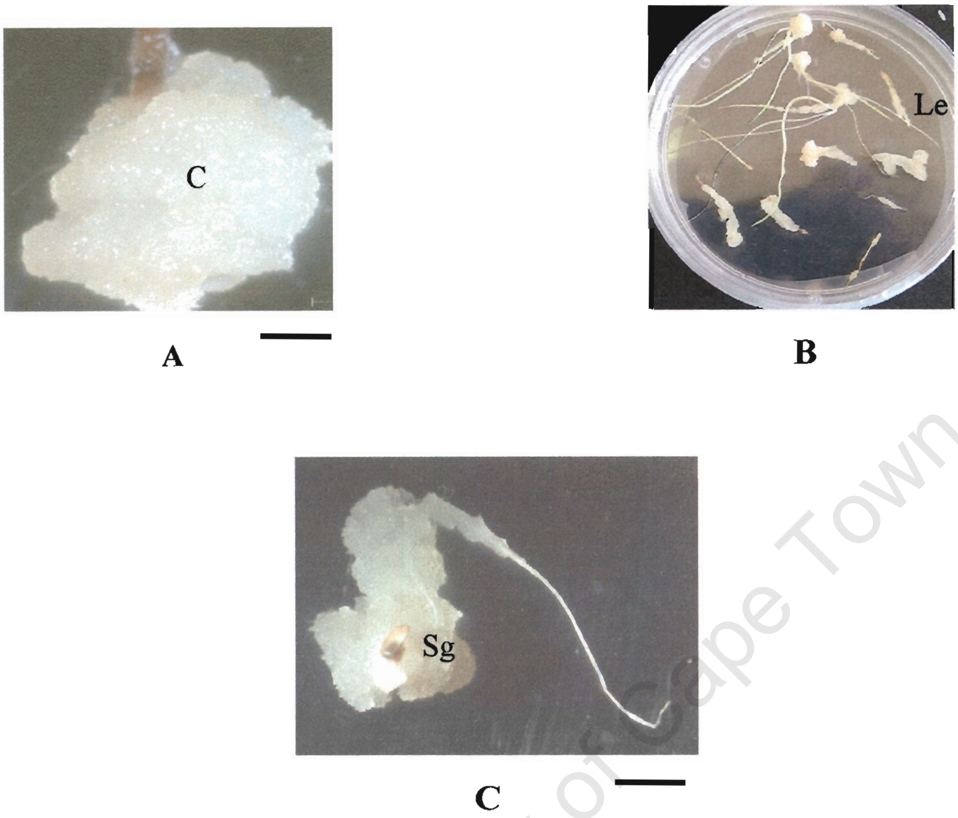


Figure 2.1. A. Callus (C) induced from immature leaf explants cultured on MS medium supplemented with 2 mg/l 2,4-D and 0.01 mg/l BAP (dark conditions). Photo taken after 2 weeks on callus induction medium (bar = 0.1 mm).

B. Frequency of callus induction from leaf explants (Le) cultured on MS medium supplemented with 2 mg/l 2,4-D and 0.01 mg/l BAP (dark conditions). Photo taken after 2 weeks on callus induction medium (bar = 0.1 mm ).

C. Callus induced from seed material cultured for 2 weeks on MS medium supplemented with 10 mg/l 2,4-D and 0.5 mg/l BAP (Sg = seed germinated and then produced callus) (dark conditions). Photo taken after 2 weeks on callus induction medium.

Figure 2.2. Callus production and regeneration of shoots from callus in *Eragrostis curvula*.

A. Callus (C) induced from immature leaves after 8 weeks of culture on MS medium supplemented with 0.01 mg/l BAP, 2 mg/l 2,4-D and 6 % (w/v) sucrose (dark conditions).

B and C. Callus producing chlorophyll (Cpl) pigment after 3 weeks of culture in the regeneration medium (same as callus induction medium above). 16 h photoperiod conditions, light intensity 7.4 µmol/m$^2$/sec, at 22 $^0$C.

D and E. Regeneration of plantlets on the shoot induction medium (same as callus induction medium) (S = shoots, C = callus). 16 h photoperiod conditions, light intensity 7.4 µmol/m$^2$/sec, at 22 $^0$C.

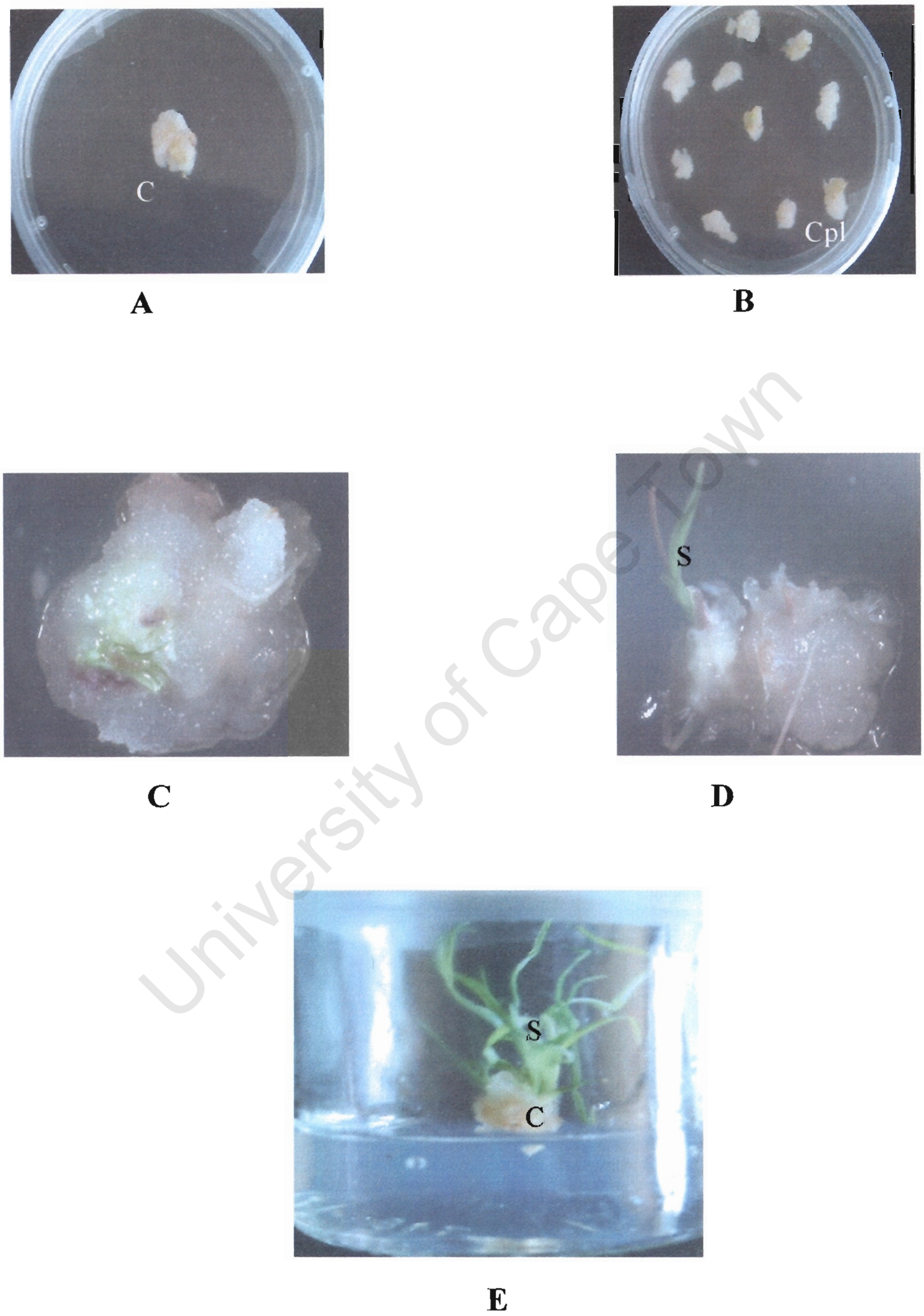


Figure 2.2. Regeneration of shoots from callus in *Eragrostis curvula*.

Figure 2.4. Direct organogenic regeneration from shoot explants.

A. Callus-shoot aggregates obtained from mature seeds after 4 weeks of culture on MS medium supplemented with 10 mg/l 2,4-D and 0.5 mg/l TDZ.

B. Shoots obtained as described in Figure 2.4A produced adventitious shoots (As) on MS medium supplemented with 0.5 mg/l TDZ (brown pigment represents dying callus).

C and D. As above, however, except that only shoot explants (Se) were cultured on MS medium supplemented with 0.5 mg/l TDZ (As =Adventitious shoots produced from shoot explants (Se)).

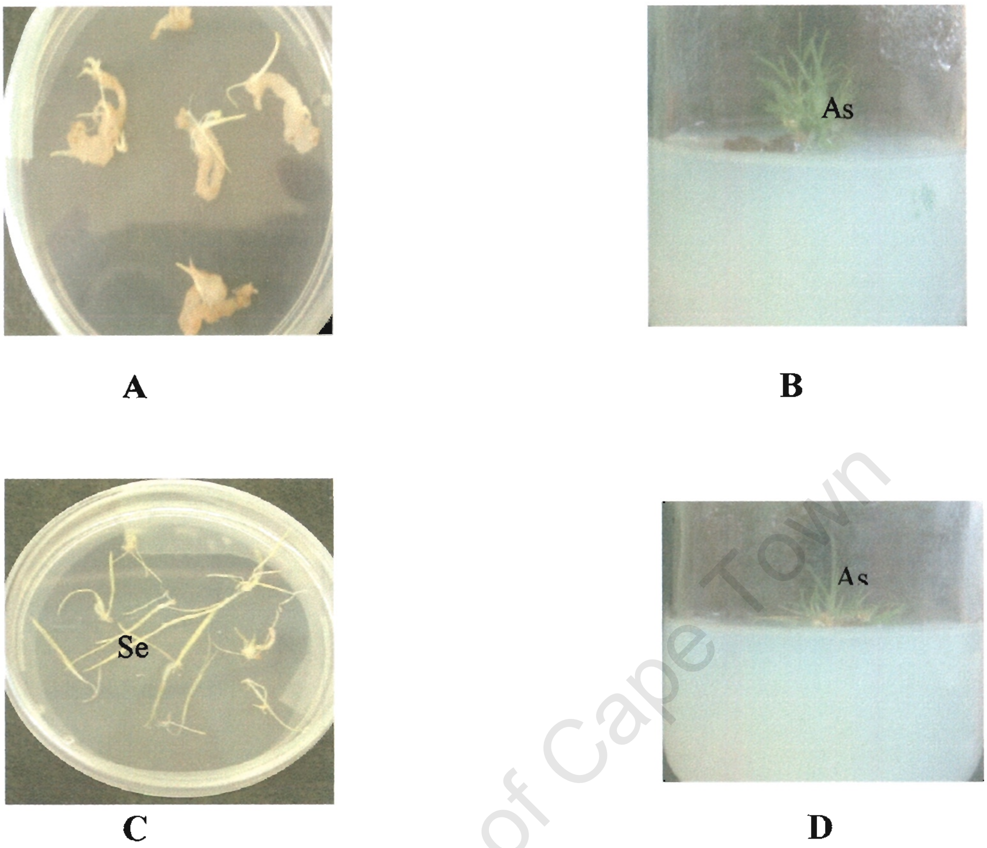
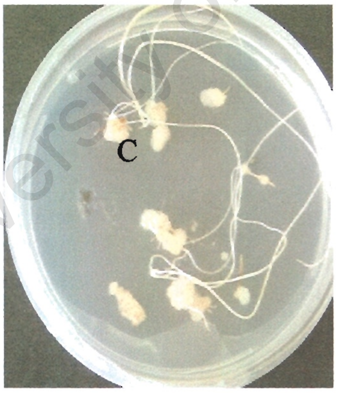


Figure 2.4. Regeneration from shoot explants.

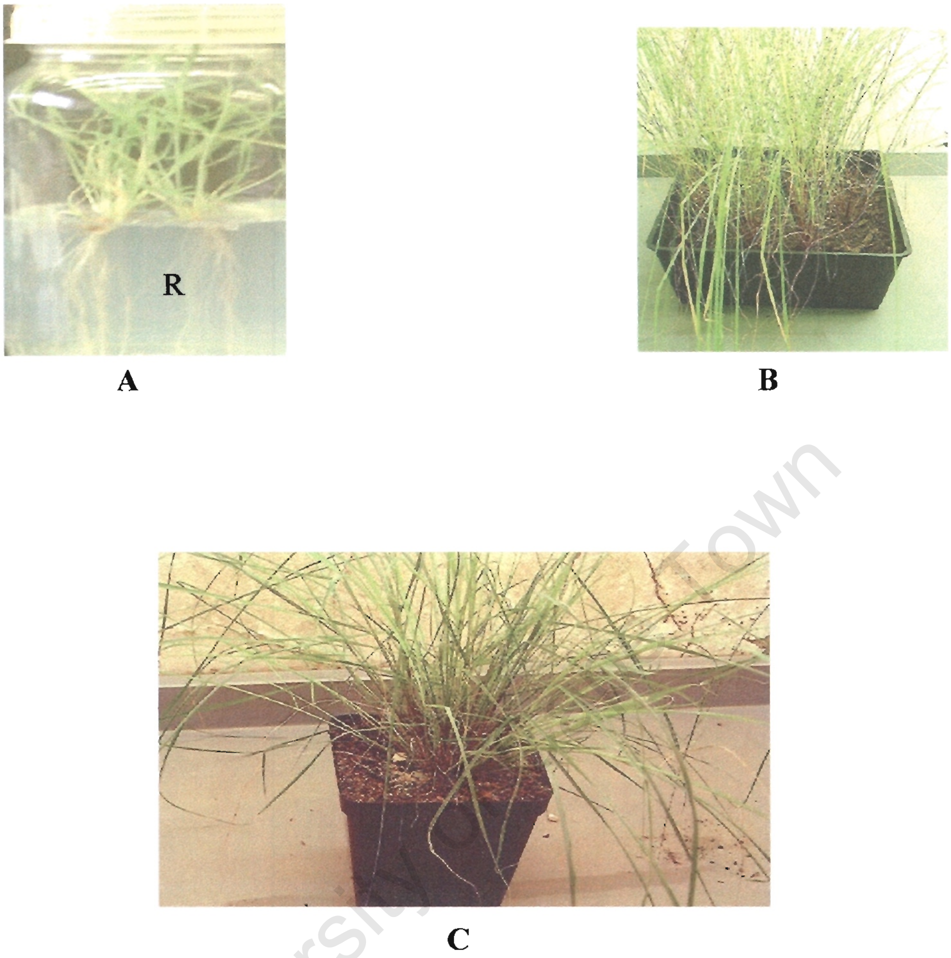
**A**



**B**

Figure 2.5. A. Plantlets obtained by direct regeneration from leaf explants on MS medium supplemented with 0.01mg/l and 2 mg/l 2,4-D (S = shoots regenerated from leaf explant).

