Agrobacterium tumefaciens mediated transformation of sweet potato (*Ipomoea batatas*) tuber and regeneration of transformed tissue

Jonathan Warren Brown

A research report submitted to the Faculty of Science, University of the Witwatersrand, in partial fulfilment of the requirements for the degree of Master of Science.

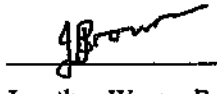
Johannesburg, February 1998.

Abstract

Sweet potato (*Ipomoea batatas*) is one of the six biggest crops in the world, its high nutritional content and large yield in tropical areas making it a useful food source, especially in developing countries. Genetic engineering has the ability to overcome factors such as insect and disease damage which are currently limiting its potential. With this in mind research has been conducted into the development of a protocol to generate transgenic sweet potato from tubers of a local South African cultivar, blesbok. A protocol has been developed which appears capable of generating transgenic plants. Transformation of blesbok tuber tissue was carried out by *Agrobacterium tumefaciens* mediated transfer of three different binary vectors containing the *uidA* gene encoding β-glucuronidase, the *nptII* gene conferring kanamycin resistance and the *bar* gene conferring L-phosphinothricin resistance. Long term, stable expression of kanamycin and L-phosphinothricin resistance was confirmed with kanamycin and L-phosphinothricin screening. Long term, stable expression of β-glucuronidase was confirmed with fluorescence histochemical studies employing ImaGene Red™. This was further confirmed with quantitative assays of β-glucuronidase activity using 4-methylumbelliferyl-β-D-glucuronic acid which showed an average activity of 2.82 nmole.min^{-1}.mg^{-1} protein. Long term, stable integration of *uidA* into the plant genome was confirmed with polymerase chain reaction amplification screening. Transformed tuber tissue was regenerated via shoot organogenesis to stem structures similarly produced from non transformed tuber tissue. This was achieved for optimised transformation conditions and focused on shoot induction with 2 mg.l^{-1} of the auxin 2,4-dichlorophenoxyacetic acid and 0.2 mg.l^{-1} of the cytokinin 6-benzylaminopurine. The stems produced still need to be stimulated to develop fully into transgenic plants. This will probably require a sharp increase in the cytokinin:auxin concentration ratio after initial shoot induction.

Declaration

The work presented in this research report was carried out by the author between the months of July 1997 and February 1998. It has not been previously submitted for any other degree, examination or research purpose. It is being submitted for the degree of Master of Science in the University of Witwatersrand, Johannesburg.



Date: 1998-02-23

Jonathan Warren Brown

Acknowledgements

The author acknowledges the following people:

Dr V. Gray for the supervision of this project.

Mrs B. Woodward and the Agricultural Research Vegetable and Ornamental Plant Institute, Roodeplaat for assistance, advice and research facilities provided.

Dr D. Mycock, Mrs S. Laminski and Mr J. Groll for assistance and advice.

Prof. R. Pienaar and the Department of Botany for the research facilities provided.

Mr P. Tshabalala of the photographic unit and Mr S. Coetzee of the microscope unit.

Table of Contents

List of Abbreviations

A_{260}	spectrophotometric absorbance at a wavelength = 260 nm
A_{280}	spectrophotometric absorbance at a wavelength = 280 nm
A_{600}	spectrophotometric absorbance at a wavelength = 600 nm
A_{620}	spectrophotometric absorbance at a wavelength = 620 nm
ABA	abscisic acid
A. tumefaciens	*Agrobacterium tumefaciens*
BAP	6-benzylaminopurine
bar	bialaphos resistance gene
BSA	bovine serum albumin
2,4-D	2,4-dichlorophenoxyacetic acid
DMF	dimethyl formamide
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
$F_{460\pm15}$	fluorescence at a wavelength = 460 ± 15 nm
GA_3	gibberellic acid
gfp	green fluorescent protein gene
GUS	β-glucuronidase
gusA	β-glucuronidase gene
hph	hygromycin phosphotransferase gene
IAA	indoleacetic acid
λ	wavelength
MU	4-methylumbelliferyl
MUG	4-methylumbelliferyl-β-D-glucuronic acid
mRNA	messenger RNA

NAA	α-naphthaleneacetic acid
NA	nutrient agar
NB	nutrient broth
nos	nopalin synthase gene
npt11	neomycin phosphotransferase 11 gene
onc	oncogenic gene
pat	L-phosphinothricin acetyl transferase gene
PAT	L-phosphinothricin acetyl transferase
PEG	polyethylene glycol
PCR	polymerase chain reaction
pNPG	p-nitrophenyl-β-D-glucuronide
PPT	L-phosphinothricin
SDS	sodium dodecyl sulphate
TBE	tris borate ethylenediamine tetraacetic acid buffer
TDNA	transferred DNA
TDZ	thidiazuron
TE	tris ethylenediamine tetraacetic acid
Ti	tumour inducing
uidA	β-glucuronidase gene
vir	virrulence
x-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
YM	yeast mannitol

1 Introduction

1.1 Sweet potato background

Sweet potato, *Ipomoea batatas*, is loosely classified as one of the tuber crops which include potato; *Solanum tuberosum*, yam; *Dioscorea batatas* and cassava; *Manihot esculenta*. Sweet potato is genetically distant from the other tuber crops however. It falls into the Dicotyledoneae class and is a member of the bindweed family Convolvulaceae (Henderson *et al.*, 1984). It is classified into the section batatas of the very large genus *Ipomoea* along with its related species which are all wild and most of whose tubers are poisonous. Its species name is also *batatas* and the original agricultural strain is classified as (L.) Lam hence the full name *Ipomoea batatas* (L.) Lam. The closest wild relatives include *Ipomoea lacunosa*, *Ipomoea trifida* and *Ipomoea triloba*, the major difference between it and these species being that it is a hexaploid whilst they are diploid and tetraploid (Nishiyama, 1991).

Sweet potato has never been documented in the wild state so its exact origin is unknown. There is strong evidence to suggest that it is a New World plant since it was being used as a staple food crop by tribes in central and southern America and the south Pacific at the turn of the millennium. A wild type *Ipomoea trifida* hexaploid was first found in Mexico in 1955 which had many similar characteristics to sweet potato. It has been postulated that sweet potato originated as a result of the production of some such hexaploid by a chance chromosome multiplication with the subsequent domestication of this strain several thousand years ago (Nishiyama, 1991). From these origins it seems to have spread further by three different routes. It spread via the prehistoric trade routes from Peru, Ecuador and Colombia to Polynesia, Easter Islands, Society Islands and Hawaii and then further to Western Samoa, Tonga and New

Zealand. Spanish explorers discovered the sweet potato as a food source of the South American Incas in 1500 and they spread it to Mexico, the Philippines and East Indies. Portuguese explorers spread it via Europe to Africa, India, China and Malaysia where it spread to Japan (Henderson *et al.*, 1984; Nishiyama, 1991).

1.2 Sweet potato cultivation

Major sweet potato crops are limited to the tropical and temperate zones, where 98 % of the economic crop is grown in the developing world (Newell *et al.*, 1995). This is due to their requirement for mean temperatures above 23.8 ^{0}C, a rainfall of 800-1300 mm per annum, a photoperiod of at least 10 hours a day and a 4-6 month frost free growing period so that maximum yield is obtained (Henderson *et al.*, 1984). One of the main advantages of the crop is its very short growing cycle, some cultivars are capable of growing fully in only 3 months, provided satisfactory environmental conditions prevail. This allows up to three crops to be grown a year giving a very high yield of food per area of land cultivated.

The sweet potato is the sixth most important crop in the world after wheat, rice, corn, white potato and barley (Figure 1.2). In developing countries it is the fifth most important crop, previously estimated to occupy 10 million hectares with a production value of 45 billion rand (FAO Year Book 1992). It is most popular in Asian, followed by African and South American countries (Peirce, 1987) but first world countries like America grow vast amounts of sweet potato which makes it an important export market for them. The large market it already commands is increasing rapidly as consumers are being exposed to a variety of sweet potato cultivars. All in all its high nutritional content and overall versatility (Bouwkamp, 1985) coupled with its high yield in tropical areas make it a useful food source with potential to become one of the most important crops of the future.

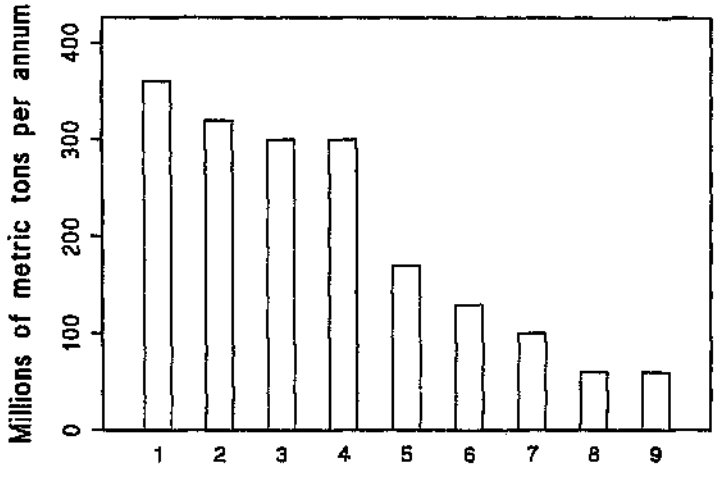


Figure 1.2: Graph of top nine crops in the world as measured by their annual production in millions of metric tons (FAO Yearbook, 1992). The crops are listed as 1 Wheat, 2 Rice, 3 Corn, 4 Potato, 5 Barley, 6 Sweet potato, 7 Cassava, 8 Grapes, 9 Soybean.

1.3 Factors limiting sweet potato market growth

The main limitation to the spread of sweet potato is at the moment due to the strict climatic requirements and high susceptibility to insect pests and disease. Insect damage to crops is a problem in the field and in storage. Over 40 different insects have been implicated in crop damage, the most important pests including the May beetle; Coleoptera *Scarabaeidae Lachnosterna,* the click beetle; Coleoptera *Elateridae Heteroderes laurenti,* the weevil; Coleoptera *Curculionidae Alcidodes orientalis,* the sweet [illegible] weevil; Coleoptera *Curculionidae Cylas formicarius* and the common red spider; Acarina *Trombidiformes tetranychus urticae* (Wyninger, 1962). The worst pest is the sweet potato weevil (Newell *et al.*, 1995).

Disease primarily affects the sweet potato in the field except for bacterial soft rot and black rot which affect the plant in storage. Diseases include a variety of viral, bacterial, fungal and nematode infections (Stevens, 1921) The worst are the mosaic virus infections, stem rot (*Fusariam* wilt) caused by *Fusariam hyperoxysporum and Fusariam oxysporum batatas*, root knot caused by the nematode *Meloidogyne incognita*, black rot caused by *Sphoeronema fimbriatum*, soft rot caused by *Rhizopus nigricans* and bacterial soft rot caused by *Erwinia carotovora* (Henderson *et al.*, 1984; Lucas *et al.*, 1992).

1.4 Selective breeding of sweet potato

As early as 1904, work began on the sexual breeding of sweet potato in order to improve cultivars and address the problems limiting their market potential. Characteristics focused on included yield, palatability, nutritional content, disease and insect resistance, ease of storage and variety of flavour (Henderson *et al.*, 1984).

Initially there was little success. It should be remembered that conventional methods of breeding are tedious and complex. Desired traits are not always present or easily detectable. Optimising desirable traits by selective, pure line, mass selection, pedigree, bulk population and backcross breeding can be time consuming (Allard, 1966). Furthermore, extensive breeding often compromises the overall fitness of the plant. The process is difficult in sweet potato because it is a hexaploid which complicates gene flow studies, causes genetic variance in desirable traits and makes prediction of resultant phenotype often impossible (Allard, 1966). The sterility of highly desirable sweet potato cultivars and cross incompatibilities are also a problem for selective sexual breeding. Some success was ultimately achieved through lengthy breeding programmes. Increased varieties were produced with improved palatability, increased nutritional content and multiple disease resistance (Henderson *et al.*, 1984).

Other characteristics such as increased yield and specific high level insect and disease resistance could not be bred. Plants do not have specific high level pest and insect resistance so breeding rarely helps in this regard but this is not the case for yield. This represents a major limitation to breeding since yield is its main goal. This limitation is probably due to the small genetic pool available to sweet potato breeders. Although its important to breed crops for cultivation conditions, extensive breeding often causes the loss of genetic variability. One needs to conserve genetic resources so that they can be utilised for other, new conditions (Marshall, 1990). Crossing with wild types has subsequently begun in an attempt to get greater germplasm genetic variability or in cases to get more stable distinct characteristics (Belarmino *et al.*, 1994).

1.5 Genetically engineered plants

Whilst normal selective breeding is necessary for optimising polygenic traits it clearly has limitations with regard to developing specific phenotypes. Genetic engineering however has the ability to introduce functional genes for novel phenotypes (Figure 1.5), phenotypes which may confer great benefit either to plants or to humankind. There are three areas of interest with regard to plants and genetic engineering: plant protection, overall physiology and biopharming.

Crop protection revolves around the actual protection of plants from disease, pests and even competition from weeds. An example of this is the inclusion of the gene for the *bacillus thuringiensis* toxin in the plant genome. When produced this toxin has the ability to kill off lepidopteran, dipteran and coleopteran insects (Fujimoto *et al.*, 1993). The inclusion of a gene for viral coat proteins can protect plants from cross viral infections (Nelson *et al.*, 1990). The gene for the enzyme phosphinothricin acetyltransferase (PAT) can be included in crops, conferring resistance to L-phosphinothricin (PPT) based herbicides, allowing weed control by spraying crops with these herbicides (Liewellyn *et al.* 1990).

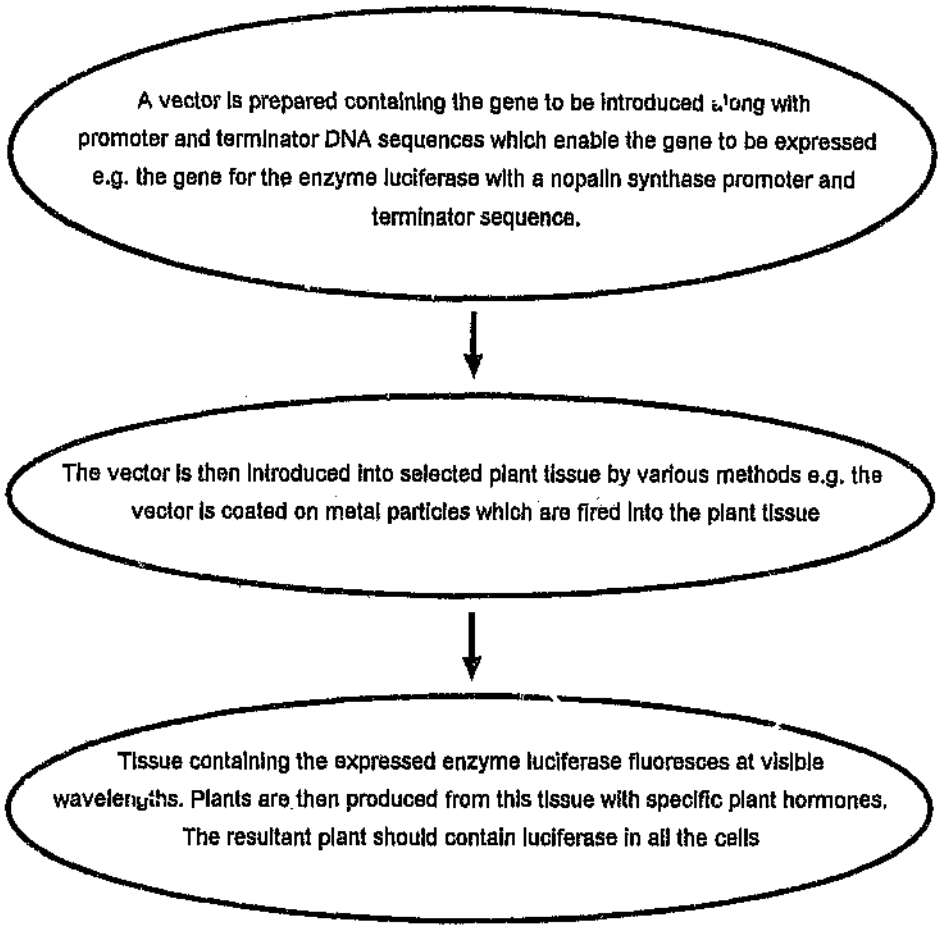


Figure 1.5: Scheme of events in the development of a genetically engineered plant from vector preparation to plant regeneration (Lehninger, 1993).

Improving physiology involves modifying the plant to give some benefit in physiology, like increased starch or sugars or longer time for ripening. An example of this is the inclusion of a gene whose messenger RNA (mRNA) is antisense to and binds mRNA from the gene for the enzyme polygalacturonase. As a result polygalacturonase cannot be synthesised. Polygalacturonase hydrolyses pectin causing ripening so its absence slows down ripening, thereby increasing shelf life (Kramer *et al.*, 1993).

Lastly, one can use plants as factories to produce useful hormones, fatty acids, enzymes or other organic substances. This technology is termed biopharming and has been used in plants to produce important antibodies for medicine by merely incorporating the gene for the antibody (Hiatt and Mostov, 1993). Artificial laurate, a 12 carbon fatty acid used for soaps and detergents, has even been produced in plants. The gene for the 12 carbon laurate producing enzyme was incorporated into rapeseed which then produced laurate in high amounts (Brown, 1996).

These of course are just some of many successes. Each project requires a great deal of time since one has to ensure that the gene undergoes stable integration and expression and that the gene product is then correctly processed. Furthermore there are requirements for the transmission of the gene to offspring and various ethical and safety considerations.

Genetic engineering could overcome a number of the problems facing the sweet potato, enabling the introduction of desirable single gene traits often novel to the genus or even to the plant kingdom. Traits for insect, viral, bacterial, fungal and nematode resistance would reduce the impact of these pests on sweet potato production. Increased size, niacin and starch content could be achieved by genetic engineering and essential amino acids like methionine and tryptophan could be introduced. The technology could in effect broaden the genetic pool of the sweet potato and engineer it specifically for varying conditions. Future prospects would then be biopharming, sweet potato being ideal for such a venture with its quick growing season.

With this in mind attempts have been made to develop a system to generate transgenic sweet potato. This process involves three important steps: the development of a transformation system, tissue transformation and regeneration and analysis of newly introduced genes. Firstly however, one needs to have a mechanism for plant regeneration in mind.

1.6 Plant tissue transformation and regeneration

The main problem with multicellular organisms like plants is that it is not possible to ensure stable transformation in all the cells with current transformation technology. One can attempt to transform a whole plant but only a small proportion of plant cells will be transformed. This is what is termed a chimeric, where there is a mixture of transformed and non transformed cells in the transgenic plant.

Even if one transforms embryos or plantlets which still have to develop there is no way of ensuring transformation of all the cells present and after the plant has reached maturity, it will still be a chimeric, just with a much higher level of transformed cells. One needs to transform single cells which can gives rise to whole plants. These cells would ideally have to be germ line, zygotic or embryo cells. This is difficult to do because in many cases plants do not readily reproduce by sexual reproduction so one cannot isolate such cells. One can however utilise the latest *in vitro* plant propagation techniques which circumvent sexual reproduction.

The latest plant propagation technology revolves around the regeneration of plants from somatic cells instead of germ line cells, relying on the totipotent nature of somatic plant cells. This technology was originally created for large scale *in vitro* production of disease and insect free plants, for agricultural purposes but it has great relevance for the regeneration of plants from transformed somatic tissue. There are some problems with this application in that regeneration protocols have only been developed for a limited number of plants In addition to this transformation often compromises the cells ability to regenerate into plantlets. The technology does provide a useful base to work from though. Regeneration requires initial morphogenesis of the respective tissue. Morphogenesis can be described as the creation of new form and organisation where previously it was lacking, technically this includes organogenesis or somatic embryogenesis which are specific types of morphogenesis (George, 1993).

Organogenesis is the process of root and shoot differentiation in plant cells. Sometimes this process is spontaneous but generally requires hormones, a high auxin:cytokinin ratio for root formation and a high cytokinin:auxin ratio for shoot formation. Organogenesis can occur directly from explants such as a root or stem cuttings under the influence of such hormones (George, 1993). However, tissue is often predetermined to a specific direction be it root or shoot development and it is difficult to force it in a different direction. In cases like these one needs to go through a phase of callus formation where highly specialised cells can dedifferentiate and then one can use hormones to stimulate specific organogenesis from the dedifferentiated callus. This is termed indirect organogenesis (Warren, 1991). Once shoot formation is induced one can then root the shoots and produce new plants. Getting roots to shoot however is often not possible. Shoot organogenesis is a useful strategy for plant regeneration but the required rooting step can be tedious and time consuming. Somatic embryogenesis is the route usually attempted since it is generally quicker and more effective.

1.7 Somatic embryogenesis

Somatic embryogenesis is the preferential method of plant regeneration since it leads to the direct and exact formation of a plant via an embryo. Somatic embryogenesis is a process whereby a root and shoot develop simultaneously in non reproductive tissue which has not undergone meiosis. It is characterised by bipolar growth at two meristems on opposite poles (George, 1993). It is similar to zygotic embryogenesis in many ways; both are initiated by cell polarity and asymmetrical cell divisions and go through similar developmental stages. Somatic embryos generally are not patterned the same though, being bigger than zygotic embryos. Meristem formation is also often abnormal in somatic embryos which can hinder germination. Furthermore, somatic embryos do not undergo a dormant stage with seed development (Dodeman *et al.*, 1997).