B. Direct regeneration accompanied by callusing of the leaf explant on MS medium supplemented with 0.01mg/l and 2 mg/l 2,4-D (C = Callus produced from leaf explant).



A

B

C

Figure 2.7. A. Plantlets rooting after 2 weeks of culture on MS medium devoid of hormones (R = roots).

B. Regenerants growing in the mixture of sand and soil in the growth room.

C. Conventionally grown plant.

Figure 2.8A. Light micrograph of callus tissue (8 weeks old) of *E. curvula*. Sections were mounted on the glass slide and stained with 1 % toluidine blue. Meristematic activity (Mer) resulting in the formation of epidermal layer (magnification X 1000).

Figure 2.8 (B-D). Transmission electron micrographs of callus tissue (8 weeks old) of *E. curvula*. Typical features of meristemoids occurring.

B. **1** = big cell containing starch; **2** = small cells having dense cytoplasm (Cy) and minimal vacuolation (magnification X 10000).
C. Highly vacuolated cells (V = vacuole, P =plastids, N = nucleus) (magnification X 33000).
D. Meristemoids resulted into matured cell containing chloroplasts (magnification 30000).

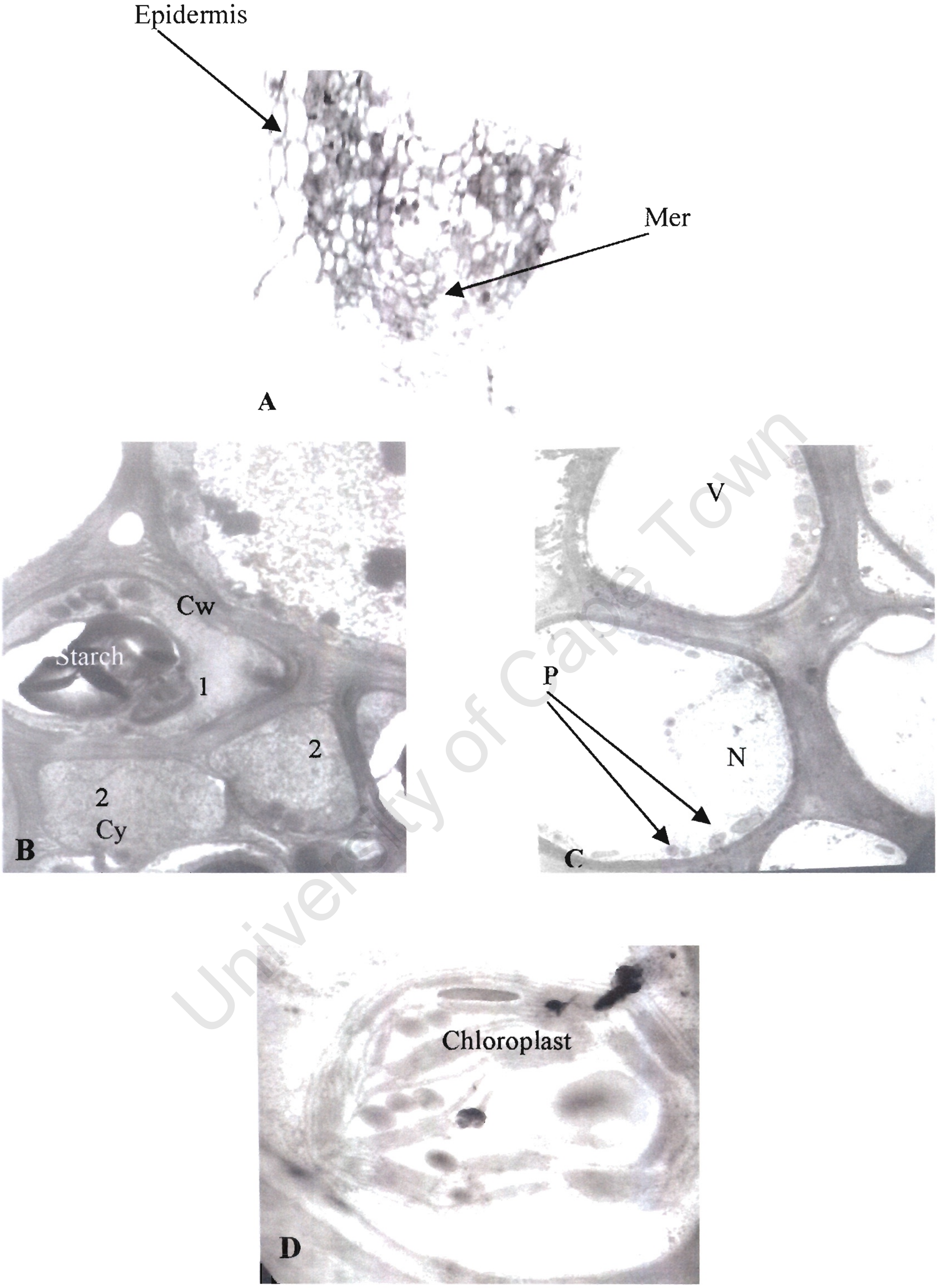


Figure 2.8. Histological evidence of indirect organogenesis (regeneration from callus) in *E. curvula.*

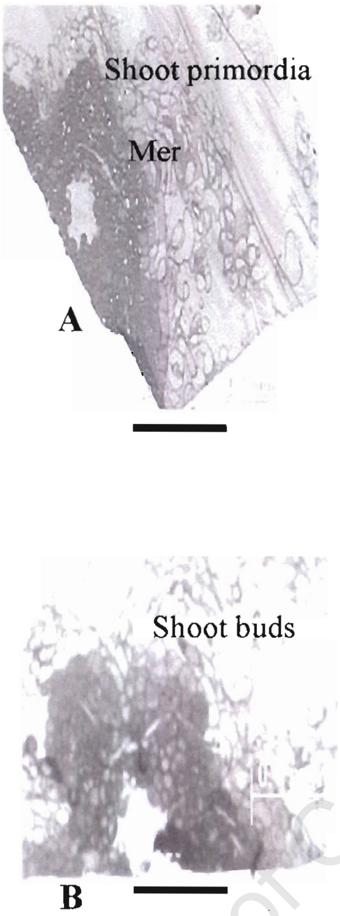


Figure 2.9. Histological evidence of direct organogenesis (regeneration from shoot explants) in *E. curvula* Figure 2.9 (A and B). Sections were mounted on the glass slide and stained with 1 % toluidine blue. Bars represent 0.1mm.

A. Shoot primordium regenerated in the meristemoids.

B. Transverse section of a developing shoot buds.

## 2.4 Discussion

### 2.4.1 Callus initiation

In the current study, callus was induced from immature leaf explants and seed material using MS macro and micronutrients as the basal medium. 5 different methods, which used various combinations of plant growth regulators, were used to initiate callus. Of the 3 methods that yielded relatively large callus fresh weight, best production of callus from seeds was achieved with a combination of 10 mg/l 2,4-D and 0.5 mg/l TDZ (Table 2.1, Method 4) whereas the most suitable concentrations of 2,4-D and BAP for callus initiation from leaf explants were 2 mg/l and 0.01 mg/l respectively (Table 2.2, Method 5). Noted from the 3 methods (Table 2.5) is that callus could still grow for up to at least 3 months with or without subculturing. However, in this study long culture was avoided to minimize changes in ploidy level from diploid to tetraploid or any other form of somaclonal variation, which leads to a reduction in regeneration potential (Nayak and Sen, 1991). Thus callus tissue was initiated and used within 4-8 weeks in culture.

### 2.4.2 Regeneration from callus

Regeneration from callus was approached using 3 different methods. Method C (Table 2.3) characterized by low concentration of BAP (0.01 mg/l) and high sucrose concentration (6% (w/v)), resulted in the best plant regeneration from callus that had been induced from immature leaf explants, the phenomenon which was also reported by Ahloowalia (1984) The noticeable factor in plant regeneration from callus in the current study is that the same medium could be used throughout the process. The inference that can be made from this is that induction of morphogenic callus and its maturation and development require the same hormonal signal in similar quantities. Alternatively the endogenous hormonal regulation enables the regeneration of shoots from callus. Using the basal part of the immature leaves, increasing the level of sucrose and lowering the level of cytokinin BAP all contributed to successful production of morphogenic callus from *E. curvula* leaf explants. High levels of BAP in particular inhibited the production of morphogenic callus, the phenomenon which was also described by Lowe *et al.* (1985) and subsequently Echenique *et al.*, (1996). In addition to plant growth regulators and carbon source, light intensity played vital role in plant regeneration since shoot regeneration was enhanced after callus exposure to high light intensity. Thus another inference that can be made from these data is that highest light intensity tested in this study (45 μmol/m$^2$/sec) (Figure 2.3) enhances plant regeneration from callus. An increase in regeneration might be associated with the suggestion that light probably facilitates sugar catabolism and respiration in the cells (Cousson and Tran Thanh Van, 1983).

In this study, it is clear that the production efficiency of morphogenic callus was not that high since only 2 to 4 pieces of callus regenerated from 10 pieces on a plate (Figure 2.2B). Furthermore, time required for development of morphogenic callus from leaf explants was long (8 weeks old callus used). Nevertheless, this callus is sufficient to conduct stable transformation studies since from each piece of callus approximately 50 shoots were obtained which could be separated into several plantlets.

Histological data on callus which was cultured on regeneration medium give a clear evidence for indirect organogenesis in *E. curvula.* It is therefore suggested that meristemoids produced from callus represent the original site of shoot formation. The observed epidermis (Figure 2.8A) presumably originated from apical meristem. Meristemoids are probably the most suitable cells for transformation studies since they can originate from single cells.

### 2.4.3 Regeneration from shoot explants

Regeneration of plants from shoot explants was achieved by using 2 different methods that differed in terms of plant growth regulators. Method 1 (Table 2.4) where MS medium was supplemented with 10 mg/l 2,4-D and 0.5 mg/l TDZ was the best method for plant regeneration from shoot explants. Method 1 can be utilized to micropropagate shoots regenerated from callus, thus increasing the number of regenerants (putative transformants). Through this morphogenic pathway one would also hope that the risk of somaclonal variation (Scowcroft *et al.*, 1987) often observed in the transformation process is minimized

Histological studies on shoot explants cultured on regeneration medium showed the presence of meristemoids and shoot primordial, therefore, regeneration probably occurred through the process of direct organogenesis. The shoot primordia presumably developed as a results of the de-differentiation of parenchyma cells or from meristematic tissue (Figure 2.9). The organization of parenchyma cells or meristemoids into shoot buds and subsequently to shoots can be attributed to hormonal factors, as it was noted previously that cytokinin TDZ resulted in more shoots than BAP.

In summary, two methods for *E. curvula* regeneration *in vitro* were developed. In the first method, callus induced from immature leaves produced shoots through the process of indirect organogenesis and fully developed plants were obtained after 5 months. In the second method, direct organogenesis from shoot explants occurs. Fully developed plants were obtained after 3 months. These methods provide sufficient regenerable tissues that can be used in transformation, with piece of callus producing more shoots than shoot explants. Thus regeneration efficiency from callus is higher than from shoot explants. However, as mentioned previously, production of morphogenic callus was low therefore future studies should concentrate on improving production efficiency of morphogenic callus.

# Chapter 3

## Construction of a vector for subsequent production of transgenic *E. curvula* plant expressing *Saccharomyces cerevisiae* Hsp 12 gene

### 3.1 Introduction

The basic procedure for the insertion of foreign genes into plants consists of 2 stages. The first stage is cloning the gene of interest into a suitable plasmid, which, in the second stage is used to transform plants (Sambrook *et al.*, 1989). As mentioned in Chapter 1, the vectors that are frequently used are those based on the naturally occurring plasmids of *Agrobacterium tumefaciens*. The Ti or tumour inducing plasmids are particularly chosen due to their natural capacity to transfer and express a particular segment of DNA in the plant cell genome (Zaenen *et al.*, 1973). The segment is called transferred or T-DNA coding for the synthesis of novel amino acids and sugar derivatives (opines). Genetic analysis of the Ti plasmid shows that two regions of this plasmid are essential for tumorigenesis, the T-DNA and the *vir* or virulence region. Any foreign DNA can be transferred to the plant nucleus provided that it is flanked by the T-regions. Therefore, experimentally, the *vir* functions are provided by a non-tumorigenic Ti plasmid derivative called a disarmed Ti plasmid or *vir* helper plasmid, in which T-DNA genes have been removed (Hood *et al.*, 1993; Torisky *et al.*, 1997). The derivatives of wild type Ti plasmid in which T-DNA genes have been removed include pPZP vector family (Hajdukiewicz *et al.*, 1994) among others.