The tissue used for somatic embryogenesis varies. Tissue highly committed to regeneration viz. nucellus, ovule, ovary or embryo tissue is preferable. This tissue has an embryogenic determination and embryogenesis sometimes occurs spontaneously. Other cells can be induced to this state, usually with exposure to auxins which is the basis of somatic embryogenesis techniques. Auxins appear to reprogram the cell into an embryogenic state by mechanisms such as kinase cascades and DNA demethylation which seem to be a prerequisite for proemberyo formation (George, 1993).

Therefore, during auxin exposure cells are induced into a state competent for embryogenesis. Upon auxin induction, tissue highly committed to regeneration can directly give rise to somatic embryos. In the case of most tissues though direct embryogenesis does not occur and the process usually involves indirect embryo formation. This occurs via the formation of a mass of unorganised, unspecialised and dedifferentiated callus tissue. A specific compact and structured callus termed embryogenic callus gives rise to somatic embryos. A problem with this technique is that callus tissue is proned to genetic mutations which can result in inferior embryo formation (George, 1993)

One can split up somatic embryogenesis into various stages but the most important is the induction phase with auxin. For the development of the embryo the auxin has to be reduced or removed completely. The embryo then passes through maturation, desiccation and finally germination stages (Merkle *et al.*, 1996). Protocols for each stage have to be optimised.

1.8 Somatic embryogenesis in sweet potato

Somatic embryogenesis in sweet potato has to date mostly been initiated with the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), although other auxins have been used. This protocol was established primarily with leaf explants and shoot tips, stems and roots being less successful in embryo formation (Liu and Cantliffe, 1984). An optimal concentration was later determined to be 2.20 $mg.l^{-1}$ 2,4-D (Chee and Cantliffe, 1988). It has been shown that a 0.22 $mg.l^{-1}$ concentration of a cytokinin like kinetin or 6-benzylaminopurine (BAP) further stimulates embryogenic callus formation, therefore increasing embryogenesis (Chee and Cantliffe, 1988). Studies optimising embryogenic callus formation subsequently showed 2.24 $g.l^{-1}$ KCl could also stimulate its formation (Bieniek *et al.*, 1995). Sweet potato embryogenic callus can be distinguished morphologically from non-embryogenic callus. Embryogenic callus is bright to pale yellow in colour with a compact and organised structure while the other is a very dull yellow, translucent or turning black with a friable structure (Liu and Cantliffe, 1984).

After about 6 weeks of callus growth, initiation of histodifferentiation seems to be most successful for sweet potato, where the callus with globular embryos can develop through heart and torpedo to mature cotyledonary stages (Bieniek *et al.*, 1995). Studies relating to these stages have found that concentrations of 2,4-D above 1.10 $mg.l^{-1}$ inhibit this process (Chee and Cantliffe, 1989). Hormones are therefore not included in culture media for this stage (Chee and Cantliffe, 1988; Liu and Cantliffe, 1984). This process takes about four weeks, hormones like 0.38 $mg.l^{-1}$ gibberellic acid (GA_3) have been shown to speed up the globular to heart stage (Mukherjee *et al.*, 1991).

Once the embryo has developed to the mature cotyledonary stage, plant formation is generally initiated via germination although there have been reports in sweet potato that up to 25 % more plantlets can be obtained from embryos at the elongated torpedo

stage (Schultheis *et al.*, 1990). Both stages give successful regeneration though (Chee and Cantliffe, 1988). Culture on hormone free medium, with desiccation by substances like polyethylene glycol (PEG), may be required prior to germination (Newell *et al.*, 1995). This seems to activate the embryo for germination, a process which furthermore requires a antibiotic free environment (Newell *et al.*, 1995). Growth through germination can be speeded up with low (0.002 $mg.l^{-1}$) amounts of auxins and cytokinin (Cavalcante *et al.*, 1994) and by temperatures of 30 ^{0}C, 16 hour photoperiod and sucrose concentrations of 30 $g.l^{-1}$ (Jarret and Gawel, 1991).

This would be the method of choice for the regeneration of transformed tissue into transgenic plantlets. New protocols are being developed continuously however and the latest developments have shown that short 2 week exposure to the optimised 2,4-D and BAP concentrations followed by exposure 2.50 $mg.l^{-1}$ abscisic acid (ABA) gives much higher levels of embryogenesis (Zheng *et al.*, 1996). Abscisic acid appears to stimulate induction of embryogenesis and increases subsequent adventitious shoot formation (Zheng *et al.*, 1996) though it also known to inhibit germination and induce non embryogenic callus conversion (George, 1993). Some protocols go the route of shoot regeneration viz. using 0.22 $mg.l^{-1}$ 2,4-D for just 3 days followed by 0.35 $mg.l^{-1}$ concentrations of the cytokinin zeatin riboside which stimulates direct shoot formation (Dessai *et al.*, 1985).

The protocols used to date have mostly been established for leaves and apical meristems as explants. The work which will be carried out in this project however will use tubers and success with these has been limited. The advantages of using tubers is their availability, ease of storage and high tissue content. In addition to this only limited work has been done with sweet potato tubers to date. It seems that tubers require longer times of exposure to auxins and cannot go straight off 2,4-D for embryo development but instead require transfer to media with indoleacetic acid (IAA) or α-naphthaleneacetic acid (NAA) at about 1 $mg.l^{-1}$ (Newell *et al.*, 1995).

Once a basic system for regenerating sweet potato tissue has been developed one needs to choose and develop a system for the transformation of tissue so that tissue transformation can commence prior to plant regeneration (Merkle *et al.*, 1996).

1.3 Plant transformation systems

By genetic engineering definitions, transformation could be defined as the introduction of functional genes into cells, resulting in chromosomal integration, functional expression and stable heritability of the functional gene. This differs from the transient expression of genes where there is no chromosome incorporation of the gene and expression is ultimately lost with time.

Attempts to produce transgenic plants were naturally preceded by work in the early 1990's to develop and optimise a viable transformation system. There are a variety of techniques for the introduction of DNA into plant cells. These include *Agrobacterium tumefaciens* (*A. tumefaciens*), *Agrobacterium rhizogenes*, liposome, PEG and calcium chloride, microlaser, pollen, viral, microinjection, fibre-mediated, electroporation or particle gun bombardment mediated transfer. (Potrykus, 1991). The most common techniques are electroporation of protoplasts, particle gun bombardment and *A. tumefaciens* mediated gene transfer.

Electroporation uses short electrical impulses from a capacitor to induce transient formation of pores in the membranes of protoplasts, plant cells with their walls removed, where DNA can enter. The technique is capable of introducing high quantities of DNA into varying cell types. It is not a direct method of gene transfer and therefore does not ensure integration of the DNA, although it seems there is a tendency for integrative transformation to occur. Furthermore it is labour intensive and regeneration of protoplasts into transgenic plants can be extremely difficult (Potrykus, 1995).

Particle gun bombardment is a widely used system where DNA is coated on heavy metal particles which are fired into the cells, and thereby may introduce some DNA into the nucleus. This technique is useful since it can be used on all plant types and cells and therefore has a broad target range. Once again it is an indirect gene transfer method and does not ensure integrative transformation and regeneration is difficult. Furthermore, there is usually a low and erratic frequency of gene delivery (Klein 1995).

A. tumefaciens is a direct method of transfer. The transfer of foreign DNA is mediated by a disarmed *A. tumefaciens* bacteria which introduces the DNA into the plant genome by its specialised transfer mechanism upon wounding of the plant. It is a highly reproducible method since there is a definite trend for integration into areas of expressed plant DNA (Hooykaas and Schilperoort, 1992). Its main limitation is that it only works optimally with dicotyledonous plants which exhibit a wounding response but this makes it suitable for sweet potato (Hooykaas, 1995).

The successful transformation of sweet potato has been achieved with all three of the common plant transformation techniques; electroporation (Nishiguchi *et al.*, 1992), particle gun bombardment (Prakash and Varadarajan, 1992) and *A. tumefaciens* mediated transfer (Al-Juboory and Skirvin, 1991; Newell *et al.*, 1995; Gama *et al.*, 1996).

Electroporation introduced the β-glucuronidase (GUS) gene (*uidA* or *gusA*) and hygromycin phosphotransferase gene (*hph*) into protoplasts with a 1% success rate but the transformed cells were not regenerated into plants. Particle gun methods achieved stable integration of *gusA* and neomycin phosphotransferase II gene (*nptII*) in leaf tissue, there was a 13% success rate for transformation and many transformed cells developed roots. However, there was a very poor success rate with another plasmid introduced with particle gun methods which indicated a gross lack of reproducibility. Also no whole plants could be regenerated from the transformed tissues.

The success using *A. tumefaciens* has been by far the most promising. Genes introduced include *uidA*, cowpea trypsin inhibitor, snowdrop lectin and *npt11*. In all three cases plant regeneration of the transformed tissue has been achieved from tuber, leaf and callus tissue, using the international cultivar jewel. For this reason this study will use the *A. tumefaciens* system of transformation.

1.10 *Agrobacterium tumefaciens* transformation system

A. tumefaciens is a gram negative soil bacterium which causes crown gall disease in plants. The mechanism of infection is through a tumour inducing plasmid (Ti) containing a virulence (*vir*) operon which facilitates the transfer of a section of the Ti plasmid known as the transferred DNA (TDNA). The TDNA encodes oncogenic gene (*onc*) products like tryptophan monooxygenase as well as opines which are used as a carbon, nitrogen source by the bacterium. The vir operon encodes proteins with varying functions. Phenolics like acetosyringone activate vir A which activates vir G which causes other vir proteins to be expressed. The protein vir D causes nicking of TDNA, vir C replicates it, vir E binds it and vir B is involved in the transfer of the replicated, bound TDNA to the plant cell (Hooykaas and Schilperoort, 1992).

In the case of transformation applications, the Ti plasmid is used to deliver TDNA without any *onc* products i.e. disarmed. All that is essential is the 24 base pair (b.p) left and right border repeats (recognition signals), the rest of the DNA can be replaced by homologous recombination. The two transformation systems used are the cis system where vectors are introduced into artificial TDNA on the Ti plasmid and the binary system where the Ti plasmid lacks TDNA and artificial TDNA containing vectors are introduced separately as another plasmid (Hooykaas and Schilperoort, 1992).

1.11 Transgenic gene analysis

Once tissue has been transformed the fate of newly introduced genes have to be monitored in order to evaluate the success of transformation. One way to do this is with transgenic marker genes which if functional and expressed produce a detectable product. Transformed tissue can be selected on the basis of it producing the novel gene product. The benefit of this system is that it requires no genetic techniques.

A common transgenic marker in plants is β-glucuronidase (GUS) encoded by *uidA* or *gusA* (Jefferson, 1987). This is because GUS is unique but non toxic to the plant. Furthermore, GUS is very stable and can be detected easily with a variety of substrates allowing histochemical and quantitative enzymatic analysis (Martin *et al.*, 1992)

The basis of all GUS detection reactions is the presence of the sugar D-glucopyranosiduronic acid (glucuronide) which is attached by a glycosidic bond to a hydroxyl group of a chromogenic or fluorogenic detectable molecule. Functional GUS cleaves the glycosidic bond and the detectable molecules are released.

A commonly used histochemical GUS substrate is 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (x-gluc) which yields a blue product (Martin *et al.*, 1992). The substrate p-nitrophenyl-β-D-glucuronide (pNPG) gives a product which can be measured spectrophotometrically at a wavelength (λ) = 405nm, allowing one to quantitate GUS activity (Martin *et al.*, 1992). The 4-methylumbelliferyl-β-D-glucuronic acid (MUG) substrate gives a product which can be detected by fluorescence at λ = 460nm, its fluorescence makes it a very sensitive assay and allows one to quantitate GUS activity (Martin *et al.*, 1992).

Another useful GUS substrate is ImaGene dye (ImaGene Red™ and Green™). It is similar in structure to resorufin-β-D-glucuronic acid but contain a 12 carbon aliphatic chain which makes it lipophilic and facilitates the uptake and localisation of the dye into the cell membrane. The cleaved product fluoresces red or green in the membrane of cells which contain functional GUS, allowing highly specific histochemical studies where GUS can be imaged in living cells (MPE ImaGene Manual).

Transgenic marker genes conferring antibiotic or herbicide resistance are also useful e.g. PPT and neomycin (kanamycin) resistance. PPT, used in commercial herbicide, inhibits glutamine synthetase which causes the toxic accumulation of ammonia (De Block *et al.*, 1987). Kanamycin, an aminoglycoside antibiotic, inhibits the ribosome small subunit thereby inhibiting protein synthesis (McDonnel *et al.*, 1987). Resistance to these compounds is conferred by *npt II* and phosphinothricin acetyltransferase (*pat*) or bialaphos resistance (*bar*) genes which encode the enzymes neomycin phosphotransferase (NPT) and PAT respectively (Wehrmann *et al.*, 1996).

The enzymes NPT and PAT inactivate kanamycin and PPT by phosphorylation and acetylation reactions respectively (McDonnel *et al.*, 1987; De Block *et al.*, 1987). Using these marker genes, one can select or detect transformed tissue by pressurisation with the antibiotic or herbicide. One can also carry out specific assays to detect and quantitate PAT and NPT activity which involve using radioactively labelled C^{14} and P^{32} to follow acetylation and phosphorylation reactions or by physically separating acetylated and non acetylated or phosphorylated and non phosphorylated antibiotic or herbicide (Spencer *at al.* ,1990; McDonnel *et al.*, 1987). Phosphinothricin resistance is also useful as a lot of the commercial herbicides have PPT as the active component e.g. basta, bialaphos®, herbiace®, ignite® and glufosinate. The *bar* or *pat* gene can therefore be used as a dominant gene for engineering weed control in crops as well as a transgenic marker in initial stages transformation (De Block *et al.*, 1987).

The marker genes need to be analysed over a period of at least four weeks in plants to allow for the loss of transient expression so that one can determine the level of expression arising from integrated genes only. Once plants have been established one can then do genetic analysis including polymerase chain reaction (PCR) and southern blots to screen for transgenic genes in the plant genome. These techniques will enable one to confirm if there is integration of the genes in the plant genome One can also carry out northern or dot blots to see if the genes are being transcribed and western blots to determine if the genes are being expressed.

Finally protein studies need to be done to determine the levels of transgenic protein and its stability. The final test would be if the genes of interest are conferring the desired phenotypic effect, this could be achieved by field trials or in the case of resistance, screening against the pathogen or pest of interest. Furthermore, one needs to determine the inheritance patterns of the newly introduced phenotype in order to asses transmission of the gene.

1.12 Conclusion

The various steps involved in the production of a transgenic plant need to be integrated so that the whole process is successful. A transformation system is required which can ensure the stable integration of functional genes into areas where they will be functionally expressed and passed to offspring. This transformation system must be capable of introducing genes specifically into the tissue type one has chosen to use. There has to be a reproducible system for regenerating plants from transformed tissue keeping in mind that transformed material may differ from non transformed material in its ability to be regenerated. The transformation system then must not have an adverse effect on the stage of regeneration. Finally one needs an effective method of monitoring the fate of the introduced genes in the early and late stages of the process so that one can optimise the protocol to increase the level of transformation.

In this study, *A. tumefaciens* has been the method of gene transfer used. A binary vector system was used to transform sweet potato tuber with the transgenic marker genes *uidA*, *npt11* and *bar*. Non transformed controls were also kept so that the effects and levels of transformation could be accurately assessed. Transformed tissue and non transformed controls were then screened for evidence of the introduced genes. The gene products of *npt11* and *bar* were screened for by pressurising transformed tissue with kanamycin and PPT respectively. The GUS enzyme was screened for histochemically with x-gluc, ImaGene, and MUG and GUS activity was determined using MUG. Elements of *uidA* were screened for by PCR.

Plant regeneration of the transformed and non transformed tissue was attempted primarily via somatic embryogenesis using the auxin 2,4-D for induction. The morphogenesis observed was not strictly classified however. Secondary stages of plant regeneration focused on the continued development of induced structures.

1.13 Aim

To develop and test protocols for the generation of transgenic sweet potato from tubers.

1.14 Objectives

1. Develop a protocol for the regeneration of plantlets from the tubers of local cultivars of sweet potato.
2. Transform sweet potato tubers with *npt11*, *uidA* and *bar* using *A. tumefaciens* mediated transfer.
3. Determine the success of transformation experiments.
4. Regenerate plantlets from transformed tuber material.

2 Materials and Methods

2.1 Sweet potato tissue culture

2.1.1 Culture techniques

Experimental work involving plant tissue culture was carried out using sterile techniques in a laminar flow bench. All glassware, equipment and media was sterilised by autoclaving at a temperature of 121 °C and a pressure of 15 p.s.i. for 20 minutes. To maintain sterility, equipment was periodically rinsed in 100 % ethanol and flamed and the glassware and bench were sprayed periodically with 80 % ethanol. The basal growth medium for all tissue culture was Murashige and Skoog (MS) salts and vitamins (Unilab) (Murashige and Skoog, 1962) (Section 7.2) + 30 $g.l^{-1}$ sucrose (Unilab) + 3 $g.l^{-1}$ gelrite (Unilab), pH 5.8. Plant tissue was incubated at 30 °C in a light intensity of 120 μmole $quanta.m^{-2}.s^{-1}$ for a 13 hour photoperiod except where specified.

2.1.2 Plant tissue material

Leaves were obtained from blesbok single node cultures. These cultures were started *in vitro* by a 15 minute sterilisation with 1 % $v.v^{-1}$ commercial sodium hypochlorite prior to culture on growth media + 5 $mg.l^{-1}$ GA_3 (Sigma). They were subcultured every 4 weeks. Blesbok root tubers were obtained fresh for *in vitro* culture. They were peeled, cleaned under tap water and sterilised for 30 minutes in 2 % $v.v^{-1}$ commercial sodium hypochlorite containing 0.1 % $v.v^{-1}$ triton x-100 (Univar). The tubers were then rinsed three times in sterile distilled water before discs were cut using a 14 mm or 8 mm borer.

2.2 Induction of morphogenesis in sweet potato

2.2.1 Auxin experiments on leaves

Concentrations of 0.0, 0.5, 1.0, 2.0, 3.0, and 10.0 mg.l^{-1} 2,4-D (BDH) were include growth media. Five Petri plates were prepared for each concentration. Six leaf secti were cultured on each Petri plate. The experiment was run for six weeks in the dar which point observations were made.

2.2.2 Auxin experiments on tubers

Concentrations of 0.0, 0.2, 1.0, 1.5, 2.0, 2.5 and 3.0 mg.l^{-1} 2,4-D were include growth media. Five Petri plates were prepared for each concentration. Seven d were cultured on each Petri plate. The experiment was run for six weeks in the dar which point observations were made.

2.2.3 Auxin:cytokin experiments on tubers

The following concentrations of 2,4-D:BAP (Fluka) were included in growth medi mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP (10:1), 2 mg.l^{-1} 2,4-D + 0.4 mg.l^{-1} BAP (5:1), 2 m 2,4-D + 1.0 mg.l^{-1} BAP (2:1), 2 mg.l^{-1} 2,4-D + 2.0 mg.l^{-1} BAP (1:1), 1 mg.l^{-1} 2,4-D 2.0 mg.l^{-1} BAP (1:2), 0.4 mg.l^{-1} 2,4-D + 2.0 mg.l^{-1} BAP (1:5), 0.2 mg.l^{-1} 2,4-D + mg.l^{-1} BAP (1:10). Five Petri plates were prepared for each ratio. Seven discs w cultured on each Petri plate. The experiment was run for 6 weeks at which p observations were made.

2.2.4 Long term auxin induction studies with tubers

Concentrations of 0.0, 1.0, 2.0 and 3.0 mg.l^{-1} 2,4-D were included in growth media. Ten Petri plates were prepared for each concentration. Five discs were cultured on each Petri plate. Tissue development was compared by written observation at 1, 2, 3, 4 and 6 weeks. Similarly, the effects of 2 mg.l^{-1} 2,4-D, 2 mg.l^{-1} NAA (BDH) and 2 mg.l^{-1} IAA (Sigma) were compared. Similarly, the effects of 2 mg.l^{-1} 2,4-D, 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP and 2 mg.l^{-1} 2,4-D incubated in the dark were compared.

2.3 Subsequent stages of morphogenesis

2.3.1 Second stage of morphogenesis

Concentrations of 0.5, 1.0 and 2.0 mg.l^{-1} ABA (Sigma); 0.5, 1.0 and 2.0 mg.l^{-1} NAA and 2.0 mg.l^{-1} NAA + 0.2 mg.l^{-1} BAP were included in growth media. Fifteen plates were prepared for each concentration as well as 15 plain growth medium plates. Morphogenic structures induced with 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP, at 2,4 and 6 weeks were transferred to these plates. Five plates were used for each of 2,4 and 6 week stages. Morphogenic structures larger than 20 mm were transferred to growth media + 1 mg.l^{-1} BAP or to growth media + 1 mg.l^{-1} NAA in tubes. Observations were made over 8 weeks.

2.3.2 Alternative second stages of morphogenesis

A range of varying protocols were carried out in an attempt to generate plantlets. Protocols used 2,4-D, BAP, ABA, NAA and thidiazuron (TDZ) (Sigma) in different ratios in growth media for different lengths of time (Table 2.3.2).

Experiment	Stage 1	Stage 2	Stage 3	Stage 4
10 plates, 6 root tuber discs per plate	1 week: 1 mg.l^{-1} TDZ + 1 mg.l^{-1} 2,4-D	1 mg.l^{-1} TDZ + 0.1 mg.l^{-1}2,4-D	same	same
10 plates, 6 root tuber discs per plate	1 week: 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	1 week: 0.1 mg.l^{-1}2,4-D + 0.5 mg.l^{-1} BAP	1 mg.l^{-1} BAP + 0.1 mg.l^{-1}2,4-D	same
10 plates, 6 root tuber discs per plate	1 week: 0.5 mg.l^{-1}2,4-D + 0.1 mg.l^{-1} BAP	1 week: 0.5 mg.l^{-1}2,4-D + 0.1 mg.l^{-1} BAP	1 week: 0.5 mg.l^{-1}2,4-D + 0.5 mg.l^{-1} BAP	1 mg.l^{-1} BAP + 0.1 mg.l^{-1}2,4-D
10 plates, 6 root tuber discs per plate	4 weeks: 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	4 weeks: 2 mg.l^{-1} ABA	2 mg.l^{-1} ABA + 1 mg.l^{-1} BAP	same
10 plates, 6 root tuber discs per plate	8 weeks: 2 mg.l^{-1} NAA + 0.2 mg.l^{-1} BAP	4 weeks: 1.0 mg.l^{-1} NAA + 1.0 mg.l^{-1} BAP	same	same
10 plates, 6 root tuber discs per plate	6 weeks: 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	1 week: 1 mg.l^{-1} TDZ + 1 mg.l^{-1} 2,4-D	0.2 mg.l^{-1} 2,4-D + 2.0 mg.l^{-1} BAP	same
10 plates, 6 root tuber discs per plate	1 week: 2 mg.l^{-1} 2,4-D	1 mg.l^{-1} BAP	same	same

Table 2.3.2: Alternative hormone concentrations, ratios and exposure times used in an attempt to generate plantlets from sweet potato root tuber. Hormones were included in growth media.

2.4 Transformation of *Agrobacterium tumefaciens*

2.4.1 Vectors used for transformation

A. tumefaciens strains underwent triparental mating for the uptake of the binary vectors: pBISN1, pE1120 and pCAS1 (BRDC) contained in the *Escherichia coli* (*E. coli*) strains E1226, E1120 and E1257 respectively (Figures 2.4.1.1-2.4.1.3).

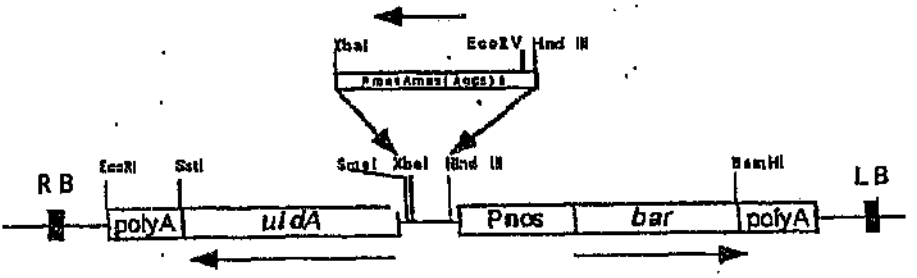


Figure 2.4.1.1: pCAS1 contains the PPT resistance marker gene *bar* with a nopalin synthase gene (*nos*) promoter and the GUS marker gene *uidA* with the Gelvin® superpromoter. pCAS1 is contained in the *E. coli* strain E1257.

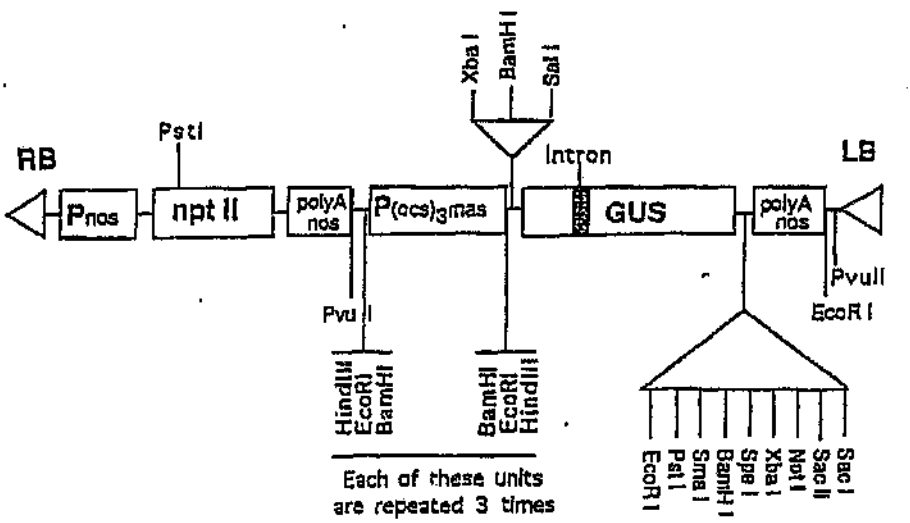


Figure 2.4.1.2: pBISN1 contains the kanamycin resistance marker gene *nptII* with a *nos* promoter and the GUS marker gene *uidA* with the Gelvin® superpromoter. An operon is included in the GUS gene which regulates its expression in bacteria. pBISN1 was contained in the *E. coli* strain E1226.

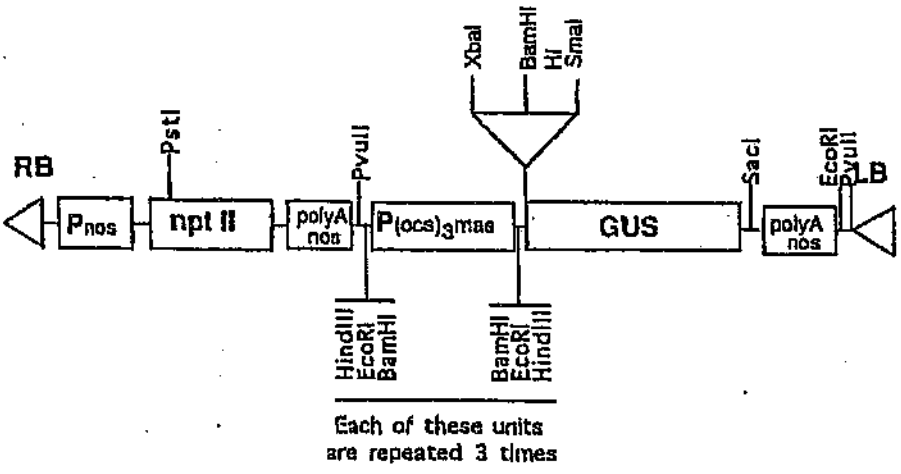


Figure 2.4.1.3: pE1120 contains the kanamycin resistance marker gene *nptII* with a *nos* promoter and the GUS marker gene *uidA* with the Gelvin® superpromoter. It does not include an operon in the GUS gene and therefore is not regulated in bacteria. pE1120 is contained in the *E. coli* strain E1120.

2.4.2 Triparental mating

All bacteria culture was carried out under sterile conditions. The *E. coli* strains E1257, E1226 and E1120 were grown up in nutrient broth (NB) (Biolab) with 100 $\mu g.ml^{-1}$ kanamycin sulphate (kanamycin) (Boehringer) at 30 °C. The *E. coli* strain HB101 containing the fertility plasmid pRK2013 was grown up in NB with 100 $\mu g.ml^{-1}$ kanamycin at 30 °C. The *A. tumefaciens* strain LBA4404 was grown up at room temperature in yeast mannitol (YM) media (4 $g.l^{-1}$ yeast extract, 100 $g.l^{-1}$ mannitol, 1 $g.l^{-1}$ NaCl, 2 $g.l^{-1}$ $MgSO_4.7H_2O$ and 5 $g.l^{-1}$ $K_2HPO_4.3H2O$ (Unilab), pH 7.0) with 50 $\mu g.ml^{-1}$ rifampicin (Boehringer). All strains were stored in cryopreserve beads (Pro-Lab Diagnostics Microbank™) at -70 °C.

The conjugative transfer of these vectors from the respective *E.coli* strains to LBA4404 was carried out by triparental mating as done by Van Haute and associates (Van Haute *et al.*, 1983). For the triparental mating, 1 ml of LBA4404, HB101 and the respective binary plasmid containing bacteria, obtained from single cell c[illegible]s and grown to mid log phase, were grown up in YM media at room temperature for 24 hours. The triparental cross was then grown up in YM media with 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin.

2.4.3 Testing the triparental cross for uptake of the vectors

The triparental was plated on plates of nutrient agar (NA) (Biolab) with 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamyc[illegible] enabled the selection of transformed colonies of *A. tumefaciens*. In order [illegible]creen for GUS, 80µl 10 $mg.ml^{-1}$ x-gluc (Sigma) in dimethyl formamide (DMF) was added to the surface of the plates. Colonies containing GUS turned blue.

2.5 Transformation of sweet potato

Growth curves were carried out for all three triparental crosses. They were grown up in YM media with 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin at 19 °C. The spectrophotometric absorbance at $\lambda = 600$ nm (A_{600}) was measured every hour for 30 hours (Varian DMS 90). For each strain the A_{600} at mid log growth phase was determined. For transformation experiments, the strain was grown to its mid log phase as determined by its A_{600}. Bacteria was then centrifuged for 15 minutes at 3000 r.p.m (Hettich Universal). The pellet was resuspended to the same A_{600} in MS salts and vitamins + 30 $g.l^{-1}$ sucrose + 40 $\mu g.ml^{-1}$ $3^1,5^1$ dimethoxy-4-hydroxy-acetophenone (acetosyringone)(Aldrich), dissolved in methanol, pH 5.8. Tuber discs or callus were

In order to remove the bacteria after incubation the material was plated on the media of choice with carbenicillin disodium salt (carbenicillin) (ICN) at 50-250 $\mu g.ml^{-1}$ or cefotaxime disodium salt (cefotaxime) (Sigma) at 50-350 $\mu g.ml^{-1}$ and 20 $\mu g.ml^{-1}$ benlate® (500 $g.kg^{-1}$ benzimidazole)(DuPont) was added to remove any fungal infections. Transformed and non transformed tissue was then cultured according to protocols optimised in Sections 2.2 to 2.3.

2.6 Antibiotic and herbicide resistance screening with sweet potato

2.6.1 Testing tissue susceptibility to screening agents

Kanamycin concentrations of 0, 50, 100, 150, 200 and 250 $\mu g.ml^{-1}$ were included in growth media + 0.2 $mg.l^{-1}$ 2,4-D. Ten plates of each concentration were prepared, five for blesbok leaves and five for blesbok tubers. Four leaf sections or seven tuber discs were used per plate. Observations were made at six weeks. Racemic D,L-phosphinothricin (gluphosinate ammonium, gluphosinate or glufosinate) (Greyhound) concentrations of 0.0, 10.0, 20, 20, 40 and 50 $\mu g.ml^{-1}$ were similarly tested.

2.6.2 Screening transformed tissue

Concentrations of gluphosinate or kanamycin found to affect sweet potato tissue were tested on transformed tissue and the non transformed controls. For comparison purposes some of the transformed tissue and non transformed controls were maintained on media free of gluphosinate or kanamycin.

2.7 Histochemical analysis of transformation

The ImaGene Red™ (C_{12}GGlcU) and Green™ (C_{12}FDGlcU) GUS gene expression kits were used (MPE) as well as MUG (Sigma). Transformed tissue and non transformed controls were placed in 1 ml MS salts and vitamins + 30 $g.l^{-1}$ sucrose. To this was added 5µl of a 10 mM C_{12}GGlcU, C_{12}FDGlcU or MUG (in 100mM Na_2HPO_4/NaH_2PO_4, pH 6.5) solution. After 1 hour at 37 °C the reaction was stopped with 10 µl 20 mM D-glucaric acid 1,4-lactone for ImaGene dyes or 50 µl 200 mM Na_2CO_3 for MUG. Slides of the tissue were prepared in a MS salts and vitamins + 30 $g.l^{-1}$ sucrose solution. Tissue fluorescence was viewed with a confocal microscope (Zeiss LSM 410 invert laser scan) with a 40 x 1.2 NA magnification.

2.8 Quantitative analysis of transformation

2.8.1 4-methylumbelliferyl-β-D-glucuronic acid assay

For each MUG assay 1g of tissue was homogenised in 1 ml of lysis buffer (50 mM Na_2HPO_4/NaH_2PO_4, pH 7.0, 10 mM β-mercaptoethanol, 10 mM ethylenediamine tetraacetic acid (EDTA), 0.1 % $w.v^{-1}$ sodium dodecyl sulphate (SDS) and 0.1 % $v.v^{-1}$ triton x-100 (Univar)). This solution was then centrifuged for 5 minutes on a benchtop centrifuge (Hagar) to remove cell debris. The supernatant was incubated with 10µl 10 mM MUG for 60 minutes at 37 °C. The reaction was stopped by adding 150 µl 200 mM Na_2CO_3. The fluorescence at $\lambda = 460 \pm 15$ nm ($F_{460\pm15}$) of a 5x dilution was measured on a fluorometer (Hoefer DyNA Quant™ 200) with an excitation bandpass of $\lambda = 365$ nm. The fluorescence reading was converted to 4 methylumbelliferyl (MU) concentration with a MU (Sigma) standard curve constructed under the same conditions in triplicate (Section 7.1). The GUS activity of transformed tissues and non transformed controls was calculated as $\mu mole(MU).min^{-1}$.

2.8.2 Protein determination

The protein concentrations of plant extracts used for the MUG assay were measured so that specific GUS activity ($\mu mole.min^{-1}.mg^{-1}$ protein) could be calculated. The Bio-Rad™ protein micro assay was used for protein determinations. For each measurement 400µl Bio-Rad was added with 80µl of the plant extract and the solution was made up to 2 ml with Millipore water and mixed. This was repeated in duplicate for each protein measurement. The spectrophotometric absorbance was read at λ = 620 nm (A_{620}) (Varian DMS 90) using protein free blank. The A_{620} was converted to protein concentration using a protein standard curve constructed with bovine serum albumin (BSA) under the same experimental conditions in duplicate (Section 7.1).

2.9 Genetic analysis of transformation

Polymerase chain reaction (PCR) reactions were carried out on three experimental tissue transformations and the non transformed controls to screen for *uidA* in the plant genome. DNA was extracted from the tissue using the method of Xiong (1993) (Section 7.3). DNA concentration was determined by its spectrophotometric absorbance at λ = 260 nm (A_{260}) (Beckman DU 64) (A_{260} = 1 for 50 $\mu g.ml^{-1}$ DNA). The spectrophotometric absorbance at λ = 280 nm (A_{280}) was measured (Beckman DU 64) to ensure there was no substantial DNA contamination ($A_{260}.A_{280}^{-1}$ above 1.8). DNA was run on a 0.5 % agarose (Promega) tris borate ethylenediamine tetraacetic acid buffer (TBE) (10 mM Tris-borate, pH 8.0 and 1 mM EDTA (Univar)) gel (Sambrook *et* al., 1989a). This was to confirm the presence of the DNA and to confirm DNA was not damaged (evident as a single band).

Two 25 b.p primers (MWG), for a 900 b.p fragment including the GUS intron, were used (Section 7.4). The initial reaction cocktails used were 10 µl mixtures including 1x Taq buffer, 1 µmole.min^{-1}.mg^{-1}protein Taq DNA polymerase, 1.5 mM $MgCl_2$, 0.1 mM dNTP (Takara) with 0.5 µM of each primer and 40 ng plant DNA. The initial PCR reaction used a 64 °C annealing temperature. The reaction was run on a PCR machine (Hybaid OmniGene) as follows: 94.5 °C for 30 seconds, 64 °C for 30 seconds and 72 °C for 45 seconds for 35 cycles followed by 94.5 °C for 30 seconds, 64 °C for 30 seconds and 72 °C for 5 minutes for 1 cycle. To optimise conditions: $MgCl_2$ concentration, DNA content and primer concentration were lowered and annealing temperature was increased. 10µl of the reaction mixtures were run on 2% TBE gels using 50, 150, 300, 500, 750 and 1000 b.p PCR markers (Promega)

3. Results

3.1 Induction of morphogenesis in sweet potato

3.1.1 The effect of auxin on tubers and leaves

Leaves responded positively to all concentrations of 2,4-D except 10.0 $mg.l^{-1}$, as compared to the control (Table 3.1.1.1). A yellow cream, compact and organised embryogenic type callus (Liu and Cantliffe, 1984) was observed for 2.0-3.0 $mg.l^{-1}$ 2,4-D after 3 weeks (Plate 3.1.1.1). Small white globular formations resembling globular embryos were observed after 6 weeks. These organised structures were classified as a form of morphogenesis and the percentage of explants with these formations was determined (Table 3.1.1.1). Optimum morphogenesis was at 2.0 $mg.l^{-1}$ 2,4-D.

Tubers responded positively to all concentrations of 2,4-D, as compared to the control (Table 3.1.1.2). Callus formation did not appear to follow any definite morphological trend but some embryogenic type callus was observed for 1.5-3.0 $mg.l^{-1}$ 2,4-D (Liu and Cantliffe, 1984). The morphogenesis observed was in the form of large amounts of green cream structures which became evident after 3 weeks (Plate 3.1.1.2). Optimum morphogenesis was achieved with 1.0-3.0 $mg.l^{-1}$ 2,4-D. These structures were produced in large amounts, four times more frequent than structures on leaf tissue. The structures were four times larger than those observed on leaf tissue.

[2,4-D] (mg.l^{-1})	Tissue	Callus	Morphogenesis
0	pale green	very little	0 %
0.5	pale green	clear	11 %
1.0	clear green	clear yellow	37 %
2.0	clear yellow	yellow	94 %
3.0	clear yellow	green yellow	58 %
10.0	black	very little	0 %

Table 3.1.1.1: The effect of various 2,4-D concentrations on sweet potato leaf observed after 6 weeks in the dark. 2,4-D concentrations were included in growth media.

[2,4-D] (mg.l^{-1})	Tissue	Callus	Morphogenesis
0	cream	clear, friable	0 %
0.2	cream, some brown	shiny yellow	40 %
1.0	brown	shiny yellow	86%
1.5	cream, some brown	yellow cream	54 %
2.0	brown	yellow cream	71 %
2.5	cream brown	yellow cream, in ridges	79 %
3.0	brown	yellow cream	60 %

Table 3.1.1.2: The effect of various 2,4-D concentrations on sweet potato tuber observed after 6 weeks in the dark. 2,4-D concentrations were included in growth media.

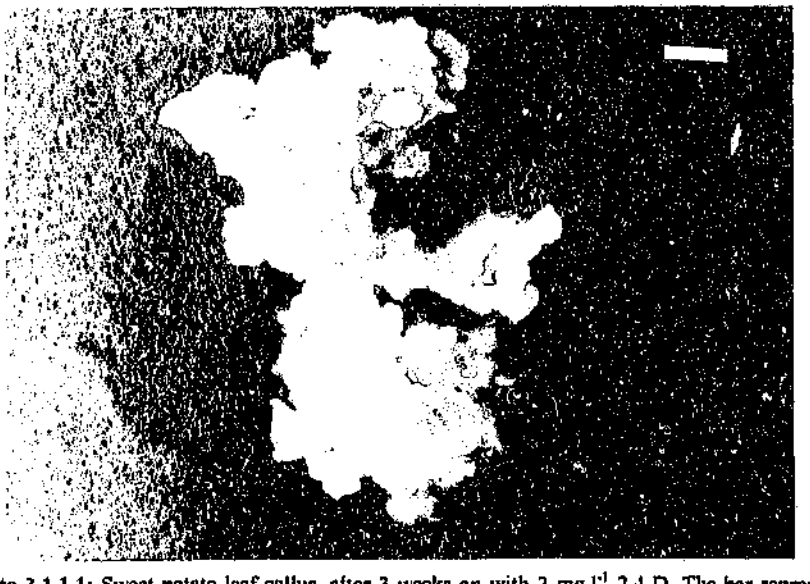


Plate 3.1.1.1: Sweet potato leaf callus, after 3 weeks on with 2 mg.l^{-1} 2,4-D. The bar represents 2.0 mm.

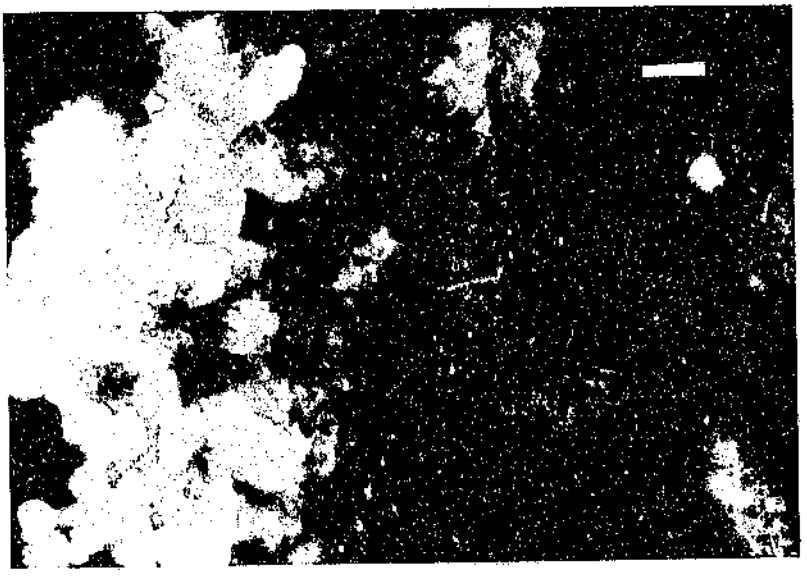


Plate 3.1.1.2: Sweet potato tuber callus and morphogenic structures after 3 weeks with 2 $mg.l^{-1}$ 2,4-D. The bar represents 1.9 mm.