In this project, the vector pCAMBIAUbeeQ (see Appendix C.1) was used. This vector is a modification of the pCAMBIA1201 vector (Mundree) to include the ubiquitin promoter upstream from the multiple cloning sites (MCS) in order to drive the expression of the inserted gene of interest. This promoter which is based on the maize ubiquitin gene resulted in increased gene expression compared with expression driven by the Cauliflower Mosaic virus 35 S RNA (CaMV35S) promoter. It was suggested that maize ubiquitin promoter was effective in monocotyledons (Christensen and Quail, 1996). The pCAMBIA1201 vector contains chloramphenicol and hygromycin resistance genes, which confer resistance to bacterial and plant cell growth and can be used as selectable markers. These resistance genes are under the control of the CaMV35S promoter. The vector also contains the $\beta$-glucuronidase (GUS) gene as a reporter gene which can be used to assess the efficiency of transformation. Moreover, there is an intron present in the GUS coding sequence which ensures that the gene is not expressed in bacteria, but only upon transfer into plants. The multiple cloning site is between the GUS gene (proximal to the right border of T-DNA transfer) and hygromycin gene (proximal to the restriction sites *EcoR* I, *Sac* I, *Kpn*, *Sma* I, *BamH* I, *Xba* I, *Sal* I, *Pst* I and *Hind* III). Other features include a hexahistidine sequence engineered at the carboxyl end of the GUS gene, allowing for protein purification by immobilized metal ion affinity chromatography (IMAC); blue/ white beta- galactosidase selection as well as high copy replication in *E. coli.*

In this study, the yeast Hsp12 gene was cloned into the *Sac* I restriction site of the MCS. The new construct, pCAMBIAUbeeQHsp12 (Appendix C.2) containing the Hsp 12 gene was replicated in *E. coli* and mobilized into *A. tumefaciens.*

## 3.2 Materials and Methods

### 3.2.1 Amplification of Hsp 12

The polymerase chain reaction (PCR) was used to amplify Hsp 12 *in vitro* using *Saccharomyces cerevisiae* chromosomal DNA as the template. The following sequence specific primers comprising *Sac* I restriction sites were used:

Forward primer: 5'ATGAGCTCACTGACGCAGGTAGAAAAG3'
Reverse primer: 5'CTGAGCTCTTACTTCTTGGTTGGGTCTTC3'.

The *Sac* I restriction site is underlined.

The amplification reaction was performed in a Sprinter programmable thermocycler (Hybaid) in a 50 µl reaction mixture containing 1 U *Taq* DNA Polymerase (Promega) dNTPs, primers, 50 ng template DNA (Appendix A.1.5.1). Amplification products were separated by electrophoresis on a 1.5 % agarose gel in TAE buffer (Appendix A.1.2). The resultant 330 bp band was gel purified using a QIAGEN kit (Appendix A.1.5.2) and stored at –20 $^{0}$C until required.

### 3.2.2 Subcloning of PCR product and transformation of competent cells

The gel purified PCR product was ligated into pGEMT-Easy (Promega) according to the manufacturer's protocol (Appendix A.1.5.2). The ligation mixture was used to transform competent *E.coli* JM109 cells. 100 µl of competent cells were mixed with 4 µl of the ligation mixture. The cells were then heat-shocked at 37 $^{0}$C for 5 min, and returned to ice for further 5 min. 900 µl of Luria Broth (LB) (Appendix D.3.1) was added to the cells, which were incubated at 37 $^{0}$C for 1 h under constant agitation. The cells were plated onto LB agar (Appendix D.3.2) supplemented with 100 µg/ml ampicillin, 0.5 mM isopropyl β-D-thiogalactopyranosidase (IPTG) and 80 µg/ml X-gal. The plates were incubated at 37 $^{0}$C for 18 h to allow selection of transformants. 5 ml of LB medium containing 100 µg/ml ampicillin was inoculated with one white colony picked using a sterile toothpick and grown at 37 $^{0}$C for 18 h. To verify that transformants contained the Hsp 12 gene, plasmid DNA was isolated using a High Pure Plasmid Isolation Kit (Roche) or by alkaline lysis (Appendix A.1.1). Isolated plasmid DNA was digested with *Sac* I (Appendix A.1.5.3) and electrophoresed on a 1.5 % agarose gel.

### 3.2.3 Cloning into pCAMBIAUbeeQ vector

Two different methods were used to construct pCAMBIAUbeeQHsp12

1) Hsp 12 was excised from pGEMT-EasyHsp12 by digesting the plasmid with *Sac* I. The digest was then electrophoresed, the Hsp12 fragment excised from the gel, and purified using a QIAGEN kit. The pCAMBIAUbeeQ vector was digested with the same restriction endonuclease and gel purified using the same methodology. The gel purified linear plasmid and the Hsp 12 insert were ligated at a ratio of plasmid to Hsp 12 of 1:3. (Appendix A.1.5.4)
2) The gel purified PCR product (the amplified Hsp 12 gene) was digested with *Sac* I and gel purified. This was then ligated to pCAMBIAUbeeQ as above.

The ligation mixture from either method was used to transform *E.coli* JM109 cells. Transformants were selected on LB medium containing 100 μg/ml chloramphenicol. Putative transformants were screened by PCR (Appendix A.1.5.1) and also by restriction endonuclease digestion.

### 3.2.4 Verification of correct orientation of Hsp 12 in pCAMBIAUbeeQ

PCR was used to confirm the correct orientation of Hsp 12 in the pCAMBIAUbeeQ vector using the plasmid DNA as a template. The reaction mixture was performed using a Ubiquitin specific primer as the forward primer:
5'CTGCAGGTCGACTCTAGAGGATCC3' and a Hsp12 specific reverse primer:
5'CTGAGCTCTTACTTCTTGGTTGGGTCTTC3'.
PCR conditions were the same as those used for amplification of the Hsp 12 gene (section 3.2.1). Plasmids containing the correct orientation of Hsp 12 were maintained in *E. coli* JM109 cells in LB broth containing chloramphenicol. The *E. coli* cultures were routinely cultured at 37 $^0$C. For mid-term and long-term storage, *E. coli* cells were stored in glycerol solution (at ratio of 1:1, glycerol to *E. coli* cells) and then kept at –70 $^0$C until

required. The cells were recovered by culturing in LB medium and plasmid isolated as above.

### 3.2.5 Transformation of *A. tumefaciens* with pCAMBIAUbeeQHsp12

2 μg of pCAMBIAUbeeQHsp12 was added to 100 μl of competent *A. tumefaciens* C58C1 cells. Cells were thawed by incubating at 37 $^0$C in a waterbath for 5 min. 1 ml LB was added to the cells which were then incubated at 30 $^0$C for 4 h with gentle shaking. After centrifugation for 30 s, the pellet was resuspended in 0.1 ml LB. The cells were spread onto LB plates containing 10 μg/ml chloramphenicol and the plates then incubated at 30 $^0$C for 72 h. 5 ml LB was then inoculated with a single white colony and incubated at 30 $^0$C for 2 days.

To confirm the presence of the Hsp 12 in *A. tumefaciens*, plasmid DNA was isolated from the *A. tumefaciens* cells grown for 2 days in LB medium using High Pure Plasmid Isolation Kit (Roche). 50 ng of the purified plasmid was used as a template DNA in a PCR reaction (Appendix A.1.5.1). Confirmation was also performed by *Sac* I endonuclease digestion (Appendix A.1.5.3). *A. tumefaciens* cells containing the Hsp12 insert were propagated in LB medium and stored in glycerol solution at –70 $^0$C until further use.

## 3.3 Results

### 3.3.1 Construction of pCAMBIAUbeeQHsp12

PCR was used to amplify Hsp 12 *in vitro* using *S. cerevisiae* chromosomal DNA as the template and Hsp 12 specific primers. A 330 bp fragment, the expected size of the PCR product, was present upon electrophoresis of an agarose gel. In the control, where template DNA was not added, no product was present on the gel (Figure 3.1).

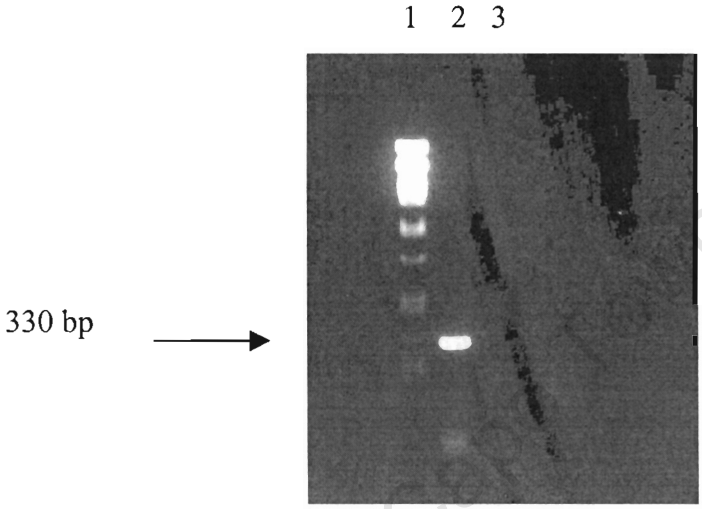


Figure 3.1. Electrophoresis on 1.5 % agarose of the PCR products after amplification of *S. cerevisiae* DNA using Hsp 12 specific primers. Lane 1: *Pst* I digested bacteriophage λ DNA , lane 2: PCR product, lane 3: negative control (no *S. cerevisiae* DNA present).

The PCR product was gel purified and cloned into pGEMT-Easy. The ligation mixture was used to transform *E. coli* JM109 cells. About 11 white colonies were found on the LB plate as well as many blue colonies (in each plate). These white colonies were screened for the presence of the Hsp 12 insert by restriction endonuclease digestion of the isolated plasmid. Successful cloning was shown by the presence of a 330 bp band after *Sac*1 digestion (Figure 3.2). 3 of the 11 colonies were positive. This construct was named pGEMT-EasyHsp12.

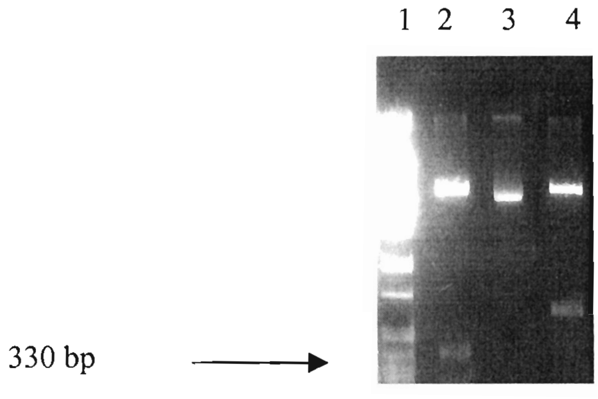
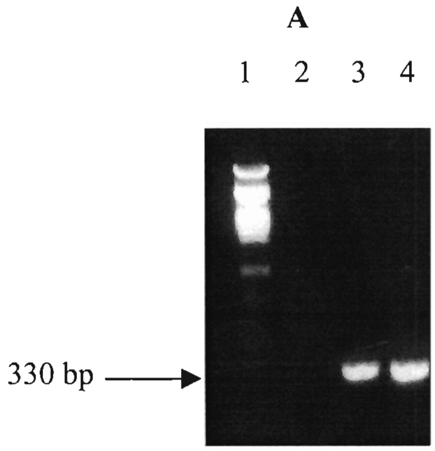


Figure 3.2. *Sac* I restriction endonuclease digestion of plasmid isolated from typical white colonies. Lane 1: *Pst* I digested bacteriophage λ DNA, lane 2: putative transformant (white colony), lane 3: non-transformed colony (blue colony), lane 4: undigested pGEMT-Easy.

The 330 bp Hsp 12 DNA excised from pGEMT-EasyHsp12 with *Sac* I (Figure 3.2) was gel purified and ligated into pCAMBIAUbeeQ linearised with *Sac* I. The ligation mixture (section 3.2.3) was used to transform *E. coli* JM109 cells. Plasmids were isolated from 20 of the approximately 60 white colonies present on the LB plate. The presence of the Hsp 12 insert was probed by PCR using Hsp 12 specific primers (Figure 3.3A). Of the 20 colonies screened for the presence of the Hsp 12 insert, only 1 was found to be a positive clone. To confirm that this colony indeed contained the Hsp 12 gene, the plasmid was isolated and digested with *Sac* I. A 330 bp fragment (Figure 3.3B) confirmed the positive PCR result. The resulting construct was named pCAMBIAUbeeQHsp12 (Appendix C.2).



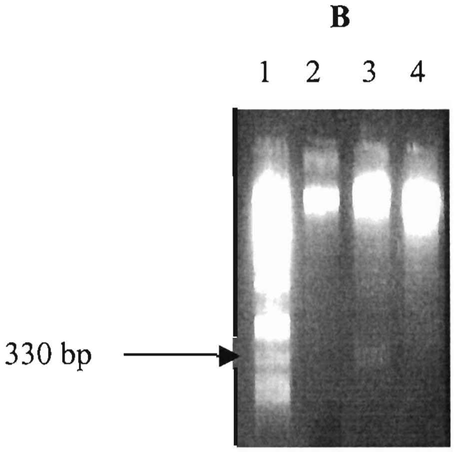


Figure 3.3A. PCR screening for the presence of the Hsp 12 insert in pCAMBIAUbeeQ . Lane 1: *Pst* I digested bacteriophage λ DNA, lane 2: non-transformant (white colony), lane 3: putative transformant (white colony), lane 4: positive control (using pGEMT-EasyHsp12 as the template).

Figure 3.3B. *Sac* I restriction endonuclease digestion of the single white colony containing Hsp 12. Lane 1: *Pst* I digested bacteriophage λ DNA, lane 2: non-transformant, lane 3: putative positive transformant, lane 4: non-transformant.

The correct orientation of Hsp 12 in pCAMBIAUbeeQ was next confirmed by PCR. The ubiquitin promoter specific forward primer together with the Hsp 12 specific reverse primer were used to amplify the recombinant plasmid. A 330 bp fragment, the expected size, was present upon electrophoresis on the agarose gel (Figure 3.4) suggesting that the plasmid contained the Hsp 12 insert in the correct orientation.

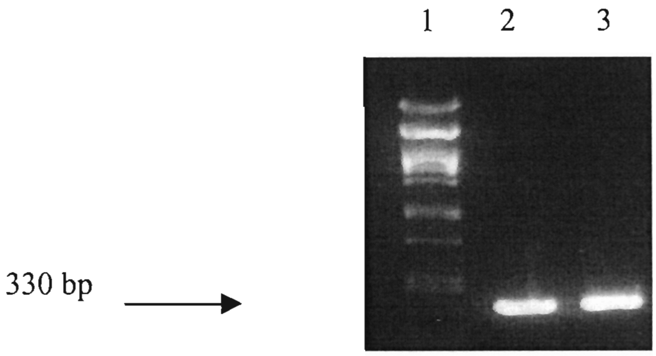
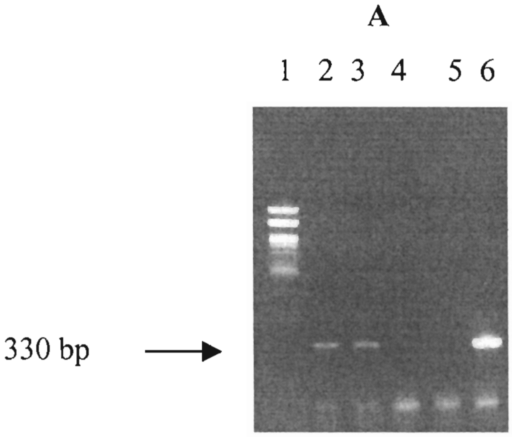


Figure 3.4. Testing the correct orientation of Hsp 12 in pCAMBIAUbeeQ. Lane 1: *Pst* I digested bacteriophage λ DNA, lane 2: PCR products (putative positive transformant), lane 3: positive control (using pGEMT-EasyHsp12 as the template).

### 3.3.2 Transformation of *A. tumefaciens* with pCAMBIAUbeeQHsp12

pCAMBIAUbeeQHsp12 recovered from *E. coli* cells was used to transform *A. tumefaciens* C58C1 cells. 10 white colonies were present on the LB plate. Plasmids were isolated from these colonies. The presence of Hsp 12 was probed by the PCR using Hsp 12 specific primers (Figure 3.5A). Of 10 colonies screened for the presence of Hsp 12 insert, 4 colonies were found to be positive. These PCR results were confirmed by digesting the plasmid with *Sac* I. A 330 bp fragment confirmed the positive PCR results (Figure 3.5B).



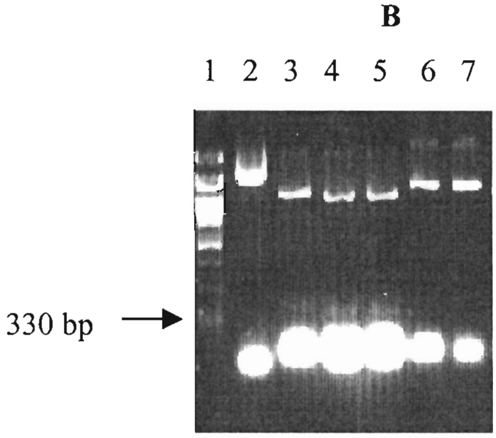


Figure 3.5A. PCR screening for the presence of the Hsp 12 insert in *A. tumefaciens*. Lane 1: *Pst* I digested bacteriophage λ DNA, lane 2: putative transformant (white colony), lane 3: putative transformant (white colony), lane 4: non-transformant, lane 5: non-transformant, lane 6: positive control (using pGEMT-EasyHsp12 as the template).

Figure 3.5B. *Sac* I restriction endonuclease digestion of plasmid isolated from the white colony containing Hsp 12. Lane 1: *Pst* I digested bacteriophage λ DNA, lane 2: plasmid DNA (uncut), lane 3: putative positive transformant, lane 4: putative positive transformant, lane 5: putative positive transformant, lane 6: non-transformant, lane 7: non-transformant.

# Chapter 4

## Transient and subsequent stable transformation of *E. curvula*

### 4.1 Introduction

Genes are transferred into other organisms using several different methods. These include *Agrobacterium*-mediated transformation as well as several direct gene transfer methods including microprojectile bombardment or biolistics (Sanford, 1987), electroporation, silicon carbide fibers, and microinjection (Songstad *et al.*, 1995) (see Chapter 1). Dicotyledonous plants are commonly transformed by the use of *A. tumefaciens*, the strain of *Agrobacterium* that naturally infects dicotyledonous plants by transferring its own DNA into the plant cell. One of the disadvantages of using *A. tumefaciens* as a vector is that monocotyledonous plants, including maize, cereal, and rice, are not naturally infected by this bacterium (Greenberg and Glick, 1993). However, this method has been sufficiently improved to allow the transformation of many monocotyledonous plant species (Birch, 1997). The improvements include the use of phenolic compounds such as acetosyringone to enhance the virulence of *Agrobacterium* (Stachel *et al.*, 1985). There are numerous recent reports of *Agrobacterium*-mediated transformation of monocotyledonous plants (McGranaham, 1988), including rice (Hiei *et al.*, 1994) and wheat (Cheng *et al.*, 1997) which are not considered to be natural hosts for this bacterium.

Biolistics is generally effective regardless of plant, tissue or cell type (Kartha *et al.*, 1989; Vasil and Vasil, 1996). This method uses rapidly propelled tungsten or gold microprojectiles that have been coated with DNA. The foreign DNA transferred using biolistics is often incorporated at random into the plant DNA. One of the disadvantages of this method is possible damage to the DNA which is caused by the bombardment process (Greenberg and Glick, 1993). The biolistics method has been modified to try and improve the intake of foreign DNA by plant DNA by binding the foreign DNA to Histone H1 to facilitate its entry into the nucleus (Chen *et al.,* 1998).

Although the transformation methods currently available have been proven successful, they generally result in low rates of transformation (Shillito *et al*., 1985). To monitor the transformation process, reporter genes are generally included in the vector construct (Nottingham, 1988). Reporter genes such as the GUS gene are expressed within days after transformation and indicate that the transgene has penetrated the cell wall, that the cell has survived the transformation, and that the transgene trait has been expressed (transient expression). The GUS gene encodes *β*-glucuronidase, a hydrolase that catalyses the cleavage of a wide variety of *β*-glucuronides, many of which are available commercially as spectrophotometric and histochemical substrates (Jefferson *et al*., 1987). GUS enzymatic activity is often visualized as a blue precipitate after histochemical staining with 5-bromo-4-chloro-3-indolyl *β*-glucuronide (X-Gluc) and thus blue spots represent a transformed cell or group of transformed cells. As reviewed in Chapter 1, the stable integration of the transgene is determined by Southern blotting (Southern, 1975; Sambrook *et al*., 1989) and PCR (Mullis and Faloona, 1987; Higgins *et al*., 2000). The expression of a transgene can also be evaluated at the RNA level by means of Northern blot analysis and/or RT-PCR. Translation of a transgene into encoded protein can be demonstrated with SDS-PAGE, Western blotting, ELISA, or MALDI-TOF mass spectrometry.

The focus of this section was to establish a transformation protocol for *E. curvula*, a monocotyledonous plant that has not been reported to have been transformed to date and is presumably not susceptible to *Agrobacterium* infection. Thus biolistics was used to transform *E. curvula* with the *S. cerevisiae* Hsp 12 gene using gold particles coated with either DNA alone or complexed with chicken erythrocyte histones.

## 4.2 Materials and methods

### 4.2.1 Biolistic-mediated transformation of *E. curvula*

#### 4.2.1.1 Preparation of target tissue for bombardment

A requirement for biolistics is that the target cell material must contain rapidly dividing cells, such as callus, or a tissue that is capable of regeneration. Callus (8 weeks old) used was induced from immature leaves on the MS medium supplemented with 2 mg/l 2,4-D and 0.01 mg/l BAP. Shoot explants used were obtained from shoots induced from seeds on MS medium supplemented with 10 mg/l. 2,4-D and 0.5 mg/l TDZ (see Chapter 2). For each bombardment, callus or shoots explants were spread in a circle over a 30 mm diameter in the center of a Petri dish containing either MS high osmoticum medium (callus induction medium supplemented with mannitol and myo-inositol (Appendix D.1.1, D.1.2) or "standard" medium (same as the above medium but not supplemented with mannitol and myo-inositol). Tissues bombarded on osmoticum medium were cultured on this medium for 24 h prior to and for 24 h after bombardment. During the pre-bombardment period, cultures to be bombarded were incubated in the dark at 25 $^0$C.

#### 4.2.1.2 Preparation of gold particles for bombardment

Plasmid DNA precipitation onto 60 mg sterile gold particles (sterilized by washing with absolute ethanol and resuspended in 50 % sterile glycerol) was performed according to the method of Klein *et al* (Klein *et al.*, 1987). 10 μg of plasmid DNA pCAMBIAUbeeQHsp12 isolated using High Pure Plasmid Isolation kit (Roche) (see Chapter 3) was precipitated with 2.5 M $CaCl_2$ and 0.1 M spermidine onto a sterile gold particle suspension by vortexing for 3 min. After centrifugation (12000 rpm for 10 s at 20 $^0$C), the supernatant was discarded and the DNA-coated particles were resuspended in 75 μl absolute ethanol. 10 μl aliquots were dried on sterile macrocarriers (Biorad) for bombardment.

DNA was also precipitated using a modified "Taxi" protocol (Chen *et al.*, 1998). Plasmid DNA (6 μg in 75 μl water) was linearised with *Hind* III (Appendix A.1.5.3), and then denatured by incubation at 100 $^0$C for 10 min. The DNA was then mixed with a chicken erythrocyte total histone extract (50 μg in 25 μl water) and incubated on ice for 30 min before being added to the sterile gold particles. After mixing at 20 $^0$C for 45 min, the particles were centrifuged at 12000 rpm for 10 s, the supernatant discarded and DNA-coated gold particles resuspended in 60 µl sterile water.

### 4.2.1.3 Bombardment of shoot explants and callus tissue

A PDS 1000/He Biolistic gun (Biorad, Richmond, CA, USA) was used for transformation according to the manufacturer's instructions (Biorad). Tissues were bombarded with 1 µm diameter gold microprojectiles. Four bombardment experiments were performed, at which various helium pressures (6300 and 9100 kPa) determined by use of the rupture discs (900 and 1300 psi respectively), numbers of bombardments (target tissue bombarded once or twice), and microcarrier travel distance to the stopping plate (7 or 10 cm), were evaluated. These experiments are summarized in Table 4.1 Each treatment contained 5 replicates. The bombarded tissues were left overnight in the osmoticum medium before being transferred to regeneration media.

As a comparative study, *Nicotiana tabacum* was transformed with Hsp 12 gene using *Agrobacterium*-mediated transformation. Transformation of *N. tabacum* leaf discs and regeneration of transgenic plants by co-cultivation with *A. tumefaciens* containing Hsp 12 gene and selection on the media containing 25 µg/ml hygromycin was as described by De Block *et al* (De Block *et al.*, 1987). Callus was induced from *N. tabacum* leaf discs infected with *A. tumefaciens* cultured on MS regeneration medium supplemented with 0.1 mg/l NAA, 0.5 mg/l BAP and 25 µg/ml hygromycin under 16 h photoperiod conditions. Shoots were induced from hygromycin resistant *N. tabacum* callus under the same conditions as above except that hygromycin selection was not performed.

Table 4.1. Bombardment conditions tested (rupture disc pressure, number of bombardments and microprojectile travel distance). The DNA alone or complexed to chicken erythrocyte histones* was coated on gold particles before biolistic bombardment.

| | **Pressure kPa** | **Target tissue** | **Travel distance (cm)** | **No. of bombardment** |
|---|---|---|---|---|
| **Experiment 1** | 6300 | shoot explant | 7 | single |
| | 6300 | shoot explant | 7 | double |
| | 6300 | shoot explant | 10 | single |
| | 6300 | shoot explant | 10 | double |
| **Experiment 2** | 9100 | shoot explant | 7 | double |
| **Experiment 3** | 9100* | shoot explant | 7 | double |
| **Experiment 4** | 9100 | callus | 7 | double |

### 4.2.1.4 Transient expression of GUS gene in shoot explants

Two alternative methods were used to determine GUS activity. In the first method, transient expression was visualized 72 h and 4 weeks after bombardment by staining shoot explants for GUS activity with 1 mM X-Gluc in 1 mM $Na_2EDTA$, 0.5 % (w/v) sarcosyl, 50 mM $NaH_2OPO_4$ pH 7 (Jefferson *et al.*, 1987). Alternatively, explants were incubated for 1 h in 0.07 % (v/v) Liqui-Nox in 0.1 M phosphate buffer pH 6.8, washed twice with 0.1 M phosphate buffer pH 6.8 before being incubated in assay buffer (0.5 mM $K_3Fe(CN)_6$, 2.0 mM X-Gluc, 10 mM $Na_2EDTA$, 0.1 M phosphate pH 6.8). The X-Gluc substrate was infiltrated into the tissue by applying a partial vacuum for 4 min (Rueb and Hensgens, 1989). Samples were then incubated at 37 $^0$C overnight. After staining, tissue sections were cleared with 70 % ethanol solution to improve contrast. Samples were checked for blue colouration under light microscopy.

#### 4.2.1.5 Culture of bombarded tissues

Bombarded tissues were cultured as described in Chapter 2. 24 h after bombardment, shoot explant tissues were transferred to regeneration medium consisting of MS medium supplemented with 0.5 mg/l TDZ, whereas callus tissues were transferred to MS medium supplemented with 0.01 mg/l BAP and 2 mg/l 2,4-D. Cultures were transferred to fresh medium every 2 weeks. Fully developed shoots were rooted on MS medium without hormones. Molecular analysis was used to verify the stable transformation of the *E. curvula* and the comparative plant, *N. tabacum*.