3.1.2 The effects of varying auxin:cytokinin on tubers

Callus formation induced with varying 2,4-D:BAP ratios differed greatly and gave no indication of the effectiveness of the hormone ratios. The only callus which slightly resembled embryogenic callus (Liu and Cantliffe, 1984) was that observed with a 10:1 2,4-D:BAP ratio but it was greatly reduced. Judging by levels of morphogenesis the optimal ratio of 2,4-D:BAP was in the 5:1 to 10:1 range (Table 3.1.2). Callus formation with 10:1 was reduced as compared to the 5:1 and structures were larger and in greater numbers. The optimal of 2,4-D:BAP for morphogenesis was therefore 10:1.

Ratio	Tissue	Callus	Morphogenesis
10:1	cream, some green	some green yellow	86 %
5:1	cream, some green	yellow	89 %
2:1	yellow cream	yellow, friable	45 %
1:1	cream	clear	12 %
1:2	green	yellow	13 %
1:5	clear green	brown yellow	67 %
1:10	green	yellow	23 %

Table 3.1.2: The effect of various 2,4-D:BAP ratios on sweet potato tuber observed at 6 weeks. Hormones were included in growth media.

3.1.3 Long term auxin induction studies on tuber

Three different types of callus could be differentiated in the experiments carried out (Tables 3.1.3.1-3.1.3.3). A friable clear cream callus was the first type of callus to be observed during induction. The second type of callus was observed primarily at 3-6 weeks and was often associated with morphogenic structures. It was a yellow cream to green colour with a compact, structured appearance, corresponding to embryogenic callus (Liu and Cantliffe, 1984). This callus was evident only in some cases and in specific areas. Finally, in the latter stages of experiments, a clear or white callus became predominant, this was highly prolific and friable.

The sequence of events in the induction of morphogenesis only became visible after about one and a half weeks. After approximately 2 weeks there was visible growth in the form of a clear cream callus. This was accompanied by growth of the tuber (Plate 3.1.3.1). Within a week of this some areas of the tuber disc gave rise to clumps of small clear cream structures about 3 mm in size (Plate 3.1.3.2). Within another week these structures had reached a size of 6 mm and had become green in colour (Plate 3.1.3.3). They appeared to have 2 areas of organised growth in many cases, these areas being separated by connective tissue resembling a hypocotyle. Formations containing 2-5 of the structures defined often occurred together. By 4 weeks clumps had developed all

over the tuber discs, originating at specific points within the tuber tissue, the number of these points varying greatly between tuber discs (Plate 3.1.3.4). The structures ultimately became predominantly green and reached about 1 cm in size after 5 weeks, often closely fused (Plate 3.1.3.5).

Prolonged culture lead to the rapid dedifferentiation of tissue (Table 3.1.3.1) and by 8 weeks the callus was predominantly clear, white and friable. At this point structures had decreased in size and become a clear brown colour (Plate 3.1.3.6). After 12 weeks the callus was no longer present and only a few stem like structures remained (Plate 3.1.3.7). Explant tissue went from a clear cream to a cream colour at 2 weeks to a yellow colour at 3 weeks to a green yellow at 4 weeks. Over the 6 weeks explants generally underwent a 50 % increase in size

Comparisons of varying 2,4-D concentrations found that the maximum morphogenesis was obtained with 2 $mg.l^{-1}$ 2,4-D (Table 3.1.3.1). Higher concentrations led to erratic development of structures and pronounced amounts of clear or white friable callus. Lower levels gave rise to smaller structures in fewer numbers and required longer incubation times. The optimum incubation time was determined to be 4 weeks after which some levels of dedifferentiation were observed which ultimately led to high amounts of reversion of structures to callus(Table 3.1.3.1). In terms of comparisons with other auxins, 2,4-D was the most effective (Table 3.1.3.2). The other auxins used, IAA and NAA were effective but required longer incubation times and did not give rise to the same amount of morphogenic structures. Incubation in the light as opposed to dark improved morphogenesis two fold as well as producing larger structures in a shorter time (Table 3.1.3.3).

The optimal conditions for large scale induction of the largest morphogenic structures were determined to be incubation for 4 weeks in the light with 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP.

Time	Hormone	Tissue	Callus	Morphogenesis
1 week	0 mg.l^{-1} 2,4-D	clear cream	none	0 %
	1 mg.l^{-1} 2,4-D	clear cream	none	0 %
	2 mg.l^{-1} 2,4-D	clear cream	none	0 %
	3 mg.l^{-1} 2,4-D	clear cream	none	0 %
2 weeks	0 mg.l^{-1} 2,4-D	clear cream	none	0 %
	1 mg.l^{-1} 2,4-D	cream	low levels of clear callus	0 %
	2 mg.l^{-1} 2,4-D	cream yellow	some clear callus	0 %
	3 mg.l^{-1} 2,4-D	cream green	high levels of clear callus	0 %
3 weeks	0 mg.l^{-1} 2,4-D	cream	none	0 %
	1 mg.l^{-1} 2,4-D	yellow cream	clea cream callus	50 % discs with 1 clump of 5 structures about 2 mm big
	2 mg.l^{-1} 2,4-D	yellow cream with some green	clear cream callus, some white callus	50 % discs with 2 clumps of 5 structures about 3 mm big
	3 mg.l^{-1} 2,4-D	green cream	massive clear and white callus	40% discs with variable structures 1-4 mm big
4 weeks	0 mg.l^{-1} 2,4-D	cream	some clear callus	0 %
	1 mg.l^{-1} 2,4-D	green cream	cream callus	80 % discs with 1 clump of 5 structures about 3 mm big
	2 mg.l^{-1} 2,4-D	green cream	cream yellow callus with minor white callus	80 % discs with 2 clumps of 5 structures about 5 mm big
	3 mg.l^{-1} 2,4-D	green cream	massive white friable callus	50 % discs with variable clumps of structures about 2-8 mm big
6 weeks	0 mg.l^{-1} 2,4-D	cream	some clear callus	0 %
	1 mg.l^{-1} 2,4-D	green cream	yellow cream callus	70% of discs with 1 clump of 5 structures about 6 mm
	2 mg.l^{-1} 2,4-D	green cream	yellow white callus	50% reach 10 mm, 20% die and the rest revert to callus
	3 mg.l^{-1} 2,4-D	green cream	white friable callus	massive dedifferentiation, 10% of the structures reach 20 mm

Table 3.1.3.1: The effect of various 2,4-D concentrations on sweet potato tuber observed over 6 weeks. Hormones were included in growth media.

Weeks	Hormone	Tissue	Callus	Morphogenesis
1 week	2 mg.l^{-1} 2,4-D	clear cream	none	0 %
	2 mg.l^{-1} NAA	clear cream	none	0 %
	2 mg.l^{-1} IAA	clear cream	none	0 %
2 weeks	2 mg.l^{-1} 2,4-D	cream with some yellow areas	some clear cream callus	0 %
	2 mg.l^{-1} NAA	cream with some dull yellow growth	none	0 %
	2 mg.l^{-1} IAA	cream with some dull yellow growth	none	0 %
3 weeks	2 mg.l^{-1} 2,4-D	yellow cream with some green	cream yellow callus	50 % discs with 2 clumps of 5 structures about 3 mm big
	2 mg.l^{-1} NAA	cream yellow	clear cream callus	0 %
	2 mg.l^{-1} IAA	cream yellow	clear cream callus	0 %
4 weeks	2 mg.l^{-1} 2,4-D	green cream	yellow cream callus, some white callus	80 % discs with 2 clumps of 5 structures about 5 mm big
	2 mg.l^{-1} NAA	yellow cream	some clear callus and yellow callus	50 % discs with 2 clumps of 5 structures about 2 mm big
	2 mg.l^{-1} IAA	yellow cream	some clear callus and yellow callus	40 % discs with 2 clumps of 4 structures about 2 mm big
6 weeks	2 mg.l^{-1} 2,4-D	green cream	green yellow callus with some white callus	50 % reach 10 mm and turn a clear brown colour, 20 % die and 30 % revert to clear callus
	2 mg.l^{-1} NAA	green cream	some cream yellow callus	50 % discs with 2 clumps of 5 structures about 4 mm big
	2 mg.l^{-1} IAA	green cream	some yellow callus and white callus	50 % discs with 2 clumps of 4 structures about 4 mm big

Table 3.1.3.2: The effect of 2 mg.l^{-1} of various auxins on sweet potato tuber observed over 6 weeks. Hormones were included in growth media.

Weeks	Hormone	Tissue	Callus	Morphogenesis
1 week	2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	clear cream	none	0 %
	2 mg.l^{-1} 2,4-D	clear cream	none	0 %
	2 mg.l^{-1} 2,4-D in dark	clear cream	none	0 %
2 weeks	2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	cream	some clear callus	0 %
	2 mg.l^{-1} 2,4-D	[illegible]am	some clear callus	0 %
	2 mg.l^{-1} 2,4-D in dark	clear cream	none	0 %
3 weeks	2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	yellow cream	some yellow cream callus	50 % discs with 2 clumps of 8 structures about 3 mm big
	2 mg.l^{-1} 2,4-D	yellow cream	clear callus and yellow callus	50 % discs with 2 clumps of 5 structures about 3 mm big
	2 mg.l^{-1} 2,4-D in dark	clear cream	clear cream callus	0 %
4 weeks	2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	yellow cream with some green	some yellow green callus	80 % discs with 2 clumps of 8 structures about 5 mm big
	2 mg.l^{-1} 2,4-D	yellow green	cream yellow callus, some white callus	80 % discs with 2 clumps of 5 structures about 5 mm big
	2 mg.l^{-1} 2,4-D in dark	clear yellow	clear cream callus	30 % with 1 clump of 5 structures about 3 mm big
6 weeks	2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP	green yellow	yellow white callus	50 % reach 10 mm, 20 % die and 30 % revert to clear callus
	2 mg.l^{-1} 2,4-D	green cream	predominantly white callus	40 % reach 10 mm, 20 % die and 40 % revert to clear callus
	2 mg.l^{-1} 2,4-D in dark	yellow	yellow callus	60 % discs with 2 clumps of 6 structures of 5 mm big

Table 3.1.3.3: The effect of 2 mg.l^{-1} 2,4-D, 2 mg.l^{-1} 2,4-D with incubation in the dark and 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP on sweet potato tuber observed over 6 weeks. Hormones were included in growth media.

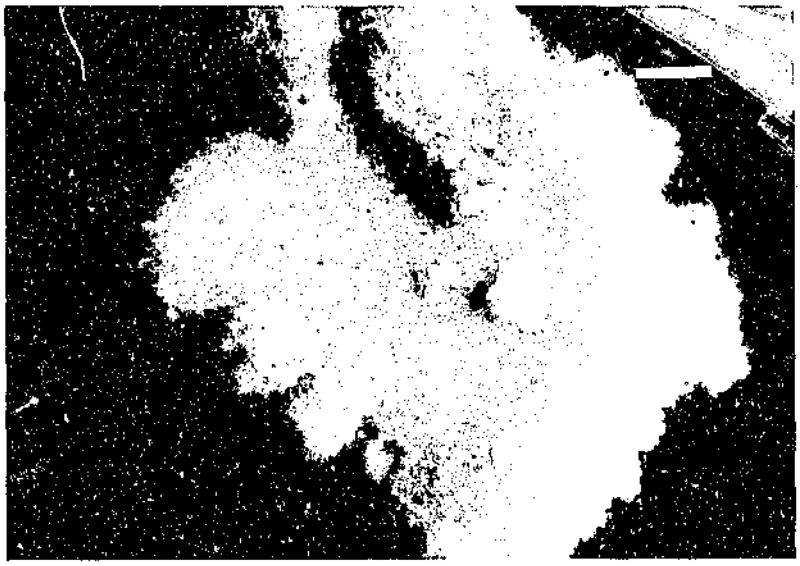


Plate 3.1.3.1: Tuber disc after 2 weeks on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP. The bar represents 1.8 mm.

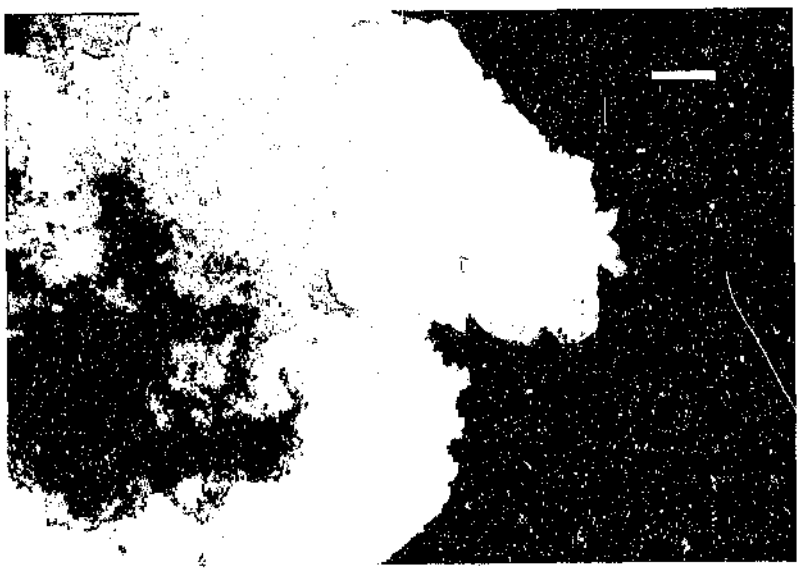


Plate 3.1.3.2: Tuber disc after 3 weeks on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP The bar represents 1.3 mm.

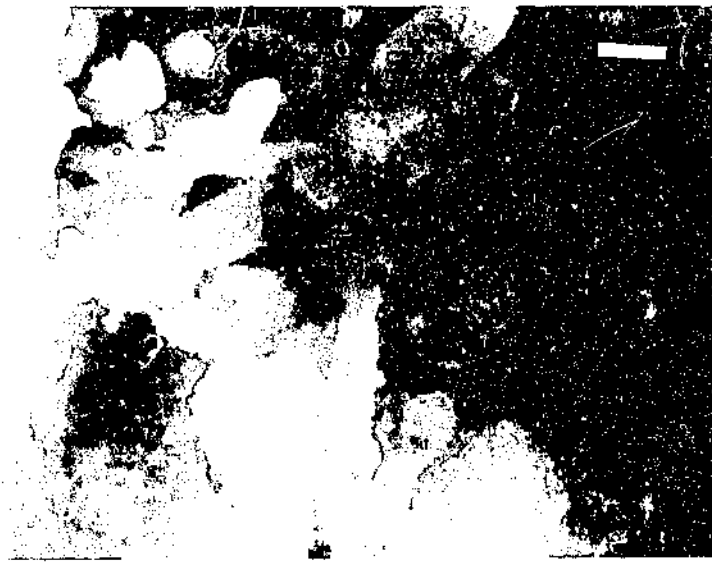


Plate 3.1.3.3: Tuber disc after 4 weeks on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP. The bar represents 1.2 mm.

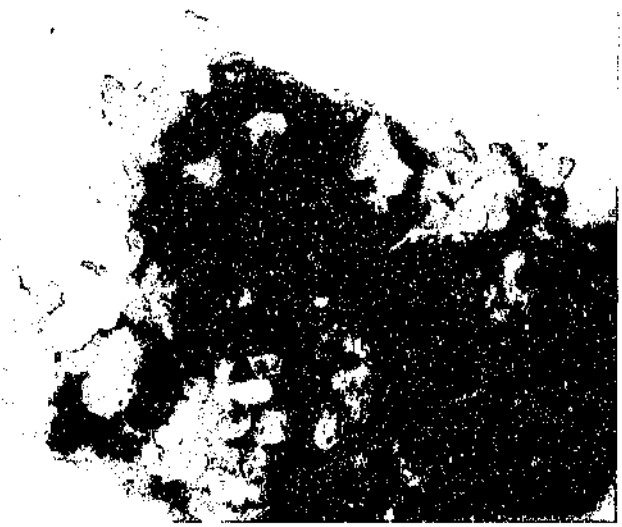


Plate 3.1.3.4: Tuber disc after 4 weeks on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP. The bar represents 2.6 mm.

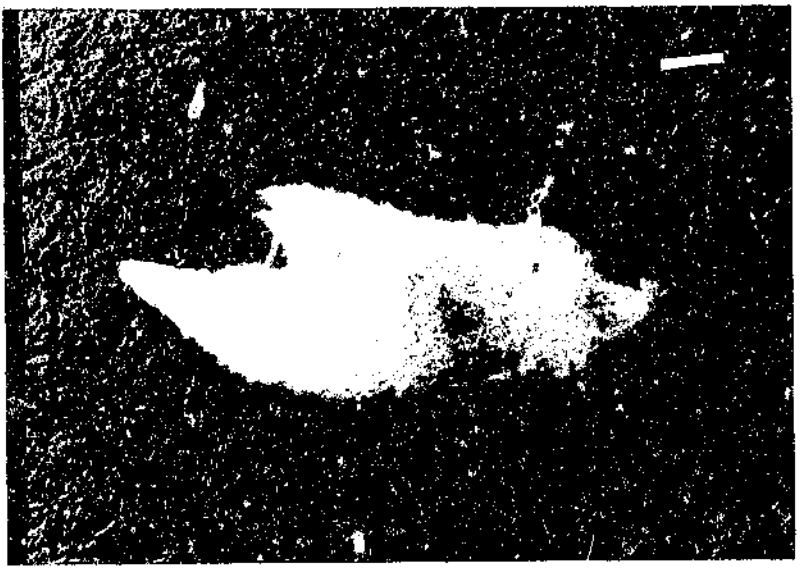


Plate 3.1.3.5: Morphogenic structure generated on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP after 5 weeks. The bar represents 1.8 mm.

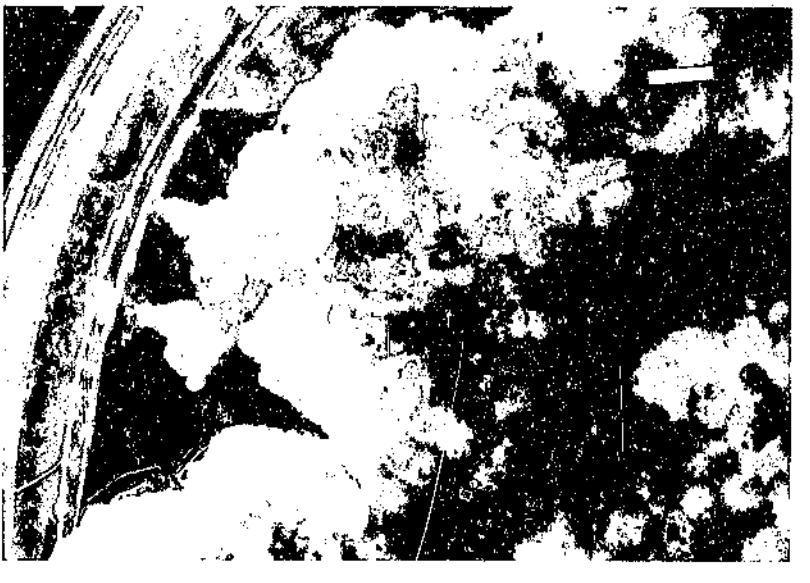


Plate 3.1.3.6: Tuber disc after 8 weeks on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP. The bar represents 2.6 mm.

Plate 3.1.3.7: Tuber disc after 12 weeks on 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP. The bar represents 2.4 mm.

3.2 Subsequent stages of morphogenesis

The exposure of 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP generated structures to NAA, BAP and ABA mixtures yielded no positive developments. Transfer after 4 and 6 weeks on 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP made no difference since the same sequence of events were subsequently observed only with a respective delay. Transfer after 2 weeks on 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP yielded no developments at all since structures were initially underdeveloped.

Transfer of 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP generated structures to ABA resulted in secondary morphogenesis, through a bright green callus, which could be observed as small white structures after 3 weeks (Plate 3.2.1). These structures differed from those observed during primary morphogenesis and over the next 5 weeks they developed into roots with clear root hair development (Plate 3.2.2). This process was most pronounced with 2 mg.l^{-1} ABA (Table 3.2).

2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP generated structures only survived on 2 mg.l^{-1} NAA and 2 mg.l^{-1} NAA + 0.2 mg.l^{-1} BAP (Table 3.2). The structures underwent secondary morphogenesis, through a yellow cream callus, to form structures which resembled those observed with primary morphogenesis to some degree (Plate 3.2.3). After a further 5 weeks some of these structures had developed into roots with root hairs while the rest remained dormant after reaching 20 mm. On 2 mg.l^{-1} NAA + 0.2 mg.l^{-1} BAP, only about 10 % of the structures formed roots while the rest remained dormant (Plate 3.2.4). Stem formation could not be induced with any of this callus using low levels of BAP and exposure to high BAP or TDZ levels resulted in the death of the tissue (Plate 3.2.5).

The 20 mm stem like structures which were transferred did not grow on NAA but underwent minor secondary morphogenesis development. On BAP these structures grew slightly but did not form roots or leaves (Plate 3.2.6). Some minor leaf formations were observed in a small number (1 %) of these structures though it was greatly irregular.

Subsequent studies focused on reducing the exposure of tuber to 2,4-D and avoiding ABA which were thought to be the reasons for subsequent root development. Short exposure of tuber for 1 week on 2,4-D was not effective in inducing morphogenesis by itself but by prolonging exposure while actively decreasing 2,4-D concentration this problem was overcome. The increased levels of cytokinin used at the same time did not have the desired effect of pushing stem like formations to increased growth and pronounced leaf development. The cytokinin TDZ was too potent and resulted in the death of all tissue. Subsequent changes therefore focused on decreasing 2,4-D as quickly as possible whilst still getting sufficient morphogenesis, concentrations from 2 mg.l^{-1} after 1 week to as low as 0.2 mg.l^{-1} after 3 weeks were used. At the same time, BAP concentrations were increased to as high as 2 mg.l^{-1} after three weeks. These conditions were still being optimised at the end of this research project.