### 4.2.2 Transgenic analysis

#### 4.2.2.1 PCR analysis for the presence of Hsp 12 gene

Plants regenerated from bombarded shoot explants and callus tissue were grown to a height of approximately 7 cm. DNA was isolated from the leaves of each plant and used for PCR analysis with primers specific to the Hsp 12 gene. Different DNA extraction methods were used for *E. curvula* and *N. tabacum*.

*DNA extraction from E. curvula plants.* DNA was extracted according to the method of Doyle and Doyle (1990). Leaf samples (150 mg) in a 2 ml Eppendorf tube were frozen using liquid nitrogen for 10 s and then ground to a fine powder. 400 μl extraction buffer (3 % (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 0.2 % (v/v) β-mercaptoethanol, 100 mM Tris-HCl pH 8.0) was added and each sample was incubated at 60 $^0$C for 30 min. An equal volume of chloroform: isoamylalcohol (24:1) was added, mixed thoroughly and centrifuged at 12 000 rpm for 5 min at 4 $^0$C. The supernatant was transferred to a clean tube, the DNA was precipitated with 250 μl isopropanol, centrifuged for 10 min at 12 000 rpm and washed with 70 % ethanol. DNA pellets were resuspended in 50 μl distilled water and the concentration of DNA in each sample was determined spectrophotometrically (Appendix A.1.4).

*DNA extraction from N. tabacum plants.* DNA was isolated according to method of Lin *et al.*, (2001), a method devised from Pich *et al.*, (1993). Leaf samples (150 mg) in a 2 ml Eppendorf tube were frozen using liquid nitrogen for 10 s and then ground to a fine powder. 600 µl extraction buffer (2 % (w/v) SDS, 500 mM NaCl, 50 mM EDTA, 2 % (v/v) β-mercaptoethanol, 1 % (w/v) PVP, 100 mM Tris-HCl pH 8.0) was added and each sample was incubated at 65 $^0$C for 15 min before being centrifuged at 12000 rpm for 10 min at 4 $^0$C. The supernatant was transferred to a new tube, 2 µl of 10 mg/ml RNAse was added and the tube incubated at 37 $^0$C for 10 min. An equal volume of phenol: chloroform: isoamylalcohol mixture (25: 24: 1) was added, mixed gently and centrifuged for 3 min at 12 000 rpm. The upper phase was transferred to a new tube and the phenol: chloroform: isoamylalcohol extraction repeated. DNA was recovered as above.

*PCR analysis.* PCR was performed in a 25 µl reaction volume containing 50 ng template DNA and the Hsp 12 specific primers. PCR conditions were identical to those used for amplification of the Hsp 12 gene (section 3.2.1). The PCR products were separated by electrophoresis on a 1.5 % agarose gel in TAE buffer (Appendix A.1.2).

#### 4.2.2.2 RT-PCR

*RNA extraction from leaves of E. curvula and N. tabacum plants.* Total RNA was isolated according to the method of Chomczynski and Sacchi (1987). All solutions used were prepared with distilled water treated with diethylpyrocarbonate (DPEC) (Sambrook *et al.*, 1989) and autoclaved unless indicated. Leaf samples (500 mg) were ground to a fine powder in liquid nitrogen with a mortar and pestle. 4 ml guanidine thiocyanate (GTC) solution (4 M GTC, 25 mM $Na_3$ citrate, 0.5 % (w/v) sarcocyl, 0.1 M β-mercaptoethanol) was added and mixed after which 0.5 ml (2 M NaOAc pH 4.0), 4.5 ml phenol and 1 ml chloroform: isoamylalcohol (24: 1) were added, mixed thoroughly and incubated on ice for 10 min. The mixture was centrifuged at 12 000 rpm for 20 min at 4 $^0$C. The aqueous phase was transferred to a new tube and the pellet re-extracted as before. RNA was precipitated after the addition of an equal volume of isopropanol, washed with 70 % ethanol, air dried briefly and resuspended in DEPC-treated water. The concentration of RNA was determined spectrophotometrically (Appendix A.1.4).

*Reverse transcription.* RNA was treated with DNAse for removal of contaminating DNA. cDNA was synthesized by mixing an aliquot of the RNA preparation with RT buffer, Oligo dT, dNTPs and MAVReverse transcriptase enzyme (Promega) in an Eppendorf tube and the tube incubated as described in Appendix A.1.5.1.1. The cDNA was then used as the template for amplification by PCR using primers specific to Hsp 12 gene. The PCR conditions were identical to those used for amplification of the Hsp 12 gene (see Chapter 3, section 3.2.1) and the products were separated by electrophoresis on a 1.5 % agarose gel in TAE buffer (Appendix A.1.2).

### 4.2.2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

*Plant protein extraction from plants.* Proteins were either extracted according to the method of Bartels *et al.* (1991) that uses Laemmli buffer or by using a buffer without a detergent sodium dodecyl phosphate (SDS) (Buffer S). Leaf material (100 mg) harvested 3 times in the period of 4 months was ground to a fine powder in a mortar and pestle in liquid nitrogen. The material was then transferred to a sterile 1.5 ml Eppendorf tube to which either 400 μl Laemmli sample buffer (2 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 0.25 M Tris-HCl pH 6.8) or buffer S (20 mM NaCl, 0.1 mM PMSF 10 mM Tris-HCl pH 7.4.) was added. The samples were mixed thoroughly for 5 min and then centrifuged at 12000 rpm for 5 min at 4 $^0$C. The supernatant was carefully removed and placed in a clean 1.5 ml Eppendorf tube. 4 μl of 100 mM phenylmethanesulfonylchloride (PMSF) (Appendix B.2.1) was added to a final concentration of 1 mM. The protein samples were stored at – 70 $^0$C until required.

*Protein extraction from S. cervisiae (yeast).* Proteins were extracted (Motshwene *et al.*, 2003) from yeast grown to stationary phase at 30 $^0$C in sterile medium (1 % yeast extract, 2 % bactopeptone, 2 % dextrone) (YPD). Cells were harvested at 4 $^0$C by centrifugation at 7 000 g using a Beckman JA 20 rotor for 10 min, the pellet washed 3 times with 20 mM NaCl, 10 mM Tris-HCl pH 7.4 and resuspended in 400 μl 0.6 M NaOH. After 1 h at 0 $^0$C the sample was centrifuged at 12000 rpm for 5 min at 4 $^0$C and the supernatant was stored at –20 $^0$C until required.

*SDS-PAGE.* All samples were heated at 100 $^0$C for 5 min and centrifuged (12000 rpm for 5 min at 4 $^0$C) before electrophoresed. SDS-PAGE was performed by the method of Laemmli (1970) using a 20 % (w/v)separating gel and 6.5 % (w/v) stacking gel (Appendix B.2.2). Gels were run at 200 V constant voltage for 4 hours, stained with Coomassie brilliant blue, and destained using 7 % (v/v) acetic acid containing 25 % (v/v) ethanol. The SDS-PAGE experiment was repeated 5 times.

### 4.2.2.4 MALDI-TOF mass spectrometry

Peptide mass fingerprinting was performed using a model Voyager DE™-Pro (PerSeptive Biosystems) MALDI-TOF mass spectrometer. The band of protein was excised from the gel and cut into small pieces. The gel pieces were washed with $CH_3CN/H_2O$ (1:1 v/v), $CH_3CN$ /50 mM $NH_4HCO_3$ (1:1 v/v) and finally with $CH_3CN$ /10 mM $NH_4HCO_3$ (1:1 v/v) and then incubated with 10 mM $NH_4HCO_3$ containing 0.1 μg trypsin (Sigma) at 37 $^0$C for 24 h. A 1 μl aliquot of the digest was added to 1 μl of 10 mg/ml sinapinic acid (3,5-Dimethoxy-4-hydroxycinnamic acid) matrix and then subjected to MALDI-TOF for analysis.

## 4.3 Results

### 4.3.1 Transient and stable GUS gene expression in *E. curvula*

Shoot explants were transformed with the pCAMBIAUbeeQHsp12 vector containing the GUS gene by employing biolistic-mediated transformation. For visualization of transient expression of the GUS gene, some of the shoot explants were stained for GUS activity with X-Gluc 72 h after bombardment. Transient transformation frequencies, determined by positive GUS activity, obtained from all experiments are presented in Table 4.2. In experiment 1, no tissues showed any visible blue colouration 72 h after bombardment since the velocity of the particles was possibly too low to allow penetration of the cell wall. Increased pressure was used in experiment 2. This resulted in transient GUS gene expression as indicated by blue colouration 72 h after bombardment (Figure 4.1A) with 3 out of 10 histochemically stained tissues being GUS positive (Table 4.2, experiment 2). It was next investigated whether the transformation frequency could be improved by complexing the DNA to chicken erythrocyte total histones prior to bombardment. However, it was found that no GUS activity was observed 72 h after bombardment (Table 4.2, experiment 3). This was attributed to the complicated process of preparing the DNA (section 4.2.1.2) and the method was therefore not used.

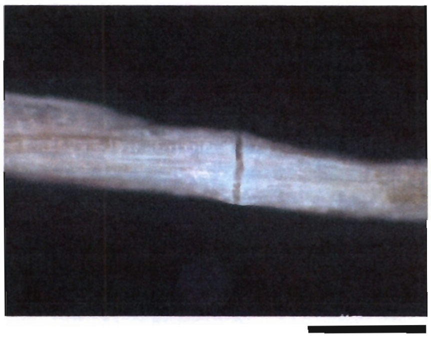
Stable GUS gene expression was visualized using microscopy by blue colouration in leaf tissue regenerated from transformed shoot explants 4 weeks after bombardment (Figure 4.1B). A higher level of stable GUS expression was observed in older leaves (Figure 4.1B) than in shoot explants (compare Figure 4.1A and 4.1B) with the highest level of GUS expression found to occur in the venation (veins and mid-ribs) of the shoot explants (Figure 4.1A) and in the leaf edges and tips of the old leaf (Figure 4.1B). No control tissues (unbombarded shoot explants) showed any visible GUS expression (not shown).

Some of the *E. curvula* shoot explants bombarded as per experiment 2 but not used for histochemical staining were regenerated on MS medium supplemented with 0.5 mg/l TDZ. An average of 6 out of 10 transformed shoot explants on a plate regenerated to mature plants that were subsequently probed using molecular techniques for the presence of the Hsp 12 gene.

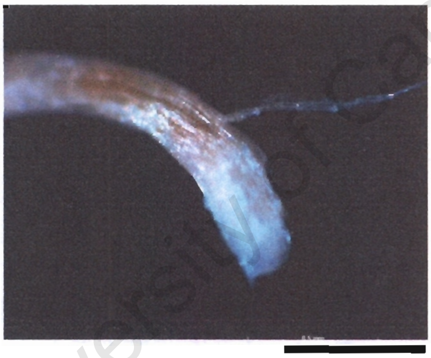
*E. curvula* callus tissue was also bombarded on osmoticum medium using the same methodology used for the shoot explants. Callus tissue was regenerated on MS medium supplemented with 2 mg/l 2,4-D and 0.01 mg/l BAP. An average of only 1 out of 10 pieces of callus on a plate regenerated (Figure 4.2). The frequency of regeneration from shoot explants did not show any significance decrease after bombardment whereas the frequency of regeneration from callus decreased considerably (compare Figure 2.2B and Figure 4.2). The comparative plant, *N. tabacum,* transformed using *A. tumefaciens* containing the Hsp 12 gene was also regenerated *in vitro* (Figure 4.3). Since regenerated plants were obtained from *E. curvula* shoot explants, callus tissue and *N. tabacum*, the next step was to use molecular techniques to investigate whether the Hsp 12 gene was present, and whether it was transcribed and translated.

Table 4.2. Transient GUS expression as a function of rupture disc pressure, number of bombardments and microprojectile travel distance. The DNA alone or complexed to chicken erythrocyte histones* was coated on gold particles before biolistic bombardment.

| | **Pressure kPa** | **Target tissue** | **Travel distance (cm)** | **No. of bombardment** | **Frequency of Transient GUS expression** |
|---|---|---|---|---|---|
| **Experiment 1** | 6300 | shoot explant | 7 | single | 0/10 |
| | 6300 | shoot explant | 7 | double | 0/10 |
| | 6300 | shoot explant | 10 | single | 0/10 |
| | 6300 | shoot explant | 10 | double | 0/10 |
| **Experiment 2** | 9100 | shoot explant | 7 | double | 3/10 |
| **Experiment 3** | 9100* | shoot explant | 7 | double | 0/10 |



A



B

Figure 4.1A. Expression of the GUS gene in bombarded *E. curvula* shoot explants. The blue colour represents a transient transformation event visualised by staining with X-Gluc 72 h after bombardment (bar = 0.5 mm).

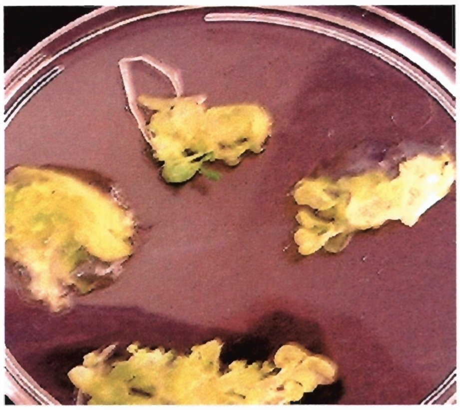
Figure 4.1B. Expression of the GUS gene in *E. curvula* mature leaf tissue regenerated from bombarded shoot explants. The blue colour represents a stable transformation event visualised by staining with X-Gluc 4 weeks after bombardment (bar = 0.5 mm).

A

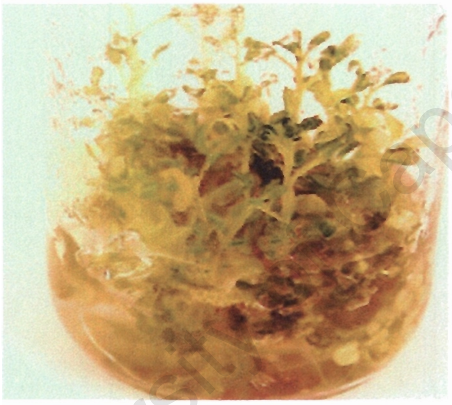
B

Figure 4.2A *E. curvula* callus tissue showing regeneration of plantlets. Callus was cultured on MS medium supplemented with 2 mg/l 2,4-D and 0.01 mg/l BAP incubated under 16 h photoperiod conditions.

Figure 4.2B. *E. curvula* plant regenerated from bombarded callus tissue.



A



B

Figure 4.3A. Hygromycin resistant callus induced from *N. tabacum* leaf discs. Leaf discs were cultured on MS regeneration medium supplemented with 0.1 mg/l NAA, 0.5 mg/l BAP and 25 μg/ml hygromycin. Callus was produced after 3-4 weeks of incubation under 16 h photoperiod conditions.

Figure 4.3B. Shoots induced from hygromycin resistant *N. tabacum* callus. Callus was cultured on MS shooting medium supplemented with 0.1 mg/l NAA and 0.5 mg/l BAP. Shoots regenerated after 1 month of incubation under 16 h photoperiod conditions.

### 4.3.2 Transgenic analysis

#### 4.3.2.1 PCR

To probe the presence of the Hsp 12 gene in plants regenerated from bombarded shoot explants and callus tissue, genomic DNA was extracted from *E. curvula* leaves. The genomic DNA was amplified by PCR using Hsp 12 specific primers. A 330 bp fragment, the expected size of the PCR product, was present upon electrophoresis (Figure 4.4A and 4.4B) of the PCR products from independent lines or plants. PCR analysis also showed that the Hsp 12 gene had been introduced into the genome of *N. tabacum* (Figure 4.4C) by *Agrobacterium*-mediated transformation. In control untransformed plants, no 330 bp PCR product was observed. Only 1 out of 25 plants regenerated from shoot explants was found to be positive for the Hsp 12 gene whereas 5 out of 20 plants regenerated from callus were found to be positive. Transformed plants were probed by RT-PCR to determine whether Hsp 12 gene was transcribed.

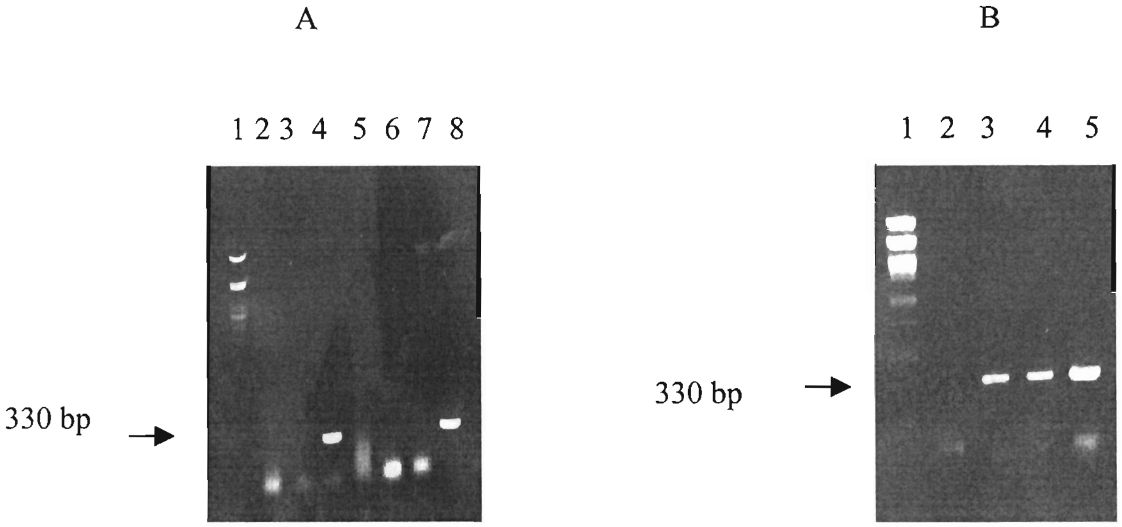


Figure 4.4. Electrophoresis on a 1 % agarose gel of the products of PCR amplification using Hsp 12 specific primers of genomic DNA extracted from *E. curvula*. The arrow indicates 330 bp Hsp 12 DNA. A) Genomic DNA extracted from leaves regenerated from bombarded shoot explants was used as the template. Lane 1: *Pst* I digested bacteriophage λ DNA; lane 2 to 6: PCR products from transformed plants; lane 7: PCR product from untransformed plant (negative control); lane 8: PCR product from plasmid pGEMT-EasyHsp12 (positive control). B) Genomic DNA extracted from leaves regenerated from bombarded callus was used as the template. Lanes 1: *Pst* I digested bacteriophage λ DNA; lane 2: PCR product from untransformed plant (negative control); lane 3 and 4: PCR products from transformed plants; lane 5: PCR product from plasmid pGEMT-EasyHsp12 (positive control).

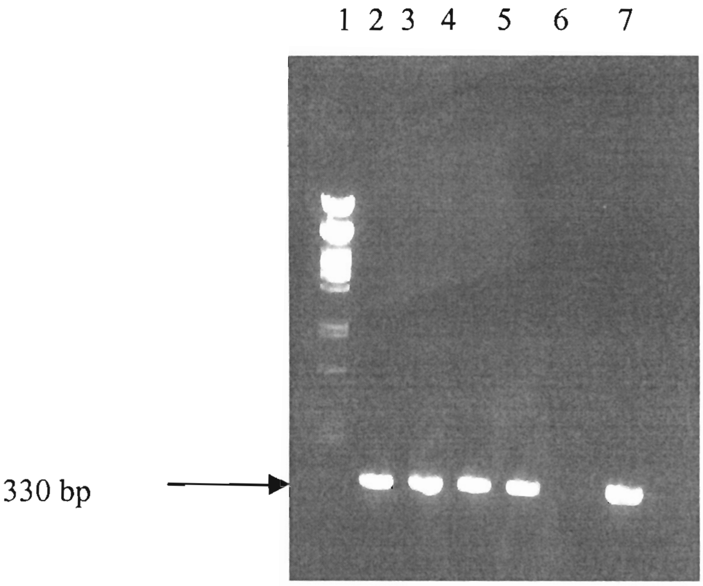


Figure 4.4C. Electrophoresis on a 1 % agarose gel of the products of PCR amplification using Hsp 12 gene specific primers of genomic DNA extracted from *N. tabacum* leaves. Lane 1: *Pst* 1 digested bacteriophage λ DNA; lane 2-5: PCR products from transformed plants; lane 6: PCR product from untransformed plant (negative control); lane 7: PCR product from plasmid pGEMT-EasyHsp12 (positive control).

### 4.3.2.2. RT-PCR

Since the Hsp 12 gene was detected in some *E. curvula* and *N. tabacum* plants using PCR, the next step was to investigate whether this gene was transcribed. Total RNA was extracted from the leaves of transformed plants and reverse transcribed to produce cDNA, which was then used as a template in a PCR reaction using Hsp 12 specific primers. A 330 bp fragment, the expected size of the PCR product, was present upon electrophoresis (Figure 4.5) of cDNA prepared by RT-PCR from both *E. curvula* and *N. tabacum*. The Hsp 12 gene introduced in the genome of the plants was therefore transcribed. The possibility of DNA contamination in the assay was checked by PCR without the reverse transcription step. No product was present on the gel (Figure 4.5).

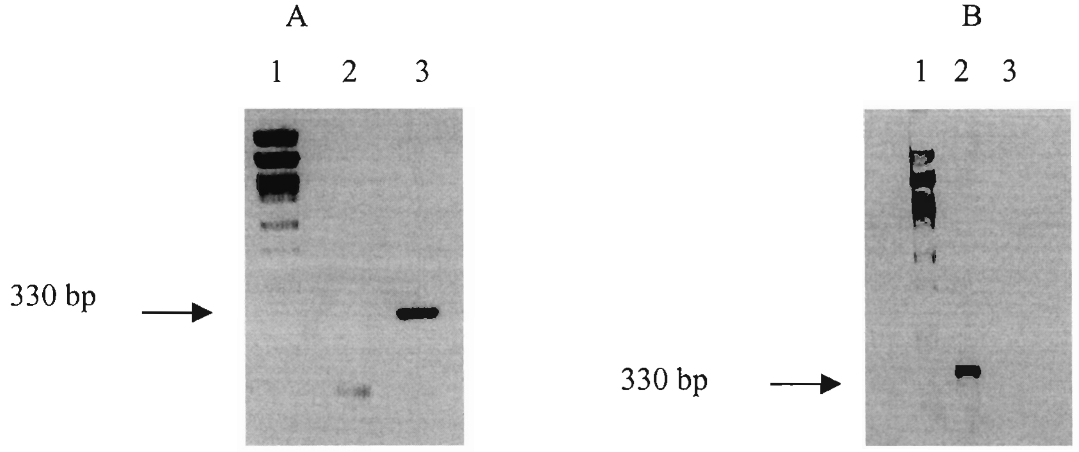


Figure 4.5 Electrophoresis on a 1 % agarose gel of the products of PCR amplification using Hsp 12 specific primers of cDNA synthesized from RNA extracted from *E. curvula* (A) and *N. tabacum* (B) leaves. Lane 1: *Pst* I digested bacteriophage λ DNA; lanes 2A and 3B: no reverse transcription performed (negative control); lanes 3A and 2B: PCR products after reverse transcription.

### 4.3.2.3 SDS-PAGE

The presence and transcription of the Hsp 12 gene warranted the investigation of the presence of the Hsp 12 protein in transformed plants. To guard against selective extraction of proteins, proteins were extracted from untransformed *E. curvula* leaves using 2 different methods. Both methods used a salt-based extraction buffer, but that of Bartels *et al.* (1991) included the anionic detergent SDS. The results of these extractions (Figure 4.6A) showed that there was no difference in the banding pattern of the proteins on the gel. The method of Bartels *et al.* (1991) was used in subsequent extractions. Proteins were extracted from *E. curvula* and *N. tabacum* plants that had tested positive by RT-PCR, as well as from *S. cerevisiae*, and the extracts electrophoresed on SDS-PAGE gels. A 12 kDa protein, the known size of the Hsp 12, was observed in the *S. cerevisiae* extract, but was not observed in the extracts of transformed *E. curvula* or *N. tabacum*. Instead, a band approximately 13.8 kDa was obtained (Figure 4.6B). To verify whether this protein was Hsp 12 with a larger molecular weight due to modification of the transcribed Hsp 12 gene sequence (e.g. the addition of primers and/or the presence of a point mutation during integration that resulted in mutation of the stop codon), the 13.8 kDa band was subjected to MALDI-TOF mass spectrometry for analysis.

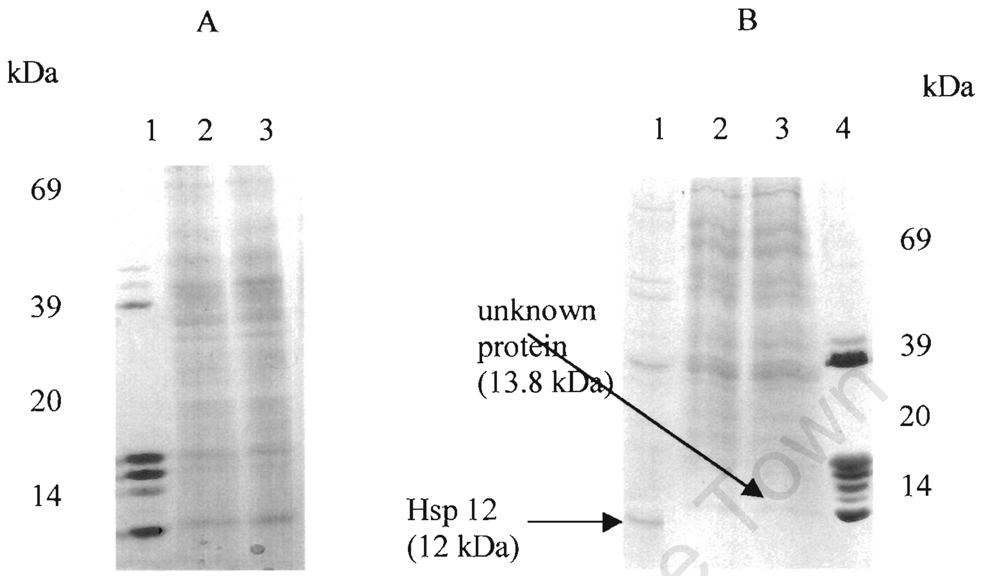


Figure 4.6. SDS-PAGE of total proteins extracted *E. curvula* and *S. cerevisiae*. A) Total proteins extracted from untransformed *E. curvula* plants. Lane 1: chicken erythrocyte histone marker; lane 2: proteins extracted without detergent; lane 3: proteins extracted using method of Bartels *et al.* (1991). B) Total proteins extracted using method of Bartels *et al.* (1991) from untransformed and transformed *E. curvula* plants. Lane 1: soluble proteins extracted from *S. cerevisiae* (positive control); lane 2: proteins extracted from untransformed *E. curvula*; lane 3: proteins extracted from transformed *E. curvula*; lane 4: chicken erythrocyte histone marker. The arrows indicate the unknown 13.8 kDa protein and Hsp 12.