Transfer	Concentration ($mg.l^{-1}$)	Development of morphogenic structures
ABA	0.5	2nd morphogenesis through green callus, some root development from initial morphogenic structures
ABA	1.0	2nd morphogenesis through bright green callus, some root development
ABA	2.0	2nd morphogenesis through bright green callus, massive root development
NAA	0.5	2nd morphogenesis initialised through crystalline callus but death in 2 weeks
	1.0	2nd morphogenesis through opaque cream callus with tissue death in 3 weeks
	2.0	2nd morphogenesis through callus, callus remains pale yellow with 60% death and predominant root formation from morphogenic structures
NAA, BAP	2.0, 0.2	2nd morphogenesis through pale yellow callus with 70 % death, morphogenic structures seem less proned to root development
Plain MS		death of all tissue in 1 week

Table 3.2: Transfer of 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP generated morphogenic structures to new media for secondary stages of development observed over 8 weeks. Hormones were included in growth media.

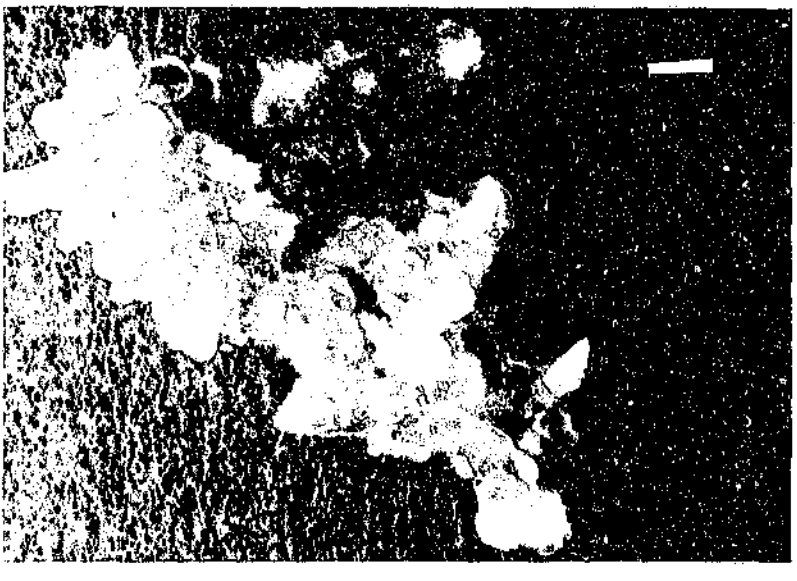


Plate 3.2.1: The development of 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP induced structures on 2 $mg.l^{-1}$ ABA after 3 weeks. The bar represents 2.4 mm.

Plate 3.2.2: The development of 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP induced structures on 2 mg.l^{-1} ABA at 3, 6 and 8 weeks from left to right. The bar represents 4.8 mm.

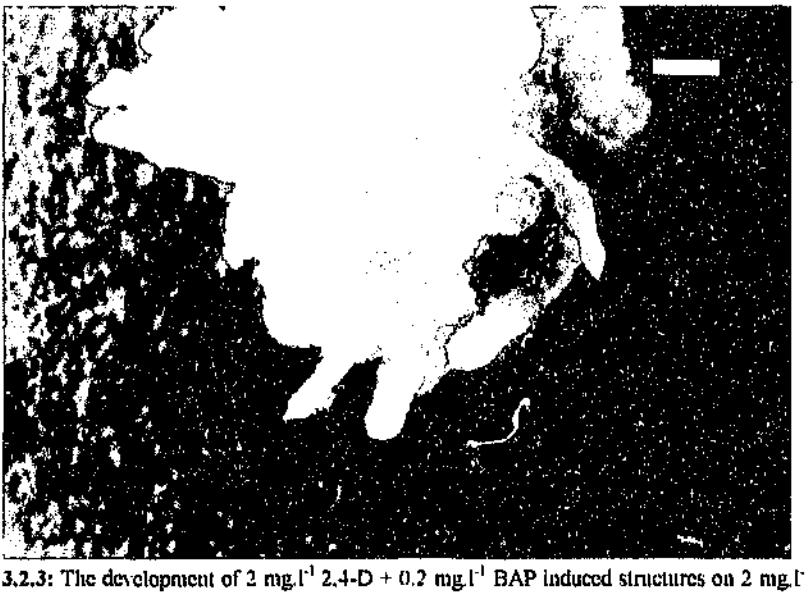


Plate 3.2.3: The development of 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP induced structures on 2 mg.l^{-1} NAA + 0.2 mg.l^{-1} BAP after 3 weeks. The bar represents 1.4 mm.

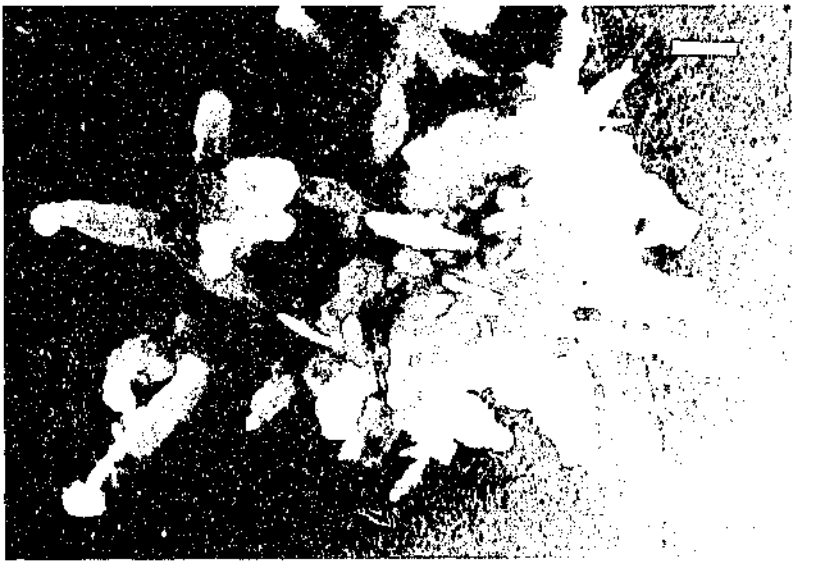


Plate 3.2.4: The development of 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP induced structures on 2 mg.l^{-1} NAA + 0.2 mg.l^{-1} BAP after 8 weeks. The bar represents 3.8 mm.

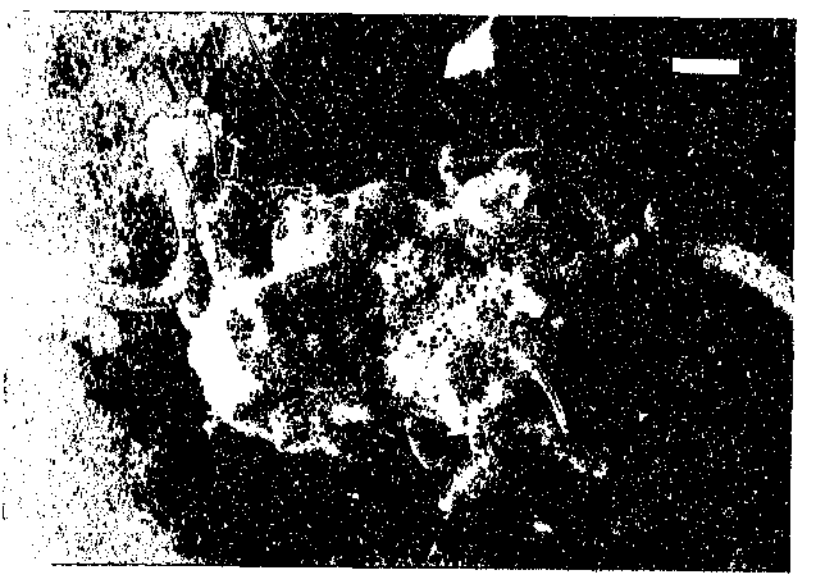


Plate 3.2.5: Effect of 1 mg.l^{-1} TDZ on callus with morphogenic structures. The bar represents 3.2 mm.

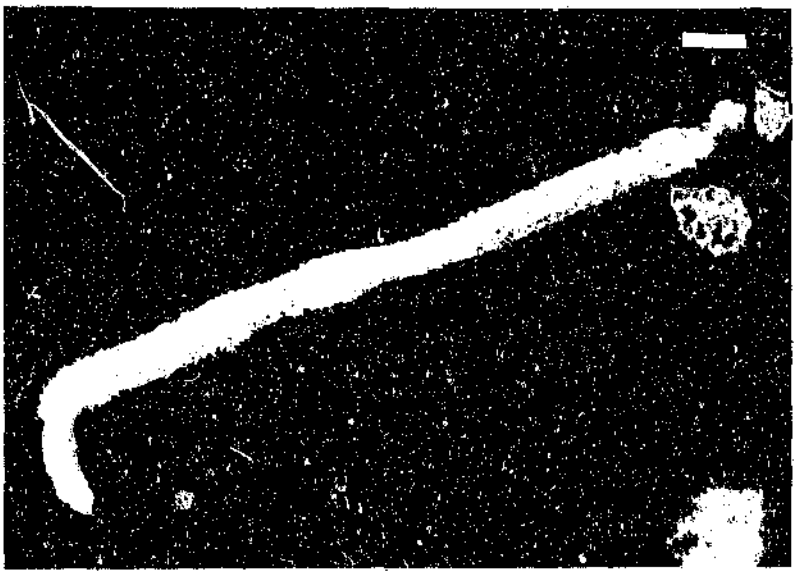


Plate 3.2.6: The development of a 2 $mg.l^{-1}$ 2,4-D + 0.2 mg.l BAP induced structure on 0.2 $mg.l^{-1}$ BAP after 6 weeks. The bar represents 7.2 mm.

3.3 *Agrobacterium tumefaciens* mediated sweet potato transformation

The growth curves of the LBA4404 which had undergone uptake of pCAS1, pBISN1 and pE1120 were all determined under selective pressure with rifampicin and kanamycin (Figures 3.3.1-3.3.3). The LBA4404 with pCAS1 showed the best log growth where mid log phase was at A_{600} = 0.5 which occurred after 20 hours of growth (Figure 3.3.1). The other two had mid log phases at A_{600} = 0.4 which was after 20 hour of growth (Figures 3.3.2, 3.3.3) Transformed *A. tumefaciens* was selected by kanamycin and rifampicin pressurisation on agar plates and these selected strains were tested for GUS expression with x-gluc. *A. tumefaciens* containing pCAS1 and pBISN1 gave strong responses to the GUS assay (Plates 3.3.1, 3.3.2), but pE1120 showed a greatly reduced response to the GUS assay. Selected transformed strains were grown up under selective pressure to mid log phase for sweet potato transformations.

The incubation time of tuber with *A. tumefaciens* was varied and subsequent survival was monitored. The removal of the *A. tumefaciens* selection media was necessary prior to incubation with tissue otherwise the tuber tissue died within 2 weeks. The media was therefore removed and *A. tumefaciens* was resuspended in MS, sucrose and acetosyringone. This resulted in the survival of tuber tissue after transformation. Removal of the *A. tumefaciens* after transformation was achieved with a minimum of 300 $mg.l^{-1}$ cefotaxime with 20 $mg.l^{-1}$ benlate, 100 $mg.l^{-1}$ carbenicillin with 20 $mg.l^{-1}$ benlate or 300 $mg.l^{-1}$ carbenicillin. These compounds did not affect the development of the tissue as determined by controls which were also exposed to these compounds.

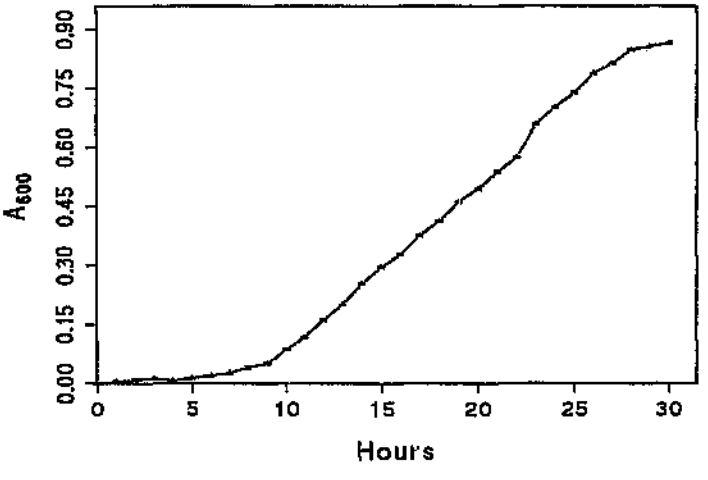


Figure 3.3.1: Graph of growth of LBA4404 transformed with pCAS1, as absorbance at λ = 600 nm versus time in hours after the initiation of culture at 19 °C in YM media with 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin. Vertical error bars represent the standard deviation of the average of 3 points.

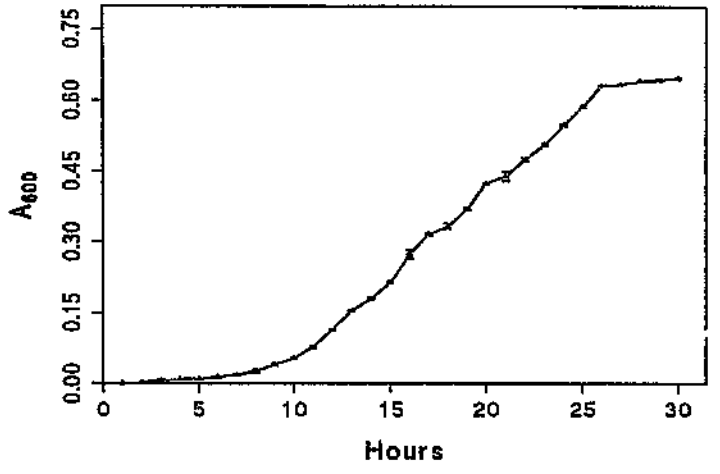


Figure 3.3.2: Graph of growth of LBA4404 transformed with pBISN1, as absorbance at λ = 600 nm versus time in hours after the initiation of culture at 19 °C in YM media with 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin. Vertical error bars represent the standard deviation of the average of 3 points.

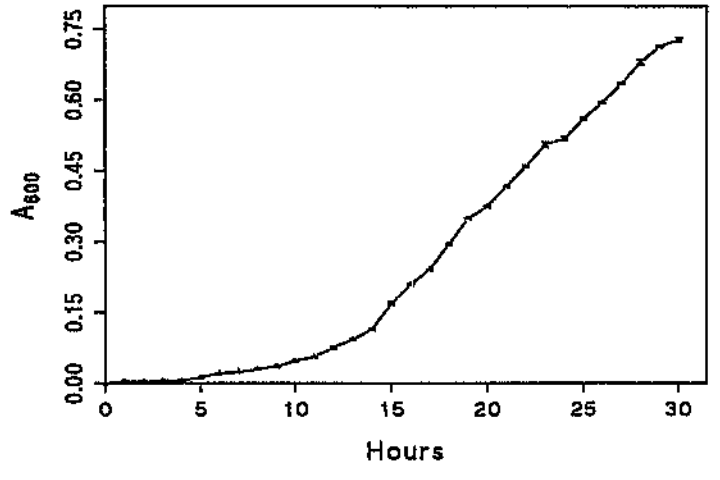


Figure 3.3.3: Graph of growth of LBA4404 transformed with pE1120, as absorbance at λ = 600 nm versus time in hours after the initiation of culture at 19 °C in YM media with 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin. Vertical error bars represent the standard deviation of the average of 3 points.

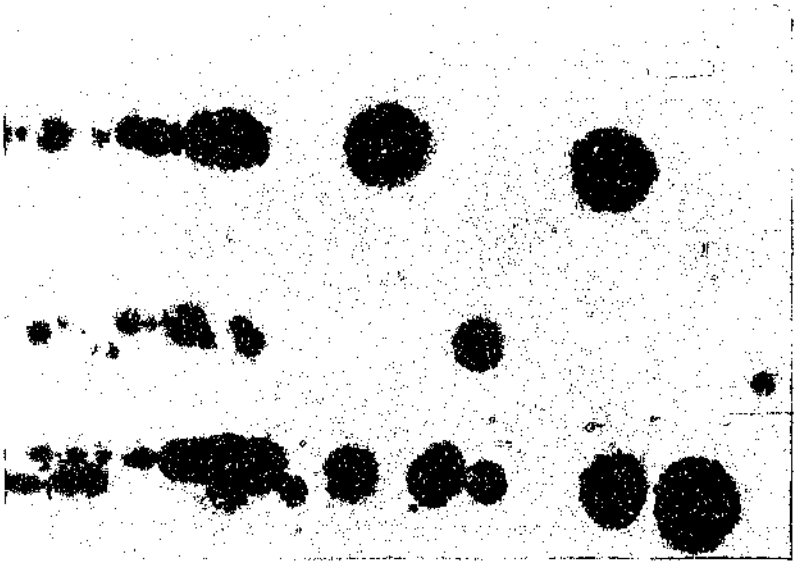


Plate 3.3.1: Colonies of LBA4404 transformed with pCAS1, grown on 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin, which have been screened for GUS activity with x-gluc. The bar represents 3.0 mm.

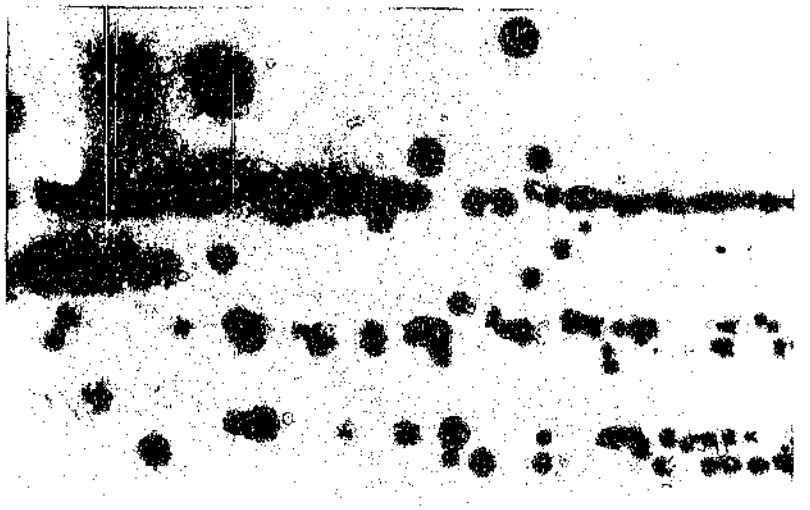


Plate 3.3.2: Colonies of LBA4404 transformed with pBISN1, grown on 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin, which have been screened for GUS activity with x-gluc. The bar represents 3.4 mm.

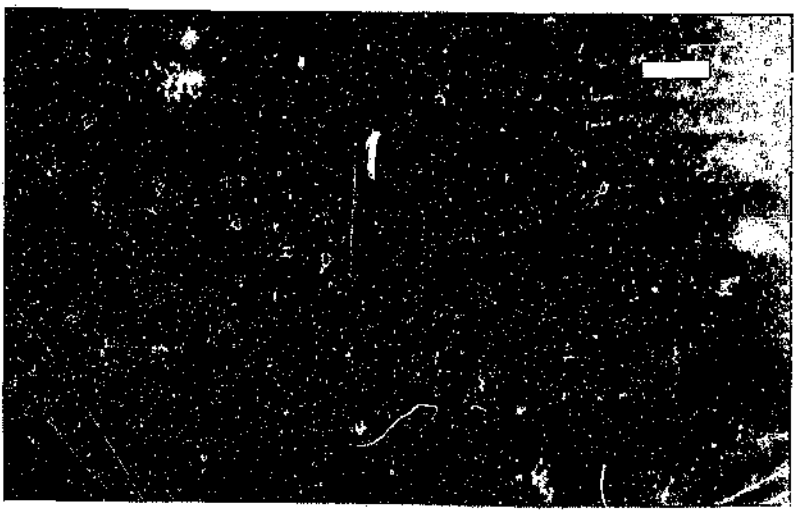


Plate 3.3.3: Colonies of LBA4404 transformed with pE1120, grown on 50 $\mu g.ml^{-1}$ rifampicin and 100 $\mu g.ml^{-1}$ kanamycin, which have been screened for GUS activity with x-gluc. The bar represents 4.2 mm.

3.4 Antibiotic and herbicide resistance screening for transformation

None of the kanamycin concentrations tested killed sweet potato tissue but there was an increasing retardation of growth and callus formation (Table 3.4.1). Tuber tissue transformed with pE1120 was screened with 100 $mg.l^{-1}$ kanamycin. After 4 weeks it was evident that the transformed tissue had obtained resistance as compared to the non transformed control (Plate 3.4.1). Screening pE1120 transformed tuber tissue with 250 $mg.l^{-1}$ kanamycin, the resistance of transformed tissue as compared to non transformed controls was even more evident (Plate 3.4.2). Screening transformed material with kanamycin reduced its development as compared to transformed non screened material

The gluphosinate concentrations used were effective against both leaves and tubers even at low levels, although tubers showed an enhanced resistance to gluphosinate (Table 3.4.2). A concentration of 50 $mg.l^{-1}$ gluphosinate was used to screen for

transformation in callus and tubers transformed with pCAS1. A definite gluphosinate resistance was observed in the transformed tissues screened as compared to the non transformed controls which died completely (plates 3.4.3, 3.4.4). Resistances to both kanamycin and gluphosinate (PPT) were evident for the 12 weeks screened. Screening of transformed material with gluphosinate reduced its development as compared to transformed non screened material. Resistances was evident for the 12 weeks observed.