### 4.3.2.4 MALDI-TOF mass spectrometry

The mass fingerprints of Hsp 12 and the unknown 13.8 kDa protein from transformed *E. curvula* plants were compared. Each protein was excised from the gel, treated with trypsin, mixed with sinapinic acid and subjected to mass spectrometry for peptide analysis. The mass fingerprint of the *S. cerevisiae* 12 kDa protein (Figure 4.7A) was indicative of *S. cerevisiae* Hsp 12 and analysis of the peptide masses by MS-FIT (Ludwig) showed that these masses were consistent with those produced by tryptic digestion of Hsp 12. The list of some common peptides have been summarised in Table 4.3. When the MALDI-TOF spectrum of tryptic peptides derived from Hsp 12 was compared to that derived from the 13. 8 kDa protein extracted from transformed plants (Figure 4.7A and 7B) it was apparent that no peptides of identical mass were present (Table 4.3). In the absence of homology, it was suggested that the unknown protein was not Hsp 12 but rather an endogenous plant protein. This is re-enforced by the presence of a protein of similar SDS-PAGE migration in untransformed *E. curvula*.

Table 4.3. Comparison of the tryptic peptides masses obtained by MALDI-TOF analysis of *S. cerevisiae* Hsp 12 and transformed *E. curvula* 13.8 kDa proteins. P denotes the presence, X denotes the absence of common peptide masses in the range of 1000 to 2600 daltons.

| *S. cerevisiae* Hsp 12 Theoretical digest | *S. cerevisiae* 12 kDa protein Actual digest | Transformed *E. curvula* 13.8 kDa protein |
|---|---|---|
| 1005 ±2 | P | X |
| 1138 ±2 | P | X |
| 1173 ±2 | P | X |
| 1223 ±2 | P | X |
| 1272 ±2 | P | X |
| 1437 ±2 | P | X |
| 1777 ±2 | P | X |

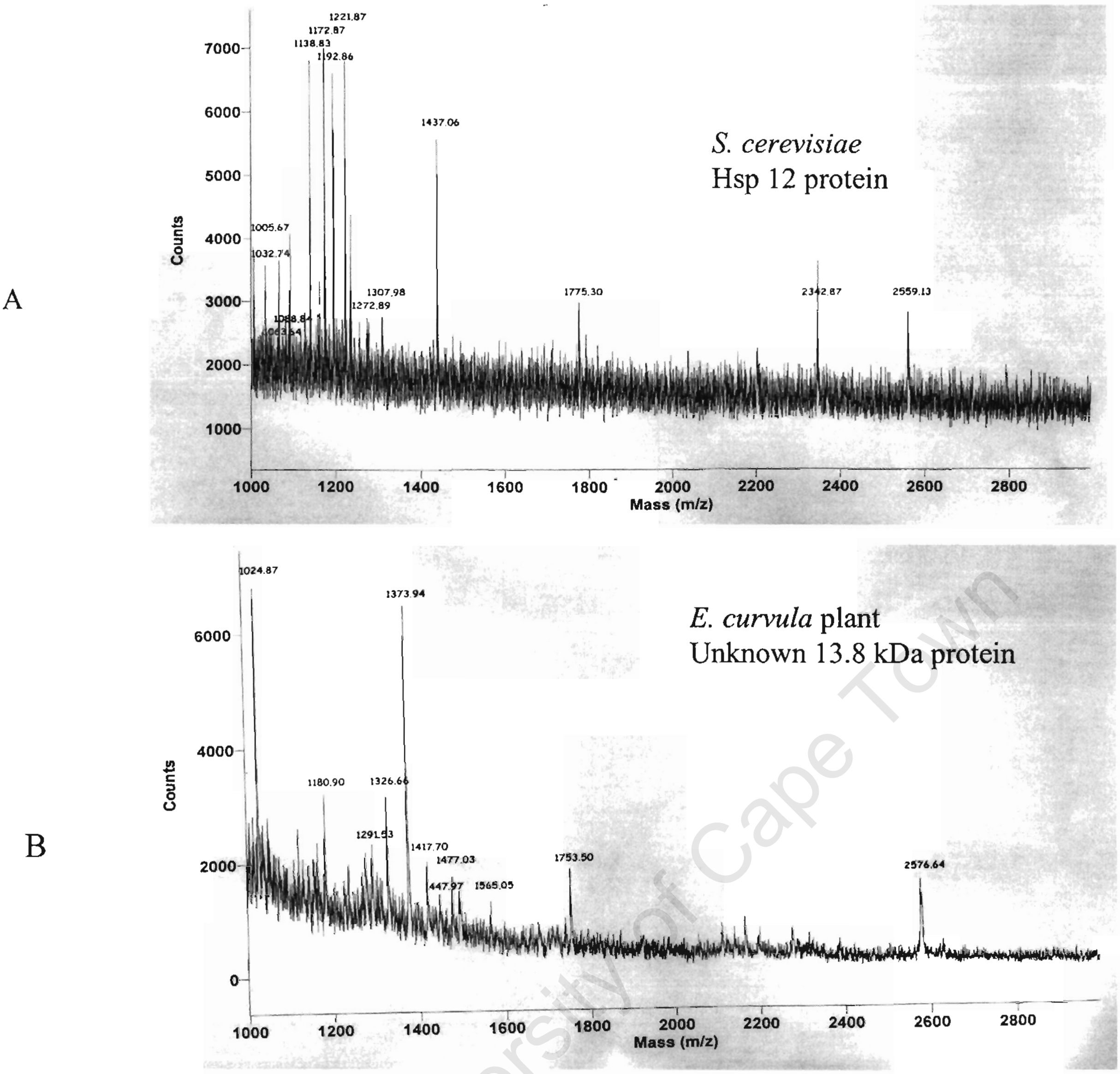


Figure 4.7. Analysis of MALDI-TOF spectra of the 12 kDa protein from *S. cerevisiae* (A) and unknown 13.8 kDa protein from transformed *E. curvula* (B). The mass range of 1000 to 2600 daltons is shown.

## 4.4 Discussion

### 4.4.1 Biolistic-mediated transformation of *E. curvula*

Biolistics was used to transform *E. curvula* with the Hsp 12 gene from *S. cerevisiae*. Target tissues were prepared as described in the regeneration protocol except that they were cultured on osmoticum medium for 24 h prior to bombardment. This change was to lower the turgor pressure in the cells and prevent bursting during bombardment. Various helium pressures, numbers of bombardment, and the distance travelled by the microprojectiles were all evaluated using GUS gene transient expression studies with the ultimate aim of achieving stable integration of the Hsp 12 gene in the *E. curvula* genome. The transient transformation of *E. curvula* shoot explants was optimum using 9100 kPa pressure, double bombardment, and a target distance of 7 cm. Lower pressure or DNA complexed to chicken erythrocyte histones did not result in transient GUS gene expression, implying that shoot explants transformation was sensitive to the experimental conditions. Bombarded tissues were regenerated without antibiotic selection since studies performed using *N. tabacum* (our comparative study) showed that callus did not produce shoots in a medium supplemented with hygromycin at the recommended concentration of 25 μg/ml. Selection of transgenic plants therefore solely relied on the identification of the Hsp 12 gene in transformed plants using PCR.

Although the Hsp 12 gene was shown to be transcribed using RT-PCR, Hsp 12 protein was not detected by SDS-PAGE in extracts of either *E. curvula* or *N. tabacum* plants (Figure 4.6). The absence of this protein in transformed plants might be due to several factors including low expression frequencies, mRNA instability, codon usage and proteolytic activity. It is also possible that the absence of protein might be due to the existence of chimeric transgenic plants. These occur when a single cell is transformed within the meristemoidal center and that this gives rise to a plant containing both transformed and non-transformed cells. A low expression frequency is therefore observed depending on the ratio of transformed cells to untransformed cells. Since RT-PCR analysis indicated Hsp 12 expression occurred, the lack of Hsp 12 protein might be due to problems at a posttranscriptional level. An alteration in the 5′ cap structure and/or the length of the poly A tail have been implicated to affect mRNA stability (Jacobson and

Peltz, 1996; Brennan and Steitz, 2001). Alternatively, the lack of expressed foreign proteins can also be attributed to codon usage. Since some amino acids have more than one codon (degeneracy) and the preference of usage of these codons has been reported to vary between species, it is possible that the *S. cerevisiae* Hsp 12 gene uses codons seldom used by *E. curvula*. This would result in no or low translation. Finally, another problem associated with expression of foreign genes in the plant is that proteolytic activity in the plant cells might have resulted in degradation of the Hsp 12 protein which, as a foreign protein, might not be resistant to *E. curvula* or *N. tabacum* cell proteases. No conclusive statement can be made regarding translation of the Hsp 12 mRNA in either plant until the above questions have been investigated further. Thus at this stage the Hsp 12 gene in transformed plants cannot be characterized (e.g. by growing the transformed plants in saline or dry conditions to test the impact of Hsp 12 gene). The future work should focus on:

- the investigation of the pathways involved in the degradation of mRNA in order to understand the principle of mRNA stability. Moreover, sequencing the transgene and/or performing site-specific mutagenesis in an effort to analyse the structural requirements of instability sequences can be performed.
- the use of other genes from resurrection plants that have been implicated to confer desiccation tolerance. This may avoid the problem associated with codon usage or proteolytic activity since system of genes to proteins in plants is presumably the same.

# Summary and conclusions

In this study, an attempt was made to transform *E. curvula* with the Hsp 12 gene, known to play a role in desiccation tolerance in *S. cerevisiae* cells (Sales *et al.*, 2000). Though the efficiency of transformation was low, the method established here opens the way for the genetic engineering of *E. curvula* and other important crops with genes conferring desiccation tolerance and other useful traits. Successful genetic transformation of *E. curvula* depended on a large number of parameters which included appropriate methods of tissue culture, recombinant DNA Technology, selection of and regeneration from transformed cells.

An *in vitro* plant regeneration system is generally known as the prerequisite for transformation systems in plants. It was therefore a first parameter to be evaluated. A tissue culture protocol was developed in this study to provide a sufficient and suitable regenerable material to be used in transformation experiments. Plant regeneration was achieved from shoot explants, and also from callus induced from immature leaves, via the process of direct and indirect organogenesis respectively. Frequency of regeneration from pieces of callus was higher than from shoot explant. Nevertheless, both shoot explants and callus were used as target tissues for bombardment. Frequency of regeneration from shoot explants did not decrease after bombardment whereas the frequency of regeneration from callus decreased considerably. However transformation efficiency was relatively high when callus tissue was used as compared to shoot explants under the same bombardment conditions. Such results confirm the studies reported by Fukuoka *et al.* (1994) among others that callus or embryogenic callus is more suitable for transformation studies than intact tissue, and that efficiency of transformation may depend on the cell stages (Kartzke *et al.*, 1990). Vigorous cell division probably increases gene transfer efficiency. Histological studies performed in this study showed meristems and this could account for increased gene transfer efficiency.

Another important parameter which warrants further investigation is an appropriate vector construct which normally includes promoters and selectable and reporter genes.

The vector pCAMBIAUbeeQ was used in this study to carry Hsp 12 gene to the plant genome. This vector was chosen on the basis that it could be further engineered to include the desired promoter to drive the expression of the Hsp 12 gene. Noticeable in the whole process of making a new construct was the single digestion of Hsp 12 insert and the vector with *Sac* I (Chapter 3), thus giving two possible ways of ligation (either same or opposite direction to the ubiquitin promoter). Nevertheless, Hsp 12 was successfully cloned into the vector in the correct orientation (same direction as the promoter). The expression of Hsp 12 gene in both *E. curvula* and *N. tabacum* plants as detected by RT-PCR was an evidence for successful cloning of Hsp 12 gene in the sense orientation. For future research, more promoters need to be used for studying transient and subsequently stable expression of GUS gene and gene of interest (Hsp 12). The use of many promoters should facilitate the establishment of efficient transformation protocols and also minimize variables associated with gene expression and silencing. A good vector should also contain a reporter gene. In this study, GUS gene was successfully utilized in optimization of parameters for bombardment. The optimal transformation of *E. curvula* was achieved using callus tissue, 9100 kPa pressure and double bombardment.

Another important aspect that was important for the establishment of the transformation protocol for *E. curvula* is selection of and regeneration of transformed tissues or cells. Generally transformed tissues or cells are regenerated on a medium supplemented with a lethal concentration of selection agent (antibiotics or herbicides) to ensure that only transformed cells differentiate into meristemoids and subsequently to plants (Hansen and Wright, 1994). In this study, the selection of putative transformants using antibiotics was not performed (Chapter 4). Instead PCR was used for detection of the Hsp 12 gene in transformed *E. curvula* and the expression of this gene was confirmed by RT-PCR. The drawback with PCR is that either false negative or positive results are possible. To account for this problem all experiments were coupled with controls. For instance, genomic DNA isolated from untransformed plants was also PCR amplified. This was to ensure that PCR product, the 330 bp fragment, obtained from the transformed plants was due to transformation. In addition, the method chosen for DNA isolation, for instance, must ensure that contaminating polysaccharides and polyphenolic compounds, which interfere with Southern blot and PCR analysis respectively, are removed. This was accounted for in the current study.

In addition to future work recommended in previous chapters, the future studies should concentrate on:

- improving the transformation frequency of *E. curvula*
- the use of selectable markers that are not detrimental for human consumption
- determining occurance of Mendelian principles in subsequent generations of transformed plants and
- full characterization of introduced gene of interests (e.g. by subjecting transformed plants to saline stress or water deficit-stress).

# Chapter 5

## References

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# Chapter 6

# Appendix

## Appendix A: Protocols

## A.1 General protocols

### A.1.1 Mini prep by alkaline lysis

A 5 ml culture was grown overnight in LB medium (Appendix D.3.1) with the relevant antibiotic. 2 ml of this culture was centrifuged at 12 000 rpm for 1 min and resuspended in 0.2 ml of solution 1 (Appendix B.3.1.1). After incubation on ice for 10 min, 0.5 ml of solution 2 (Appendix B.3.1.2) was added. The mixture was thoroughly mixed by shaking and then incubated on ice for 10 min. 0.3 ml of ice-cold solution 3 (Appendix B.3.1.3) was added, and the sample shaken before being incubated on ice for a further 10 min. The sample was then centrifuged for 10 min at 4 $^0$C at 12 000 rpm to remove flocculated material. 0.9 ml of supernatant was recovered and transferred to a 1.5 ml Eppendorf tube to which 0.6 ml of isopropanol was added. After 2 min at room temperature, DNA was recovered by centrifuging at 12 000 rpm for 10 min. The pellet was washed with 70 % ethanol, dried and resuspended in 40 µl of sterile water.

### A.1.2 Agarose gel electrophoresis of DNA

DNA was separated according to size using electrophoresis on either 1 % or 1.5 % agarose gels depending on the size of the fragment to be recovered. Agarose was mixed with TAE (Tris-acetate-EDTA) buffer (Appendix B.1.1), which was then heated in a microwave oven and mixed thoroughly. After cooling to 40 $^0$C, ethidium bromide (3 µl of a 10 mg/ml solution (Appendix B.1.2)) was added. The gel was poured and allowed to set. Samples were prepared by adding 5 µl of loading buffer (B.1.3) to 10 µl of DNA containing solution. Electrophoresis was allowed to proceed for 2 h at 60 V. The tank buffer was TAE. The gel was visualized on a UV transilluminator (Protein Lab Services) and then photographed using a Pharmacia Biotech Gel Display System.

### A.1.3 Agarose gel electrophoresis of RNA

As per method used for DNA (A.1.2).

### A.1.4 Determination of concentrations of nucleic acid

The DNA concentration was determined by UV spectrophotometry with samples diluted up to 1000 times. The absorbances were read at 260 nm. The concentration of DNA was determined by using the following equation:

O.D x Dilution factor x Extinction coefficient x Total volume = µg/Total volume

O.D = Optical density

Extinction coefficient = 50 µg/ml of double stranded DNA,

Extinction coefficient = 40 µg/ml of single stranded RNA

### A.1.5 Nucleic acid manipulations

#### A.1.5.1 Standard PCR Reaction

Unless specified, all PCR reactions were performed using Super-Therm DNA Polymerase (SR PRODUCT, Kent, UK, supplied by Southern Cross Technology (PTY) LTD).

| | |
|---|---|
| Buffer (10 X) | 5 µl |
| $MgCl_2$ (25 mM) | 3 µl |
| dNTPs (2.5 mM) | 0.7 µl |
| Forward primer (10 µM) | 3 µl |
| Reverse primer (10 µM) | 3 µl |
| *Taq* Polymerase (5U/µl) | 0.4 µl |
| DNA template (50 ng) | 1 µl |
| $dH_20$ | 33.9 µl |
| Total | 50 µl |

The cycles used were as follows:

| **Step number** | **Denaturing** | **Annealing** | **Extension** |
|---|---|---|---|
| 1 | 94 $^0$C for 5 min | | |
| 2 | 94 $^0$C for 30 s | | |
| 3 | | 55 $^0$C for 30 s | |
| 4 | | | 72 $^0$C for 60 s |
| 5 | | | 72 $^0$C for 5 min |

Steps 2 to 4 were repeated 30 times.

### A.1.5.1.1 RT-PCR

First strand cDNA synthesis reaction

| | |
|---|---|
| RNA (5 µg) | 5 µl |
| 5 X RT buffer | 10 µl |
| dNTPs (2.5 mM) | 3 µl |
| Oligo dT (500 µg/ml) | 1 µl |
| $dH_2O$ | 11 µl |

The mixture was incubated at 65 $^0$C for 5 min and then kept on ice for 10 min. MAV reverse transcriptase enzyme 2.5 µl was added to the mixture, incubated at 42 $^0$C for 60 min and then at 75 $^0$C for 10 min.

### A.1.5.2 Gel purification

DNA was separated according to size by agarose gel electrophoresis (Appendix A.1.2). The required band was excised with a sterile blade and transferred to a sterile 1.5 ml Eppendorf tube. The DNA was purified from gel piece using a QIAGEN kit according to the manufacturer's recommendations.

### A.1.5.3 DNA restriction digests

| | |
|---|---|
| Restriction enzyme buffer 10X | 2.5 µl |
| DNA sample | 10 µl |
| Restriction enzyme 10U/µl | 1.5 µl |
| $dH_2O$ | 11 µl |
| Total | 25 µl |

Unless specified, incubate at 37 $^0$C for minimum 4 h.

### A.1.5.4 Ligation reaction

| | |
|---|---|
| Ligase Buffer 10X | 5 µl |
| Plasmid DNA | 10 µl |
| Insert DNA | 30 µl |
| Ligase | 3 µl |
| $dH_2O$ | 2 µl |
| Total | 50 µl |

Incubate at 4 $^0$C overnight.

# APPENDIX B: Buffers, solutions and media

## B.1 Buffers and solutions for nucleic acids

### B.1.1 TAE buffer (50X)

Tris 60.5 g

Acetic acid 14.3 ml

EDTA (0.5 M) 25 ml

Make to 250 ml with distilled water.

### B.1.2 Ethidium bromide (10 mg/ml)

For 1 ml:

0.5 ml 1M Tris-HCl, pH 8.0

0.1 ml 0.5M EDTA

10 mg ethidium bromide

Add ethidium bromide to Tris-HCl, EDTA and 0.2 ml water, and stir to dissolve. Make up to 1 ml with water. Store in a brown bottle at room temperature.

### B.1.3 Loading buffer

50 mM NaOH

1 mM EDTA

50 % Glycerol

0.25 % Bromophenol blue

Make to 100 ml with distilled water.

## B.2 Solutions for proteins and SDS-PAGE

### B.2.1 PMSF

Prepare a 100 mM solution in isopropanol

Aliquot and store at –20 $^0$C.

### B.2.2 Separating gel (20 %)

| | |
|---|---|
| Bisacrylamide (40 % acrylamide, 0.2 % bisacrylamide | 12.5 ml |
| 1.125 M Tris-HCl pH 8.8 | 8.35 ml |
| SDS (10 %) (w/v) | 0.250 ml |
| AMPS (10 %) (w/v) | 0.250 ml |
| $dH_2O$ | 3.65 ml |
| TEMED | 25 µl |

### Stacking gel (6.5 %)

| | |
|---|---|
| Bisacrylamide (40 % acrylamide, 0.2 % bisacrylamide | 2 ml |
| 0.375 M Tris-HCl pH 6.8 | 4 ml |
| SDS (10 %) (w/v) | 0.125 ml |
| AMPS (10 %) (w/v) | 0.300 ml |
| $dH_2O$ | 5.65 ml |
| TEMED | 20 µl |

### AMPS (10 %) (w/v)

AMPS 5 g

Make up to 50 ml with distilled water.

Kept at 4 $^0$C

Make fresh every 2 weeks.

### SDS (10 %) (w/v)

SDS 10 g

Make up to 100 ml with distilled water.

### 1 X Running buffer

Tris 3 g

Glycine 30 g

SDS 1 g

Make up to 1000 ml with sterile distilled water.

### Sample application buffer

125 mM Tris-HCl pH 6.8

SDS 4 % (w/v)

Glycerol (20 %) (v/v)

Make up to 10 ml with sterile distilled water.

## B.3 General solutions and buffers

### B.3.1 Solutions for mini-prep method by alkaline lysis

#### B.3.1.1 Solution 1

0.25 M Tris-HCl pH 8.0

0.5 M Glucose

0.1 M EDTA

Make to 500 ml with distilled water.

#### B.3.1.2 Solution 2

0.2 M NaOH

1 % (w/v) SDS

Make to 100 ml with distilled water.

Make fresh every week.

#### B.3.1.3 Solution 3

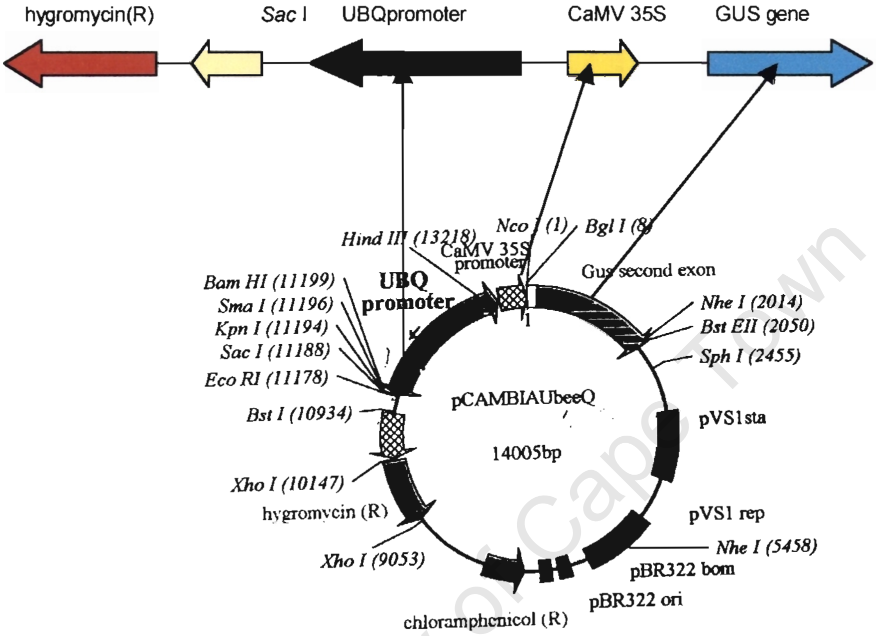
3 M Potassium acetate

2 M Acetic acid

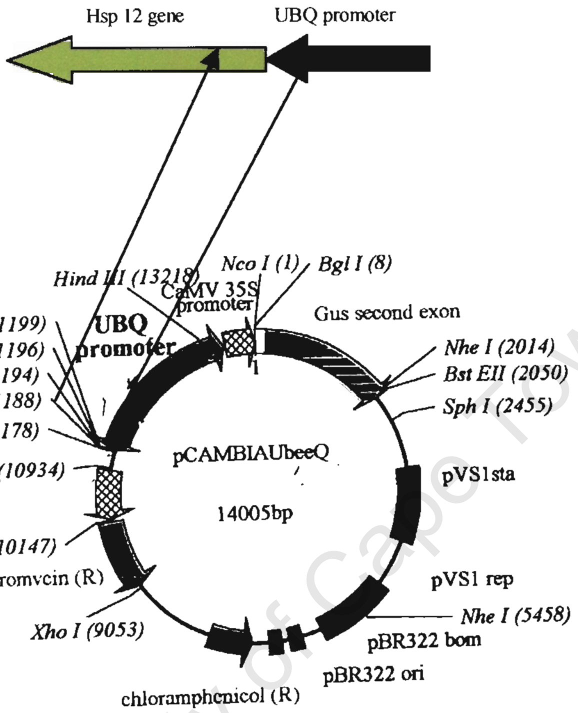
Add 300 ml of 5 M Potassium acetate and 57. 5 ml glacial acetic acid into a 500 ml beaker and make up to 500 ml with distilled water.

# Appendix C: Plasmids

## C.1 pCAMBIAUBeeQ



### C.2. pCAMBIAUbeeQHsp12



C.2.1 Hsp 12 gene was cloned into the *Sac* I restriction site of the vector pCAMBIAUBeeQ resulting into a new construct pCAMBIAUBeeQHsp12.

## Appendix D: Media and solutions

### D.1 *E. curvula* tissue culture media

#### D.1.1 MS medium high osmoticum (pre- bombardment of *E. curvula* shoot explants)

| | |
|---|---|
| MS nutrients | 4.32 g/l |
| MS vitamins (D.2) | 10 ml/l |
| Sucrose | 30 g/l |
| 2,4-D (D.2) | 25 ml |
| TDZ (D.2) | 2.28 ml |
| Mannitol (final concentration 0.2 M) | 9.11 g/l |
| Myo-inositol | 0.05 g/l |
| $dH_2O$ | 1000 ml |
| pH to 5.8 with 1 M KOH | - |
| Agar | 8 g/l |

#### D.1.2 MS medium high osmoticum (pre- bombardment of *E. curvula* callus)

| | |
|---|---|
| MS nutrients | 4.32 g/l |
| MS vitamins (D.2) | 10 ml/l |
| Sucrose | 30 g/l |
| 2,4-D (D.2) | 2 ml |
| BAP (D.2) | 100 μl |
| Mannitol (final concentration 0.2 M) | 9.11 g/l |
| Myo-inositol | 0.05 g/l |
| $dH_2O$ | 1000 ml |
| pH to 5.8 with 1 M KOH | - |
| Agar | 8 g/l |

## D.2 Tissue culture stock solutions

### MS vitamins (stock)

| | |
|---|---|
| Glycine | 50 mg |
| Nicotinic acid | 125 mg |
| Thiamine-HCl | 250 mg |
| Pyridixine-HCl | 250 mg |
| Myo-inositol | 2.5 mg |
| dH$_2$O | 500 ml |

Filter sterilize, aliquot 20 ml volumes in standard containers and store at –20 $^{0}$ C.

### 2,4-D stock solution (1mg/ml)

Dissolve 100 mg in 1 ml 1M KOH (heat in microwave oven for 5 s if insoluble).

Add 100 ml distilled water.

Aliquot into 10 ml volumes and store at –20 $^{0}$ C.

### NAA stock (0.1 mg/ml)

Dissolve 10 mg in 1 ml of 1 M KOH.

Add 100 ml of distilled water.

Store at 4 $^{0}$ C.

### BAP stock (1 mg/ml)

Dissolve 100 mg in 1 ml of 1 M NaOH (heat in microwave oven for 3 s to dissolve).

Add 100 ml of distilled water.

Store at 4 $^{0}$ C.

### TDZ stock (0.14 mg/ml)

Dissolve TDZ in a few drops of 1 M NaOH and then add distilled water to the required volume. Store at 4 $^{0}$ C.

## D.3 Bacteria media

### D.3.1 Luria Broth

10 g Tryptone

5 g Yeast extract

5 g NaCl

Make up to 1 l with distilled water and autoclave.

### D.3.2 Luria Agar

Add 15 g bacterial agar per 1 l of LB.

Autoclave.