Explant	[Kanamycin] ($mg.l^{-1}$)	Appearance
Leaves	0	100 % explants green with callus formation
	50	100 % explants green with callus formation
	100	100 % explants green with reduced callus formation
	150	100 % explants green with no callus formation
	200	100 % explants green with no callus formation
	250	100 % explants yellow brown with no callus formation
Tubers	0	100 % explants cream with white friable callus
	50	100 % explants smaller with some callus
	100	100 % explants even smaller with limited callus
	150	100 % explants cream, no growth and no callus
	200	100 % explants yellow with no callus
	250	14 % explants brown

Table 3.4.1: Susceptibility of non transformed blesbok leaves and root tuber to varying kanamycin concentrations after 4 weeks. Kanamycin was included in growth media + 0.2 mg.l-1 2,4-D.

Explant	[Gluphosinate] ($mg.l^{-1}$)	Appearance
Leaves	0	100 % explants green with some callus
	10	100 % explants clear green with no callus
	20	100 % explants clear with no callus
	30	100 % explants brown with no callus
	50	100 % explants brown with no callus
Tubers	0	100 % explants cream with callus
	10	90 % explants partially browned with some callus
	20	90 % of explants browned with no callus
	30	100 % of explants browned
	40	100 % explants brown
	50	100 % explants brown

Table 3.4.2: Susceptibility of non transformed blesbok leaves and root tuber to varying gluphosinate concentrations after 4 weeks. Gluphosinate was included in growth media + 0.2 mg.l-1 2,4-D.

Plate 3.4.1: Comparison of tissue transformed with pE1120 on the left and the non transformed control after 4 weeks on 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP with 100 mg.l^{-1} kanamycin. The bar represents 2.7 mm.

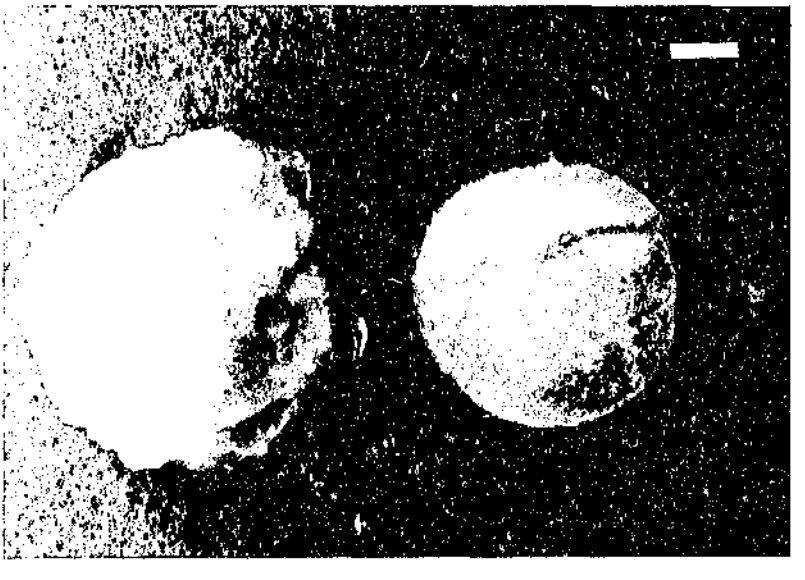


Plate 3.4.2: Comparison of tissue transformed with pE1120 on the left and the non transformed control after 3 weeks on 2 mg.l^{-1} 2,4-D + 0.2 mg.l^{-1} BAP with 250 mg.l^{-1} kanamycin. The bar represents 3.2 mm.

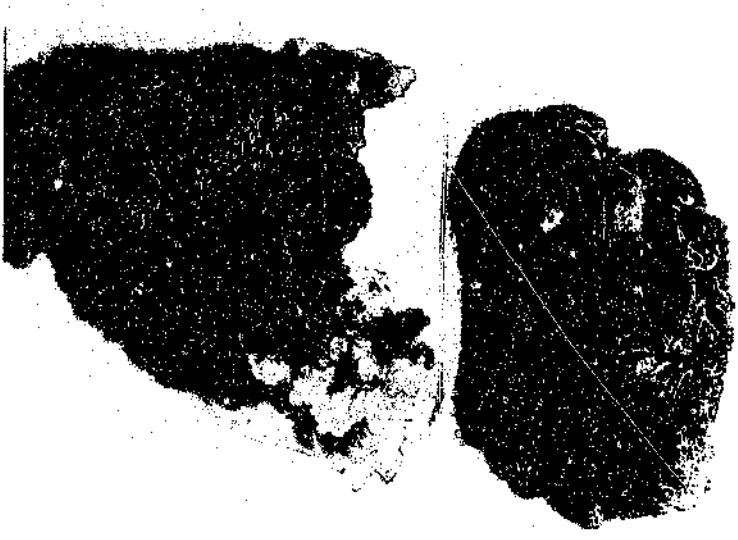


Plate 3.4.3: Comparison of tissue transformed with pCAS1 on the left and the non transformed control after 3 weeks on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP with 50 $mg.l^{-1}$ gluphosinate. The bar represents 2.9 mm.

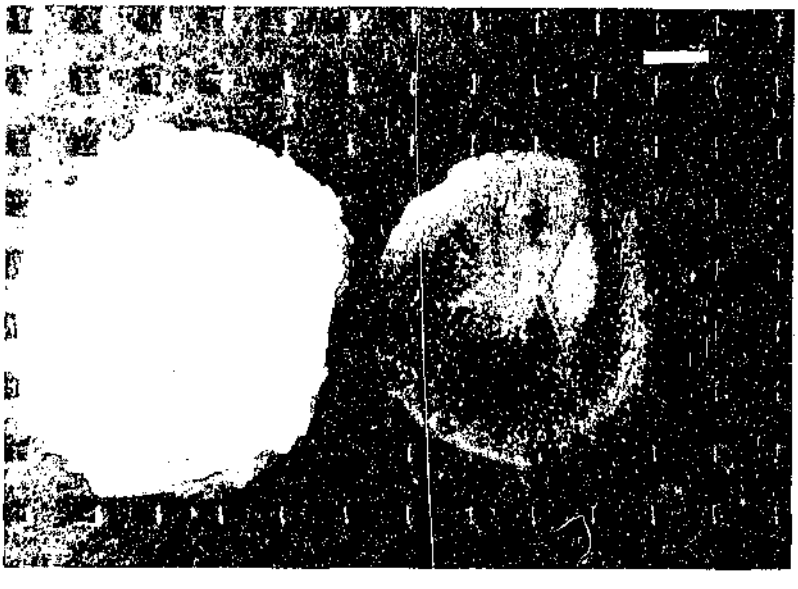


Plate 3.4.4: Comparison of tissue transformed with pCAS1 on the left and the non transformed control after 4 weeks on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP with 50 $mg.l^{-1}$ gluphosinate. The bar represents 3.1 mm.

3.5 Histochemical analysis of transformation

Histochemical analysis was carried out using a variety of substrates whose cleaved products fluoresced. ImaGene Green™, whose cleaved product fluoresces in the λ = 500-550 nm green range, was not useful because of the very high levels of λ = 500-550 nm green autofluoresence which sweet potato tuber exhibits, especially at λ = 500-515 nm (Plate 3.5.1). As a result the green fluorescent substrate could not be detected even with quenching agents and narrow range filters.

MUG was also used as a substrate. This gave positive results but levels of non specific background were observed as the 4-methylumbelliferyl (MU) product was detected in the λ = 515-560 nm range (Plate 3.5.2), where some autofluoresence occurred (Plate 3.5.1). MU has a peak emission at λ = 455 nm so it is not useful with the confocal microscope which can only detect from λ = 500 nm. Furthermore, the MU product does not allow for histochemical study since there is a lack of localisation of the dye, instead the product is spread throughout the tissue, and cellular detail is not visible.

ImaGene Red™ was the most useful substrate. Its cleaved product fluoresced at λ = 650-700 nm where there was minor λ = 650-700 nm autofluoresence(Plate 3.5.1). By shifting the detection λ to 568 nm this background was removed, and the difference in fluorescence between transformed and non transformed could be clearly seen (Plate 3.5.3). The benefit of the dye was that it could be clearly viewed in the membrane of cells which had GUS activity. This localisation was not always clearly visible in large clusters of cells where the mass fluorescence tended to blur detail to some degree (Plate 3.5.3), but it was clearly visible when individual cells where focused on (Plate 3.5.4).

Transformation of cells with GUS from pCAS1, pBISN1 and pE1120 was confirmed using the ImaGene Red™ substrate. These reactions were carried out at between 4 and 10 weeks after transformation to avoid detection of transient expression.

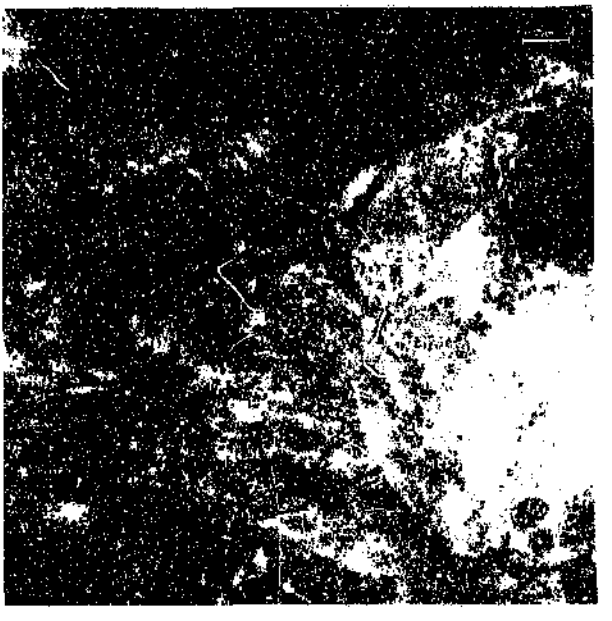
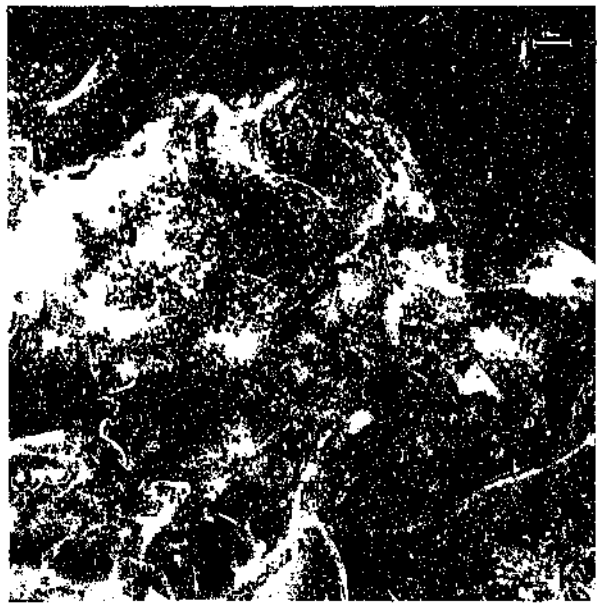


Plate 3.5.1: The autofluoresence of tuber tissue. Predominant fluorescence is in the green 500-550 nm range (coloured green), predominantly between 500-515 nm, while there is some in the red 650-700 nm range (coloured red).

a.



b.

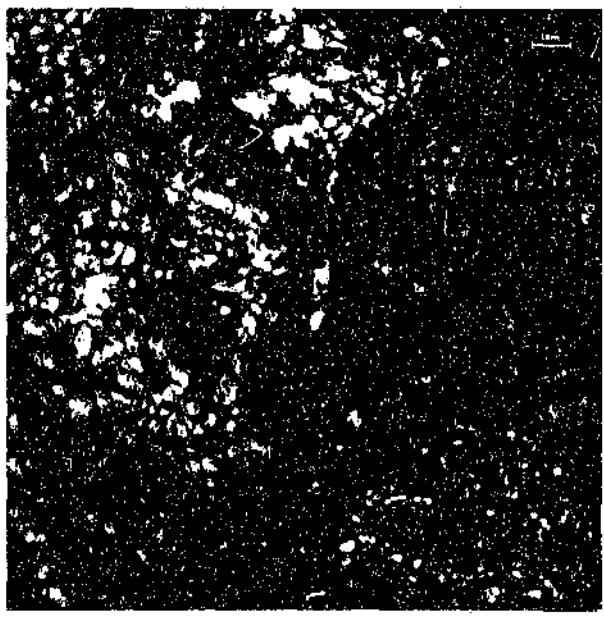
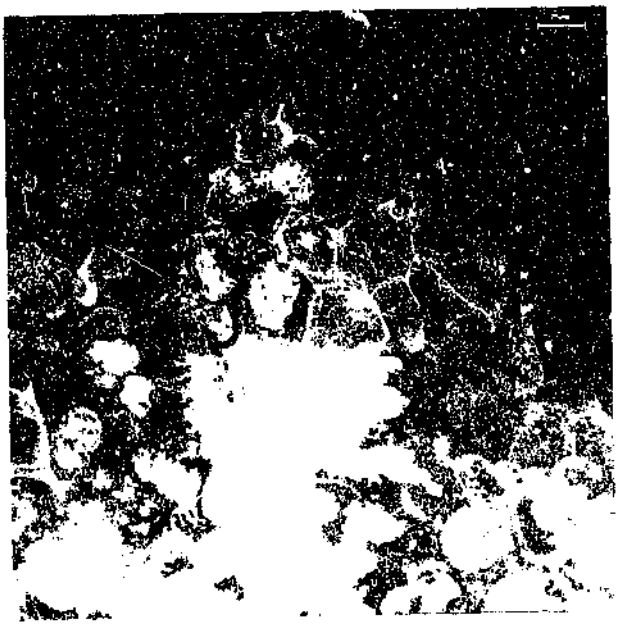


Plate 3.5.2: The 515-560 nm fluorescence of **a.** pCAS1 transformed and **b.** non transformed control tuber tissue after 4 weeks, after incubation with MUG substrate, showing a large section of tissue.

a.



b.

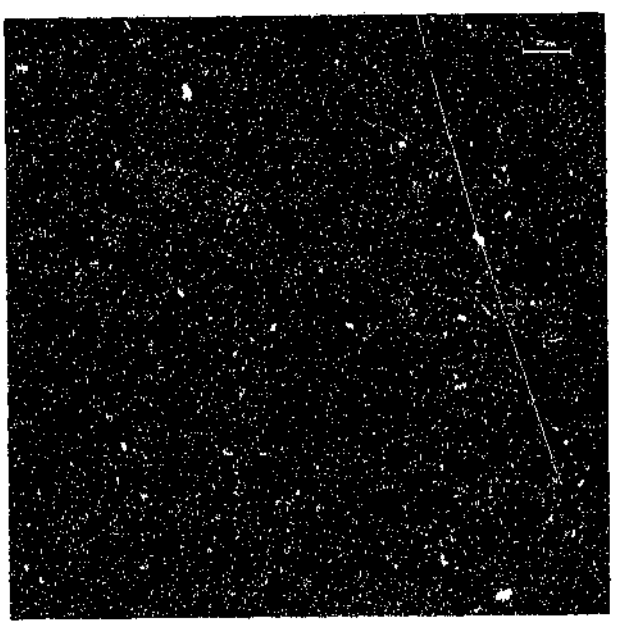
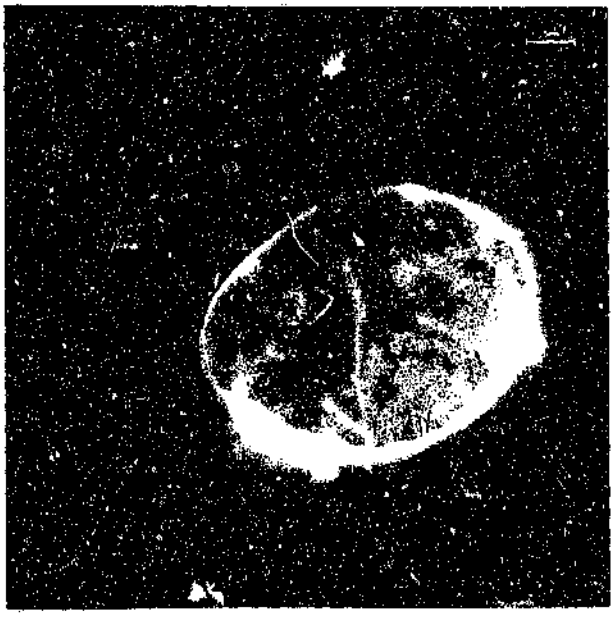


Plate 3.5.3: The 568 nm fluorescence of a. pBISN1 transformed and b. non transformed control tuber tissue after 6 weeks, after incubation with ImaGene Red™ substrate, showing a large section of tissue.

a.



b.

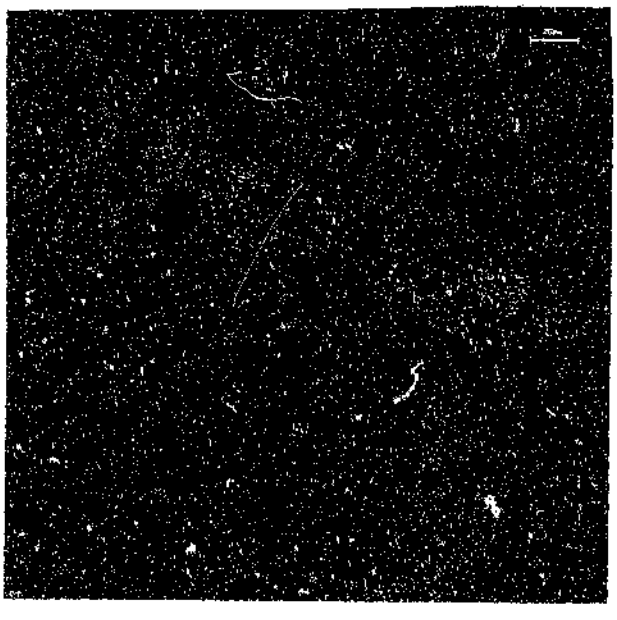


Plate 3.5.4: The 568 nm fluorescence of a. pBISN1 transformed and b. non transformed control tuber tissue after 6 weeks, after incubation with ImaGene Red™ substrate, showing individual cell detail

3.6 Quantitative analysis of transformation

The GUS activities of transformed tissues were measured in two random sections of each tissue. The GUS activities of non transformed control tissue were similarly measured. These measurements were carried out at least 4 weeks after transformation to avoid the detection of transient expression. There were relatively high levels of fluorescence in control tissue, on average giving a theoretical GUS activity of about 0.50 nmole.min^{-1}.mg^{-1}protein. This theoretical GUS activity is the result of sweet potato tissue autofluoresence (Plate 3.5.1) at 460 ± 15 nm where MU fluorescence was measured. The control activity was therefore subtracted from the activity of the transformed tissue for each measurement.

The values calculated were averaged for each section of tissue (Table 3.6) but the standard deviation of these points was high because of the random nature of tissue used. The levels calculated were variable and gave no clear indication as to whether longer transformation times gave higher levels of transformation and therefore higher GUS activity. The 24 hour transformations appeared to give consistently higher GUS activity though. GUS activity was detected in all transformed tissue assayed which included tuber and tuber callus tissues transformed with pCAS1, pBISN1 and pE1120 (Table 3.6). In the 20 assays carried out, with non transformed control activities subtracted for each assay, there was an average GUS activity of 2.82 nmole.min^{-1}.mg^{-1} protein (Table 3.6). Transformation time definitely affected survival of tissue, only callus was able to survive the longer incubation periods. Shorter incubation periods with *A. tumefaciens* were more effective as they resulted in higher tissue survival whilst still getting GUS activity in the tissue

Vector	Tissue transformed	Time transformed (hours)	Tissue survival	GUS assay (weeks)	GUS activity (nmole.min^{-1}. mg^{-1} protein)(n=2)
pE1120	tuber	1	90 %	7	2.40 ± 2.64
pBISN1	tuber	2	90 %	7	3.04 ± 0.45
pE1120	tuber	2	90 %	4	2.13 ± 0.35
pBISN1	tuber	4	80 %	5	0.85 ± 0.66
pCAS1	tuber	6	80 %	4	1.63 ± 0.13
pE1120	tuber	12	50 %	6	2.70 ± 3.13
pBISN1	tuber	24	10 %	4	5.90 ± 4.74
pCAS1	callus	24	30 %	6	4.05 ± 1.32
pBISN1	callus	24	30 %	6	1.87 ± 0.23
pBISN1	tuber	24	10 %	5	3.62 ± 0.40

Table 3.6: Various GUS activities and survival rates of sweet potato tuber tissue transformed for various lengths of time with various vectors using *A. tumefaciens*.

3.7 Genetic analysis of transformation

The PCR reaction carried out initially yielded several fragments of DNA including a fragment whose size was calculated to be 950 b.p. This fragment was evident only in the transformed tissue and was assumed to be the 900 b.p. fragment including the GUS intron (Plate 3.7.1) The fragment which was being preferentially amplified was present in the transformed and non transformed tissue and was calculated to be 1200 b.p. big. By raising the annealing temperature of the PCR reaction to 70 °C all amplification was inhibited (Plate 3 7.1). An increase in annealing temperature from 66 °C to 68 °C inhibited most non specific amplification reactions and resulted in the preferential amplification of the GUS intron fragment (Plate 3.7.2). At the same time results were optimised with a drop in template DNA to 10 ng and a drop in $MgCl_2$ concentration to 1 mM By halving the primer concentration to 0.25 µM the remaining non specific reactions were primarily inhibited (Plate 3.7.3). The PCR reactions therefore showed that elements of *uidA* from pBISN1 were definitely present in isolated plant DNA between 5 and 14 weeks after transformation as compared to non transformed controls which did not show the presence of *uidA* elements.

a. b.

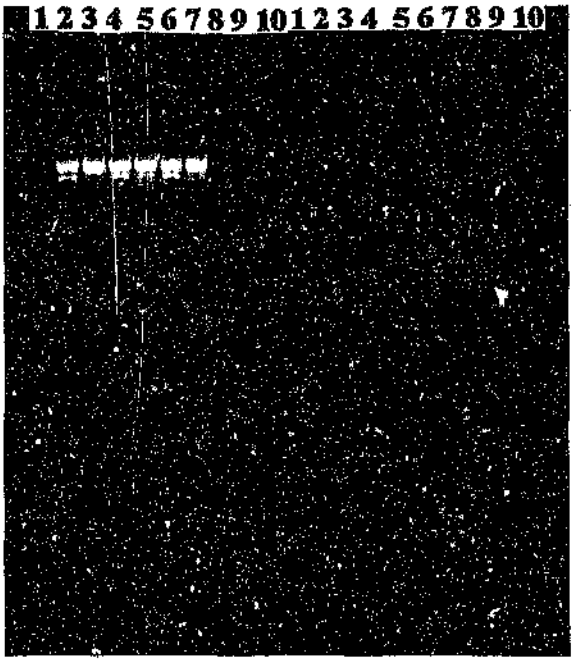


Plate 3.7.1: A 2 % agarose gel showing PCR reactions carried out at **a.** 64 °C and **b.** 70 °C respectively. The lanes are loaded as follows **1.** PCR marker (1000, 750, 500, 300, 150 and 50 b.p.). **2.** pBISN1 transformed callus after 6 weeks. **3.** Control for 2. **4.** pBISN1 transformed after 5 weeks. **5.** Control for 4. **6.** pBISN1 transformed after 14 weeks. **7.** Control for 7. **8.** Left primer only control **9.** Right primer only control **10.** Template free control

a. b.

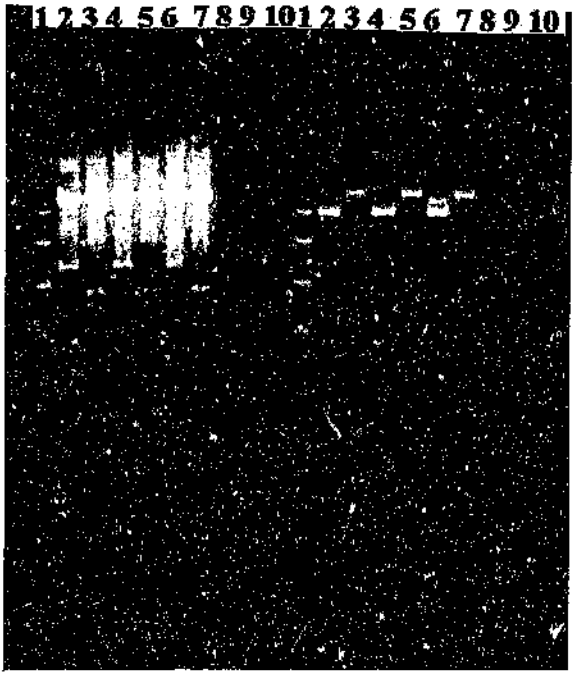


Plate 3.7.2: A 2 % agarose gel showing PCR reactions carried out at **a.** 66 °C and **b.** 68 °C respectively. The lanes are loaded as follows **1.** PCR marker (1000, 750, 500, 300, 150 and 50 b.p.). **2.** pBISN1 transformed callus after 6 weeks. **3.** Control for 2. **4.** pBISN1 transformed after 5 weeks. **5.** Control for 4. **6.** pBISN1 transformed after 14 weeks. **7.** Control for 7. **8.** Left primer only control **9.** Right primer only control **10.** Template free control.

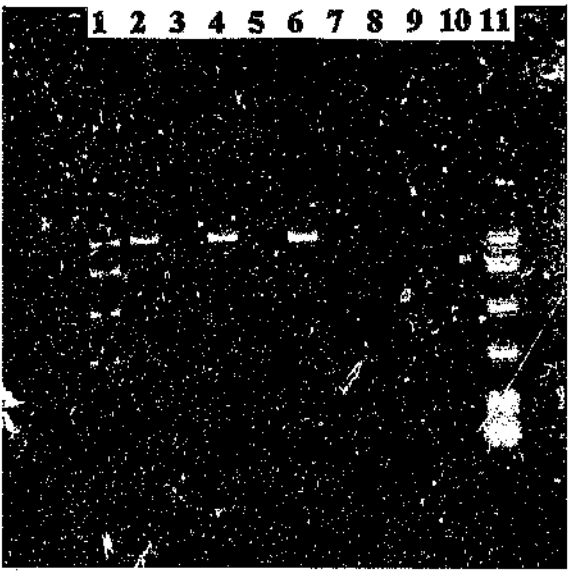


Plate 3.7.3: A 2 % agarose gels showing PCR reactions carried out at 68 °C. The lanes are loaded as follows **1.** PCR marker (1000, 750, 500, 300, 150 and 50 b.p.). **2.** pBISN1 transformed callus after 6 weeks. **3.** Control for 2. **4.** pBISN1 transformed after 5 weeks. **5.** Control for 4. **6.** pBISN1 transformed after 14 weeks. **7.** Control for 7. **8.** Left primer only control **9.** Right primer only control **10.** Template free control

3.8 Regeneration of transformed material

Tissues were compromised in their ability to regenerate by transformation as compared to non transformed controls (Plate 3.8.1). Similarly callus formed prior to secondary morphogenesis was compromised in its ability to undergo secondary morphogenesis by transformation as compared to non transformed controls. These controls underwent the same treatment as transformed tissue besides the *A. tumefaciens* exposure.

The *A. tumefaciens* growth and selection media resulted in death of both transformed and non transformed tissue and therefore had to be removed. Cefotaxime, carbenicillin and benlate at the concentrations used did not affect tissue, as judged by comparison of transformed and non transformed tissue exposed to these compounds and transformed and non transformed control tissue not exposed to these compounds. Gluphosinate and kanamycin did inhibit growth of transformed tissue as compared to transformed controls not exposed to these compounds and so was not used in regeneration experiments (Section 3.4).

Ultimately with 2 weeks of culture prior to a 2 hour transformations and 1 week on antifungal and antibiotic agents to remove infections, followed by culture in media free of antibiotics and herbicides, transformed material was able to develop the same structures as the controls on 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP, only in reduced numbers (Plate 3.8.2). This was achieved with pBISN1 and pE1120 transformed tissue.

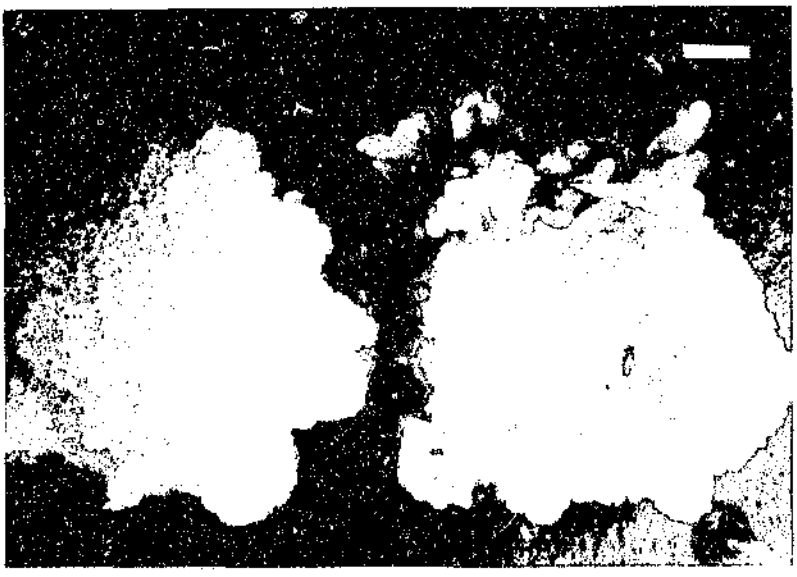


Plate 3.8.1: Comparison of material transformed with pF1'20 on the left and the non transformed control. The bar represents 2.8 mm.

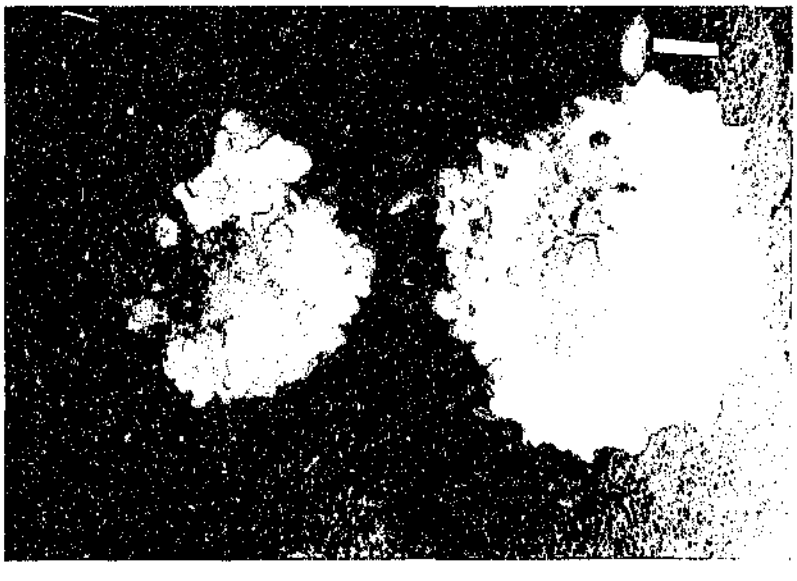


Plate 3.8.2: *Comparison of material transformed with pBISN1 on the left and the non transformed* control. The bar represents 2.0 mm.

4 Discussion

Since leaves, together with apical meristems, have been the most successful tissue source in regeneration experiments carried out on sweet potato(Liu and Cantliffe, 1984; Chee and Cantliffe, 1988; Bieniek *et al.*, 1995), initial induction of regeneration was carried out with them to obtain a frame of reference for tuber studies. The morphogenesis observed with leaves exposed to 2,4-D corresponded to available literature both in the indirect type embryogenesis observed and in the optimal 2.2 $mg.l^{-1}$ range 2,4-D concentration required.(Chee and Cantliffe, 1984) (Section 3.1.1). No further studies were carried out however so no definite conclusions can be made, especially since the cultivar used, blesbok, has never been regenerated before.

The focus of this project was the transformation and regeneration of sweet potato tuber. Tubers were chosen as a novel source which are easily available, providing large amounts of tissue and having a high degree of genetic stability (George, 1993; George, 1996). Tuber tissue responded most positively to 2,4-D concentrations in the 2.2 $mg.l^{-1}$ range as has been shown to be optimal for somatic embryogenesis(Section 3.1.1, 3.1.3). Morphogenic structures arose directly from very specific points within the tissue though i.e. direct morphogenesis. Furthermore, these structures did not seem to resemble any embryogenic stage and were much larger than usual (Section 3.1.3). So although induction conditions were similar to those required for embryogenesis, some other type of morphogenesis was observed for tubers as compared to leaves.

Subsequent optimisation of this induction was based only on the levels and size of morphogenic structures (Section 3.1.3). Observed callus colour and structure was not a reproducible technique for evaluating the effectiveness of morphogenic induction, although a yellow green embryogenic type callus was usually associated with the

morphogenic structures. The auxin 2,4-D was shown to be more effective than auxins NAA and IAA (Section 3.1.1) which was expected since 2,4-D is more potent and is therefore the choice for embryogenesis induction. The cytokinin BAP did enhance morphogenesis further and also resulted in an increased proportion of embryogenic type callus, largely due to a reduction in friable callus (Section 3.1.2, 3.1.3). Once again the optimised BAP concentration of 0.22 $mg.l^{-1}$ was in correspondence with published data for the induction of embryogenesis although the morphogenesis observed differed from embryogenesis (Chee and Cantliffe, 1988).

Incubation in the dark appeared less effective than exposure to light (Section 3.1.3). This may well be related to phenomenon of photomorphogenesis where light can induce certain developments through the activation of phytochromes which induce some change in structure or form (George, 1993). The optimum incubation time of 4 weeks was primarily due to the fact 2,4-D caused dedifferentiation after this point (Section 3.1.3). From these studies then the conditions optimised for the morphogenesis observed with sweet potato tuber were exposure to 2 $mg.l^{-1}$ 2,4-D + 0.2 $mg.l^{-1}$ BAP and incubation in the light, for 4 weeks. However, concentrations of 2,4-D as low as 0.2 $mg.l^{-1}$ still resulted in morphogenesis even with incubation in the dark. Although the optimal concentrations were subsequently used it was noted that they may not be the most useful since their long term effects still needed to be determined

The morphogenic structures themselves were not histochemically analysed so no definite conclusions can be made about them which is why they were classified only as being morphogenic structures. What was certain was that they arose in clumps from specific areas within the tissue. These areas may well be the procambium tissue within the tuber since this tissue has a high regenerative capability (George, 1993). The earlier stages did appear to have two areas of organised tissue growth, suggesting bipolar growth characteristic of embryos, joined by a non specialised hypocotyle type area (Section 3.1.3), similar to the structure of germinating plants (Keeton, 1980) The structures did

resemble shoots in structure and colour and the presence of some misformed leaves further backed up this view (Section 3.2). The morphogenesis observed therefore was assumed to be shoot organogenesis. Regeneration of shoots from roots is fairly common, cytokinin is almost always required but auxin can induce this in a very few cases.(George, 1996). With sweet potato the induction of shoots from leaf cultures has been shown to require only 2,4-D to initiate shoot regeneration (Dessai *et al.*, 1995). Shoot organogenesis is a viable method of regenerating tissue and so initial successes were promising. The next emphasis then was to get the shoot like structures to develop fully and then to root.

Secondary morphogenesis occurred with both NAA and ABA (Section 3.2). Since NAA is an auxin it can be expected that renewed exposure to auxin gives rise to another process of morphogenesis. The levels of root formation observed after prolonged incubation signal an over exposure to auxin. This problem was reduced by the inclusion of BAP but decreases in NAA resulted in tissue death as the drop in auxin could not be tolerated. The only way of decreasing root formation may the be to greatly increase cytokinin concentration during this stage.

ABA is often deemed essential for somatic embryo development which has been shown in sweet potato (George, 1993, Zheng *et al.*, 1996). However it has also been shown to inhibit embryogenic callus formation at high concentrations. Its mechanism of action has been proposed to be an active inhibition of cytokinins which then synergistically aids auxins which would explain how it aids induction of somatic embryogenesis (George, 1993). Thus it could actually inhibit shoot formation and push auxin driven root formation. For these studies it is not useful at all.

Secondary morphogenesis could be useful in that it provides a mechanism to bulk up on structures. The highly morphogenic callus produced would be a good source of tissue to transform since its high regenerative ability would mean that a large number of

transformed cells would be regenerated. For this reason some of this tissue was transformed (Section 3.8) but the transformed callus had a greatly reduced ability to form morphogenic structures and since the structures formed had a tendency to form roots anyway this protocol would require optimisation if it is at all possible. The yields with primary morphogenesis were high enough anyway. Furthermore, primary morphogenesis avoids a callus phase. Callus formation involves massive dedifferentiation related to the reprogramming of the genome and this is very often associated with genetic mutations which can cause major physiological or structural deformations or which cause chimerics and so a loss of somoclonal strains (Georg 93). It was decided then to keep the focus on enhancing the development of stem like structures to plants instead of going through stages of secondary morphogenesis.

The emphasis at the end of this study and probably the direction required for future studies was the use of cytokinins to enhance shoot development after the morphogenic induction had been initialised with auxin. Cytokinin concentrations were increased. An optimal cytokinin level between 0.2 mg.l BAP which is not enough and 1 mg.l. TDZ, the most active adenine type cytokinin (Mok *et al.*, 1987), will still have to be determined. At the same time the auxin levels were reduced as low as possible to reduce their apparent long term root inducing effects while still inducing shoot formation. These changes will hopefully stimulate further developments so that leaves develop fully and rooting can take place. The protocols employed do appear to be close to regenerating plants from blesbok tubers though.

The resistance of *A. tumefaciens* to kanamycin after the triparental mating for the uptake of the vectors required, already indicated that conjugative transformation had been successful since *A. tumefaciens* had acquired antibiotic resistance. The GUS assay further confirmed transformation (Section 3.3). The greatly reduced response with pE1120 was ascribed to the absence of the GUS intron which seemed to play a role in induction as opposed to inhibition of GUS activity (Wilson *et al.*, 1992).

The PPT and kanamycin screening was only used to assay for the phenotypic expression of *bar* and *nptII* in this study. It showed conclusively that there was phenotypic expression of *nptII* and *bar* from pE1120 and pCAS1 respectively in the tubers transformed as compared to non transformed controls (Section 3.4). The screening was carried out straight after transformation and therefore did not differentiate against transient expression. The resistances remained for the 12 weeks they were monitored though. Screening was not used to actively select transformed tissue during regeneration since it greatly reduced this process (Section 3.4).

The levels of autofluoresence limited the substrates which could be used for histochemical GUS analysis to those whose products fluoresced in the 550-650 nm λ range where autofluoresence is minimal (Section 3.5). This would also limit the use in sweet potato of marker genes like the green fluorescence protein gene (*gfp*), whose product fluoresces, which is becoming a popular marker for transformation studies. However the optimised studies with ImaGene Red™ were sufficient and useful for monitoring levels of GUS expression in transformed tissues (Section 3.5). The studies confirmed GUS expression from pCAS1, pBISN1 and pE1120 at least 4 weeks and up to 10 weeks after transformation.

There were large variations in the GUS activities measured, due largely to the random nature of transformation coupled with the lack of active selection of transformed tissue with antibiotic and herbicide resistance screening. (Section 3.6). However the assay was actually more just to confirm observations made with histochemical studies and GUS activity from pCAS1, pBISN1 and pE1120 at least 4 weeks and up to 7 weeks after transformation was clearly confirmed (Section 3.6). Furthermore, it showed that with acetosyringone even 2 hour transformations were sufficient in getting stable transformation.

The *uidA* was then itself detected within the plant genome. These tests showed there was integration of at least pBISN1 (Section 3.7). The initial problems with the PCR can be related to non specific reactions and point to the primers not being unique enough. However, they were still specific for the GUS intron fragment and optimisation could be carried out by exploiting this specificity with increases in annealing temperature and decreasing $MgCl_2$, primer and template DNA concentration (Sambrook *et al.*, 1989b). This optimisation inhibited non specific reactions and resulted in the GUS intron fragment being the only clearly observed fragment amplified. This fragment was only detected in transformed tissue as compared to non transformed tissue. The GUS gene from pBISN1 was shown to be integrated in the plant genome between 5-14 weeks after transformation (Section 3.7).

So the long term expression of *npt11* and *bar* (from pE1120 and pCAS1) was confirmed with kanamycin and PPT screening. Long term expression of *uidA* (from pCAS1, pBISN1 and pE1120) was confirmed with ImaGene Red™ and MUG assays. The fact there was a noted long term expression of genes implies strongly there was a stable integrative transformation and expression as opposed to transient transformation and expression. Long term integration of the *uidA* (from pBISN1) in the plant genome was further confirmed with PCR.

No differences in the effectiveness of the different plasmids was noted. Since they all had the same promoters and employed the same binary system this might be expected. The only difference was between pBISN1 and pE1120 in GUS expression in *A. tumefaciens*, the lacking GUS intron in pE1120 seemed to reduce its inducibility. These studies were unique in that they showed stable transformation of a South African cultivar, blesbok, whereas most studies have been done on the jewel and white star.

The transformed material then needed to be regenerated. Since transformation inhibited tissues ability to regenerate (Section 3.8), emphasis was placed on optimisation of regeneration with the following focus:

1. Creating the optimum incubation conditions which was achieved in MS salts. The addition of 40 $\mu g.ml^{-1}$ acetosyringone increased the level of transformation and allowed incubation times to be reduced to 2 hours (Section 3.6).
2. Optimal removal of bacteria which was achieved with a 1 week exposure to 300 $mg.l^{-1}$ carbenicillin, which did not affect tissue development at all (Section 3.3).
3. Reducing long term stress placed on tissue by removing all antibiotics and herbicides (Section 3.4, 3.8).
4. Having a 1-2 week preculture before initiating transformation to allow the tissue to initialise development and undergo initial induction of morphogenesis.

The 2 week preculture prior to transformation seemed the most effective in overcoming the detrimental effects of transformation, possibly allowing for the initial cellular changes required for morphogenesis induction and allowing tuber discs to begin growth. These steps enabled the transformed tuber to be regenerated to the same point as the control (Section 3.8). Since the next stage of plant regeneration had not been optimised (Section 3.2), the process was halted at this point. Future development would probably require focus on further enhancing shoot development by increasing the concentration ratio of cytokinin:auxin as quickly as possible as was being optimised at the end of this project (Section 3.2).

Conclusion

The optimal condition for morphogenesis in blesbok sweet potato tubers, characterised as shoot organogenesis, was found to be exposure to 2 $mg.l^{-1}$ 2,4-D with 0.2 $mg.l^{-1}$ BAP in the light for 4 weeks,. The structures probably require a sharp increase in the cytokinin:auxin concentration ratio after the initial induction to develop further. Blesbok sweet potato tubers were transformed with *npt11*, *uidA* and *bar* from the plasmids pBISN1, pE1120 and pCAS1. Stable, long term phenotypic expression of *bar*, *npt11* and *uidA* was confirmed with PPT screening, kanamycin screening and ImaGene Red™ and MUG assays respectively. Using PCR, long term integration of *uid*A in the plant genome was confirmed. Transformed material was regenerated via shoot organogenesis to the same point achieved for non transformed tissue. This was achieved with exposure to 2 $mg.l^{-1}$ 2,4-D with 0.2 $mg.l^{-1}$ BAP in the light for 2 weeks prior to 2 hour transformation with *A. tumefaciens* in MS + 40 $\mu g.ml^{-1}$ acetosyringone, followed by 1 week under the same conditions + 300 $mg.l^{-1}$ carbenicillin followed by 4 weeks without carbenicillin. Since the next step of plant regeneration was still being optimised this was the final stage achieved in this project.

The protocol developed appears to be close to regenerating transgenic sweet potato plants from transformed blesbok tubers. Transformation uses *A. tumefaciens* mediated transfer of binary vectors and stable integrative transformation has been confirmed with this mechanism. Transformed tissue is regenerated to the point of non transformed material via shoot organogenesis. The stems produced need only to be stimulated to develop further so that leaves form and rooting can take place, which will probably require high cytokinin levels. This work is unique as it used a local cultivar, blesbok whereas most studies used the international cultivars jewel and white star.

6 References

Allard, R.W. (1966). Pure-line breeding and mass selection, Pedigree method of plant breeding, Bulk population breeding, Backcross breeding. pp 109-165. Inheritance in autopolyploids. pp 385-399. In: *Principles of Plant Breeding.* John Wiley and Sons Incorporated. New York.

Al-Juboory, K.H., and Skirvin, R.M. (1991). *In vitro* regeneration of *Agrobacterium* transformed sweet potato (*Ipomoea batatas* L.). *Plant Growth Regulator Society of America* **19(2):** 82-89.

Belarmino, M.M., Abe, T., and Sasahara, T. (1994). Plant regeneration from stem and petiole protoplasts of sweet potato (*Ipomoea batatas)* and its wild relative, *I. lacunosa. Plant cell, Tissue and Organ Culture* **37:** 145-150.

Bieniek, M.E., Harrell, R.C., and Cantliffe, D.J. (1995). Enhancement of somatic embryogenesis of *Ipomoea batatas* in solid cultures and production of mature somatic embryos in liquid cultures for application to a bioreactor production system. *Plant cell, Tissue and Organ Culture* **41:** 1-8.

Bouwkamp, J.C. (1985). *Sweet Potato Products: A Natural Resource For The Tropics.* CRC Press Incorporated. Boca Raton.

Brown, K.S. (1996) Life on the molecular farm. *BioScience* **46(2):** 80-83.

Cavalcante Alves, J.M., Sihachakr, D., Allot, M., Tizroutine, S. ,Mussio, I., Servaes, A., and Ducreux, G. (1994). Isozyme modifications and plant regeneration through somatic embryogenesis in sweet potato (*Ipomoea batatas* (L.) Lam.). *Plant Cell Reports* **13:** 437-441.

Chee, R.P., and Cantliffe, D.J. (1989). Inhibition of somatic embryogenesis in response to 2,3,5-triiodobenzoic acid and 2,4-dichlorophenoxyacetic acid in *Ipomoea batatas* (L.) Lam. cultured *in vitro. Journal of Plant Physiology* **135:** 398-403.

Chee, R.P., and Cantliffe, D.J. (1988). Selective enhancement of *Ipomoea batatas* Poir. embryogenic and non-embryogenic callus growth and production of embryos in liquid culture. *Plant cell, Tissue and Organ Culture* **15:** 149-159.

Chee, R.P., and Cantliffe, D.J. (1988). Somatic embryony patterns and plant regeneration in *Ipomoea batatas* Poir. *In Vitro Cellular and Developmental Biology* **24(9):** 955-958.

De Block, M., Botterman, J., Van der Wiele, M., Dockx, J., Thoen, C., Gossele, V., Movva, N.R., Thompson, C., Van Montague, M., and Leemans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. *The EMBO Journal* **6(9):** 2513-2518.

Dessai, A.P., Gosukonda, R.M., Blay, E., Dumenyo, C.K., Medina-Bolivar, F., and Prakash, C.S. (1995). Plant regeneration of sweetpotato (*Ipomoea batatas* L.) from leaf explants *in vitro* using a two-stage protocol. *Scientia Horticulturae* **62:** 217-224.

Dodeman, V.L., Ducreux, G., and Kreis, M. (1997). Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany* **48(313):** 1493-1509.

Fujimoto, H., Itoh, K., Yamamoto, M., Kyozuka, J., and Shimamoto, K. (1993) Insect resistant rice generated by the introduction of a modified δ-endotoxin gene from *Bacillus thuringiensis. Biotechnology* **11:** 1151-1155

Gama, M.I.C.S., Leite, R.P., Cordeiro, A.R., and Cantliffe, D.J. (1996). Transgenic sweet potato plants obtained by *Agrobacterium tumefaciens* mediated transformation. *Plant Cell, Tissue and Organ Culture* **46:** 237-244.

George, E.F. (1993). Plant tissue culture techniques. pp 3-36. Variation in cultures and regenerated plants. pp 67-91. Factors affecting growth and morphogenesis. pp 184-271. Plant growth regulators. pp 420-476. In: *Plant Propogation by Tissue Culture, Part 1: The Technology.* 2nd edition. Exegetics Limited. Edington.

George, E.F. (1996).Appropriate regulants and media. pp 577-778. In *Plant Propogation by Tissue Culture, Part 2: In Practice.* 2nd edition. Exegetics Limited. Edington.

Henderson, J.H.M., Phills, B.R., and Whatley, B.T. (1984). Sweet potato. pp 302-307. In: *Handbook of Plant Cell Culture, Volume 2: Crop Species.* Editors: Sharp, W.R., Evans, D.A., Ammirato, P.V., and Yamada, Y. Macmillan Publishing Company. New York.

Hiatt, A., and Mostov, K. (1993). Assembly of multimeric proteins in plant cells: characteristics and uses of plant derived antibodies. pp 221-238. In: *Transgenic Plants: Fundamentals and Applications.* Editor: Hiatt, A Marcel Dekker Incorporated. New York

Hooykaas, P.J.J (1995). *Agrobacterium tumefaciens,* a natural vector system. pp 3-4. In: *Gene Transfer to Plants.* Editors: Potrykus, I., and Spangenberg, G. Springer-Verlag. Berlin.

Hooykaas, P.J.J., and Schilperoort, R.A. (1992). *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology* **19:** 15-38.

Jarret, R. L., and Gawel, N. (1991). Chemical and enviro. .al growth regulation of sweet potato *(Ipomoea batatas* (L.) Lam.) *in vitro. Plant cell, Tissue and Organ Culture* **25:** 153-159.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reports* **5:** 387-405.

Keeton, W.T. (1980). Development of an angiosperm plant. pp 719-723. In: *Biological Science.* Third edition. W.W. Norton and Company. New York

Klein, T. (1995). The biolistic transformation system. pp 115-117. In: *Gene Transfer to Plants.* Editors: Potrykus, I., and Spangenberg, G. Springer-Verlag. Berlin.

Kramer, M., Sanders, R.A., Bolkan, H., Waters, C.M., Sheesy, R.E., and Hiatt, W.T. (1993). Postharvest evaluation of transgenic tomatoes with reduced levels of polygalacturonase: processing, firmness, and disease resistance. pp 283-300. In: *Transgenic Plants: Fundamentals and Applications.* Editor: Hiatt, A. Marcel Dekker Incorporated. New York.

Lehninger, A.L., Nelson, D.L., and Cox, M.M. (1993). Cloning in plants is aided by a bacterial parasite. pp1002-1004. In: *Principles of Biochemistry.* Second edition. Worth Publishers. New York.

Liu, J.R., and Cantliffe, D.J. (1984). Somatic embryogenesis and plant regeneration in tissue cultures of sweet pot *(Ipomoea batatas* Poir.). *Plant Cell Reports* **3:** 112-115.

Llewellyn, D., Lyon, B.R., Cousins, Y., Huppatz, Dennis, E.S., and Peacock, W.J. (1990). Genetic engineering of plants for resistance to the herbicide 2,4-D. pp 67-78. In: *Genetic Engineering of Crop Plants.* Editors: Lycett, G.W., and Grierson, D. Butterworths Printing. London.

Lucas, G.B., Campbell, C.L., and Lucas, L.T. (1992). Rhizopus soft rot. pp 240-242. Bacterial soft rot. pp 272-273. In: *Introduction to Plant Diseases Introduction and Management.* Second editon. Editors: Lucas, G.B., Campbell, C.L., and Lucas, L.T. Chapman and Hall. New York.

Marshall, D.R. (1990). Crop genetic resources: current and emerging issues. pp 367-388. In: *Plant Population Genetics, Breeding, and Genetic Resources.* Editors: Brown, H.D., Clegg, M.T., Kahler, A.L., and Weir, B.S. Sinauer Associates Incorporated. Sunderland.

Martin, T., Wohner, R., Hummel, S., Willmitzer, L., and Frommer, W.B. (1992) The GUS reporter system as a tool to study plant gene expression. pp 23-30. In: *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression.* Academic Press Incorporated. London.

McDonnel, R.E., Clark, R.D., Smith, W.A., and Hinchee, M.A. (1987). A simplified method for the detection of neomycin phosphotransferase 11 activity in transformed plant tissues. *Plant Molecular Biology Reports* **5:** 380-386.

Merckle, S.A., Parrot, W.A., and Flinn, B.S. (1996). Morphogenic aspects of somatic embryogenesis. pp 156-190. In: *In vitro embryogenesis in plants.* Editor: Thorpe, T.A. Kluwer Academic. Dordrecht.

Mok, M.C., Mok, D.W.S., Turner, T.E., and Maser, C.V. (1987). Biological and biochemical effects of cytokinin active phenylurea derivatives in tissue culture systems. *Horticultural Science* **22:** 1194-1197.

Mukherjee, A., Unnikrishnan, M., and Nair, N.G. (1991). Growth and morphogenesis of immature embryos of sweet potato (*Ipomoea batatas* L.) *in vitro*. *Plant cell, [illegible]ue and Organ Culture* **26:** 97-99.

Mu[illegible]ge, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Plant Physiology* **15:** 473-497.

Nelson, R.S., Powell, P.A., and Beachy, R.N. (1990). Coat protein mediated protection against virus infection. pp 13-24. In: *Genetic Engineering of Crop Plants.* Editors: Lycett, G.W., and Grierson, D. Butterworths Printing. London.

Newell, C.A., Lowe, J.M., Merryweather, A., Rooke, L.M., and Hamilton, W.D.O. (1995). Transformation of sweet potato (*Ipomoea batatas* (L.) Lam.) with *Agrobacterium tumefaciens* and regeneration of plants expressing cowpea trypsin inhibitor and snowdrop lectin. *Plant Science* **107:** 215-227.

Nishiguchi, M., Uehara, Y., and Komaki, K. (1992). Stable transformation of sweet potato by electroporation. *In Vitro Cellular and Developmental Biology* **28:** 126.

Nishiyama, I. (1991). Ploidy aspect of the origin of sweet potato. pp 315-324. In: *Chromosome Engineering in Plants: Genetics, Breeding, Evolution. Part B.* Editors: Tsuchiya, T., and Gupta, P.K. Elsevier Science Publishers. Amsterdam.

Peirce, L.C. (1987). Sweet potato. pp 300-308. In: *Tuber and Tuberous Rooted Crops.* John Wiley and Sons Incorporated. New York.

Potrykus, I. (1991) Gene transfer to plants: assessment of published approaches and results. *Annual Review of Plant Physiology and Plant Molecular Biology* **42:** 205-225.

Potrykus, I. (1995). Transformation by tissue electroporation. p 74. In: *Gene Transfer to Plants.* Editors: Potrykus, I., and Spangenberg, G. Springer-Verlag. Berlin.

Prakash, C.S., and Varadarajan, U. (1992). Genetic transformation of sweet potato by particle bombardment. *Plant Cell Reports* **11:** 53-57.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989a). Agarose gel electrophoresis. pp 6.3-6.13. In: *Molecular Cloning: A Laboratory Manual Part 1.* Editor: Nolan, C. Cold Spring Harbor Laboratory Press. New York

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989b). Applications of PCR amplification. pp 14.14-14.19. In: *Molecular Cloning: A Laboratory Manual Part 2.* Editor: Nolan, C. Cold Spring Harbor Laboratory Press. New York

Schultheis, J.R., Cantliffe, D.J., and Chee, R.P. (1990). Optimizing sweet potato (*Ipomoea batatas* (L.) Lam.) root and plantlet formation by selection of proper embryo developmental stage and size, and gel type for fluidized sowing. *Plant Cell Reports* **9:** 356-359.

Spencer, T.M., Gordon-Kamm, W.J., Daines, R.J., Start, W.G., and Lemaux, P.G. (1990). Bialaphos selection of stable transformants from maize cell culture. *Theoretical Applied Genetics* **79:** 625-631.

Stevens, F.L., and Hall, J.G. (1921). Sweet potato. pp 245-251. In: *Diseases of Economic Plants.* Second edition. Editor: Stevens, F.L. Macmillan Publishing Company. New York.

Van Haute, E., Joos, H., Maes, M., Warren, G., Van Montagu, M., and Schell, J. (1983). Intergenetic transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy fo: the reversed genetics of the Ti plasmids of *Agrobacterium tumefaciens*. *The EMBO Journal* **2(3):** 411-417.

Warren, G. (1991). The regeneration of plants from cultured cells and tissues. pp 82-100. In: *Plant Cell and Tissue Culture*. Editors: Stafford, A., and Warren, G. Open University Press. Buckingham.

Wehrmann, A., Van Vliet, A., Opsomer, C., Botterman, J., and Schulz, A. (1996). The similarities of *bar* and *pat* gene products make them equally applicable for plant engineers. *Nature Biotechnology* **14:** 1274-1278.

Wilson, K.J., Hughes, S.G., and Jefferson, R.A. (1992) The *Escherichia coli* GUS operon: induction and expression of the GUS operon in *E. coli* and the occurrence and use of GUS in other bacteria. pp 7-22. In: *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression*. Academic Press Incorporated. London.

Wyninger, R. (1962). Sweet potato. pp 205-215. In: *Pests of Crops in Warm Climates and their Control*. Friedrich Reinhardt AG. Basel.

Xiong, Z. (1993). Extraction of cotton DNA for CCLV detection. *University of Arizona Plant Virology Laboratory* **3(10)**.

Zheng, Q., Dessai, A.P., and Prakash, C.S. (1996). Rapid and repetitive plant regeneration in sweet potato via somatic embryogenesis. *Plant Cell Reports* **15:** 381-385.

7 Appendix

7.1 Standard curves

Standard curves were constructed for the protein determination (Figure 7.1.1) and MU determination (Figure 7.1.2) of plant tissue extracts used for GUS assays. These graphs were required to determine GUS activity as nmole(MU).min^{-1}.mg^{-1} protein.

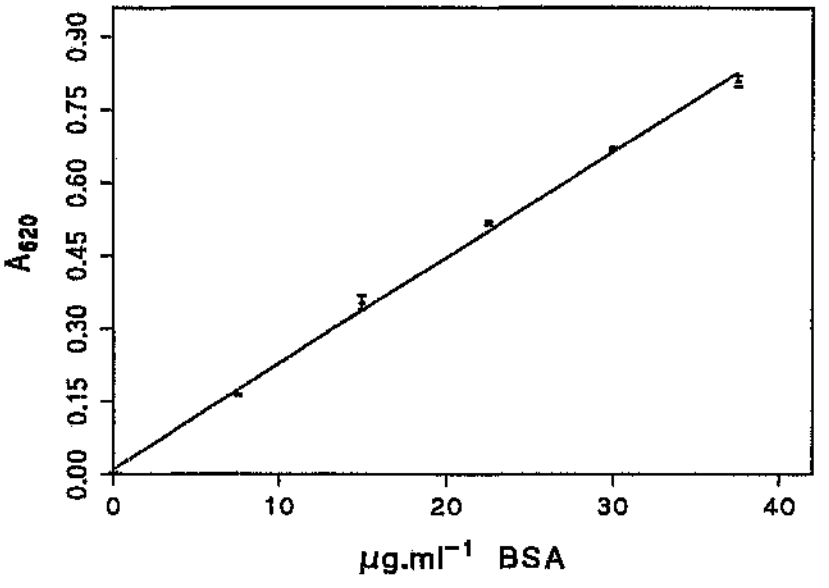


Figure 7.1.1: Graph of A_{620} versus protein concentration in µg.ml^{-1} of a BSA standard. The line has a regression coefficient of 0.9989 and its equation is $y = 0.0218x + 0.0109$. Vertical error bars represent the standard deviation of the average of 2 values. The graph was used to determine the protein concentration of plant tissue extracts used for GUS assays.

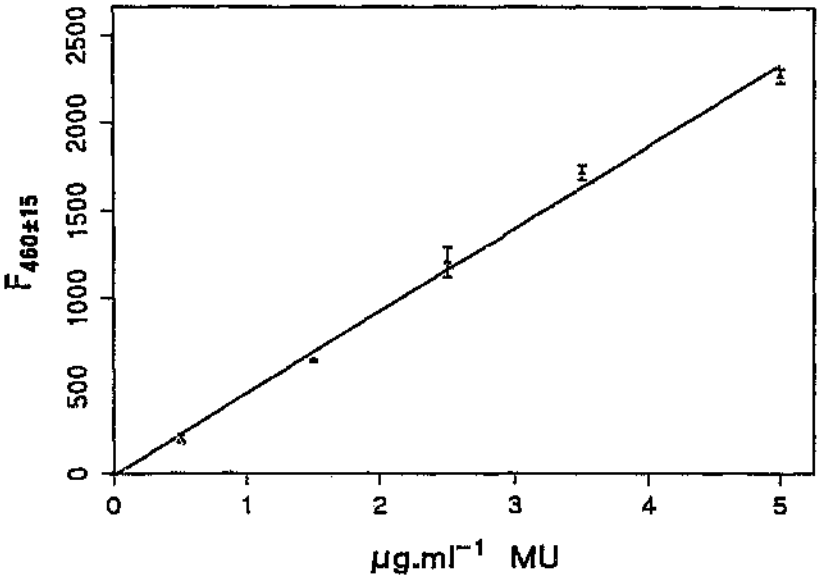


Figure 7.1.2: Graph of $F_{460 \pm 15}$ versus MU concentration in µg.ml^{-1} of a MU standard. The line has a regression coefficient of 0.9980 and its equation is y = 469.6x - 9.3. Vertical error bars represent the standard deviation of the average of 3 values. The graph was used to determine the MU concentration of plant tissue extracts used for GUS assays.

7.2 Murashige and Skoog growth medium

The growth media used in all experiments was made up according to the protocol of Murashige and Skoog (Table 7.3).

Chemical	Final Concentration ($mg.l^{-1}$)
Stock 1	
NH_4NO_3	165.000
KNO_3	190.000
Stock 2	
$MgSO_4.7H_2O$	370.000
$MnSO_4.4\ H_2O$	15.600
$ZnSO_4.\ 7H_2O$	8.600
$CuSO_4.5\ H_2O$	0.025
Stock 3	
$CaCl_2.2\ H_2O$	440.000
KI	0.830
$CoCl_2.6\ H_2O$	0.025
$Na_2MO_4.2\ H_2O$	0.250
Stock 4	
KH_2PO_4	170.000
H_3PO_4	6.200
Stock 5	
$FeSO_4.7\ H_2O$	37.300
Na_2EDTA	27.800
Stock 6	
Inositol	100.000
Glycine	2.000
Nicotinic acid	0.050
Pyridoxine Hcl	0.500
Thiamine HCl	0.050

Table 7.3: Chemical composition of Murasghige and Skoog growth media stock solutions and the final concentration of chemicals (Unilab) in the growth medium.

7.3 Xiong method of DNA extraction

1. 50mg samples of plant tissue were ground down in 50ml extraction buffer (2% N-cetyl-N,N,N-trimethyl ammonium bromide, 20 mM EDTA, 1.4 M NaCl, 100mM Tris, 0.1 % $v.v^{-1}$ β-mercaptoethanol (Unilab)).
2. The mixtures were incubated at 65 °C for 30 minutes.
3. The mixtures were mixed with an equal volume of a 24:1 ratio of chloform:isoamyl alcohol (24:1) (BDH).
4. The mixtures were centrifuged for 5 minutes at 5000g (Sigma 201 M) at room temperature and the aqeous phases were extracted.
5. Steps 4 and 5 were repeated three times.
6. DNA was precipitated with an equal volume of isopropanol (BDH).
7. DNA was pelleted by centrifugation at 10000g for 10 minutes(Sigma 201 M).
8. DNA was washed with 70% ethanol.
9. DNA was resuspended in 50 µl Tris EDTA (TE) (10mM Tris, pH 8.0, 1mM EDTA (Univar)) with 20 $\mu g.ml^{-1}$ Rnase A.

7.4 Polymerase chain reaction primers

GUS-intron-left

5^1-AAT TGA TCA GCG TTG GTG GGA AAG C-3^1

GUS intron right

5^1-GAG GTT AAA GCC GAC AGC AGC AGT T-3^1